

The Development of Therapeutic Monoclonal Antibody Products

A Comprehensive Guide to CMC Activities from Clone to Clinic

A Report Prepared by BioProcess Technology Consultants, Inc. Editors: Howard L. Levine, PhD Brendan R. Cooney SECOND EDITION

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Foreword

Foreword

s the pharmaceutical market in the United States and the rest of the world continues to expand, biopharmaceutical products have taken on increasing importance in the treatment of disease. From 2005 through 2015, the global pharmaceutical market has grown from approximately \$6.5 billion to nearly \$11 billion, driven in large part by the introduction of more and more monoclonal antibody products. Sales of this segment of the pharmaceutical market have grown at a compound annual growth rate of approximately 10% for the last 10 years making biologics approximately nearly 15% of the total pharmaceutical market. As more and more exciting monoclonal antibody products for treatment of cancer, autoimmune diseases, cardiovascular disease, and others are introduced, the growth of monoclonal antibodies is predicted to continue with expectations that sales of these products expected to reach almost \$250 million by 2020.

When The Development of Therapeutic Monoclonal Antibodies was originally released in 2010, it quickly became an indispensable tool for those involved in the development or financing of monoclonal antibodies. It served as a guide to the complex technical, regulatory, and strategic Chemistry, Manufacturing, and Controls (CMC) activities necessary to successfully advance new monoclonal antibody products to clinical trials and the market as quickly as possible. This Second Edition has been fully revised and updated for 2016, with the addition of new content addressing advancements in Quality by Design (QbD), analytical development, and process validation, and more. Since publication of the First Edition of this book, regulatory agencies in the US, Europe, and the rest of the world have updated or issued all of the major guidances and regulations related to biopharmaceutical products. This new Second Edition includes a discussion of these new guidance documents from FDA, EMA, and ICH.

The Second Edition takes an updated look at, and provides recommendations for, all aspects of CMC necessary for the development of monoclonal antibody products from discovery through First In-Human Trials. The regulatory framework in which developers of monoclonal antibodies must operate is complex and constantly evolving. This report provides an overview of the most up to date regulatory thinking and the course that it may take going forward.

The Development of Therapeutic Monoclonal Antibodies Second Edition goes beyond other reports by incorporating the latest technical developments and integrating strategic and regulatory considerations with these technical requirements. This report will serve as a guide to product development companies, service providers, investors, and analyst as they work their way through the complex and rapidly evolving world of therapeutic monoclonal antibodies.

Howard L. Levine, Ph.D. Brendan R. Cooney June 2017



CHAPTER 1:

The Therapeutic Monoclonal Antibody Market

n 1984 Kohler and Milstein received the Nobel Prize in Medicine for their pioneering work on the production of monoclonal antibodies.¹ One of the most significant advantages of this new technology over traditional techniques for producing antibodies was the creation of an immortalized cell line creating a continuous source of the same antibody with a single antigen specificity. This enabled the development of highly specific monoclonal antibodies directed toward a single epitope on the target antigen. Initially monoclonal antibodies were used as laboratory reagents but their use was quickly adopted as clinical diagnostic reagents. In the early 1980s, commercial development of monoclonal antibodies as therapeutic agents commenced so that by 1986 the first therapeutic monoclonal antibody, Orthoclone OKT3, was approved for prevention of kidney transplant rejection.

1. Antibody Structure

Antibodies are a component of the immune system whose ability to bind targets, activate other immune system functions, and reside in the circulation for weeks has been harnessed to create effective therapeutic products. The power of antibodies as effective therapeutics resides in their specificity, their bivalency, and their modular structure, which has enabled this category of therapeutic products to emerge as a leading component of the biopharmaceutical market. All antibodies have the same basic structure, which consists of two identical heavy chains and two identical light chains as shown in Figure 1.1. Early structural studies revealed that antibodies could be enzymatically digested into two regions, the Fab region which contains the antigen binding site, and the Fc region, which contains sequences that interact with other components of the immune system to activate additional functions.² This modularity is the basis for many of today's discovery methods, which focus on discovery of suitable antigen binding sequences and then use molecular techniques to add an Fc region that is appropriate for the intended indication.

Antibodies in human sera can be divided into five different classes based on the sequence of the heavy chain. These classes are known as IgA, IgD, IgE, IgG, and IgM, and each class has a different function within the overall immune system. Some classes can be further subdivided, such as the IgG class, which contains the four subclasses IgG1, IgG2, IgG3, and IgG4. Most therapeutic monoclonal antibodies are IgG, with most approved antibodies falling in the IgG1 or IgG4 subclass.³ Some products in development are IgM, which consist of pentamers or hexamers of the four chain basic antibody structure, but most discovery and development efforts continue to focus on IgG antibodies and this class is the primary focus of this report. Two types of light chain are also found in human antibodies, kappa (κ) and lambda (λ) with the κ chain being far more common in therapeutic monoclonal antibodies. A typical IgG antibody with either type of light chain contains approximately 1,080 amino acids and has a total molecular weight of approximately 146 kDa prior to posttranslational modification.



Figure 1.1 Antibody Structure

$V_{H}=heavy\ chain\ variable\ region;\ V_{L}=Light\ chain\ variable\ region;\ C_{H2},\ C_{H2}=Heavy\ chain\ constant\ regions,\ C_{L}=Light\ chain\ constant\ region\ Source:\ BPTC$

As shown in Figure 1.1, IgG antibodies usually have four interchain disulfide bonds, two connecting each light chain with a heavy chain and two connecting the heavy chains to enable dimerization. This feature of the Fc region of the heavy chain can be utilized to form dimers of other therapeutic proteins by creating a fusion between the protein of interest and the IgG heavy chain Fc sequence. Among the potential therapeutic benefits of these fusion proteins is a longer serum half-life of the fusion protein compared to the monomer used without linkage to the Fc region and bivalent functionality.

Intra-chain disulfide bonds are also found in the variable and constant regions. The intra-chain bonds in the variable regions help create the three dimensional structure that enables proper antigen binding. Low levels of free sulfhydryl groups from disulfide bonds that did not form properly can be found in recombinant antibodies and can create product stability problems.⁴

Antigen Binding

The antigen binding function of an antibody is located within the 110 amino acid variable region at the N-terminus of each chain. Within the variable regions, three surfaceexposed hypervariable amino acid loops, known as complementarity determining regions (CDR), are embedded in a relatively conserved framework structure.5 The six combined CDRs from the heavy and light chains form the antigen binding site, and slight changes to CDR sequences can significantly alter affinity and specificity for the target antigen.⁶ Because the antigen binding function of an antibody is localized in such a specific region of the protein, molecular engineering tools can be used to introduce novel variability in the CDRs of one or both chains followed by in vitro selection for improvements in target binding.⁷ Binding at the antigen binding sites on each arm of the antibody can occur independently so that the antibody can also be engineered to contain two different antigen binding domains. Such bi-specific antibodies are currently under development by several companies.8 Also, if the variable region of an antibody is cloned independently and expressed as a soluble monomer it will retain the ability to bind to the target antigen.⁹ These monovalent products are also under development by several companies.¹⁰

Effector Functions

In addition to antigen binding function, antibodies contain oligosaccharides on the constant region that can interact with other components of the immune system to activate effector functions such as antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). If immune system activation is important for the therapeutic activity of an antibody, the oligosaccharide structure is often critical to the clinical behavior of the molecule.¹¹ For IgG antibodies, an N-linked biantennary oligosaccharide is attached to a highlyconserved asparagine. The core structure contains three mannose residues and two N-acetyl-glucosamine residues (GlcNAc) as shown in Figure 1.2.¹² In some monoclonal antibodies, the carbohydrate structure may also contain fucose. If present, the fucose residue is linked to the proximal GlcNAc residue, and additional terminal sugar residues including galactose and sialic acid are also present. Occasionally GlcNAc is added to the central mannose to form a structure known as a bisecting GlcNAc, which has a significant impact on antibody function.¹³



Figure 1.2. IgG Oligosaccharide Structure

complex-type N-linked oligosaccharides

The oligosaccharide structure of N-linked glycans in the CH2 domains is shown. Individual sugar moieties may or may not be present in all molecules (indicated by \pm in the figure), however sialic acid can only be present if galactose is also present in the oligosaccharide structure. (Figure adapted and reprinted with permission from References 14 and 15)

Source: GlycoWord

Variation in the terminal sugar residues is the basis of most of the glycan heterogeneity seen in purified, recombinant monoclonal antibodies. This can influence which, if any, effector functions are activated. For example, the oligosaccharide can contain either no (G0), one (G1), or two (G2) terminal galactose residues (see Figure 1.2); increased galactose content can increases CDC activity while ADCC activation is not known to be affected by the galactose content of the oligosaccharide.¹⁶ Likewise, if fucose is not present on the core GlcNAc, the antibody exhibits enhanced ADCC activity compared to the fully fucosylated form but no impact on CDC has been observed.¹⁷ In addition, variation in the oligosaccharide structure in the binding protein of an Fcfusion may greatly impact overall half-life in a way not generally seen with whole antibodies. For example, sialic acid content in the binding protein may greatly affect half-life or efficacy of the product.

Glycan variability is primarily influenced by clone selection and cell culture conditions, but should also be considered during discovery and lead candidate identification, especially when choosing a heavy chain constant region for a particular target product profile. If effector functions are not required for the intended therapeutic mode of action, it may be most effective to develop an IgG4 antibody that has less effector function. For example, for monoclonal antibodies whose therapeutic activity is entirely based on blocking another protein from binding to the target, effector function and oligosaccharide structure are not critical to therapeutic function.

2. Therapeutic Applications of Monoclonal Antibodies

Following the approval of Orthoclone OKT3, there was a long gap before any new antibody products were approved. During this time, new approaches to discovering and developing antibody products emerged and enthusiasm for therapeutic monoclonal antibodies returned. Several additional monoclonal antibody products were approved in the US and Europe in the mid to late 1990's, while the 2000's ushered in the next wave of antibody products generally being developed as anti-cancer and anti-inflammatory agents. Today, monoclonal antibody products, including fragments, conjugates, and full length entities are a mainstay in the pharmaceutical industry. Utilizing today's novel technologies and enhanced targeting, they continue to be discovered, developed, and approved to treat many different diseases¹⁸.

As of October 31, 2016, there were 71 monoclonal antibodyrelated products on the market in the US and/or Europe for the treatment of a variety of diseases including autoimmune disorders, cardiovascular indications, infectious diseases, and oncology (see Table 1). These approved monoclonal antibody products, which include full length monoclonal antibodies as well as antibody fragments (Fab fragments), Fc-fusion proteins, antibody-drug conjugates, and other conjugated antibody products, have been approved for diseases with patient populations ranging from a few thousand or fewer for such orphan indications as paroxysmal nocturnal hemoglobinuria, or the cryopyrin-associated periodic syndromes, to hundreds of thousands of patients for some cancers and multiple sclerosis, to millions of patients for diseases such as asthma and rheumatoid arthritis. In some cases, the market penetration of monoclonal antibody products in the US and Europe is quite large with most of the potentially treatable patients receiving the appropriate antibody therapy. However, for some diseases such as asthma, there are a large number of potentially treatable patients who are not receiving monoclonal antibody therapy due to the variety of treatment options currently available and the fact that the approved monoclonal antibody product in this indication is not the typical first line of treatment for the disease. On the other hand, access to monoclonal antibody therapies in emerging

or developing countries is often much lower than in the US or Europe due to lack of availability of the monoclonal antibody products, higher prices, or other regulatory hurdles. the development of these products are similar to those for fulllength monoclonal antibodies.

Table 1.1 Applications of Therapeutic Monoclonal Antibody Products			
Disease Category	Example Product	Specific Indication(s)	Additional Products
Allergy	Xolair	Asthma, moderate to severe, chronic idiopathic urticaria	2
Bone disease	Prolia	Osteoporosis	1
Cardiovascular	Praluent	Primary hyperlipidemia	1
Hematologic	Reopro	Anti-platelet prevention of blood clots in high-risk percutaneous transluminal angioplasty, and in refractory angina when percutaneous coronary intervention is planned	3
Immune and autoimmune diseases	Humira	Rheumatoid arthritis, juvenile idiopathic arthritis, psoriatic arthritis ankylosing spondylitis, Crohn's disease, ulcerative colitis, plaque psoriasis, hidradenitis suppurativa and uveitis	27
Infectious diseases	Synagis	Prevention of respiratory syncytial virus (RSV) infections in children	3
Macular degeneration	Lucentis	Neovascular (wet) age-related macular degeneration, macular edema following retinal vein occlusion, diabetic macular edema and diabetic retinopathy in patients with diabetic macular edema	1
Oncology	Rituxan ^b	Non-Hodgkin's lymphoma, chronic lymphocytic leukemia (also for rheumatoid arthritis and Wegener's granulomatosis)	25

^a Number of other approved products in each therapeutic indication area

^b Rituxan is approved for both oncology and immune and autoimmune indications

Among the monoclonal antibody-related products, one very important class is the Fc-fusion proteins, which incorporate or contain the Fc region of an antibody within their structure. Products in this category, which include Enbrel, AlprolIX and Nplate, generally combine the antibody Fc region with a binding protein whose half-life in the body is usually too short to allow the binding protein alone to be therapeutically beneficial. In these products, the primary function of the Fc region is to prolong the half-life of the product thereby increasing its efficacy and bioavailability rather than to activate the complement system. While these antibody-related products combine two very different protein moieties in a single molecule, many of the Chemistry, Manufacturing and Control (CMC) strategies, activities, costs, and timelines for the development of Fc-fusion proteins are similar to those of full-length antibodies. For example, the presence of the Fc region in the fusion protein allows Protein A affinity chromatography to be used as a primary capture step in the downstream processing of these products (see Chapter 7). As a result, the CMC strategies, activities, costs, and timelines for

Similarly, the development, manufacturing, and quality control of the monoclonal antibody portion of an antibody-drug conjugate follow much the same CMC strategies as for fulllength antibodies. For these products, however, the preparation and conjugation of the toxic drug moiety adds additional complexity and cost to the development of these products.

For monoclonal antibody fragments, many of which are produced in microbial hosts rather than mammalian cell culture, the development strategies, costs, and timelines may vary compared to full-length monoclonal antibodies. For example, since these products lack the Fc region of a full-length antibody, they cannot be purified using Protein A affinity chromatography and much of the discussion in this report regarding platform processes is not relevant to these products.

3. Growth of the Monoclonal Antibody Market

Following the approval of the first monoclonal antibody product in 1986, sales growth and approval of additional products was slow until the late 1990s when the first chimeric monoclonal antibodies were approved. With the approval of these products, followed by the approval of humanized and then fully human monoclonal antibodies, the rate of product approvals and sales of monoclonal antibody products has increased dramatically so that in 2015, global sales revenue for all monoclonal antibody products was nearly \$90 billion,19 representing nearly 60% of the total sales of all biopharmaceutical products. Among biopharmaceutical products currently on the market, seven of the top ten selling products in 2015 were monoclonal antibody products (see Table 1.2).

As shown in Figure 1., the number of monoclonal antibody products approved for commercial sale in the US and Europe has grown steadily. Since 2011, between three and eleven products have been approved, with an average of seven new products approved per year and since 2014 an unprecedented average of ten products per year have been approved. While a total of 82 monoclonal antibody products have been approved in Europe and/or the US since 1986, eleven of these products have been withdrawn for various reasons, leaving 71 approved monoclonal antibody products currently on the market.^{19, 20, 21}

Table 1.2. 2015 Sales of the Top Ten SellingBiopharmaceutical Products^a

US Product Name	2015 Sales (\$ Billions) $^{\rm b}$
Humira	14.0
Enbrel	8.7
Remicade	8.4
Rituxan	7.0 ^b
Lantus	7.0 ^b
Avastin	6.7 ^ь
Herceptin	6.5 ^b
Neulasta	4.7
Novolog	4.6 ^b
Eylea	4.0 ^b

^a Non-monoclonal antibody products are listed in italics
 ^b Values converted to USD from reporting currency with a strong USD in 2015.

Figure 1.3. Annual Approvals of Monoclonal Antibody Products^{20, 21}



Source: BPTC

Table 1.3. Commercially Marketed Therapeutic Monoclonal Antibody Products ^a				
Product Name (INN Name)	Original BLA/MAA Applicant	Company Reporting US Sales	Company Reporting EU Sales	Year of First Approval
Abthrax (raxibacumab)	Human Genome Sciences	GlaxoSmithKline	N/A ^b	2012
Actemra (tocilizumab)	Roche	Roche	Roche	2009
Adcetris ° (brentuximab vedotin)	Seattle Genetics	Seattle Genetics	Takeda Pharmaceutical	2011
AlprolIX ^d (Factor IX Fc-fusion protein, eftrenonacog alfa)	Biogen Idec	Biogen	Biogen	2014
Amjevita ^e (adalimumab-atto [biosimilar])	Amgen	Amgen	N/A	2016
Anthim (obiltoxaximab)	EluSys Therapeutics	EluSys Therapeutics	N/A	2016
Arcalyst ^f (rilonacept)	Regeneron Pharmaceuticals	Regeneron Pharmaceuticals	N/A	2008
Arzerra (ofatumumab)	GlaxoSmithKline	GlaxoSmithKline/ Novartis ^g	GlaxoSmithKline/ Novartis ^g	2009
Avastin (bevacizumab)	Genentech	Roche	Roche	2004
Benepali ^h i (etanercept [biosimilar])	Samsung Bioepis	N/A	Biogen	2016
Benlysta (belimumab)	Human Genome Sciences	GlaxoSmithKline	GlaxoSmithKline	2011
Blincyto ^j (blinatumomab)	Amgen	Amgen	N/A	2014
Cimzia ^k (certolizumab pegol)	UCB	UCB	UCB	2008
Cinqair (reslizumab)	Teva	Teva	Teva	2016
Cosentyx (secukinumab)	Novartis	Novartis	Novartis	2015
Cyramza (ramucirumab)	Eli Lilly	Eli Lilly	Eli Lilly	2014
Darzalex (daratumumab)	Jannsen Biotech	Johnson & Johnson	Johnson & Johnson	2015
Eloctate ¹ (Factor VIII Fc-fusion protein, efmoroctocog alfa)	Biogen Idec	Biogen	Biogen	2014
Empliciti (elotuzumab)	Bristol-Myers Squibb	Bristol-Myers Squibb	Bristol-Myers Squibb	2015
Enbrel ^h (etanercept)	Immunex	Amgen	Pfizer	1998

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Table 1.3. Commercially Marketed Therapeutic Monoclonal Antibody Products ^a				
Product Name (INN Name)	Original BLA/MAA Applicant	Company Reporting US Sales	Company Reporting EU Sales	Year of First Approval
Entyvio (vedolizumab)	Takeda Pharmaceutical	Takeda Pharmaceutical	Takeda Pharmaceutical	2014
Erbitux (cetuximab)	ImClone Systems	Eli Lilly ^m	Merck KGaA	2004
Erelzi ^{hi} (etanercept-szzs [biosimilar])	Sandoz	Novartis	N/A	2016
Eylea " (aflibercept)	Regeneron Pharmaceuticals	Regeneron Pharmaceuticals	Bayer Healthcare Pharmaceuticals	2011
Flixabi ⁿ (infliximab [biosimilar])	Samsung Bioepis	N/A	Biogen	2016
Gazyva (obinutuzumab)	Genentech	Roche	Roche	2013
Herceptin (trastuzumab)	Genentech	Roche	Roche	1998
Humira (adalimumab)	Abbott Laboratories	AbbVie	AbbVie	2002
llaris (canakinumab)	Novartis Pharmaceuticals	Novartis	Novartis	2009
Inflectra °p (infliximab [biosimilar])	Hospira	Pfizer	Pfizer	2013
Kadcyla ª (ado-trastuzumab emtansine)	Genentech	Roche	Roche	2013
Keytruda (pembrolizumab)	Merck & Co.	Merck & Co.	Merck & Co.	2014
Lartruvo (olaratumab)	Eli Lilly	Eli Lilly	N/A	2016
Lemtrada (alemtuzumab)	Genzyme	Genzyme	Sanofi	2013
Lucentis ^r (ranibizumab)	Genentech	Roche	Novartis	2006
Nplate ^s (romiplostim)	Amgen	Amgen	Amgen	2008
Nucala (mepolizumab)	GlaxoSmithKline	GlaxoSmithKline	GlaxoSmithKline	2015
Nulojix ^t (belatacept)	Bristol-Myers Squibb	Bristol-Myers Squibb	Bristol-Myers Squibb	2011
Opdivo (nivolumab)	Bristol-Myers Squibb	Bristol-Myers Squibb	Bristol-Myers Squibb	2014
Orencia " (abatacept)	Bristol-Myers Squibb	Bristol-Myers Squibb	Bristol-Myers Squibb	2005
Perjeta (pertuzumab)	Genentech	Roche	Roche	2012

Table 1.3. Commercially Marketed Therapeutic Monoclonal Antibody Products ^a				
Product Name (INN Name)	Original BLA/MAA Applicant	Company Reporting US Sales	Company Reporting EU Sales	Year of First Approval
Portrazza (necitumumab)	Eli Lilly	Eli Lilly	Eli Lilly	2015
Praluent (alirocumab)	Sanofi Aventis	Sanofi	Sanofi	2015
Praxbind (idarucizumab)	Boehringer Ingelheim	Boehringer Ingelheim	Boehringer Ingelheim	2015
Prolia ^v (denosumab)	Amgen	Amgen	Amgen ^w	2011
Remicade (infliximab)	Centocor	Johnson & Johnson	Merck & Co.	1998
Removab ^x (catumaxomab)	Fresenius Biotech	N/A	Neovii Biotech	2009
Remsima ° ¤ (infliximab [biosimilar])	Celltrion	N/A	Celltrion	2013
ReoPro ^y (abciximab)	Centocor	Eli Lilly	Eli Lilly	1994
Repatha (evolocumab)	Amgen	Amgen	Amgen	2015
Rituxan (rituximab)	Genentech	Roche	Roche	1997
Simponi (golimumab)	Centocor Ortho Biotech	Johnson & Johnson	Merck & Co.	2009
Simulect (basiliximab)	Novartis Pharmaceuticals	Novartis	Novartis	1998
Soliris (eculizumab)	Alexion Pharmaceuticals	Alexion Pharmaceuticals	Alexion Pharmaceuticals	2007
Stelara (ustekinumab)	Janssen-Cilag International	Johnson & Johnson	Johnson & Johnson	2009
Strensiq ^z (asfotase alfa)	Alexion Pharmaceuticals	Alexion Pharmaceuticals	Alexion Pharmaceuticals	2015
Sylvant (siltuximab)	Janssen Biotech	Johnson & Johnson	Johnson & Johnson	2014
Synagis (palivizumab)	Abbott Laboratories	AstraZeneca	Abbvie	1998
Taltz (ixekizumab)	Eli Lilly	Eli Lilly	Eli Lilly	2016
Tecentriq (atezolizumab)	Genentech	Roche	N/A	2016
Trulicity ªª (dulaglutide)	Eli Lilly	Eli Lilly	Eli Lilly	2014
Tysabri (natalizumab)	Biogen Idec	Biogen	Biogen	2004

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Table 1.3. Commercially Marketed Therapeutic Monoclonal Antibody Products ^a				
Product Name (INN Name)	Original BLA/MAA Applicant	Company Reporting US Sales	Company Reporting EU Sales	Year of First Approval
Unituxin (dinutuximab)	United Therapeutics	United Therapeutics	United Therapeutics	2015
Vectibix (panitumumab)	Amgen	Amgen	Amgen	2006
Xgeva ^v (denosumab)	Amgen	Amgen	Amgen ^w	2010
Xolair (omalizumab)	Genentech	Roche	Novartis	2003
Yervoy (ipilimumab)	Bristol-Myers Squibb	Bristol-Myers Squibb	Bristol-Myers Squibb	2011
Zaltrap ^{ab} (ziv-aflibercept)	Sanofi Aventis	Sanofi	Sanofi	2012
Zevalin ^{ac} (ibritumomab tiuxetan)	IDEC Pharmaceuticals	Spectrum Pharmaceuticals	Spectrum Pharmaceuticals	2002
Zinbryta (daclizumab (high yield))	Biogen	Biogen	Biogen	2016
Zinplava (bezlotoxumab)	Merck Sharp Dohme	Merck & Co.	N/A	2016

- a Products approved as of October 31, 2016.
- b N/A denote product not available in this region.
- c Antibody-Drug Conjugate, MMAE
- d Fc-Fusion Protein, Fc-Factor IX
- e Biosimilar Antibody, Humira Originator
- f Fc-Fusion Protein, Fc-IL1R
- g In August of 2015, Novartis acquired all remaining rights to ofatumumab from GlaxoSmithKline.
- h Fc-Fusion Protein, Fc-TNFR (p75)
- i Biosimilar Fc-Fusion Protein, Enbrel Originator
- j Bispecific Antibody
- k Fab Conjugate, PEG (produced by microbial fermentation)
- I Fc-Fusion Protein, Fc-Factor VIII
- m In April of 2015, Eli Lilly acquired all North American rights to cetuximab from Bristol-Myers Squibb.
- n Fc-Fusion Protein, Fc-VEGFR (1,2)
- o Biosimilar Antibody, Remicade Originator
- p Inflectra and Remsima are considered as two individual products; see text.

- q Antibody-Drug Conjugate, DM1
- r Fab (produced by microbial fermentation)
- s Fc-Fusion Protein, Fc-TPO-R binding peptide (produced by microbial fermentation)
- t Fc-Fusion Protein, Fc-CTLA-4 with amino acid substitutions
- u Fc-Fusion Protein, Fc-CTLA-4
- v Prolia and Xgeva are considered separate products even though they contain the same bulk monoclonal antibody.
- w In December of 2015, Amgen reacquired all rights to denosumab from GlaxoSmithKline.
- x Bispecific, Tri-functional Antibody
- y Fab, produced by papain digestion of full length monoclonal antibody
- z Fc-Fusion Protein, Fc-TNSALP
- aa Fc-Fusion Protein, Fc-GLP-1
- ab Fc-Fusion Protein, Fc-VEGFR
- ac Antibody Conjugate, Y-90

Those products still on the market as of October 31, 2016, are listed in Table 1.3 along with the year of first approval in the US or Europe. Of these products approved and marketed in the United States and Europe, three are produced in *E. coli* while all of the other products are produced in mammalian cells. Of the products produced in mammalian cell culture, 49 are full-length naked monoclonal antibodies, including four biosimilars; two are bispecific antibodies, two are antibody-drug conjugates, one is a radio-labeled antibody conjugate, two are antigenbinding fragments (Fab), and 12 are Fcfusion proteins containing the antibody constant region fused to another non-antibody-related protein domain, including two biosimilars. Two of the three products produced in *E. coli* are Fabs, one of which is a Fab conjugate while the third is an Fcfusion protein.

Of the full-length monoclonal antibodies, two products, Prolia and Xgeva are considered separate products even though they are manufactured from the same biologically active substance. This distinction is based on the fact that Prolia and Xgeva are presented in different formulations and container/ closure systems, and separate Biological License Applications (not Supplemental Applications) were filed in the US for each product. The list of 71 approved monoclonal antibody products also includes the first biosimilar monoclonal antibodies approved in Europe, Inflectra and Remsima. The bulk monoclonal antibody used to produce these biosimilars is manufactured by a single supplier (Celltrion). However, as with Prolia and Xgeva, these are considered separate products since the final drug product for each is manufactured by a separate entity and two separate manufacturers are responsible for final batch release of the commercial products. Furthermore, separate European Marketing Authorization Applications were submitted for each product.

From a review of historical success and turnover rates (i.e., the length of time required for a product to move from one stage of development to the next) of biopharmaceutical product development candidates, approximately 26% of the monoclonal antibody products entering Phase 2 human clinical trials in recent years will ultimately achieve market approval with an average time from the start of Phase 2 clinical trials to approval of approximately seven years.^{25, 26, 27} Based on this data and the number of monoclonal antibody product candidates currently in development, it is expected that the number of products approved each year for the coming years will be approximately the same or more than it has been for the last several years. In fact, as of October 31, 11 monoclonal antibody products were granted first approvals in 2016, the greatest number of monoclonal antibody products in a year to date. Based on an approval rate of approximately five monoclonal antibody products per year, we anticipate that there will be nearly 100 monoclonal antibody products on the market by 2021.

For the last five years, the growth in the sales of monoclonal antibody-related products has been significantly higher than any other class of biopharmaceutical products. This is illustrated in Figure 1., where the sales of the following six different classes of biopharmaceutical products are compared for the five-year period from 2010 through 2015:

- 1. Recombinant protein products produced in mammalian cell culture
- 2. Monoclonal antibody products produced in mammalian cell culture
- 3. Antibody conjugates, antibody-fusion proteins and fragment products produced in mammalian cell culture
- 4. Recombinant protein products produced by microbial fermentation
- 5. Antibodyfusion proteins and fragment products produced by microbial fermentation
- 6. Recombinant proteins produced by plant cell culture

As seen from these data, sales of all monoclonal antibody products, regardless of the production system, have grown from approximately \$50 billion in 2010 to almost \$90 billion in 2015, an approxiantely 1.8fold increase. By comparison, sales of other recombinant protein therapeutics have only increased approximately 18% in the same time period.



Figure 1.4. Sales of Biopharmaceutical Products by Product Type and Class

Total annual sales of biopharmaceutical products are shown as a function of product type. Note that recombinant proteins produced by microbial fermentation includes recombinant human insulin products which represent nearly 50% of the sales and >90% of the material produced in this category. *Source: BPTC*

Corresponding to the increasing sales of monoclonal antibody products, there has been an increase in the total quantities of these products produced annually to meet the market demand. As shown in Figure 1.4, an estimated 15.3 metric tons of mammalian-based monoclonal antibody products (including Fc-fusion proteins and antibody fragments) were produced in 2015 compared to approximately 12.2 metric tons of all other recombinant protein products. The demand for monoclonal antibody products has resulted in a significant amount of global manufacturing capacity devoted to their production as well as to significant improvements in methods and approaches to monoclonal antibody manufacturing process design and optimization.^{28, 29, 30, 31, 32}

Of the 71 monoclonal antibody-related products on the market as of October 31, 2016, ²⁴ (Humira, Enbrel, Remicade, Rituxan, Avastin, Herceptin, Eylea, Lucentis, Soliris, Stelara, Xolair, Simponi, Tysabri, Orencia, Erbitux, Perjeta, Actemra, Xgeva, Synagis, Prolia, Cimzia, and Yervoy) achieved annual sales of over \$1 billion. Six products (Humira, Enbrel, Remicade, Rituxan, Avastin, and Herceptin) had sales of greater than \$6.5 billion. The top selling monoclonal antibody product, Humira, recorded sales of \$14 billion, the highest sales figure ever recorded for a biopharmaceutical product.

To further highlight the growth of monoclonal antibody products during the last ten years, the sales growth profiles of the top six selling monoclonal antibody products (Avastin, Enbrel, Herceptin, Humira, Remicade, and Rituxan) are plotted along with those of the two top-selling recombinant protein products produced in mammalian cell culture (the cytokines Avonex and Rebif) in Figure 1.5. The average compound annual growth rate for these six monoclonal antibody products over this period is 11% while that of the two mature recombinant protein products was essentially flat.



Figure 1.5. Sales Growth for Commercial Monoclonal Antibody Products

Source: BPTC

Based on a review of historical sales data, company annual reports, and sales projection data collected by BioProcess Technology Consultants in our proprietary bioTRAK® database, we forecast that the monoclonal antibody market will continue to grow at a compound annual growth rate of 9% or more for the next several years. At this growth rate, sales of currently approved monoclonal antibodies plus sales from new products approved in the coming years will drive the world-wide sales of monoclonal antibody products to approximately \$110 billion by 2018 and nearly \$150 billion by 2021. Our projections are consistent with those of others, such as a recent report from BCC Research that predicts the market for monoclonal antibody products will be nearly \$123 billion by 2019.³³

4. Factors Contributing to Growth of the Monoclonal Antibody Market

The continued interest in antibody product development is partially driven by the rapid advancement of our understanding of disease at a molecular level. Although failing to meet some observers' initial high expectations, genomics, proteomics, and other systems biology tools continue, in fact, to provide important new targets for modulating disease.³⁴ In addition, antibodies have been successfully used to deliver cytotoxic drugs or radiation specifically to target disease sites, minimizing side effects and improving efficacy for the small molecule drugs that could not otherwise be delivered in sufficient doses to be effective.

Early Development Advantages of Monoclonal Antibodies

With many extracellular targets emerging from research and discovery programs, the most rapid route towards a clinical proof-of-concept related to the value of activating or inhibiting these targets is through the use of monoclonal antibodies. Since monoclonal antibodies have conserved structural elements, they have been amenable to efficient platform-based approaches for development, particularly at larger companies with multiple antibodies in the pipeline. This platform approach has enabled improved ability to advance novel antibodies from concept into clinical trials with impressive speed.³⁵ As with all new medicines, there is some risk of unexpected safety issues with monoclonal antibodies, as seen with Tegenero AG's anti-CD28 product candidate TGN1412³⁶, and with an increased risk of progressive multifocal leukoencephalopathy (PML) with certain marketed antibody products.³⁷ However, since antibodies are generally well tolerated and highly specific, the risk of unexpected safety issues in human clinical trials of monoclonal antibodies is lower than with many other types of therapeutic products. Therefore, for many novel targets, monoclonal antibodies are often the first product candidates advancing to clinical trials. If proof-of-concept studies are successful, these products can move rapidly towards commercialization, providing a "first-to-market" advantage.

Increasing Treatable Patient Population

Also fueling the growth in monoclonal antibody product sales is the global market expansion of the pharmaceutical market in general resulting from the increasing and aging worldwide population and the increasing standard of living in emerging markets.³⁸ In addition, the continued evaluation of monoclonal antibody products in new and expanded clinical indications results in continued demand for product for clinical studies and subsequent sales in newly approved indications.

As the biopharmaceutical industry matures, the number and types of diseases that will be economically treatable with monoclonal antibody products will increase. Driven in part by the need for cost-effective supply of large quantities of biopharmaceutical products for such cost-sensitive indication as rheumatology and asthma,³⁹ significant improvements in bioprocess technology have substantially reduced actual manufacturing costs for antibodies. As a result, there is an ever-increasing opportunity for these products to penetrate more cost-sensitive indications and markets, providing additional stimulus for substantial industry-wide revenue growth.

Improved Process Economics for Production from Mammalian Cells

Of the 68 approved and marketed monoclonal antibody products produced in mammalian cell culture, a majority of products (41, %) are produced using Chinese Hamster Ovary (CHO) cells as the production host. Other productions hosts include SP2/0 cells (10 products, 15%), NS0 cells (13 products, 19%) Human Embryonic Kidney (HEK) cells (2 products 3%). Baby Hamster Kidney (BHK) Cells (1 product, 2%) and a rat/mouse hybridoma (1 product, 2%). With the explosive growth of the antibody market over the past decade and the increasing focus on containing manufacturing costs, the industry has focused on improving productivity from

CHO cells and on developing new cell lines that can meet or exceed current CHO cell line productivities. Whereas twenty years ago, most production cell lines yielded no more than 0.51 g/L crude antibody in the bioreactor, process and cell line improvements have led to a reasonable expectation of 57 g/ $L^{40, 41, 42, 43, 44, 45, 46}$ and industry leaders are forecasting levels even higher than this. Coupled with improved automation and enhanced performance of downstream processing components, the cost of producing monoclonal antibodies has dramatically decreased. These improvements, along with the emergence of platform manufacturing processes and widespread industry knowledge on antibody production, have enabled the biopharmaceutical industry to meet the market demand for many monoclonal antibody products and have helped open the market to cost-effective development of monoclonal antibodies for numerous diseases.

5. Biosimilars

Many of the first biopharmaceutical products, including monoclonal antibodies, were protected by patents that have recently expired or will expire in the coming years. As the patents governing the exclusive rights to many of the high profile and blockbuster biopharmaceutical products begin to expire (see Table 1.4), the pharmaceutical industry worldwide has shown a growing interest in developing biosimilar products.

Table 1.4. Patent Expiration Dates for KeyMonoclonal Antibody Products47

Product	Patent Expiration Date		
	United States	Europe	
Adalimumab	Dec 2016	Oct 2018	
Bevacizumab	Jul 2019	Jan 2022	
Cetuximab	Feb 2018	Sep 2014	
Etanercept	Nov 2028	Aug 2015	
Infliximab	Sep 2018	Feb 2015	
Natalizumab	Mar 2015	Aug 2015	
Omalizumab	Jun 2017	Aug 2017	
Ranibizumab	Jun 2020	Apr 2022	
Rituximab	Dec 2018	Nov 2013	
Trastuzumab	Nov 2019	Jul 2014	

While most of the regulatory authorities worldwide have now established regulations for the review and approval of biosimilars, the regulation of these products is progressing in the United States. In Europe, the approval pathway for biosimilars was set forth in 2004 and nearly 25 biosimilar products are currently marketed there. In September 2013, the first biosimilar monoclonal antibody products, sold under the brand names Remsima and Inflectra, were approved for commercial sale in Europe. These biosimilar versions of the blockbuster monoclonal antibody product Remicade were the first of many biosimilar monoclonal antibodies that will undoubtedly be approved for commercial sale in Europe in the coming years. To this point, in 2016 Europe also approved the first Fcfusion protein biosimilar, Benepali, a biosimilar to Enbrel.

In the US, the 2010 Biologics Price Competition and Innovation Act (BPCIA), a critical component of the Affordable Care Act (ACA), authorized the FDA to develop a new regulatory framework for approving biosimilars and a trio of guidance documents were released in draft form in February of 201248. Three years later, in 2015, the guidances were finalized⁴⁹ and Zarixo, a filgrastim product, was the first biosimilar approved by the FDA⁵⁰. Since then, four additional biosimilars have been approved, three of which are monoclonal antibody products. As patent expiry on key monoclonal antibody products begins to occur, we expect an increase in the number of biosimilar monoclonal antibody products in development and ultimately an increase in the number of biosimilar approvals.

Although the current impact of these and other biosimilar monoclonal antibody products in US and European biopharmaceutical marketplace cannot be gauged at this early stage, we anticipate modest acceleration of the sales growth of all monoclonal antibodies as these biosimilar products gain market acceptance. In parallel with the development of biosimilar monoclonal antibodies in the US and Europe, there is a surging interest in biosimilar monoclonal antibodies in the developing markets of Latin America, China, Southeast Asia, India, and Russia. The introduction of biosimilars in these markets is likely to have a very large impact on the growth in sales of monoclonal antibody products as biosimilar monoclonal antibodies are approved in geographies that are currently unable to access expensive innovator products.

6. Microbial Expression Technologies for Monoclonal Antibody Production

The development of alternate expression systems and hosts for antibody production, especially microbial systems such as E. coli and yeast, represent significant technological advances that broaden the available technologies for manufacturing monoclonal antibody products, especially antibody fragments and those products that may not require glycosylation for activity. Two currently approved monoclonal antibody products, Lucentis and Cimzia, are produced by microbial fermentation with many more in development. Additionally, technologies like Alder Biopharmaceutical's technology for expression of full length antibodies in yeast,⁵¹ Ablynx's eukaryotic expression of antibody fragments (Nanobodies[®]),⁵² and recently acquired by Cell Medica,⁵³ Delenex Therapeutics whose PENTRA®bodies consist of single chain antibody fragments expressed in *E. coli*⁵⁴ demonstrate the potential for microbial expression technologies to contribute to the growth of new monoclonal antibodies in the future.55,56

While the original drivers for development of these newer expression technologies may have been production of monoclonal antibody-based products or lower manufacturing costs, these factors are not necessarily the drivers for implementation and adoption of these technologies. Rather, other drivers such as intellectual property or utilization of existing fermentation capacity may be more important than cost of manufacturing for the adoption of these new technologies in the future.

7. Summary

As the development and commercialization of monoclonal antibody products continues with no limit in sight, lessons learned from the early monoclonal antibody products along with the use of advanced and novel technologies for their production and the increasing familiarity of global regulatory authorities with therapeutic monoclonal antibody products will contribute to their continued dominance as the major class of biopharmaceutical products worldwide. The increasing adoption and use of technology platforms for the discovery, development, and manufacturing of monoclonal antibody products and advances in high throughput automation have enabled the development of high yielding, reliable processes.^{40, 41, 42, 43, 44} Similarly, continued advances in analytical methods for characterizing biopharmaceutical products and processes, including the development and implementation of process analytical technologies (PAT) for online monitoring and control, will provide better and more sophisticated tools to enhance and facilitate process qualification and continuous process verification.

This report is intended to provide a general roadmap for the development of monoclonal antibody products from initial discovery through the filing of an IND or Investigational Medicinal Product Dossier (IMPD) or equivalent for first in human clinical trials. The primary focus is on those specific issues related to process development, manufacturing, quality control, and analysis of full length single specificity antibody products produced in mammalian cell culture although much of the discussion is relevant to the production of antibodyrelated products such as Fcfusion proteins and antibody-drug conjugates as well as to antibody products produced in non-mammalian hosts. Where appropriate, the report also discusses activities and issues, such as comparability, process validation, large scale manufacturing, which become more important in the later stages of development and commercialization of monoclonal antibody products. Not included in this report are discussions on target identification, selection of appropriate disease indications or antibody formats, nor designing preclinical or clinical programs.

Each chapter in this report describes the required or recommended activities needed for the development of a monoclonal antibody product along with potential risks, alternative approaches, and high level discussions regarding timing and costs for these activities. Wherever possible, appropriate references have been provided to assist the reader in learning more about specific topics. However, it is important to note that the science and technology of monoclonal antibody development and manufacturing is advancing rapidly and the regulatory landscape for these products continues to evolve. As a result, many of the references included in this report are derived from conference proceedings where the newest, cuttingedge technologies are initially presented. Copies of these conference presentations are often available through the conference organizer or the author. Also, while we have made an attempt to include references to the most current regulatory guidelines and regulations regarding monoclonal antibody development, the reader should check with the regulatory agencies to ensure that a more current version of the guidance documents or regulations has not been issued since the publication of this report. Due to the rapidly advancing science and regulatory policies worldwide and the continued harmonization of regulations through the ICH, specific regulatory documents referenced in this report may have been superseded.

References

- 1 Novartis AG [Internet] Basel (CH): Novartis AG; c2016. Novartis acquires all remaining rights to GSK's ofatumumab to develop treatments for MS and other autoimmune indications; 2015 Aug 21 [cited 2016 Oct 14]; [about 7 screens]. Available from: https://www.novartis.com/news/media-releases/novartis-acquires-all-remaining-rights-gsks-ofatumumab-develop-treatments-ms-and.
- 2 Bristol-Myers Squibb [Internet]. New York City (NY): Bristol-Myers Squibb Company; c2016. Lilly, Bristol-Myers Squibb Restructure Erbitux® (cetuximab) Collaboration in North America; 2016 Apr 16 [cited 2016 Oct 14]; [about 12 screens]. Available from: http://bms.newshq. businesswire.com/press-release/rd-news/lilly-bristol-myers-squibb-restructure-erbitux-cetuximab-collaboration-north-a.
- 3 Amgen [Internet]. Thousand Oaks (CA): Amgen Inc.; c2016. Amgen Reacquires All Product Rights To Prolia® (denosumab), XGEVA® (denosumab) And Vectibix® (panitumumab) From GSK In 48 Countries; 2015 Dec 14 [cited 2016 Oct 14]; [about 22 screens]. Available from: http://wwwext. amgen.com/media/news-releases/2015/12/amgen-reacquires-all-product-rights-to-prolia-denosumab-xgeva-denosumab-and-vectibixpanitumumab-from-gsk-in-48-countries/
- 4 Derbyshire M. Patent expiry dates for best-selling biologicals. GaBI J. 2015 Apr; 4(4):178-179.
- 5 Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975 Aug;256(5517):495-7.
- 6 Porter RR. The hydrolysis of rabbit gamma globulin and antibodies with crystalline papain. Biochem J. 1959 Sep;73:119-26.
- 7 Reichert JM. Monoclonal antibodies as innovative therapeutics. Curr Pharm Biotechnol. 2008 Dec;9(6):423-30.
- 8 Zhang W, Czupryn MJ. Free sulfhydryl in recombinant monoclonal antibodies. Biotechnol Prog. 2002 May-Jun;18(3):509-13.
- **9** De Pascalis R, et al. Grafting of "abbreviated" complementarity-determining regions containing specificity-determining residues essential for ligand contact to engineer a less immunogenic humanized monoclonal antibody. J Immunol. 2002 Sep;169(6):3076-84.
- 10 Zhou Y, et al. Impact of single-chain Fv antibody fragment affinity on nanoparticle targeting of epidermal growth factor receptor-expressing tumor cells. J Mol Biol. 2007 Aug;371(4):934-47.
- 11 Hoogenboom HR. Selecting and screening recombinant antibody libraries. Nat Biotechnol. 2005 Sep;23(9):1105-16.
- 12 Carter P. Bispecific human IgG by design. J Immunol Methods. 2001 Feb;248(1-2):7-15.
- 13 Huston JS, et al. Antigen recognition and targeted delivery by the single-chain Fv. Cell Biophys. 1993 Jan-Jun;22(1-3):189-224.
- 14 Huston JS, et al. Medical applications of single-chain antibodies. Int Rev Immunol. 1993;10(2-3):195-217.
- 15 Kubota T, et al. Engineered therapeutic antibodies with improved effector functions. Cancer Sci. 2009 Sep;100(9):1566-72.
- **16** Wang F, et al. Structural and functional characterization of glycosylation in an immunoglobulin G1 to Cryptococcus neoformans glucuronoxylomannan. Mol Immunol. 2006 Mar;43(7):987-98.
- 17 Raju TS. Terminal sugars of Fc glycans influence antibody effector functions of IgGs. Curr Opin Immunol. 2008 Aug;20(4):471-8.
- 18 Glycoforum [Internet]. Japan: Glycoforum; c2009. Fig.1 General structure of N -linked oligosaccharides in Fc region of human IgG in Enhancement of ADCC of antibodies by glycoengineering; 2006 Sep 26 [cited 2016 Oct 14]; [about 1 screen]. Available from http://www.glycoforum.gr.jp/science/word/immunity/IS-A06E.html.
- 19 Clark M. Mike's Immunoglobulin Structure/Function Home Page [Internet]. [updated 2015 Nov 6; cited 2016 Nov 21]. Available from http://archive. li/NHOoJ
- 20 Shinkawa T, et al. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human lgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem. 2003 Jan 31;278(5):3466-73.
- 21 Shields RL, et al. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J Biol Chem. 2002 Jul 26;277(30):26733-40.
- 22 Doig AR, et al. Monoclonal antibodies targets and indications. Amer Pharma Rev. 2015 Jul/Aug;18(5):72-75.
- 23 Bioprocess Technology Consultants [Internet]. Woburn (MA): Bioprocess Technology Consultants, Inc. bioTRAK® database; 2016 [cited 2016 September 14]; [about 2 screens]. Available from: http://www.bptc.com/pipeline-databases.php.
- 24 Drugs@FDA [Internet]. Silver Spring (MD): U.S. Food and Drug Administration, Center for Drug Evaluation and Research. 1939- [cited 2016 September 14]. Available from: http://www.accessdata.fda.gov/scripts/cder/daf12.
- 25 European public assessment reports, human medicines [Internet]. London: The European Medicines Agency. 1995- [cited 2016 September 14]. Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/landing/epar_search.jsp&mid=WC0b01ac058001d124.
- 26 Ecker DM, et al. The state of mammalian cell culture biomanufacturing. Woburn (MA): BioProcess Technology Consultants, Inc.; 2011 Dec 12. 150 p. Available from: http://www.bptc.com/ECommerce/Reports.
- 27 DiMasi JA, et al. Trends in risks associated with new drug development: success rates for investigational drugs. Clin Pharmacol Ther. 2010 Mar;87(3):272-7.
- 28 Pavlou AK, Reichert JM. Recombinant protein therapeutics-success rates, market trends and values to 2010. Nat Biotechnol. 2004 Dec; 22(12):1513-9.
- 29 Greb E, Drakulich A. Platform technologies. Pharm Technol. 2012 Mar;36(3):46-52.
- 30 Rameez S, et al. High-throughput miniaturized bioreactors for cell culture process development: reproducibility, scalability, and control. Biotechnol Prog. 2014 May-Jun;30(3):718-27.
- 31 Bhambure R, et al. High throughput process development for biopharmaceutical drug substances. Trends Biotech. 2011 Mar;29(3):127-35.
- **32** Chollangi S, et al. Accelerating purification process development of an early phase MAb with high-throughput automation. Part 1. Bioprocess Int. 2014 Mar;12(3):48-52.
- **33** Chollangi S, et al. Accelerating purification process development of an early phase MAb with high-throughput automation. Part 2. Bioprocess Int. 2014 Apr;12(4):32-41.

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- 34 BCC Research. Antibody Drugs: Technologies and Global Markets. Wellesley (MA): BCC Research, LLC; 2015 Jan. 141 p. Available from: http://www. bccresearch.com/market-research/biotechnology/antibody-drugs-market-bio016j.html
- 35 Bilello JA. The agony and ecstasy of "OMIC" technologies in drug development. Curr Mol Med. 2005 Feb;5(1):39-52.
- **36** Shukla AA, et al. Downstream processing of monoclonal antibodies--application of platform approaches. J Chromatogr B Analyt Technol Biomed Life Sci. 2007 Mar;848(1):28-39.
- 37 Suntharalingam G, et al. Cytokine storm in a phase I trial of the anti-CD28 monoclonal antibody TGN1412. N Engl J Med. 2006 Sep;355(10):1018-28.
- 38 Kuehn BM. Rare neurological condition linked to newer monoclonal antibody biologics. JAMA. 2009 Apr;301(14):1423-4.
- **39** United Nations Development Programme. Human development report 2015: Work for Human Development [Internet]. NY (NY): United Nations Development Programme; c2015 [cited 2016 September 14]. 288 p. Available from: http://hdr.undp.org/sites/default/files/2015_human_development_report_1.pdf.
- 40 Wurm FM. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol. 2004 Nov;22(11):1393-8.
- 41 Charlebois TS. Achieving 10+ grams/liter: challenges in process development. Presented at IBC Life Sciences' BioProcess International Conference and Exhibition. 2008 Sep 26; Anaheim, CA.
- 42 Ryll T. How do we get to 30g/L using fed-batch technology? What are the limitations? Presented at IBC Life Sciences' 22nd International Antibody Development and Production Conference; 2009 Mar 4-6; Carlsbad, CA.
- 43 Chen J. High-titer fed-batch cell culture process development for CHO cell lines. Presented at American Chemical Society National Meeting; 2010 Mar 20-24; San Francisco, CA.
- 44 Lu F, et al. Automated dynamic fed-batch process and media optimization for high productivity cell culture process development. Biotechnol Bioeng. 2013 Jan;110(1):191-205.
- 45 Yang WC, et al. Perfusion seed cultures improve biopharmaceutical fed-batch production capacity and product quality. Biotechnol Prog. 2014 May-Jun;30(3):616-25.
- **46** Povey JF, et al. Rapid high-throughput characterization, classification and selection of recombinant mammalian cell line phenotypes using intact cell MALDI-ToF mass spectrometry fingerprinting and PLS-DA modelling. J Biotechnol. 2014 Aug 20; 20(184):84-93.
- 47 Liu B, et al. The availability of glucose to CHO cells affects the intracellular lipid-linked oligosaccharide distribution, site occupancy and the N-glycosylation profile of a monoclonal antibody. J.Biotechnol. 2014 Jan 20;(170):17-27.
- 48 RAPS: Regulatory Affairs Professionals Society [Internet]. Rockville (MD): RAPS: Regulatory Affairs Professionals Society; c2016. Gaffney A: FDA Releases Long-Awaited Guidances on Biosimilar Price Competition and Innovation Act; 2012 Feb 9 [cited 2016 Oct 14]; [about 5 screens]. Available from: http://www.raps.org/focus-online/news/news-article-view/article/874/fda-releases-long-awaited-guidances-on-biosimilar-price-competition-and-innovat.aspx
- 49 RAPS: Regulatory Affairs Professionals Society [Internet]. Rockville (MD): RAPS: Regulatory Affairs Professionals Society; c2016. Gaffney A: After Three-Year Delay, FDA Finalizes Guidance Documents on Biosimilarity; 2015 Apr 28 [cited 2016 Oct 14]; [about 7 screens]. Available from: http://

www.raps.org/Regulatory-Focus/News/2015/04/28/22058/After-Three-Year-Delay-FDA-Finalizes-Guidance-Documents-on-Biosimilarity/

- 50 FDA: U.S. Food & Drug Administration [Internet]. Washington DC: U.S. Department of Health & Human Services. FDA approves first biosimilar product Zarxio; 2015 Mar 6 [cited 2016 Oct 14]; [about 5 screens]. Available from: http://www.fda.gov/newsevents/newsroom/ pressannouncements/ucm436648.htm.
- 51 Alder Biopharmaceuticals [Internet]. Bothell (WA): Alder Biopharmaceuticals, Inc; c2014. Antibody production: Alder's MabXpress Antibody Production System; c2016 [cited 2016 Oct 14]; [about 4 screens]. Available from: http://www.alderbio.com/technologies/antibody-production/.
- 52 Ablynx [Internet]. Ghent (BE): Ablynx NV; c2015-2016. Nanobodies competitive features; c2015-2016 [cited 2016 Oct 14]; [about 7 screens]. Available from: http://www.ablynx.com/technology-innovation/nanobodies-competitive-features/.
- 53 Cell Medica [Internet]. London (GB): Cell Medica; c2008-2016. Cell Medica acquired Delenex Therapuetics; 2016 Jul 12 [cited 2016 Oct 14], [about 4 screens]. Available from: https://cellmedica.co.uk/press-releases/cell-medica-announces-acquisition-delenex-therapeutics/
- 54 deals.bio: Available Pharmaceutical M&A and Licensing Opportunities for Life Science Professionals [Internet]. New York (NY): one.bio, LLC; c2016. 2015-June-HomepageSlides.pdf: Delenex Therapeutics: The PENTRA®Body Company; 2015 Jun [cited 2016 Nov 17]; 29 p. Available from http://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=0ahUKEwjW-7fuirDQAhVI54MKH YPkDW8QFggbMAA&url=http%3A%2F%2Fdeals.bio%2Fhome%2Fdownload%3Fdoc_id%3D1777&usg=AFQjCNHxWtV0CS1WrP66FoV-DNh2iJ9K2Q&bvm=bv.139138859,d.eWE.
- 55 Andersen DC, Reilly DE. Production technologies for monoclonal antibodies and their fragments. Curr Opin Biotechnol. 2004 Oct;15(5):456-56
- 56 Spadiut 0, et al. Microbials for the production of monoclonal antibodies and antibody fragments. Trends Biotechnol. 2014 Jan;32(1):54-60.



CHAPTER 2:

Overview of Chemistry, Manufacturing, and Control Activities for Monoclonal Antibody Product Development

hen a decision is made to pursue clinical development of a therapeutic monoclonal antibody product, a complex, multi-faceted development program must be set in motion, including the CMC activities required to develop a reliable manufacturing process and associated quality control test methods. These activities include the following major areas, each covered in detail later in this report:

Analytical Method Development	Chapter 4
Upstream Process Development	Chapter 5
Scale-up and Bulk Drug Substance Manufacturing	Chapter 6
Downstream Process Development	Chapter 7
Formulation Development	Chapter 8
Drug Product Manufacturing (Fill/Finish)	Chapter 9

The overall goal of a monoclonal antibody CMC development program is to develop a reliable, robust, and reproducible process to manufacture a product that is safe to administer to humans. As stated in ICH Q8(R2), Pharmaceutical Development, "The aim of pharmaceutical development is to design a quality product and a manufacturing process to consistently deliver the intended performance of the product. The information and knowledge gained from pharmaceutical development studies and manufacturing experience provide scientific understanding to support the establishing of the design space, specifications,

and manufacturing controls."1 Achieving such a process generally occurs in stages as the product meets certain clinical or regulatory milestones. The process, which begins at small-scale due to the relatively low demand for product, should be scalable and in compliance with all relevant regulatory guidelines.^{1, 2}As development of the monoclonal antibody product proceeds from pre-clinical testing through human clinical trials and ultimately to commercialization, the regulatory requirements increase in scope and depth. In conjunction with the development of this manufacturing process, suitable analytical methods should be developed that permit the full characterization of the monoclonal antibody product and provide appropriate in-process controls and quality control release specifications for the product. While the cost of manufacturing a monoclonal antibody product for first-in-human clinical trials is generally not overly important at this early stage of product development, the manufacturing process should also be economically viable to

produce early stage clinical trial materials. High titer platform processes – i.e., "generic" processes used again and again with minimal modification between development projects – can greatly enable the early stage evaluation of multiple potential antibody candidates for a given indication.

The development of a successful therapeutic monoclonal antibody product can potentially be enhanced using a Quality by Design approach, and using high throughput methods. The principles of QbD, which are discussed in detail in Chapter 3, are embodied in the ICH guidelines Q8(R2) Pharmaceutical Development, Q9 Quality Risk Management, Q10 Pharmaceutical Quality System, and Q11 Development and Manufacture of Drug Substance. These guidelines, along with FDA's 2011 Guidance on Process Validation, establish a framework that aligns closely with CMC activities.

Proper documentation of all activities carried out during process and analytical development is critical to the success of the overall development program. All development activities performed in support of the monoclonal antibody manufacturing process, analytical test methods to characterize and release the product, and pre-formulation and formulation work, should be fully described in formal technical reports that follow Good Documentation Practices, since they may ultimately be included in or used to prepare regulatory submissions. This documentation will enable regulatory agencies and others to assess the full range of parameters that were evaluated and will be essential in supporting regulatory filings, scale-up, and potential transfer of the process to a large-scale manufacturing facility, whether it be in-house or to a contract manufacturer or partner.

In addition to the technical and regulatory aspects of monoclonal antibody product development, there are also strategic considerations regarding the timing of certain development activities and the degree of regulatory compliance applied to the development process. As discussed below, the CMC development of a monoclonal antibody product from clinical candidate nomination to first in human (FIH) clinical trials generally requires approximately eighteen months and nearly \$7 million. Many companies will move to this initial FIH benchmark with a less robust "INDenabling process;" in fact, such an approach is acceptable and even acknowledged by the regulatory agencies.³ Nevertheless, some companies choose to develop a more mature process meeting later stage regulatory compliance requirements even at this early stage. This strategic approach may require more time and investment to accomplish. Regardless of which strategic approach a company adopts, rigorous project management is required to coordinate the wide range of complex CMC activities necessary for successful development of a monoclonal antibody product.

1. Analytical Method Development

Selective, sensitive, and reproducible testing methods are critical tools used throughout the entire program, from initial process development and characterization through product release to the marketplace.4,5 At the initiation of the development program, some basic analytical methods must be in place to guide cell line selection and purification optimization so that the right product is produced and purified. As the program progresses, additional analytical methods must be developed to support process development, quality control testing and characterization of the bulk drug substance, to aid in demonstrating comparability of material produced as a result of future process scale-up, optimization, or improvement, and to map the clearance of process impurities through the process as well as to demonstrate stability of the product. Each assay must be developed and optimized for the particular application. Some methods are generic and can be applied to new products with very little effort, but other methods require considerable development and optimization work before they can be used for the particular product or process.

Analytical methods generally must be qualified for bulk drug substance (BDS) release testing prior to production and testing of clinical trial material to demonstrate that the analytical methods have the required specificity, sensitivity, accuracy, linearity, precision, and suitability for each particular application as dictated by ICH Q2(R1) entitled; Validation of Analytical Procedures: Text and Methodology.⁶ Later in development, and prior to process validation activities, assays used for inprocess and release testing of bulk drug substance and drug product must also be validated to additionally demonstrate reproducibility of the assay over time, and to confirm that the assay can be reliably and reproducibly performed by different operators, in different laboratories.7 Analytical Method Validation can be viewed as a process, akin to the manufacturing process, which allows it to employ the model laid out for Process Validation in FDA's 2011 guidance.8 To support the development of suitable analytical methods for the testing and characterization of a monoclonal antibody product, it is also critical to produce, characterize, and store sufficient quantities of an appropriate reference standard to ensure that all batches of the product manufactured throughout development can be analyzed using a standard that meets all release specifications and whose quality attributes are those that are intended to be in the clinical product. At the start of the development program, a laboratory reference standard is normally obtained from the discovery laboratory. This standard is used to support the initial development activities and may be used for the testing and release of initial manufacturing batches for preclinical safety studies and Phase 1 human clinical trials. The laboratory reference standard should be replaced as soon as possible by a fully qualified reference standard produced using the manufacturing process intended to produce Phase 1 clinical trial material. This standard is often obtained from the initial batch produced after scale-up of the manufacturing process to an appropriate scale for production of clinical supplies. This reference standard should be fully characterized and produced in sufficient quantity to support the product development at least through early stage human clinical trials if not beyond.^{9, 10} The reference standard should be stored under suitable conditions to maintain its integrity and should also be divided into small volumes for storage so that a single vial or tube is only used once for testing. For most monoclonal antibody products, the reference standard is stored frozen at -70oC or below.

To support process development, analytical methods are required to measure the concentration and activity of the monoclonal antibody product as well as the concentration of impurities and potential contaminants that must be removed by the process. These impurities include both productrelated impurities such as aggregates or fragments of the antibody molecule as well as process-related impurities such as host cell proteins or cell culture media components.

Analytical methods used to support product characterization include methods used for the detailed analysis of the physical/chemical structure of the monoclonal antibody product, such as determining the extent of glycosylation, specific carbohydrate sequences and sites of attachment, identification of product related species, i.e., active species, and product related impurities, i.e., inactive

species, as well as bioassays for the activity of the product in all modes of action in the intended indications. These characterization methods are generally not used during routine process development but are used at critical points in the development program, such as characterization of the product to support the initial regulatory filing or after any major process changes. While full characterization of a monoclonal antibody product is not necessary to support an initial regulatory filing such as an IND application or IMPD; it is required to support regulatory filings for product approval. In addition, characterization of monoclonal antibody products produced during these early stages of development can serve as a baseline for assessing comparability of the early clinical product with product manufactured later in development after additional process optimization, changes in production scale, changes in production cell line, or other significant process changes.

Before manufacturing of a monoclonal antibody product begins, an appropriate subset of the analytical methods developed and used during process development must be qualified for use in raw material and in-process testing, lot release and stability testing of the bulk drug substance and final drug product. Additional analytical methods may need to be developed after initiation of production of clinical material to support in-process or release testing or characterization, but this additional method development occurs in response to the identification of a specific impurity or contaminant, which must be controlled during the manufacturing process.

As early as possible in the development of a monoclonal antibody product, the stability of both the bulk drug substance and final drug product under defined storage conditions should be demonstrated. The primary routes of degradation should be determined using forced degradation, and analytical methods should be developed so that the expected degradation products are readily detected and measured over time. Additionally, stability following repeated freeze/thaw cycles or short-term storage at elevated temperatures should be demonstrated if the product or intermediates are to be stored frozen or exposed to elevated temperatures. Lastly, the compatibility of the monoclonal antibody product with any diluent or administration set to be used in a human clinical trial setting should be demonstrated. The formal stability programs required for monoclonal antibody products (bulk drug substance and final drug product) are defined in ICH Q5C,¹¹ which outlines the different stability studies required in support of marketing applications for biotechnological/biological products. ICH guidance for all product types can be found in Q1A(R2).¹²

2. Upstream Process Development

A critical aspect of the development of any monoclonal antibody product is the development of a suitable genetically modified cell line capable of producing the product at a sufficiently high titer to meet clinical and eventually commercial demands. Many technologies are available to improve the speed of cell line development or the specific productivity levels that can be achieved¹³ including the application of mini- and micro-bioreactors as detailed in Chapter 5.

In addition to the production cell line, a suitable cell culture process that enables production of sufficient quantities of the monoclonal antibody product in an appropriate sized bioreactor must be developed. Upstream process development encompasses all of the activities related to the development of the DNA expression vector, the production cell line and the cell culture process for production of the monoclonal antibody product.

Expression Vectors and Cell Lines for Monoclonal Antibody Production

The expression vector for a monoclonal antibody product is the DNA construct, which is inserted into a suitable host cell line to enable the production of the monoclonal antibody of interest. The process development program itself begins with the design, construction, production, and sequencing of expression vectors, which include the coding sequence for the antibody as well as selectable markers and various control sequences designed to enable high expression and secretion of the product by the cells at the appropriate point in the cell growth cycle.

The first monoclonal antibody products that entered the market were produced in several different mammalian host cell lines including the original hybridoma or the murine myeloma cell lines SP/0 and NS0.¹⁴ However, as more monoclonal antibody products were developed, the biopharmaceutical industry focused its efforts on the use of various CHO cell lines for production of these products.¹⁵

As a result, CHO cells are the dominant host cell line in use today for the production of monoclonal antibody products. CHO cell lines suitable for use in production of human therapeutic products can be obtained from many commercial suppliers, and most contract manufacturing organizations offer their own parental CHO cell lines to assist their clients. In addition to CHO, some alternative cell lines such as the Crucell (now J&J/Janssen) PER.C6 line can be used to develop a monoclonal antibody production cell line.¹⁶

To generate the production cell line, the host cells are transfected with the expression vector and cell clones are screened to find antibody producing cells. The goal of this transfection and screening process is to select a cell line that grows well and is genetically stable in culture, and that produces high levels of the product in its active form (properly assembled, folded, glycosylated and not fragmented or aggregated). Once a cell line is established, it must be fully characterized and banked for use in long-term production.¹⁷

Cell Banks

For manufacturing purposes, regulatory agencies require the preparation of well controlled and characterized cell banks. A two-tiered cell banking system, consisting of a Master Cell Bank (MCB) and a Working Cell Bank (WCB), is commonly used to assure an adequate supply of equivalent cells tested to be free of adventitious agents for use over the entire life span of the product. These Master and Working Cell Banks also ensure consistent and reproducible production of comparable product each time a batch of product is manufactured. These cell banks must be produced in full compliance with current Good Manufacturing Practices (cGMP) and should be fully characterized.¹⁷ Some companies will delay production of a WCB for their product until clinical efficacy has been demonstrated. This is acceptable under current regulation, but it is strongly recommended that a WCB be produced relatively early in development so that the MCB can be preserved and can be used to support late stage and commercial production through generation of additional WCBs.

Cell Culture Process

After the production cell line is established, a process for growing the cells and producing the monoclonal antibody product at the desired scale must be developed. Several different approaches can be developed for expanding the cells from the initial vials up to a sufficient biomass to inoculate the bioreactor, growing the cells to maximum density, and viability, and supporting maximum productivity. As discussed in detail in Chapter 6, upstream process development includes the systematic optimization of the growth and production medium, as well as the bioreactor conditions over the course of the culture process to produce the maximum amount of active product of desired quality with the minimum amount of resource consumption (bioreactor volume, media, and time). During process development, laboratory studies on a small scale are used to evaluate different cell culture conditions. As the process is scaled up, additional process development may be required to solve problems that arise or to make process improvements.

3. Downstream Process Development

Following cell culture, the monoclonal antibody produced by the production cell line in a suitable bioreactor must be separated from the cells, purified to remove impurities and potential contaminants to an acceptable level, formulated at the correct concentration with the proper excipients, and placed in storage in preparation for final formulation and drug product manufacturing. The downstream processing operations necessary to purify and formulate the bulk drug substance typically includes a step to remove the cells and cell debris, a capture step to selectively concentrate the monoclonal antibody product and remove a majority of the impurities, one or more intermediate and polishing purification steps to remove the remaining impurities, and steps to inactivate or remove endogenous or potential adventitious viruses. The purified monoclonal antibody is then normally concentrated and exchanged into an appropriate buffer for storage and/or formulation to produce the bulk drug substance. To develop a downstream process, small-scale studies are used to evaluate the performance of different purification options, to optimize the conditions of a selected purification approach, and to integrate the different steps of the process. Separate studies are also required prior to initiating human clinical trials that demonstrate the ability of the process to remove and/or inactivate endogenous or potential adventitious viruses to acceptable levels.¹⁸

4. Formulation Development

The bulk drug substance is formulated to ensure that the proper dose of active antibody reaches the proper site of action in the body upon administration to the patient and that the drug product is stable during storage. Formulation development should be initiated as soon as product is available, even product from process development activities, which may not be fully representative of the final process. To select an optimized formulation, the monoclonal antibody is prepared in a number of different formulations and subjected to accelerated storage conditions. Analytical methods that are stability-indicating are used to determine which formulations are best for maintaining antibody structure and function. While formulation development is complex and highly specific for any given monoclonal antibody product and its intended indication, there are many similarities among monoclonal antibody product formulations. This enables the formulation development process to be focused on those formulations that are already known to support monoclonal antibody stability and activity.¹⁹ Chapter 8 discusses formulation development in more detail, including high throughput methods for formulation development.

5. Scale-up and Bulk Drug Substance Manufacturing

The material requirements for a monoclonal antibody bulk drug substance, as well as the quality of this material, will vary throughout the development of a monoclonal antibody product. During the initial discovery and lead optimization phases, the quantities required are generally small (<1 g) and production of this material is not subject to any regulations. Such quantities can easily be produced using conventional laboratory-scale equipment. A myriad of cross-departmental activities begin once a company identifies a particular monoclonal antibody for pre-clinical development. These activities, ranging from upstream and downstream process development, analytical, formulation development to pre-clinical animal toxicology, pharmacokinetic and pharmacodynamic studies; all require increased quantities of the monoclonal antibody product. The quantities required are orders of magnitude greater than during the discovery phase with several hundred grams of product often required for large animal studies, high concentration formulation development, and other activities. As most of this material is produced for development purposes, there are little to no regulatory requirements for the material, other than for drug substance produced for IND-enabling

GLP toxicology studies. For these studies it is important that the manufacturing process be well documented, that the product be adequately characterized, and that the material be representative of that to be used in the proposed or anticipated FIH studies.²⁰

Companies developing monoclonal antibodies are often working against aggressive timelines to FIH clinical trials, and one of the major gating activities is IND enabling GLP toxicology testing. Performing and properly documenting the toxicology testing results can require up to six months. Regulatory guidance specifies that toxicology assessment be performed using product that is manufactured using the same cell line and essentially the same manufacturing process that will be used to produce clinical trial material (CTM). There is no requirement that the antibody used in GLP toxicology be produced at the same scale intended for production of human clinical material, and the material does not need to be produced under cGMP conditions. Companies may use material from the first batch produced in the cGMP facility, which is typically termed an "engineering" batch, but is generally not produced under cGMP conditions. However, the engineering batch is produced shortly before the cGMP clinical batch, and therefore the CTM would be manufactured and available prior to completion of the GLP toxicology testing. To accelerate the time to produce GLP toxicology material, an alternative strategy, that has been adopted by many companies, is to use material produced in the process development laboratory for GLP toxicology testing.

In most cases, process development is completed with the performance of one or more end-to-end runs of the process in the development laboratory. These runs are referred to as "development" or "consolidation" runs and are typically run at scales ranging from 5 L to 200 L, using the same raw materials that are used in cGMP production. Data from the development runs supports generation of batch records that will be used to transfer the process to the cGMP manufacturing site. While the material resulting from the development runs is from the final cell line and process, it is generally not at the intended CTM production scale and is not produced under the highlycontrolled conditions found in the cGMP facility. However, if the development runs are well controlled to limit endotoxin and bioburden and to ensure similar process performance to the final at-scale process, this material is acceptable for GLP toxicology studies.

The risks of using material from the development lab are primarily related to the requirement to demonstrate comparability of material used in toxicology testing to the CTM. Comparability is a well-defined regulatory requirement (see Chapter 10). If any process changes are implemented following the development runs and prior to CTM production at full scale, regulatory authorities will be more concerned about comparability. Further, if the impurity profiles of the GLP toxicology material is significantly lower than the clinical trial material, or if there are significant differences in any critical quality attributes (glycosylation, deamidation, aggregation, etc.) that may impact a claim of comparability. This risk can be mitigated by doing limited animal testing of the CTM, including PK and acute toxicity, which do not require as much time to complete and can provide supporting data in the IND filing. However, since monoclonal antibodies are a wellunderstood class of biopharmaceuticals and the initial manufacturing processes are most often platform processes, the risk that there will be significant changes between the development and the cGMP runs are minimal.

Manufacture of Clinical Trial Material

While GLP toxicity studies are underway and before the filing of an IND or IMPD, cGMP clinical trial material is manufactured for use in FIH Phase 1 clinical studies to assess the safety of the product in humans. The quantities of drug substance required for these studies are usually not much greater than that required for pre-clinical and process development activities. Consequently, the increase in manufacturing scale may be modest, especially for high titer processes. Prior to initiation of the first cGMP clinical manufacturing run, one or more engineering runs are typically performed in the same equipment that will be used to manufacture the clinical lots to test out the process, procedures, and batch records that will be used for cGMP manufacturing. These engineering runs typically supply drug substance for supporting stability studies and inprocess streams for initial process validation studies such as virus clearance studies. Although the manufacturing scale may be similar or slightly greater to manufacture antibody drug substance for Phase 1 clinical studies, the regulatory requirements are substantially increased due to the general requirement to comply with cGMP. It should be noted that in 2008 the FDA announced in the Federal Register a Final Rule amending the cGMP regulations to exempt most Phase 1

investigational drugs from complying with these regulations and issued a guidance document to clarify expectations for GMPs for Phase 1 clinical supplies.³ By contrast, the European regulations do not have this exemption for Phase 1 materials. Any investigational medicinal product to be tested in human clinical trials in Europe must be made in compliance with cGMP regulations.²¹

Despite the guidance from FDA relaxing the regulatory requirements for cGMP compliance, in practice most companies adhere to full compliance, or close to it, even for the manufacture of monoclonal antibodies even for early stage clinical trials. Since many monoclonal antibody products are manufactured for clinical trials in the United States and Europe, where full cGMP compliance is required even for Phase 1 manufacturing, relaxing cGMP compliance may lead to difficulties with the European regulatory authorities. In addition, as described in the FDA guidance, the cGMP requirements are largely unchanged for products requiring sterile processing. As most monoclonal antibody products are provided as sterile products for parenteral administration, drug product manufacturing for these products must still comply with virtually all of the provisions in the FDA guidance on aseptic processing for sterile products.²² Moreover, since the manufacturing environment used for the production of a monoclonal antibody drug substance is highly susceptible to contamination and propagation of microbial agents, bioburden control is essential, thereby requiring the strict procedural and quality controls required by the cGMP regulations. In addition, the host cells expressing the monoclonal antibody as well as some of the raw materials used to cultivate these cells are susceptible to viral contamination. This merits additional consideration in the FDA guidance document, especially for multi-product facilities, to ensure the safety of patients from potential viral contamination. Consequently, cGMP requirements for the demonstration and documentation of the ability of the cleaning protocols to prevent crosscontamination and the demonstration of the process to inactivate or remove viruses are largely intact.

For companies manufacturing their own products only for Phase 1 trials in the United States, there are some concessions in the FDA guidance that reduce the potential investment required for manufacturing. These include less stringent requirements for equipment and assay qualification and validation, the recognition that raw material and product specifications, while required, may not be as comprehensively defined at this phase of development as they will be at a later point, some flexibility in documentation, and the ability to use the same personnel for some manufacturing and quality functions.

As production proceeds through the various clinical phases, the process may be scaled up further to meet increasing product requirements depending on the required patient dosing regimen and clinical trial size. For relatively low dose antibody drug conjugates developed for oncology indications, only a few hundred grams may be required for pivotal clinical trials, whereas a high multi-dose treatment regimen for a chronic, non-life threatening indication with existing therapies may require up to 50 kilograms of drug substance. Changes in scale during clinical development require careful engineering and planning to ensure consistent product quality between the scales. In many cases, the process is transferred from one facility (or even organization) to another over the course of clinical production, and this process transfer itself entails a significant time and resource expenditure. Often process improvements are introduced between the end of Phase 2 clinical trials and the initiation of Phase 3 or pivotal clinical trials, especially if a process scale-up or a change of manufacturing site is required to supply the increased quantity of material required for the pivotal clinical trials. Analytical comparability between products manufactured at different scales, different manufacturing sites or with different processes must be executed and submitted to regulatory agencies before the drug substance from the scaled-up process can be introduced into the clinic.

As the demand for drug substance increases with the progression through Phase 2 and Phase 3 clinical trials, so too do the expectations for regulatory compliance to full cGMP.²³ Release assays that were formerly only qualified are expected to be fully validated prior to the release of material for Phase 3 clinical trials. Raw material and bulk drug substance release specifications are expected to be refined and tightened as product and process understanding increases at the later stages of development. Most significantly, prior to or concurrent with the initiation of manufacturing for Phase 3 clinical material, acceleration of a comprehensive program to characterize both the

product and the process is expected since the process and facility used to supply material for the pivotal clinical trials is anticipated to be the process and facility to be filed with regulatory authorities as the commercial process, at least initially. These expectations are in line with the current regulatory approach to process validation and product life cycle management, described in more detail in Chapter 11.

Commercial Manufacturing

A critical step in the transition from the manufacture of clinical trial material to commercial manufacturing involves the validation of the manufacturing process and the production of Process Performance Qualification (PPQ) lots. These PPQ lots can be manufactured as part of the Phase 3 clinical manufacturing program provided process characterization activities have sufficiently progressed to enable their initiation. Alternatively, these lots may be manufactured after completion of the clinical trials and can be stockpiled as potentially commercially saleable material once the product is approved for sale. Regardless of their intended use – clinical or commercial – data from these PPQ lots must be submitted as part of the final process validation package included with the submission of BLA, MAA, or equivalent request for marketing approval of the product.

For monoclonal antibody products, marketing approval does not typically signal the end of the evolution of the manufacturing process and depends on the relative success of the commercial product relative to expectations during clinical development. Ideally, the scale of the process used to manufacture Phase 3 material will be sufficient to satisfy the commercial demand through the product's lifecycle. This assumes that the mature product demand 5 to 10 years in the future can be accurately forecasted during late phase clinical development. However, such an approach can carry a large strategic risk. If the Phase 3 manufacturing process is scaled to accommodate a very large anticipated peak market demand starting 3 to 4 years after approval, but the product is not approved, this very large expenditure of money, time and resources to support launching at such a large scale could be financially disastrous. An alternative strategy that is more risk adverse would scale Phase 3 manufacturing and initial commercial launch at a more modest, economically viable scale. Then, if analysis of the clinical data suggests a high likelihood of regulatory approval, efforts can commence to procure additional and potentially larger manufacturing

capacity that could come on line a year or two after launch. Note that this strategy is not without its risks, either. If supply is planned incorrectly, resulting in challenges meeting demand or even stock-outs, patients could be lost to competing treatments. The launch of Enbrel by Immunex in 1998 is illustrative of the inherent difficulties and uncertainties in attempting to match launch capacity with speculative market demand.²⁴ The demand for Enbrel soon after launch vastly exceeded Immunex's capability to manufacture, led to lost revenue and lost market share to competing products launched in Enbrel's wake, such as Humira. Immunex was eventually acquired by Amgen in 2002 because it was unable to supply Enbrel as a stand-alone entity.

While poor forecasting can cause significant problems, poor manufacturing management and execution can be devastating as well, as was the case for Genzyme. In May 2009, Genzyme received an FDA warning letter identifying manufacturing deficiencies at its Allston, Massachusetts plant, which led to the facility shutdown and severe shortages of key drugs Cerezyme and Fabrazyme. Due to the Cerezyme shortage, Shire and Protalix, two companies developing drugs competitive to Cerazyme, received fast track designation from FDA for their experimental treatments, accelerating the regulatory review and approval of these products. Meanwhile, Genzyme was unable to adequately address the issues raised by FDA in the warning letter, resulting in FDA issuing a consent decree to the company. This forced Genzyme to move filling and packaging out of its Allston facility and the remaining Cerezyme and Fabrazyme manufacturing was placed under oversight of a third party. While Genzyme was still struggling with supply shortages, Sanofi-Aventis initiated a takeover of the company that led to their eventual acquisition of Genzyme.

6. Final Drug Product Manufacturing

Regardless of whether a monoclonal antibody product is in early stage clinical trials or already commercial, it must be filled in an appropriate container closure system for final dosage delivery to the patient. For most monoclonal antibody products in early stage clinical trials, as well as for many late stage and commercial products, this container/ closure system is either a glass vial or a syringe, as discussed further in Chapter 9. However, monoclonal antibodies are also being marketed in a variety of alternative delivery systems, such as autoinjectors, with increasing frequency for both clinical and commercial applications. Often, monoclonal antibody products are fully formulated at the drug substance stage so that no additional formulation is necessary prior to sterile filtration and aseptic filling into the vial or syringe. However, it is occasionally necessary to adjust the monoclonal antibody product concentration, change buffer salts, and/or add additional excipients or stabilizers as part of the drug product manufacturing process.

Following filling into a suitable container, the vial is stoppered and capped if it is to be stored as a liquid, or lyophilized (freeze dried) if it is to be stored as a dry powder. A fill/finish process must be developed and tested before clinical production can begin. Fill/finish processes are frequently developed and carried out in a different facility, or possibly a different organization, from the development and manufacture of the drug substance. The drug product manufacturer may be capable of running some of the product specific analytical methods that are used to test and release the drug substance. An alternative approach widely used for early stage products is to send samples of the drug product to the drug substance testing location for release and stability testing of the product specific methods. This reduces the cost and effort of having the same methods established at two different manufacturing sites.

Drug product release testing must include sterility testing according to appropriate compendial methods. Many pharmaceutical products or excipients used in formulations can inhibit the growth of bacteria or fungi that could potentially contaminate a product, therefore it is a requirement that the product in its final formulation be tested for bacteriostasis and fungistasis activity.

7. Strategic Considerations in Early Process Development

The CMC development program described in this chapter outlines the basic steps that must be completed to produce a monoclonal antibody product suitable for use in human clinical trials and in compliance with regulatory guidelines for early stage clinical products. However, as with any development program, many different strategies can enable successful CMC development. A balance of cost, time, and risk will lead each company to make decisions at each development step that are most aligned with the company's overall business strategy and risk tolerance. For a more detailed discussion on different strategies employed in monoclonal antibody development and manufacturing, see Chapter 12.

At the highest level, there are two critical business milestones related to the CMC development activities of monoclonal antibodies for early stage clinical trials, namely the initial decision to pursue clinical development of a lead monoclonal antibody product candidate from research and the successful filing of an IND or IMPD for initiation of Phase 1 human clinical trials. The first milestone represents the initiation of the development program and the CMC activities discussed here as well as a commitment to the financial, technical, and human resources associated with successfully achieving the second milestone. The filing of an IND, especially for early stage or small biopharmaceutical companies, is often the trigger for an increase in a company's valuation by investors, an important step in securing adequate financing to support continued development.

A great deal of time and cost is incurred between initiation of the development program and the initiation of human clinical trials. However, the technical and business risks of a product development program are not reduced in any significant way until efficacy of the drug candidate is actually demonstrated in humans during the later stage Phase 2 and Phase 3 clinical trials. For this reason, it is of tremendous strategic importance for any biopharmaceutical company, whether they are a small start-up company or large multinational pharmaceutical company, to evaluate development options and to consider ways to minimize the time and cost required for the CMC activities prior to initiating Phase 1 human clinical trials, while minimizing the risks of regulatory or commercial failure. Perhaps the most important challenge facing all biopharmaceutical companies is how to effectively decrease the initial development time and cost for a monoclonal antibody product to free up resources to move more product candidates through the development pipeline without sacrificing product quality or incurring unnecessary costs or risks.

In an effort to move more product candidates from research to clinical development with the same or fewer resources, many companies chose to focus on the speed with which they can develop a "good enough" manufacturing process to support initial clinical development in order to reduce overall commercialization timelines and delay the larger spending required to develop a robust, commerciallyenabling process.^{25, 26} Such a development strategy employs techniques to rapidly identify high producing cell lines for use in established platform cell culture and purification processes, while utilizing, as much as possible, existing analytical methods and product formulations for use in preparing materials for early stage clinical trials. Incorporation of such platform approaches can accelerate the CMC development path for a new monoclonal antibody product allowing the filing of an IND approximately 18 months from the start of cell line development. Platform approaches are an area of active exploration and optimization within many biopharmaceutical firms, with an emphasis on reduction in time and cost required to sufficiently complete the CMC development and manufacturing to initiate clinical trials. Some companies that routinely develop antibody products and have developed streamlined platforms in-house claim that the CMC development path can be executed in as little as 15 months, but this would be an excessively aggressive timeline for smaller companies or companies without in-house development expertise and capability.

The use of platform and/or unoptimized processes and methods or pilot facilities to produce initial clinical material is consistent with the Phase 1 cGMP Guidance, but patient safety should always be considered. Initiating clinical development with a cell line that has sub-optimal expression levels or a purification process with an overall process yield of less than 50% is not unreasonable if this initial "good enough" process can be developed much more quickly than a fully optimized and validated process. Following this approach, a new monoclonal antibody product candidate may enter clinical trials sooner and the critical milestone of proof of efficacy (or lack of efficacy) will be reached sooner. Once there is some indication that the product candidate has efficacy in humans in the intended indication, the risk of product failure is reduced sufficiently, thus justifying the additional time and cost of further development and optimization. This approach is not without some risk, however, as there are strict regulatory requirements that product comparability be maintained throughout development and commercialization of a biologic product requiring potentially expensive and time-consuming comparability programs to support manufacturing process changes during and after clinical development.²⁷ Because a change in cell line is a very significant change, the ability

to quickly develop cell lines suitable for eventual use in commercial product manufacture is an increasingly desirable element in any accelerated product development path. New technologies to make cell selection faster and cell line attributes better are continually investigated and evaluated in early stage development to further benefit later stage development by eliminating the need for a second round of cell line development. An accelerated development plan such as that outlined here uses pre-defined drug substance and drug product manufacturing processes, along with pre-defined drug product formulations. These platform processes are designed for use with minimal modification to provide adequate production yields and product quality for preparation of non-clinical study supplies and for the manufacture of Phase 1 and Phase 2 clinical trial supplies. The drug substance and drug product should be sufficiently characterized and evaluated during this early development to establish key product attributes and product stability to support nonclinical studies, reference standard qualification, preparation of CMC sections for regulatory submissions and to establish comparability during subsequent development.

The risk inherent in the rapid approach to process development described above for monoclonal antibody products is somewhat mitigated by the use of platform technologies, in which a common process framework, e.g., similar or identical process steps, equipment, or media, is used across multiple products. The use of a platform approach can increase the likelihood that an early-stage, sub-optimal process will produce product quality similar to a well-optimized commercial process. In addition, platform approaches can reduce the development resources required both to reach the clinic and to implement a commercial process and can assist the incorporation of QbD with lower up-front investment. Monoclonal antibodies are uniquely suited to the platform approach due to the high degree of similarity between the individual product molecules and the types of processes that may be used to produce them.

Despite the availability of platform processes and a regulatory pathway for process changes accompanied by comparability programs, some companies choose to fully reduce risk by spending the necessary time and money to develop a cell line that has sufficient expression to serve throughout development and commercialization, and to fully develop the manufacturing process prior to any human clinical experience. The full development approach means that there is significantly less risk that process changes introduced later in development will have an impact on the product quality or function since these changes will only be in scale and not in process parameters, cell line, cell bank, or other critical process steps. A hybrid strategy, in which a commercially enabling production cell line is generated at the initiation of the development program, but the process is not fully optimized until later in development, is often chosen by companies.²⁸ A change in cell line introduces greater regulatory and technical risk than a well-understood improvement in the cell culture or purification process, so this hybrid strategy does provide reasonable risk reduction.

Once a product has achieved marketing approval from the regulatory agencies, several other strategic considerations become important. It is, of course, critical that the manufacturing process be robust and reliable and capable of routinely producing product with the required safety and efficacy profile. Efforts to expand the operating ranges of process parameters that regulatory authorities may have limited at filing due to insufficient process characterization data, will enable more efficient operations by reducing the number of investigations triggered by out of range operations. Harmonizing operating ranges between regions that may have approved different ranges for some process variables would also enable a more robust and efficient global commercial supply chain. Additional process characterization experiments with small scale processes verified to be representative of the commercial scale process along with the mining of commercial process data can support such efforts. Scale-up and process economics may be vital, as many monoclonal antibody therapeutics require high doses and/or chronic use and may treat multiple indications or large patient populations.

The ability to easily make process changes and improvements is also important, especially as companies manage large and ever-changing product portfolios across multiple manufacturing sites, including contract manufacturers and partners. The FDA and other regulatory agencies are also seeking new ways to reduce the risks to patients without increasing regulatory resources by insuring that producer companies have better understanding and control of their manufacturing processes. At these later stages of development and commercialization, different manufacturing controls and approaches are required, but for the early stage development there is significant flexibility and the right approach will depend on business objectives, investment goals, and risk tolerance.

8. Timelines and Costs for CMC-Related Activities

Managing the CMC activities of an antibody development program requires a coordinated and integrated project management approach that reaches back to the discovery function and forward to commercial manufacturing, and considers the requirements and impact of each activity as it relates to the timeline and dependencies of other activity. Project management is essential to coordinate these numerous, interdependent activities and to ensure that the overall development program is consistent with the risk tolerance and strategic initiatives within the sponsor company. Certain development tasks are dependent on completion of previous activities whereas others can be initiated prior to finalization of previous steps or activities. The project management activities associated with each key task in the development of a monoclonal antibody product and the integration of these tasks in an efficient and effective manner and the considerations required for each task are described below.

The following is a general framework for understanding how the different program activities relate to each other, how long these activities can be expected to take for a typical monoclonal antibody, and how much they can be expected to cost for a representative program. The timeline and costs assume that development is carried out in the context of a reasonably well-established "platform process" for analytical methods and upstream and downstream processing, as discussed in detail in later chapters, and assumes the company wants to move quickly to the production of product suitable for initial clinical evaluation and that the process will be fully optimize later in development.

There are a range of possible times and costs for each task, depending upon the specific product, the clinical indications, the particular resources available and the technical issues encountered as development unfolds. The times and costs given below represent typical current values, and should be taken only as a general guideline. The technical and business details of any given monoclonal antibody product development program may result in reasonable differences in these time and costs estimates.

Major Tasks and Timelines for Monoclonal Antibody Development

A comprehensive listing of all the individual tasks required to develop a therapeutic monoclonal antibody product is beyond the scope of this report. However, the major CMC related tasks required to advance a monoclonal antibody product candidate from discovery to first in human clinical trials are outlined in Figure 2.1. This list provides a useful framework for understanding the interdependencies of the various tasks that must be completed prior to filing an IND and initiating human clinical trials for a monoclonal antibody product. Each of the major groups of tasks is generally performed by distinct resources either within a development company or at a contractor. While outsourcing can greatly reduce capital costs and requirements for internal staffing, the timeline to key milestones is generally longer due to the outsourcing and process transfer activities.





A fully integrated CMC project plan to develop a monoclonal antibody from cell line development to first in human clinical trials. Source: BPTC

Estimated Costs for the Development of a Monoclonal Antibody Product

A summary of the estimated costs and time for each of the major CMC-related tasks involved in the development of a monoclonal antibody product from initial development of a production cell line, through all phases of process development and manufacturing up to and including the filing of an IND, is provided in Table 2.1. While this table includes all of the major CMC-related tasks required for monoclonal antibody product development, it does not include the additional costs for pre-clinical animal

studies and other non-CMC activities required for the development of a new therapeutic product. These costs are product-specific and can vary significantly depending on the intended use of the monoclonal antibody product and other factors and are beyond the scope of this report.

Development and manufacturing activities beyond early stage clinical trials are highly product-dependent and often relate to the company's strategic plans for commercial manufacturing, product partnering, and other business considerations.

Table 2.1. Estimated GMC-Related Costs for Monocional Antibody Development				
Task	Cost (\$000)	Time (Months)	Critical Predecessors	
1. Analytical Methods Development				
Preliminary Development Assays and Standards	\$100	2		
Analytical Methods Development	\$500	6	Cell Line Selection and Optimization	
Assay Qualification and Transfer to QC	\$150	2	In Process & Product Release Assays	
Product Characterization	\$150	3	Preclinical/Engineering Drug Substance Production	
Clinical Reference Standards	\$150	2	Preclinical/Engineering Drug Substance Production	
Subtotal	\$1,050			
2. Upstream Process Development				
Expression Vector	\$50	1		
Cell Line Selection and Characterization	\$150	5	Expression Vector	
Cell Banking and Characterization	\$150	4	Cell Line Selection and Characterization	
Initial Media and Process Development	\$250	5	Cell Line Selection and Characterization	
Subtotal	\$600			
3. Downstream Process Development				
Purification Process Development	\$750	6	Cell Line Selection and Characterization	
Initial Viral Validation Study	\$150	3	Purification Process Development	
Subtotal	\$900			
4. Formulation Development	1	1	1	
Pre-Formulation Development	\$200	4	Cell Line Selection and Characterization	
Clinical Formulation Development	\$100	4	Pre-Formulation Development	
Subtotal	\$300			

Table 2.1 Estimated CMC Delated Casta for Managlanal Antibady Development

Table 2.1. Estimated CMC-Related Costs for Monoclonal Antibody Development					
Task	Cost (\$000)	Time (Months)	Critical Predecessors		
5. Drug Substance Manufacturing					
Process and Analytical Documentation	\$100	3	Initial Bioreactor Process and Media Development Purification Process Development		
Preclinical/Engineering Drug Substance Production	\$1,300	3	Process and Analytical Documentation		
Phase 1 (cGMP) Drug Substance Production	\$1,700	3			
Preliminary Drug Substance Stability Studies	\$200	1a	Preclinical/Engineering Substance Production Phase 1 (cGMP) Drug Substance Production		
Subtotal	\$3,300				
6. Drug Product Manufacturing					
Clinical Fill/Finish Process Development	\$50	1			
Process and Analytical Documentation	\$50	1			
Preclinical/Engineering Drug Product Production	\$50	1			
Phase 1 (cGMP) Drug Product Production	\$100c	1			
Preliminary Drug Product Stability Studies	\$300	1b	Preclinical/Engineering Drug Product Production Phase 1 (cGMP) Drug Product Production		
Subtotal	\$550				
7. Regulatory, Validation & Clinical					
IND Preparation-CMC Section	\$100	2	All Drug Substance and Drug Product tasks		
Project Total	\$6,800				

^a While stability studies must run for the proposed or anticipated shelf life of the drug substance, a minimum of one month of stability data is required for an IND filing. The time shown here covers the initial testing required to support the IND filing and not the full timing of a drug substance stability program.

^b While stability studies must run for the proposed or anticipated shelf life of the drug product, a minimum of one month of stability data is required for an IND filing. The cost and time shown here covers the initial testing required to support the IND filing and not the full timing of a drug product stability program.

° Assumes production in a 1,000 L bioreactor. Larger bioreactors are employed to produce late stage clinical and commercial scale drug substance material.

^d Project costs shown in this table cover only CMC-related tasks necessary to file an IND. Additional CMC tasks and costs required for commercialization are not included nor are non-CMC tasks such as the cost of pre-clinical safety and toxicity studies and the cost of human clinical trials.

The overall CMC costs for a monoclonal antibody development program presented in Table 2.1 represent a relatively conservative cost that includes sufficient process development to support clinical production through Phase 2. Companies willing to accept some risk in their development program often minimize the upfront process development work performed for a new monoclonal antibody product, shaving up to \$1 million off the cost estimate in Table 2.1. These cost savings come from doing little to no analytical method development and relying on platform methods, minimal cell culture and downstream process development, and using a simple phosphate buffered saline-based liquid formulation with minimal pre-formulation work.

9. References

- International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Pharmaceutical development Q8(R2) Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2009 Aug 13 [cited 2016 Nov 21]. 28 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q8_R1/Step4/Q8_R2_Guideline.pdf.
- 2 Food and Drug Administration, Center for Biologics Evaluation and Research (US). Points to consider in the manufacture and testing of monoclonal antibody products for human use [Internet]. Washington, DC: Department of Health and Human Services; 1997 Feb 28 [cited 2016 Nov 21]. 50 p. Available from: http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/OtherRecommendationsfor-Manufacturers/UCM153182.pdf.
- 3 Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Office of Regulatory Affairs (US). Guidance for industry cGMP for phase 1 investigational drugs [Internet]. Washington, DC: Department of Health and Human Services; 2008 Jul [cited 2016 Nov 21]. [20 p.] Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070273.pdf.
- 4 Zabrecky JR. Why do so many biopharmaceuticals fail? BioProcess Int. 2008 Dec;6(11):26-33.
- 5 Apostol I, Kelner DN. Managing the analytical lifecycle for biotechnology products: a journey from method development to validation, part one. BioProcess Int. 2008 Sep;6(8):12-19.
- 6 nternational Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Validation of Analytical Procedures: Text and Methodology Q2(R1) Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2005 Nov [cited 2016 Nov 21]. 17 p. Available from: http://www.ich. org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf.
- 7 Chamberlain P. An integrated approach to characterization in early phase development. Presented at International Business Communications Life Sciences' Comparability and Immunogenicity of Biologicals Conference; 2005 Jun 14-15; Vienna, Austria.
- 8 Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Center for Veterinary Medicine (US). Guidance for industry: Process validation: General principles and practices. Revision 1 [Internet]. Washington, DC: Department of Health and Human Services; 2011 Jan [cited 2016 Nov 21]. 22 p. Available from: http://www.fda.gov/downloads/Drugs/.../Guidances/UCM070336.pdf.
- 9 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Specifications: test procedures and acceptance criteria for biotechnological/biological products Q6B Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1999 Mar 10 [cited 2016 Nov 21]. 16 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf.
- 10 World Health Organization. Procedures for the preparation, characterization and establishment of international and other biological reference standards [Internet]. Geneva: World Health Organization; 2011 Nov 3 [cited 2016 Nov 21]. 1 p. Available from: http://www.who.int/biologicals/ reference_preparations/establishment/en/.
- 11 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Quality of biotechnological products: stability testing of biotechnological/biological products Q5C Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1995 Nov 30 [cited 2016 Nov 21]. 8 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5C/Step4/Q5C_Guideline.pdf.

- 12 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Stability testing of new drug substances and products Q1A(R2) Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2003 Feb 6 [cited 2008 Dec 3]. 18 p. Available from: http://www.ich. org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2__Guideline.pdf.
- 13 Jones SD. Technologies to improve cell line development and engineering. Presented at Cambridge Healthtech Institute's Peptalk; 2009 Jan 11-16; San Diego, CA.
- 14 Zhou W, et al. Fed-batch culture of recombinant NSO myeloma cells with high monoclonal antibody production. Biotechnol. Bioeng. 1997 Sep;55:783-92.
- **15** Ransohoff TC, et al. (BioProcess Technology Consultants Inc., Acton, MA). Cell culture manufacturing capacity: trends and outlook through 2013. Springfield (VA): Pharmsource Information Services; 2008 Dec. 126 p.
- 16 Jones D, et al. High-level expression of recombinant IgG in the human cell line Per.C6. Biotechnol Prog. 2003 Jan-Feb;19(1):163-68.
- 17 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Quality of biotechnological products: analysis of the expression construct in cells used for production of r-DNA derived protein products Q5B Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1995 Nov 30 [cited 2016 Nov 21]. 4 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5B/ Step4/Q5B_Guideline.pdf.
- 18 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin Q5A(R1) Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1999 Sep 23 [cited 2016 Nov 21]. 27 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5A_R1/Step4/ Q5A_R1__Guideline.pdf.
- 19 Daugherty AL, Mrsny RJ. Formulation and delivery issues for monoclonal antibody therapeutics. Adv Drug Deliv Rev. 2006 Aug;58(5-6):686-706.
- 20 European Medicines Agency (GB). Guideline on strategies to identify and mitigate risks for first-in-human clinical trials with investigational medicinal products [Internet]. London (GB): The European Medicines Agency; 2007 Jul 19 [cited 2016 Nov 21]. 12 p. Available from: http://www. ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002988.pdf.
- 21 European Commission. EudraLex Vol. 4, EU Guidelines to Good Manufacturing Practices: Medicinal Products for Human and Veterinary Use, Annex 13: Investigational Medicinal Products. Feb 2010. [Internet]. Brussels (BE): The European Medicines Agency; 2011 Jul 2 [cited 2016 Nov 21]. 19 p. Available from: http://ec.europa.eu/health/files/eudralex/vol-4/2009_06_annex13.pdf
- 22 Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Office of Regulatory Affairs (US). Guidance for industry: sterile drug products produced by aseptic processing - current good manufacturing practice [Internet]. Washington, DC: Department of Health and Human Services; Sep 2004 [cited 2016 Nov 21]. 63 p. Available from: http://www.fda.gov/downloads/Drugs/.../ Guidances/ucm070342.pdf.
- 23 Hoiberg CP. Guidance on CMC for Phase 1 and Phases 2/3 Investigational New Drug Applications. Presented at DIA China Conference, Beijing, China. May 16-18, 2011.

- 24 Dario B, et al. The role of marketing. Evanston (IL): Kellogg School of Management, Northwestern University; 2004 Apr 11 [cited 2015 Jan 15]. 21 p. Available from: http://www.kellogg.northwestern.edu/biotech/faculty/articles/role_of_marketing.pdf
- 25 Slaff G. Early process development: it's almost all about speed. Presented at Manupharma Congress; 2004 Apr 4-5; Boston, MA.
- 26 Seymour P, Emery E. Strategies for CMC excellence playbooks for product development from first in human through commercialization. Presented at the 18th International IBC Antibody Development and Production Conference; 2007 Feb 28-Mar 2; Carlsbad, CA.
- 27 U.S. Food and Drug Administration [Internet]. Silver Spring, MD: U.S. Food and Drug Administration; c2009. Demonstration of comparability of human biological products, including therapeutic biotechnology-derived products; [updated 2009 May 5; cited 2009 Jun 26]; [about 1 screen]. Available from: http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122879.htm.
- 28 Kraichely DM. Expression development interface: guiding successful transition of biological new molecular entities from discovery research to pharmaceutical development. Presented at: International Business Communications Life Sciences' Fifth Annual Cell Line Development and Engineering Conference; 2009 Jun 22-24; La Jolla, CA.



CHAPTER 3:

Quality by Design

uality by Design (QbD) is a modernized approach to pharmaceutical development that is intended to provide regulatory flexibility, increased development and manufacturing efficiency, as well as provide greater room to innovate and improve the manufacturing process within defined ranges without obtaining prior regulatory approval. QbD requires more product and process understanding than traditional development approaches, and significant focus is placed on understanding the relationships between process inputs and the product's quality characteristics.¹

The goal of pharmaceutical development is the design of a drug and a manufacturing process that can yield a high quality, safe, effective product on a consistent basis.² Prior to 2000, biopharmaceutical development and manufacturing tended to be plagued by inefficiencies that resulted in limited innovation throughout the product lifecycle and significant product waste due to lost batches. In many cases, there appeared to be an inability to connect product attributes to the manufacturing process, despite the high risk for waste generated by mistakes.¹ FDA's 2002 initiative entitled; Pharmaceutical cGMP for the 21st Century: A Risk Based Approach, sought to update how companies interact with regulators and to shift course to focus on a science and risk-based approach to development. This document states: "continuous improvement is an essential element in a modern quality system and it aims at improving efficiency by optimizing a process and eliminating wasted efforts in production. In the current system continuous

improvement is difficult, if not impossible. Reducing variability provides a 'win-win' opportunity from both public health and industry perspectives, therefore continuous improvement needs to be facilitated."³ The shift towards risk-based development has resulted in the use of iterative risk assessments along with QbD methodologies that contribute to the growth of product and process understanding and innovative solutions to manage risk associated with the variability inherent in pharmaceutical manufacturing.

The QbD approach provides a set of tools that support both developmental and manufacturing activities, and align with the phases of clinical development, enhancing the pharmaceutical development process. ICH Guidelines Q8(R2), Q9, Q10, and Q11 provide the framework for QbD. A summary of these documents is shown in Table 3.1.

Table 3.1. ICH Guidelines Related to Quality by Design

ICH Q8(R2): Pharmaceutical Development - This document provides guidelines for drug product development. ICH Q8 defines QbD as, "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management."¹² This guideline outlines the principles for potentially achieving increased regulatory flexibility.

ICH Q9: Quality Risk Management - This guideline provides principles and examples of tools for quality risk management that can be applied to all aspects of pharmaceutical quality including development, manufacturing, distribution, and inspection and submission/review.²⁰ This document states that: risk assessment should be based on sound scientific knowledge; and the level of risk assessment activities should be a function of the level of risk.^{4,20}

ICH Q10: Pharmaceutical Quality System - This document applies to pharmaceutical drug substances and drug products throughout their lifecycles and provides a comprehensive model for pharmaceutical quality based on ISO standards. It is intended to promote innovation and continual improvement in pharmaceutical manufacturing.⁸ It outlines a pharmaceutical company's responsibilities and ICH expectations.⁴ This guideline introduces the concept of "phase appropriate" development.

ICH Q11: Development and Manufacture of Drug Substances - This guidance covers the development and manufacturing process of drug substances.⁵ It provides an explanation of what should be included in the Common Technical Document submission.

QbD is a systematic developmental approach that starts with a clear goal in mind and emphasizes understanding of how variability in both process and materials impacts the final product.⁷ Historically, product quality has been assured by either end product testing (drugs) or by strict and narrow control of the manufacturing process without a comprehensive understanding of the link between process parameters and product quality attributes (biologics). According to a FDA guidance released in January of 2011, product quality cannot be assured by testing alone.⁶ In the QbD paradigm, quality is built into the process rather than being tested into the product.

For biopharmaceuticals, QbD includes identifying critical process steps and parameters, and designing the operation of these steps to reduce risk and increase quality. Since manufacture of biopharmaceuticals involves production of the desired product in a living host organism, particular attention should be focused on the development and characterization of the production host cell line. The product is then purified from host and culture related impurities using a series of orthogonal unit operations. At each step of the cell culture and purification process those materials or operations that may impact product quality and safety should be identified. A long history of process knowledge, along with the relationship of specific process parameters to product quality, is available for monoclonal antibody products, and therefore the implementation of QbD into a new antibody manufacturing process is more straightforward than for other biopharmaceuticals.

A Lifecycle Approach to Pharmaceutical Development

QbD is a lifecycle approach to biopharmaceutical product development that encompasses the development, optimization, and validation of a manufacturing process from the initial identification of a potential new product through all stages of clinical development and commercialization, until the withdrawal of the product from the marketplace. It is an iterative, lifecycle-based approach to development that focuses heavily on leveraging prior knowledge and experience to both improve the process and reduce overall risk. FDA's Janet Woodcock is frequently credited with saying that QbD is derived from a combination of prior knowledge, experimental assessment, and a causeand-effect model that links critical process parameters and critical quality attributes.⁷ In 2011 FDA introduced the Guidance for Industry, Process Validation: General Principles and Practices, which introduced a new, lifecyclebased, approach to process validation, aligned with QbD. This guidance and its implications for process validation are discussed in more detail Chapter 11.

The overall goal of QbD is to maintain a state of control for a product and its manufacturing process over the course of the product lifecycle, through the design, definition, and implementation of a control strategy. A control strategy is "a planned set of controls, derived from current product and process understanding that assures process performance and product quality. The controls can include parameters and attributes related to drug substance and drug product materials and components, facility and equipment operating conditions, in-process controls, finished product specifications, and the associated methods and frequency of monitoring and control."⁸ An outline of the QbD approach, aligned with the phases of clinical development and product/process development, is shown in Figure 3.1.²⁶

Quality by Design in Industry

Genentech's Gazyva (obinutuzumab) is the first monoclonal antibody to be approved by FDA using a full QbD filing.⁹ There are many key lessons that can be learned from Genentech's experience, not only with the approval of Gazyva, but with the non-approval by FDA and EMA of the QbD filing for Perjeta's (pertuzumab), another monoclonal antibody developed by Genentech. According to Dr. Lynne Krummen, every bit of flexibility that QbD offers must be earned and everything must be justified.¹⁰ In improving a manufacturing process over the course of the product lifecycle, information must be readily available and well documented. Quality

Figure 3.1. The Quality by Design Approach

The QbD approach aligns with the Product and Process Development Stages and Clinical Development Phases. QbD provides milestones and assessments to facilitate product development and ensure quality.



Source: BPTC

Effective product lifecycle management requires the engagement of cross-functional teams for all QbD steps. Diversity in the makeup of the development team is essential to successful product realization and a variety of subject matter experts from multiple disciplines should be included. A range of perspectives and opinions should be sought out and embraced. documentation of research and of process performance must be employed in order to take full advantage of the knowledge available and to meet the relevant regulatory standards. Internal policy and practice should be established in recognition of the importance of thorough documentation. The use of risk management tools and iterative risk assessments is essential to developing a control strategy and design space that global regulatory agencies will accept. Engaging regulatory agencies early and being upfront about knowledge gaps are also key to a successful QbD filing, according to Dr. Krummen.

While a full QbD filing will provide increased flexibility in commercial manufacturing of a monoclonal antibody product, the cost associated with completing all the necessary work for such a filing makes the cost of filing for approval for a new product significantly higher than a filing without QbD. In Genentech's Gazyva filing, the extensive documentation and testing associated with the QbD-related work resulted in a much larger CMC section of the company's BLA than for other monoclonal antibody product filings and may had added as much as \$1 million to the overall development of the product.⁴

1. TPP/QTPP and Critical Quality Attributes

In the QbD paradigm, development starts with a Target Product Profile (TPP). This dynamic summary of the desired product attributes is used to enhance dialogue between the sponsor and regulatory agencies and establishes the foundation for the Quality Target Product Profile (QTTP), which is a summary of the product's quality characteristics. Following the creation of the QTPP, critical quality attributes are proposed via risk assessment. CQAs are characteristics or properties that must be maintained within a specific range to assure quality.

Target Product Profile

The TPP is usually defined by the discovery functions within a company and later transferred to Process Development; the TPP is the foundation of product development and can serve multiple purposes, depending on the needs of the company. The TPP is meant to facilitate interactions with global regulatory authorities. According to the FDA guidance: "the TPP embodies the notion of beginning with the goal in mind. That is, the sponsor specifies the labeling concepts that are the goals of the drug development program, documents the specific studies intended to support the labeling concepts, and then uses the TPP to assist in a constructive dialogue with FDA."11 The TPP states the intent of the product and can provide a snapshot of the prospective drug at a given moment in time. The TPP includes information amount the drug and its desired features. It includes the drug description, indication, desired efficacy and safety claims, desired drug product format and container-closure (e.g. liquid prefilled syringe or lyophilized

vial), route of administration (e.g. SC or IV) and other desired attributes of the product³³.

FDA currently does not require the use of a TPP and the overall interpretation of the draft guidance is left to the judgment of the applicant. As a TPP is not a required element of the filing, it does not represent a commitment to follow all stated product goals. Submitting a TPP does not represent an obligation by the sponsor to submit draft labeling in an NDA or BLA that is identical to that in the TPP. Any endorsement of the TPP by FDA during the dialogue process does not necessarily represent intent of approval of the final label.¹¹ The TPP is not meant to constrain a company or impose restrictions, but rather to provide a useful tool to facilitate discussions between the sponsor and FDA. In cases where a TPP is not used, similar documentation should be employed stating the overall intent of the drug, which will be used to inform the QTPP.

Quality Target Product Profile

The QTPP is based on the foundation laid by the TPP, or similar documentation. The QTPP is a prospective summary of the quality characteristics of a pharmaceutical product that will ideally be achieved to ensure the desired quality, taking into account both safety and efficacy.¹² Attributes represented in the QTPP relate to the intended use of the drug product and are those that impact the patient. Considerations for the QTPP should include: route of administration, dosage form, bioavailability, strength, and stability.¹² While the TPP indicates what the drug will do, the QTPP indicates the quality targets necessary to do it.

The QTPP should be defined when a drug candidate has been identified as viable for development. The QTPP is meant to serve as a guide for future product and process development activities. Due to the iterative nature of QbD, the QTPP should be revisited and updated as necessary at phase appropriate intervals over the course of the product lifecycle.^{11,13}

The long history and abundant data available for therapeutic monoclonal antibodies provides a good template for the initial definition of the QTPP for a new antibody product. The correlation between specific functions of antibodies and the quality attributes that contribute to these functions are well understood for this class of molecule, and therefore designing the QTPP in advance of clinical trials is less challenging than it is for other types of biopharmaceuticals. For example, ADCC, the mechanism by which antibodies recruit other immune system components to kill target cells, is known to be enhanced by lower fucose levels, higher galactose content, reduced sialyated glycan content, and/or a bisecting GlcNac on the single glycan structure found on each heavy chain.^{14, 15, 16} Cell lines and cell culture conditions can be developed to increase or decrease the levels of these quality attributes depending on the desired function of the antibody product, thereby directly applying the QTPP to the process development activities.

Critical Quality Attributes

A CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure that the product has the desired safety and efficacy in the patient, as defined by the QTPP.¹² CQAs are selected from all of the product's quality attributes though risk assessment, as outlined in ICH Q9. The inputs to the CQA risk assessment are shown in Figure 3.2. The CQA risk assessment considers prior knowledge about the target molecule, toxicology studies, and any available clinical or non-clinical studies.

Figure 3.2. CQA Risk Assessment

CQA are determined by assessing the product quality attributes in a risk assessment. Clinical and non-clinical studies, toxicology studies, and prior knowledge elements are taken into consideration in this process.



The CQA risk assessment considers two factors: the severity of something going wrong; and, the level of certainty of it going wrong. These factors are scored using a severity score and an uncertainty score. The severity score measures the impact of a failure while the uncertainty score measures the level of confidence in the information used to determine the severity score. The CQAs should be sorted using a criticality continuum, with attributes having the most impact on safety and efficacy given the highest priority.

Prior knowledge is used in scoring the CQA risk assessment and may include: molecule design, non-clinical studies, in vivo and cell-based assays, relevant platform products or quality attributes, and relevant published literature.^{7, 13} Prior knowledge elements are weighted differently in determining the uncertainty score as shown in Figure 3.3.¹⁷ CQAs for a new monoclonal antibody product are based on the extensive literature and knowledge regarding how this type of molecule interacts with the patient's immune system and the therapeutic target (antigen). As product knowledge evolves throughout clinical development, the criticality of certain attributes may change and the CQA criticality assessment should be repeated at phase appropriate intervals.

Figure 3.3. Prior Knowledge Elements

Prior knowledge elements are essential to the CQA risk assessment. They are weighted to determine the uncertainty score. As the figure shows, a product with no preexisting knowledge would result in the highest uncertainty score whereas a product with existing clinical data would result in a low uncertainty score. (Reprinted with permission from Reference 17)



While many biopharmaceutical companies are implementing QbD methodologies as standard business practice, they have not been routinely applied early in product development, partly due to the lack of sufficient process data to adequately provide a correlation between process steps and product quality attributes and lack of analytical data to identify the product and process CQAs. Production of clinical trial material is always on the critical path in early development and the initial step for any biopharmaceutical production is the generation of a production cell line and cell culture/ fermentation process. Most cell line development technologies focus on obtaining high titers quickly with little to no attention paid to post-translational modifications and potential product heterogeneity. A more effective approach would be to use cells specifically engineered to produce the desired posttranslational modifications as well as achieve high titers. This approach to early development activities reflects the goals of QbD by enabling a rational approach to the production of product with desired quality attributes.

2. Design Space

Defining a process design space is critical to establishing a suitable control strategy for the process and is a crucial step in achieving the regulatory flexibility inherent in the QbD guidelines. ICH Q8(R2) defines design space as: "the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality. Working within the design space is not considered as a change. Movement out of the design space is considered to be a change and would normally initiate a regulatory post approval change process. Design space is proposed by the applicant and is subject to regulatory assessment and approval."¹² The parameters and variables that are contained within the design space are determined by the CQA criticality assessment and the criticality assessment of the process parameters.³⁰ Design space describes the ranges in which the manufacturing process must operate to ensure product quality. Using design space facilitates continuous improvement and innovation by allowing a certain level of variation in the process to account for and correct variability caused by process inputs.

Design Space Modeling

A process design space consists of the ranges in which parameters for each unit operation must be maintained to assure product quality. Design spaces may be defined for an entire process or a single unit operation.³¹ Combining several unit operations in a design space may be more complex to develop, but it can provide greater manufacturing flexibility. Establishing a multiple unit operation design space requires enhanced understanding of the relationships between process steps in order to control overall impact on CQAs.

When more than one unit operation impacts a given CQA, as is often the case with monoclonal antibody processes, the overlapping ranges of the individual parameters for each step will put constraints on the design space for the overall process. Since parameters influencing process performance often interact and relate to each other in a non-linear manner, their relationship can be quite complex. Therefore, in order to properly visualize the relationship between multiple process parameters on a particular CQA, a three-dimensional graph is often used.

As an example, the design space for a hydrophobic interaction chromatography (HIC) purification step for an Fc fusion protein is shown in Figure 3.4. For this unit operation, process characterization studies show that protein loading and hydrophobicity of the chromatography media each have the impact the high molecular weight (HMW) content of the final product. Since the level of HMW contaminants in the final product is a CQA for this product, these two process parameters were defined as critical process parameters (CPPs).¹⁸

Although not a CQA, process yield is an important process performance attribute related to economic feasibility of a monoclonal antibody process. The impact of the two CPPs (protein loading and hydrophobicity of the chromatography media) on the final HMW content of the Fc fusion protein and the overall process yield are shown in Figure 3.4. Any combination of protein load and column media hydrophobicity outside the blue triangle in the lower right hand corner of Figure 3.4 results in an acceptable yield of the Fc fusion protein. Similarly, any combination of these two CPPs below the red area in Figure 3.4 results in an acceptable HMW content. By mapping the impact of protein load and column media hydrophobicity on yield and HMW content in a single graph, the design space for this process can be defined as the area below the red area of the figure but above the blue area. These combinations allow the control of HMW content to $\leq 2.5\%$ while achieving a process yield of $\geq 40\%$.

Figure 3.4. Example of a Design Space

A design space plot as determined by Jiang et al for an HIC chromatography step used to purify an Fc-fusion protein. This specific example shows the acceptable design space for protein loading and HIC resin hydrophobicity (defined in minutes of lysozyme retention time) related to HMW content (a CQA for the product) and step yield (an important process performance attribute).

Protein Load (g/L)



Source: BPTC

Process Risk Assessment

The development of manufacturing processes involves the identification, exploration and optimization, and eventual specification of multiple operating parameters. The Process Risk Assessment (PRA) identifies the operating parameters (inputs) for each process step, such as cell density and integrated cell viability for an upstream process or load temperature, load pH, and load conductivity for a column chromatography step that, if varied, have the potential to impact a CQA. PRA is generally carried out using either Failure Modes Effects Analysis (FMEA) or Failure Modes Effects Criticality Analysis (FMECA).^{19, 20}

A process risk assessment will evaluate each operating parameter (process, equipment, input materials) to determine the impact of a failure to control this parameter on both process and product (severity); the likelihood of such a failure (occurrence); and, whether this failure would be detected (detection).¹² A score is assigned to each operating parameter for each category and the resulting numbers are multiplied to calculate the Risk Priority Number (RPN).¹⁹ The severity score measures the level of harm caused to the patient should failure occur and is based on prior knowledge elements. The occurrence and detection scores measure the likelihood of an excursion, or deviation, from the proposed operating range and the likelihood of detecting that excursion in time to prevent an impact on safety and/or efficacy.1 Based on the RPN, parameters are classified as potentially critical or non-critical. These parameters will be examined further in process characterization studies.

The final selection of operating parameters and their associated acceptable ranges is based on what is required to adequately control the manufacturing process and, thus the product quality. It requires careful scientific analysis of these parameters and their effect on both process performance and product attributes. This type of analysis is often done in conjunction with experimentation using a scale-down model of the process and a "design of experiments" (DOE) approach to evaluating the effect of parameters in a statistically meaningful way.

Specifications

Developing design space includes setting specifications for in-process, drug substance, and drug product attributes. Specification are: "a list of tests, references to analytical procedures, and appropriate acceptance criteria, which are numerical limits, ranges, or other criteria for the tests described. It establishes the set of criteria to which a drug substance, drug product or materials at other stages of its manufacture should conform to be considered acceptable for its intended use."²¹ Specifications are set based on elements that link CQAs to product safety and efficacy using published literature, relevant platform knowledge, and any available clinical or non-clinical experience.

Figure 3.5. Specifications Settings

Specifications are based on elements that link a product's CQAs to its safety and efficacy. In developing specifications, information from the published literature, related products and process platform knowledge, specific process data generated during discovery and early process development, and available clinical experience.



Global regulatory agencies require the establishment of "scientifically sound specifications" that assure that components, drug product containers, in-process materials, labeling, and drug product meet the standards of identity, strength, quality, and purity.^{21, 22} Tests and acceptance criteria for appearance, identity, purity and impurities, potency, and quantity make up specifications as shown in Figure 3.5.¹³ Specifications should be updated over the course of the product lifecycle, particularly when product or process knowledge changes.

Process Characterization Studies

Process characterization studies are a key step in defining the design space. These studies examine the deliberate variation of the parameters identified as potentially critical by PRA to determine the acceptable limits of variation. These studies determine process robustness, the ability of the process to deliver acceptable quality and performance and tolerate variability in inputs.^{7,23} Process characterization studies lead to the classification of process parameters as either critical or non-critical. This distinction will determine which process parameters will be categorized as critical process parameters (CPP) and included in the design space and eventually controlled by the control strategy. CPPs are defined as a process parameter whose variability impacts a CQA and must be controlled.¹² In some cases there may be a need to further categorize process parameters beyond critical and non-critical. In these instances, key process parameter (KPP) may be used for process parameters that impact process performance, but not product quality. ICH guidelines do not include a definition for KPP and as such definitions are established at the discretion of individual organizations.²⁴ It is important to note, however, that FDA and EMA accept the use of process parameter classification beyond critical and non-critical only in the Pharmaceutical Development section of the filing and not in "Description of the Manufacturing Process and Process Controls" or "Control of Critical Steps and Intermediates" sections.²⁵

Figure 3.6. Relationship of Process Characterization Studies to Design Space

Process characterization studies examine the parameters identified as potentially critical by PRA to determine the acceptable amount of variability that can be tolerated in the process, which results in the setting of the characterization, acceptable, and operating ranges.



Process characterization studies will help to establish the characterization range, acceptable range, and operating range for each process parameter, as shown in Figure 3.6. The operating range for a parameter is the range within which a process will be run during normal operation and which has been shown to reliably and reproducibly deliver a product that meets the desired specifications. The acceptable range, which is wider than the operating range, is the range for a parameter that has been shown to have no impact on product quality. The even wider characterization range is the range to which an operating parameter has been evaluated. Process characterization studies typically examine a wider range than the operating and acceptable ranges for each parameter to determine the effect of process excursions, which may occur outside the normal operating ranges during routine product manufacturing.²⁶ The ranges established through process characterization contribute to defining the process design space.

Risk assessment, scale-down models, and design of experiment studies are all key tools in understanding how a process will respond to variability in individual operating parameters and help establish how that variability can be controlled.

Process Robustness

The evaluation of process robustness is a key element in defining the design space. Process robustness is: "the ability of a process to demonstrate acceptable quality and performance and tolerate variability in inputs at the same time."7 Process capability indices are widely used throughout a range of industries and are particularly useful in antibody development. Process capability (Cp) compares the output of an in-control process to the specification limits by using capability indices. The comparison is made by forming the ratio of the spread between the process specifications (i.e., the specification "width") to the spread of the process values, as measured by six process standard deviation units (the process "width"). Cp measures the reproducibility and consistency of a manufacturing process and represents the ability of the process to deliver a product within the defined specifications. In other words, Cp is a statistical measure of the inherent variability of a quality attribute. Cp is defined as the upper specification limit (USL) minus the lower specification limit (LSL) divided by six times the standard deviation, as shown in Equation 3.1.^{13, 27, 28}

Equation 3.1. Process Capability

$$Cp = \frac{USL - LSL}{6\sigma}$$

The process capability formula is used to determine the reproducibility and consistency of a manufacturing process. USL and LSL represents the upper and lower specification limit, respectively, for the process output while σ represents the standard deviation of the process specification mean.

A process is considered to be capable of delivering a product that meets specifications if Cp is greater than 1.33.²⁸ In this equation, it is assumed that the process mean is centered between the specification limits. If the process mean is not centered between the specifications, Cp will overestimate the process capability.

The variability of a process can be determined by calculating the process capability index (Cpk), defined as "the value of the tolerance specified for a particular characteristic divided by the process capability."¹³ This process capability index measures the relationship between the process specifications and the performance of the process, providing a measure of how close the product produced by a specific process is to the target specification and how consistently the process will deliver that product meeting its specification. Cpk, defined in Equation 3.2, represents the amount by which a process can vary without going out of specification, taking into consideration that the process mean may not be centered between the specification limits.

Equation 3.2. Process Capability Index $Cpk=Min\left(\frac{USL-\mu}{3\sigma}, \frac{\mu-LSL}{3\sigma}\right)$

The process capability index measures how close the process is to its target specification and evaluates its consistency. Cpk represents the acceptable variation of the process, σ represents the standard deviation of the process specification mean, and μ is the estimated mean of the process.

As with the process capability, Cp, when the process capability index, Cpk, is greater than 1.33 a specific process is considered to be capable; if it is less than one further action is required to reduce variability.^{13, 28}

It is possible to have little variation, with a high Cp value, but not be on target, with a low Cpk value. Therefore, a combination of Cp and Cpk should be used for process characterization studies. Process capability assessment can contribute to defining design space by determining the ability of the process to stay within specification.

Design of Experiment

Design of experiment (DOE) studies are statistical multivariate studies that can enhance process knowledge by evaluating interactions between the process parameters identified by PRA and the CQAs identified by the initial quality attribute criticality assessment. These studies can help determine the range in which operating parameter variation has no impact on product quality. DOE can be used to design worst case studies, which evaluate process robustness.²³ DOE studies are typically analyzed using statistical software such as JMP (SAS Institute, Cary, NC), which allows the analysis of multifactor spaces.^{23, 26, 29}

Defining Design Space

The design space should lead to a greater understanding of all of the variables that impact CQAs and how they come

together.³⁰ The design space includes the inputs and process parameter ranges, which define the space in which the process must operate to ensure product quality. The relationship between CQAs and CPPs are defined by design space, which specifies the acceptable ranges and operating ranges for all of the included CPPs. Design space is aligned with the acceptable range and is outside of the operating space, which is aligned with the operating range. Design space may be defined for an entire process or a single unit operation.^{5,31}

Development of design space should begin early in the development process, ideally with the conceptualization of the monoclonal antibody. The design space will evolve over the course of the product lifecycle. During initial production for an IND, potentially limited development data may be all that is available to define design space. For a monoclonal antibody produced using only an existing platform process, there may be a less well-defined design space. Following process characterization studies, however, the design space will become increasingly more defined as product knowledge evolves.

Examples of Defining Design Space

Design space is established for all processes, both upstream (Chapter 6) and downstream (Chapter 7). The application of QbD principles to biopharmaceutical products is well documented. The following examples described how PRA, scale-down models, and process characterization studies are used to define a design space.

Rathore et al describe a case study for Pichia pastoris fermentation, which explains defining design space for upstream processes. This study used a methylotrophic Mut Komagataella Pichcia pastoris (P. pastoris) expressing a recombinant protein, using a process design typical of similar processes. Parameters for process characterization were identified by PRA, using FMEA. A qualified scaleddown model was used to characterize operating parameters with an RPN greater than 50, which included: pH, temperature, DO, OD inoculum, Feed 1 start OD, Feed 1 rates during fed batch and adaption, induction start OD, and Feed 2 rates during adaption and production. With the exception of agitation, scale-dependent operational control set points were scaled down linearly. The observed process outputs from the scale-down model were within the ranges observed at pilot-scale. DOE was used to design studies

that would provide data useful for defining the design space. Resulting from the high number of parameters, three studies were designed: optical density and feed rate screening, culture parameters, and protein stability.²³ Following the execution of these studies, JMP analysis was used to determine the criticality of the studied parameters that contributed to defining the design space. In this study, none of the variables in the fermentation were found to impact the quality of the product, which resulted in none being defined as CPP and a wide design space.^{4,23}

Jiang et al describe the process of defining a design space for a hydrophobic interaction chromatography (HIC) purification of an Fc protein. Developing an understanding of how raw materials can affect the process is an important aspect of this study, as resin is a crucial raw material with the potential for variability in chromatography steps.²⁶ The study used an Fc fusion protein, expressed in a CHO cell line. The study employed a scale-down HIC column. Evaluation of HMW aggregate content was performed using size-exclusion chromatography. Host cell protein was quantified using an ELISA assay. In the study, HIC was utilized as a polishing step in the downstream process to reduce levels of host cell protein (HCP). There was significant variation in the retention times of lysozyme on the different lots of HIC resin. A PRA was conducted, using FMEA, where severity was ranked on a scale of 1-5, occurrence on a scale of 1-3,

and detection on a scale of 1-3. The overall scale for this risk assessment was 1-45. Parameters with an RPN of 8 or higher were selected for process characterization studies. DOE was used to design the process characterization studies. JMP was used for data analysis. The result was that protein loading and resin hydrophobicity were identified as CPPs.²⁶

3. Control Strategy

Control strategy is not a new concept in pharmaceutical process development. The manufacturing process for every monoclonal antibody, whether developed or designed using QbD or not, must have an adequate control strategy that is designed to ensure product quality and control product and process variability.³² In certain circumstances it may be desirable to use a hybrid approach to development, that is, to use a combination of traditional control strategy elements and QbD control strategy elements for particular CQAs.³⁰

A QbD control strategy is: "a planned set of controls, derived from current product and process understanding that assures process performance and product quality. The controls can include parameters and attributes related to drug substance and drug product materials and components, facility and equipment operating conditions, in-process controls, finished product specifications, and the associated methods and frequency of monitoring and control."⁸

Figure 3.7. Development of a Process Control Strategy

The development of a control strategy takes into consideration all of the existing product and process knowledge gained over the course of product development.



Source: BPTC

A control strategy, shown in Figure 3.7, is based on the most up to date knowledge available and serves to ensure that the product that is manufactured is within the specifications set by the design space. The complexity of monoclonal antibodies makes necessary a control strategy that can demonstrate the manner in which control over quality is maintained.³² A strong control strategy is essential to securing regulatory approval.

The CQAs and CPPs that are included in the design space must be included in the control strategy. The purpose of the control strategy is to ensure the manufacturing process remains in a state of control, inside the design space, assuring that the established quality targets are met.

Control Strategy Risk Assessment

A control strategy is based on the risk of CQAs being outside of their established ranges. The level of control or monitoring required is determined by the cumulative risk of a CQA range deviation. This determination can be made using the Control Strategy Risk Assessment, which is a cumulative FMEA. Equation 3.3 shows a potential formula for a Control Strategy Risk Assessment from the A-Mab case study published in 2009.³³

Defining the Control Strategy

The CPPs and CQAs categorized and prioritized over the course of the QbD process are essential in defining the control strategy. The knowledge gained through experimentation in the process development and process characterization stages will contribute to the rationale for each CPP included in the control strategy. The CQAs and CPPs identified by iterative risk assessments and process characterization, and included in the design space, are included in the control strategy.

Elements of the Control Strategy

Elements of a control strategy may include: input material controls, specifications, product characterization, in-process controls, process/performance parameter controls, and process monitoring.¹³ Elements of the control strategy are shown in Table 3.2.

The control strategy includes a description of how control over each CQA is maintained. It is important to keep in mind, however, that a control strategy or assay does not alter the designation of critical or non-critical for an individual CQA, but rather represents that the parameters impacting that characteristic are controlled.³⁰

Equation 3.3. The Control Strategy Risk Assessment

The Control Strategy Risk Assessment combines the CQA criticality assessment, process capability, and testing strategy to determine the Risk Priority Number for the control strategy.



Source: BPTC

Table 3.2. Control Strategy Elements				
Control Element	Description			
Input Material Control	Control over raw material inputs should be based on the risk that their variability poses to a given CQA.24			
Process Control Elements				
In-Process Controls	In-process controls (IPC) include controls of facility, equipment, and quality systems that ensure that the proper product quality is achieved.33 IPCs are inputs to the process that serve as checks to maintain the process.24			
Process Parameter Controls	Process parameter controls ensure that CPPs are within the limitations established by the design space.33			
Performance Parameter Controls	Performance parameter controls are process outputs that cannot be directly controlled but are indicative of process performance.24			
Testing Control Elements				
In-Process and Release Specifications	Specifications and acceptance criteria guarantee that the product quality targets are met.24, 33			
Product Characterization	Product characterization involves testing attributes to monitor the process.33			
Process Monitoring	Process monitoring consists of testing attributes and parameters to assure that CQAs are within the defined design space.13, 24, 33			

Control Strategy Lifecycle

A control strategy is developed in the early stages of development, as necessary for the manufacture of product for all clinical phases. The control strategy is not a static element, like all of QbD it is iterative and tied to the product lifecycle. The control strategy should be updated or revised as new product or process knowledge or understanding changes. The increased understanding gained during commercial manufacturing can allow for improvements to be made to the process, often with no regulatory hurdles provided that an adequate process design space is in place.

4. Scale-Up and Technology Transfer

Applying QbD in early development provides a strong basis for scaling up a monoclonal antibody manufacturing process from early clinical production through commercialization. To successfully scale-up, it is necessary to understand the factors that may create variations in outcome and to use production and control methods that reduce that risk to a minimum. Most development work is performed at the 5 or 10-liter scale, while commercial production will be at the 2,000 or even 20,000-liter scale, representing up to 4,000fold increase in scale. Using QbD to develop a robust process understanding and control strategy, using all available information gathered during development, therefore facilitates the transfer and scale-up of the process. Full process understanding and control requires not only process characterization but also the full characterization of the monoclonal antibody, using the most sensitive and reliable analytical methods available to identify and control critical process parameters throughout scale up.

5. Process Validation

In 2011 FDA updated its process validation guidance, aligning the principles and requirements of process validation with the lifecycle concept of QbD.³⁴ The objective of process validation is to demonstrate that a manufacturing process, when operated within established limits, reliably and reproducibly produces a product that meets its required quality standards.⁶ The process validation guidance separates the product lifecycle into three distinct stages, beginning with "Process Design" early in development of a new product and/or process, followed by "Process Qualification" as the product advances through clinical trials, and culminating in "Continued Process Verification" throughout the commercial life of the product. Process validation and its relationship to QbD is further discussed in Chapter 11.

Stage 1 – Process Design

Process Design refers to those process development and characterization activities outlined in Chapter 11, during which the manufacturing process is established and scaledup to the appropriate level to deliver a product of the desired quality. Proper documentation and knowledge management is critical at this stage.

Based on the knowledge and understanding gained through early risk assessments, experimentation, and modeling, overall process controls and controls for individual unit operations should be established.⁶ Process controls manage inherent variability in the process to assure product quality and are the subject of control strategy. Process controls may include material testing and monitoring.³⁵

Stage 2 – Process Qualification

During Process Qualification the manufacturing process is evaluated to ensure that the control strategy defined during Stage 1 is sufficient to meet the established quality requirements for the product and that all critical process parameters are maintained within their defined limits. Included in this stage of validation are facility fit and equipment qualification as well as process performance qualification.⁶

Stage 3 – Continued Process Verification

During Continued Process Verification, the manufacturing process is routinely monitored to ensure that it remains in a state of control during the course of normal commercial manufacturing operations and that the product continues to meet all established Critical Quality Attributes. Control systems must be in place to identify deviations from design performance and full documentation of process performance and all measured parameters is essential. Process performance evaluation identifies problems and dictates the actions to be taken to maintain a state of control.^{6,36}

6. Regulatory Filing

Regulatory filing follows the definition of process design space and control strategy, and the completion of Process Qualification. The filing includes detailed descriptions of the product design space, process design space and control strategy.^{1,5,7,13} Descriptions of all of the process controls used to manage each CQA should be included in the control strategy section.5 The regulatory filing is submitted using the CTD format (www.ich.org). The CTD is a harmonized approach to filing an NDA in all of the ICH regions.³⁷ Using the CTD for filing eliminates the need to generate multiple documents for different regions.

7. Process Monitoring

Immediately following approval, the manufacturing process must be continuously monitored to ensure that variability is within the limits defined by the process design space. Using the QbD approach can result in being able to make changes to the process without the need for further review or regulatory approval.¹² During the course of commercial manufacturing, process knowledge gained can be used to make adjustments to the operating space, inside the process design space, which may result in greater efficiency and reliability. Any alterations to the process design space, however, must be validated and approved by FDA.⁷

Adequate quality systems are required for oversight of changes made to the process within the process design space that will not require regulatory approval.^{1,7,13}
8. References

- 1 Rathore A, Winkle H. Quality by design for biopharmaceuticals. Nature Biotechnology 2009 Jan; 27(1): 26-34.
- 2 Parenteral Drug Association (US) [Internet]. Bethesda (MD): Parenteral Drug Association; c2016. Technical Report 56 Application of Phase Appropriate Quality Systems and cGMP to the Development of Therapeutic Protein Drug Substance, PDA technical report 56, (TR 56); c2016 [cited 2016 Nov 21]; [about 2 screens]. Available from: https://store.pda.org/ProductCatalog/Product.aspx?ID=3474
- 3 Food and Drug Administration, Center for Drug Evaluation and Research (US). Pharmaceutical Current Good Manufacturing Practices for the 21st century A Risk-Based Approach [Internet]. Washington DC: Department of Health and Human Services; 2004 Sep [cited 2016 Nov 21]. 32 p. Available from: http://www.fda.gov/downloads/drugs/developmentapprovalprocess/manufacturing/questionsandanswersoncurrentgoodmanufacturingpracticescgmpfordrugs/ucm176374.pdf.
- 6 Food and Drug Administration, Center for Drug Evaluation and Research (US). Guidance for Industry: Process Validation General Principles and Practices [Internet]. Washington DC: Department of Health and Human Services; 2012 January [cited 2016 Nov 21]. 22 p. Available from: http:// www.fda.gov/downloads/Drugs/.../Guidances/UCM070336.pdf.
- 7 Roy S. Quality by design: A holistic concept of building quality in pharmaceuticals Int J Pharm Biomed Res 2012; 3(2): 100-108.
- 8 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Pharmaceutical Quality System Q10 Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2008 June 4 [cited 2016 Nov 21]. 21 p. Available from: http://www.ich.org/fileadmin/Public_ Web_Site/ICH_Products/Guidelines/Quality/Q10/Step4/Q10_Guideline.pdf.
- 9 Zettl F. Development and Implementation of a Design Space for a New Therapeutic Antibody– a Case Study. Presented at: IBC Biopharmaceutical Development and Production Conference; 2014 Mar 24-27; San Diego, CA.
- 10 Kim S. QbD in Biologics: Genentech's Success and Failure in Design Space Approval. 2013 Nov 18. http://qbdworks.com/qbd-biologics-genentechs-success-failure-design-space-approval/, accessed July 14, 2014.
- 11 Food and Drug Administration Center for Drug Evaluation and Research (US). Guidance for Industry and Review Staff: Target Product Profile A Strategic Development Process Tool, Draft Guidance [Internet]. Washington DC: Department of Health and Human Services; 2007 March [cited 2016 Nov 21] Available from: http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm080593.pdf.
- 12 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Pharmaceutical Development Q8(R2) Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2009 Aug [cited 2016 Nov 21]. 28 p. Available from: http://www.ich.org/fileadmin/Public_Web_ Site/ICH_Products/Guidelines/Quality/Q8_R1/Step4/Q8_R2_Guideline.pdf.
- 13 Rathore A. Roadmap for implementation of quality by design (QbD) for biotechnology products Trends in Biotechnology 2009 Aug; 27(9): 546-553.
- 14 Scallon BJ, et al. Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. Mol Immunol. 2007 Mar;44(7):1524-34.
- 15 Shinkawa T, et al. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human lgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem. 2003 Jan;278(5):3466-73.

- 16 Swann PG, et al. Considerations for the development of therapeutic monoclonal antibodies. Curr Opin Immunol. 2008 Aug;20(4):493-9.
- 17 Godavarti R. Quality by Design. Presented at: MIT Professional Education Downstream Processing, 10.45s; 2013 Aug 5-9; Cambridge, MA.
- 18 Jiang C, et al. Defining Process Design Space for a Hydrophobic Interaction Chromatography (HIC) Purification Step: Application of Quality by Design (QbD) Principles. Biotech and Bioeng. 2010; 107(6): 985-997.
- 19 International Electrotechnical Commission. International Standard 60812: Analysis technique for system reliability Procedure for failure modes and effects analysis (FMEA); 2006.
- 20 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Quality Risk Management Q9 Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2005 Nov 9 [cited 2016 Nov 21]. 23 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ ICH_Products/Guidelines/Quality/Q9/Step4/Q9_Guideline.pdf.
- 21 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Test Procedures and Acceptance Criteria for Biotechnological/Biological Products Q6B Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1999 Mar 10 [cited 2016 Nov 21]. 16 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf.
- 22 Current Good Manufacturing Practices for Finished Pharmaceuticals, 21 CFR 211.160, (2013)
- 23 Rathore A, et al. Defining Process Design Space for Biotech Products: Case Study of Pichia pastoris Fermentation, Biotechnol Prog 2008; 24: 655-662.
- 24 Parenteral Drug Association (US) [Internet]. Bethesda (MD): Parenteral Drug Association; c2016. Process Validation: A Lifecycle Approach, PDA Technical Report 60, (TR 60); c2013 [cited 2016 Nov 21]; [about 2 screens]. Available from: https://store.pda.org/ProductCatalog/Product. aspx?ID=1946
- 25 European Medicines Agency and Food and Drug Administration [Internet]. EMA-FDA pilot program for parallel assessment of Quality by Design applications: lessons learnt and Q&A resulting from the first parallel assessment; August 2013. [cited 2016 Nov 21] 3 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Other/2013/08/WC500148215.pdf
- 26 Jiang, et al. Defining Process Design Space for a Hydrophobic Interaction Chromatography (HIC) Purification Step: Application of Quality by Design (QbD) Principles, Biotechnology and Bioengineering 2010 Aug; 107(6): 985-987.
- 27 Steiner et al. Understanding Process Capability Indices. Waterloo, Ontario. Available from: http://scholar.google.com/scholar_url?hl=en&q=http:// xa.yimg.com/kq/groups/20114366/26277246/name/Process%2BCapability.pdf&sa=X&scisig=AAGBfm0wb02YajMKHRVf0WWj36-Jiavf_g&oi=scholar. Accessed: July 14, 2014.
- 28 www.isixsigma.com. Process Capability (Cp, Cpk) and Process Performance (Pp, Ppk). http://www.isixsigma.com/tools-templates/capabilityindices-process-capability/process-capability-cp-cpk-and-process-performance-pp-ppk-what-difference/#defs. Accessed: July 14, 2014.
- 29 www.JMP.com
- 30 Garcia e. al. PQLI Key Topics: Criticality, Design Space, and Control Strategy, J Pharm Innov 2008; 3: 60-68.

- 31 Chatterjee S. Design Space Considerations. Presented at: AAPS Annual Meeting; 2012 Oct.
- 32 Piriou, et al. Control Strategy as the Keystone of the Product Lifecycle, from Product/Process Understanding to Continuous Process Verification and Improvement, Pharmaceutical Engineering 2012 Jan/Feb; 32(1).
- 33 CMC Biotech Working Group. A-Mab: A Case Study in Bioprocess Development; 2009 Oct 30. Available from: https://www.ispe.org/pqli/a-mabcase-study-version-2.1
- 34 Scott C. Quality by Design and the New Process Validation Guidance, Bioprocess International; 2011 May. 14-21.
- **35** Current Good Manufacturing Practice for Finished Pharmaceuticals. 21 CFR 211.110(c), 2013.
- 36 Current Good Manufacturing Practice for Finished Pharmaceuticals. 21 CFR 211.180(e), 2013.
- 37 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Organisation Of The Common Technical Document For The Registration Of Pharmaceuticals For Human Use M4. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2016 Jun 15 [cited 2016 Nov 21] 24 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/CTD/M4_R4_Organisation/M4_R4_Granularity_Document.pdf.

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CHAPTER 4:

Analytical Development

onoclonal antibodies are large complex proteins whose structural features can impact their biological activity and stability. As a result, multiple analytical methods are needed to characterize the many different structural and functional features of any monoclonal antibody product. Additionally, a variety of analytical methods are essential in the development and control of monoclonal antibody manufacturing processes including methods that can be performed on process intermediates that are in different buffers and contain more contaminants than the final product. Analytical methods used in the analysis and characterization of monoclonal antibody process intermediates and final purified products include methods to determine the protein identity, purity, safety, and potency as well as to investigate the multiple forms of the monoclonal antibody that may be present.

Some methods used in analyzing monoclonal antibodies, such as the determination of protein concentration by a dye-binding assay, by absorbance of UV light at 280nm, or SDS-PAGE are commonly used and easily applied without modification to most monoclonal antibody products. Methods that are dependent on the specific antibody sequence and structure, such as chromatographic methods to analyze identity and/or purity, require some adaption for each monoclonal antibody, while other methods, such as peptide mapping or methods to investigate post-translational modifications, often require extensive development or customization for each antibody product. Each analytical method used in the characterization or testing of a monoclonal antibody product should be chosen and optimized based on careful consideration of the anticipated critical quality attributes (CCP) of the product and the ability of the analytical method to precisely, accurately and robustly measure one or more of these attributes.

1. Structure of Antibodies

The activity of a monoclonal antibody can be significantly impacted by changes in the antibody structure making it important to understand which structural features of the protein are likely to contribute to the product's potency and stability and in developing appropriate analytical methods to monitor and assess these structural characteristics during various stages of development.

The primary structure or amino acid sequence of a monoclonal antibody product should be verified when an initial cell line is constructed or when a new cell line or process is developed. This primary sequence should not change during processing or storage of the monoclonal antibody product although post-translational processing of the heavy and/or light chain may result in product variants with an altered N- or C-terminal sequence. Some amino acid sequences are prone to spontaneous cleavage resulting in clipped forms; for example, aspartyl-proline bonds are especially sensitive to acidic conditions. Formation of these internal clips can be affected by factors such as pH and may change during storage.

Degradation of various amino acids in the primary sequence may also lead to charge variants not generally considered to have changed the primary sequence. For example, deamidation of asparagine or glutamine to form aspartic or glutamic acid will increase the net negative charge of the product. The molecule should be monitored during development and storage of the monoclonal antibody product for these changes, however, these charge variants are not considered changes in the primary structure of the product.

Monoclonal antibodies contain multiple different secondary structure elements throughout each chain of the protein, which help to minimize energy within the protein and stabilize the monoclonal antibody's three dimensional, or tertiary, structure. This complex tertiary structure in which the protein is fully folded into its most stable and active conformation brings together regions of the monoclonal antibody, which may be widely separated in the primary sequence to form the antigen binding regions of the monoclonal antibody. The antigen binding sites of the monoclonal antibody result from a juxtaposition of the different short complementarity determining regions on both heavy and light chains.

The tertiary structure involving the C-terminus of the two heavy chains forms the Fc region, which is important in binding to its target. For optimal activity, it is essential that the tertiary structure of the monoclonal antibody product be maintained to allow maximal binding to the antibody's target and provide the desired therapeutic activity. The fully active antibody is formed when the two heavy and two light chains are associated; this association of several proteins or peptide subunits is referred to as the quaternary structure. Tertiary and quaternary structure are established and maintained by a number of interactions across different parts of the protein. These interactions including hydrogen bonding, disulfide formation, and ionic interactions of the amino acid side chains. Since the activity of an antibody product is highly dependent on these higher order structures, analytical methods that can measure changes in tertiary and quaternary structures must be developed and included in antibody development programs.

Understanding the links between higher order structure (secondary, tertiary, and quaternary structure) and product

quality is key to the successful development of monoclonal antibodies and may be a critical element of the regulatory filing process. A Quality by Design (QbD) developmental approach (see Chapter 3) can facilitate the requirements for extensive product and process understanding, employing higher order structure characterization, which is a requirement in and of itself.¹ An understanding of higher order structure, and the environmental changes that may alter the structure, can help in the definition of design space and assist in the eventual implementation of changes to the manufacturing process.

In addition to the structure formed by the amino acid chains, post translational modifications of individual amino acid side chains also impact the function of antibody products. Glycosylation, a post translational modification found on most antibodies produced in mammalian cells, is important in certain antibody functions that may contribute to the therapeutic activity of a specific product. In addition, glycosylation can impact distribution and biological half-life of an antibody, so demonstrating of consistent glycosylation of antibody products throughout development is a major concern of companies and regulatory agencies.

Other posttranslational modifications include, among others, oxidation, disulfide shuffle, cleavage of the N-terminal amino acids during production, and deamidation of asparagine and glutamine. Each protein strand of a monoclonal antibody may be modified in a different manner leading to a significant variability in the structure of the 'pure' monoclonal antibody. Kozlowski and Swann have estimated that there may about 108 variants of a monoclonal antibody based on combinations of possible post-translational variation.² It is the impact of this inherent structural variability on the behavior of a monoclonal antibody drug that makes the application of suitable analytical methodologies so challenging and necessary. This variability requires the use of many orthogonal analytical methods to examine the structure of a monoclonal antibody during development, manufacturing, release, and stability testing, and in examining the degradation pathways of the antibody in the potential product formulations.

2. Regulatory Requirements for Analytical Methods

The complete analysis and characterization of a monoclonal antibody product is a requirement for development of these products worldwide. Regardless of the stage of development of a monoclonal antibody product, sufficient testing of the product must be performed on each batch to demonstrate the identity, purity, safety, and potency of the drug and the consistency of the product between different batches that are manufactured to support pre-clinical and clinical development. The exact level of testing required and the details of the characterization of the monoclonal antibody product expected will vary throughout the development of these products, with greater emphasis placed on detailed product characterization during later stages of development.

In the US, the requirement for analytical testing and characterization of a new monoclonal antibody product is outlined in 21CFR Part 610 as it is for all investigational products.³ For approved products, the requirements for inprocess and final release testing are further defined in 21CFR210 and 21CFR211.^{4,5} Recently, FDA acknowledged that during the development of a new drug, full compliance with the regulations in 21CFR210 and 21CFR211 is not required for Phase 1 investigational materials and should be applied later in the development process and, in July, 2008, issued a guidance specifying that 21CFR211 no longer applies to Phase 1 investigational drugs.⁶

The Phase 1 guidance document places emphasis on the early validation of analytical methods used to assure the safety of the product, including sterility and the control of endotoxins to low levels. While acknowledging that many of the methods used to analyze a new drug may not be validated during early product development, FDA requires manufacturers or sponsors of new products to control any aspect of manufacturing that is reasonable, including exercising control over raw materials used in the manufacturing process.

For monoclonal antibody products under development in the EU as well as the US, the requirements for the development and validation of analytical methods required for testing and characterization of a new monoclonal antibody product are outlined in a series of ICH guidance documents listed in Table 4.1.^{7, 8, 9, 10, 11, 12} These guidance documents are intended to harmonize countryspecific regulations and provide a common format for regulatory submissions.

Table 4.1. ICH Guidance Documents
Covering the Testing and Characterization of
Monoclonal Antibody Products

Document Number	Document Title
ICH Q2	Validation of Analytical Procedures: Text and Methodology
ICH Q5A	Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin
ICH Q5B	Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of r-DNA Derived Protein Products
ICH Q5C	Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products
ICH Q5D	Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products
ICH Q6B	Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products

ICH Q6B outlines the setting of product specifications for biologic products, including monoclonal antibodies. The specifications for a monoclonal antibody product will include a list of specific analytical procedures to be used to test the bulk drug substance and final drug product along with appropriate numerical limits, ranges, or other criteria for each of these tests. During the early stages of product development, these specifications for a monoclonal antibody product may have wide ranges or broad limits due to the limited number of batches produced and the limited amount of data available for setting the specification. As the development of the product proceeds these specifications may be adjusted, either by reducing or changing the spread of acceptable test limits, to reflect the better understanding of the product, the larger dataset available on which to finalize specifications, and improvements in the analytical methods used in early product development that improve the accuracy or precision of the method. It is also likely that additional analytical methods and specifications may be introduced for testing and release of the product. Recently the FDA and EMA issued additional guidance for biosimilars.^{13, 14}

In addition to conforming to all the recommendations and requirements outlined in the ICH guidance documents, as of July 2009, monoclonal antibody products under development in the EU must also conform to the EMA guidelines on development, production, and characterization of monoclonal antibody products.¹⁵ This document provides detailed recommendations for the characterization of a new monoclonal antibody product and the setting of specifications for release testing. In addition, the European Pharmacopoeia (EP) now includes a general monograph that provides guidance on the inprocess and final product release testing required for monoclonal antibody products.¹⁶

In the US, there is no comparable United States Pharmacopoeia (USP) guideline or monograph specifically for monoclonal antibody products. However, many of the tests that may be applied to the characterization and testing of monoclonal antibody products are now defined in various chapters of the USP. An outline of testing requirements and references to the specific USP chapters describing the tests for biotechnology derived drug substances and drug products, are contained in chapter charts 2 and 5 of the USP.^{17, 18} The USP methods below <1000> are considered to be pre-validated and may, therefore, be used for the release testing of a new monoclonal antibody product based on a verification of suitability testing rather than full validation.^{19,} ²⁰ Compendial methods above <1000> provide guidance for characterization of monoclonal antibodies.

Analytical methods used to characterize and release a monoclonal antibody product during early-stage clinical development must be suitable to provide information on the quality, safety, and potency of the product.²¹ Which analytical methods are required varies with the critical quality attributes (CQA) of the monoclonal antibody and to a lesser extent with the stage of development.^{22, 23, 24} Normally, method validation is not performed prior to Phase 1 clinical trials though some companies validate methods for potency assessment early. Early in method (and product) development, regulatory expectations can be met sufficiently by demonstration that an analytical method is fit for its intended purpose as outlined by the Eurachem working group.²⁵ For use in early-stage development of a monoclonal antibody product, an analytical method may be considered fit for its purpose if it has been demonstrated to be scientifically sound and precise, even if the method has not been fully validated.

3. Analytical Method Development and Validation

The complexity and diversity of analytical methods available to support control of monoclonal antibody products at all stages of process development and manufacturing is continually increasing. Therefore, the development and execution of analytical methods is an important core competency for biopharmaceutical companies or contract manufacturing organizations that develop and manufacture monoclonal antibody products. The development of suitable analytical methods for the testing and characterization of monoclonal antibody products provides the foundation for other critical development and manufacturing activities, including process development, formulation development, quality control, and stability assessment, (see Figure 4.1). All of these critical activities require robust and reliable analytical methods to ensure their proper and timely execution, making the development of suitable analytical methods an essential part of any monoclonal antibody development program. Inadequate analytical method development can result in a bottleneck in the development of a monoclonal antibody product and delay the initiation of first-in-human clinical trials.

Analytical Method Development and Qualification

The goal of analytical method development is to "conduct the right science at the right time", while being able to implement any necessary changes during development. Analytical methods must be adequately developed, qualified, and subsequently validated, so that they meet their pre-established goals on a consistent basis. Not all methods require qualification, and what might be called qualification can sometimes occur during development. There is no universally accepted definition for qualification, while there is for validation. Analytical methods can be developed using a traditional one factor at a time (OFAT) or QbD approach, or a combination of the two. While all method development is iterative, analytical QbD is a lifecycle approach that mirrors QbD for process development, embracing the concept of starting with a clear goal in mind. To speed method development, using an enhanced QbD approach to method development, is recommended for most monoclonal antibody development programs.

Figure 4.1 Analytical Methods Lifecycle

The critical activities needing robust and reliable analytical methods to ensure proper and timely execution. The development of suitable analytical methods is an essential part of any monoclonal antibody development program.



Source: BPTC

Analytical Method Selection

Method selection, and subsequent development activities, should be a collaborative effort between those developing the method and those who will eventually use it.^{27,28} Method selection should take into consideration the capability of the method in regards to the intended use and phase of development.

Method development occurs in parallel with process development and the criteria of the method should reflect the needs of the antibody development program. Method development starts with the identification of the method design intent, which establishes what the method must do. Design intent should be based on prior knowledge elements, including literature and prior knowledge based on similar drug substances and products.

Method performance criteria and method operational intent are critical components of method design.²⁷ CQAs and specification limits are used to establish method performance criteria. These criteria are developed through understanding the product and control requirements.

Method Development

The development stage is based on the method performance criteria and intended function of the method. It is essential for analytical methods to meet the established method performance criteria over the course of their lifecycle. Elements of the method that need to be controlled must be identified so that risk can be reduced. Risk assessment tools should be used to ensure that the method will meet all of the required criteria. Risk assessment can be used to establish method CQAs, or critical method attributes, and method CPPs, critical method parameters.

During the development stage, capability limits for the method are set, which will be used to create test method limits and controls and support setting specifications for the product.²⁹ Risk assessment is part of this process. Cross-functional teams, representing those who will be using the method during normal operations, should be involved in all risk assessment activities.

Development data should be gathered under conditions as close to cGMP as possible, recognizing that most development labs do not operate under full cGMP. Good Documentation Practices (GDP) should be followed as well. Properly trained personnel working with appropriately qualified equipment are essential.

Method optimization is frequently part of method qualification. This activity identifies the critical method parameters and determines limits for the method or sample. DOE studies performed during method development may be used to support appropriate criteria for more detailed DOE analysis.³¹

Method Qualification

Preparation of a formal analytical method qualification report is not required, but may be performed prior to analytical method validation. Understanding the difference between qualification and validation is essential. Qualification determines whether a method is suitable for it intended purpose based on limited performance criteria. Analytical method validation, a regulatory requirement, assures that the method is suitable for its intended purpose on a routine basis based on pre-determined performance criteria.³² Qualification studies may take place in the development lab, while validation studies are often performed under cGMP by QC or dedicated validation personnel.³⁰

Analytical Method Validation

Analytical method validation (AMV) is defined as the process of assuring that an assay is suitable for its intended purpose on a routine basis based on pre-defined assay performance criteria.³² Analytical methods are validated similarly to manufacturing processes. Analytical method validation (AMV) includes the evaluation of documentation gathered from method development through routine quality control testing.³³ This process of evaluation assures that an analytical method is capable of consistently performing as it is supposed to, based on strong scientific evidence.

The operational intent of an analytical method can be considered starting with the definition of the product Quality Target Product Profile (QTPP). The intended use should be taken into account over the course of the method's lifecycle. It is important to note that the intended use may change as the product evolves.³⁴

Assessment of Validation Readiness

The assessment of validation readiness consists of risk assessments that determine whether or not an analytical method is ready for validation. The validation readiness assessment considers the operational intent, patient risk, production process, process capability and desired method performance of a developed and qualified (if applicable) method. These elements are evaluated to determine the readiness of a method to move forward into validation and provide the foundation for AMV studies. Figure 4.2 shows the validation readiness process.

Figure 4.2 Method Validation Readiness Flow Path

The flow of requirements and overall process for analytical methods validation. Validation readiness assessment considers the operational intent, patient risk, production process, process capability and desired method performance of a developed and qualified method. Reprinted with permission of PDA, Inc.: Technical Report No. 57, "Analytical Method Validation and Transfer for Biotechnology Products"



The upper limit of the AMV protocol acceptance criteria is compared to past method performance using development reports, qualification reports (if available), transfer reports, assay control charts and any other available data. The method is considered to be ready for validation if its past performance exceeds the maximum performance limits and the chances of not meeting the acceptance criteria are low.

Specifications, regulatory requirements, and prior product and process knowledge are evaluated to determine the intended use of the method. The AMV acceptance criteria are established based on this document. The validation risk assessment evaluates the method based on its ability to meet the intended use. Data representing the ICH Q2(R1) validation characteristics, including, accuracy, precision, specificity, detection limit (DL), quantitation limit (QL), linearity, and range are considered along with robustness data, standards and controls, and stability and samples to create a summary of the method performance characteristics.7 This report, along with the AMV acceptance criteria, is used to execute the validation risk assessment.³⁴

Risk assessments play a key role in determining the amount of AMV studies required. The goal of risk assessments in

evaluating method readiness for validation is to; determine the amount of AMV studies needed, and determine the method performance level required as determined by the acceptance criteria.

The of AMV study required is determined by assessing the method by categorizing it as class A through E. Methods assessed as class A, the highest risk category, require the most study. Class A methods pose the most risk to patient safety, and are marked by high levels of uncertainty. Methods are considered to be class B-D if they are previously validated or based on platform technology. Class E methods are compendial methods and require the least amount of study.³⁴

Validation Acceptance Criteria

Acceptance criteria are; "Numerical limits, ranges, or other suitable measures for acceptance of the results of analytical method validation that is satisfied to determine suitability of test method performance."¹² There are two key considerations that should be kept in mind when setting validation acceptance criteria. The desire to show a high level of process/method capability within the target specifications may lead to setting the acceptance criteria too narrow,

making it difficult to meet the criteria. Conversely, the desire to assure compliance by passing all of the acceptance criteria may result in the acceptance criteria being set overly wide. A careful balance must be established in order to set appropriate acceptance criteria. Potential sources of variation and uncertainty should be taken into careful consideration when determining the acceptance criteria.³⁴

AMV protocol acceptance criteria that is risk-based should come from target specifications, prior knowledge elements, and, when relevant, regulatory expectations. Acceptance criteria should be set to guarantee the lowest acceptable limit of method performance.34

Validation

ICH Q2(R1) describes the validation procedure for the four most common analytical methods. These are: identification tests, quantitative tests for impurities content, limit tests for the control of impurities, and quantitative tests of the active moiety in samples of the drug substance or drug product.⁷ Table 4.2 shows the validation parameters for each type of test as prescribed by ICH Q2(R1).

Table 4.2 Minimum AMV Characteristics from ICH Q2(RT)				
ICH Q2(R1) Category (Test)	l (Identification Test)	ll (Quantitation of Impurities)	III (Qualitative Limit Test for Impurities)	IV (Quantitation of Active Ingredient)
Accuracy	No	Yes	No	Yes
Repeatability Precision	No	Yes	No	Yes
Intermediate Precision	No	Yes	No	Yes
Specificity	Yes	Yes	Yes	Yes
Linearity	No	Yes	No	Yes
Range	No	Yes	No	Yes
Limit of Detection	No	No	Yes	No
Limit of Quantitation	No	Yes	No	No

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Accuracy describes an analytical method's ability to represent the relationship between the true value, which is accepted as a conventional truth, and the found value of a sample.⁷ Accuracy is often determined through the use of a reference sample. There can be significant variation in the ways in which accuracy is measured during AMV, depending on the type of method.³⁴

4. Analytical Methods for In-Process, Release, and Stability Testing

Since no single attribute can completely demonstrate the safety, potency, and quality of a monoclonal antibody product, many of the attributes of the product must be measured using a combination of analytical methods. Some of these analytical methods will be used for in-process testing during bulk drug manufacturing, some will be used only for release testing of the bulk drug substance and final drug product, and some will be used in the stability testing of the monoclonal antibody drug substance or drug product. Additional analytical methods may also be used more infrequently to characterize the protein, for example to determine the detailed structural features of a monoclonal antibody product or to fully characterize the reference standards used for testing and release of materials.

Like all pharmaceutical products, each batch of monoclonal antibody product intended for use in clinical trials must be tested for identity, purity, safety, and potency. The identity may be demonstrated using a single assay, as long as the methods are sufficient to confirm that the product is the intended product, if not a combination of two or more analytical methods may be required. The level of specific impurities of a monoclonal antibody is demonstrated through a variety of assays that measure the purity and/or the level of specific impurities that may be present. Safety is assessed primarily by measurement of the microbiological purity of the product, the level of bacterial endotoxin present, and the level of particulates present in the final drug product. These essential assays are required by all regulatory agencies and in some cases additional safety assays may be required. Potency is measured using one or more methods that relate to the biological activity of the product, including those that demonstrate binding to the target18 or cell-based methods that assess biological impact in a reporter cell based system.¹⁹ Potency assays should relate to the biological activity of the monoclonal antibody. While a binding assay may be acceptable in early development, if the biological activity of the monoclonal antibody is cytotoxicity then the regulatory expectation is the development of an assay, which measures cytotoxicity.

Identity Tests for Monoclonal Antibodies

Identity testing of a monoclonal antibody is intended to show that the antibody that was manufactured is the intended monoclonal antibody and may require the use of several different analytical methods.7 Identity testing is normally performed on the drug substance as well as on the drug product. Many of the methods used to determine identity include comparison of the sample to a suitably characterized reference standard analyzed at the same time and under the same conditions. Some of the analytical methods normally used for identity testing of monoclonal antibody products along with the typical specifications for release of these products for Phase 1 human clinical trials are listed in Table 4.3. Note that not all of the analytical methods listed in Table 4.3 will necessarily be used for each monoclonal antibody product. The specific analytical methods used for a given monoclonal antibody product will depend on the specific product, its intended use, and prior experience with monoclonal antibody products for similar clinical indications. In addition to the methods listed, Western blotting to confirm identity is sometimes used for monoclonal antibodies, but is more often used for other types of recombinant protein products.

Table 4.3 Some Methods Used for Identity Testing of Monoclonal Antibody Products		
Method	Physicochemical Information	Typical Specification for Early Stage Clinical Trial Material
SDS-PAGE, reduced	Number of chains and approximate MW; comparison to reference material, purity	Two major bands corresponding to approximately 50 kDa and 25 kDa and comprising ≥95% of total stained area Major bands correspond to reference standard
SDS-PAGE, non-reduced	Approximate MW of intact antibody, purity	One major band corresponding to reference standard
Peptide Map	Number, size and nature of peptides released by limited enzymatic or chemical digestion	Corresponds to reference standard

Polyacrylamide gel electrophoresis (PAGE) can separate the antibody product into the heavy and light chains after treatment of the product with sodium dodecylsulfate (SDS) and a reducing agent such as dithiothreitol (DTT), which disrupts the inter-and intra-chain disulfide bonds.¹³ The SDS binds along the length of the protein chains and imparts a strong negative charge. The amount of the negative charge is proportional to the number of SDS molecules bound and in turn, this is proportional to the size of the protein chain. The treated chains are separated by means of an electrical field applied across a gel bed; the rate of migration is proportional to the charge and hence to the molecular weight of the protein(s). The gel bed consists of cross-linked polyacrylamide strands and the amount of cross-linking and polyacrylamide can be adjusted resulting in gels that are suitable for different molecular weight ranges. The proteins are visualized in the gel by reacting with a stain specific for proteins and the molecular weight determined by comparison to the migration of standard proteins of known

molecular weights run at the same time. The relative purity of the bands can be estimated by calculating the proportion of the product band(s) relative the total stained bands.

While SDS-PAGE is commonly used for the determination of the molecular weight of proteins, the method does have some disadvantages. The molecular weight determined by this method is only approximate. Control of the method to ensure consistent and accurate results requires control of the protein concentration of the sample, the gel utilized for separation, the strength and time of application of the electric field used, and strict control of both the staining and de-staining process. In addition, complete disruption of the three-dimensional structure of the antibody must be performed in order to obtain meaningful results. When utilized solely for identity testing, staining variability is of less concern since the reference material is run at the same time and on the same gel and is, therefore, affected by the same variations in the method. However, when the method is used to determine purity, the large number of parameters that can impact performance complicates the validation of this method and the development of suitable specifications. In particular, when this method is used for purity, it is important to have system suitability standards to control the variability of the method. Moreover, some dyes used for staining may give different staining intensities for different proteins leading to misinterpretation of the purity.

SDS-PAGE, non-reduced

When the antibody is treated with SDS without a reducing agent, the intermolecular disulfide bonds are not disrupted and the monoclonal antibody will not be dissociated into heavy and light strands. The proteins are still separated by migration in an electric field in a manner proportionally to the amount of negative charge, which is a function of the MW of the protein. Since the protein is not completely denatured because the disulfide bonds remain intact, the binding of the SDS is not as complete and determination of the molecular weight is not accurate. This method does have an advantage for visualizing aggregated forms held together by intermolecular disulfide bonds, or other covalent interactions.

As with reduced SDS-PAGE, this method has a number of disadvantages. When used for simple demonstration of identity these are overcome by inclusion of the reference standard on the same gel. SDS-PAGE, non-reduced, is also sensitive to the same kind of variability as the reduced SDS-PAGE and the development of suitably precise specifications is quite difficult. Because most monoclonal antibodies are of similar molecular weight, neither of these methods is specific enough to demonstrate that the intended monoclonal antibody is present. Despite better resolution, this method is frequently replaced by size exclusion chromatography for determination of purity due to the inherent variability of the non-reduced SDS-PAGE.

Peptide Map

Peptide mapping demonstrates that the number and characteristics of peptides released by partial digestion of a monoclonal antibody conforms to that seen when the reference material is subjected to the same digestion and separation of the peptides.^{14, 15} To perform a peptide map analysis, the protein is digested into peptides in a controlled manner using chemical and/or enzymatic digestion methods. After digestion the peptides are separated, most commonly by high performance liquid chromatography (HPLC), and the resulting pattern is compared to the results from analysis of the reference standard. For more detailed structural information on the monoclonal antibody product, the separated peptides in the peptide map may be subjected to Mass Spectrometry (MS) to determine the mass of each peptide for confirmation of the protein structure.

The digestion conditions used for peptide mapping must be well controlled to ensure consistent digestion and to minimize the assay-to-assay variability of the method. Identification of suitable digestion conditions includes selection of an enzyme, to provide enough peptides to allow the protein to be well covered, but not so many peptides that separation would be difficult. The most common enzyme used for peptide mapping is trypsin, which cleaves the protein on the N-terminus of arginine or lysine residues. Since trypsin is used so commonly, peptide mapping is often referred to as tryptic mapping. Including reference material digestion in each analysis provides some control over variability, but validation of a peptide map is still complicated by the variability inherent in the method.³⁷

Analytical Methods for Measuring Purity and Product-Related Impurities

The demonstration of purity as well as the levels of specific impurities is required for all monoclonal antibody products. The analytical methods used for determining product purity should include methods for quantifying the different product-related substances (active and inactive variants that may be present in the final product) as well as both process- and product-related impurities.¹² Product-related impurities in a monoclonal antibody product may include oxidized and deamidated forms of the antibody, high molecular weight aggregates, and low molecular weight degradants of the product. Some of these variants are the result of heterogeneity in the monoclonal antibody while others may be formed during the production or storage of the monoclonal antibody product.

The similarity of product-related impurities to the monoclonal antibody product requires the use of methods that can separate and quantify proteins of very similar structure. Chromatographic methods are frequently used for the measurement of product related impurities. However, since no single chromatographic method can separate all of the different potential product-related impurities that may be present in a monoclonal antibody product, multiple tests may be required to sufficiently demonstrate the purity of the monoclonal antibody product. Some of the most commonly used analytical methods used to determine the purity of a monoclonal antibody product and product-related impurities are listed in Table 4.4 along with the typical specification for each test of clinical trial material for early stage human clinical trials.

The analytical methods used to measure the purity and impurities of a particular monoclonal antibody product will be specific for each monoclonal antibody product so that not all the tests listed in Table 4.4 are necessary for demonstration of the purity of every monoclonal antibody product. Identification and quantitation of a product related form does not provide information on whether it is an active form of the product or inactive, resulting from degradation of the product. However, by measuring the forms present and correlating this information with measurements of potency some indication of the activity the variants can be obtained.

Nonoclonal Antibody Products		
Method	Physicochemical Information	Typical Specification for Early Stage Clinical Trial Material
Size Exclusion Chromatography	High molecular weight forms, low molecular weight forms and monomer	≥95% Monomer
Reverse Phase Chromatography	Purity, especially oxidized forms	≥95% Monomer
lon Exchange Chromatography	Charge variants	≥95% Main peak
Isoelectric Focusing	Charge variants	Conforms to reference standard
SDS-PAGE, reduced	High molecular weight forms, low molecular weight forms and monomer	Two major bands corresponding to approximately 50 kDa and 25 kDa and comprising \geq 95% of total stained area
SDS-PAGE, non-reduced	High molecular weight forms, low molecular weight forms and monomer	\geq 95% Main band of the total stained area
Carbohydrate Content	Monosaccharide content	Report result
	Sialic acid content	Report result

Size Exclusion Chromatography

Size exclusion chromatography (SEC) separates proteins according to their hydrodynamic radius, which for most globular proteins is directly related to molecular weight. This method is routinely used to determine the amount of monomer as well as high molecular weight aggregates and low molecular weight fragments of the monoclonal antibody product.¹⁶ In contrast to SDS-PAGE, which separates on the basis of molecular weight, SEC does not require disruption of the three-dimensional structure of the molecule and therefore is suitable to separate and quantify non-covalent aggregated forms. SEC has become a workhorse technique during process development because the method has a fairly high throughput and can be robust and easy to validate. Different detectors can be applied; commonly a UV detector is used, but light scattering or fluorescence detection can also be used providing a relatively simple method to quantify the varying forms.

While SEC is routinely used to demonstrate the purity of monoclonal antibodies, there is a limit to the concentration that can be injected in a single sample. With the high concentration formulations currently in use, the need to dilute samples may cause disassociation of aggregated antibody, resulting in an underestimation of the amount of aggregate present. Therefore, the amount of aggregate present should be confirmed by one or more orthogonal characterization method such as analytical ultracentrifugation, multi-angle laser light scattering or fast flow fractionation, discussed below.

Reverse Phase and Hydrophobic Interaction Chromatography

Analytical reverse phase high performance liquid chromatography (RP-HPLC) is a powerful chromatographic technique with good resolution and precision that is capable of separating the heavy and light chains of the monoclonal antibody for analysis.¹⁷ RP-HPLC separations are based on the relative hydrophobicity of the protein chains of the monoclonal antibodies. The sample dissolved in an aqueous solution is applied to a column and the protein is eluted with an increasing gradient of an organic buffer containing an ion pairing reagent to minimize non-specific interaction with the resin. The column contains particles, usually of silica, from 3 to 5 micrometers, that have been coated with carbon chains of a defined size; the carbon chains can range from C2 to C18 offering very different column selectivity. By combining the selectivity of the column and the gradient of the organic buffer, it is possible to obtain separations of closely related antibody forms that cannot be readily separated by other methods. However due to the nature of the buffers used in RP-HPLC, organic and aqueous solutions at very low pH, antibodies may dissociate into heavy and light chains resulting in two major peaks in the chromatogram making it more difficult to use this method to determine purity.³⁹

Similar to RP-HPLC, hydrophobic interaction chromatography (HIC) also separates proteins based on their relative hydrophobicity. HIC is performed in an aqueous system in which a high salt content, such as ammonium sulfate, is gradually reduced. Since HIC doesn't require utilization of an organic buffer nor an extreme buffer pH to achieve separation the heavy and light chains, the monoclonal antibody is less likely to dissociate during the separation so it elutes as a single peak.¹⁸ RP-HPLC or HIC can be used to examine the purity of the antibody, while RP-HPLC is commonly be used to separate the peptides in a peptide map.

Both RP-HPLC and HIC most commonly use UV absorbance of the column eluant for detection and quantitation of the proteins eluted from the column. The chromatographic elution conditions must be developed carefully to ensure that the various peaks eluted are sufficiently separated and that there is little peak broadening, which interferes with the quantitation of the peak. If UV absorbance is not sensitive enough to detect the proteins eluting from the column or if the solvents used interfere with detection by this method, other detection methods, such as refractive index or fluorescence can be used.

Ion Exchange Chromatography

Ion exchange chromatography is a powerful analytical method where separation is based on charge differences.¹⁹ The method requires sample loading at low conductivity and in a buffer at least one pH unit away from the pI of the protein. Separation can be done using either anion exchange or cation exchange columns. In ion exchange chromatography, the positive or negative charges on the protein are bound to the oppositely charged resin. The proteins are eluted from either anion or cation columns by gradually disrupting this binding either by slowly changing the pH of the buffer or, more commonly, by adding a gradient of a counter ion that displaces the bound

protein. The most common counter ion used is sodium chloride, which can be used with either anion or cation chromatography. The technique is well suited for separation of charge variants of the monoclonal antibody with high resolution and good precision. The charge variants may be due to deamidation, oxidation, protein hydrolysis, changes at the C or N terminus, loss of three-dimensional structure, or reduction of disulfide bonds.

Sample preparation for ion exchange chromatography may require dilution or a buffer exchange of the sample to lower the conductivity and adjusting the pH so that the protein will bind to the column. Additionally, the dilution of the sample may lower the protein concentration so that the sample must be concentrated before applying to the column. These sample manipulations may alter the protein and reduce the accuracy of the results of the analyses.

Isoelectric Focusing

Charge differences may also be analyzed using isoelectric focusing (IEF), which is used to examine charge heterogeneity, an important quality attribute of a monoclonal antibody. Using either flatbed IEF or the newer capillary IEF (cIEF), the various proteins are separated based on their net charge. The flatbed method uses a gel similar to that used in SDS-PAGE, but containing ampholytes, chemicals which create a pH gradient in the gel when the electrical current is passed through. The proteins move through the gel until reaching the region where the pH is near their pI where they concentrate (focus). This method can separate proteins of the same size, but with very small differences in charge, such as when an asparagine converts to an aspartic acid adding one positive charge to the molecule. In flatbed IEF the separated bands in the gel are stained in a manner like that used in SDS-PAGE. In cIEF the proteins are swept out of the capillary after focusing and detected with a UV detector. The result of both methods is a series of closely related bands (IEF) or peaks (cIEF), which indicate the number of differently charged proteins present. By comparison to a reference material run at the same time, it can be determined if the antibody is degrading to form more basic or acidic compounds.

As with IEC, use of IEF is very sensitive to the nature of the sample, especially the presence of relatively high concentrations of salt. Since the IEF separation is based on the pI of the protein it often provides better separation of proteins with small differences in charge than does IEC, which is more dependent of the molecules surface charge. IEF also allows the pI of the bands to be determined by comparison to a series of pI standards run in the same gel. IEF can be a difficult method to validate, though the newer cIEF methods have overcome this problem to some extent.

SDS-PAGE

Product purity and product-related impurities can also be measured using reduced and non-reduced SDS-PAGE. The uses and limitations of this method for determining purity as well as identity have already been discussed above. SDS-PAGE, as with most methods, which evaluate productrelated impurities, provides information on both the level of specific impurities as well as the purity of the antibody.

Carbohydrate Content

All monoclonal antibodies have a conserved N-linked oligosaccharide on Asn297 of the heavy chain as well as several other potential sites for glycosylation. Variability in the structure and content of the oligosaccharides present at each potential glycosylation site results in microheterogeneity of the monoclonal antibody product. Each monoclonal antibody product may contain productrelated variants ranging from forms of the antibody that contain no glycosylation at a particular site to variants resulting from the presence or absence of sialic acid or fucose. Therefore, it is important to determine the total carbohydrate of a monoclonal antibody product as part of the overall product characterization.

Carbohydrate content is determined by first cleaving the carbohydrate from the protein with the enzyme PNGase F ((peptideN4Nacetyl-beta-D-glucosaminyl) asparagine amidase) or other endoglycosidases, treatment of the released glycan with 2 N acidic acid to remove sialic acid, and then hydrolysis of the glycan with exoglycosidases or with acid (2 N trifluoroacetic acid or 6 N HCl) to release the monosaccharides. The individual monosaccharides are then separated and quantified by RPHPLC. For analysis, the monosaccharides may be derivatized with anthranilic acid, which provides a fluorescent tag for detection20, or analyzed directly by high performance anion exchange chromatography with pulsed amperometric detection.21 The sialic acid content of the monoclonal antibody product is determined by derivitization and separation of the sialic

acid residues from the glycan in a manner analogous to the analysis of the monosaccharides.

Differential Screening Calorimetry

Differential screening calorimetry (DSC) is a thermal analysis technique that examines how a protein's heat capacity (Cp) is altered by temperature. When a protein is heated, its structure begins to alter, starting at different points, until it is fluid. DSC measures the difference in heat required to raise the Cp of a protein sample in solution alongside a reference sample, with or without the protein, linearly as a function of temperature. The signal from the sample and the reference are heated at the same rate while being continuously monitored. The signal from both samples is converted to Cp as a function of temperature. The higher the melting point (Tm) of the protein, the more stable it is considered to be. Unlike small molecules, proteins denatured by DSC do not return to the original structure upon cooling and a sample cannot be subjected to repeated DSC analyses.

DSC has been used to examine protein thermodynamics, folding, and interactions, as well as protein thermal stability, overall conformation, and domain folding integrity.²² DSC can be used for structural analysis, purity testing, and stability testing for monoclonal antibody development and manufacturing. DSC is an accurate and precise tool for developing specific formulations and for supporting biosimilar decisions.

Analytical Methods for Measuring Process-Related Impurities

In contrast to product-related impurities, process-related impurities are impurities in the final product that are derived from the manufacturing process. Such impurities may include:

- Protein A leaching off the Protein A column
- Residual host cell protein and DNA
- Materials used during cell culture that may not be completely removed during purification;
- Materials which may leach from the container/closure system used to store either the drug substance or drug product
- Filter or other materials used during aseptic processing of the monoclonal antibody drug product

Acceptable levels for each impurity in the final drug substance or drug product should be specified and

appropriate analytical methods developed to measure their levels during final release testing of the drug substance or drug product. Testing for process-related impurities is generally limited to drug substance release testing unless a potential process-related impurity could be introduced during drug product manufacturing.

Determining the amount of process-related impurities present in a monoclonal antibody product requires the use of highly specific and sensitive methods for quantifying each impurity. For many of these impurities there are commercially available test kits that are suitable for use in measuring process-related impurities during process development and for testing clinical trial material for early stage human clinical trials. For the manufacture of material later in development and commercial sale, more specific tests designed to measure the processrelated impurity in the particular monoclonal antibody product may be required. If the removal of a specific processrelated impurity is validated at any stage of development, the routine testing of each batch of monoclonal antibody product may be eliminated. It is unusual to validate removal at very early stages of development.

A list of common process-related impurities and the analytical methods used to determine them are listed in Table 4.5 along with the typical specification for each test for clinical trial material for early stage human clinical trials.

Residual Protein A

Residual levels of Protein A in a monoclonal antibody product can typically be determined down to 1 ppm using commercially available ELISA test kits.23 However, before using a Protein A ELISA for measuring residual levels of this process-related impurity, it may be necessary to ensure that Protein A has been fully dissociated from the monoclonal antibody product to allow its detection. This dissociation is accomplished either by heating the sample prior to the ELISA, incubating the sample at acidic pH, or a combination of both. If heat treatment is applied consideration must be given that the analyte might co-precipitate with the IgG, resulting in poor precision and accuracy of the assay. It is also important to use the appropriate standard when measuring residual levels of Protein A using ELISA. Different Protein A affinity chromatography media use different forms of the Protein A ligand so that use of an inappropriate standard may lead to a bias (either positive or negative) in the assay. A recent USP monograph on Protein A attributes describes the appropriate reference standard to be used for measuring levels of the most common Protein A ligands used in large scale manufacturing today.24

Host Cell Protein

ELISA kits for measuring residual host cell protein (HCP) from CHO cells are commercially available that can measure levels of residual HCP as low as 2 ng/mL.25 For monoclonal antibody products produced in CHO cells,

Table 4.5 Some Methods Used for Measurement of Some Process Related Impurities		
Impurity	Method	Typical Specification for Early Stage Clinical Trial Material
Protein A	Protein A ELISA	<10 ng Protein A/mg product
Host Cell Protein	ELISA	<10 ng HCP/mg product
	Western Blot using Host Cell Protein specific antibodies	Report results
Host DNA	qPCR, DNA Threshold™ System	<50 pg DNA/mg product
Other Process- related Impurities	Specific to impurity being measured	Report results

these commercially available kits can be used to measure residual HCP during early process development and the manufacture of monoclonal antibody products for early stage clinical trials. During later stages of development, the regulatory expectation is that a product-specific ELISA will be developed using HCP derived from the production host cell line. Such a product-specific HCP assay should have a level of detection and quantitation at least as good as the commercially available kits. However, it is not uncommon to have an apparent increase of residual HCP by a factor of 10-20 when shifting to a product specific assay. Some manufacturers avoid this by having a well-characterized in house developed platform ELISA that can be qualified for all their products.

Host Cell DNA

Test kits for measuring residual DNA as low as 3 fg/sample are also commercially available.²⁶ These kits are based on either a quantitative PCR detection or the proprietary DNA Threshold System. As with the measurement of residual HCP, the measurement of residual DNA in monoclonal antibody products during process development and manufacture of early stage clinical trial materials is generally done using one of these assays. During later stages of development, the removal of residual DNA from monoclonal antibody products is generally validated to show reliable and consistent removal of the potential process-related impurity by the drug substance manufacturing process has been validated, the test for residual DNA in a monoclonal antibody product can usually be removed from the product's specifications.

Other Process-Related Impurities

Analytical methods for determining the amount of other process related impurities, such as antibiotics or insulin used during cell culture, may require significant analytical method development. Regardless of whether a commercially available test kit or a specifically developed analytical test method is used to quantitate levels of the process-related impurities in the final product, the method must be shown to be suitably sensitive and free interference from the product in detecting the impurity.

Safety Tests for Monoclonal Antibody Products

Throughout of the life cycle of a monoclonal antibody product it is imperative to demonstrate that the drug product is sterile and does not contain excessive levels of endotoxin or particulates. The monoclonal antibody drug substance is not usually sterile; however, it is necessary to demonstrate that the bioburden (microbial content) of the drug substance is low. The low bioburden provides assurance that the drug substance is not likely to be degraded or altered by microbial activity on the protein. The specific test methods for all of the safety tests required for monoclonal antibody products are defined in the various pharmacopeia and are summarized in Table 4.6 along with the typical specification for these tests for product intended for early stage clinical trials.

Table 4.6 Some Methods Used for SafetyTesting of Monoclonal Antibody Products		
Safety Test	Compendial References	Typical Specification for Early Stage Clinical Trial Material
Bioburden ^a	USP <61> EP 2.6.12	<10 CFU/10 mL
Sterility⁵	USP <71> EP 2.6.1	No evidence of microbial growth
Particulate Matter ^b	USP <787> EP 2.9.19	By Light Obscuration: $\leq 6000 \text{ per container at} \geq 10 \ \mu\text{m}$ $\leq 600 \text{ per container at} \geq 25 \ \mu\text{m}$
Endotoxin ^c	USP <85> EP 2.6.14	≤1.5 EU/mg
^a Test performe	ed on the drug subst	tance

b Test as aformed on the days and det

^b Test performed on the drug product

 $^{\rm c}$ Test performed on both drug substance and drug product

Potency Tests for Monoclonal Antibody Products

Monoclonal antibody products are tested to determine both the concentration of the protein in the final formulation and the potency, or biological activity, of the product (Table 4.7). Each of these parameters is measured separately using a variety of methods.

Table 4.7 Methods Used for Potency Testingof Monoclonal Antibody Products			
Method	Physico- chemical Information	Typical Specification for Early Stage Clinical Trial Material	
Concentration:			
Absorbance at 280 nm Dye binding assay	Concentration Concentration	Target concentration ± 10% mg/mL ^a Target concentration ± 10% mg/mL ^a	
Potency:			
Receptor binding Cell or animal based bioassay	Affinity for receptor Potency	Product specific Product specific	
^a Specific protein concentration will vary from product to product			

Protein Concentration

Measurement of protein concentration can be done by determining the absorbance at 280 nm and calculating the protein concentration according to the Beer-Lambert law and the extinction coefficient of the monoclonal antibody product. Early in development, the extinction coefficient of the product may not have been determined and either a calculated extinction coefficient is determined based on the amino acid sequence of the antibody or a standard curve using BSA or other protein is constructed and the concentration determined from the curve. The latter has the advantage of being very simple but may be inaccurate due to differences in absorbance between the monoclonal antibody and the protein used to create the standard curve. Later in development, the extinction coefficient of the monoclonal antibody product should be experimentally determined using quantitative amino acid analysis.

The protein concentration in the drug substance or drug product can also be determined by a dye-binding method such as the Bradford method or colorimetric methods such as BCA method or Lowry.27 In addition, protein concentration can be determined by quantitative amino acid analysis, though this method is less common as a routine method for concentration.

Potency

The potency of a monoclonal antibody product is often measured using multiple analytical methods, including both antigen binding and functional assays.² One method used to measure binding to the target antigen is enzyme-linked immunosorbant assays (ELISA). To perform an ELISA, the target antigen is attached to the surface of a 96 well plate and the test antibody or reference standard antibody is added to the well under conditions in which it can bind to the antigen. Excess antibody is removed and a reporter antibody that binds to the test antibody and that is linked to an enzyme (or other detection moiety) is allowed to bind. Finally, a substrate for the enzyme is added and binding is quantified by measuring a detectable signal released from the substrate. A more sensitive, but less widely used approach, to determine antigen binding is analysis using a Biacore or other sensitive instrumentation that deliver high quality data on kinetics, affinity and specificity for the target antigen.

Bioactivity assays that measure a specific response in cultured cells may be used as a surrogate assay to determine potency in place of costly and highly variable animal studies that are used in initial product discovery and lead optimization but are unsuitable for product release in a cGMP environment. Cell-based potency assays for monoclonal antibody products are generally specific to the antibody and often require significant effort to develop. Complement dependent cytolysis (CDC) can be measured by a non-radioactive method in which cells are plated and then treated with a reporter molecule.28 The treated cells are then transferred to 96-well microtiter plates and the antibody added. After further incubation, the supernatant from each well is transferred to another plate and the released reporter concentration determined by time-resolved fluorescence. Since this method requires specialized techniques and equipment to perform it may be run in a bioanalytical laboratory rather than the QC laboratory. These functional assays are normally required by the regulatory agencies unless a strong case for using antigen binding as the sole potency assay can be presented.

Other Tests for the Release of Monoclonal Antibody Products

The testing of monoclonal antibody drug products includes tests for the overall appearance of the product²⁹ as well as general properties of the solution such as pH³⁰ and

osmolality.³¹ These general tests are generally done using compendial methods so little or no development of the test is required before use. The exceptions to this are analytical methods used to determine excipient concentrations in monoclonal antibody drug products. Analytical test methods for some commonly used excipients are described in the various pharmacopeia. For those excipients that do not have an established compendia test, an appropriate analytical method, often HPLC or a colorimetric assay, must be developed to measure the concentration of the excipient. The general tests used for monoclonal antibody products, along with the typical specifications for these tests for products intended for Phase 1 human clinical trials, are listed in Table 4.8.

Table 4.8. Methods Used for Testing GeneralAttributes of Monoclonal Antibody DrugSubstance and Drug Product

Method	Physicochemical Information	Typical Specification for Early Stage Clinical Trial Material
Appearance	Description of the solution	Clear to slightly yellow solution essentially free of visible particulates
рН	pH of the solution	Target ± 0.5ª
Osmolality	Description of the solution	Target ± 10% mOsmª
Excipient Concentration ^b	Solution concentration	Target concentration ± 10% mg/mL°
Extractable Volume⁵	Content of vial	≥Label Volume

^a Range of specification may vary from product to product ^b Test performed on Drug Product only, may not be required

^c Excipient concentrations will vary from product to product

5. Analytical Methods for the Characterization of Monoclonal Antibody Products

In addition to the routine release tests performed on each batch of monoclonal antibody, further biochemical, and biophysical characterization of the product manufactured using the process intended to produce clinical trial material is essential to fully understand the detailed structure of the monoclonal antibody and to identify critical quality attributes of the product. This detailed characterization data can also be used to support formulation development for the product and assist in the demonstration of comparability of the monoclonal antibody product following scale-up, process changes, or facility changes. It does not need to be performed on each batch of antibody, and not all methods described herein are performed prior to Phase 1 studies.

The analytical methods used for the characterization of a monoclonal antibody product should provide as complete a description of the product's structure and variations as possible. As can be seen from the partial list of the methods commonly used to characterize monoclonal antibody products shown in Table 4.9, some analytical methods may be used for both product characterization as well as release testing. For example, peptide mapping may be used as a routine identity test since it provides a confirmation of primary structure of the monoclonal antibody product. However, peptide mapping can also provide more detailed characterization information on monoclonal antibody such as a confirmation of disulfide bonds in the antibody, presence or absence of such degradants as oxidized or deamidated forms of the protein, and the location and structure of various post-translational modifications such as glycosylation. Another method commonly used for both release and characterization is mass spectroscopy. In addition to the confirmation of the correct mass, protein sequence, and post-translational modifications, mass spectroscopy can also be used to identify other proteins, product-related or unrelated, present in the monoclonal antibody product and provide an orthogonal confirmation of the structure information determined by peptide mapping.

Table 4.5. Analytical methods used to characterize monocional Antibudy brugs		
Test Method	Structural Information Provided	
1. Characterization of Protein Structure		
Amino acid analysis	Primary structure	
N- and C-terminal sequencing	Confirm terminal sequences	
Peptide map, including identification of the peptides by MS or sequencing	Confirmation of post-translation modifications	
Free sulfhydryl and disulfide bridges	Examine disulfide scrambling	
Intrinsic Fluorescence Spectroscopy	Tertiary structure	
Extrinsic Fluorescence Spectroscopy	Tertiary Structure	
Mass Spectrometry, MALDI-TOF/Electrospray/LC-MS	Primary structure, post translation modifications, intact molecular weight identify impurities by mass	
Isoelectric focusing	Charge heterogeneity, deamidation, oxidation, amount of sialylation	
Fourier Transform IR Spectroscopy (FTIR)	Percentages of helical, $\beta\mbox{-sheet}$ and other structures	
Circular Dichroism (CD)	Percentages of helical, $\beta\mbox{-sheet}$ and other structures	
Nuclear Magnetic Resonance (NMR)	Conformational integrity	
Differential scanning calorimetry (DSC)	Structural stability	
2. Characterizati	on of Aggregation	
Laser light scattering, multi-angle (MALLS)	Identify aggregates and low molecular weight forms	
Field Flow Fractionation (FFF)	Identify aggregates and low molecular weight forms	
Analytical Ultracentrifugation (AUC)	Identify aggregates and low molecular weight forms	
3. Characterization of Glycosylation		
Demonstration of site(s) of glycosylation, occupancy of those sites and whether the oligosaccharides are N-or O-linked	Confirmation of post translational modification	
Carbohydrate content and structure	Structure and micro-heterogeneity of the oligosaccharides	

Table 4.9. Analytical Methods Used to Characterize Monoclonal Antibody Drugs

Characterization of Protein Structure

Amino Acid Analysis

Amino acid analysis identifies and quantifies the majority of the amino acids present in a protein sample after acid hydrolysis is used to break the peptide bonds between the individual amino acids.³² Acid hydrolysis is usually performed with 6 N HCl at 100°C for 16-24 hours. After release of the individual amino acids, they are derivatized either before or after separation by chromatography and then detected on-line. The amino acids are identified and quantified by comparison to a standard run at the same time. While this is a routine method available in most protein analytical laboratories, it has the disadvantage of inaccuracy for some amino acids. The amide-containing amino acids, asparagine and glutamine, are converted to the corresponding acid forms, aspartic acid and glutamic acid, and cannot be determined separately. Some other amino acids (serine, threonine, cysteine, and methionine) are partially degraded by the harsh digestion conditions and cannot be accurately quantified. One amino acid, tryptophan, is destroyed by the common acid hydrolysis method.

N- and C-Terminal Sequencing

Demonstration of the correct N-termini and C-termini for a monoclonal antibody is another means of confirming the primary structure of the product as well as determining if there are forms of the antibody that have been processed differently. C-terminal lysine variability and incomplete cleavage of the N-terminal leader sequence are frequent variants seen in monoclonal antibodies, and the presence of such variants will be detected using N-terminal and C-terminal sequencing. These are acceptable variants but they should be measured to insure consistency across monoclonal antibody batches produced at different scales or by different processes throughout development. N-terminal sequencing can usually be done on the intact monoclonal antibody using the standard Edman degradation method,³³ if the N-terminus is not blocked by pyroglutamatic acid formed by cyclization of the N-terminal glutamic acid residue. Normally the N-terminal five to ten amino acid residues are determined for both the heavy and light chain and the resulting sequences compared to the expected sequence of the monoclonal antibody product. Determination of the C-termini usually requires digestion of the protein into small peptides and sequencing the peptides, which represent the C-termini of the heavy and light chains. This is often performed as part of the peptide mapping of the entire monoclonal antibody.

The presence of two protein chains in each antibody can make interpretation of the sequence data more difficult than for a single chain protein. Also, it is not possible to sequence through pyroglutamic acid using the Edman degradation so no N-terminal sequence will be detected for heavy chains containing pyroglutamic acid by this method, but can be determined in the peptide map as is the C-terminal sequence. The absence of a sequence for the heavy chain does not confirm the presence of pyroglutamic acid but this is the most likely cause. Therefore, N-terminal sequencing is generally used as a qualitative test to confirm that the expected sequence(s) are present rather than for quantitation of the amount of each sequence present. In those cases where some or all of the heavy chain contains pyroglutamic acid at the N-terminal, the sequence can be confirmed by peptide mapping.

Peptide Map

As discussed above, peptide mapping provides confirmation of the primary structure of the antibody, but it may also allow the identification of single amino acid changes resulting from degradation such as deamidation or oxidation. Peptide mapping is also used to determine the location of disulfide bonds in a monoclonal antibody product and the sites of glycosylation. The peptide map of a monoclonal antibody product may be viewed as a fingerprint of the protein, which provides a comprehensive understanding of the protein.

When used for characterization, peptide mapping should confirm at least 90% of the protein sequence and mapping of a monoclonal antibody may require more than one enzyme be used to digest the protein. Depending on the sequence, portions of an antibody may not be suitably digested by an enzyme resulting in peptides too large to be fully characterized. Other portions may be thoroughly digested to individual amino acids, which are not retained by the column used to separate the peptides. The presence of posttranslational modifications may also make some portions of the antibody resistant to enzymatic digestion and the protein may need to be deglycosylated before digestion and mapping.

Free sulfhydryl and disulfide bonds

Under normal conditions, an IgG1 monoclonal antibody will have four inter-chain disulfide bonds, two connecting each light chain with a heavy chain and two connecting the two heavy chains to each other. Other IgGs have slightly different numbers of interchain bonds, but all IgGs are held together by disulfide bonding. Additional intra-chain disulfide bonds are also found in the variable and constant regions which help to define the three-dimensional structure of the monoclonal antibody. While all of the cysteine residues on a monoclonal antibody should be paired in disulfide bonds, one or more of the disulfide bonds may be reduced resulting in low levels of free sulfhydryl groups in the monoclonal antibody product. These free sulfhydryl groups can react with free sulfhydryl groups in another antibody molecule leading to dimerization and aggregation, limiting the stability of the monoclonal antibody product.34,35

The pairing of cysteine residues in a monoclonal antibody product into disulfide bonds can be determined by analysis of a peptide map of the product under reducing and nonreducing conditions. Following deglycosylation of the antibody to remove carbohydrate from the protein backbone, one sample of the monoclonal antibody is alkylated with 4-vinylpyridine, digested with trypsin or another suitable enzyme and analyzed by HPLC with detection by mass spectroscopy (LC/MS). A second sample of the deglycosylated antibody is then reduced with dithiothreitol (DTT), alkylated with 4-vinylpyridine, digested with the same enzyme and analyzed by LC/MS. From a comparison of the resulting two peptide maps, those peptides containing cysteine residues paired through disulfide bonds can be identified. For a monoclonal antibody, which has many disulfide bonds, determining which cysteine residues are paired in disulfide bonds may be determined more easily by collecting the separated peptides before reduction. Mapping the two peptides, which are released by the reduction, provides a clear indication of which two cysteine resides form a particular disulfide linkage.

Free sulfhydryl groups should not be present in a properly folded monoclonal antibody product. The extent to which any are present in the product can be confirmed by treating the monoclonal antibody product with Elman's reagent (DTNB). The reaction of Elman's reagent with free sulfhydryl groups in the monoclonal antibody releases p-nitrophenol, which absorbs very strongly at 412 nm. Prior to treatment with DTNB, the three-dimensional structure of the monoclonal antibody product must be disrupted by treatment with a mild denaturing agent to expose any free sulfhydryl groups to the Elman reagent. Following this reaction, the absorbance at 412 nm is measured and the amount of free sulfhydryl groups present is determined by dividing the background-corrected absorbance at 412 nm by 13,600 (the extinction coefficient of the p-nitrophenol). Elman's reagent can be replaced by a number of other reactants, which bind specifically to free thiols and undergo a colorimetric or fluorometric conversion allowing the product to be measure in the presence of excess unbound reagent.

Intrinsic and Extrinsic Fluorescence Spectroscopy

Intrinsic fluorescence spectroscopy (IFS) provides quantitative information on the tertiary structure of a monoclonal antibody product. In this method, the protein is excited with laser light in the near UV to blue wavelengths causing the tryptophan residues within the monoclonal antibody to emit a characteristic fluorescence, which is a reflection of the microenvironment surrounding each tryptophan residue.³⁶ As a result, the intrinsic fluorescence of a monoclonal antibody product is very sensitive to the tertiary structure of the protein and any changes to this structure resulting from denaturation, degradation, or aggregation will cause a shift in either the wavelength maximum or the intensity of the intrinsic fluorescence. Since there is no absolute effect of tertiary structure on the intrinsic fluorescence, it is not possible

to derive information on the antibody structure from the fluorescence. However, this method is very useful in evaluating changes in the tertiary structure under stress conditions and is frequently used in analysis of forced degradation samples as part of the stability assessment of the monoclonal antibody product. This method may be used both in formulation studies and in evaluating biosimilars.

It is possible to determine the extrinsic fluorescence by using fluorescent probes that bind specifically to certain amino acids such as tryptophan. Binding of a probe may disrupt the monoclonal antibody structure so that intrinsic fluorescence measurement is preferred as a method to investigate the tertiary/quaternary structure of a monoclonal antibody.

Nuclear Magnetic Resonance

Nuclear magnetic resonance spectroscopy (NMR) identifies interacting structures by examining the interaction of their nuclei. NMR provides atomic resolution of protein structures by analyzing the magnetization caused by the spinning of each proton. NMR analysis is highly technical and software dependent, requiring expensive equipment and significant computer analysis to deconvolute the data and provide an identification of which amino acid side chains are interacting for antibodies, which are large molecules. Study of an antibody is facilitated if the antibody can be labeled with carbon13 or nitrogen.¹⁵ Labeling of the protein may be achieved by producing it in a microorganism in a growth media enriched in compounds enriched in either of these isotopes. While not commonly used to study the structure of monoclonal antibodies, NMR can be used to study the interaction of an antibody bound to its target molecule.³⁷ Comparison of the NMR spectra before and after binding can help identify which amino acid side chains are in close proximity to the binding region.

A key benefit of NMR is that it can be applied to protein structures in solution, although the concentration of the solution must be sufficiently high (.05 - 1.0 mM) for useful results.38 This is a non-destructive methodology, meaning that samples have the potential to be re-analyzed. NMR results can be delivered rapidly, which, while partial, can be extremely useful. Presently NMR is not capable of analyzing proteins larger than 40 kDa molecular weight and as a result is usually applied to small proteins.³⁹

Mass Spectrometry

Mass spectrometry or mass spectroscopy (MS) separates antibodies by inducing a charge on the protein by a number of ways and then accelerating the charged protein by means of a magnetic and/or electric field.40 The protein is accelerated in proportion to its mass to charge ratio and be captured in the detector. Ionization of the protein may be done by bombardment with noble gas atoms (fast atom bombardment, FAB), ionization with a laser (matrix assisted laser desorption ionization, MALDI), or electrospray ionization (ESI). There are other means of imparting a charge to the antibody protein, but these are the most common. The charged ions are accelerated through the analyzer, which is, most commonly, either time-of flight (TOF) or quadrupole type. Time-of-flight (TOF) refers to mass spectrometers, which accelerate the charged protein in an electric field so that each molecule with the same charge has the same energy. The speed of the molecule is determined solely by the mass to charge of the protein. Each type has different ranges and is suitable for certain ionization methods, for example TOF analyzers are suitable for either ESI or MALDI, while the quadrupole analyzer is not suitable for MALDI ionized samples. The detector is usually a photomultiplier or electron multiplier. The type of mass spectrometry technique employed is usually described by both the ionization and analyzer type, for example MALDI-TOF MS. The detector registers the ions and depending on the mode of acceleration, the mass to charge (m/z) ratio determined. By applying suitable computer software this can be converted into the mass of the original molecule. MS can be combined with separation methods such as high performance liquid chromatography (HPLC) to separate a sample into its components and determine the molecular weight of each. Historically this method was time consuming and labor intensive limiting its usefulness.41 However, modern mass spectrometers and the software developed for interpreting the results have made this method much more accessible and currently it is frequently applied. Hydrogendeuterium exchange (HDX) functional labeling and fragment separation methods can be combined with MS to identify surface residues.

Mass spectroscopy can provide accurate information on the molecular weight of the antibody, but care must be taken to choose a suitable method to ionize the protein and conditions for acceleration of the ionized species.42, 43 Ionization with too high an energy level may result in disruption of the monoclonal antibody into heavy and light chains as well as possible fragmentation of the protein. The output of the mass spectrometer consists of a series of peaks representing the masses detected, but the peak heights are not proportional to the amount of any individual protein and therefore the method does not provide quantitative results. Peak height in a mass spectrogram is proportional to the amount of ions but this is a function of how well the protein ionizes, how stable the ionized form is as well as how well it is accelerated through the mass spectrometer. To be able to quantify the various proteins the MS method must be combined with a method whose output is proportional to the protein contents such as reverse phase HPLC. The separation method must be developed so that it is suitable for use with MS detection as well as UV detection used for most HPLC methods. Trifluoroacetic acid, commonly used for reverse phase HPLC, is not suitable for MS samples and other buffer systems have been developed.

In addition to determining the intact mass of an antibody, sequence data can be obtained by using the excitation energy to fragment the protein. Using two mass spectrometers directly connected (MS/MS) the protein is bombarded with sufficient energy to produce multiple protein fragments from the antibody. These fragments are accelerated and individual fragments are subjected to additional energy resulting in additional fragments, which are accelerated and detected in the second MS. Using specialized software, the peptide sequence can be determined by examining the fragments detected and finding those fragments, which differ by the known molecular weight of individual amino acids. This can be done rapidly enough so that most fragments created in the first excitation can be examined in the second. The high mass sensitivity of today's MS/MS equipment provides information on oxidation or deamidation as well as the primary structure.

Isoelectric Focusing

Isoelectric focusing, described above, is often used for protein characterization studies if it is not part of the product release testing. The technique is capable of extremely high resolution and is capable of separating proteins differing by a single charge into separate bands. As a result, the method is well suited for measuring charge heterogeneity in monoclonal antibody products and also monitoring deamidation, oxidation, and the degree of sialylation of the product.^{44, 45} While this method can measure charge heterogeneity, it is not useful for demonstrating the site of heterogeneity and must be combined with another method such as peptide mapping to identify the sites of oxidation or deamidation.

Circular Dichroism

Circular Dichroism (CD) is an absorptive phenomenon that is represented as the differential absorption of left and right circularly polarized light.⁶⁰ CD is based on differences in how a molecule, such as an antibody, affects right and left polarized light in the far UV region (195250 nm) or near UV region (250350 mm). These differences arise since monoclonal antibodies, as all proteins, are structurally asymmetric. A monoclonal antibody, which has no ordered structure, will produce no intensity; ordered structures may have either a positive or a negative CD signals. The information obtained from CD spectra in these regions is different. The far UV CD is more sensitive to secondary structure while the near UV CD spectra is more sensitive to tertiary structure.

CD can be used to examine protein secondary structure. It is commonly used to determine the effects of folding or mutation on a protein and to study protein interactions.⁴⁶ In the far UV, the α -helix, β -sheet or random coil have well defined and unique CD spectra. The far UV CD spectrum of a monoclonal antibody reflects the amount of each of these spectra present in the protein structure. By applying specific software, the spectra can be deconvoluted to determine the relative amount of each secondary structure present in the protein. This method can also be applied to evaluating how altering the antibody's environment affects the secondary structure. Some of the environmental changes include different buffer pH or osmolality of the solution. Unlike the far CD there is no correlation of the near UV CD spectra to specific structural motifs. The near UV spectrum is determined by the absorption and environment of the aromatic amino acids (tyrosine, phenylalanine and tryptophan) and cysteine. While not commonly used for release, CD can be used to compare the secondary structure of different lots of a monoclonal antibody and is a useful tool for formulation development.

CD is a non-destructive analytical technique that allows sample to be re-analyzed. It requires relatively high concentration samples (0.5-1.0 mg/mL). CD can deliver

rapid results and studies can be executed over a wide range of conditions such as pH and temperature.^{60, 68} Analysis of antibody samples is significantly reliant on empirical models developed from reference spectra derived from proteins of known structure. As such, successful analysis depends on the reference database used.⁴⁷

Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is also used to examine the secondary structure of a protein. Infrared spectroscopy measures the incident infrared radiation $(.78-1000 \,\mu\text{m})$ absorbed at a particular energy when it interacts with electric dipoles within a molecule.⁶⁰ FTIR relies on the foundation of IR spectroscopy, which is the correlation between protein secondary structure and the position of the IR bands. FTIR instrumentation combined with advanced mathematical analysis methods allows the separation of overlapping IR bands. FTIR provides information on the presence and relative amount of highly structured areas, such as α -helices or β -sheets, of the antibody protein chains. The structured areas affect absorption in the amide bond regions of the infrared spectrum of the antibody. By applying the result of studies with proteins of known secondary structure content, it is possible to correlate the FTIR data and the content of various structural motifs. One advantage of FTIR to other methods for examining secondary structure is its applicability to solid formulations. This method may be applied to lyophilized or spray-dried samples to evaluate multiple formulations.

Multi-angle Laser Light Scattering

Multi-angle laser light scattering (MALLS) provides information on the amount and nature of aggregated and clipped forms of the antibody. For antibodies coupled to polymers such as PEG, MALLS can give a good estimate of the true size, which may not be seen by SDS-PAGE or SEC.

Differential Scanning Calorimetry

Monoclonal antibodies may also be characterized by differential scanning calorimetry (DSC). As in FTIR, DSC provides information on the thermal stability of an antibody. Determining the amount of energy needed to affect a phase change in the sample, DSC provides comparative information on monoclonal antibody in different formulations. A monoclonal antibody, which is folded into the most thermodynamically stable structure, requires more energy to unfold than does an antibody that is less compact or has a less stable tertiary/quaternary structure. The transition midpoint (Tm), where 50% of the molecules in the sample have unfolded is a function of the tertiary structure stability. The folding and unfolding of a monoclonal antibody occurs in multiple locations in the molecule resulting in multiple thermal transitions. Since the Tm of a monoclonal antibody product can be affected by the specific formulation used for the product, DSC is an important tool in screening and optimizing monoclonal antibody product formulation.

Additional Methods to Characterize Protein Structure

Several other spectrophotometric methods are used to characterize monoclonal antibodies, although these methods are rarely used prior to Phase 1. Examining the second derivative of the UV spectra provides information on the purity of the antibody. Fourier Transform Infrared spectroscopy (FTIR) and circular dichroism (CD) provide information on the secondary structure of the antibody. The content of aromatic amino acids (tyrosine, tryptophan and phenylalanine) can differ for each monoclonal antibody resulting in differences in the antibody's unique spectrum in the near UV range (250-350nm). By examining the second derivative of the spectrum it is possible to determine whether the spectrum is for a single pure molecule or is the result of the combination of two or more spectra indicating the presence of an impurity.

Another common degradation pathway is asparagine deamidation, which can be measured by using a commercially available kit.⁴⁸ The method determines the amount of isoaspartic acid present by enzymatic conversion of the isoaspartic residue with protein isoapartyl methyltransferase (PIMT) and the conversion of S-adenosyl-methionine (SAM) to S-adenosyl-homocysteine (SAH). The SAH, which is equal to the amount of isoaspartic acid converted, is then separated and detected by HPLC. The concentration of SAH is determined by comparison to a standard curve run at the same time. This method is suitable for quantitation of asparagine deamidation since isoaspartic acid is produced primarily by the deamidation. Care should be taken since the solution pH and the surrounding sequence may affect the ratio of isoaspartic and aspartic acid produced.

Characterization of Aggregation

Aggregation in a monoclonal antibody drug substance or drug product is a critical quality attribute that can impact potency and safety. Size exclusion chromatography and SDS-PAGE, discussed above, are used for routine measurement of monoclonal antibody aggregation. For more detailed characterization of a monoclonal antibody product, more sophisticated methods such as multi-angle laser light scattering (MALLS),⁴⁹ analytical ultracentrifugation (AUC),⁵⁰ and asymmetric fast flow filtration (FFF)⁵¹ are used.

MALLS makes use of the known behavior of light to be scattered by dissolved protein in solution. The amount of light scattered from the zero angle, in direct line with the light source, is proportional to the mass of the dissolved protein. Since it is not possible to measure zero angle light which is overwhelmed by the light from the source, a MALLS instrument measures the light scattered at a number of angles (>3) and uses this data to determine the average molecular weight of the dissolved molecule. This method is capable of identifying molecules of a number of different molecular weights in a single solution so that it is not necessary to previously separate the antibody, for example by SEC. MALLS can also be used to determine the molecular weight of the antibody peaks from an SEC separation. This provides information on whether the higher molecular weight forms separated are dimers, trimers or higher order forms. To provide the most information on the nature and amount of aggregated antibody forms, combining SEC with both UV and MALLS detection provides both quantitation of the protein peaks and identification of the molecular weight of the material in each peak.

AUC is based on the migration, in a centrifugal field, of a protein or other polymer through a gradient solution. This migration is a function of the molecular radius of the antibody being studied. While not amenable to quality control or easily validated due to poor precision, AUC can be considered to be a gold standard in determination of molecular mass of a monoclonal antibody and related forms and can be used to determine the binding constant. Analysis of AUC data is based on the premise that the molecular radius is directly related to the molecular weight. Requiring little sample preparation, AUC can examine both covalent and non-covalent aggregates. Despite the large range of molecular weights, which can be determined by AUC, at high antibody concentrations the protein behaves in a non-ideal manner requiring dilution of the sample, which may result in disassociation of reversible aggregation. Analysis of AUC data requires specialized software to convert the sedimentation velocity into molecular mass and interpretation requires specialized training.

Asymmetric fast flow fractionation can also see both covalent and non-covalent aggregates. FFF separation is accomplished by application of a field perpendicular to the flow of sample through a narrow channel, layering the solutes against a membrane. The movement of the molecules along the flow path is a function of the molecular weight providing molecular mass based separation. Since there is no column for separation this method does not sieve out extremely large molecules and can see aggregates and particles in the range of 0.150 μ m.⁷³ As in AUC, this method has poor precision making it difficult to validate and unsuitable for QC use. Interaction of the sample with the membrane is not well understood or controlled and may contribute to inaccuracy in the results.

Characterization of Glycosylation

One critical aspect of the characterization of monoclonal antibody products is the determination of the carbohydrate content of the molecule (amount and type of monosaccharides), the mapping of the carbohydrate chains on the protein and the oligosaccharide pattern of the various glycans present on the monoclonal antibody product, and the determination of the amount and type of sialic acid present on as the terminal glycosyl residue in each carbohydrate structure.^{52, 53, 54} It is also important to determine whether the oligosaccharides present on the monoclonal antibody product are N-or O-linked.55 Monoclonal antibodies produced in mammalian cells typically have an N-linked carbohydrate structure at Asn297 in the CH2 region on each heavy chain. However, the extent of this glycosylation will vary among different production cell lines and can be dependent on the culture conditions used to produce the monoclonal antibody product. Furthermore, CHO cells can also add sugars or incorporate sugar linkages in a monoclonal antibody that are not found normally on human antibodies, resulting in non-active or immunogenic products.⁵⁶ Similar N-linked carbohydrates can also be found on other asparagine residues in monoclonal antibodies. For example, approximately 15-20% of the currently approved monoclonal antibody products, such as ceruximab (Erbitux), have N-linked glycosylation at Asn88 of the VH region.⁵⁷ As antibody glycosylation is a major cause

of batch-to-batch variability during production, regulatory authorities require full characterization of the nature and extent of glycosylation in monoclonal antibody products.

Full characterization of a monoclonal antibody product includes an analysis of the percent occupancy of each potential glycosylation site in the molecule as well as the determination of the structures of the different oligosaccharides. As discussed above, peptide mapping can be used to identify the sites of glycosylation in a monoclonal antibody product as well as the site occupancy. Analysis of the peptides released during peptide mapping by MS/MS or N-terminal sequence analysis allows for the identification of the definitive location of each glycosylation site in the product. Site occupancy at each of these sites is determined by measuring the relative amounts of the peptide containing the glycosylation site with and without carbohydrate. When performing such an analysis, care must be taken to ensure that the presence of the oligosaccharides does not adversely affect the cleavage of the protein. This may be compensated for somewhat by sequencing each peptide released so that difference in cleavages can be identified.

Demonstration of the structure of the oligosaccharides at each glycosylation site is complicated by the variety of oligosaccharide structures that may be present at each site since each glycoform may contain varying amounts of sialic acid, may be biantennary, or triantennary, may be differentially fucosylated, etc. Determination of the structure of each glycoform is accomplished by cleavage of the intact oligosaccharide from the monoclonal antibody using PNGase F. The different oligosaccharide structures are then separated by HPLC and sequenced using one or more methods. The sequence of each oligosaccharide can be determined by comparison of the elution time of the sample to a series of standard oligosaccharides run at the same time under the same chromatographic conditions. Alternatively, the structure of each oligosaccharide may be determined directly by mass spectrometry in a manner similar to the amino acid sequencing of the peptide backbone of the monoclonal antibody product. Combining the sequencing data determined in this manner with the previously determined monosaccharide composition provides a high level of assurance that the sequence determined is accurate.

Quantitation of Subvisible Particulates

Control of the number and size of potential particulates present in a monoclonal antibody product is essential for product consistency and safety as FDA has become increasingly concerned about particulates smaller than 10 µm in size. The compendial methods for measuring particulates (USP <788> and EP 2.9.19) are less accurate in determination of particulates in this size range. No regulatory expectation has been set for control of particulates in this size range yet, but companies are being asked to determine the concentration of particulates in this range and report the result. The inaccuracy of the current compendial methods when used to measure these small particulates requires additional analytical methodologies to be applied to this determination.⁵⁸ New methodologies include image analysis, laser diffraction, and polarization intensity diffraction (PID). Image analysis captures an image of the particulates suspended in solution as they pass a sensing area. Application of image analysis software provides a determination of the concentration and size of the particles. Laser diffraction is based on the same principal as used in MALLS; differential scattering of incident light as a function of the size of particulates in the solution. Polarization intensity diffraction also uses the differential scattering of light as a function of the particulate size, but the incident light is vertically and horizontally polarized and the scattered light is measured at many wavelengths to increase the information obtained. At this time, none of these methods are suitable for implementation in the QC laboratory, but each can provide information on the size and concentration of small, subvisible $(\leq 10 \mu m)$ particulates present in monoclonal antibody drug products.

6. Characterization of Reference Standard

Many of the analytical methods used for release testing or characterization of a monoclonal antibody product require the use of a reference standard for comparison to the sample of interest. As with other biologic products, reference standards used for monoclonal antibody products should be fully characterized as part of the qualification of the standard. During the development of a monoclonal antibody product, a laboratory standard, prepared in the research laboratory with minimal characterization, is often used to support process development. However, as the manufacturing process is developed and larger quantities of material are prepared for characterization and pre-clinical animal studies, a reference standard should be prepared from either a non-GMP batch of product prepared during scale-up (e.g., an engineering run) or a sample of the initial GMP batch intended to be used in the Phase 1 human clinical trial. This reference standard should be fully characterized and, if possible, sufficient reference standard should be prepared to allow it to be used as the standard for release of all clinical trial material. At the time the marketing authorization is submitted for the monoclonal antibody product, a new reference standard should be prepared material produced by the proposed commercial manufacturing process. This reference standard should be fully characterized with regards to its structure and biological activity.

7. Analytical Methods Used to Test and Release Raw Materials

The extent and type of testing required for each raw material used in the manufacture of a monoclonal antibody product will depend on the nature of the raw material and where it is used in the manufacturing process. At a minimum, each raw material must be tested to confirm the identity of each lot of the raw material (or EMA each container must be analyzed). Manufacturers must also demonstrate that the raw material conforms to the vendor's Certificate of Analysis and is acceptable for use in the manufacturing process. The manufacturer or sponsor should have performed an audit to qualify the vendor and confirmed the vendor has a suitable quality system.

Wherever possible, cGMP-grade raw materials should be used in the manufacture of a monoclonal antibody product to ensure that the material is safe for use in the manufacture of products for human use. For those raw materials, which meet the compendial standards, such as buffer salts used during drug substance manufacturing or sugar alcohols used as excipients in the final drug product, no additional testing other than that specified in the relevant pharmacopeia are usually required.

For raw materials that do not have a pharmacopeia monograph, such as antifoams used in the cell culture process to produce a monoclonal antibody product, suitable analytical methods must be developed for the testing of the raw material. As with the compendial methods, the methods used to test a non-compendia raw material should be sufficient to uniquely identify the raw material and determine its purity and safety for use in monoclonal antibody product manufacturing. If raw materials derived from animal sources are used in a manufacturing process (including the preparation of cell banks), the material must be tested and certified to be free of viral or adventitious agent contamination. The supplier of the raw material should provide certification attesting to the origin and safety of the source of the raw material, e.g., fetal bovine serum should be certified as coming from herds of cattle from countries with no known incidence of bovine spongiform encephalitis. Prior to the use of any raw material derived from animal sources, a risk assessment should be conducted to identify potential risks to patients that may result from the use of the particular animal derived material and steps should be taken to eliminate or minimize these risks.⁵⁹

8. Analytical Methods for Release and Stability Testing

Prior to release for use in human clinical trials, the monoclonal antibody drug substance and drug product must be analyzed using methods sufficient to demonstrate the identity, purity, safety, and potency of the product and the product must meet pre-established specifications for each analytical method. Additionally, every drug product used in human clinical trials must be demonstrated to be stable throughout the trial. Analytical methods used for product release and determination of product stability are the same and those commonly used have been discussed in detail above and are summarized in Table 4.10. Some of the analytical tests listed in Table 4.10, such as residual DNA or host cell protein, are only performed on the monoclonal antibody drug substance as there are no steps in the drug product manufacturing process that would alter the amount of these process related impurities in the drug product. Other tests are only performed on the drug product as the test does not measure an attribute of the drug substance, but rather an attribute of the drug product solution, such as the extractable volume in a filled vial or the concentration of excipients added during the drug product manufacturing process. These measurements provide an additional check on the proper formulation of the drug product and assurance that the monoclonal antibody product will conform to the prescribed dose. If the drug product manufacturing process may result in changes of a particular attribute of a monoclonal antibody product from the drug substance to the drug product, or if the attribute is stability indicating, a release test may be performed on both the drug substance and drug product. For example, filtration of the drug substance may result in an increase in oxidized forms of the monoclonal antibody product, which can be detected by reverse phase HPLC analysis. For certain monoclonal antibodies, other analytical methods may be used in addition to or in place of those listed in Table 4.10. For example, for a monoclonal antibody in

which the glycosylation is known to affect mode of action, additional tests related to the monosaccharide content and/ or glycan structure may be required.

Table 4.10. Common Release Tests for

Monoclonal Drug Substance and Drug Product			
Attribute	Test	Drug Substance	Drug Product
General	Appearance	Y	Y
	рН	Y	Y
	Osmolality	N	Y
Identity	SDS-PAGE, reduced	Y	Y
	SDS PAGE, non-reduced	Y	Y
	Peptide Map	Y	N
Strength	Concentration	Y	Y
	Container Volume (Extractable volume)	N	Y
	Concentration of Excipients	N ^a	Ya
Potency	Receptor Binding	Y	Y
	Bioassay	Y	Y
Product Purity	SDS-PAGE, reduced and non- reduced	Y	N
	Ion Exchange Chromatography	Y	N
	Size Exclusion Chromatography	Y	Y
	Reverse Phase Chromatography	Y	N
	Isoelectric focusing	Y	N
	Monosaccharide Content	Y	N
Impurities	Residual Protein A	Y	N
	Host Cell Proteins	Y	N
	Residual DNA	Y	N
Safety	Endotoxin	Y	Y
	Bioburden	Y	N
	Sterility	N	Y
	Particulate Matter	N	Y

^a May be analyzed in formulated drug substance instead of drug product

For each monoclonal antibody product, the exact analytical methods that are used for the testing and release of the drug substance and the specifications for each method are determined based on process development history, product characteristics, and regulatory requirements. Since multiple analytical methods may be available for assessing different product attributes, not all analytical methods are used for every product. While analytical methods for measuring those product attributes, which may impact the safety or efficacy of the product are required, Sponsors have flexibility in determining which analytical method will be used for to determine the identity, purity, or potency of the product. Multiple chromatographic methods such as reverse-phase HPLC, ion exchange HPLC, and SEC may be included in the list of purity tests for a monoclonal antibody product if these methods have been shown to provide useful information about the product. A sample set of analytical methods and specifications for a monoclonal antibody drug substance intended for use in early stage clinical trials is shown in Table 4.11.

For the testing and release of a monoclonal antibody drug product, the analytical methods used will typically include some of the drug substance release tests as well as some additional analytical methods specifically required for drug products. The details of drug product release tests and specifications are discussed in Chapter 10.

10. Identification of Critical Process Parameters

Identification of critical and key process parameters is dependent on the output of inprocess and final product release testing as well as stability testing of the drug substance and drug product. During process development, the results of all analytical tests performed on the monoclonal antibody product are used to identify which process parameters must be controlled tightly and which parameters have little impact on product quality. The identification of these critical process parameters helps to define the process design space for a monoclonal antibody product and provide valuable information on potential critical attributes that must be addressed during process validation.

Table 4.11. An Example of QC Release Methods and Specifications for a Monoclonal Antibody Product in Early Clinical Development		
Attribute	Method	Specification
рН	pH meter	Target pH ± 0.2
Appearance	Visual inspection	Clear colorless solution; essentially free of visible particulates
Concentration	Absorbance at UV280	Target ± 10%
Identity	Peptide Map	Conforms to reference standard
Identity/purity	SDS PAGE (reduced)	Two major bands of molecular weight corresponding to the molecular weight of the reference standard Two major bands constituting ≥95% of total stained area
ldentity/purity	SDS PAGE (non-reduced)	One major band of molecular weight corresponding to the molecular weight of the reference standard One major band constituting \geq 95% of total stained area
Purity (aggregates)	Size Exclusion HPLC	Monomer peak represents \geq 95% to the total peak area Total high molecular weight peaks represent \leq 5% of the total peak area Total low molecular weight peaks represent c5% of the total peak area
Purity	Cation Exchange HPLC	Retention time of the main peaks correspond to those seen in the reference standard Main peaks comprise ≥95% of the total peak area Report % of minor acidic and basic forms
Purity	Isoelectric focusing	Conforms to reference standard Report pl range
Attribute	Method	Specification
Potency	Antigen-binding ELISA	75-125% of reference standard
Potency	Cell based bioactivity assay	60-140% of reference standard
Monosaccharide content	HPLC analysis of monosaccharides released from the protein by treatment with trifluoroacetic acid	Report result
Host cell protein	CHO-specific ELISA	<10 ng/mg antibody
Host cell DNA	qPCR	<50 pg/mg antibody
Residual Protein A	ELISA	<10 ng/mg antibody
Endotoxin	USP <85> EP 2.6.14	≤1 EU/mL
Bioburden	USP <61> EP 2.6.12	<10 CFU/ 100 mL

References

- 1 Wei Z, The Role of Higher-Order Structure in Defining Biopharmaceutical Quality, BioProcess International, 2011 April 58-66
- 2 Kozlowski S, Swann P. Current and future issues in the manufacturing and development of monoclonal antibodies. Adv Drug Delivery Rev. 2006 Aug;58(5-6):707-22.
- **3** IND Content and Format, 21 CFR Part 610 (2015).
- 4 Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs; General, 21 CFR Part 210 (2008).
- 5 Current Good Manufacturing Practice for Finished Pharmaceuticals, 21 CFR Part 211 (2008).
- 6 Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Office of Regulatory Affairs (US). Guidance for industry cGMP for phase 1 investigational drugs [Internet]. Washington, DC: Department of Health and Human Services; 2008 Jul [cited 2009 Nov 9]. 20 p. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070273.pdf.
- 7 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Validation of analytical procedures: text and methodology Q2(R1) Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2005 Nov [cited 2016 Nov 21]. 17 p. Available from: http://www.ich. org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf.
- 8 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin Q5A(R1) Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1999 Sep 23 [cited 2016 Aug 8]. 31 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5A_R1/Step4/Q5A_R1_Guideline.pdf.
- 9 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Quality of biotechnological products: analysis of the expression construct in cells used for production of r-DNA derived protein products Q5B Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1995 Nov 30 [cited 2016 Aug 8]. 6 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5B/ Step4/Q5B_Guideline.pdf.
- 10 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Quality of biotechnological products: stability testing of biotechnological/biological products Q5C Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1995 Nov 30 [cited 2016 Nov 21]. 10 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5C/Step4/Q5C_Guideline.pdf.
- 11 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Derivation and characterisation of cell substrates used for production of biotechnological/biological products Q5D Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1997 Jul 16 [cited 2016 Nov 21]. 17 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5D/Step4/Q5D_Guideline.pdf.
- 12 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Specifications: test procedures and acceptance criteria for biotechnological/biological products Q6B Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1999 Mar 10 [cited 2016 Nov 21]. 20 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf.

- 13 United States Pharmacopeial Convention. The United States Pharmacopeia-National Formulary (USP32-NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008. <1056> Biotechnology-derived articles polyacrylamide gel electrophoresis; p. 5005.
- 14 United States Pharmacopeial Convention. The United States Pharmacopeia-National Formulary (USP32-NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008. <1055> Biotechnology-derived articles peptide mapping; p. 496500.
- 15 Dougherty J, et al. Using peptide maps as identity and purity tests for lot release testing of recombinant therapeutic proteins. BioPharm Int. 2003 Apr;16(4):54-8.
- 16 Hale G. Size exclusion chromatography (SEC). In: Shepherd P, Dean C, editors. Monoclonal antibodies: a practical approach. New York: Oxford University Press; 2000. p. 168-9.
- 17 Woodward C, Ricker RD. Optimization of the rapid HPLC analysis of monoclonal intact antibodies, light chains, and heavy chains using ZORBAX poroshell columns. Technical overview [Internet]. Santa Clara (CA): Agilent Technologies; 2004 Feb 20 [cited 2016 Nov 21]. 8 p. Available from: http://www.chem.agilent.com/Library/applications/5989-0604EN.pdf.
- **18** Kostareva I, et al. Purification of antibody heteropolymers using hydrophobic interaction chromatography. J Chromatogr A. 2008 Jan 11;1177(2):254-64.
- 19 Hale G. Ion exchange chromatography. In: Shepherd P, Dean C, editors. Monoclonal antibodies: a practical approach. New York: Oxford University Press; 2000. p. 162-6.
- 20 Du P, Anumula KR. Quantitative analysis of monosaccharides from glycoproteins by fast HPLC with highly sensitive fluorescence detection. J Biomolecular Tech. 1998 Dec;9(4):7-12.
- 21 Dionex Corporation. Technical note 30: monosaccharide and oligosaccharide analysis of glycoproteins electrotransferred onto polyvinylidene fluoride (PVDF) membranes [Internet]. Sunnyvale (CA): Dionex Corporation; c1999 [cited 2016 Aug 8]. 8 p. Available from: http://www.dionex.com/ en-us/webdocs/110764-Dionex-TechPubs-web-16Sep2011-LPN034690-45.pdf.
- 22 Wen et al. Applications of Differential Scanning Calorimetry for Thermal Stability Analysis of Proteins: Qualification of DSC. J Pharm Sci. 2012 Mar; 101(3):995-964.
- 23 Assay Designs, Stressgen. Protein A 96 well enzyme immunoassay kit, catalog #900-057. Kit insert [Internet]. Ann Arbor (MI): Assay Designs; 2008 Nov 21 [cited 2016 Aug 8]. 16 p. Available from: http://www.enzolifesciences.com/fileadmin/redacteur/pdf/adi/ADI-900-057.pdf.
- 24 United States Pharmacopeial Convention. The United States Pharmacopeia-National Formulary (USP32-NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008. <130> Protein A quality attributes; p. 118-23.
- 25 Cygnus Technologies, Inc. Immunoenzymetric assay for the measurement of CHO host cell proteins commonly found in conditioned media, catalog # CM015. Product insert [Internet]. Southport (NC): Cygnus Technologies, Inc.; 2008 Jun [cited 2016 Aug 8]. 6 p. Available from: http://www. cygnustechnologies.com/CM015_CH0%20CM%20ELISA%20PI%2006-08.pdf.
- 26 Applied Biosystems. resDNASEQ quantitative CHO DNA system. Product bulletin [Internet]. Foster City (CA): Applied Biosystems; 2009 Apr [cited 2016 Aug 8]. 4 p. Available from: https://tools.thermofisher.com/content/sfs/manuals/cms_060698.pdf.
- 27 Walker JM, editor. The protein protocols handbook. Second ed. Totowa (NJ): Humana Press Inc.; c2002. 1147 p.

- 28 Zell S, et al. Down-regulation of CD55 and CD46 expression by anti-sense phosphorothioate oligonucleotides (S-ODNs) sensitizes tumour cells to complement attack. Clin Exp Immunol. 2007 Dec;150(3):576-84.
- 29 United States Pharmacopeial Convention. The United States Pharmacopeia-National Formulary (USP32-NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008. <631> Color and achromicity; p. 238-9.
- 30 United States Pharmacopeial Convention. The United States Pharmacopeia-National Formulary (USP32-NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008. <791> pH; p. 313-4.
- 31 United States Pharmacopeial Convention. The United States Pharmacopeia-National Formulary (USP32-NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008. <785> Osmolality and osmolarity; p. 305-7.
- **32** United States Pharmacopeial Convention. The United States Pharmacopeia-National Formulary (USP32-NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008. <1052> Biotechnology-derived articles amino acid analysis; p. 48190.
- 33 Allen G. Sequencing of proteins and peptides. Second ed. New York: Elsevier Science Publishing Company; c1989. Chapter 6, Determination of peptide sequences; p. 207-93. (Laboratory techniques in biochemistry and molecular biology; vol. 9).
- 34 Zhang W, Czupryn MJ. Free sulfhydryl in recombinant monoclonal antibodies. Biotechnol Prog. 2002 May-Jun;18(3):509-13.
- **35** Girard M. Biopharmaceuticals by capillary electrophoresis. In: Cazes J, editor. Encyclopedia of chromatography. New York: Marcel Dekker; c2001. p. 87-91.
- **36** Weichel M, et al. Probing thermal stability of MAbs by intrinsic tryptophan fluorescence BioProcess Int. 2008 Jun;6(6):42-52.
- 37 Tugarinov V, et al. NMR structure of an anti-gp120 antibody complex with a V3 peptide reveals a surface important for co-receptor binding. Structure. 2000 Apr 15;8(4):385-95.
- **38** Ravi et al., Techniques for the measurement of the structure of proteins in solution, and their complementarity to circular dichroism. NPL Report. 2005. 22 p.
- 39 Ravi et al. High Order Protein Structure Measurements for Biopharmaceutical Quality Control. NPL Report. August 2007. 81p.
- 40 van den Bremer ETJ, Heck AJR. Mass spectrometry in protein conformational analysis and molecular recognition. In: Jiskoot W, Crommelin D, editors. Methods for structural analysis of protein pharmaceuticals. Arlington (VA): American Association of Pharmaceutical Scientists; 2005. p. 435-64.
- 41 Englander J, et al. Protein structure change studied by hydrogen-deuterium exchange, functional labeling and mass spectrometry. PNAS. 2003 June; 100(12): 7057-7062.
- 42 Ji QC, et al. Mass spectrometric approaches for protein quantitation in drug development. Am Pharm Rev. 2004 May-Jun;7:84-9.
- 43 Flatman S, et al. Process analytics for purification of monoclonal antibodies. J Chromatogr B Analyt Technol Biomed Life Sci. 2007 Mar;848(1):79-87.
- 44 Lee H. Rapid high-performance isoelectric focusing of monoclonal antibodies in uncoated fused-silica capillaries. J Chromatogr A. 1997 Nov;790(1-2):215-23.
- 45 Costello M, et al. Characterization of humanized anti-Tac monoclonal antibody by traditional separation techniques and capillary electrophoresis. J Liq Chromatogr Related Technol. 1992 Apr;15(6-7):1081-97.
- 46 Greenfield N. Using circular dichroism spectra to estimate protein secondary structure. Nat Protoc. 2006; 1(6): 2876-2890.
- 47 Whitmore L, Wallace BA. Protein Secondary Structure Analyses from Circular Dichroism Spectroscopy: Methods and Reference Databases. Biopolymers. 2007 September; 89(5): 392-400.
- 48 Promega Corporation. ISOQUANT® isoaspartate detection kit, catalog #MA1010. Kit insert [Internet]. Madison (WI): Promega Corporation; 2009 Mar [cited 2016 Aug 8]. 9 p. Available from: https://www.promega.com/~/media/files/resources/protocols/technical%20bulletins/101/isoquant%20isoaspartate%20detection%20kit%20protocol.pdf.
- **49** Wen J, et al. Size-exclusion chromatography with on-line light-scattering, absorbance, and refractive index detectors for studying proteins and their interactions. Anal Biochem. 1996 Sep;240(2):155-66.
- 50 Philo JS. Analytical ultracentrifugation. In: Jiskoot W, Crommelin D, editors. Methods for structural analysis of protein pharmaceuticals. Arlington (VA): American Association of Pharmaceutical Scientists; 2005. p. 379-412.
- 51 Liu J, et al. A critical review of analytical ultracentrifugation and field flow fractionation methods for measuring protein aggregation. AAPS J. 2006 Sep 22;8(3):E580-9.
- **52** Adamo M, et al. Evaluation of oligosaccharide methods for carbohydrate analysis in a fully human monoclonal antibody and comparison of the results to the monosaccharide composition determination by a novel calculation. J Pharm Biomed Anal. 2009 Feb;49(2):181-92.
- 53 Hoffstetter-Kuhn S, et al. Profiling of oligosaccharide-mediated microheterogeneity of a monoclonal antibody by capillary electrophoresis. Electrophoresis. 1996 Feb;17(2):418-22.
- 54 Kakehi K, et al. Analysis of glycoproteins and the oligosaccharides thereof by high-performance capillary electrophoresis significance in regulatory studies on biopharmaceutical products. Biomed Chromatogr. 2002 Apr;16(2):103-15.
- 55 Beck A, et al. Trends in glycosylation, glycoanalysis and glycoengineering of therapeutic antibodies and Fc-fusion proteins. Curr Pharm Biotechnol. 2008 Dec;9(6):482-501.
- 56 Jefferis R. Glycosylation as a strategy to improve antibody-based therapeutics. Nat Rev Drug Discov. 2009 Mar;8(3):226-34.
- 57 Jefferis R. Glycosylation of natural and recombinant antibody molecules. In: Axford JS, editor. Glycobiology and medicine, proceedings of the 7th Jenner glycobiology and medicine symposium. The Netherlands: Springer Dordrecht; c2005. p. 143-8. (Axford JS, editor, Advances in experimental medicine and biology; vol 564).
- 58 Krishnamurthy R, et al. Emerging analytical technologies for biotherapeutics development. BioProcess Int. 2008 May;6(5):32-42.
- 59 European Directorate for the Quality of Medicine and Healthcare (FR). European Pharmacopoeia (Sixth edition, Supplement 6.5). Strasbourg (France): Directorate for the Quality of Medicine and Healthcare of the Council of Europe; c2008. 1/2008:50208: Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products; p. 558-67.

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CHAPTER 5:

Antibody Production Cell Line Development

s of October 31, 2016, there were more than 70 therapeutic monoclonal antibody and antibody-related products on the market in the US, Europe, and elsewhere.¹ With the exception of just two antibody-fragment products, Cimzia and Lucentis, Fab antibody fragments produced in microbial (E. coli) cell lines, all commercial antibody products today and the vast majority of monoclonal antibody products in development are expressed in mammalian cell culture. Production of whole monoclonal antibodies generally requires mammalian cell culture to ensure proper folding, pairing of heavy and light chains, dimerization of the heavy chain, and proper glycosylation necessary for activity and function. The dominant mammalian cell line used to produce monoclonal antibodies today is the Chinese Hamster Ovary (CHO) cell, although cell lines of murine origin were used to produce some of the earliest antibodies on the market and cell lines of human origin have recently become available. As a result of the dominance of CHO cells for production of therapeutic antibodies, much effort has been focused on developing optimized parental CHO host cell lines, cell culture media designed to support these CHO host cell lines, and other process and equipment components that support this specific host cell line. Many expression technologies are available to improve the speed of CHO cell line development and the specific productivity levels that can be achieved.²

During the candidate selection phase of an antibody discovery program, many candidate antibodies are typically produced to screen for affinity to the therapeutic target, off-target interactions, activity in cell based assays that reflect the intended mode of action, and in many cases, animal models of disease, and overall manufacturability. The candidate antibodies are often produced using transient transfection of human embryonic kidney cells that contain virally-derived transcriptional activators (HEK293) and that produce relatively large quantities of antibody transiently. However, antibodies produced in HEK293 cells may not contain the same post-translational modifications or other properties as antibodies produced at larger scale in a stable, production cell line suitable for GMP manufacture of clinical trial material (IND/IMPD-enabling cell line). Methods and reagents to enable transient transfection of the more applicable CHO cell line have been developed and these are useful for producing small amounts of antibodies at the stage where there are still tens of antibodies to screen.^{3,4} However, the most representative candidate antibodies will come from stably-transfected pools of CHO cells or from clonal production cell lines.

There are many technologies available today that enable generation of research-enabling CHO cell pools in three weeks and clonal CHO cell lines in as little as six weeks.^{5,6,7,8,9} Clonal cell lines generated rapidly, intended for use in selection of the lead monoclonal antibody, will not necessarily have the documentation, genetic and phenotypic stability, or high titer that are required for manufacturing initial material for clinical trials. Therefore, upon identification of the lead monoclonal antibody, a fully characterized, stable production cell line capable of expressing the product meeting the desired target product profile (TPP) and critical quality attributes (CQA) must be developed.¹⁰ A time-saving approach to generating the final production cell line is to use a production technology in early discovery that is also suitable for generation of the IND/ IMPD-enabling production cell line.¹¹

Monoclonal antibodies must be manufactured using reliable cell lines and cell culture processes capable of producing sufficient quantities of the antibody with the desired CQAs to meet the projected material demand. Development of the fully characterized, stable production cell line is among the most important, early CMC activity because production of the monoclonal antibody product for GLP toxicology studies and human clinical trials requires the final production cell line and the Master Cell Bank. Since generating a production cell line is always on the critical path for initiation of first in human clinical trials, companies should consider timelines, cost, and desired product characteristics when evaluating specific technologies and strategies for cell line generation. Production cell lines with very high expression levels (titers) are usually desired because the productivity will ultimately define the maximum overall process yield and will impact all subsequent development activities. However, it is equally important to select a cell line with optimal growth properties since the production cells must remain viable for many days in a production bioreactor in order to generate the quantities of antibody necessary for clinical development of the product.

Most antibody expression technologies available today routinely deliver monoclonal antibody production cell lines with titers of at least 2 g/L and sometimes as high as 57 g/L in a production bioreactor. For recently approved commercial products and those in later stages of clinical development, antibody expression levels in the bioreactor are typically in the range of 24 g/L. Proprietary technologies can often achieve genetically stable cell lines with higher titers more quickly, but any technology can be used to generate a production cell line that produces sufficient quantities of antibody and that meets regulatory expectations. Often the newer, proprietary technologies that offer superior timelines or higher titers are only accessible through a service provider or a licensing program. Higher titers for most cell lines can be obtained by additional process development (see Chapter 6) but the time and cost savings of higher titers decreases at some point due to the additional downstream processing costs.¹² As the safety, genetic and phenotypic stability, and monoclonality of the production cell line are considered critical issues by regulatory authorities, cell line development must be rigorously performed and documented and the final production cell line fully tested and characterized according to all current regulatory requirements and expectations.¹³

One critical goal of cell line development is to ensure that the selected production cell line makes the desired product with the intended CQA. The antibody must bind effectively to its target, have appropriate glycosylation, and have low levels of aggregation. Therefore, to support cell line development, it is important that appropriate analytical methods are available to measure the important CQAs and determine that the product that exhibits the desired function.¹⁴ Reference material is also valuable in insuring that the production cell line produces a product that has similar performance to the antibody used in the final candidate selection.

The quantity of monoclonal antibody product present in a production bioreactor at harvest is a function of the cellular specific productivity, the densities to which the cells are grown, and the viability of the culture during the production phase. In a typical fed-batch process with harvest criteria of 5080% viability, the culture duration can be as short as 11 days or as long as 17 days. Culture durations longer than two weeks are rarely transferred to manufacturing, however, due to the impact on overall facility operations and scheduling. To achieve the desired high product titers in the production bioreactor, a stable production cell line with a specific productivity above 30 pg/cellday and the ability to grow to high density in fed-batch cell culture is desired.

With today's highly competitive market and the resulting pressure to shorten development timelines as much as possible, cell line development should be completed as quickly and efficiently as possible to enable early entry into human clinical trials. If the productivity, product quality, and stability of the selected lead cell line are sufficient, the initial line can be used to support the entire clinical development program, and even commercial production. However, the selected cell line may not always be suitable for commercial production. In this case a second production cell line can be generated during clinical development. Once first-inhuman studies have been performed and have shown that the product has a therapeutic benefit in a small set of patients, additional time and funding often becomes available and a new production cell line capable of meeting commercial production goals can be developed. A transition to using material from this new cell line in clinical studies will require significant characterization and comparability testing of the product from the original and new cell line and so must be carefully planned and agreed to in advance with the appropriate regulatory authorities. While the burden for such characterization and comparability testing is high, there is precedent for regulatory acceptance of such cell line changes without the requirement to repeat clinical studies if there is suitable biochemical and biophysical characterization and suitable animal data to demonstrate that the product CQAs are not significantly affected by the cell line change. Examples of cell line changes that have occurred late in development include the high-profile change in cell lines just prior to approval of Avonex and the more recent cell line change for canakinumab prior to Phase 3 trials.^{15,16} This latter product is now approved and marketed as Ilaris by Novartis.

Despite the precedent for cell line changes during development, many organizations strive to reduce the technical risk of this change by having the cell line used for first-inhuman studies the same as the ultimate production strain. This mitigates potential comparability issues due to a cell line change later in development. Technological innovation in expression technologies has made this goal attainable for many programs but there is no regulatory or technical reason to choose either approach. Whether to invest in an IND/IMPDenabling cell line only or a commercially-enabling cell line depends on the company's business risk tolerance and other financial and strategic considerations.

1. Host Cell Lines Used for Monoclonal Antibody Product Production

The selection of a parental host cell line should, for production of a given monoclonal antibody product, be based on the projected development and commercial product requirements, regulatory acceptance of the host cell line, and desired product properties. Since the host cell line used will impart specific characteristics to the products, such as glycosylation, carboxylation, hydroxylation, sulfation, and amidation,^{17, 18} which may affect the half-life of the product in the patient's serum, immunogenicity, or the biological activity of a monoclonal antibody (effector function),^{19, 20} the properties of the host cell line should be carefully considered at the outset of an antibody development program.

The technical and regulatory risks associated with the development of biopharmaceutical products are significantly reduced if cell line development is performed using a fullycharacterized and documented host cell line, known to be free of adventitious agents such as bovine and porcine viruses and TSE transmissible spongiform encephalopathy. Further, the parental cell line should be adapted for growth in suspension culture using media that does not contain serum or other animal-derived components. Today, a variety of well characterized mammalian host cell lines with full documentation and traceability of the cell line's origin and exposure to potential adventitious agents are readily available. Given this, most smaller companies choose to use one of these commercially available host cell lines rather than develop a new host cell line for their use. Most larger companies have developed an in-house host cell line and expression technology that is applied to their pipeline of antibodies and other biopharmaceutical products.²¹

As noted above, the vast majority of monoclonal antibody products on the market and in development today are produced in a CHO cell line. Many different parental CHO cell clones are available from ATCC or other repositories or from companies that offer cell line development services or technologies. While each of these particular CHO cell lines may have different properties, any can generally be used for antibody production. A sampling of the different parental CHO cell lines used to produce currently marketed antibody products are listed in Table 5.1.

Table 5.1. CHO Species Used in Monoclonal Antibody Production				
CHO Cell Line	e Properties	Sample Products Produced in Cell Line		
CHO-DuxB11	DHFR negative (one deletion, one mutation)	Rituxan, Herceptin, Enbrel, TPA		
CHO-DG44	DHFR negative (full deletion)	Avastin		
CHO-S	Suspension adapted and serum free	Products in clinical development		
CHO-K1 and CHO-K1SV	Suspension adapted and serum free	Zenapax, Synagis, Solaris		
СНО-М	Derived from CHO-S	Products in clinical development		

From a regulatory perspective, the use of CHO parental cell lines for antibody development has been reduced, but not eliminated, with much of the concern regarding potential immunogenicity of the desired therapeutic product due to the long history of antibody products produced in these cell lines with little or no significant immunogenicity or related issues.²² Although other cell lines are available for therapeutic antibody production, the most time and costeffective approach for a new monoclonal antibody product is to use one of the available CHO cell lines in conjunction with an appropriate expression system.

Expression Technologies for CHO Cells

There are a number of well characterized expression technologies and strategies to consider when initiating a cell line development program for a monoclonal antibody. Currently, almost all suppliers of parental CHO production cell lines have pre-adapted their cell lines to grow and produce product in serum-free, animal component-free media. In some cases, companies have developed chemically defined media that support mammalian cell growth. The ability to move rapidly through process development and into initial production of clinical trial material is greatly enhanced by initiating cell line development with host cell lines that have been developed or selected for attributes that contribute to good growth and productivity in the bioreactor.

The selection of an expression system should be based on the amount of product needed (near-term and estimated commercial requirements), the company's risk tolerance, and compatibility of the expression system with existing manufacturing operations within the company or at contract manufacturing organizations (CMOs). While some service providers advertise very short timelines to clonal production cell lines, the actual timelines are typically 4 – 5 months from project initiation to identification of the lead, genetically stable, production cell line although candidate clones can be available much more quickly.

Non-proprietary Technologies

Viral Promoters in Expression Vectors Some of the strongest promoters for mammalian cells are derived from viruses, which must divert cellular transcription factors away from cellular genes and toward the viral promoters to initiate the infection process. The human cytomegalovirus (CMV) promoter is one such viral promoter that is routinely used in mammalian expression vectors. The CMV promoter is part of the immediate-early regulatory DNA sequence that controls high level expression of CMV early viral genes following infection of a host cell.^{23, 24} Like many viral promoters, the CMV promoter functions in a variety of different cell types and has been widely used in expression vectors for many years. When used in an expression vector to drive expression of a recombinant protein or antibody, the CMV promoter has been shown to provide a high expression level of a wide variety of proteins. It was originally developed and patented by the University of Iowa but this patent has expired and there is no license fee associated with use of the CMV promoter. US and European regulatory agencies are very familiar with CMV promoter systems, and accept that the use of this genetic element to

produce therapeutic proteins for clinical and commercial applications provides minimal risk to patients.

Dihydrofolate Reductase Selection and Amplification One of the earliest effective methods for transfection, selection, and amplification of foreign genes in mammalian cells was developed in 1981 by scientists at Columbia University using dihydrofolate reductase (DHFR) selection.²⁵ In this method, a parental mammalian cell line deficient in the enzyme DHFR is transfected with an expression vector containing the DHFR gene under control of a relatively weak promoter and the antibody (or other protein) genes under control of a strong promoter.26 By inhibiting DHFR activity in a transfected cell using methotrexate (MTX), the DHFR gene and any nearby gene sequences will be amplified to enable cell survival. The DHFRbased expression technology makes use of this observation to design expression vectors in which the DHFR gene and the gene of interest are linked. By applying increasing concentrations of MTX to the selection media and then isolating single cell clones, cell lines with amplified DHFR can be isolated. These cells generally will exhibit increased expression of the linked antibody genes as well. Through sequential rounds of increasing MTX exposure and selection of surviving clones, cells that express high levels of the antibody can be identified.

Although widely used and accepted, standard DHFRbased expression vectors have some drawbacks. Each amplification cycle typically requires about four to six weeks to complete and provides two to five-fold increase in the linked transgene expression. The entire process can take up to six months total to obtain a clone with acceptably high expression levels for today's therapeutic monoclonal antibody products (e.g., at least 30 - 50 pg/cell-day and more).

Genomic structure in the amplified region may not be stable and gene copies are often deleted when selective pressure is removed. Although it is toxic and expensive, MTX must often be used during production to maintain the copy number and transgene expression. If used, MTX must then be removed by the downstream process. In the absence of continuous MTX, some production cells will exhibit genetic instability when the cell culture process is scaled-up to commercial scales. Nevertheless, the DHFR system can be effective and has been used in conjunction with overall cell line development efforts to achieve multi-gram-perliter expression levels of monoclonal antibody. The original patents for this technology have now expired so there are no license fees associated with the use of DHFRbased expression vectors. The technology is still widely used to generate antibody production cell lines and forms a platform technology at some companies.

The system is effective and has been used in conjunction with the other aspects of cell line development to achieve grams per liter of antibody. However, the total time the DHFR amplification system requires to obtain a suitable production clone (about 6 - 12 months total) is often too long to meet the aggressive product development timelines that today's biopharmaceutical companies strive to achieve. Therefore, selection strategies and technologies that reduce the time to obtaining the final production clone have been developed by a variety of companies and investigators. Some of these systems use DHFR as the selectable marker in a single round of transfection and selection, since it provides a highly effective selection method and is cost and time effective when combined with other technologies that enable selection of high expressing cell lines without amplification.

Proprietary Technologies

Alternative expression systems that enable development of production cell lines with the desired high antibody expression levels in much shorter times than amplification of DHFR-containing vectors have been developed and are available through reagent suppliers, individual CMOs, or technology licensing programs. Some technologies work with most mammalian cell lines but others, such as CMC Biologics CHEF-1 expression system, are specific for CHO cell lines. There are CMOs and technology suppliers that have developed proprietary CHO host cell lines with beneficial properties in terms of expression and bioreactor performance or, in some cases, production of antibodies with specific quality attributes. Most of the available, proprietary technologies are capable of supporting multi-gram per liter antibody titers in large-scale cell culture processes and typically have productivities of >30 pg/cell-day in the initial shake flask measurements of individual clones, although the upper limit achievable can be product specific. Many of the technologies may also be synergistic with other expression technologies, although combinations of multiple expression technologies in a single cell line are not a common practice today. Some of the technologies can also be used as alternatives to transient transfection to rapidly generate stable pools of cells expressing the desired antibody for use in other development activities such as formulation development and preclinical testing. Table 5.2 shows examples of expression systems available from service providers for developing

monoclonal antibody production cell lines. Clearly the technologies that increase the percentage of transfectants that are high producers and, equally important, are genetically stable throughout many generations would enable companies to screen fewer clones to identify the final production cell line and therefore would reduce timelines and costs.

Company	Technology	Track Record	
Lonza	Glutamine synthetase selection, current technology is GSXceed which uses CHO-K1SV- GS-KO	GS used in at least 13 commercial products; GSXceed programs initiated recently	
Lonza	Potelligent CHO system for expression of proteins with no fucose in the glycan	Multiple products in development	
Boehringer Ingelheim	BI-HEX toolbox of promoters, enhancers, and parental CHO cell line	Technology used to produce commercial products	
Selexis	SURE CHO Cell Line ™, Selexis Genetic Elements, improve DNA structure	Technology in products globally, currently up to Phase 3 in US/EU	
Catalent	GPEx retroviral vectors, 100% transduction so no selection	Technology used to produce commercial products	
Batavia Biotech	STEP improved DHFR selection	New technology, no products in late stage	
CMC Biologics	CHEF-1, CHO-specific housekeeping promoter	Commercial product in US	
Thermo Fisher Life Technologies	PD-Direct (CHO DG44), Freedom Kit (CHO DG44 or CHO-S)	Widely used, PD-direct used for commercial products	
FujiFilm Diosynth Biotechnology	ApolloTM CHO cell line selected using Directed Evolution	New Technology, no products in late stage	
ProBioGen	GlyMaxx engineered CHO cell line that provides lower level of fucosylation	New Technology, no products in late stage	
Cellca	Proprietary selected CHO-DG44 with superior growth and stability in bioreactor	Products in late stage development	
Excellgene	CHOEXPRESS™ Proprietary selected CHO-K1, scalable Tubespin technology for screening	Products in clinical development	
SAFC	ChoZn targeted knock-out CHO cell lines with DHFR or GS deletions	New Technology, no products in late stage	

Table F. 2. Commercially Available Everyopian Systems

2. Cell Line Development Activities

Cell line development includes generation of a suitable expression vector and introduction of this vector into a well-characterized host cell line. Single cell clones are then isolated and analyzed by increasingly stringent criteria until a lead and back-up production cell line have been identified. A representative Process Flow Diagram for development of an IND/IMPD-enabling production cell line for a monoclonal antibody product is shown in Figure 5.1 The individual process steps shown in Figure 5.1 are meant to be an illustration of a typical cell line development cycle; the exact dilution and cloning procedure used, the number of clones screened at each stage, and the assessments that are performed to select the top clones at each stage will vary from company to company and often from product to product. Regardless of the exact details of this process, the critical objective is that the procedures used result in a

production cell line with reasonable expression levels, good growth in a production bioreactor, genetic stability, and a high probability of monoclonality.

Cell line development is often performed in an isolated tissue culture room that has been thoroughly cleaned and documented to have no other cell or DNA present. By strictly isolating the incubation and procedures from other laboratory activities, chances of obtaining a cell line producing the wrong product are minimized. This is especially important when companies are developing multiple antibody products, which all behave similarly in early test methods such as SDSPAGE, SEHPLC, or CIEXHPLC (see Chapter 4 for descriptions of these analytical methods). Descriptions of each stage of production cell line development are provided below.

Figure 5.1. Representative Cell Line Development Workflow.

A representative workflow for development of an IND/IMPD-enabling production cell line for a monoclonal antibody product. The individual process steps shown are meant to be an illustration of a typical cell line development cycle.



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Expression Vector Construction

The cell line development process begins with the generation of the expression vector that will be used to introduce the antibody heavy and light chain genes into the parental cell line. To generate the expression vector, genes encoding the desired heavy and light chain sequences are designed, synthesized, and inserted into the backbone plasmid vector as shown in Table 5.3.

Table 5.3. Expression Vector Construction				
Activity	Notes			
Design codon optimized genes	Outsourced to specialized service providers (GeneArt, DNA2.0, or others)			
Synthesize genes with restriction sites compatible with expression vector	Outsourced; genes normally provided in a shuttle vector			
Transfer antibody genes into one or two backbone plasmids	Confirm gene sequences after final vector(s) are constructed			

Mammalian expression vectors include selection markers (e.g., DHFR, antibiotic resistance), transcription regulatory elements, (promoters, enhancers), translation initiation sequences, translation enhancing sequences, and polyadenylation signals. In most expression vectors, the selectable marker is intentionally cloned downstream of a weak promoter while the antibody chains are placed downstream of strong promoters. Expression vectors can contain mono or bicistronic expression cassettes,²⁷ and there are reasonable justifications for either approach. Additional elements may be present in the vector that can contribute to genetic stability, improved rates of transcription or translation, or other functions that improve overall productivity. Some of the elements that are used in mammalian expression vectors can be proprietary, in which case they must be accessed through an appropriate licensing arrangement with the owner of the technology. Proprietary systems can enable more rapid identification of production cell lines with high product yields and other superior performance properties28 the benefits of which should be weighed against the license fees and royalties necessary to access the technology.

As a first step in developing a production cell line for expression of a monoclonal antibody product, the codons

used in the DNA sequences of the heavy and light chain genes to be used should be optimized to improve the likelihood of efficient translation in the host organism and minimize the formation of secondary structures in the product mRNA which might impede translation. Such codon optimization is typically outsourced to one of a few vendors that offer unique codon design and optimization programs as part of their DNA synthesis services. Since codon utilization can often dramatically impact the rate of translation of an antibody, it may be useful to evaluate multiple DNA sequences coding for the same antibody before selecting the gene sequence to use in cell line development. Mismatches between translation rate, posttranslational processing, and secretion can lead to significant product quality issues such as misfolding or aggregation, which can impact downstream processing.^{29,30}

Once the specific DNA sequence has been chosen and an expression vector has been constructed, the heavy and light change genes inserted into the expression vector should be fully sequenced to confirm that no errors in the genetic sequence were introduced during the manipulations necessary to create the expression vector. Such sequencing of the genes inserted into the expression vector is expected by all regulatory authorities as part of the initial IND or IMPD submission. Further, it is good business practice to confirm that the time and expense of cell line development will lead to a production cell line that produces the correct protein. Failure to confirm the sequences in expression vectors has caused delays and failures in more than one antibody development program.

Transfection

Following vector construction and sequence verification, the expression vector is introduced into the parental cell line by transfection. The parental cell line is cultured until a sufficient number of viable cells in mid-log phase are obtained and the expression vector is then introduced into the cells. Most often, electroporation is used as the transfection method since this does not expose the parental CHO cell line to any new or poorly characterized reagents. Other options for transfection include cationic lipid-based reagents,³¹ Polyethylenimine (PEI),³² or calcium phosphate, although these reagents are more often used to transfect cell lines that are not intended for use in GMP production.

Some expression technologies utilize vectors that are derived

from viruses that are capable of introducing DNA into the host cell line without electroporation or other reagents. Retroviral vectors can be used without other reagents to introduce the desired genes into a parental cell line via a process called transduction. Catalent's proprietary GPex technology uses this strategy to obtain close to 100% efficiency in CHO cell transduction. This level of efficiency eliminates the requirement for selectable markers and stable clonal cell lines are produced rapidly.⁸

Table 5.4. Transfection and Selection				
Activity	Notes			
Generate population of CHO parental cells	Serum-free media, mid-log phase			
Transfect by electroporation or reagent	Two-day recovery before selective pressure			
Select using antibiotic, enzyme inhibitor, or nutrient removal	Shake flask, up to two weeks for selection			
Optional: Production	Can use enriched pool to make antibody			
Optional: RCB of pools	Freeze pools to preserve selected cells			

Selection and Optional Production from Pools

Those cells that incorporate the expression vector are preferentially enriched in a cell culture pool based on the selectable marker.³³ This initial selection is typically performed in shake flasks over approximately two weeks, resulting in stable pools of transfected cells that express some level of the selectable marker and presumably some level of the antibody. Slightly different transfection and selection strategies can be applied in a single program, in which case multiple pools may be obtained. Pools are generally preserved by freezing vials of each pool prior to analysis of the pool productivity.

In addition to providing the source culture for single cell cloning, the enriched, selected pools can be used to produce small amounts of the monoclonal antibody for use in analytical development, limited stability assessments, downstream process development, cell-based or animal testing, or evaluation of efficacy on patient tumor samples. Some expression technologies enable production of monoclonal antibodies from pools at scales up to 100 L, although production from pools at scales closer to 5 - 10 L is more common. Pools grown without selective pressure will generally be overtaken by the least productive cells in the culture since producing the antibody requires energy and therefore the high producers often grow slowly.

Antibodies produced from early cell pools can be purified using a one-step capture – elute on Protein A columns. The antibodies from pools are suitable for the above-mentioned testing but are not suitable for required GLP toxicology studies since the antibody for the toxicology studies must come from the production cell line and intended production process. However, by starting to produce the antibody from enriched pools, development scientists gain exposure to the antibody and its properties, and generate material that can support early development.

Assessing Pool Productivity

If the selection process results in multiple pools, the candidate pools can be grown in shake flasks using either fed-batch production models or the simpler overgrowth method to assess the antibody levels in each pool. One to three pools with the highest product titer are generally selected to seed plates or wells for single cell cloning and isolation of the clonal production cell line candidates. It is critical to document the source of the pool(s) used for single cell cloning so that an accurate history of the cell line can be provided in the IND or IMPD. In some cell line development workflows, pool productivity assessment is not performed and the single pool that was generated is the source for single cell cloning.

Single Cell Cloning

A major regulatory requirement in cell line development is the control and documentation of the procedures used to demonstrate that the production cell line is derived from a single parental cell (i.e., monoclonal). To be monoclonal, all cells in the production cell line must have the same integrated gene sequence, copy number, and integration site(s), all of which have been properly verified and documented.³⁴ Demonstration and verification of the monoclonality of the production cell line is important to ensure product consistency and to achieve optimal yield in bioreactors as the production is scaled up to meet late stage and commercial material requirements. The activities performed during single cell cloning are summarized in Table 5.5 and described in more detail below.

Table 5.5. Single Cell Cloning				
Activity	Notes			
Separate cells into single wells or plates using limiting dilution, FACS-based single cell deposition or ClonePixFL	Imaging on day 0 and subsequent days optional but recommended			
Evaluate productivity of hundreds of clones in static wells	Initial screen is for expression level only			
Evaluate up to 30 clones in production models.	Screen for expression in larger system, possibly screen for product quality			
Optional second round of single cell cloning	Recommended if imaging not used in first round of cloning. Can use same or different method to separate cells.			
Optional: Evaluate up to 30 clones from second round in production models	Same screening strategy as first round			
Evaluate top 4 – 6 clones	Growth, viability, genetic stability, product quality, bioreactor performance			
Select lead clone and back-up clone(s)	Based on results of final analysis			
Prepare full documentation	Documentation of preparation, reagents, and cloning required for IND/IMPD			

There are multiple technologies and approaches to separating the cell culture into single cells that will then grow into a colony of single cell origin. Limiting dilution is an established approach in which the cells are diluted and a target of 0.1 - 1.0 cell is deposited in each well of a 96-well plate. It is useful to image the cell culture before plating into wells to demonstrate that there are very few cell clusters that contain more than one cell. Often as many as forty 96-well plates are seeded since the plating efficiency will be low. During the colony expansion period the plates can be imaged using the Cellavista cell imager,³⁵ or other equivalent instrument, starting on the day of seeding.

Cell sorting using fluorescence-activated cell sorting (FACS) has become more widely used for single cell cloning as the sensitivity and accuracy of the equipment has improved. In a recent publication, the probability of monoclonality obtained by combining one single round of single cell deposition by fluorescence-activated cell sorting (FACS) with the imaging capability of the Cellavista cell imager was evaluated.³⁶ The combined overall probability of monoclonality by using one round of single cell deposition and photo documentation imaging with verification was 99.962% at the 95% confidence interval. Several pharmaceutical companies and CMO customers have products in clinical development where the production cell line was generated using single-round FACS selection with imaging. FACS sorting and selection can also be followed by another round of single cell cloning by FACS or an alternative approach.

Another approach to isolating clonal cell lines is the ClonePix 2 system from Molecular Devices. Cells from the enriched pool are diluted and plated onto a semi-solid media that contains a fluorescent antibody detection reagent. The ClonePix 2 system automatically images, selects, and picks colonies that meet predetermined criteria such as colony size, shape, and proximity to neighbors. The detection reagent also enables selection based on relative expression levels.

Initial Clone Screening

As described above, cells are isolated and single cells are deposited in a well or on a plate, an activity that must be well documented. If any animal-derived materials are used at the single cell cloning stage, documentation supporting low BSE/TSE risk must be available and included in the IND or IMPD. Generally, hundreds to thousands of cells are seeded in wells or on plates and grown for two to three weeks. For screening clones from the wells of a 96-well plate, a rapid ELISA, Protein A HPLC, or other screening method can be used to identify candidate clones that express sufficient levels of the antibody. For cells arising from the ClonePix 2 system, the intensity of fluorescence is an indicator of expression level. Typically, 100 – 300 clones are selected for expansion and further screening.

The selected candidate clonal cell lines are grown in larger wells, shaking deep wells, or in Micro Bioreactors³⁷. The

culture supernatants are analyzed for antibody productivity using ELISA or other high throughput assays. Using productivity as the initial screening enables scientists to identify up 30 clones that are then evaluated for growth as well as additional assessments of productivity.

Secondary Screening

At this stage, the selected clones are grown in small shake flasks or mini-bioreactors to determine growth rates, viability, productivity, and limited assessment of product quality attributes. Up to 30 clones identified in the initial screening are expanded into larger shake flask cultures for detailed growth and productivity assessment and up to 10 are selected for further analysis. Security cell banks (SCB) are prepared for the clones at this stage so that if the final lead and backup clones have any issues with stability, productivity, or clonality, the project does not need to start at the beginning.

During secondary screening, a model platform cell culture process may be applied to the cultures, to mimic the conditions that the cells will be subjected to during actual production. The production model uses a media and feed strategy that has been optimized for the parental host cell line and which is representative of the expected production conditions. Cell viability is monitored and those clones that are not sufficiently viable during the 14-day culture period are not progressed into final clone selection, even if the titers are high. It is critical to success in production that cell health and viability contribute to cell line selection, and that expression levels are not the only criteria applied. High growth rate during subculture is highly likely to remain heritable in recombinant cell lines, thus reducing cell line development, scale-up and large-scale production time.

Between two and ten candidate cell lines with the desired properties in growth, productivity, and product quality in small scale laboratory studies are usually isolated during secondary screening.

Most often these top clones from the first round of single cell cloning are subjected to a second round of single cell cloning by the same or a different cloning method to insure that the final cell line is of single cell origin. If a second round is performed, the same screening funnel described above is used to select clones for additional evaluation in small bioreactors to select the lead and back-up production cell lines.

Final Clone Selection

In this stage, production models in bioreactors are often used to generate material from the top 2 - 10 clones that can be subjected to extensive product quality analysis. While screening clones for high productivity is important, close attention should also be paid to ensuring that the product produced contains the desired CQA's, which may include both intrinsic structural properties and post-translational modifications. To evaluate product quality, cell culture supernatants are subjected to single-step purification using Protein A columns, and the resulting antibody is evaluated for aggregation, glycan structure and content, thermal stability, and other biophysical properties. Even though the same sequence is produced in each clonal cell line, the selection process leads to clones with different rates of processing and secretion of antibodies, so the properties of the antibody from different clones will vary. This final assessment of growth, productivity, and product quality enables selection of a lead clone and one or more back-up clones.

Productivity in bioreactors is often higher than predicted due to better cell environment control. Therefore, the top candidate clones are often evaluated in bench-scale bioreactors (from 250 mL to 10 L scale) to insure that the performance seen in the initial test culture conditions are replicated or improved in a bioreactor. Final clone selection is based on the bioreactor evaluations and concurrent evaluation of genetic stability (see below).

At the early development stage, expression levels of 30 -50 pg/cell-day in a shake-flask are considered adequate since productivity can be improved by developing appropriate cell culture conditions such as product-specific nutrient feeds.

Clone Selection Criteria

Lead clone selection is a rate limiting step in process development, resulting in frequent pressure to take shortcuts in order to advance a potential product candidate more quickly into the clinic. However, clone selection is an important decision in product development with many significant ramifications. When done properly, clone selection will lead to a high quality production cell line capable of generating the desired quantities of product in a consistent, stable, cost-effective manner. The criteria used to select a specific clone for further development include specific productivity and product yields, growth characteristics such as growth rate, maximum cell density, longevity, and viability in culture, as well as stability of cell line productivity and product characteristics, including glycosylation profile and structural fidelity. Since such post transcriptional modifications as glycosylation, especially of the Fc region which may affect the biological activity of the antibody product, and formation of truncated product forms can vary from clone to clone, it is important to define the characteristics of the desired product beforehand and use these characteristics to guide clone selection.

Automated Systems for Clone Selection

Several different automated systems for single cell cloning, identification of high expressing candidate cell lines, and selection of final clones have been developed in recent years, helping to accelerate and streamline the process of clone selection and reduce the labor and time required for these critical activities.^{38, 39} Using these automated systems for clone selection also allows for the evaluation of a large number of clones by performing many multifactorial experiments40 and facilitates running many experiments during cell line screening and selection that were traditionally postponed until upstream process development after final clone selection.⁴¹

For the initial selection, automated screening systems sort higher expressing cell lines (secretors) from lower secretors to accelerate the selection of a high expressing cell line. For example, when used in conjunction with DHFR selection in a non-proprietary vector background, automated screening systems can reduce the selection of a final clone candidate from 26 weeks to as little as 10 weeks.⁴²

Any technique that could speed the selection of high product producing cell lines and enable more rapid identification of the best cell line from among many clones could offer a faster approach to generating a production cell line of commercial interest. An intriguing paper by Povey et al,⁴³ describes the use of MALDI-TOF mass spectrometry fingerprinting and an in silico modelling method that could potentially reduce the clone selection by 30 to 40%. The method, using intact CHO cells, provides high throughput characterization, screening, and selection of cell phenotypes in a significantly lower amount of time that traditional, manual methods. Using a combination of whole cell mass spectrometry and an associated Partial Least Squares Discriminant Analysis method, the authors report reducing the time for clonal selection from 19.8 weeks to 12.4 weeks.

Genetic Stability

Genetic stability testing is needed to select the final lead and back-up clone(s). Genetic stability must be tested during cell line development or the initial process development activities. Chromosome rearrangements, loss of recombinant gene copies, and transgene silencing have been reported to cause instability in CHO cells.⁴⁴ Genetic stability is evaluated more stringently as product and clinical development proceed and stability throughout the intended number of generations in the bioreactor at commercial scale must be demonstrated for product licensure. The goal is to insure that the copy number of the inserted expression vector remains constant over (at a minimum) the number of generations that the cell line is expected to grow during a full-scale commercial production run; that the integrity of the expression vector is maintained; and that only a single messenger RNA of the expected length is expressed for the recombinant protein product. Genetic instability can be a significant problem with some transfected cell lines if the inserted heterologous gene is not maintained integrated in the genome over time or is otherwise silenced. Those genetic loci that cause genetic instability are not well characterized but are likely to be locations that exhibit strong transcriptional activity and are therefore accessible to the enzymes and cellular machinery that remove the inserted gene.

Monoclonality

Production cell line monoclonality is a major focus for regulatory authorities, and both FDA and EMA normally request significant documentation regarding the origin, cloning strategy, results, and statistical likelihood that a production cell line is monoclonal.^{45,46,47} Appropriate statistical analysis applied to the cloning procedures and data are necessary to demonstrate monoclonality. This is highlighted in many guidance documents from regulatory agencies. For example, the 1997 ICH guideline Q5D45, in section 2.1.3., states: "For recombinant products, the cell substrate is the transfected cell containing the desired sequences, which has been cloned from a single cell progenitor". The EMA/CHMP 2008 Guideline on Development, Production, Characterization and Specifications for Monoclonal Antibodies and Related Products⁴⁶ states that: "The cell substrate to be used for the production of the monoclonal antibodies should be a stable and continuous monoclonal cell *line...*". Finally, the 2013 WHO Expert Committee on

Biological Standardization⁴⁷ states the following on page 111: "In the process of cloning a cell culture, single cells should be selected for expansion. The cloning procedure should be carefully documented, including the provenance of the original culture, the cloning protocol and reagents used. Cloning by one round of limiting dilution will not necessarily guarantee derivation from single cells; additional subcloning steps should be performed ... the cloning procedure should be fully documented, with details of imaging techniques and/or appropriate statistics. For proteins derived from transfection with recombinant plasmid DNA technology, a single fully documented round of cloning is sufficient, provided that product homogeneity and consistent characteristics are demonstrated throughout the production process and within a defined cell age beyond the production process".

Historically, using statistical methods for dilution and colony outgrowth was generally considered sufficient to present a convincing case for the probability of monoclonality. Currently, however, the regulatory authorities often request documentary evidence of a single cell³⁴. Industry standard practice for a clonal production cell line is to target a 99% probability of clonality⁴⁸. The probability of monoclonality for a colony is estimated as 1 minus the probability that the colony is superimposed with another colony assuming a grey zone of twice the size of both colonies⁴⁹ Multiple cloning steps increase the probability of clonality. The equation used to calculate the cumulative probability is the following:⁵⁰

$$P_{N} = P_{N-1} + (1 - P_{N-1})P_{T}$$

 $P_{_{\rm N}}$ = cumulative probability of clonality including $n^{\rm th}$ step., i.e., the last step, in this case $P_{_{1\&2}}$

 P_{N-1} = cumulative probability of clonality before n^{th} step, in this case P_1

 $P_n =$ probability of clonality achieved by nth step only, in this case P²

Example with 95% in first cloning step and 90% in second:

$$P_{n} = 95\% + (5\% \times 90\%) = 99.5\%$$

Therefore, any combination of two cloning steps where each step provides at least a 90% assurance of clonality will greatly increase the likelihood of clonality and will also mitigate regulatory concerns with the cloning strategy. This is only true for cloning steps where a statistical and technical analysis can rigorously support the claims for clonality of the step. For the initial IND or IMPD submission, regulatory review focuses significantly on the cell line development procedures and the probability of monoclonality.

To provide a high likelihood of clonality, one round of limiting dilution single cell cloning can be sufficient if specific criteria are met. Lower target cell numbers per well (a density of 0.5 cells per well or less) or separation of single cells using FACS can be acceptable if the cloning procedures and provided documentation are sufficient. For greater assurance of monoclonality of the colony in each well, imaging can be used to monitor colony formation and distribution from the day of plating through to the day of picking the clone for further evaluation.

Frequently, companies will perform one round of single cell cloning with imaging to meet aggressive development timelines. Other companies or service providers prefer to reduce regulatory risk by performing two rounds of single cell cloning with or without imaging. No matter which approach is used to develop an initial production cell line for a new monoclonal antibody product, statistical analysis including the accuracy of the initial determination of cell concentration and the probability of monoclonality must be provided. Further, it is critical that suitable segregation of candidate cell lines and accurate tracking and handling of candidate clones is performed and documented.

Upstream process development

Once a production cell line has been developed and demonstrated to be monoclonal as outlined above, this production cell line is used to initiate upstream process development necessary to manufacture the antibody product for pre-clinical and clinical development (see Chapter 6). For monoclonal antibodies, a pre-defined platform cell culture process can be used as the starting point for process development to optimize the operating parameter ranges and to quickly define and optimize an upstream process suitable for production of clinical trial material, A platform process will consist of a defined cell culture media and one or more feeds that have been developed to work well with the host cell line and media. Some companies and service providers will perform an extensive media screen while others have already identified toolbox media and feeds that are most likely to result in good performance in the bioreactor. Early process development focuses on achieving high expression, robust and sustainable growth of the candidate cell line, and production of product with the optimal CQA. While selecting production cell lines that make product with the desired CQA is an important aspect of cell line development, cell culture conditions can also impact the quality attributes and can be a second level of control applied to the overall development program. Hossler et al. have shown that optimal and consistent glycosylation of monoclonal antibodies, which is often a CQA, is strongly influenced by cell culture conditions.⁵¹

3. Preparation and Characterization of Cell Banks

During cell line development, small research cell banks (RCB) of the top 410 clones are normally prepared as a source for further development activities. These cell banks are typically prepared in the development laboratory and the preparation and characterization of each is recorded in laboratory notebooks and development reports for future reference. After selection of the final production cell line for use in development of a monoclonal antibody product, the coding sequences of the heavy and light chain in the final clonal cell line are again verified to confirm that no mutations were introduced during the entire cell line development process. and cells from the RCB for this cell line are used to prepare a Master Cell Bank (MCB) for further development and manufacture of the product. This MCB should be manufactured under cGMP conditions and characterized as described below. Full and detailed characterization of the MCB is not only a regulatory requirement for monoclonal antibody development, it is necessary to ensure that the cell line to be used for production of clinical trial material is well-characterized and meets all technical and regulatory requirements and guidelines for such cell lines. In addition to the MCB, a Working Cell Bank (WCB) may also be prepared and characterized at this point in development. The production and characterization of the WCB is described below. Once prepared, these cGMP cell banks are used for cGMP production of the product throughout its development and lifecycle.

As discussed above, companies frequently prepared a controlled bank of the parental cell line which they intend to use for development of a portfolio of antibody products. This parental cell line is typically subjected to the full panel of tests used for master and working cell banks to ensure that the cell line is well-characterized and to reduce the risk of contamination by adventitious agents or other safety concerns that may arise from the host cell line. However, even if such a parental cell bank is prepared and characterized, it is still necessary to fully test and characterize the production cell banks

Cell Banks Required for Monoclonal Antibody Production

The manufacture of biologic products requires the use of well characterized cell banks, produced in accordance with cGMP regulations and relevant regulatory guidances, to assure an adequate supply of equivalent cells for manufacturing. A cell bank is defined in ICH Q5B as "*a collection of ampoules* [*vials*] *of uniform composition stored under defined conditions, each containing an aliquot of a single pool of cells.*"⁵² Preservation of the cells under appropriate storage conditions minimizes the potential for genetic changes, which may lead to reduction or loss of desired *phenotypic properties, including decrease in specific* productivity, or changes to the critical quality attributes of the monoclonal antibody product.

For monoclonal antibody products, regulatory authorities recommend a two tier cell bank system consisting of a Master Cell Bank (MCB) and a Working Cell Bank (WCB) to ensure long-term supply of the production cell line.¹³ Once generated, the cell banks should always be maintained frozen at temperatures below -1350C and thawed only when needed to initiate production or to generate a new cell bank.

The MCB is intended to provide long-term preservation of the production cell line and is the source material for the preparation of WCBs. The WCB is derived from one or more vials of the MCB and one or more vials from the WCB are used to produce one or multiple lots of a the desired monoclonal antibody product. To save time and money during the initial phases of product development, cells from an MCB are frequently used directly to produce product for pre-clinical and toxicology studies and initial clinical trials. In this way, the expense of the WCB preparation can be delayed until there is sufficient proof of concept for the monoclonal antibody product to justify this expense. Postponing WCB preparation and testing can also have an impact on the overall early development timeline.

In addition to the MCB and the WCB, current regulatory guidances recommend the preparation of an end-ofproduction cell bank (EPC), prepared from cells that are harvested at the end of a production bioreactor run and cultivated to the limit of *in vitro* cell age at least once during development. This EPC should be fully characterized as the MCB and WCB so as to identify any new contaminants in the product that may be introduced or induced by the growth conditions and to determine the stability of the cell clone's phenotypic and genotypic characteristics.53 The EPC should be prepared and stored under the same conditions as the MCB and WCB, although the bank will not be used in production. If the MCB is used for initial cGMP manufacturing of a monoclonal antibody product, then an EPC should be prepared from a production bioreactor using this bank and then again from a new bioreactor run using the WCB once it has been prepared. Information on the testing and characterization of an EPC should be included in all regulatory filings.

Preparation of Cell Banks

Prior to the production of the MCB and WCB, the RCB for the cell clone selected should be fully tested to ensure that it is sterile and free of mycoplasma contamination. In addition, preliminary work should be completed to determine how the cells respond to freezing, the best conditions for freezing and storing the cell banks, and to develop procedures for thawing of the cell bank to ensure consistent results in recovering healthy, viable cells which are capable of reproducible exponential growth in culture. For storage of the cell bank, cryovials which are well designed and able to withstand the intended storage conditions without impacting the cell bank quality or stability should be used.

To prepare the MCB, a small number of cells from the RCB are thawed and expanded by serial subculture up to a selected passage number, at which point the cells are harvested, and resuspended in fresh media. A suitable cryoprotectant, such as dimethylsulfoxide (DMSO) is then added and the cell suspension is dispensed into vials or other selected containers, and the vials frozen using the pre-determined freezing conditions. ^{54, 55, 56} MCB vials from

the beginning, middle, and end of the vial filling protocol should be thawed to test for viability, viable cell number, and growth in culture, to confirm that production of the MCB was successful. Following this, the MCB should be tested and characterized in accordance with current regulatory requirements and expectations. Once the MCB has been prepared and tested, a WCB can be prepared following the same procedure but starting with a vial or vials of the MCB rather than the RCB.

To minimize the risk of losing a MCB or WCB for a monoclonal antibody product due to freezer failure or other facility-related failures, vials of each cell bank should be divided into at least two portions and each portion stored in a different location.

The use of larger containers, such as plastic bags holding hundreds of milliliters of concentrated cells, has also been employed to prepare cell banks.⁵⁷ Using these larger containers for storage of the cell bank provides a large initial number of cells for use in initiating a production run and can reduce the time for preparation of sufficient number of cells to inoculate a large scale bioreactor. This interesting and time-saving strategy has not yet been widely applied in the industry but could be adapted in the future as the industry moves towards more efficient manufacturing processes.

Testing of Cell Banks

Testing of the MCB and WCB is mandated in all regulatory jurisdictions to ensure identity, consistent performance, and freedom from contamination.^{58, 59} The types and number of tests will depend on the species and origin of the cell bank, any exposure to animal-derived components during cell line development, and the intended application.⁶⁰ The most extensive testing is done on the MCB as this will be the starting material for all manufacturing throughout the product lifecycle. Additionally, each new WCB prepared from this MCB should also be tested, although the extent of testing may be less than used for the MCB. The EPC should also be tested for adventitious agents, although this testing can be delayed until after initial clinical trials are performed. Testing and characterization of cell banks is often outsourced to specialized service providers who are qualified and known to the regulatory authorities. The tests recommended for different types of cell banks are summarized in Table 5.6.

Table 5.6. Testing of Mammalian Cell Banks					
TEST		WCB	EPC		
Identity tests - isoenzymes or DNA fingerprinting		+	+		
Growth on soft agarose		-	-		
Growth kinetics and cell yields		+	+		
Production kinetics and yields		+	+		
Product quality QC release tests		+	+		
Sterility; free of bacteria and fungi		+	+		
Free of mycoplasma		+	+		
Species-specific viruses ^b E.G, MAP assay for murine hybridomas, Hamster Antibody Production Assay (HAP) for CHO cells		-	-		
Retroviruses Infectivity, electron microscopy, reverse transcriptase		-	+°		
Adventitious viruses In Vitro assay In Vivo assay	+	-	+		

^a Growth on soft agarose not required for murine hybridomas

^b Human cell banks are routinely tested for the following viruses:

HIV types 1 and 2 Cytomegalovirus (CMV) Hepatitis B (HBV) Epstein-Barr virus (EBV) Parvovirus B-19 Herpes virus 6 (HHV-6) HTLV types I and II Minute Virus of Mice (MVM)

^c Retrovirus testing not required for murine hybridomas.

4. Key Decisions and Risks Associated with Cell Line Development

One of the most important and fundamental decisions in a monoclonal antibody development program is the selection of the host cell line and expression technology to be used for production of the product. For those companies developing a portfolio of monoclonal antibody products, a single host cell line and expression technology is often used for all of the company's products. However, this approach presents a risk that alternative or newer technologies that could provide significant advantages, such as human glycosylation or improved intrinsic cell line stability, are not implemented for new product development. Nevertheless, selecting a host cell line and complementary expression technology platform is usually the best approach for companies developing a portfolio of products as it allows development efforts on multiple programs to leverage prior experience and knowledge and affords synergies between development programs which may accelerate overall product development. When choosing the platform cell line and expression technology, the potential for improving and accelerating cloning speed, higher expression, and stability using proprietary technologies should be balanced against the licensing fees and future royalty requirements of these technologies.

When developing a cell line for product development, the safety and stability of the cell line should be of paramount concern as these are essential for acceptance of the cell line by regulatory authorities. The use of an unstable cell line could result in production variability in the bioreactor which can negatively impact product titers, overall product characteristics, and impurity profiles. Therefore, cell line stability should be assessed as early as possible in a cell line development program. Furthermore, to ensure safety of the chosen cell line, cell line history as well as all manipulations performed from initial cloning of the antibody genes through preparation and selection of the final cell line should be fully documented in laboratory notebooks and development reports.

Occasionally companies initiate human clinical trials with product produced in a cell line which is subsequently

changed later in development. Such a change may be made to improve productivity and reduce manufacturing costs, take advantage of newer production technologies, or facilitate production of a monoclonal antibody with specific quality attributes that have been shown to be important for safety or efficacy. While changes in the production cell line are permitted under the current regulations for biologic products, such a change will require that the product produced by the new cell line is comparable to that produced by the original cell line. While adopting such a strategy can shorten the timeline for filing an initial IND and starting clinical trials, it also presents a risk that a suitable new cell line meeting all commercial requirements and producing a comparable product may not available in time to switch. And, the later a change in cell line is made during development, the higher the burden for demonstrating comparability between product produced from the original and the new cell lines becomes (see Chapter 10)

References

- 1 Bioprocess Technology Consultants [Internet]. Woburn (MA): Bioprocess Technology Consultants, Inc. bioTRAK® database; 2016 (cited 2016 Oct 31); [about 2 screens]. Available from: http://www.bptc.com/pipeline-databases.php
- 2 Jones SD. Technologies to improve cell line development and engineering. Presented at Cambridge Healthtech Institute's Peptalk; 2009 Jan 11-16; San Diego, CA.
- 3 ThermoFisher Scientific [Internet]. Waltham (MA): ThermoFisher Scientific, Inc.; c2016. ExpiCHO expression system; [cited 2016 Nov 28]; [about 9 screens]. Available from: https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-expression/mammalian-protein-expression/transient-mammalian-protein-expression/expicho-expression-system.html.
- 4 Mirus [Internet]. Madison (WI): Mirus Bio LLC; c2016, CHOgro® expression system; [cited 2016 Nov 28]; [about 12 screens]. Available from: https://www.mirusbio.com/products/transfection/chogro-expression-system.
- 5 Girod AP, et al. Rapid production of functional proteins of a combinatorial IgG library of CHO cells. BioProcess International; 2012 10(1): 58-61.
- **6** Running Deer J and Allison DS. High-level expression of protein in mammalian cells Using Transcription Regulatory Sequences from Chinese Hamster EF-1α Gene. Biotechnol. Prog.; 2004 20(3): 880-889.
- 7 Lonza [Internet]. Basel (Switzerland): Lonza Group Ltd; c2016. GS Xceed[™] gene expression system; [cited 2016 Nov 28]; [about 8 screens]. Available from: http://www.lonza.com/custom-manufacturing/development-technologies/gs-xceed-gene-expression-system.aspx.
- 8 Bleck GT. An Alternative method for the rapid generation of stable, high-expressing mammalian cell lines. BioProcessing Journal; 2005 Sep/Oct: 1-7.
- 9 Fujifilm Diosynth Biotechnologies [Internet]. College Station (TX): Fujifilm Diosynth Biotechnologies U.S.A., Inc.; c2016, Apollo[™] mammalian expression platform; [cited 2016 Nov 29]; [about 5 screens]. Available from: http://www.fujifilmdiosynth.com/what-we-do/cell-linestrain-develop-ment/apollo-mammalian-expression-platform/.
- 10 Okumura T Et al. Efficient enrichment of high-producing recombinant Chinese hamster ovary cells for monoclonal antibody by flow cytometry. J Biosci. Bioeng. 2015 Sep; 120(3): 340-6.
- 11 Jones SD. Multiplexing for efficient product development. Presented at IBC BioProcess International West; 2016 Mar 14-17: Oakland, CA.
- 12 Aldridge S. Brining greater efficiency to antibody manufacturing: biological production challenges form basis for debate at Annual Bioprocessing Forum. GEN; 2009 Aug 29(14) (Cited on August 4, 2016) Available from: http://www.genengnews.com/genarticles/bringinggreaterefficiencytoantibodymanufacturing/2998/
- 13 International Conference on Harmonisation.of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Derivation and characterization of cell substrates used for production of biotechnological/biological products [Internet]. Geneva: International Conference on Harmonisation.of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1997 [cited 2016 Jul 20]. 17 p. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5D/Step4/Q5D_Guideline.pdf.
- 14 Ritter N Et al. Bridging Analytical methods for release and stability testing: technical, quality and regulatory considerations. BioProcess International; 2016 Feb 14(2): 12-23.

- 15 Summary Basis of Approval for Avonex, PLA#95-0979
- 16 Chakraborty A, et al. Pharmacokinetics and pharmacodynamics of canakinumab, a human anti-interleukin-1β monoclonal antibody. Clin Pharmacokinet 2012 51(6):e1-e18
- 17 Castillo FJ. Organism selection. In: Asenjo JA, Merchuck JC, editors. Bioreactor system design. New York: Marcel Dekker Inc; 1994. p. 13-45.
- 18 Walsh G, Jefferis R. Post-translational modifications in the context of therapeutic proteins. Nat Biotechnol. 2006 Oct;24(10):1241-52.
- 19 Werner RG, et al. Glycosylation of therapeutic proteins in different production systems. Acta Paediatr Suppl. 2007 Apr;96(455):17-22.
- 20 Raju TS. Terminal sugars of Fc glycans influence antibody effector functions of IgGs. Curr Opin Immunol. 2008 Aug;20(4):471-8.
- 21 Challener C. Pfizer applies integrated technology for cell-line development of proprietary antibodies. PharmTEch.com; September 2013 (Cited August 4 2016 Available from: http://www.pharmtech.com/pfizer-applies-integrated-technology-cell-line-development-proprietary-antibodies
- 22 Kim J.Y. et al. Cho cells in biotechnology for production of recombinant proteins; current state and further potential. 2012 Appl Microbiol. Biotechnol. 93, 917-930
- 23 Stinski MF, inventor; University of Iowa Research Foundation, assignee. Transfer vectors and microorganisms containing human cytomegalovirus immediate-early promoter-regulatory DNA sequence. United States patent US 5,168,062. 1992 Dec 1.
- 24 Stinski MF, inventor; University of Iowa Research Foundation, assignee. Transfer vectors and microorganisms containing human cytomegalovirus immediate-early promoter-regulatory DNA sequence. United States patent US 5,385,839. 1995 Jan 31.
- 25 Wigler M, et al. Transformation of mammalian cells with an amplifiable dominant-acting gene. Proc Natl Acad Sci U S A. 1980 Jun;77(6):3567-70.
- 26 Schimke RT, et al. Amplification of genes in somatic mammalian cells. Methods Enzymol. 1987;151:85–104.
- 27 Li J, et al. A comparative study of different vector designs for the mammalian expression of recombinant IgG antibodies. J Immunol Methods. 2007 Jan;318(1-2):113-24.
- 28 Clarke HRG, Compton BJ. Comparing mammalian expression systems. BioProcess Int. 2008 Nov;6(Suppl 7): 24-32.
- 29 Katsnelson A. Breaking the silence. Nat Med. 2011 Dec;17(12):1536-38.
- **30** Povey JF, et al. Rapid high-throughput characterization, classification and selection of recombinant mammalian cell line phenotypes using intact cell MALT-TOF mass spectrometry fingerprinting and PLS-DA modelling. J Biotechnol. 2014 Aug;184:84-93.
- 31 ThermoFisher Scientific [Internet]. Waltham (MA): ThermoFisher Scientific, Inc.; c2016. Cationic lipid transfection; [cited 2016 Nov 29]; [about 2 screens]. Available from: https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/transfection-basics/gene-delivery-technologies/cationic-lipid-mediated-delivery.html.
- 32 Hacker DL Et al. Polyethyleneimine-based transient gene expression processes for suspension-adapted HEK-293E and CHO-DG44 cells. Protein Expr Purif; 2013 Nov; 92(1):67-76.

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- 33 Agrawal V and Manjot B. Strategies for tRapid production of therapeutic proteins in mammalian cells. BioProcess Interantional: 2012 Apr 10(4): 32-48.
- 34 Sarah Kennett, Ph.D., Review Chief, Division of Monoclonal Antibodies, OBP/OPS/CDER/FDA. 2014. Establishing Clonal Cell Lines A Regulatory Perspective. Black Cell, Blue Cell, Old Cell, New Cell?
- 35 Synentec [Internet]. Elmshorn (Germany): Synentec GmbH; c2016. Cellavista; [cited 2016 Nov 29]; [about 3 screens]. Available from: http://synentec.com/products/cellavista.
- **36** Evans K, et al. Assurance of monoclonality in one round of cloning through cell sorting for single cell deposition coupled with high resolution cell imaging. Biotechnol Prog. 2015 Sep-Oct;31(5):1172-8. doi: 10.1002/btpr.2145. Epub 2015 Sep 18
- 37 Sartorius Stedim Biotech [Internet]. Royston (United Kingdom): Sartorius Stedim Biotech Group; c2016. Ambr® 15 cell culture system overview; [cited 2016 Nov 29]; [about 2 screens]. Available from: http://www.tapbiosystems.com/tap/cell_culture/ambr.htm
- 38 Sleiman RJ, et al. Accelerated cell line development using two-color fluorescence activated cell sorting to select highly expressing antibodyproducing clones. Biotechnol Bioeng. 2008 Feb;99(3):578-87.
- 39 Lee CJ, et al. A clone screening method using mRNA levels to determine specific productivity and product quality for monoclonal antibodies. Biotechnol Bioeng. 2009 Mar;102(4):1107-18.
- 40 Carrier T. High-throughput process development, promises, myths and truths. BioProcess Int. 2008 Apr;6(4):54-8.
- 41 Gryseels T. Considering cell culture automation in upstream bioprocess development. BioProcess Int. 2008 Dec;6(11):12-6.
- 42 Cheng L. High-throughput screening for antibody-producing cell lines. Presented at International Business Communications Life Sciences' Third International BioProcess International Asia Pacific Conference; 2008 Oct 20-22; Mumbai, India.
- **43** Povey JF. et al. Rapid high-throughput characterisation, classification and selection of recombinant mammalian cell line phenotypes using intact cell MALDI-ToF mass spectrometry fingerprinting and PLS-DA modelling, J Biotechnol, 2014 184: 84-93.
- 44 Chusainow J, et al. A study of monoclonal antibody-producing CHO cell lines: what makes a stable high producer? Biotechnol Bioeng. 2009 Mar;102(4):1182-96.
- 45 Ho SCL et al. Generation of monoclonal antibody-producing mammalian cell lines. Pharm. Bioprocess; 2013 1(1): 71-87.
- 46 EMA/CHMP, 2008. Guideline on development, production, characterization and specifications for monoclonal antibodies and related products
- 47 WHO, 2013. WHO Expert committee on biological standardization. Sixty-first report. Technical Report Series No. 978
- 48 Hawley-Nelson, P. Challenges, approaches, and lessons learned. Presented at IBC Cell line development and engineering conference 2013 June 21-22 La Jolla, CA
- 49 Gerritsen, J. Optimal selection of monoclonal cell lines producing high amounts of biopharmaceutical human IgGs. Presented at CHI Bioprocessing summit 2011 Aug 22-25 Boston, MA

- 50 Ahmed O. et al. Using ClonePix FL to Assess Monoclonality. GEN Tutorials: Nov 1, 2009. Vol 29, No.19
- 51 Hossler P. et al. Optimal and consistent protein glycosylation in mammalian cell culture. 2009 Glycobiology 19:9, 936 949
- 52 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Quality of biotechnological products: analysis of the expression construct in cells used for production of r-DNA derived protein products Q5B Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1995 Nov 30 [cited 2016 Jul 20]. 4 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5B/ Step4/Q5B_Guideline.pdf.
- 53 Food and Drug Administration, Center for Biologics Evaluation and Research (US). Points to consider in the manufacture and testing of monoclonal antibody products for human use [Internet]. Washington, DC: Department of Health and Human Services; 1997 Feb 28 [cited 2016 Jul 20]. 50 p. Available from: http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/OtherRecommendationsfor-Manufacturers/UCM153182.pdf.
- 54 Glazer AN, Nikaido H. Microbial biotechnology: fundamentals of applied microbiology. First ed. New York: W.H. Freeman and Company; 1995. 640 p.
- 55 Hay RJ. Preservation and characterization. In: Freshney RI, editor. Animal cell culture: a practical approach. New York: Oxford University Press USA; 1989. p. 71-112.
- 56 Wiebe ME, May LH. Cell banking. In: Lubinecki AS, editor. Large scale mammalian cell culture. New York: Marcel Dekker Inc; 1990. p. 147-60.
- 57 Kleman MI, et al. Optimal conditions for freezing CHO-S and HEK293-EBNA cell lines: Influence of Me2SO, freeze density, and PEI-mediated transfection of revitalization and growth of cells, and expression of recombinant protein. Biotechnol Bioeng. 2008 Aug;100(5):911-22.
- 58 Avis KE, Wagner CM, editors. Cryopreservation applications in pharmaceuticals and biotechnology. Englewood (CO): Interpharm Press; 1999. 500 p. (Avis KE, Wagner CM, editors. Drug manufacturing technology series; vol. 5).
- 59 Simione FP. Cryopreservation: storage and documentation systems. In Avis KE, Wagner CM, Wu VL, editors. Biotechnology quality assurance and validation. Boca Raton (FL): CRC Press; 1999. p. 7-31. (Avis KE, Wagner CM, editors. Drug manufacturing technology series; vol 4).
- 60 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin Q5A(R1) Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1999 Sep 23 [cited 2008 Dec 3]. 27 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5A_R1/Step4/Q5A_R1__Guideline.pdf.
- 61 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Comparability of biotechnological/biological products subject to changes in their manufacturing process Q5E Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2004 Nov 18 [cited 2008 Dec 3]. 12 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5E/Step4/Q5E_Guideline.pdf.



CHAPTER 6:

Cell Culture Development and Scale-up

evelopment of a cell culture process to produce a monoclonal antibody (also known as the upstream process) is initiated once a production cell line has been developed and cloned, a sub-clone selected and a primary cell bank prepared. At the earliest development stage, this activity will focus on increasing the viable cell concentrations and increasing the time at which the cells are at high cell viability to maximize antibody titers. Since cell culture development is normally initiated prior to the availability of the Master and Working Cell Banks, the transferability and reproducibility of a developed process must be verified using the intended production cell bank prior to scale up and cGMP manufacturing. As described in Chapter 5, most parental cell lines used to develop monoclonal antibody production cell lines are CHO cells that have been adapted to grow in suspension in serum-free media and have been banked and characterized for identity and purity. Using such adapted parental cell lines in the preparation of production cell lines is an effective strategy that reduces the risk of impacting antibody productivity during medium and suspension adaptation, and shortens the overall development timeline by enabling direct progression from cell line development to cell culture development and initial manufacturing.

The upstream process for the manufacture of a monoclonal antibody starts with the preparation of an inoculum, traditionally using cells from one or more vials, or bags, of a working cell bank, and the subsequent continuous propagation of cells in different types and increasing sizes of growth vessels, which may include plastic bags, T-flasks, shake flasks, spinners and bioreactors of varying volumes, up to hundreds of liters. The inoculum is then used to seed a production bioreactor where the cells are grown and maintained to produce the corresponding antibody. Growth and productivity profiles depend primarily on intrinsic characteristics of the production cells, but are also greatly influenced by many physical, chemical and nutritional parameters discussed below. During culture, the monoclonal antibody product synthesized by the cells is secreted into the culture medium so the output of a bioreactor consists

of spent culture medium containing the antibody product, cells (viable and dead) and cell debris, plus process related impurities such as host cell proteins, nucleic acids, medium additives and selective agents if used, endogenous retroviruslike particles, and impurities related to the product including heavy and light chains, antibody fragments, aggregates, and denatured molecules.

Upstream process development has as a primary objective the definition of a robust, reproducible, cell culture process that is based on the best conditions identified to consistently generate high product yield and quality, and minimal amounts of impurities and contaminants. During upstream process development, media composition and bioreactor conditions are studied to select the optimal for both. To these objectives, screening and factorial experiments are usually done to determine the effects of multiple interacting variables on cell growth and viability, culture longevity, specific productivity, antibody yields, product consistency, and impurity levels.

Process development is expected to be done by increasingly adopting principles of QbD, as discussed in Chapter 3.^{1,2} One output of QbD is the generation of an understanding of the relationship between CQAs of the product, such as the glycan structure, and critical process parameters (CPP) that impact the CQAs of the product. By developing this relationship information early in development, future process optimization efforts can focus on minimizing changes to these critical parameters and therefore minimizing changes in the product.

A comprehensive cell culture process development plan consists of a number of related activities, all undertaken to improve the overall production of the monoclonal antibody product in the bioreactor, while maintaining control over the product CQAs. A major activity that impacts all other stages of upstream and downstream processing is media optimization, including the design of strategies for nutrient supplement(s) feeding. Often, the lead production cell line clone and a back-up clone are progressed into this stage of cell culture development to ensure no loss of development time if the primary clone fails to achieve sufficient growth and productivity, in any cell culture condition, or is not stable. Basal media from multiple suppliers, and/or proprietary media formulations, are evaluated and compared to select the best one for each production cell line. Following confirmation of the lead production cell line's suitability and stability for large-scale manufacturing and the selection of the optimal basal medium, studies to identify limiting nutrients begin. Using a DOE approach,³ these studies are used to guide the selection and evaluation of corresponding media feeds containing combinations of amino acids, lipids, inorganic salts, and micronutrients (essential nutrients such as trace minerals or vitamins, required by the cells in minute amounts), that may also include energy sources such as glucose and glutamine. In addition, non-nutritional small molecule additives that may stimulate production, such as sodium butyrate, dimethyl sulfate, and others are tested to determine their impact on overall antibody expression and quality.

A second major activity involves the evaluation of bioreactor conditions aimed to increase productivity and yield of antibody product, and the data generated helps support the choice for bioreactor conditions. Evaluation of parameters such as pH, osmolality of medium for initial growth and during product accumulation phases (which often have different optima), dissolved oxygen and CO2, temperature (including temperature shifts during culture), sparging and agitation (mixing for mass transfer and hydrodynamic effects), are included in the optimization of bioreactor conditions. Often these experiments can be performed in micro-scales, using qualified models such as shaking deep well plates, tube-spin bioreactors, or micro-bioreactors such as the ambr[™] system,⁴ the micro-24 from Pall Inc,⁵ and others discussed below. The availability of micro-scale systems enables wider multivariate analysis to be performed at this early stage and facilitates exploration of larger areas of potential design space than was previously possible. In the absence of a qualified micro-scale development platform, small shake flasks are often used as an intermediate development scale. The selected conditions from any smallscale model system should then be confirmed in bench scale bioreactors ranging in size from 1 L to 10 L.

To expedite initial product development, and for the production of monoclonal antibody products for preclinical studies and cGMP manufacture of Phase 1 clinical trial material, proprietary cell culture process platforms are increasingly employed by CMOs and larger biotechnology companies. Cell culture process platforms may include the use of a common parental cell line to generate the new production cell lines, favored media compositions and feeds, and standardized bioreactor conditions. Such platform processes have evolved from the greater understanding of CHO and other cells, metabolism and nutrient requirements, as well as through accumulated experience with production of a variety of antibodies. Recently, proteomic and transcriptomic analysis of CHO cell metabolism has enabled the introduction of parental CHO cells specifically engineered to improve antibody expression through activation of genes responsible for translation, secretion, or cell viability.⁶ The use of a platform process and/or an engineered parental cell line provides a high level of assurance during early process development that an appropriate upstream process can be developed within acceptable timelines, and at reasonable cost, but does not assure that the maximal productivity for the specific cell

line will be achieved. Companies using platform processes to enable rapid progression for production of material for Phase 1 clinical trials will continue to engage in cell culture development and optimization prior to production of Phase 3 clinical trial material. At this point, the upstream process should be fully defined and suitable for scale-up to appropriate levels for commercial manufacturing.

1. Growth and Productivity Assessment

The first steps in cell culture development should include analysis of cell growth kinetics and productivity of the selected cell line. Mammalian cells divide by binary fission and exhibit exponential growth kinetics. When growth data is plotted as viable cell counts against time, from the thawing a vial from a cell bank to start the preparation of an inoculum, a typical batch growth curve is observed that consists of the following six phases^{7,8}

- Lag
- Accelerating growth
- Exponential (logarithmic) growth
- Decelerating growth
- Stationary phase
- Decline or death phase.

A lag period is always observed when starting cultures from frozen stocks because, upon thawing, the cells go through an initial period of adhaptation. Maximum exponential growth occurs when the growth conditions are ideal (e.g. no nutrient limitations, no accumulation of metabolites to toxic levels, and no changes in pH or osmolality to inhibitory levels). During normal exponential growth, the doubling time (t_{at} also known as generation time) is at the minimum and the specific growth rate (μ), which is the rate of growth per unit amount of biomass, is maximum (μ max, expressed in h⁻¹). The μ max is related to the doubling time as shown in Equation 6.1.

Equation 6.1. Relationship of Doubling Time to Growth Rate

$$td = \frac{ln2}{\mu max} = \frac{0.693}{\mu max}$$

When a culture is maintained beyond the exponential phase before passaging (that is to say before sub-culturing, which consists of transferring a fraction of the culture into fresh medium in another vessel or diluting the culture two or more-fold with fresh medium), a lag is often observed upon sub-culturing and the cells are said to have been "overgrown." The longer cells are maintained beyond the exponential phase, the more likely that a lag period will occur upon sub-culturing. Ultimately, there will be a point of "overgrowth," past which all cells will die due to one or a combination of causes including starvation, toxic metabolite (e.g. lactate and ammonia) build-up, and/or extreme pH and osmolality so that the culture can no longer be recovered when sub-cultured.⁹

For most monoclonal antibody-producing mammalian cell lines, doubling times during exponential growth range between 14 and 36 hours, corresponding to specific growth rates (μ) of 0.05 to 0.02 h-1, respectively.^{10, 11} The doubling time for CHO production cell lines is frequently in the range of 2432 hours, and one goal of both clone selection and cell culture development is to obtain a clone and upstream process that delivers a doubling time at the lower end of this range. Shorter doubling times will translate into shorter overall processes, which may reduce production costs.

Cells can be cultured in steady state conditions in chemostats, where fresh medium is fed at constant flow rate (F, in liters/hour) and the culture is harvested at the same rate, thus maintaining the culture volume (V) constant. In chemostat cultures the dilution rate (flow rate per unit volume of bioreactor: F/V = D) which has units of reciprocal hours [h-1]) is equivalent to the specific growth rate μ . The maximum growth rate (μ max) is equal to the critical dilution rate (Dc) where the flow is maximum and the steady state biomass is reduced to zero.7 While in theory the specific growth rate can be regulated at any value below maximum, in studies of chemostat cultures at dilution rates ranging from critical (Dc), to less than ten percent of critical, an intrinsic minimum specific growth rate of approximately 0.02 h-1 was found for all types of mammalian cells examined.^{12, 13, 14, 15} Viability decreases with decrease in dilution rate^{16, 17, 18} and death is attributed mostly to apoptosis induced by reduced protein synthesis. A practical implication of this minimal μ for continuous perfusion culture processes at high cell density, which are used for production of several monoclonal antibodies, is that the fraction of dead cells will increase with decreases in the dilution rate, which is equivalent to purge rate and

thus the growth rate in perfusion cultures (Purge rate is the flow [volume per unit time] of high-density culture removed from the bioreactor divided by the volume of the bioreactor).¹⁴ The decrease in the ratio of viable/dead cells will result in decreased productivity and final yield of the culture by decreasing the monoclonal antibody concentration in the harvest and reducing the longevity of the culture. This decreased ratio can also affect the product's profile and quality attributes.

Monoclonal antibodies are expressed only by viable cells during culturing.^{19, 20} There are four patterns in monoclonal antibody production observed in hybridomas and recombinant monoclonal antibody producing cells, namely: growthassociated,^{21, 22, 23} partly growth-associated,^{17, 24, 25} non-growthassociated,²⁶ and negative or inversely growth-associated.²⁴

The specific production pattern of a given expression cell needs to be considered in defining the type of culture and harvest times for optimal production. For instance, with cells exhibiting a growth associated pattern, once growth slows or stops monoclonal antibody production will slow or stop and little will be gained from extending the culture. Alternatively, for cells where production is non-growth associated, it will be advantageous to extend the longevity of the culture because the cells will produce when quiescent, and the final product yield will be a function of the number of viable cells and the time they are kept in culture. This is the main feature of fed-batch culture, which can extend culture longevity and overall product yield.

Specific monoclonal antibody productivities in hybridomas and recombinant monoclonal antibody producing cells range from less than 10 pg/cell-day to over 100 pg/cell-day achieved in optimized fed-batch culture.^{28, 29, 11} High specific productivities in recombinant cells are often associated with high transgene copy numbers,^{30, 31} with a with a heavy chain to light chain gene ratio optimized for maximal expression,^{32, 33, 34} increased heavy and light chain gene transcription levels,31 and increases in mRNA stability.³⁵

The key to successful monoclonal antibody upstream manufacturing process development is to appropriately leverage the cell growth and antibody production characteristics into a process that is implementable, robust and cost effective.

2. Media and Feed Optimization

Optimizing the composition of the culture media and feeds and the feeding strategies for monoclonal antibody production are key aspects of upstream development that offers significant potential to increase productivity.36 In addition, to support high viabilities, specific product vities, and cell densities, which translate into high product titers, medium development and selection requires the consideration of additional process factors. These factors include those related to culture performance, simplicity and robustness, product quantity and quality, compatibility with downstream process, regulatory compliance, and cost of goods.³⁷ Cell culture media and feed development are highly amenable to a QbD approach because the interaction of many variables combine to give the highest growth and productivity, and QbD enables exploration across ranges of these variables.³⁸

Basal Media

The use of serum-free media has become standard in industrial production of monoclonal antibodies, and protein-free media are used in a growing number of applications. Older commercial processes may still use serum, but newer CHO parental cell lines and cell culture processes are expected to be serum free to reduce risk of adventitious agents arising from the serum sources.³⁹ There are multiple formulations of cell culture media commercially available that support growth and production under serum-free conditions of all parental cell lines used in the manufacture of monoclonal antibodies, with a dominant industry focus on designing, developing, and marketing media that is specific to various CHO cell lines typically used in the biopharmaceutical industry today. Because each of these media will most likely perform differently for a given production cell line, the selection of a basal medium is done by comparing different media for their capacity to sustain cell growth and generate product. Often combinations of these commercial formulations can give higher productivities. Integrating the selection of lead clone and basal medium is highly efficient, can save substantial development time, and result in increased productivities and product yields.⁴⁰ This integrated approach is often taken by CMOs and by companies with large antibody development pipelines, and is preferable to sequential clone selection and media optimization in both time and cost. The use of microbioreactors is a strategy that can accelerate this process.⁴¹

Media Components

Identifying the effects and interdependence of media components on growth, productivity, and product attributes like glycosylation, and determining which components become limiting in batch culture, is critical for the optimization of the basal production medium, and for defining the composition of media additions and their timing of addition.^{42, 43, 44} Different groups of media components have relevant effects on cell physiology and monoclonal antibody production that can be useful in determining which components are most likely to improve product quality and cell line productivity. The numerous potential interactions between media components and their impact on cell growth, productivity, and product quality can be assessed using a factorial design of experiments approach,⁴⁵ by metabolite profiling,⁴⁶ by comparative metabolic analysis of cell lines,^{47,48} and by media blending.49, 50

Energy Sources

Mammalian cells utilize glucose and glutamine as the preferred sources of energy. Glucose utilization rates in hybridomas and recombinant monoclonal antibody producing cell lines range from <1 pmol/cellday to >10 pmol/cellday.^{34, 51} In the absence of glucose (or some substitutable sugar such as fructose), mammalian cells do not grow.^{52, 53} Glucose metabolized through glycolysis generates lactic acid, which accumulates in the medium of the bioreactor. In later stages of a cell culture process, the lactic acid is frequently utilized by the cells. However, excessive lactate accumulation inhibits cell growth and monoclonal antibody production.^{54, 55} The levels of lactate can be controlled by nutrient feeds and even cause a shift in lactate metabolism from net lactate production to net lactate consumption.^{56,47} The enzyme lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate into lactate. In monoclonal antibody-expressing CHO cells that had LDH and pyruvate dehydrogenase kinase down-regulated, resulted in reduced lactate production, increased specific productivity and increased volumetric antibody production by approximately 90%, 75% and 68% respectively without appreciable impact on cell growth.⁵⁷ Better understanding of the metabolic profiles and their regulatory mechanisms of low and high producing cells would help to develop very productive cell culture processes.48

Glutamine is typically used along with glucose to support

growth and to help maintain cell viability in mammalian cell culture. In hybridomas, the utilization rate of glutamine ranges from <1 pmol/cell day to >5 pmol/cell day, depending on its concentration in the media.³⁴ CHO cells have a lower dependency on glutamine than hybridomas or murine myelomas so that less is required in media to support their growth.⁵⁰ In recent metabolic analyses of CHO cells, glutamine was found to be more efficiently utilized than glucose for anaplerotic replenishment and contributed more to lactate production during the exponential phase of growth.⁴⁸ The consumption of glutamine by the cells generates ammonia, which accumulates in the medium and can be inhibitory for growth and production, as well as affect the glycosylation of monoclonal antibodies by inhibiting terminal sialylation.⁵⁸ The terminal sialylation of monoclonal antibody is also affected by the concentration of both glucose and glutamine in the media. Low concentrations (<0.7 mM glucose or <0.1 mM glutamine) result in decreased sialylation of the product.⁵⁹ Addition of some amino acids such as threonine, proline, and glycine has been reported to mitigate the toxic effects of ammonia.⁶⁰

The potentially negative impacts of glucose and glutamine consumption can be minimized by using appropriate nutrient feeding strategies to control the levels of these nutrients and redirect their metabolism to minimize lactate and ammonia production.^{61,62} Application of such approaches in fed-batch culture can greatly increase the number of cells and monoclonal antibody concentration in the bioreactor.^{63,64}

Amino Acids

Proteins are the most abundant components of mammalian cells and represent approximately 18% of the cellular weight.⁶⁵ Cells transfected to produce a monoclonal antibody have the additional burden of synthesizing heterologous proteins and require sufficient quantities of the corresponding amino acid building blocks.

Mammalian cells are unable to synthesize certain essential amino acids, which must be provided in the medium for the cells to grow. Although non-essential amino acids can be synthesized by the cells, they are also routinely added in media formulations. Addition of these amino acids results in lower energy requirements to the cells because the need to synthesize them is eliminated and the cell can instead divert additional energy to production of the monoclonal antibody.

Glutamine is an essential nutrient in cell cultures for energy production as well as protein and nucleic acid synthesis, but in cell culture media it is highly labile and degrades spontaneously, generating ammonia and pyrrolidone carboxylic acid as byproducts.⁶⁶ Therefore, glutamine is frequently provided as a dipeptide because in this form it is much more stable.

Supplemental additions of glutamine and asparagine to cultures of CHO-GS cell lines cultures were found effective in buffering the culture, reduce lactate generation, maintain a higher viability profile and improve antibody productivity.⁶⁷

Tyrosine is poorly soluble in aqueous medium and tends to precipitate. Starvation of the cells for this amino acid can cause unwanted tyrosine sequence variants in the monoclonal antibodies produced, where the cell replaces tyrosine with another amino acid. The practice of feeding tyrosine-containing dipeptides allows the introduction of higher concentrations of the amino acid, therefore preventing the nutritional limitation and eliminating unwanted primary structure changes in the product.⁶⁸

In addition to their nutritional function, several amino acids including threonine, proline, and glycine have been shown to have protective effects for cells exposed to stresses such as starvation, hyperosmolality, and elevated CO2 or ammonia.^{58,69} conditions which are likely to occur during extended fed-batch culture and in highly productive, high cell density processes.

Lipids

Lipids are essential structural elements of all cell membranes for the proliferation, growth and survival of all cells, and represent 5% of the mammalian cell weight. Sixty percent of the lipids are phospholipids, the most abundant components of membranes.⁷⁰ In whole CHO cells the three main lipids correspond to phospholipids, present at 30 fmol/ cell, cholesterol at 10.8 fmol/cell and sphingomyelin at 1.9 fmol/cell, and the plasma membranes contain 49% of the total phospholipids, 64% of the cholesterol and 69% of the sphingomyelin.⁷¹ Starting from ethanolamine and fatty acids, mammalian cells can synthesize phosphatidylethanolamine from which they can then derive other phospholipids. While mammals require two essential fatty acids (linoleic and α -linoleic) for growth, this requirement does not appear to apply to mammalian cells grown in culture. Nevertheless, these fatty acids, as well as lipoic acid and some phospholipids (i.e. phosphatidylcholine) are frequently included in basal media formulations.^{15, 72} Some cell lines are incapable of synthetizing certain lipids and require their presence in the medium to grow. One example is the requirement for cholesterol shown by the murine myeloma NS0, which has lost an enzyme in the cholesterol synthesis pathway.73 Supplementation of cell cultures with nonessential phospholipids, fatty acids, and sterols reduces the need for their biosynthesis. While mammalian cells respond differently to lipid supplements, improvements in cell growth and monoclonal antibody yields are common^{67, 74} and lipids are frequently used as part of the feeds employed in fedbatch cultures.⁷⁵ If the intent is to produce the monoclonal antibody using single-use bioreactors, one factor to consider is the potential that the added lipids may adhere to the bioreactor surface and become inaccessible to the cells. To address this issue, lipid supplementation and availability should be studied in small-scale single use bioreactors prior to scale-up and the supplementation should be adjusted to insure availability of adequate lipid to the cells.^{76,77}

Lipids are not water-soluble and require dispersion aids when added to culture media. In addition to serum extracts and bovine serum albumin, a traditional carrier, lipid dispersion technologies include emulsions, micelles, and lyposomes. In serum-free media the use of cyclodextrins, which are circular polymers of glucopyranose that increase the solubility of lipids, is highly successful.⁷⁸ Cyclodextrins provide a dual function in one: it effectively solubilizes lipids and maintains them in the media by molecular encapsulation.

The optimal concentration of cyclodextrin and its ratio to cholesterol-lipids in the medium need to be determined for each cell line, especially when growing cells in single use bioreactors. Okonkowski et al⁷⁹ found that the concurrent presence of the carrier methyl-beta-cyclodextrin in the culture medium and the linear low-density polyethylene film in Wave Bioreactors was sufficient to inhibit growth of cholesterol dependent NS0 cells. By reducing the excess cyclodextrin added to the culture medium, they were able to cultivate in Wave Bioreactors using a cholesterolcyclodextrin complex as the sole source of exogenous cholesterol. They proposed that the mechanism of growth inhibition involves the extraction of cholesterol from cell membranes by the excess cyclodextrin in the medium, followed with the irreversible adsorption or entrapment of the cholesterol-cyclodextrin complexes to the linear lowdensity polyethylene surface of the Wave Bioreactor bag.

Inorganic Ions

Inorganic ions are required in variable amounts by mammalian cells and can comprise up to 1% of the total cell weight.⁶⁷ As a result, these ions are routinely added to basal media in sufficient quantities and normally are not limiting in batch cultures, but may become limiting in extended highdensity cultures. Inorganic ion salts are present in media in six relative concentration levels: (1) very high concentration (grams/L), as is the case with sodium chloride, which is added to adjust the osmolality of the medium, and sodium bicarbonate, which is added to control the pH; (2) high concentrations (10100 mg/L), including calcium, lithium, magnesium, potassium and phosphate salts; (3) low concentration (hundreds of micrograms per liter), including iron and zinc salts; (5) very low concentration (tens of micrograms per liter), such as salts of aluminum, barium, cobalt, chromium, copper, fluoride, selenium and titanium; and (6) extremely low (below 1 μ g/L), such as salts of silver, germanium, bromine, iodine, manganese, molybdenum, vanadium, nickel, rubidium and tin. These inorganic salts are normally components of basal media and/or feeds that are added to the culture.

Phosphorus is a structural component of nucleic acids, phospholipids and other cell components. Phosphate feeding has been shown to increase cell and monoclonal antibody yields in some high cell concentration cultures.⁸⁰

Iron can be supplied in the form of chelated compounds using, for instance, citrate or EDTA, which eliminates the need for adding transferrin, the iron transport protein. It has been observed that selenium can serve as a highly effective iron carrier allowing high-density growth (>10 x 106 cells/ mL) and monoclonal antibody titers (approximately 3 g/L) in fed batch culture and in shake flasks.⁸¹

The addition of zinc ions to protein-free culture media can fully replace the need for addition of recombinant insulin for the cultivation of some hybridoma, NS0 and CHO cells, and support good growth and monoclonal antibody production.^{82, 83}

Vitamins

Vitamins are required by mammalian cells and sufficient amounts are included in most media formulations.¹⁵ While limitations are not normally observed under typical nonhigh density growth conditions, vitamins are frequently added as concentrated supplements for high productivity applications.⁷³ Vitamin concentrations above 35% of normal have been reported to diminish growth of some CHO lines.⁴⁴

Supplements

A common supplement employed frequently in cell culture in research laboratories and occasionally in the earliest stages of production cell line development is animal serum, although regulatory and safety concerns have significantly reduced or eliminated serum use even in early development. Serum contains nutrients, hormones, proteins and many factors that directly or indirectly stimulate cell growth or are otherwise protective and therefore provides a superb growth media for mammalian cells. However, for clinical and commercial manufacturing, the use of animal-derived serum presents practical and regulatory issues that include variability in quality and growth promotion capability, and the concern of potential contamination with prions and animal viruses. Because of these very valid concerns, regulatory authorities have issued guidelines aimed at controlling and, in effect, discouraging the use of animal serum in any stage of development for the production of biologics.39

Serum can be replaced by combinations of supplements, such as insulin, transferrin, ethanolamine and selenium, which are most frequently employed in serum-free formulations. Protein hydrolysates from animal, yeast, and plant sources are also used as components of basal media and supplements. However, concerns with prions and adventitious viruses also apply to the use of animal-derived hydrolysates and proteins, and even plant derived hydrolysates have been shown to contain animal viruses capable of propagation in CHO and other cells.^{84,} ⁸⁵ Therefore, the current trend is towards fully chemically defined media which, while more acceptable from a regulatory and safety point of view, provides a more challenging growth environment for mammalian cells in culture. As a result of industrial experience with contaminated raw materials including culture media, new procedures to insure the safety of all components of the supply chain have been required by regulatory authorities and implemented by companies to reduce the risk of contaminating products during manufacturing. These procedures include revised raw material management and testing, risk evaluation, evaluation of technologies, which may inactivate viruses in raw materials (e.g., UV treatment and gamma irradiation), and implementation of nucleic acid testing for process surveillance.

When proteins such as insulin, transferrin and albumin are added to the culture medium, the use of recombinant material, instead of animal-derived, is preferred. The addition of unwanted proteins that can interfere with downstream processing and that can be present in small amounts in the final purified product is reduced when utilizing serum-free medium, and is eliminated with proteinfree formulations. Many serum-free and protein-free media are commercially available in powder or liquid forms, including concentrates. Commercially available or custommade supplements provide concentrated nutrients and growth factors that can be used to constitute nutrient feeds for the fed-batch process.⁷³

Other Additives

Additions of some compounds to the medium, such as sodium propionate and sodium butyrate, have been reported to enhance product yields in hybridomas and CHO cells by mechanisms not entirely clear, although increased transcription of heavy and light chain genes by improvement of gene accessibility seems to be involved.^{86, 87, 88, 89}

Aurintricarboxylic acid has also been introduced recently as a synthetic alternative to transferrin and was also found to exert insulin-like growth stimulating effects in CHO cells,⁹⁰ and is used in some manufacturing processes.

Hexanohydroxamic acid was shown to increase monoclonal antibody production in CHO cells by 40%,⁹¹ and pentanoic acid was reported to enhance protein production in CHO cultures.⁹²

Valproic acid (VPA, 2-propylpentanoic acid) is a branchedchain carboxylic acid inhibitor of histone deacetylase. When added late in culture was found to increase monoclonal antibody titers on two (one strongly and the second moderately) out of three CHO cell lines that stably express monoclonal antibodies.⁹³

Surfactants such as Tween 80 and Pluronic F-68 are often added to cell culture media to protect cells from hydrodynamic damage caused by shear and surface bubble bursting during agitation and sparging in the bioreactor.^{94, 95}

Development of the cell culture process involves testing various commercially available or custom media formulations that contain some or all of these additives, in conjunction with supplement feed strategies, to identify the best combination for the specific production cell line. Even clonal cell lines with a common origin can behave differently in culture due to unknown epigenetic or integration effects of the transgene, and therefore dedicated process development will generally provide a superior process compared to a platform that is more generally based on the parental cell line.

3. Media Preparation and Storage

Most manufacturing operations prepare cell culture media by reconstituting powder formulations of the desired media or diluting concentrated liquid formulations of the media using purified water or WFI, or by weighing individual medium components into a suitable medium preparation tank and dissolving all components by adding purified or WFI water. Once prepared, the liquid medium is sterilized by filtration through 0.1 μ m or 0.2 μ m filters into a presterilized bioreactor or media holding tank. For small and medium scale operations, the use of liquid media directly from vendors, supplied in plastic bags, is common, as it is for concentrated supplements. Obviously, this approach simplifies operations for media preparation, but introduces the need for cold storage of the media supplies required to maintain uninterrupted operations.

Cell culture media has components that are labile at room temperature or culture conditions and that therefore spontaneously degrade, more so when in liquid form. To slow media degradation, all media should be stored cold and in the dark and powdered media should be held in containers that prevent exposure to moisture.

Feed development and feeding strategies The use of nutrient feeding prevents the depletion of

important medium components and results in improved culture longevity and high cell and product yields. There are multiple formulations of cell culture feeds commercially available for CHO and other production cell lines.

Feed development can involve analysis of spent media to calculate the specific consumption rates for nutrients such as amino acids and vitamins. Concentrated nutrient feeds can then be formulated such that the ratio of each and every pair of nutrients is equivalent to their specific consumptions rates. Formulated in this way, the idea is that restoration of one nutrient to its basal level through feeding will restore all nutrients to their basal levels.

Current industry practices for large-scale mammalian cell cultures typically employ a standard platform fed-batch process with fixed volume bolus feeding at intervals.⁹⁶ Recent work on the development of feeding strategies involves automatic monitoring and dynamic feeding employing algorithms to alter feed rates. Lu et al. described a simple strategy for fed-batch optimization, combining the development of a feed medium based on spent media analysis and the establishment of a feeding strategy that consists of adding variable volumes of feed media at specific intervals after off-line measurement of the concentration of a reference nutrient.⁹⁷

4. Cell Culture Process Development, Optimization, and Scale-up

As with media development and optimization, the development and optimization of a cell culture process for monoclonal antibody production is the other key aspect of upstream development that offers significant potential to increase productivity.

Perfusion versus Fed-batch Processes

Two basic types of cell culture modes, perfusion and fed-batch, are employed in the production of clinical and commercial monoclonal antibodies.⁹⁸ Perfusion culture is a continuous process in which cells are often cultured for weeks and spent medium, containing product, is continuously removed from the bioreactor and replaced with fresh medium. Because of the continuous media replacement that take place during a perfusion process, these cultures achieve higher volumetric productivities (i.e. grams of product per liter of bioreactor per day) than batch cultures but at the expense of lower product titers per liter of medium consumed. The changing cellular micro-environment and long culture times required for perfusion production may lead to inconsistent production results, including variable glycosylation and other post-translational modifications in the product. In addition, because of the extended times of operation, there is a higher risk of contamination than for a batch or fed-batch process. Perfusion operations tend to be more complex, difficult to scale-up, and generally less robust than fed-batch processes.⁹⁹ Nonetheless perfusion operations are used for the commercial manufacturing of several monoclonal antibodies and other biopharmaceuticals.

In a fed-batch process, cells are maintained within a bioreactor for a fixed period of time with no media or product removed at any stage other than the final batch harvest. During the culture period, required nutrients are added to maintain cell growth, viability, and culture longevity, resulting in increased overall productivity. This approach is generally more robust and easier to implement and has a lower risk of contamination than perfusion cultures. Fed batch processes using high producing cell lines can enable total bioreactor productivities that rival those of perfusion cultures so that very high cell concentrations (>107 viable cells/mL) and monoclonal antibody titers of >5g/L can be routinely obtained.^{100, 101, 102}

Because of the larger bioreactor volumes required for fed-batch culture compared to perfusion culture, the capital investment required for a manufacturing facility based on fed-batch culture are higher than those for a perfusion culture-based facility. However, the overall cost of manufacturing for a monoclonal antibody product using either fed-batch or perfusion culture is generally comparable.^{103, 104, 105} While both fed-batch and perfusion cultures are successfully used today by companies producing commercial monoclonal antibody products, fed-batch culture has been adopted as the primary method of choice for robust, reproducible, and reliable monoclonal antibody manufacturing. Fed-batch processes are also more compatible with multi-product facility operations and are therefore generally preferred by CMOs offering cGMP production services to multiple customers.

Integrated continuous production of recombinant proteins including monoclonal antibodies, comprised of high-density

perfusion cultures directly coupled with continuous capture steps are increasingly being explored and implemented throughout industry,¹⁰⁶ and may become widely adopted manufacturing platforms replacing the current, discontinuous ones.

Bioreactor options

A large variety of in vitro culture bioreactors can be used to grow monoclonal antibody producing cells, ranging from laboratory systems such as multi-well plates, T-flasks, roller bottles, and spinner flasks to larger scale production vessels including hollow fiber cartridges, bag, air-lift, and stirred-tanks. For large scale production, the stainless steel stirred-tank bioreactor is the most common bioreactor type used, ranging in scale up to 25,000 L.¹⁰⁷ Single-use (disposable) bioreactors at scales ranging from 50 L to 2,000 L are increasingly used for inoculum preparation and/or production of clinical trial material.^{108, 109, 110, 111, 112} There is no technical or regulatory reason to prevent the use of single-use bioreactors for production of commercial product, but the technology is relatively recent so there are very few products in the market that are manufactured this way. Nonetheless, it is anticipated that more commercial products will be produced using singleuse bioreactor technology, as long as the available scale of bioreactors is sufficient to meet the market demand.

The bioreactor provides a physicochemical environment in which key variables for cell growth and productivity, such as pH, temperature, O2, CO2, sparging, agitation, and osmolality can be carefully monitored and controlled as discussed below.

Process variables

pН

Mammalian cells can tolerate a range of pH (i.e., 6.4 to 8) if adapted gradually, but viability is strongly affected by sudden pH changes and extended pH perturbations.¹¹³ Uptake and utilization of nutrients is affected by pH. For instance, glucose and glutamine utilization rates increase at alkaline pH.15 Maximum viability and growth rates are usually observed at pH values between 6.9 and 7.3, but the values are different for each cell type and affected by other conditions such as cell density, nutrient feeds, and oxygen levels. Cell growth and monoclonal antibody production are frequently optimal at different pH values so that a production process will typically consist of a growth phase in which the pH is controlled at the best value for growth followed by a production phase where the pH is adjusted to a value that enhances antibody production and or maintains viability.¹¹⁴

The glycosylation of a monoclonal antibody can be affected by the pH of the medium.¹¹⁵ Thus, the effect of pH on growth, productivity, monoclonal antibody yields, and product attributes, such as glycosylation profile, must be studied to maximize yields, product quality and consistency.

Cell cultures trend towards acidic pH during growth due mainly to the production of lactic acid and CO2. To control the pH, sodium bicarbonate when used as a buffering agent is initially added to media in concentrations of several grams per liter. Organic buffers such as 2[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 2-morpholinoethanesulfonic acid (MES), 3-morpholinopropanesulfonic acid (MOPS) or others are also used at concentrations of 10 mM or higher.15

Temperature

Optimal growth temperature for mammalian cell growth is about 37°C, but the cells can tolerate short exposures to slightly higher temperatures. CHO, myelomas, hybridomas, and other cells are less sensitive to lower temperatures and can be maintained at room temperature for several days.

Culturing of mammalian cells at sub-physiological temperatures has multiple effects including decrease in growth rate, delayed apoptosis, maintenance of cell viability for longer periods, reduced glucose and glutamine consumption, and others.¹¹⁶ Lowering temperature of cultivation after the growth phase has been reported to enhance monoclonal antibody production for some processes,¹¹⁷ and is frequently used in monoclonal antibody production. However this approach had little effect on other manufacturing processes, indicating the effects of temperature on productivity is cell line and product specific.¹¹⁸

Oxygen

Oxygen has limited solubility in aqueous solutions and must be supplied nearly continuously during cultivation. In many instances, cells will be unaffected by a wide range of oxygen concentrations. However, high oxygen concentrations (i.e. >100% of air saturation) can inhibit growth rates and induce cell lysis in later culture stages, presumably through the production of damaging oxygen radicals. Specific oxygen consumption rates have been reported to range from 1 to 40 pg/cell-hour.¹¹⁹ Optimal concentrations of dissolved oxygen for growth and production may be different in some cells and the glycosylation pattern of monoclonal antibodies can be affected by the levels of dissolved oxygen.^{120, 121} Oxygen is one of the main limiting factors in the maintenance of viable populations in high density perfusion cultures. Limited oxygen can induce the increase of lactate to levels that will have a negative impact on growth and productivity.

Carbon Dioxide

During growth the cells produce CO2, some of which exits the off-gas while the rest dissolves into the medium. Dissolved CO2 acts to reduce the pH of the culture medium and the intracellular pH, which affects the activity of intracellular enzymes and cell metabolism. The partial pressure of CO2 in the culture medium produced by highdensity cultures may reach 150 to 200 mm Hg and can inhibit growth and affect monoclonal antibody production and glycosylation.122, 123 Removal of CO2 that builds up in large-scale bioreactors is an important issue, but excessive stripping of the CO2 can also be detrimental to cell growth. Thus, optimal levels of CO2 for growth and production need to be determined and are often cell line dependent.¹²⁴

Sparging

The design of adequate, scalable sparging strategies to control dissolved oxygen and CO2 in cell culture development is important. Micro-bioreactors can be used to define the optimal concentrations for sparging and control of dissolved gases but scale-up of these strategies often requires additional fine-tuning at the large scale. Oxygen is often used to supplement air sparging. It is desirable to keep the superficial velocity of the sparge gasses similar on scale-up. Mathematical models can often be used to help adjust the agitation and sparge rates to achieve consistent gas levels at large scale.¹²⁵

Agitation

Agitation in the bioreactor creates the mixing needed to achieve culture homogeneity in cell density, gas dispersion, temperature, pH, nutrients, and waste metabolites. Excessive

agitation produces hydrodynamic shear (as does bubble surface bursting) which may result in cell damage^{126, 127} which, in addition to reducing production yields, can cause effects on product attributes such as disulfide bond reduction¹²⁸ and changes in the abundance and composition of host cell proteins.¹²⁹ To protect the cells in culture from potential damage by hydrodynamic forces inherent to suspension culture, surfactants are often added to the medium. Poloxamer 188 (Pluronic F68) is the primary additive in cell culture media used industrially to protect cells from hydrodynamic damage caused by agitation and sparging. The concentrations of Poloxamer 188 in cell culture media range typically from 0.5 to 3 g/L (0.05 to 0.3% w/v). Addition of low concentrations of Poloxamer 188 (i.e. 0.02 g/L) can cause a reduction in oxygen mass transfer in cell culture but this effect is generally not observed when the Poloxamer 188 concentration exceeds 0.1 g/L.¹³⁰

Osmolality

Mammalian cells grow optimally in physiological osmolality values ranging from 260300 mOsmol/Kg H2O, and commercial basal media are usually formulated to have these initial values by adjusting the NaCl concentration. Osmolality increases during cultivation, mostly due to feeding concentrated nutrients, and high values inhibit growth rate and cell yields. Hyperosmolality, has been shown in several cases to induce higher monoclonal antibody production at later stages of cultivation.^{131, 132, 133} Thus, some manufacturing processes include a shift to higher osmolality at the start of the production stage of cultivation either deliberately or as a result of adding concentrated feed solutions. Hyperosmotic stress and elevated CO2 levels can alter the monosaccharide content and isoelectric point of some monoclonal antibodies.¹³⁴ Therefore, increased yield must be balanced with the negative consequences of introducing changes in the antibody CQAs during process optimization.

5. Optimization and Scale-up of Cell Culture Processes

There are multiple equipment alternatives for doing cell culture development and optimization. Process development laboratories commonly use shake flasks of less than one liter and bench-top bioreactors (e.g., spinner vessels and bioreactors with dimensional and design characteristics of larger bioreactors) with working volumes in the range of 15 L to perform the experiments needed to develop a cell culture process, with the 2 L bioreactor being a common bench-top platform. While traditional shake-flask culture methods are used to generate useful information and have an important place in cell culture development, they are limited in their ability to adequately simulate larger scale bioreactors in key aspects, such as oxygen transfer and monitoring and control of key process parameters, including DO and pH. On the other hand, bench-top bioreactors, as well as larger bench-top systems require significant amounts of labor and resources to set-up and monitor, although automated on-line sampling systems such as the SegFlow^{®135} (a product of Flownamics®) can facilitate the monitoring tasks. Because of the time needed for each bench-top bioreactor experiment, it is often difficult or cost-prohibitive to run the necessary number of experiments required to minimize process variability and fine-tune a cell culture process. In addition, the extensive data analysis associated with these cell culture experiments often prevents the full characterization and optimization of a cell culture process necessary to define a robust commercial manufacturing process.

Use of Mini-bioreactors for Cell Culture Process Development and Optimization

Steadily increasing business demands to reduce product development time and costs as well as expectations from the regulatory authorities for better process understanding, characterization and validation¹³⁶ has caused companies to search for more efficient process development tools that help reduce development time and cost while providing better process insight and understanding. One approach to reaching these goals is the use of high throughput or mini-system experimental technologies that rely on either significantly scaled-down experimental methods or on other miniaturized systems that can generate greater information about a particular unit operation in shorter periods of time while consuming less material.^{137, 138, 139}

In most cases, a strategy that combines the use of high throughput methods with traditional larger scale development methods is likely to be the most appropriate means to ensure the generation of reliable and useful data while maximizing productivity of process development resources. This combination of miniaturized, high throughput development technologies to rapidly and efficiently examine many process alternatives and variables, combined with traditional laboratory-scale process development to confirm the details of individual unit operations, can expand the total knowledge base on which a monoclonal antibody manufacturing process is developed and increase process reliability, thereby leading to a more robust and fully controllable manufacturing process.

High throughput techniques have been used in the small molecule drug discovery field for decades and have been introduced more recently in the field of biologic process development. The successful application of high throughput screening and mini-system experimental techniques have been reported in many areas related to the development of bio-manufacturing processes, such as clone screening and selection^{140, 141, 142}

Mini-bioreactors with working volumes of 0.5250 mL, that incorporate similar mixing and mass transfer parameters as larger bioreactors, as well as suitable monitoring and control software to allow a comparable level of control as a production bioreactor, have been developed to allow simulation of larger bioreactors without the time and expense required for the larger systems.

The Advanced Microscale Bioreactors (ambr[™]) ambr15 system from Sartorius Stedim Biotech, is an automated workstation that provides individual monitoring and control of culture dissolved oxygen and pH in 24 or 48 single-use, stirredtank bioreactors at a working volume of 10-15 mL of capacity each offering parallel processing and evaluation of multiple experiments in an automated benchtop system.¹⁴³ Sartorius Stedim has also introduced the ambr250 system that provides for slightly larger working volumes of 100 – 250 mL.¹⁴⁴

The DASGIP Parallel Bioreactor System 145 is capable of handling 4, 8, 12 or more bioreactors in the range of 35 mL to 4 L that can be operated on a parallel basis with a single integrated controller.

An intermediate scale system called the Biopod is also available from Fogale Nanotech.¹⁴⁶ This system is capable of running eight different mini-bioreactors (either 80 mL or 800 mL in working volume) at once.

Additional systems and designs for running multiple minibioreactors are also available from M2p-labs¹⁴⁷ and other vendors. Some monoclonal antibody product development
companies have also built their own high throughput minibioreactor systems for process development.¹⁴⁸

Several investigators have shown the usefulness and reliability of mini-bioreactors systems in the development of cell culture processes. For example, Legmann et al.¹⁴⁹ used data from 700 µL SimCell mini-bioreactors to develop and optimize production of an anti-interleukin 1ß monoclonal antibody, which was then scaled to a conventional 3 L bioreactor. In this study, data collected in the minibioreactors was compared to data from the conventional bioreactor, showing excellent agreement between the two systems. Regression analysis of the data showed that the correlation factors for each parameter measured was greater than 0.84 and the product quality profiles were very similar. Amanullah et al¹⁵⁰ described the use of the SimCell micro-bioreactor technology for fed-batch cultivation of a GS-CHO transfectant expressing a model IgG4 monoclonal antibody. Cell growth, process parameters, metabolic and protein titer profiles were also compared to those from shake flask, bench-top, and pilot scale bioreactor cultivations and found to be within +/-20% of the historical averages. Xiao et al¹⁵¹ optimized a chemically defined medium and feed in a single fed-batch study using the SimCell[™] system, and the secreted protein titers increased three to six-fold.

Using the ambr[™] system, Hsu et al compared the performance of four recombinant CHO cell lines in a fedbatch process in parallel ambr[™], shake flasks, and 2-L benchtop bioreactors. Cultures in ambr[™] matched 2-L bioreactors in controlling the environment (temperature, dissolved oxygen, and pH) and in culture performance (growth, viability, glucose, lactate, Na+ osmolality, titer, and product quality). Cultures in shake flasks did not show comparable performance to the ambr[™] and 2-L bioreactors.¹⁵²

Rameez et al.¹²⁶ compared the ambr[™] system to conventional bioreactor systems for their performance in the production of a monoclonal antibody in a CHO cell line. The ambr[™] system was found to produce cell culture profiles that matched across scales to 3 L, 15 L, and 200 L stirred tank bioreactors. The processes used included complex feed formulations, perturbations, and strict process control within the design space, in-line with processes used for commercial scale manufacturing of biopharmaceuticals. Changes to important process parameters in ambr[™] resulted in predictable cell growth, viability and titer changes, which were in good agreement to data from the conventional larger scale bioreactors. The ambr[™] was found to successfully reproduce variations in temperature, DO, and pH conditions similar to the larger bioreactor systems. Additionally, the miniature bioreactors were found to react well to perturbations in pH and DO through adjustments to the Proportional and Integral control loop.

The data reported in these studies demonstrates the utility of the high throughput systems for cell culture process development. It also demonstrates that conventional bioreactors can be adequately modeled using a minibioreactor system and that such systems allow the investigation of greater than one hundred different culture conditions simultaneously at greater statistical depth than it can be performed with a conventional bioreactor. High throughput cell culture technologies can be an effective tool for the development and optimization of cell culture processes as well as in troubleshooting cell culture problems, and their use will continue to increase.

Scale-up of Cell Culture Processes

A cell culture process is typically transferred to bench scale bioreactors for final process development activities and then scaled-up for production of clinical trial material in the pilot plant. When initially scaling-up a cell culture process, there is always some unavoidable variation between the smaller bench-scale bioreactors used for final process development and the larger scales used in cGMP manufacturing. While process optimization is typically done at small scale, some refinements of the cell culture process may be required at the larger production scale prior to using the scale-up process in cGMP manufacturing. As a result, it is strongly recommended that non-cGMP pilot, or engineering, runs at scale be run prior to initiating the manufacture of clinical trial material under cGMP conditions. By running at least one non-cGMP batch at scale and adjusting some parameters based on the data from this run, the risk of failure of the initial cGMP batches is reduced and makes it more likely that subsequent production runs at the same scale will be reproducible. Companies who have developed a robust platform process and have scaled the process up to production scale for multiple products can generally progress directly to cGMP production but the first batch is produced at risk. Time pressures on smaller companies often leads them to progress directly from bench scale to cGMP production as well, but again the first production run is done at a higher risk of failure than subsequent runs at scale.

Cell culture processes continue to be scaled-up throughout development to meet increasing material needs for clinic development of a new monoclonal antibody product and the eventual commercial requirements for the product. While the quantity of product required throughout development of a monoclonal antibody may increase by an order of magnitude or more between each phase of development, the production scale need not increase in proportion to these product requirements. This is because various strategies can be used to accommodate the increased demand, including the use of parallel bioreactors of the same scale and the use of the equipment through the production of multiple batches of product in a single campaign. The ratio of the size of the equipment used at the commercial manufacturing scale to the size used at each of the phases during product development typically does not exceed three orders of magnitude range. Phase 1 and Phase 2 clinical development may require multiple kilograms of product, including samples to be retained as reference. However, the production of this clinical material can often be accomplished in a campaign of a few batches. For instance, the campaign could involve the production of 2-5 batches of product in a 2,000 L bioreactor at a product concentration of 12 g/L. For Phase 3 clinical trials, larger quantities of product may be required so either more production campaigns using the same size bioreactor as used for production of early stage clinical trial materials are required or scale-up to a larger bioreactor is necessary.

One fundamental phenomenon that occurs during bioreactor scale-up is the decrease in surface area (a square function) per unit volume (a cubic function). Since surface area per unit volume decreases with increasing equipment size, in larger bioreactors there should be fewer product quality problems related to any undesirable phenomena occurring on various interfaces, such as non-specific adsorption and/or surface induced precipitation. However, other critical parameters for optimal cell growth and productivity may be more difficult to control at larger scales, such as heat transfer for heating and cooling due to the relative decrease in surface area, high localized shear associated with the higher tip speeds of impellers, and poorer gas exchange (e.g., oxygen supply and CO2 removal).

Such parameters as the specific power input, impeller speed and shear rate, impeller circulation rate (specific pumping rate or mixing time), and oxygen mass transfer coefficient (kLa) required for the cell culture process should be considered and addressed during scaling up. Whenever possible, one should maintain geometric similarity between bioreactors (keeping ratio of major dimensions constant) when scaling-up from a smaller bioreactor to a larger one. For geometrically similar bioreactors, all of the scale-up factors cannot be kept constant during scale-up so some adjustments must be made with scale.¹⁵³ For example, to maintain the same impeller circulation rate in geometrically similar vessels of 200 L and 10,000 L, the larger bioreactor would require 13.60 times the power input, resulting in 5.45 and 3.68 times higher values of the mass transfer coefficient and the impeller tip speed than in the smaller bioreactor, respectively. To formulate the best approach to scale-up, different combinations of these factors should be considered.¹⁵⁴ By understanding the controlling factors for a particular cell culture process though process development, the most critical factors can be scaled geometrically and the other parameters adjusted accordingly.

The Impact of Increased Cell Densities on Scale-up

Advances in production cell lines used for monoclonal antibody product production, media and feed composition used in cell culture processes, and bioreactor design have enabled the development of high cell density cell culture processes maintained over longer periods of time that produce higher amounts of product than before. The scale-up of these high cell density processes presents a greater challenge than scale-up of older processes because the high cell density process may be more sensitive to such factors as mixing time, oxygen transfer, and CO2 removal. These process parameters must be carefully controlled during scale-up so that the high productivity obtained during development can be maintained or improved at the larger production scale. Poor mixing can result in localized nutrient and pH control (i.e., acid or base) gradients within the bioreactor leading to reduced cell growth and productivity. At the same time, a low power input mixing is recommendable because of sensitivity of mammalian cells to shear stress. Mixing also affects both oxygen transfer and dissolved CO2 removal. Consequently, a key strategy to successful cell culture scale-up entails optimizing oxygen supply and CO2 removal while avoiding cell shear damage,

and while these considerations are important for any cell culture scale-up, they are essential to scaling up today's high yield, high cell density processes.

6. Options for Production of Monoclonal Antibodies in High Concentrations

Increasing monoclonal antibody concentrations in cultures have increased manufacturing options for clinical and commercial manufacturing. Monoclonal antibody concentrations of greater than 25 g/L have been reported in perfusion or perfusion-like cell culture processes.^{155, 156} Monoclonal antibody concentrations of over 10 g/L have been reported for fed-batch processes and products manufactured by such processes are currently in clinical development and moving towards commercialization. With such high concentrations, smaller bioreactor runs can meet product requirements that in the past required bioreactors of several thousand litres. If smaller bioreactors are feasible for a particular product, the challenges of scaling up and the number of scale changes that need to be performed are minimized, enabling a more seamless transition from the first production run to commercial production.

As mentioned above, an alternative to conventional stainless steel bioreactors for the manufacture of clinical trial materials that is gaining increasing popularity among product development companies and CMOs are singleuse or disposable bioreactors (SUB). SUBs are currently available in a variety of configurations and sizes up to 2,000 L.101 Productivity in SUBs can be comparable to that of stainless steel bioreactors and product concentrations of 10 g/L have been reported in 250 L SUBs.¹⁴⁴ The operational flexibility offered by SUBs can be an advantage during the manufacture of material for early stage clinical trials while the reduced changeover time and the elimination of the need for cleaning between production runs for different products offered by these bioreactors can be a benefit to CMOs and Sponsors producing multiple products for clinical trials.

SUBs can also be considered for commercial production of monoclonal antibodies because of the high productivity of today's cell lines and cell culture processes and the relatively modest quantities of product that are likely to be needed even for a commercially successful monoclonal antibody product. A survey of monoclonal antibody products currently on the market and those close to commercialization shows that most monoclonal antibody products will require less than a few hundreds of kilograms of product per year. This demand could easily be met with today's SUBs. For example, four 100 L SUBs producing 10 g/L of product in a 14 day cell culture process, combined with a single downstream line based on one 20 L protein A column, and one 5 L anion exchange column (two-step process), could deliver 80 kg of antibody per year assuming an 80% overall process yield. While the flexibility provided by use of SUBs is of less concern for commercial manufacturing than for clinical manufacturing, other potential benefits of single-use technology, such as reduced validation costs and reduced CIP requirements, have resulted in SUBs being used in selected commercial manufacturing applications as well as clinical applications.

The balance of bioreactor volume and the concentration of the monoclonal antibody product at the end of the cell culture process can be used to help choose between SUBs and conventional stainless steel bioreactors at various production scales.

7. Planning for Process Changes

In order to expedite product development and enable the manufacture of Phase 1 clinical trial material as rapidly as possible, the development of an upstream process for a new monoclonal antibody product is often limited to the implementation of a platform process without full process characterization and optimization. As a result, many cell culture processes are modified at some point later in the product development cycle to improve the overall productivity of the manufacturing process or to produce a more consistent product. As with changes in the production cell line discussed in Chapter 5, changes to the cell culture process are permitted under the current regulations for biologic products both during development as well as post-approval. As discussed in more detail in Chapter 8, the manufacturing processes for many of the most successful monoclonal antibody products, including Enbrel,^{157, 158} Herceptin,^{159, 160} Rituximab/MabThera,^{161, 162} and Synagis.^{163, 164} have been altered during scale-up and post-approval.

Any process change has the potential to affect the critical quality attributes of the product. Therefore, prior to implementing a process change, the comparability of the post-change product to the pre-change product must be evaluated. Additionally, a risk assessment of the change on the quality attributes should be done to determine the potential impact on the safety and efficacy of the monoclonal antibody product and to determine whether or not additional animal or clinical studies are required. The risk of requiring additional animal studies or human clinical trials prior to implementing a process change increases the later such a change is introduced.

8. The Future of Cell Culture Processes

A number of technical and engineering developments are being made in biopharmaceutical process technologies that may affect cell culture process development, manufacturing operations, and productivity in the near to long term, including integrated continuous production platforms. To optimize the upstream processes, the use of mini-bioreactor technology, including high throughput bioreactors capable of conducting large numbers of experiments quickly is likely to increase. The use of these mini-bioreactors will reduce development time and costs, while expanding the number and combination of variables that can be evaluated.^{165, 126} Metabolomic analysis of specific production cell lines is emerging as another tool to enable better and more focused process optimization and improved antibody expression at the cellular and culture level, and may become more widespread in the future.166 Advances on several fronts are rapidly creating the potential to increase

monoclonal antibody manufacturing productivity and to reduce manufacturing costs. These improvements include perfusion-based high density cell banking processes that reduce the number of steps in the preparation of inocula for production,^{167, 168} the use of frozen high density perfusion seed cultures,^{169,170} the use of high cell densities to accelerate and reduce the length of the production process,^{171, 172} methods to increase the maximum concentration of viable cells in a culture,^{173, 174} the use of small molecule additives and heterologous transcription factors to enhance specific monoclonal antibody productivity,^{175, 176} the prolongation of cell culture processes by controlling apoptosis signaling genes and expressing inducible anti-apoptotic genes,^{20, 177, 178} using glutamine synthetase gene knockout cells and the use of weakened SV40E promoter to improve the efficiency of CHO cell line generation,^{179, 180} and a variety of other techniques to extend the longevity and productivity of monoclonal antibody producing cultures.^{181, 182}

Finally, the higher productivities of today's cell lines and cell culture processes will enable commercial demand to be met by smaller scale production runs, and increasingly by singleuse bioreactors. The immediate impact of reduced volumetric demand is that the industry possesses excess manufacturing capacity; the longer term impact will be seen as new facilities are designed with smaller bioreactors and larger purification suites to accommodate the changing parameters of antibody and other biopharmaceutical production.

References

- 1 Horvath B, et al. Characterization of a monoclonal antibody cell culture production process using a quality by design approach. Mol Biotechnol. 2010 Jul;45(3):203-6.
- 2 Looby M, et al. Application of quality by design principles to the development and technology transfer of a major process improvement for the manufacture of a recombinant protein. Biotechnol Prog. 2011 Nov-Dec;27(6):1718-29.
- 3 Box GE, et al. Statistics for experimenters: design, innovation, and discovery. Second ed. Hoboken (NJ): John Wiley & Sons; c2005. 664 p.
- 4 http://www.tapbiosystems.com/tap/cell_culture/ambr.htm
- 5 http://www.pall.com/main/biopharmaceuticals/product.page?id=52961
- **6** Jones SD, Ecker DM. Using genomic analysis to improve mammalian cell culture for biopharmaceutical production. Pharm Processing. 2013 Oct; 28(8):24-6.
- 7 Pirt SJ. Principles of microbe and cell cultivation. Oxford: Blackwell Scientific Ltd; c1985. 284 p.
- 8 Freshney RI. Culture of animal cells: a manual of basic technique. Fifth ed. Wilmington (DE): Willey-Liss; c2005. 672 p.
- 9 Wong DC, et al. Transcriptional profiling of apoptotic pathways in batch and fed-batch CHO cell cultures. Biotechnol Bioeng. 2006 Jun;94(2):373-82.
- 10 Sauer PW, et al. A high-yielding, generic fed-batch cell culture process for production of recombinant antibodies. Biotechnol Bioeng. 2000 Mar;67(5):585-97.
- 11 Fan L, et al. A high-yielding, generic fed-batch process for recombinant antibody production of GS-engineered cell lines. J Microbiol Biotechnol. 2009 Dec;19(12):1695-702.
- 12 Tovey MG, Brouty-Boye D. The use of the chemostat to study the relationship between cell growth rate, viability, and the effect of interferon on L 1210 cells. Exp Cell Res. 1979 Feb;118(2):383-8.
- 13 Ljunggren J, Haggstrom L. Specific growth rate as a parameter for tracing growth-limiting substances in animal cell cultures. J Biotechnol. 1995 Sep;42(2):163-75.
- 14 Martens DE, et al. A combined cell-cycle and metabolic model for the growth of hybridoma cells in steady-state continuous culture. Biotechnol Bioeng. 1995 Oct;48(1):49-65.
- 15 Castillo FJ. Hybridoma, antibody production. In: Flickinger M, Drew S, editors. The encyclopedia of bioprocess technology: fermentation, biocatalysis & bioseparation. Vol. 5. Hoboken (NJ): John Wiley & Sons; 1999. p. 1405-18.
- 16 Konstantinov K, et al. The "push-to-low" approach for optimization of high-density perfusion cultures of animal cells. Adv Biochem Eng Biotechnol. 2006 Aug;101:75-98.
- 17 De Alwis DM, et al. Statistical methods in media optimization for batch and fed-batch animal cell culture. Bioprocess Biosyst Eng. 2007 Mar;30(2):107-13.

- 18 Ducommun P, et al. The role of vitamins and amino acids on hybridoma growth and monoclonal antibody production. Cytotechnology. 2001 Oct;37(2):65-73.
- 19 Mercille S, Massie B. Apoptosis-resistant E1B-19K-expressing NS/0 myeloma cells exhibit increased viability and chimeric antibody productivity under perfusion culture conditions. Biotechnol Bioeng. 1999 Jun;63(5):529-43.
- 20 Figueroa B Jr, et al. Enhanced cell culture performance using inducible anti-apoptotic genes E1B-19K and Aven in the production of a monoclonal antibody with Chinese hamster ovary cells. Biotechnol Bioeng. 2007 Jul;97(4):877-92.
- 21 Merten O-W, et al. Specific kinetic patterns of production of monoclonal antibodies in batch cultures and consequences on fermentation processes. In: Spier RE, Griffiths JB, editors. Modern approaches to animal cell technology. Oxford: Butterworth-Heinemann; 1987. p. 381-96.
- 22 Castillo FJ, et al. Perfusion cultures of hybridoma cells for monoclonal antibody production. Ann N Y Acad Sci. 1992 Oct;665:72-80.
- 23 Gong X, et al. Fed-batch culture optimization of a growth-associated hybridoma cell line in chemically defined protein-free media. Cytotechnology. 2006 Sep;52(1):25-38.
- 24 Jang JD, Barford JP. Effect of feed rate on growth rate and antibody production in the fed-batch culture of murine hybridoma cells. Cytotechnology. 2000 Mar;32(3):229-42.
- 25 Yoon SK, et al. Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. Biotechnol Bioeng. 2003 May;82(3):289-98.
- **26** Takuma S, et al. Dependence on glucose limitation of the pCO2 influences on CHO cell growth, metabolism and IgG production. Biotechnol Bioeng. 2007 Aug;97(6):1479-88.
- 27 Lee FW, et al. Engineering Chinese hamster ovary (CHO) cells to achieve inverse growth-associated production of a foreign protein, betagalactosidase. Cytotechnology. 1998 Nov;28(1-3):73-80.
- **28** Hartman TE, et al. Derivation and characterization of cholesterol-independent non-GS NS0 cell lines for production of recombinant antibodies. Biotechnol Bioeng. 2007 Feb;96(2):294-306.
- 29 Chusainow J, et al. A study of monoclonal antibody-producing CHO cell lines: what makes a stable high producer? Biotechnol Bioeng. 2009 Mar;102(4):1182-96.
- **30** Jiang Z, et al. Regulation of recombinant monoclonal antibody production in Chinese hamster ovary cells: a comparative study of gene copy number, mRNA level, and protein expression. Biotechnol Prog. 2006 Jan-Feb;22(1):313-8.
- 31 Lee CJ, et al. A clone screening method using mRNA levels to determine specific productivity and product quality for monoclonal antibodies. Biotechnol Bioeng. 2009 Mar;102(4):1107-18.
- 32 Smales CM, et al. Comparative proteomic analysis of GS-NSO murine myeloma cell lines with varying recombinant monoclonal antibody production rate. Biotechnol Bioeng. 2004 Nov;88(4):474-88.
- **33** Schlatter S, et al. On the optimal ratio of heavy to light chain genes for efficient recombinant antibody production by CHO cells. Biotechnol Prog. 2005 Jan-Feb;21(1):122-33.

- **34** Khoo SH, Al-Rubeai M. Detailed understanding of enhanced specific antibody productivity in NSO myeloma cells. Biotechnol Bioeng. 2009 Jan;102(1):188-99.
- 35 Oguchi S, et al. pH Condition in temperature shift cultivation enhances cell longevity and specific hMab productivity in CHO culture. Cytotechnology. 2006 Nov;52(3):199-207.
- 36 Grosvenor S. The role of media development in process optimization: an historical perspective. BioPharm Int. 2008 Jun;6(Suppl):28-36.
- 37 Decaria P, et al. Many considerations in selecting bioproduction culture media. BioProcess Int. 2009 Jan;7(1):44-51.
- 38 http://www.genengnews.com/insight-and-intelligence/implementing-a-full-quality-by-design-strategy/77900063/?page=1
- **39** European Medicines Agency, Committee for Proprietary Medicinal Products (GB). Guideline on the use of bovine serum in the manufacture of human biological medicinal products [Internet]. London: The European Medicines Agency; 2013 May 30 [cited 2016 Nov 22]. 8 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/06/WC500143930.pdf.
- 40 Gerber MA, et al. Integrated strategies for clone and media formulation selection. BioProcess Int. 2008 Jan;6(1):58-63.
- 41 Legmann R. et al. A strategy for clone selection under different production conditions. Biotechnol Prog. 2011 May-Jun:27(3):757-765.
- 42 Fletcher T. Designing culture media for recombinant protein production. BioProcess Int. 2005 Jan;3(1):30-6.
- **43** Allison DW, et al. Development and optimization of cell culture media. BioProcess Int. 2005 Jan;3(1):38-45.
- 44 Landauer et al. Development of a chemically defined CHO medium by engineering based on a feed solution. BMC Proceedings 2011 5(Suppl 8):41-42.
- 45 Sandadi S, et al. Application of fractional factorial designs to screen factors for antibody production by Chinese hamster ovary cells. Biotechnol Prog. 2006 Mar-Apr;22(2):595-600.
- **46** Sellick CA, et al. Metabolite profiling of recombinant CHO cells: designing tailored feeding regimes that enhance recombinant antibody production. Biotechnol Bioeng. 2011 Dec:108(12):3025-31.
- 47 Luo J, et al. Comparative metabolite analysis to understand lactate metabolism shift in Chinese hamster ovary cell culture process. Biotechnol Bioeng. 2012 Jan:109(1):146-56.
- 48 Dean J, Reddy P. Metabolic analysis of antibody producing CHO cells in fed-batch production. Biotechnol Bioeng. 2013 Jun: 110(6):1735-47.
- 49 Jordan M, et al. Cell culture medium improvement by rigorous shuffling of components using media blending. Cytotechnology. 2013;65:31-40.
- 50 Rouiller Y, et al. A high-throughput media design approach for high performance mammalian fed-batch cultures. MAbs. 2013 May-Jun;5(3):501-11.
- 51 Zhang J, Robinson D. Development of animal-free, protein-free and chemically-defined media for NS0 cell culture. Cytotechnology. 2005 Jun;48(1-3):59-74.
- 52 Sun X, Zhang Y. Glutamine cannot support recombinant CHO cell growth and maintenance in the absence of glucose. Process Biochem. 2004 Feb;39(6):719-22.

- **53** Hwang SO, Lee GM. Nutrient depravation induces autophagy as well as apoptosis in Chinese hamster ovary culture. Biotechnol Bioeng. 2008 Feb;99(3):678-85.
- 54 Burky JE, et al. Protein-free fed-batch culture of non-GS NS0 cell lines for production of recombinant antibodies. Biotechnol Bioeng. 2007 Feb;96(2):281-93.
- 55 Pascoe DE, et al. Proteome analysis of antibody-producing CHO cell lines with different metabolic profiles. Biotechnol Bioeng. 2007 Oct;98(2):391-410.
- 56 Ma N, et al. A single nutrient feed supports both chemically defined NSO and CHO fed-batch processes: Improved productivity and lactate metabolism. Biotechnol Prog. 2009 Sep-Oct:25(5):1353-63.
- 57 Zhou M, et al. Decreasing lactate level and increasing antibody production in Chinese Hamster Ovary cells (CHO) by reducing the expression of lactate dehydrogenase and pyruvate dehydrogenase kinases. J Biotechnol. 2011 Apr:153(1-2):27-34.
- 58 Yang M, Butler M. Effects of ammonia and glucosamine on the heterogeneity of erythropoietin glycoforms. Biotechnol Prog. 2002 Jan-Feb;18(1):129-38.
- 59 Wong CF, et al. Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures. Biotechnol Bioeng. 2005 Jan;89(2):164-77.
- 60 Chen P, Harcum SW. Effects of amino acid additions on ammonium stressed CHO cells. J Biotechnol. 2005 May;117(3):277-86.
- 61 Glacken MW et al. Reduction of waste product excretion via nutrient control: Possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. Biotechnol Bioeng. 1986 Sep;28(9):1376-89.
- 62 Glacken MW. Catabolic control of mammalian cell culture. Biotechnology 1988;6: 1041-50.
- 63 Selvarasu S, et al. Elucidation of metabolism in hybridoma cells grown in fed-batch culture by genome-scale modeling. Biotechnol Bioeng. 2009 Apr;102(5):1494-504.
- 64 Spens E, Haggstrom L. Defined protein and animal component-free NS0 fed-batch culture. Biotechnol Bioeng. 2007 Dec;98(6):1183-94.
- **65** Alberts B, et al. Molecular biology of the cell. Fifth ed. New York: Garland Science; c2007. 1392 p.
- 66 Tritsch, GL, Moore GE. Spontaneous Decomposition of Glutamine in Cell Culture Media. Experimental Research. 1962;28:360–64.
- **67** Xu P, et al. Effects of glutamine and asparagine on recombinant antibody production using CHO-GS cell lines. Biotechnol Prog. 2014 Nov;30(6):1457-68.
- 68 Feeney L, et al. Eliminating Tyrosine Sequence Variants in CHO Cell Lines Producing Recombinant Monoclonal Antibodies. Biotechnol. Bioeng. 2012 Apr;110(4):1087-97.
- 69 deZengotita VM, et al. Selected amino acids protect hybridoma and CHO cells from elevated carbon dioxide and osmolality. Biotechnol Bioeng. 2002 Jun;78(7):741-52.

- 70 Henneberry AL, et al. The major sites of cellular phospholipid synthesis and molecular determinants of fatty acid and lipid head group specificity. Mol Biol Cell. 2002 Sep;13(9):3148-61.
- 71 Warnock DE, et al. Determination of plasma membrane lipid maps and composition in cultured Chinese hamster ovary cells using high gradient magnetic affinity chromatography. J Biol Chem. 1993 May;268(14):10145-53.
- 72 Ertola RJ, et al. Design, formulation, and optimization of media. In: Asenjo JA, Merchuck JC, editors. Bioreactor system design. New York: Marcel Dekker Inc; 1994. p. 89-137.
- 73 Gorfien S, et al. Growth of NSO cells in protein-free, chemically defined medium. Biotechnol Prog. 2000 Sep-Oct;16(5):682-7.
- 74 Whitford WG. Lipids in bioprocess fluids. BioProcess Int. 2005 Jan;3(1):46-56.
- 75 Pence B. Process supplements for optimized fed-batch culture systems. BioProcess Int. 2008 Jul;6(7):26.
- 76 Okonkowski J, et al., "Cholesterol Delivery to NSO Cells: Challenges and Solutions in Disposable Linear Low-Density Polyethylene-Based Bioreactors," J. BioSci. and BioEng. 2007;103(1): 50-59.
- 77 Tao Y, et al. Novel cholesterol feeding strategy enables a high-density cultivation of cholesterol-dependent NSO cells in linear low-density polyethylene-based disposable bioreactors. Biotechnol Lett. 2012 Aug;34(8):1453-8.
- **78** Keen MJ, Steward TW. Adaptation of cholesterol-requiring NSO mouse myeloma cells to high density growth in a fully defined protein-free and cholesterol-free culture medium. Cytotechnology. 1995 Oct;17(3):203-11.
- 79 Okonkowski J, et al. Cholesterol delivery to NSO cells: challenges and solutions in disposable linear low-density polyethylene-based bioreactors. J Biosci Bioeng. 2007 Jan;103(1):50-9.
- 80 De Zengotita VM, et al. Phosphate feeding improves high-cell-concentration NSO myeloma culture performance for monoclonal antibody production. Biotechnol Bioeng. 2000 Sep;69(5):566-76.
- **81** Zhang J, et al. A novel function for selenium in biological system: selenite as a highly effective iron carrier for Chinese hamster ovary cell growth and monoclonal antibody production. Biotechnol Bioeng. 2006 Dec;95(6):1188-97.
- 82 Wong VT, et al. Evaluation of insulin-mimetic trace metals as insulin replacements in mammalian cell cultures. Cytotechnology. 2004 Jul;45(3):107-15.
- 83 Wong VT, et al. Zinc as an insulin replacement in hybridoma cultures. Biotechnol Bioeng. 2006 Feb;93(3):553-63.
- 84 Kidd RD. Practicing Safe Cell Culture: Applied Process Designs for Minimizing Virus Contamination Risk. PDA Journal of Pharmaceutical Science and Technology. 2011 June;65(6):715-29.
- 85 Eltermann J. Adventitious Agents: Issues and Considerations during Pre-Approval Reviews and Inspections. PDA Journal of Pharmaceutical Science and Technology. 2011 June;65(6):694-97.
- **86** Chun BH, et al. Enhanced production of recombinant B-domain deleted factor VIII from Chinese hamster ovary cells by propionic and butyric acids. Biotechnol Lett. 2003 Feb;25(4):315-9.

- 87 Yee JC, et al. Genomic and proteomic exploration of CHO and hybridoma cells under sodium butyrate treatment. Biotechnol Bioeng. 2008 Apr;99(5):1186-204.
- 88 Jiang Z, Sharsftein ST. Sodium butyrate stimulates monoclonal antibody over-expression in CHO cells by improving gene accessibility. Biotechnol Bioeng. 2008 May;100(1):189-94.
- **89** Ghorbaniaghdam A, et al. A kinetic-metabolic model based on cell energetic state: study of CHO cell behavior under Na-butyrate stimulation. Bioprocess Biosyst Eng. 2013 Apr;36(4):469-87.
- **90** Liu C, et al. Aurintricarboxylic acid exerts insulin-like growth stimulating effects on Chinese hamster ovary cells under serum-free conditions. J Bioscience Bioeng 2001 Jun;91(6):576-80.
- **91** Allen MJ, et al. Identification of novel small molecule enhancers of protein production by cultured mammalian cells. Biotechnol Bioeng. 2008 Aug;100(6):1193-204.
- 92 Liu C, et al. Pentanoic acid, a novel protein synthesis stimulant for Chinese hamster ovary (CHO) cells. J Biosci Bioeng. 2001 Jan;91(1):71-5.
- 93 Yang WC, et al. Addition of Valproic Acid to CHO Cell Fed-Batch Cultures Improves Monoclonal Antibody Titers. Mol Biotechnol. 2014 May;56(5):421-28.
- **94** Michaels JD, et al. Analysis of cell-to-bubble attachment in sparged bioreactors in the presence of cell-protecting additives. Biotechnol Bioeng. 1995 Aug;47(4):407-19.
- **95** Na M, et al. Quantitative studies of cell-bubble interactions and cell damage at different Pluronic F-68 and cell concentrations. Biotechnol Prog. 2004 Jul-Aug;20(4):1183-91.
- 96 Industrialization of mAb production technology: The bioprocessing industry at a crossroad. MAbs 2009 Sep-Oct;1(5):443-52.
- **97** Lu F, et al. Automated dynamic fed-batch process and media optimization for high productivity cell culture process development. Biotechnol Bioeng. 2013 Jan;110(1):191-205.
- 98 Chu L, Robinson DK. Industrial choices for protein production by large-scale cell culture. Curr Opin Biotechnol. 2001 Apr;12(2):180-7.
- **99** Meuwly F, et al. Conversion of a CHO cell culture process from perfusion to fed-batch technology without altering product quality. J Biotechnol. 2006 May;123(1):106-16.
- **100** Whitford WG. Fed-batch mammalian cell culture in bioproduction. BioProcess Int. 2006 Apr;4(4):30-40.
- 101 Lu F, et al. Automated dynamic fed-batch process and media optimization for high productivity cell culture process development. Biotechnol Bioeng. 2013 Jan;110(1):191-205.
- 102 Li F, et al. Cell culture processes for monoclonal antibody production. MAbs. 2010 Sep-Oct;2(5):466-79.
- **103** Butler M. Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. Appl Microbiol Biotechnol. 2005 Aug;68(3):283-91.

- 104 Lim AC, et al. A computer-aided approach to compare the production economics of fed-batch and perfusion culture under uncertainty. Biotechnol Bioeng. 2006 Mar;93(4):687-97.
- 105 Pollock J, Ho SV, Farid SS. Fed-batch and perfusion culture processes: economic, environmental, and operational feasibility under uncertainty. Biotechnol Bioeng. 2013 Jan;110(1):206-19.
- 106 Warikoo V, et al. Integrated continuous production of recombinant therapeutic proteins. Biotechnol Bioeng. 2012 Dec;109(12):3018-29.
- 107 Birch JR, Racher AJ. Antibody production. Adv Drug Deliv Rev. 2006 Aug;58(5-6):671-85.
- 108 Wilson JS. A fully disposable monoclonal antibody manufacturing train. BioProcess Int. 2006 Jun;4(Suppl 4):34-6.
- 109 Fuller M, Pora H. Introducing disposable systems into biomanufacturing. BioProcess Int. 2008 Nov;6(10):30-6.
- **110** Rao G, et al. Disposable bioprocessing: the future has arrived. Biotechnol Bioeng. 2009 Feb;102(2):348-56.
- 111 Eibl R, et al. Disposable bioreactors: the current state-of-the-art and recommended applications in biotechnology. Appl Microbiol Biotechnol. 2010 Mar:86(1):41-49.
- 112 Shukla AA, Gottschalk U. Single-use disposable technologies for biopharmaceutical manufacturing. Trends in Biotechnol. 2013 Mar:31(3):147-54.
- 113 Osman JJ, et al. The response of GS-NS0 myeloma cells to single and multiple pH perturbations. Biotechnol Bioeng. 2002 Aug;79(4):398-407.
- 114 Kim HS, Lee GM. Differences in optimal pH and temperature for cell growth and antibody production between two Chinese hamster ovary clones derived from the same parental clone. J Microbiol Biotechnol. 2007 May;17(5):712-20.
- 115 Muthing J, et al. Effects of buffering conditions and culture pH on production rates and glycosylation of clinical phase I anti-melanoma mouse IgG3 monoclonal antibody R24. Biotechnol Bioeng. 2003 Aug;83(3):321-34.
- **116** Al-Fageeh MB, et al. The cold-shock response in cultured mammalian cells: harnessing the response for the improvement of recombinant protein production. Biotechnol Bioeng. 2006 Apr;93(5):829-35.
- 117 Shi M, et al. Controlled growth of Chinese hamster ovary cells and high expression of antibody-IL-2 fusion proteins by temperature manipulation. Biotechnol Lett. 2005 Dec;27(23-24):1879-84.
- 118 Marchant RJ, et al. Metabolic rates, growth phase, and mRNA levels influence cell-specific antibody production levels from the in vitro-cultured mammalian cells at sub-physiological temperatures. Mol Biotechnol. 2008 May;39(1):69-77.
- **119** Wohlpart D, et al. Effects of cell density and glucose and glutamine levels on the respiration of hybridoma cells. Biotechnol Bioeng. 1990 Sep;36(6):630-5.
- 120 Kunkel JP, et al. Dissolved oxygen concentration in serum-free continuous culture affects N-linked glycosylation of a monoclonal antibody. J Biotechnol. 1998 Jun;62(1):55-71.
- 121 Kunkel JP, et al. Comparisons of the glycosylation of a monoclonal antibody produced under nominally identical cell culture conditions in two different bioreactors. Biotechnol Prog. 2000 May-Jun;16(3):462-70.

- 122 deZengotita VM, et al. Characterization of hybridoma cell responses to elevated pCO2 and osmolality: intracellular pH, cell size, apoptosis, and metabolism. Biotechnol Bioeng. 2002 Feb;77(4):369-80.
- 123 Zhu MM, et al. Effects of elevated pCO2 and osmolality on growth of CHO cells and production of antibody-fusion protein B1: a case study. Biotechnol Prog. 2005 Jan-Feb;21(1):70-7.
- 124 Mostafa SS, Gu X. Strategies for improved dCO2 removal in large-scale fed-batch cultures. Biotechnol Prog. 2003 Jan-Feb;19(1):45-51.
- 125 Sieblist C, et al. Insights into large-scale cell-culture reactors: II. Gas-phase mixing and CO2 stripping. Biotechnol J. 2011 Dec;6(12):1547-56.
- 126 Hu W et al. The potential of hydrodynamic damage to animal cells of industrial relevance: current understanding. Cytotechnology. 2011; Oct;63(5):445-60.
- 127 Velez-Superbie ML et al. Impact of aeration strategy on CHO cell performance during antibody production. Biotechnol Prog. 2013 Jan; 29(1):116-26.
- **128** Trexler-Schmidt M, et al. Identification and prevention of antibody disulfide bond reduction during cell culture manufacturing. Biotechnol Bioeng. 2010 Jun:106(3):452-61.
- 129 Tait AS. Host cell protein dynamics in the supernatant of a mAb producing CHO cell line. Biotechnol Bioeng. 2012 Apr:109(4):971-81.
- 130 Sieblist C, et al. Influence of Pluronic F68 on oxygen mass transfer. Biotechnol Prog. 2013 Sep-Oct;29(5):1278-88.
- **131** Kim NS, Lee GM. Response of recombinant Chinese hamster ovary cells to hyperosmotic pressure: effect of Bcl-2 overexpression. J Biotechnol. 2002 May;95(3):237-48.
- **132** Kim MS, et al. Biphasic culture strategy on hyperosmotic pressure for improved humanized antibody production in Chinese hamster ovary cell culture. In Vitro Cell Dev Biol Anim. 2002 Jun;38(6):314-19.
- **133** Shen D, Sharfstein S. Genome-wide analysis of the transcriptional response of murine hybridomas to osmotic shock. Biotechnol Bioeng. 2006 Jan;93(1):132-45.
- 134 Schmelzer AE, Miller WM. Hyperosmotic stress and elevated pCO2 alter monoclonal antibody charge distribution and monosaccharide content. Biotechnol Prog. 2002 Mar-Apr;18(2):346-53.
- 135 http://www.flownamics.com/automated_bioreactor_sampling_Seg-Flow.html
- 136 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Pharmaceutical Development Q8(R2) Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2009 Aug [cited 2014 Jul 14]. 28 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/ Guidelines/Quality/Q8_R1/Step4/Q8_R2_Guideline.pdf.
- **137** Ramezz S, et al. High-throughput miniaturized bioreactors for cell culture process development: Reproducibility, scalability, and control. Biotechnol Prog. 2014 May-Jun;30(3):718-27.
- 138 Chhatre S, et al. A microscale approach for predicting the performance of chromatography columns used to recover therapeutic polyclonal antibodies. J Chomatogr A. 2009 Nov;1216:7806-15.

- **139** Long Q, et al. The development and application of high throughput cultivation technology in bioprocess development. J Biotechnol. 2014 Dec;192 Pt B:323-38.
- 140 Legmann R1, Benoit B, Fedechko RW, Deppeler CL, Srinivasan S, Robins RH, McCormick EL, Ferrick DA, Rodgers ST, Russo AP. A strategy for clone selection under different production conditions. Biotechnol Prog. 2011 May-Jun;27(3):757-65.
- 141 https://www.moleculardevices.com/systems/cell-screening-and-bioproduction
- 142 http://www.tapbiosystems.com/tap/applications/cell_line_selection.htm
- 143 http://www.tapbiosystems.com/tap/cell_culture/ambr.htm
- 144 http://www.tapbiosystems.com/tap/cell_culture/ambr_250.htm
- 145 http://www.dasgip.com/catalog/category/Systems_systems/
- 146 http://www.fogalebiotech.com/PHP/applications-bioreactor.php
- 147 https://online-shop.eppendorf.us/US-en/Bioprocess-44559/Bioprocess-Systems-60767/DASGIP-Parallel-Bioreactor-Systems-PF-133597.html
- 148 Cheung L, et al. Developing an automated high-throughput system for cell culture process development. Presented at International Business Communications Life Sciences' BioProcess International Conference; 2008 Sep 22-26; Anaheim, CA.
- 149 Legmann R, et al. A predictive high-throughput scale-down model of monoclonal antibody production in CHO cells. Biotechnol Bioeng. 2009 Dec 15;104(6):1107-20.
- 150 Amanullah A1, Otero JM, Mikola M, Hsu A, Zhang J, Aunins J, Schreyer HB, Hope JA, Russo AP Novel micro-bioreactor high throughput technology for cell culture process development: Reproducibility and scalability assessment of fed-batch CHO cultures. Biotechnol Bioeng. 2010 May 1;106(1):57-67.
- **151** Xiao Z, et al. Screening and optimization of chemically defined media and feeds with integrated and statistical approaches. Methods Mol Biol. 2014;1104:117-35.
- **152** Hsu WT, Aulakh RP, Traul DL, Yuk IH. Advanced microscale bioreactor system: a representative scale-down model for bench-top bioreactors. Cytotechnology. 2012 Dec;64(6):667-78.
- 153 Murakami S, Walter P, Ishihara T, and Yamamoto S, Pharmaceutical Engineering Process modeling proposition in biopharmaceutical manufacturing, 2005 Nov-Dec;25(6):8-19.
- **154** Xing Z, Kenty BM, Li ZJ, and Lee SS, Scale-up analysis for a CHO cell culture process in large scale bioreactors. Biotechnol Bioeng. 2009 Jul 1;103(4):733-46.
- 155 Hof R., "High Titer cell culture using the PER.C6® cell line", presented at the ISPE Conference on Applying a Science and Risk-based Approach, Barcelona, Spain, 2008 1–2 December 2008 (unpublished).
- 156 Kelley BD. Very large scale monoclonal antibody purification: the case for conventional unit operations. Biotechnology Progr. 2007 Sep-0ct;23(5):995-1008.

- 157 European Medicines Agency (GB). Enbrel: scientific discussion [Internet]. London: The European Medicines Agency; c2006 [cited 2016 Nov 22]. 42 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000262/WC500027358.pdf.
- 158 Food and Drug Administration, Center for Biologics Evaluation and Research (US). CMC review of BLA 980286, Immunex's TNFR:Fc for RA [Internet]. Rockville (MD): Department of Health and Human Services; 1998 Jun 12 [cited 2009 Jul 1]. 26 p. Available from: http://www.fda.gov/ downloads/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/ ucm088695.pdf.
- 159 European Medicines Agency (GB). Herceptin: scientific discussion [Internet]. London: The European Medicines Agency; c2005 [cited 2016 Nov 21]. 42 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000278/WC500049816.pdf.
- 160 Kuhne W. Site transfer and process validation in case of Herceptin API. Dev Biol (Basel). 2003;113:47-50.
- 161 European Medicines Agency (GB). Mabthera: scientific discussion [Internet]. London: The European Medicines Agency; c2006 [cited 2016 Nov 22]. 29 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000165/WC500025817. pdf.
- 162 Garnick RL. The product development continuum: the evolution and application of the concept of comparability. Dev Biol (Basel). 2002;109:3-8.
- **163** European Medicines Agency (GB). Synagis: scientific discussion [Internet]. London: The European Medicines Agency; c2005 [cited 2016 Nov 22]. 19 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000257/WC500056731.pdf.
- **164** Schenerman MA, et al. Comparability testing of a humanized monoclonal antibody (Synagis) to support cell line stability, process validation, and scale-up for manufacturing. Biologicals. 1999 Sep;27(3):203-15.
- 165 Neway JO. Process excellence: five critical elements of quality by design. BioProcess Int. 2008 May;6(5):18-22.
- 166 Ahn WS, Antoniewicz MR. Towards dynamic metabolic flux analysis in CHO cell cultures. Biotechnol J. 2012 Jan;7(1):61-74.
- **167** Tao Y. et al. Development and implementation of a perfusion-based high cell density cell banking process. Biotechnol Prog. 2011 May-Jun:27(3):824-29.
- 168 Clincke, M. et al. Very high density Chinese hamster ovary cells in perfusion by alternating tangential flow or tangential flow filtration in WAVE bioreactorTM -Part II: Applications for antibody production and cryopreservation. Biotechnol Prog. 2013 May:29(3):768-77.
- **169** Seth G, et al. Development of a new bioprocess scheme using frozen seed train intermediates to initiate CHO cell culture manufacturing campaigns. Biotechnol Bioeng. 2013 May:110(5):1376-85.
- 170 Yang WC, et al. Perfusion seed cultures improve biopharmaceutical fed-batch production capacity and product quality. Biotechnol Prog. 2014 May-Jun;30(3):616-25.
- 171 Pohlscheidt M, et al. Optimizing capacity utilization by large scale 3000 L perfusion in seed train bioreactors. Biotechnol Prog. 2013 Jan-Feb:29(1):222-29.
- 172 Padawer I, Ling WLW, Bai Y. Case study: An accelerated 8-day monoclonal antibody production process based on high seeding densities. Biotechnol Prog. 2013 April:29(3):829-32.

- 173 Xing Z, et al. Identifying inhibitory threshold values of repressing metabolites in CHO cell culture using multivariate analysis methods. Biotechnol Prog. 2008 May-Jun;24(3):675-83.
- 174 Clincke M, et al. Very high density of CHO cells in perfusion by ATF or TFF in WAVE bioreactorTM. Part I: Effect of the cell density on the process. Biotechnol Prog. 2013 April:29(3):754-57.
- 175 Allen MJ, et al. Identification of novel small molecule enhancers of protein production by cultured mammalian cells. Biotechnol Bioeng. 2008 Aug;100(6):1193-204.
- 176 Becker E, et al. An XBP-1 dependent bottle-neck in production of IgG subtype antibodies in chemically defined serum-free Chinese hamster ovary (CHO) fed-batch processes. J Biotechnol. 2008 Jun;135(2):217-23.
- 177 Wong DC, et al. Targeting early apoptotic genes in batch and fed-batch CHO cell cultures. Biotechnol Bioeng. 2006 Oct;95(3):350-61.
- 178 Lim SF, et al. RNAi suppression of Bax and Bak enhance viability in fed-batch cultures of CHO cells. Metab Eng. 2006 Nov;8(6):509-22.
- **179** Fan L, et al. Improving the efficiency of CHO cell line generation using glutamine synthetase gene knockout cells. Biotechnol Bioeng. 2012 Apr;109(4):1007-15.
- **180** Fan L, et al. Development of a highly-efficient CHO cell line generation system with engineered SV40# promoter. J. Botechnol. 2013 Dec;168(4):652-58.
- 181 Heath C, Kiss R. Cell culture process development: advances in process engineering. Biotechnol Prog. 2007 Jan-Feb;23(1):46-51.
- 182 Sharfstein ST. Advances in cell culture process development: tools and techniques for improving cell line development and process optimization. Biotechnol Prog. 2008 May-Jun;24(3):727-34.

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CHAPTER 7:

Purification Development and Scale-up

ollowing production of a monoclonal antibody in cell culture, the crude antibody must be purified to homogeneity through a series of recovery and purification steps often referred to as the downstream process. In this phase of production, the antibody produced in the production bioreactor is separated from impurities (substances related to the desired monoclonal antibody product or process that are undesirable in the final product) and potential contaminants (substances that are not intended to be in the process, product, or intermediates but which may be present and require removal) to levels sufficient for delivery to a patient. The purified monoclonal antibody product is then concentrated to a suitable concentration and transferred into an appropriate buffer for storage prior to final formulation and drug product manufacturing. The purified monoclonal antibody solution resulting from the recovery and purification process is referred to as the bulk drug substance.

The development of a suitable recovery and purification process for a monoclonal antibody focuses on two main technical aspects – the effectiveness of the removal of impurities and contaminants (i.e. the product purity) and the yield of the product (the percent of the active antibody product present in the bioreactor batch that is recovered in the final bulk drug substance). All processes involve tradeoffs between yield and purity so that the major overall goal of downstream process development is to ensure that the purity is sufficient for the monoclonal antibody product to be safe for its intended use, while simultaneously maximizing yield. Meeting the necessary purity levels is an absolute regulatory requirement, while product yield is primarily an economic concern for the Sponsor.

In addition to producing a product of desired purity and potency, a purification process must also be robust and reproducible so that the resulting monoclonal antibody product consistently meets all release specifications and conforms to the critical quality attributes of the product. Robust manufacturing processes will generally scale from the laboratory to full-scale commercial production without problems and information gathered during process development to demonstrate robustness can be used to support process validation and changes in the manufacturing process both during clinical development and after commercialization.

In 2011 FDA introduced a three stage, lifecycle-based, approach to process validation, 1 in alignment with the QbD principles discussed in Chapter 3. The process validation lifecycle is based on the principles that quality cannot be tested into a product and must be built in from the start. The concept of ongoing process evaluation, from discovery through commercial manufacturing, promotes the leveraging of knowledge gained through all stages of development and the continuing improvement of the process. The process validation lifecycle is an increasingly important framework for protein development and is discussed further in Chapter 11.

Unlike other recombinant proteins that have been produced for therapeutic applications, the commonality in molecular structure of different monoclonal antibody products can facilitate the development of purification processes for these products and allow the use of platform technologies or processes for both upstream and downstream processing. These platform processes allow companies producing monoclonal antibody products to develop common process unit operations that can be used to rapidly produce products for human clinical evaluation and then to fine-tune or optimize these platform processes for a specific monoclonal antibody product during later stages of development.

The antibody-related products in development and on the market are based on a wide variety of structural frameworks (e.g., Fabs, IgGs, IgMs) and produced in a wide range of host cell lines (e.g., mammalian cells, yeast, bacteria, plant cells), with each product requiring different approaches to downstream processing. However, this chapter focuses on purification of monoclonal antibodies of the IgG isotype produced in mammalian cell culture since these represent the vast majority of antibody products currently in development and on the market.

1. Removal of Contaminants and Product-related Impurities

The primary goal of the recovery and purification process is to achieve sufficient product purity (i.e., reduce process and product-related impurities to sufficiently low levels). The bioreactor harvest, which contains the desired monoclonal antibody product, is a complex mixture of diverse substances, ranging from low molecular weight metabolites and media components to higher molecular weight host cell proteins and other species, present at a wide range of concentrations. All of these substances, except for those intended to be in the bulk drug substance, are considered impurities that must be removed during the downstream process. These impurities must be separated from the product below a threshold level to produce a safe drug. Additionally, there are potential contaminants that may enter into biopharmaceutical processes, either through materials, equipment or the environment, which must also be removed. These potential contaminants include adventitious agents, such as viruses and bacteria, reagents used in cell culture or purification, or material that may leach from chromatography media.

Cells and Cellular Debris

One obvious group of contaminants in the bioreactor harvest is micro-particulate material such as the host cells themselves and any debris from dead cells that may be present in the bioreactor at the end of the cell culture process. Monoclonal antibody products are secreted by the production cell line into the surrounding media so that separation of the cells from the liquid phase containing the product is typically the first step in a recovery process. This liquid/solid separation can be performed by filtration or centrifugation, or a combination of both, and results in a solution that is essentially free of any living or dead cells or cellular fragments. This step should be completed rapidly since the monoclonal antibody product is generally more stable in the cell-free broth than in the crude (unfiltered) bioreactor harvest.

Mammalian cells used for monoclonal antibody production are relatively uniform in diameter (typically 1020 µm) and are typically fairly easy to separate from the liquid phase of the bioreactor harvest. However, debris from dead cells in the harvest range in size from diameters close to a viable cell to the low submicron range, making removal of dead cell debris a major challenge. Also, mammalian cells are relatively fragile and may be lysed during the pumping and other manipulations necessary to separate them from the liquid phase, creating smaller particles and releasing proteases, which may degrade the monoclonal antibody product and other proteins which will increase the levels of impurities in the harvest. As cell density in the bioreactor is increased in order to improve product titer, the fraction of dead or nonviable (and highly fragile) cells and cell debris also typically increases, exacerbating the problem.

Among the cellular debris, cell membranes, membrane fragments, and some internal elements of the cell which are composed of lipids and lipid-related molecules can pose particular problems in clarifying monoclonal antibody-containing bioreactor harvests. Lipids are very hydrophobic and often poorly soluble in water and can often form aggregates or precipitates (between themselves and with other molecules) that behave as small particulates, sometimes downstream in a process after chemical or temperature conditions change. Fouling or plugging of membranes and chromatography media used in purifying monoclonal antibody products is a frequent result of process lipids making effective removal of lipids early in the recovery process important.

Protein Impurities

Other proteins besides the monoclonal antibody product present in the feedstream represent a critical set of impurities. There are several potential sources of protein impurities, including the proteins and peptides introduced into the cell culture medium (if any), proteins secreted into the medium by the cells along with the antibody product, and proteins secreted by the cells or released when cells are lysed. Proteins introduced into the cell culture medium are often minimal in modern, chemically defined media, but may include significant amounts of highly bioactive proteins (such as insulin or transferrin) that must be removed to very low levels in the final product. Since antibody products are almost always secreted by the producer cells, it is theoretically possible for the product to be relatively free of host protein impurities, aside from the culture medium proteins. However, some cell lysis (with release of the many intracellular proteins) is inevitable, and will almost certainly increase as bioreactor conditions are optimized to maximize product concentration. Thus, much of the purification process is devoted to robust removal of a very diverse range of protein impurities.

Nucleic Acids

Nucleic acids, DNA and RNA, from the host cells represent another important class of impurity to be considered in developing a monoclonal antibody purification process. The concern with nucleic acid contamination is the potential introduction of active, foreign genetic material into the patient, with possible adverse consequences such as oncogenesis.² Early regulatory standards regarding residual DNA in recombinant protein products were extremely rigorous and often difficult to achieve. More recently, these standards have been relaxed somewhat as clinical trials of gene therapy products (where the objective is to deliberately introduce DNA into the patient through carefullydesigned vectors and delivery systems) have shown just how difficult it is to incorporate and express foreign DNA. For therapeutic products derived from mammalian host cells, the generally accepted standard is the World Health Organization recommended limit of 10 ng of foreign DNA per dose of final product.² The most recent FDA guidance on the characterization and qualification of cell substrates specifically references this WHO recommended limit as an acceptable target for removal of host DNA by a purification process.³

Viruses

All products produced in mammalian cell culture, including monoclonal antibodies, are potentially susceptible to contamination by viruses. Additionally, many mammalian cells contain some endogenous viruses incorporated over the course of evolution into their genome that are normally expressed during the cell culture process. The level of endogenous retrovirus present will depend on the cell line and process conditions and can be quantified using transmission electron microscopy or by real-time polymerase chain reaction PCR, which is also referred to as quantitative real time polymerase chain reaction (qPCR).⁴ Of more concern are so-called adventitious viruses, which are introduced from outside the host cells either through contact with the human operators or through raw materials used in the process. The rapidly reproducing host cells could potentially serve as hosts for replication of these viruses. Concern about adventitious viruses and other diseasecausing agents like prions, has, in part, led to major efforts in recent years to reduce or eliminate animal-derived raw materials in the production of pharmaceuticals.

Although the chances of viral contamination of a monoclonal antibody product are low, the effects of a single incident could be devastating as was the case in the largescale infection of hemophilia patients with human immune deficiency virus (HIV) and hepatitis from contaminated blood plasma used to produce Factor VIII before the problem was understood or steps taken to minimize this potential risk. Designing a recovery and purification process to ensure that no viruses are present in the final product is challenging and validating its effectiveness requires special consideration. Demonstration of viral clearance is so critical to product safety that all regulatory agencies require a complete risk assessment of the potential for viral contamination in a monoclonal antibody product and at least some validation of viral clearance or removal by the proposed manufacturing process prior to initiation of Phase 1 clinical trials.

Endotoxins

Endotoxins are lipopolysaccharides produced by certain types of bacteria, which cause a fever response (pyrogenesis) when injected into the bloodstream. Unlike bacterial host systems, mammalian host cells do not produce endotoxins themselves, so they are not an intrinsic impurity in the feedstream. However, some elements of the plant operation, particularly the water systems, can become sources of endotoxin if not maintained and operated correctly, so purification processes usually include polishing steps designed to remove endotoxins together with other trace contaminants.

Bioburden

An absolute requirement for any injectable pharmaceutical product such as a monoclonal antibody drug is that the final drug product be free of live infectious microorganisms. While the bulk drug substance does not need to be free of bioburden (number of live microorganisms per unit volume), it is a practical and regulatory requirement that the bulk drug contain very low bioburden levels to prevent degradation of the product, prevent an increase in endotoxin levels in the product during storage of bulk drug substance, and to ensure that a sterile drug product can be readily achieved through standard aseptic processing. Since monoclonal antibodies are generally purified in aqueous buffer solutions, which can support microbial growth, it is critical to control bioburden during downstream processing. During downstream processing, control of bioburden is achieved through a variety of approaches, including the proper design and operation of the facility (i.e., air handling, clean-room operations, and water and other utility systems), tight control over the time and temperature for holding in-process intermediate solutions, and the design and operation of processing equipment to prevent microbial contamination. In particular, the use of closed pre-sterilized disposable equipment, with connections made through sterile welds or sterile connectors, is increasingly practiced in downstream bioprocessing operations.

Prior to packaging of the bulk drug substance and any stored intermediates, it is common to filter the solution through a $0.2 \mu m$ filter to ensure reduction of bioburden. The resulting

bulk drug substance will have a low bioburden specification that must be met. Note that this is distinct from the sterility specification that must be met by the final formulated and packaged drug, which is in place to ensure that the product will not cause infection upon injection.

Mycoplasma represents a particular type of bioburden contamination that may be introduced as a contaminant during the cell culture process itself, with devastating effects on the entire process. Generally, control of mycoplasma is an issue for the upstream process, and all cell banks and end of production cells are tested for mycoplasma.

Other Process-related Impurities

Other materials besides proteins are often deliberately introduced into the process stream (either upstream or downstream) for important purposes but must not be present in the final, purified product. These include a wide range of small molecules (salts, buffers, acids, bases, solvents, carbohydrates, amino acids, peptides, vitamins, etc.) used in both the cell culture media and the downstream processing solutions. Most of these process-related impurities are non-toxic and easily removed to acceptable levels, but some are highly bioactive and must be removed to trace or undetectable levels in the bulk drug substance.

Another potential source of process-related impurities are the various materials of construction that come in contact with the product stream during the process in the bioreactor, tanks, piping and process equipment (including the bags, connectors and flexible tubing used in disposable-type process approaches). These materials have the potential to leach impurities into the process stream, some of which (such as heavy metals or plasticizers) can be toxic. Generally these concerns are addressed through very careful selection of materials for product contact early in process development and through appropriate validation studies with actual or representative process streams under process conditions prior a filing a BLA or equivalent.⁵

Chromatographic separation media represent a special class of process-contact materials. These are highly complex materials composed of a porous solid matrix (usually a polymer) that has been chemically derivatized with surface functional groups or ligands to provide the appropriate binding selectivity. The support matrix itself may partially break down (particularly under the harsh chemical conditions used for cleaning the column between runs), potentially releasing some matrix material and the surface groups into the product stream.

The most critical process-related impurity of concern in the purification of antibodies is the ligand Protein A, very often used in the primary capture step because of its highly selective binding characteristics. Protein A leaching can occur either through partial breakdown of the resin backbone or coupling chemistry, or through proteolysis of the Protein A ligand itself. Protein A itself is both immunogenic and a potent immune system stimulator, so steps downstream of the Protein A capture column must be designed to remove leached Protein A ligand to very low levels and these levels must be measured with highly sensitive assays.⁶

Product-related Impurities

Although impurities are usually thought of as molecules and materials that are non-product, some critical impurities are variant forms of the product molecule itself. These productrelated impurities can fall into several classes.

Aggregates are a potentially serious impurity in any monoclonal antibody product. Aggregates are typically large, tangled clusters of denatured antibody molecules that are irreversibly formed either during the cell culture, as a result of downstream processing, or over long periods of time during storage. The causes of aggregation of monoclonal antibodies are varied and are influenced by the biochemical and biophysical properties of the monoclonal antibody itself as well as the chemical and physical environment to which the monoclonal antibody is exposed during processing and storage.⁷

Aggregation can cause the product to become turbid and may expose normally unexposed epitopes, causing problems with immunogenicity. Typically, the level of aggregation in the final product must be as low as possible and is generally less than 12%. Note that the relatively large, irreversible aggregates are different from the often reversibly-formed dimers and trimers of antibody molecules that are generally less of a concern from a product safety and immunogenicity standpoint.^{8,9}

Individual monoclonal antibodies vary widely in their propensity to form aggregates. Aggregates formed during

cell culture are an increasing problem as steps are taken to increase the amount of antibody produced per cell to enhance productivity.⁹ Extremes of pH, ionic strength, temperature, concentration, shear forces and other processing conditions can sometimes lead to increased aggregate formation, so aggregation must be monitored throughout the development of a recovery and purification process.

Other product-related impurities are much more specific to the particular monoclonal antibody product or manufacturing process. One type is fragments of the antibody molecule, including free or dimerized light and heavy chains, or fragments produced by improper assembly or digestion with proteases. Fragments may be a greater problem with antibody-type products produced in nonmammalian hosts. Protease levels in mammalian cell culture are also typically low unless there is a great deal of cell lysis.

Product-related impurities can also be specific to the isotype of the antibody.¹⁰ For example, antibody molecules of the subclass IgG4 have a strong tendency for incomplete formation of the disulfide bonds between the two heavy chains, giving rise to a high proportion of "half antibody" molecules with one light and one heavy chain. The half antibodies reversibly form structures similar to whole antibodies (i.e., a structure containing two heavy and two light chains) with other half antibodies (referred to as "half molecule exchange") or through interactions other than the intended disulfide bond to create product-related impurities that are difficult to separate from the correctly bonded whole antibodies. If removed, these may represent a major yield loss. Most commercial antibodies to date are not of the IgG4 subclass due in part to this additional manufacturing challenge.

Another set of important product-related species are glycosylation variants resulting from variations in the complex array of diverse, branched carbohydrate structures on the Fc portion of the monoclonal antibody (and in the variable region for some monoclonal antibodies). These various glycosylation forms can potentially result in altered potency of the antibody product (effect Fc function), change the clearance of the monoclonal antibody from the body, or affect the immunogenicity of the product.¹¹ The glycosylation profile of a monoclonal antibody product is strongly influenced by the production cell line and the cell culture conditions and, to a lesser extent, the purification processes used to manufacture the monoclonal antibody product. Glycosylation variants warrant significant consideration in the development of biosimilar proteins, where even minor changes can impact product quality and the assessment of similarity to the innovator product.¹² While new technologies are under development to enable more precise and independent control of glycosylation within the host cells,^{13, 14} these technologies have not yet been widely adopted so that the downstream process must be robust enough to handle potential variations in the glycosylation pattern of the monoclonal antibody product and still produce a consistent product with a well-defined range of glycoforms.

Finally, monoclonal antibodies are subject to the same variety of chemical modifications of individual amino acid side chains as other recombinant proteins, including deamidation, oxidation, glycation, etc. described in more detail in Chapter 8. Unless these modifications occur in the binding site itself, they generally do not affect the potency or immunogenicity of the drug, and are thus usually not an issue for purification. Monoclonal antibodies almost always have variant forms with the amino acid lysine added to some portion of the C-terminal ends of the heavy chains, which is rarely, if ever, an issue in purification. Control of the ratio of various expected modifications is important in retaining a comparable product throughout development, and introductions of new variants from a modified process later in development may be cause for regulatory agencies to require additional safety testing or bridging clinical studies to confirm biological equivalence of product used early in development with that used in later clinical trials. Some control of the variants of the purified antibody can occur during purification but the primary step for control of variants is the cell culture process.

Product Yield

In addition to removal of contaminants and impurities, another objective of the recovery and purification process is to maximize the overall yield of active product. While yield is largely an economic concern, it can be critical as overall product yield will have an influence on the size of the production facility required to produce the product to meet market demand. Since many monoclonal antibody products are administered in high doses for chronic indications and/ or for large patient populations, the annual demand for most monoclonal antibody-related products is generally very large. A low yielding recovery process would thus necessitate a very large manufacturing facility, which may have unacceptably high capital costs.

When considering yield, it is important to realize that antibodies are large, complex molecules, subject to denaturation, aggregation, and other degradation mechanisms that lead to loss of therapeutic activity. Thus, yield is not simply the percent of the mass of total antibody recovered from the process, but the percent of relevant activity recovered. As a rule antibody molecules are reasonably stable so that steps taken to improve mass yield generally also result in improved activity yield. However, care must sometimes be taken to minimize activity yield loss, particularly when process steps are incorporated to inactivate viruses, which might inactivate the antibody product as well.

Because of the sometimes-conflicting objectives of high product purity and high product yield in a downstream process, there is often a tradeoff between purity and yield during process development.¹⁵ This is particularly the case when the purification process involves unit operations that rely on small differences between the molecules being separated to achieve high purity. Generally, yield will be improved by minimizing the number of overall process steps in a manufacturing process, by using as many steps as possible in which the product flows through a device onto which contaminants and impurities are bound, and where affinity-based product-binding steps are used to maximize the binding selectivity.

3. Specific Unit Operations for Monoclonal Antibody Purification

The specifics of purification processes for monoclonal antibody products differ from company to company but virtually all processes share certain common approaches and contain the basic elements shown in Figure 7.1. Most purification processes begin with a product specific capture step, such as Protein A affinity chromatography, followed by well-established unit operations for virus inactivation, contaminant and impurity removal, virus clearance, and product formulation. A well-developed platform purification process can generally be used with each new monoclonal antibody product developed to enable rapid process development and speed to clinic. During later stages of development, the platform purification process may be further optimized or refined to maximize the yield of a specific monoclonal antibody product, improve the robustness of the process, and consistently meet the desired endpoints of the process by efficiently purifying all of the desired monoclonal antibody product from bioreactor harvests that may change over the course of product development.

Figure 7.1. Typical Unit Operations Used in Monoclonal Antibody Purification

The typical process sections and unit operations used in a monoclonal antibody recovery and purification process are shown in blue boxes with specific unit operations most commonly used for each step listed in the adjacent text.



Single-use Processing

"Single-use" processing represents a major trend in recent years of the shift from fixed, stainless steel processing equipment to a "single-use" or disposable plant approach, replacing fixed tanks, piping, and other process fluid contact plant elements with plastic bags, tubing, filters, sensors, etc. that are designed to be replaced after every run or batch. While there are many potential benefits to this style of manufacturing, the most obvious are speed of development and cost reduction.¹⁶ Employing this approach can eliminate or reduce critical utilities such as highly purified water and steam, as well as cleaning and cleaning validation, which is time-consuming and costly. Single-use processing can deliver faster deployment times and reduced risk, by eliminating the need to construct costly, product specific manufacturing facilities prior to approval.

Many of the disposable and single-use technologies currently available can be implemented in today's manufacturing facilities and can, in many cases, reduce the overall capital cost of a new or renovated facility; the actual savings achieved using single-use technologies will depend on the specifics of the manufacturing process and the mix of technologies used.¹⁷

Originally, in the downstream processing of monoclonal antibodies, single-use technologies were used primarily for buffer preparation and storage and in the final polishing steps of a purification process, including flow-through anion exchange membrane cartridges for trace contaminant removal, nanofiltration for virus removal, and microfiltration for bioburden reduction. Within the past 5-10 years, single-use technologies have evolved to the point where there are single-use or disposable options available for all conventional unit operations in monoclonal antibody downstream processing.¹⁸

Cell and Cellular Debris Removal

The first step in any monoclonal antibody purification process is to remove cells and cellular debris from the bioreactor feedstream, producing a particulate-free solution suitable for subsequent downstream steps. The cell separation step is essentially a solid/liquid separation, with the cells and cell debris being the solid phase and the antibody product contained in the liquid phase.

The clarification of the bioreactor harvest prior to further purification is generally accomplished through a combination of centrifugation, depth filtration, and microfiltration.¹⁹ Alternative processes in which centrifugation is eliminated or the centrifugation and depth filtration steps are replaced with tangential flow filtration (TFF) are also sometimes used, especially at smaller production volumes.

Centrifugation

Since centrifugation is very efficient at removing the large bulk of the cells themselves, and is relatively easily scaled to handle very large volumes, it is by far the most frequently used technology as a first step for clarification. In centrifugation, a high acceleration factor created by a spinning rotor is used to separate the solid and liquid phases based on the difference in their densities, with the cells and debris being denser than the liquid phase.

Centrifuges used for cell separation are complex and expensive systems and require sophisticated engineering to ensure good results. The most common type used in biopharmaceutical production is the disk-stack centrifuge, which operates essentially continuously, with periodic discharge of the accumulated packed solids, and has the capacity to handle a wide variety of feedstocks.²⁰ During the development of a centrifugation process, a discharge cycle must be set up in which the bowl of the centrifuge is flushed briefly to remove antibody product and the solids are fully or partially discharged. The discharge cycle must be carefully optimized for a bioreactor feedstream (primarily based on the packed cell density) to minimize yield loss and dilution of the monoclonal antibody product and lysis of the cells. Particular concerns in centrifugation processes include control of the intense shear forces that can occur where the flowing feedstream enters the centrifuge rotor (which can cause cell lysis and denaturation and aggregation of the monoclonal antibody product), aseptic or low bioburden equipment design and operation, easy solid discharge, ability to clean in place and appropriate scale for the particular process. In recent years, vendors of centrifuge systems have done a good job responding to the needs of the biopharmaceutical industry, and a number of excellent systems are available.

The efficiency of a centrifugation step will depend on the solids volume fraction, the effective clarifying surface and the acceleration factor $(\omega 2r/g)$ within the centrifuge being used. Typically, accelerating factors of 1,500 g are used for clarifying the harvest from a mammalian cell culture bioreactor. Once an effective clarification process has been developed, the process is scale-up by keeping the sigma factor (Σ), determined by multiplying the effective clarifying surface and the acceleration factor, constant. Σ represents the equivalent area of the centrifuge and is unique for each disc stack centrifuge and the angular velocity. For continuous operation, the ratio between flow rate through the centrifuge

and Σ should be kept constant during the scale-up. Σ can also be used to scale disc stack centrifuges from a laboratory bottle centrifuge, by replacing one of the flow rates in the above-mentioned ratio with centrifuged volume divided by time of the centrifugation. In designing a bioreactor clarification by centrifugation, care should be taken in choosing all of the equipment associated with the separation to minimize the amount of shear in the system since high shear forces can cause aggregation of degradation of the monoclonal antibody product and may also cause release of lysis of cells, releasing impurities into the product stream.

Centrifugation is one of the most challenging unit operations to convert to single-use technologies due in part to the high forces and speeds of rotating equipment required for this step. In recent years, several disposable or single-use centrifugation technologies have been introduced for bioprocess applications, including the kSep[®] (kSep Systems, Durham, NC) and the Unifuge (PneumaticScaleAngelus, Stow, OH).

Depth Filtration

Typically, secondary clarification using depth filter after centrifugation is required to further clarify the bioreactor harvest prior to further downstream processing. In depth filtration, the feedstream passes through a thick filter "bed", which traps cell and debris particles within the body of the filter itself rather than on the surface.

Depth filtration can be a complex process due to the very broad size range of the debris particles that may be present in the feed stream and their tendency to plug the filters used. Depth filters are composed of either a sheet filter matrix (usually made from mats of cellulosic or polypropylene fibers) or a packed bed of so-called filter aid particles (usually based on highly purified diatomaceous earth). Pure packed bed depth filters are used when a large fraction of solids must be removed (e.g. when depth filtration is used for cell removal). However, in monoclonal antibody production where centrifugation is used to remove the cells, the most common depth filters incorporate both filter matrix and filter aid in a composite sheet material. Graded sheets with decreasing trapped particle sizes are packaged in series to prevent plugging and enhance filter life. In order to handle the large volumes of bioreactor harvest being clarified and/or the volumetric flow requirements of the manufacturing process, multiple depth filters operated in parallel may be used.

The mechanism of depth filtration is complicated, including both physical entrapment of particles in the filter bed and adsorption to the filter matrix or filter aid particles. Additional binders and surface coatings are often incorporated into the depth filtration materials to enhance adsorption of contaminants such as lipids, DNA, viruses, etc. These specialized depth filters can be very effective for protecting the chromatography columns downstream and enhancing final product purity.

As cell culture titers have increased in recent years, the relative concentration of small-particle debris to be removed by filtration has also tended to increase. This can lead to a dramatic increase in the depth filter area required to handle the debris load, or to an unacceptable increase in the turbidity of the final supernatant or even of the Protein A pool. One effective approach to this problem, when it occurs, is to use polymeric flocculating agents, which, when mixed with the feedstream²¹, cause the very small debris particles to aggregate together into much larger particles that are filtered out more easily.

Single-use processing options for depth filtration have advanced significantly over the recent years. EMD Millipore's Millistak+® Pod system was the earliest product launch in this area, including self-contained disposable pods that eliminate the messy change-out associated with traditional filter housing as well as the need for cleaning. Scalable singleuse offerings from other depth filter manufacturers are now available (e.g., Cuno, Pall, and Sartorius).

Microfiltration

The final filtration of the bioreactor harvest generally includes an absolute microfilter, with a pore size typically in the range of 0.10.5 μ m for bioburden reduction and removal of the final traces of particulate material in the feedstream. To facilitate the combined depth filtration and microfiltration operation and to minimize the hardware necessary for these operations, depth filters, which incorporate a microfilter, are often used.

Tangential Flow Filtration

As an alternative to centrifugation and depth filtration for the clarification of a bioreactor harvest, tangential flow microfiltration is sometimes used to remove both the cells and the vast majority of the debris in a single step. Tangential flow filtration of a cell culture harvest is done using membrane filters with pore sizes in the range of 0.52 μm . In tangential flow microfiltration, the crude bioreactor harvest is pumped parallel to an open-channel membrane filter, which is capable of retaining the cells and debris. By applying backpressure to the recalculating flow across the filter surface, clarified cell culture supernatant containing the monoclonal antibody product is forced through the membrane while the solid debris is left behind. To prevent fouling or plugging of the filter by the retained solids, the harvest solution is pumped at high flow rate across the membrane surface.

As in a process incorporating centrifugation, the clarified harvest following TFF may be further clarified using either depth filters incorporating microfilters or membrane filters to remove the final trace quantities of small debris particles and bioburden in the feedstream. Several technologies exist for disposable or single-use applications of microfiltration, including hollow-fiber filtration and cassette style microfiltration devices that are designed with disposable product contact surfaces.

Despite the apparent ease and simplicity of tangential flow microfiltration, the systems required for handling large bioreactor volumes are quite large and complex to operate and the membrane area must be scaled with the bioreactor volume, so this technology has not been widely adopted in large-scale monoclonal antibody production.

Product Capture

Once the cells and cell debris are removed, the next step in most platform purification processes is the so-called capture purification. In the feedstream at this point (which is often referred to as a "clarified cell culture supernatant"), it is common that most proteins are impurities (although with very high titer cell line and cell culture conditions that are currently more prevalent this may not be the case). The general goal of capture purification is to bind the desired antibody product as selectively as possible and to wash away and remove the great majority of the impurities and contaminants. The bound antibody product is then eluted in a concentrated and purified form suitable for the subsequent steps of the downstream process.

By far the most common capture purification step for monoclonal antibodies is Protein A affinity chromatography.²²

Protein A is a 40 kD protein found on the surface of Staphylococcus aureus bacteria where it selectively binds IgG molecules in the host bloodstream to aid the bacteria in evading the immune system. Engineered versions and variants of Protein A are manufactured using recombinant methods for use as affinity ligands in the production of antibodies. Protein A is quite selective in binding a wide array of IgG subclasses from a number of different species through a specific binding site on the Fc portion of the antibody molecule. Binding of monoclonal antibodies to Protein A occurs under a fairly broad range of pH and ionic strengths. Therefore, once the antibody is bound to the affinity matrix, many of the impurities and contaminants present in the column load can be removed from the antibody by washing the column with solutions at a variety of pH and ionic strengths as well as with such other solutions as detergents to significantly increase the purity of the monoclonal antibody once it is eluted from the column. Elution of monoclonal antibodies from Protein A generally occurs at low pH under conditions that do not inactivate the antibody, as long as the pH is adjusted to neutral relatively quickly.

For monoclonal antibody purification, Protein A is immobilized to a porous chromatography support matrix, packed in a column and used as a purification medium. A wide range of different support matrices have been successfully used and are offered as products by different vendors, including cross-linked agarose, synthetic polymers, controlled-pore glass and various composite materials. Vendors optimize the pressure/flow and mass transport characteristics and immobilization chemistry to maximize throughput and dynamic binding capacity, while maximizing the potential useful life of the resin and minimizing the amount of the Protein A ligand leaching into the product stream.

Protein A affinity chromatography has many advantages as a capture purification step for monoclonal antibody production. It works well for the vast majority of human and humanized monoclonal antibodies. The notable exception is monoclonal antibodies of the IgG3 subclass which do not bind to Protein A. However, since monoclonal antibodies of this subtype are rarely used for therapeutic monoclonal antibody products, this limitation is minimal. Product purity after a Protein A affinity chromatography step is usually very high (95 – 99%) and, due to the binding selectivity of the Protein A ligand, is relatively unaffected by changes in the bioreactor harvest used as feedstream. Binding occurs under a broad range of conditions, so the clarified bioreactor feedstream can be applied to the capture column without buffer exchange, dilution or usually even pH adjustment. Although the affinity ligand is a protein, it is unusually robust and column lifetimes of several hundred cycles are common. Recently, variants of Protein A have been developed that are more stable to high pH conditions, so cleaning and sanitization of the column with sodium hydroxide solutions has become a possibility. The leading example of such a novel ligand is the MAbSelect SuRe[™] ligand (GE Healthcare Bio-Sciences, Uppsala, Sweden), which is a tetrameric form of an engineered variant of a Protein A domain to confer improved alkaline stability.²³

Another advantage of Protein A affinity chromatography as a capture step is that it typically requires little in the way of process optimization. Most antibodies can be bound at high capacity from the clarified cell culture supernatant. One or more wash steps are then applied which typically include increased ionic strength and potentially reduced pH and additives such as detergents to remove bound impurities. Once developed for a particular host cell type, however, these wash procedures typically do not need to be optimized for each individual antibody product. Therefore, since most companies developing monoclonal antibody products are using the same production host for all their antibody products, it is relatively easy to develop a Protein A affinity chromatography process that can be utilized across a complete portfolio of monoclonal antibody products with little to no modification.

One area for optimization of Protein A affinity chromatography that is generally required for each product is the elution step. The different IgG subclasses vary somewhat in the pH required for elution and individual antibodies can vary considerably in their stability under low pH conditions. Fine tuning of such elution conditions as the precise elution pH and ionic strength and the addition of additives to the elution buffer can sometimes be used to improve recovery in cases where it is needed. In addition, the selection of the buffer ion used for elution can have a major effect on the amount of neutralization buffer and the subsequent dilution factor that may be required for the next step downstream.²⁴

Modern Protein A affinity chromatography media typically

have relatively low leaching (at least due to matrix or coupling breakdown) but are still susceptible to leaching via proteolysis from proteases present in the feedstream. Any Protein A which leaches from the affinity column is a potential impurity that must be removed from the monoclonal antibody product to a sufficiently low level, typically <5 µg Protein A per gram monoclonal antibody (<5 ppm), by subsequent purification steps. Leached Protein A ligand tends to bind strongly to the monoclonal antibody product under most conditions, making removal a potential challenge. However, Protein A is relatively acidic with an isoelectric point of 5.1 compared to the vast majority of monoclonal antibodies, which generally have isoelectric points of 7 or greater. Protein A is also typically more hydrophobic than the product antibodies. Because of these differences in molecular characteristics, antibody-bound Protein A can typically be removed to below the levels required in the intermediate and polishing purification steps.

Perhaps the largest disadvantage of Protein A affinity chromatography in the purification of monoclonal antibodies is the high cost of the media, which can range from \$5,000 to \$15,000 per liter or more, even when purchased in process-scale quantities. The cost of Protein A affinity media can make it the most expensive raw material used in a monoclonal antibody production process.²⁵ Concerns about the cost of Protein A affinity media have led to careful engineering and process designs that maximize the utilization of the material and thus minimize the cost. including media reuse strategies that lower the effective cost of the Protein A affinity media per gram of product purified to less than a few dollars per gram resulting in overall costs of production of monoclonal antibodies using Protein A affinity chromatography of less than \$10/g.²⁶ Nevertheless, some companies have sought to use much less expensive capture steps (such as ion exchange chromatography) or developed less expensive alternative ligands to lower the cost of raw materials for monoclonal antibody production. However, in most cases, the many advantages of Protein A affinity chromatography as a capture step outweigh the disadvantages, so that the majority of companies developing monoclonal antibody products use some form of Protein A affinity chromatography in their purification process.

An increasing array of options for pre-packed disposableformat chromatography columns exists for bioprocess applications. The earliest entries to this market included the ReadyToProcess columns (GE Healthcare Bio-Sciences, Uppsala, Sweden), GoPure[™] columns (Life Technologies, Carlsbad, CA), Opus[®] columns (Repligen Corporation, Waltham, MA), and MaxiChrome[®] columns (Atoll GmbH, Weingarten, Germany). New technologies are introduced regularly in this area, so the range of technology options available to users is likely to increase, including increasing use of membrane adsorbers. Importantly, cost-effective application of disposable chromatography columns generally requires cycling the column, either by running multiple batches or through continuous processing, to amortize the cost of the resin across a sufficient quantity of purified protein.

Process Cycling

One approach to improving the economics of downstream process operations, particularly those involving expensive separation media, is to utilize process cycling.^{27, 28} In the cycling approach, the process is designed so that the complete separation step can be run as rapidly as possible, and the step is then repeated (or cycled) multiple times in order to process the entire batch of feedstream. Since the costs of utilizing separation media (column packing or filter) are inversely proportional to the number of cycles the media are in operation, cycling can dramatically reduce overall manufacturing costs in some cases. The extreme example of cycling is a continuous or semi-continuous process, which is the norm for many large-scale processing industries such as food, beverages or chemicals.

The primary quality and regulatory concerns with cycling are maintaining batch integrity, product quality and traceability. These are critical to ensure that any given dose of the final drug product can be traced back through all of its specific process steps and raw materials in order to determine the root cause in the event of a problem. On the one hand, if the products from multiple cycles of a process are pooled, it is possible that critical traceability might be lost. On the other hand, if the products from each cycle must be maintained and tested as separate "sub-batches," the economic benefits of cycling may be lost.

It is clear that the regulatory agencies allow producers to incorporate cycling as they see fit, provided that batch integrity, product quality and traceability are properly maintained. The question largely revolves around risk analysis -the real risks of failure of a sub-batch in a cycling process and the consequences of such a failure (both for safety and cost). Validation of the re-use of the separation media over many cycles (either in the same batch or in multiple batches) provides the basis for a "lifetime" of the separation media is a critical element of enabling cycling. Chromatography column and membrane lifetime studies are typically performed as part of process validation during development using scale-down models. While this provides a solid basis for cycle limits during manufacturing, confirmation of column lifetimes can also be incorporated into continuous process verification programs²⁹ as part of a life cycle approach to process validation 1. As the experience of the industry has grown over the years, the risks associated with process cycling have become better understood and approaches to mitigate these risks have been established. As a result, the use of process cycling is now commonplace and process scientists and engineers are developing standardized approaches to cycling and continuous or semi-continuous processing.

Virus Inactivation

A critical function of any downstream process is to inactivate and/or remove potential viral contamination using at least two methods that have different modes of action. Since the low pH conditions used to elute monoclonal antibodies from a Protein A affinity chromatography column can be very effective for inactivating certain major classes of viruses, most platform processes for monoclonal antibody purification include a timed hold step at controlled temperature following the elution from a Protein A affinity chromatography column. This step must be optimized to maximize the inactivation of the target viruses while minimizing the denaturation of the antibody product. While other proven approaches can be used to inactivate viruses, such as exposure to solventdetergent solutions, the low pH hold method is by far the most commonly used approach in monoclonal antibody processes. The solvent-detergent method is an alternative for products that may be unstable at low pH.³⁰

There has been considerable activity in developing new approaches to viral inactivation. The general challenge for viral inactivation is to remove or destroy the biological activity of the virus without inactivating or denaturing the therapeutic product itself. The key to this is that the activity of the virus is dependent upon the RNA or DNA present, while (in the case of antibodies) the therapeutic is a protein. Unfortunately, nucleic acids and proteins are similar enough in their stability with respect to various inactivation approaches to make exploiting differences difficult.

Several technologies have been investigated as complements, or alternatives, to low pH and nanofiltration, which are commonly used today for viral inactivation and clearance. These technologies include rapid heating and cooling using microwaves, exposure to electromagnetic radiation (visible, UV or gamma) in the presence of protective agents, and specific chemical inactivation agents.^{31, 32, 33, 34, 35} While effective in some cases, to date none of these technologies have been widely adopted and interest in their development may be waning. If any of these alternative technologies are used for virus inactivation, the specific methods must be tested carefully for their effectiveness with model viruses in the actual process feedstream and for their effects on the monoclonal antibody product itself must be determined along with the cost and scale-up and integration of the method into existing manufacturing processes and facilities must also be considered.

Intermediate and Polishing Purification

Following the capture purification and low pH hold steps, the concentration of the monoclonal antibody product is relatively high compared to other contaminants and impurities, which are typically present at only a few percent or less. These contaminants and impurities include host cell and media protein impurities, some level of leached Protein A ligand (assuming Protein A affinity chromatography was used as the capture step in the purification process), product-related impurities such as modified antibody forms or aggregates, and trace levels of host cell DNA. Protein impurities must generally be reduced to less than a few hundred parts per million (µg contaminant per gram IgG) or lower, leached Protein A to less than 5 ppm, and monoclonal antibody dimers and higher molecular weight aggregates to less than a few percent. Nucleic acids must typically be reduced to less than 10 ng/ dose.² In addition to removal of these impurities, additional viral clearance is also required beyond what the capture step and low pH hold may have provided.

The greatest variability between different platform processes and also between the specific processes developed for individual monoclonal antibody products occurs in the intermediate and polishing purification step(s). These steps are generally chromatographic separations based on specific physicochemical characteristics of the antibody, such as surface charge and/or hydrophobicity. However, a range of different approaches and specific media and conditions may be used.

Modes of Chromatography Used in Intermediate and Polishing Purification

Three basic modes of chromatography are used for intermediate and polishing purification: ion exchange, hydrophobic interaction, and mixed mode (including hydroxyapatite) chromatography. Less frequently used is size exclusion chromatography, which typically requires lower flow rates and larger column sizes, reducing its attractiveness for large-scale process use.

Chromatographic separations used for intermediate and polishing purification can be run either as bind/elute (B/E)operations or flow-through (FT) operations. In the B/E mode, the antibody is bound to the column under one set of conditions, the column is washed under these same or different conditions to remove the contaminants and impurities, and then the monoclonal antibody product is selectively eluted from the column under another set of conditions, leaving more tightly bound contaminants and impurities behind on the column. FT separations are designed so that the antibody does not bind to the column but the contaminants and impurities do bind and are retained on the column. Conditions for FT operation must be optimized for each antibody so that the maximum possible amount of contaminants and impurities are bound and removed from the solution while the maximum amount of antibody flows through into the eluate. B/E steps are generally more flexible for removing a wider range of contaminants and impurities, but require large columns to bind the large mass of the antibody product and are relatively complex to develop and operate. FT steps are operationally much simpler and, because only the trace levels of contaminants bind to the column, usually require much smaller column volumes.

Ion Exchange Chromatography

By far the most common chromatographic mode used in the intermediate purification of monoclonal antibody products, and often as an alternative to Protein A affinity chromatography for initial product capture, is ion exchange chromatography, in which a resin with immobilized charge groups is used to bind molecules in the feedstream with the opposite surface charge through electrostatic forces. The bound molecules are then selectively washed off and eluted by changing the pH and/or ionic strength. The process may be done with either anion exchange media (in which positive charge groups such as quaternary amines on the column bind negatively charged molecules in the feedstream) or cation exchange media (which are the reverse, in which negatively charged groups such as sulfonates on the column bind positively charged molecules in the feedstream).

Antibodies in general tend to be more basic (i.e. positively charged) than other proteins (including most host and media proteins and Protein A) and other impurities and contaminants such as DNA and viruses. Many (although by no means all) therapeutic monoclonal antibodies are highly basic (pI >8). Therefore, ion exchange is a useful purification modality, and cation exchange is often used in a bind/elute mode as an intermediate purification step. The cation exchange column is loaded at the highest pH and ionic strength that support high capacity binding of the monoclonal antibody product (to minimize the binding of impurities), and generally washed and eluted with increasing ionic strength, sometimes with a pH shift. Modern process cation exchange media available from a wide range of vendors have high dynamic binding capacity, good flow characteristics, and increasingly are available with enhanced mass transport to enable high throughput.

Interestingly, cation exchange chromatography can often be optimized to remove aggregates of the antibody, even though the aggregates are composed of the same polypeptide chains as the monomeric product. This is because the molecules in the aggregate are partially denatured and unfolded, which exposes different amino acids on the surface of the aggregate from the native monomer. With large protein molecules, the strength of binding to ion exchangers is determined by groups of charged amino acid side chains on the surface of the molecule, rather than the overall net charge. This surface charge distribution may be very different for denatured aggregates than for the native monomer, enabling an effective separation.

The same general charge difference between antibody products and the impurities and contaminants can also

be exploited by using anion exchange chromatography for impurity removal. This general charge difference, between the antibody product and the other impurities and contaminants, enables anion exchange chromatography to be utilized very effectively in flow-through mode. In this case, the pH is made as high as possible and the ionic strength as low as possible to enhance binding of impurities and contaminants without significantly binding the monoclonal antibody product. Flow-through anion exchange is particularly effective for removing highly negative charged impurities and contaminants, including Protein A, DNA and endotoxins. This approach is so powerful that virtually all platform antibody purification processes include a flowthrough anion exchange step.³⁶

Because the amount of bound contaminants and impurities in the polishing step is so small, very little bed volume is actually required for binding capacity in flow-through anion exchange. However, with conventional packed bed chromatography, mass transport limitations with the porous resin particles necessitate a residence time in the column (column volume divided by flow rate) of several minutes to enable complete binding of the impurities as the feedstream passes through. This means that fairly large columns are still needed if reasonable flow rates and throughput are to be achieved.

In recent years, this problem has been overcome through the use of membrane adsorbent devices, which have enhanced mass transport and thus can be operated at very high flow rates relative to the adsorbent volume and still achieve efficient capture of the contaminants and impurities. These devices are now made available as disposable cartridges, eliminating the need for cleaning validation activities³⁷ resulting in their increasing use in monoclonal antibody purification processes. Drawbacks for membrane adsorbers have included low capacity, which limits applications outside of "flow through" steps and the need to operate at very low conductivities to achieve the desired separation. Recently, membrane adsorbers with greater salt tolerance (e.g., Sartorius STIC-PA products) have been introduced to enable effective separations with less dilution. Additionally, devices such as the Natriflo HD Q, from Natrix Separations (Burlington Ontario, Canada) have been developed to offer higher capacity adsorbers in a membrane format. Finally, as already described above, pre-packed columns containing modern ion exchange resins

with improved mass transfer properties can also be used to provide rapid and efficient removal of trace impurities and contaminants.^{38, 39, 40}

Recently a number of other approaches have been taken to optimizing anion exchange chromatography as an intermediate and polishing purification step for antibodies, one being the "weak partitioning" mode. This approach relies on careful adjustment of the pH and ionic strength of the binding step to maximize the binding strength of the impurities and contaminants, even if this results in weak binding of the product antibody. By maximizing the product load and including an optimized brief wash step, this "weak partitioning chromatography" approach can dramatically improve the log removal of impurities and contaminants with negligible loss of product yield, potentially enabling a two-column process for antibody purification.⁴¹

Hydrophobic Interaction Chromatography

A second common chromatography mode used for intermediate and polishing purification is hydrophobic interaction chromatography (HIC), in which a resin with immobilized hydrophobic groups is used to bind proteins in the feedstream on the basis of their surface hydrophobicity. Commonly used HIC media, include media with propyl, butyl or phenyl functional groups that are immobilized on conventional chromatography backbones, such as agarose or methacrylate beads. In order to insure good recovery of proteins from the column, the hydrophobic groups are weak enough and immobilized at a low enough concentration so that in the presence of high concentrations of certain salts, such as ammonium sulfate, the protein is bound to the chromatography media through hydrophobic interactions between the hydrophobic ligand and hydrophobic regions on the monoclonal antibody. Elution of the monoclonal antibody product is then performed by reducing the salt concentration to break these hydrophobic interactions.

HIC steps are often developed with a primary objective of reducing aggregate levels in the monoclonal antibody preparation as high molecular weight aggregates of monoclonal antibodies generally bind more tightly to these media than the monomeric monoclonal antibody due to the exposed hydrophobic groups in the denatured chains of the aggregate. The use of very high salt concentrations makes hydrophobic interaction chromatography considerably less convenient and more expensive than ion exchange and can make integration with downstream steps challenging, but sometimes the enhanced selectivity of HIC media, particularly if removal of aggregates from the monoclonal antibody product is an issue makes it worthwhile to incorporate such a step in a monoclonal antibody purification process.

Mixed Mode Chromatography

A third mode of chromatography used for intermediate and polishing purification involves so-called "mixed mode" ligands which incorporate combinations of charge groups, hydrophobic groups and potentially other functionalities to impart unique selectivity through mechanisms such as hydrogen bonding. A number of commercial mixed mode media have been found to be very effective for or have been developed specifically for use in therapeutic monoclonal antibody purification.

Several mixed mode media have the potential to provide the benefits of hydrophobic interaction chromatography while reducing the practical limitations of these media. One such approach has been to develop mixed mode anion exchange ligands with enhanced binding strength for the key impurities, typically through the addition of hydrophobic functionality.^{42, 43} This can increase the range of ionic strength and pH for effective binding of impurities and can dramatically enhance the removal of the more hydrophobic aggregates.⁴⁴ By appropriately adjusting the pH and ionic strength of a mixed mode chromatography column, monoclonal antibodies can be bound to the media through hydrophobic interaction and selectively eluted by changing the pH and/or ionic strength of the elution buffer resulting in very good separation of a wide range of monoclonal antibodies from critical impurities and contaminants present in the feedstream.

Another category of mixed mode resins is based on hydroxyapatite, made from calcium phosphate, which has both positive and negative charge and interacts with proteins through a combination of electrostatic interactions and coordination complex formation.⁴⁵ Binding is typically at neutral to slightly acidic pH, with elution by increasing ionic strength. In recent years, a more robust ceramic hydroxyapatite media that has enhanced physical stability has been developed and commercialized for use in large-scale process applications.46 Hydroxyapatite has been demonstrated to be able to separate monoclonal antibodies effectively from host and media proteins, aggregates, DNA and Protein A, all of which tend to bind more tightly. Its effectiveness for separation of aggregates has been particularly noted.⁴⁴

Development of Intermediate and Polishing Steps Regardless of whether a product binding or flow-through approach is used or which type of chromatography media is selected for intermediate purification, a significant amount of process optimization must be done for each specific antibody and feedstream to obtain the required results. In all cases, the pH, ionic strength (salt concentration) and product load must be simultaneously optimized for the binding, wash and elution steps to maximize both effective capacity and selectivity or final purity. Buffer ion, salt type and other additives at each step may also significantly affect the separation. Media of the same type from different vendors will behave differently, so this must also be factored into the development program. Finally, once a set of conditions that works well is discovered, it is important for process robustness to determine the critical parameters and to assess what range of those parameters will produce acceptable results. All of the process variables are interactive with each other (e.g. changes in the pH will affect the optimal range of ionic strength), further complicating development.

The number of intermediate and polishing steps required to achieve the desired purity of a monoclonal antibody product will vary from product to product and will depend on the extent of the development effort applied to optimizing each individual unit operation. In some cases, it is clearly feasible to obtain the required purity with a single purification step following the initial capture purification column, sometimes even with a simple flow-through anion exchange step. Such a two-column process is very attractive from the perspectives of operational simplicity and low manufacturing cost, and this may be enabled in a number of ways, including the "weak partitioning" approach to anion exchange described above (see Ion Exchange Chromatography section). However, optimization of such processes can be difficult and it is often necessary to add an additional step to remove the last traces of contaminants such as adventitious viruses, and product-related impurities, especially high molecular weight aggregates.47

As a result, a "three column" process is much more commonly used for monoclonal antibody purification. In such a process, bind and elute capture and intermediate purification steps, such as Protein A and cation exchange chromatography, are followed by a flow-through anion exchange step. This type of process is often used for production of preclinical and early stage clinical trial materials since this approach will virtually guarantee a rapidly developed, robust, effective process for all monoclonal antibody products. Later in development, when the manufacturing process has been better characterized and optimized, the potential to eliminate one step from the process and develop a robust two column process, specific for a particular monoclonal antibody, for production of Phase 3 clinical trial and commercial material can be investigated.

One additional factor to be considered in the development of intermediate and polishing purification steps is the integration with each other and with the rest of the process. Ideally, the product feedstream coming from the capture purification step can be loaded on the intermediate column with minimal processing for adjustment of solution conditions (i.e., pH and buffer and/or salt concentration). If buffer exchange (removal and replacement of buffer or salt components) is required, this will entail an additional process step (usually ultrafiltration), which adds significant capital expense and operating cost. Similarly, it is desirable if the product of the first intermediate and polishing purification steps can be integrated with the subsequent purification step. This type of integration requires very careful design of the process, including the order of the steps.

Nanofiltration for Virus Removal

In addition to the typical low pH hold for virus inactivation, most purification processes will also include an additional step specifically focused on removal of small viruses resistant to chemical inactivation, such as parvovirus. For this step, nanofiltration using specifically designed membranes with nominal pore sizes down to 15 nm, is often used as it employs size rather than chemical sensitivity as a removal mechanism for viruses. Due to the relative sizes of monoclonal antibodies compared to most viruses, a monoclonal antibody product will pass through a nanofilter while the vast majority of the virus particles are retained by the membrane. The added advantage of nanofiltration over chemical inactivation methods is that nanofiltration is capable of removing all potential viral contaminants (both infectious and non-infectious) whose size is greater than the pore size of the filter used while chemical and physical inactivation technologies will potentially leave inactive viral particles or fragments in the final product. Nanofiltration has become commonly accepted as a very robust viral clearance step and is included in the final stages of nearly all monoclonal antibody production processes.

Considerable care must be taken in the manufacturing of the filters used for nanofiltration and their handling. Nondestructive testing of nanofilters for integrity is considerably more challenging than for microfilters, and vendors are continuing to develop new and more effective techniques. Proper operation of nanofilters is also critical for achieving the validated level of viral clearance in each production batch. One critical parameter to monitor is the decay in membrane flux or flow rate as the process proceeds. Plugging of the membrane by retained materials tends to occur preferentially in the smaller pores, so that a greater proportion of the flux occurs through larger pores and potential pinhole defects, reducing the effective clearance. Processes are typically designed and validated with a controlled level of flux decay to prevent this problem.

Bulk Drug Substance Formulation

After the stages of purification described above, the antibody product has reached its final purity level with respect to the key impurities and contaminants. The only remaining process objective is to place the antibody product into the proper conditions of pH, ionic strength and concentration for storage as bulk drug substance. In some cases excipients that are needed in the final drug product formulation are also added at this step.

The primary means of concentration and buffer exchange is ultrafiltration, in which a membrane filter is used with pores small enough to retain the antibody product and large enough to pass water, salts and other small molecules. The layer of retained protein on the membrane surface, commonly referred to as a "polarization layer," will quickly slow the flow through the membrane if steps are not taken to prevent this. The most common approach is to pump the feedstream at high flow across the membrane surface, causing back mixing and reducing the thickness of a polarization layer. This so called tangential flow is almost always used in conjunction with ultrafiltration, often referred to as tangential flow filtration.⁴⁸ In a typical TFF process, the antibody product is first concentrated by removal of water and buffer salts by the application of pressure across the membrane. The buffer salts can then be exchanged for different buffer salts by pumping the new buffer solution into the feed tank while the filtration continues. The new buffer will eventually wash out the old buffer in a process called diafiltration. Single-use tangential-flow filtration devices are available for a wide range of bioprocess applications, including the ultrafiltration and diafiltration (UF/DF) processes used in bulk drug substance formulation with Novasep's SiusTM (Pompey, France) line of single-use TFF cassettes being an early example of such product offerings.

Bioburden Reduction

Following the formulation of the bulk drug substance, the last step in a monoclonal antibody manufacturing process is normally microfiltration, usually through a 0.1 or 0.2 μ m filter, which is capable of very effectively removing virtually all microorganisms. Although great care is usually taken to prevent microorganisms from entering the product stream throughout the process, a final microfiltration step into a sterile bag or other storage vessel is usually regarded as a vital step to ensure that there is low bioburden in the final bulk drug substance.

4. Platform Purification Process

Monoclonal antibodies are unusual among biopharmaceutical products in that antibodies as a class share a large fraction of common molecular structure, with the primary variability for antibodies within a specific subclass being in the two binding sites on the Fab portion of the molecule. Because of this commonality of structure, it is possible to develop a common platform purification process, which can be used, with only minor variations and optimization, for a wide range of antibody products.^{49, 50}

The platform purification process approach is so powerful, and the potential benefits so significant, that it has been adopted in recent years by most of the major therapeutic antibody producers and contract manufacturers. The application of QbD principles can both facilitate and enhance these benefits. An obvious advantage is the use of common facilities, equipment, materials, and unit operations for all products, which speeds process development and facilitates the introduction of new products. QbD requires strong product and process understanding gained through iterative experimentation and risk assessment and encourages the leveraging of existing platform knowledge, principles that align closely with the platform purification approach. Using a QbD approach, it is possible to establish process design space for a new product based on the understanding gained from similar platform products. Regulatory support for this type of approach to process development has recently been further strengthened with the publication of ICH Q11, a comprehensive regulatory guidance for the development and manufacture of drug substances.⁵¹

A subtler but equally important advantage is that because process performance and optimization information gathered for one antibody product in a platform process can often be applied to other antibodies in the same process, it is worthwhile to do the kind of detailed characterization and optimization that result in more robust processes for all the antibody products. Finally, some organizations with many antibody products in development or on the market have been able use process validation information for one product to support validation of subsequent products.

A number of key common characteristics of monoclonal antibodies have enabled the development of extremely robust and effective platform purification processes that work for the vast majority of individual products. These include the relative stability of most antibodies to low pH (which enable both the use of Protein A affinity chromatography as a very effective capture step and the use of a low pH hold for viral inactivation) and the relatively high positive charge compared to the major contaminants and impurities (which enables both "flow through" anion exchange and "bind and elute" cation exchange to be very effective as intermediate and polishing purification steps). This platform approach can dramatically reduce the time and material required to develop an effective downstream purification process, with the primary effort being required to optimize the loading and elution conditions used in the intermediate and polishing purification steps for the individual antibody to achieve the required final purity level. Most of the other steps in the platform process can be run with similar results for most antibodies with little optimization. The unit operations typically used in platform purification processes for monoclonal antibodies and their purpose from a monoclonal antibody purification perspective are summarized in Figure 7.2.



Figure 7.2. Basic Elements of a Platform Purification Processes

A wide variety of impurities are present in the cell culture supernatant for monoclonal antibody production. The ability of each step in a typical monoclonal antibody purification process to remove specific impurities is shown. Each light blue box in line with a process step indicates that the impurity is normally efficiently removed as an intended consequece of the process step or section of the downstream process. In addition, although not specicically shown in the figure, there are usally 0.2 μ filters included in the column chromatography steps and between stages to both protect columns and to provide bioburden reduction for process intermediates. Finally, the low pH hold, while primarily intended for virus inactivation, also can kill many microbes.

Rigor in the Platform Process

Although the advantages of the platform process approach are many, one cautionary note should be considered. Monoclonal antibody production is still relatively early in its development as a routine industrial process and many exciting and valuable new products and technologies are still emerging. The widespread use of platform processes in the industry provides many benefits but creates the challenge of how to incorporate new, and potentially cost-saving, technologies into the platform. An overly rigorous approach in the application of the platform process can create issues in responding to problems that may arise or in taking advantage of new developments.

Customized Process Approaches

Although the general platform purification processes described above are well-proven and can be effectively optimized for virtually all monoclonal antibody products, there are many alternatives for the purification of monoclonal antibody products. For example, the plasma fractionation industry uses selective precipitation and (sometimes) ion exchange chromatography to produce polyclonal antibody therapeutics at the metric ton scale. While the feedstream composition and technical requirements for plasma-derived polyclonal antibody products are very different from cell culture-derived monoclonal antibody products, some of these techniques can and have been used in the purification of monoclonal antibodies. However, for the reasons discussed above, the general platform strategy described here is currently employed by most sponsors for the production of monoclonal antibodies.

Because of the cost of Protein A affinity chromatography media and the relative sensitivity to cleaning and reusing the media, some companies have developed processes to reduce the level of contaminants and impurities in the column feedstream thereby protecting the Protein A affinity chromatography column but still achieving high levels of purity with minimal reduction in yield. One such approach has been borrowed from the plasma protein industry and is based on the observation that at pH 45, low concentrations of octanoic (caprylic) acid will cause most contaminating proteins in a cell culture harvest to precipitate, leaving the partially purified monoclonal antibody in solution.52 This is a particularly interesting method, as the precipitation step can potentially be performed prior to the cell and debris removal steps allowing the precipitated protein to be removed without the need for an additional liquid/solid separation. It is also possible to selectively precipitate the antibody product itself using various salts or PEG, but this leads to a more operationally complex process.

Another approach is to avoid the use of Protein A affinity chromatography entirely and develop alternative platform processes using only non-affinity media. In one example of such a process, a cation exchange column is used as an alternative capture step to Protein A affinity chromatography.53 For monoclonal antibodies with relatively high isoelectric points such a process can have sufficiently high selectivity to produce an intermediate of the same purity as Protein A affinity chromatography. Following the cation exchange chromatography step, the monoclonal antibody can be further purified using mixed-mode or hydrophobic interaction chromatography followed by a highly optimized flow-through anion exchange polishing step. In more recent work, novel clarification⁵⁴ and chromatographic approaches dubbed steric exclusion chromatography (SCX)⁵⁵ and void partitioning anion exchange (VPAX)⁵⁶ have been adapted for the development of a bench-scale non-affinity antibody purification process.

Deciding Between Platform versus Customized Processes

There are many advantages to using the platform process approach for the production of monoclonal antibodies. The reasons for seeking an alternative process design include an attempt to reduce manufacturing cost, or specific characteristics of the antibody that make the typical platform approach unsuitable. The latter is somewhat rare, except in the case of alternative antibody-related products (such as fusion proteins, antibody fragments, etc.) or products using atypical IgG isotypes, which may not be amenable to the platform approach. Economic gains from alternative processes are possible, but some caution must be exercised. Process development costs and time will almost certainly increase, and there will be increased risk that the process may not meet one or more of the complex, highly interrelated performance requirements. Cases where the product has extraordinary requirements for low manufacturing costs are unusual, and sometimes modifications can be made to the basic platform process that will achieve much the same end with lower risk. Investment in alternative process options generally is more common - and more feasible from an economic standpoint - in later stages of development. As an antibody product enters late-stage development, more specific information about product-related impurities, critical quality attributes, commercial supply requirements, or other parameters may provide adequate justification for exploring alternative process approaches to address product-specific issues, which are generally less clear in early-stage development.

5. High Throughput Process Development Approaches

In the past, process development laboratories have relied on laboratory scale columns (>1 mL) and automated or manual chromatography systems to perform experiments in series for purification process development. The two main drawbacks of this approach were the significant amount of sample needed for these experiments and the time required to complete the experiments. The first was related to the size of columns used and the second to the intrinsic non-parallel character of most automated chromatography systems that required performing one chromatography run at a time.

In recent years, many groups have developed high throughput experimental approaches that rely on both significantly scaleddown chromatography columns and batch adsorption methods. Both of these approaches have the advantage of requiring 10-50 fold less sample and, if performed in parallel, experiments can be completed in dramatically reduced time compared to traditional process development.⁵⁷

High throughput purification development techniques can be used for primary screening of chromatography media (e.g. in order to determine their static and dynamic binding capacity and choose the process resins), or other rapid yes/ no types of experiments to evaluate a large variety of process options or parameters in a short period of time. These high throughput techniques can also be used to guide process optimization and the mapping of a purification design space to fully understand the interactions between critical process parameters and product quality attributes. The preliminary design and operating space are then typically verified by a series of runs on the smallest columns that can model the chromatographic operation.

Scale-down Model Concepts

As with scale-down models used for cell culture development, careful attention must be paid to the design and operation of scale-down models for purification development to ensure that the model is as representative as possible of the downstream unit operation at process scale. While there are challenges in developing scale-down models for all possible unit operations used in the purification of monoclonal antibodies, the scale-down of chromatography is perhaps the most difficult. Chromatography is a multi-step operation requiring careful control and optimization of a number of process and operational parameters to optimize the recovery of the monoclonal antibody product and ensure reliable and reproducible removal of contaminants and impurities. Those parameters which are important in the development and optimization of a chromatography process are listed in Table 7.1 along with an indication of which of these parameters have been, or can be, studied or developed using the high throughput techniques described in this chapter. slopes and step elution strategies; and the effectiveness of cleaning solutions; and resin chemical stability can also be studied using high throughput methods. Parameters not appropriate for investigation using high throughput methods include the effect of flow rate on the mechanical stability of a packed bed, physical fouling of a chromatography column due to the accumulation of sample components on the resin, and the effect of packing quality on the separation factor for a given process. These factors are important considerations

Process Parameter or Feature	Compatible with High Throughput Screening?
Equilibrium constant for interaction between resin and sample constituents at different process conditions	Yes
Maximum binding capacity for sample constituents at different process conditions	Yes
Dynamic binding capacity for sample constituents at different process conditions	Yes
Feed composition on the capacity and strength of interaction	Yes
Buffer composition including additives, pH and ionic strength on binding, wash and elution efficiency	Yes
Gradient strength and composition	Yes
Packing quality and its effect on column performance	No
Resin life time due to physical fouling	No
Resin life time due to chemical fouling	Yes
Cleaning efficiency and its effect on life time	Yes
Mechanical stability of a resin	No

Table 7.1. Parameters to be Considered in Chromatography Step Development

As is evident from Table 7.1, most or the process parameters necessary for operation and control of a chromatography process, such as the determination of equilibrium constants and maximum (static) binding capacities, can be studied using high throughput methods. These are two parameters that are best suited for evaluation using high throughput plate-based methods. Additionally, such other process variables as the effect of buffer compositions and additives on the effectiveness of wash and elution steps; gradient in choosing a resin and in process scale-up. The bed packing, mechanical, and physical stability are determined on linearly scaled down columns and the results later verified at scale.

In general, the high throughput information is used to establish the operating conditions and design space, which is then refined using a column that is an appropriate scaledown model of the manufacturing column. Later in the development process, the design and operating space is verified using a qualified or validated manufacturing model.
Formats for High Throughput Purification Methods

The parallel experimental techniques for development/ characterization of chromatographic process steps include methods based on micro-columns and batch adsorption. The micro-column methods can be further divided into those based on pre-packed columns and those based on pipette tips filled with chromatography resins. Micro-columns, ranging in size from 50 μ L to 600 μ L, and in a 200 μ L and 600 μ L format, can be obtained from Atoll GmbH (Wingarten, Germany) and Bio-Rad (Hercules, CA), respectively. The Atoll columns can be purchased packed with any resin whereas the Bio-Rad columns are available with only their resins. Pipette tips pre-filled with 10 to 500 μ L of chromatography media are available from PhyNexus, Inc. (San Jose, CA).

High experimental throughput is achieved with the microcolumn and pipette tips by operating several micro-columns or tips, typically multiples of eight, in parallel by using a standard laboratory multi-channel liquid handler, such as a Freedom EVO® system from Tecan Group Ltd (Männedorf, Switzerland) or the Perkin Elmer Janus® BioTX Pro (Waltham, MA), as a multi-pump delivery system, where each channel of the liquid handler delivers liquid to one of the micro-columns (or micro wells). Several other liquid handlers (from Gilson, Hamilton, Eppendorf and Beckman, to name a few), could also be used as the foundation of a high throughput chromatography system by adding in the ability to buffer exchange, centrifuge, filter and vacuum. Experiments using micro-columns or pipette tips can also be performed using manual pipettes but these devices do not provide the same degree of flow control as the automated system and require much more time.

Batch adsorption combined with the high throughput capacity of microtiter plates can be used as an alternative to micro-columns for high throughput purification development. In this case, microtiter plates either filled with chromatography media in-house or purchased pre-filled can be used in conjunction with appropriate instrumentation to automate and control the addition and removal of liquid from the wells of the microtiter plate. If in-house filled microtiter plates are used, it is important that a reliable method be developed for filling the plates to ensure a uniform distribution of chromatography media across the plate and to ensure reproducibility of results.58 A drawback too many pre-filled plates, however, is that one cannot purchase plates containing multiple vendors' resins. GE Healthcare Bio-Sciences (Uppsala, Sweden), Thermo Scientific (Rockford, IL) and Bio-Rad (Hercules, CA) are examples of suppliers that produce pre-filled plates that contain their resins. Microtiter plates are also available with ion-exchange membranes (PALL Scientific, Port Washington, NY) and ion-exchange and hydrophobic membranes (Sartorius, Goettingen, Germany).

A qualitative comparison of the capabilities and attributes of different high throughput formats used in the development of chromatographic separations is shown in Table 7.2.⁵⁹ While the use of micro-columns may provide a more representative scale-down model for certain chromatography operations, microtiter plates have greater flexibility and lower cost for developing and optimizing the details of a chromatography operation. Furthermore, because the specialized hardware and experimental protocols necessary for developing a chromatographic separation are readily available from vendors of robotic systems commonly used in high throughput screening,⁶⁰ the microtiter-based systems and methodology generally represent the easiest and most cost-effective approach to chromatography development and optimization. Finally, the fact that experiments using microtiter plates can be performed manually using readily available equipment increases the attractiveness of this method.

Table 7.2. Comparison of High Throughput Methods for the Development of Chromatogra	phic
Separations ⁶⁰	

Attribute	Micro-pipette	Microtiter plates	Micro-columns
Binding capacities	Dynamic	Static	Dynamic
Automation	Easy	Difficult	Easy
Flexibility	High	Very High	High
Cost	High	Very Low	Very High

The Application of High Throughput Technologies in Downstream Processing

Development and Optimization of a Monoclonal Antibody Purification Process

Many examples of successful application of high throughput techniques in downstream process development have been reported in the literature. These include investigations of a second step in a monoclonal antibody purification process by cation exchange,61 weak partitioning,⁶² multimodal anion exchange,⁶³ or HIC chromatography,⁶⁴ screening of purification conditions,⁶⁵ characterization of a multi-component adsorption system,⁶⁶ estimation of dynamic binding capacities,⁶⁷ and purification of virus like particles.⁶⁸

In addition to the development of monoclonal antibody chromatographic processes^{69, 70, 71, 72} high throughput techniques have been coupled to high throughput analytics^{73, 74} or used to develop non-chromatographic separations such as two-phase aqueous systems,⁷⁵ and in devising and optimizing target formulations.⁷⁰ The use of high throughput screening and development tools represent a key technology platform for rapid development of monoclonal antibody purification processes and are essential for efficiently applying the principles of QBD to downstream processing.

A comprehensive example of the use of microtiter plate high throughput method for the development of a monoclonal antibody purification process has recently been reported by Lacki et al.⁷⁶ One major objective of this study was the reduction in high molecular weight aggregate content in the final monoclonal antibody product from 15% in the bioreactor harvest used as starting material for purification to less than 1% in the final purified monoclonal antibody while still achieving high overall yields. This study included the testing of nine different chromatography resins and the evaluation of over 1,000 different operating conditions. All of the initial screening and optimization experiments were completed in 63 working days by one scientist, demonstrating the efficiency of the high throughput methodology.

For the initial capture step, the relationship between residence time and dynamic binding capacity was determined from uptake curves describing the rates of adsorption of the monoclonal antibody to various media under different conditions using 6 µL of chromatography media per well.⁷⁶ A characteristic time constant for the adsorption step was then determined by fitting a mass transfer model to the data. The model was then adjusted to describe the same adsorption step as it would occur in a chromatography column and the effect of residence time on dynamic binding capacity was estimated. These dynamic binding capacities were then verified in several actual column chromatography runs. The efficiency of different elution buffers was then investigated using microtiter plates with 20 µL of resin per well where it was found that the salt concentration of the elution buffer had no effect on either the overall step yield or monomer content of the recovered monoclonal antibody. However, the overall yield and monomer content of the recovered monoclonal antibody were affected by changes in the pH of the elution buffer. Decreases in pH increased the step yield up to 100% but also resulted in an increase in aggregate content.

Following optimization of the initial Protein A affinity

capture step, Capto adhere media (GE Healthcare Biosciences, Uppsala, Sweden) was evaluated for removing aggregates still present in the elution pool from the Protein A affinity column. Capto adhere PreDictor plates with 6 µL resin per well were used in this study. Initially, a 0.2 mL aliquot of pH and salt adjusted Protein A affinity column elution pool was added to each well and binding capacities for monoclonal antibody monomer and aggregates were determined from the difference in aggregate concentration before and after incubation for 60 minutes to mimic a flowthrough chromatography operation. In all cases examined, the binding capacity for monoclonal antibody aggregates was always lower than binding capacity for monomer. From an analysis of the binding capacity data, it was determined that the desired values of monoclonal antibody yield and purity could not be achieved under any of the tested conditions. For instance, a purity of >99% could be obtained in some cases but at a yield of only 65%. In order to increase the yield while maintaining acceptable purity, the selective elution of bound monomer was next investigated. In this experiment, a plate containing 20 µL resin per well was used. The resin in each well was incubated with elution pool from the Protein A affinity column at the conditions where the highest purity for the flow through step was found (pH 7 and 50 mM NaCl), and, after the incubation and removal of unbound sample, the effect of salt type, salt concentration, and buffer pH on the efficiency of selective elution of monomer was investigated. The data obtained clearly showed that it was possible to increase the overall step yield and to keep the desired purity of the monoclonal antibody if the elution conditions were properly optimized. Using the optimal elution conditions determined from the microtiter plate experiment, a column run was performed which resulted in a step yield of 87% and a purified monoclonal antibody product containing only 0.5% aggregates.

The level of process understanding and optimization achieved in such a short time in this study was only possible through the use of high throughput methods for development and optimization of each chromatography step.

Optimization of Cleaning Protocols for a Protein A Affinity Column

High throughput screening techniques have been used to study the effect of various clean-in-place (CIP) conditions on the extent of product carry-over in a Protein A affinity chromatography column.⁷⁷ For this study, CIP conditions were investigated using MabSelect SuRe and MabSelect affinity media (GE Healthcare Biosciences, Uppsala, Sweden, 50 μ L media/well, 1 plate per media) artificially fouled by incubation with E. coli lysate spiked with polyclonal IgG, followed by incubation in 2.9 M ammonium sulphate in 0.6 M phosphoric acid, pH 2.5. A variety of potential CIP agents were evaluated for cleaning efficiency by incubating the resins with the CIP agents for 15 minutes. After incubation, samples of the media were removed from the wells and the extent of fouling proteins still bound to the media was determined.

The effect of CIP solutions on the binding capacity of MabSelect and MabSelect SuRe was also determined by monitoring the change in binding capacity before and after exposure to the cleaning solution in PreDictor plates containing 6 µL of either MabSelect or MabSelect SuRe per well. By storing the media in CIP solutions for 18 hr, conditions equivalent to the total CIP exposure time during 180 cycles on a chromatography column cleaned with a 30 min CIP/Sanitization-in-place every fifth cycle, were mimicked and the IgG binding capacities of each media sample was measured and compared with the reference capacities measured on untreated resins. These results showed that the alkali stabilized affinity media, MabSelect SuRe, maintained the IgG binding capacity at higher sodium hydroxide concentrations compared to the recombinant Protein A affinity media, MabSelect.

6. Separation Media as Raw Materials

Most of the materials used in a recovery and purification process for a monoclonal antibody product are simple chemicals such as acids, bases, salts, buffers, detergents, etc. However, the separation media (chromatography media and filtration membranes) used in the process are also considered raw materials, which must be properly qualified and controlled.

One complicating factor is that the chemical composition of separation media can be very complex, involving polymers, inorganic materials, complex organic coupling agents and/or ligands which may be synthetic or themselves be the product of a complex biotech production and purification process. The complete chemical composition and critical quality attributes for these complex materials is often proprietary to the vendor, but may have substantial effects on the monoclonal antibody product and production process.

One solution to this dilemma developed by media vendors has been the use of a Drug Master File (DMF) to provide critical and proprietary information regarding their product composition, manufacturing process, quality control, etc. in a separate regulatory submission kept on file with the regulatory agencies. The producer companies are able to reference the vendor's drug master file in their own submission documents, so that the regulatory body can find the required information without it being made public. Since this approach is not available in Europe, other regulatory support mechanisms must be used for European submissions. These include vendor regulatory support files, which can be provided by vendors to producer companies, typically under a confidentiality agreement. Regulatory support files can also be used to support the use of raw materials in the US in lieu of a DMF.

A second challenge for separation media is that they are generally single-source materials. In order to deal with this concern, some biopharmaceutical manufacturers are beginning to qualify several different chromatography media and filtration membranes used in critical steps to establish secondary sources of supply.

With the increased use of single-use technologies described throughout this chapter and book, another emerging challenge is the management of these single-use components and materials from a supply-chain and quality assurance perspective. One aspect of this challenge is ensuring that the impact of leachates from any single-use component or material do not adversely impact the quality of the biopharmaceutical product being purified. This assurance involves two major components: first, the profile of leachables and extractables from any single-use component must be understood, and second, the risk of the leachates from any component to adversely impact the product quality of the biopharmaceutical drug substance must be assessed under the actual conditions of use for that component (i.e., solution conditions, time of exposure/contact, temperature, etc.). In some cases, it may be sufficient to use extractables and leachables data supplied by the vendor combined with a risk assessment demonstrating that, even under worst case conditions, any leachates from the component would be cleared from the

biopharmaceutical product to levels below concern. In other cases, more detailed and intensive studies (i.e., extractable and leachable studies under worst-case process conditions) may need to be conducted along with a risk assessment to verify the acceptability of a particular single-use component or material in a biopharmaceutical process.

Finally, it is an important responsibility for the user of a particular separation media product to verify that the vendor has appropriate controls on their own raw materials, manufacturing process and product quality, and that there is a system in place to insure complete traceability of the media products in the event of a problem. Quality assurance of suppliers of materials and components is a critical element of biopharmaceutical manufacturers' quality systems. This is often accomplished by a quality assurance audit of the media vendor's operation. Materials and components should only be specified from suppliers that are familiar with the requirements of biopharmaceutical production and can demonstrate compliance with them.

7. Scale-up of Downstream Processes

In order to meet requirements for clinical and commercial product demand, all manufacturing processes require scaleup after development. Ideally, scale-up of the production process should be done on a fully developed and optimized purification process. However, scale-up and process optimization are often performed concurrently or iteratively, in which a process is scaled-up to an intermediate scale, further optimized, and then scaled-up again. The challenge of process scale-up is that there are often unavoidable variations between different scales. Surface area per unit volume decreases with increasing equipment size, so larger equipment should result in fewer product quality problems related to any interfacial phenomena, such as non-specific adsorption and/or surface induced precipitation. Effects of system dead volume will also generally be reduced at larger scales.

The scale at which the final production process will be operated must to be defined before scaleup calculations or studies are done. The final scale will depend on expected market demand which, together with the cell culture technology employed, will dictate the number of batches needed per year. With the mass per batch defined, it is a fairly simple task to scale all unit operations in the manufacturing process, as their size can be calculated taking into account mass produced in a single batch, cycling philosophy for a given unit operation, and yield of preceding steps.

The number of cycles per batch involves a trade-off between equipment size and processing time. The smaller the equipment the more cycles will be needed to process the mass to be produced in a single batch. However, since the cycle time is largely independent of the scale of operation, more cycles will take longer to complete. Therefore, facility constraints such as shift patterns and process scheduling need to be considered in order to make sure that the total time for all the cycles does not exceed the allocated process time. Similarly, facility constraints related to the size of available equipment must be evaluated, as the size of this equipment will determine the minimum number of cycles that will be needed to process an entire batch of product. If the minimum number of cycles requires more time than is allocated for the step, then larger equipment will be required.

A typical large-scale process for purification of monoclonal antibodies consists of three main types of unit operations, namely liquid/solid handling, filtration, and chromatography. Liquid handling includes storage, transfer and mixing of process solutions, including product pools, buffers and cleaning solutions. For antibody processes, solid handling refers to removal of biomass, which is typically done by centrifugation. Filtration includes all the procedures where either filters or membranes are used, and can be further divided into normal flow filtration and tangential flow, respectively. Chromatography includes both batch and column separations that are either based on adsorptive separations, molecular size, or both. Membrane chromatography is also included in this category. For scale-up for each unit operation in a manufacturing process, detailed studies should always be performed to develop a thorough understanding of relevant scale-up parameters and to ensure that the process behaves as expected at the final scale. The following guidelines are generally applicable to the scale-up of all monoclonal antibody purification process.

Scale-up of Liquid Handling Operations

Liquid handling operations include the storage of buffers, in-process intermediates, and the bulk drug substance, liquid transfer of buffers to various purification operations or product from one tank to another, and mixing of solutions.

Hold Steps

The simplest approach to scaling up hold tanks, used to store intermediate product solutions in a downstream process, is based on product concentration. By setting a minimum final concentration of a product in a tank at any stage of the process, the maximum operating volume for each tank can be easily calculated. The final size (volume) of a tank will be larger as the operating range of volumes (i.e., "working volume") for typical tanks is around 80-90 % of the total tank volume. Additionally, in order to ensure consistent control of product quality and bioburden, the time and temperature of the hold steps should be maintained during scale-up.

In recent years, use of single-use bags for buffer distributions and intermediate hold has become commonplace and has changed the way processes are designed. It has been shown that significant savings can be realized when replacing a stainless steel tank farm with single-use bags.78 Additionally, as noted above in the Bioburden impurity section, disposable hold tanks can be obtained in presterilized form with sterile connectors for introduction and removal of liquids. Due to limitations associated with bags manufacturing technology, there is a practical limit on the scale at which the single-use bags can be used. Currently, the single-use bags available for manufacturing of biologics range from 10 to 2,500 L in volume. These bags can be used as an alternative to reusable stainless steel tanks in manufacturing processes operated at 500-2,000 L scale. For larger process volumes, several smaller bags can be used to reach larger total volumes (e.g., >6,000 L), but this approach increases the labor requirements for a manufacturing process and increases the overall manufacturing risk.

Mixing and Liquid Transfer

For both mixing and liquid transfer, care must be taken so shear stress is minimized to avoid unnecessary losses due to unfolding or aggregation of the monoclonal antibody product⁷⁹ since shear rates as low as 10,000 s-1 can induce unfolding of monoclonal antibodies.⁸⁰

In the case where a process step requires adjustment of liquid composition which cannot be done through inline adjustments, scale-up must take into account the potential effects of mixing efficiency as variation in local composition within a tank can affect product quality and the overall process yield. For instance, during pH adjustment for low pH viral inactivation after a Protein A affinity chromatography step, a local decrease in pH below the target value can cause irreversible aggregation of an antibody.

From the perspective of mixing efficiency, the criterion for scale-up are similar to those applicable to the scale-up of cell culture processes (see Chapter 6) except that cells are not present in the solution and mixing intensities can be much higher. Ideally, a successful scale up would maintain the same mixing time at the pilot and full scale. However, keeping blend time constant is rarely practical with any significant scale change because the power inputs necessary to achieve this are often not practical, nor necessary. Scale-up of mixing can be simplified by maintaining the same ratio of each tank dimension. In this case, the only variables that must be evaluated during scale-up are size and rotational speed of the impeller.

Of importance throughout a monoclonal antibody manufacturing process is the accurate and correct preparation, mixing, and delivery of buffer solutions. At the laboratory and pilot scale, buffers are usually prepared at the strength in which they will be used and delivered directly to the process step without further adjustment. At large scale, however, the use of buffer concentrates and inline dilution of solutions as they are delivered to their point of use can reduce the number and size of buffer holding tanks required in a manufacturing facility. Studies have shown that the use buffer concentrates and in-line dilution through static mixers can reduce tank sizes two-fold, reduce the number of buffers that must be prepared by 30%, lower clean-in-place requirements 30% and reduce labor required for buffer preparation operations by 31%.^{81,82}

Scale-up of Filtration Operations

Filtration is one of the most frequently used unit operations in biopharmaceutical manufacturing and is used for clarifying bioreactor harvests (depth filtration and microfiltration), concentrating product solutions (ultrafiltration), buffer exchange (diafiltration), sterilization and virus reduction. From the scale-up perspective, filtration can be divided into normal flow filtration and tangential flow filtration (sometimes referred to as cross flow filtration).

Normal Flow Filtration

In normal flow filtration applications, the filtration operation can be either flux or capacity limited.83 The flux through the filter depends on filter permeability, which in turn is dependent on pore size distribution and filter thickness, and on liquid properties such as viscosity and density, which in turn will depend on process temperature. The capacity of a particular filter is related to the rate of fouling of the filter, which depends on the composition of the sample as well as on the process conditions. The fouling increases pressure over the filter if the filtration process is operated at a constant filtrate flux, and causes a decrease in filtrate flow rate if the filtration is performed at a constant pressure.

Sizing of a normal flow filter will depend on the relative amount of filter area required to process all liquid if there was no fouling of the filtration media so that the process is flux limited (A_0) and the minimum filter area required to assure that the entire process volume can be filtered before the filter becomes impermeable (Amin). When A0 is much greater than Amin the filtration time has a significant effect on filter size. However, when A_0 is much less than Amin reducing flow rate will not significantly reduce filter size. For all other combinations of Amin and A_0 both capacity and operating pressure will influence the optimal filter size.

Scale-up in an application where the filtration is flux limited is fairly simple since assumption that filter performance scales linearly with filtration area is typically correct and the filter is sized based on the total volume to be processed and the processing time available for the filtration step.

In case of the capacity limited filtration, there are different methods for determining the filter capacity, which differ in terms of time and sample required and reliability of scale-up information.^{81, 84} Regardless of the method used to find out filter capacity, the scale-up is accomplished by assuming that between 50 to 80% of filter capacity scales linearly with the filter area. Based on this assumption, a minimum filtration area necessary to accomplish a given filtration task within the time specified when operating at a given constant pressure can be calculated. With the minimum filtration area known, the final size of the filtration unit is determined by applying a safety factor, typically of 1.5, to account for feed and membrane variability. Larger safety factors can be applied if a more variable feed stream, such as harvested cell

culture fluid is used. Since the normal flow filters are usually available in finite size cartridges, the final sizing of a filtration step must account for available cartridge configuration including the filter housing aspect.

In the case of scale-up of virus filters, the same basic scale-up strategy as for sterile filtration is applied, but it is recommended to extend the capacity study beyond the expected manufacturing scale by a factor of 1.5 to 2 to deal with feed stream and membrane lot to lot variability.⁸⁴

Tangential Flow Filtration

Typical tangential flow filtration applications include ultrafiltration and diafiltration (UF/DF) processes for concentration and buffer exchange as well as microfiltration. As with normal flow filtration, the size of a TFF filter will depend on the filter capacity defined as the volume of feed that can be processed per unit membrane area before the membrane fouls and must be replaced or regenerated. Depending on whether the filtration is operated at constant flux or constant pressure conditions, this volume will be linked to a moment at which the pressure drop in the system reaches maximum value or the permeate flow rate drops to an unacceptable level, respectively. For the latter, the permeate flux at approximately 80% of the maximum flux is generally selected for process operation.⁸⁵

Scale-up of TFF steps is simplified when the membrane

cartridges selected (cassettes or hollow-fibres) are linearly scalable. This linear scalability is achieved by the geometrical similarity of the membrane cartridges at different scales provided that the channel length is held constant and, the same hydrodynamic regime within the channels is maintained. In combination, these two factors guarantee that trans-membrane pressure, local flux, pressure drop across the channel and protein concentration at the membrane wall are as close as possible at all scales of operation.⁸⁶

Sizing of a UF/DF system to process a specific volume within a given processing time to reach a desired concentration factor and a final composition is performed based on average fluxes measured at process conditions. With the fluxes known, the minimum filter area for a UF/DF step for different processing times can be quickly estimated following the procedures outlined in Figure 7.3. For a given average flux, a ratio between the volume to be processed and the average flux is calculated. The intersection between a desired process time, (e.g., ultrafiltration time, tUF), and a line representing the calculated ratio is found. The ordinate of the intersect point gives the minimum membrane area necessary to process the volume in the desired time. A reverse procedure is then used to calculate diafiltration time, that is the intersection between the line representing membrane area and the line representing a ratio between total volume of diafiltration buffer is found and the abscissa the intersect point gives the duration of the diafiltration operation.

Figure 7.3. Effect of Processing Time on Membrane Area for a UF/DF Process

The solution volume to be processed (V_{proc}) and the average flux measured under normal process conditions (Javg) can be used to determine the minimum filter area requirements for a UF/DF system. Based on these data, the minimum ultrafiltration membrane area can be calculated for a given processing time. Conversely, the same parameters can be used to estimate the processing time for the unit operation for a given filter area. (Figure courtesy of GE Healthcare).



With the minimum membrane area calculated for a given UF/DF operation, and subsequently adjusted to account for process variability, the cartridge configuration necessary to accomplish the filtration task can be found.

Scale-up of Chromatography

Sizing of a chromatography operation requires consideration of both chromatographic and non-chromatographic factors but can be accomplished by following fairly simple guidelines. The simplest approach to the scale-up of a chromatography process is direct or linear scale-up in which the column bed height, product residence time on the column, sample concentration, and the ratio of gradient volume to column volume remain constant at all scales. In such a scale-up, the sample load, volumetric flow rate, and column cross-sectional area are increased by the same scaling factor.⁸⁷ Direct linear scale-up of a chromatography process is outlined in Figure 7.4.

Figure 7.4. Principle of Linear Scale-up of a Chromatography Column

The basic principles of linear scale up for chromatography columns include maintenance of bed height, loading ratio, and flow velocity. Various approaches to implementation of these principles are illustrated in this figure. The scale factor (SF) for the columns needed in scale up depends on the strategy employed. (Figure courtesy of GE Healthcare.)



As illustrated in in Figure 7.4, linear scale up of a chromatography column can be accomplished by either proportionately scaling a column to a single column of appropriate dimensions or by scaling to multiple columns of intermediate size, which will be operated in parallel. The guidelines for linear scale-up of a chromatography column are summarized in Table 7.3.

Table 7.3. Guidelines for Linear Scale-up ofChromatography

Maintain	 Bed height Eluent velocity Sample concentration Gradient slope/bed volume
Increase	 Column diameter Volumetric flow rate in proportion to column volume Sample volume in proportion to column volume Gradient volume in proportion to column volume
Check	 Reduction in wall support (increased pressure drop) Sample distribution Piping and system dead volumes

Although direct scale-up of a chromatography operation is relatively simple, non-chromatographic factors that can impact the success of the scale-up should also be considered. One drawback of the direct scale-up approach is the availability of suitable hardware to match the scaleup requirements. Since chromatography columns are available only in discrete dimensions, the appropriately sized column may not be available to meet the necessary column geometry to achieve proper scale-up. Typically production columns diameters are available with diameters 20 to 200 cm; larger diameter columns must be special ordered. Column heights of 10 to 50 cm, depending on the column type, are commonly available but columns with bed heights up to 100 cm are also available. This range of bed heights in combination with the wide range of column diameters gives flexibility in obtaining the desired column volume. However, this flexibility can only be exploited if scale up is performed based on a different criterion than the linear scale up, or if process development is done using bed height representative for the final large scale process. If the linear

scale up is employed columns are typically slightly oversized or multiple columns are used.

While direct or linear scale-up is the most commonly used method for scale-up of chromatography operations, an alternative scale-up method known as constant residence time can also be used. Constant residence time scale-up is based on the concept of maintaining constant product residence time on the column during scale-up. Residence time is defined as the ratio of column height, or column volume, to liquid velocity, or flow rate. With constant residence time scale-up it is possible to scale-up a chromatographic process at constant productivity while changing both the bed height and the column diameter, allowing more flexibility in choosing the right hardware for large-scale manufacturing.⁸⁸

Constant residence time scale-up is often used during initial development of chromatography protocols. In the majority of cases, the constant residence time scale-up criterion will hold for heavily loaded columns, gradient and isocratic elution.⁸⁹ For a typical monoclonal antibody purification process, constant residence time scale-up is applicable, especially for bind elute steps such as Protein A affinity chromatography, cation exchange, or HIC steps. However, in case of convection-governed adsorption where the slowest mass transfer step depends on the local velocity, the separation may not be the same if the column height is varied even when data for identical residence times are compared. For instance, clearance of critical impurities during a chromatography step operated in flow through mode may require performing both the optimization and the scale-up by applying the constant bed height and the constant velocity criterion, i.e., direct or linear scale-up. This is especially true when very large impurities such as DNA, viruses and certain host cell proteins are present in the product stream. Since these large molecules cannot access intraparticle pores of commonly used chromatography resins, the overall rate of their adsorption onto the surface of these resins will be more or less all dependent on the local liquid velocity in the proximity of the surface. Therefore, in the case of separations where large molecules are to be adsorbed, in order to keep the same separation performance at different scales, the liquid velocity and the residence time should be kept constant. In these cases, the constant bed height criterion is more appropriate.

As described above, the rules of linear scale-up - increasing column cross-sectional area in proportion to the process volume (i.e., keeping the bed height constant) - should be enough for a successful scale-up. In practice however, an increase in column diameter above 30 cm will lead to a decrease in column wall support for the resin. Depending on the resin type, this effect may result in a need to decrease the highest possible flow rate for a chromatographic step established at smaller scales in order to minimize bed compression and all chromatographic effects associated with it. The effect of wall support on compressibility of a packed bed have been investigated both by academic groups^{90, 91, 92} and by industry.93,94 A simple yet very useful approach looks at the effect of aspect ratio (column length to column diameter) on the maximum operating velocity in a chromatography column.89 Using this tool, the effect of column wall support on column performance at the final scale can be investigated at smaller scales and potential corrective actions can be taken.

Regardless of the scale-up method chosen for a particular chromatography operation, the use of modeling can be effective in understanding the impact of different process parameters on scale-up and the best means of optimizing and scaling up a chromatography operation. With a proper model, sensitivity analyses can be performed that all the identification of critical process parameters and their impact on the separation at different scales. For instance, model simulations can be used to predict the effect of column packing quality and residence time on the separation of monomer and aggregate species.95 However, it is important to remember that, just as each separation task is different, the results of any model must be verified before it can be used for scale-up by comparing the model's predictions against the results of small-scale column experiments.

8. Planning for Process Changes

The potential impact of changes in the recovery and purification process for a monoclonal antibody product should always be considered as the product moves through human clinical trials and the manufacturing process is refined, optimized, and scaled-up. As discussed further in Chapter 10, there is now a clear regulatory path for evaluating comparability of products manufactured by different processes and for the implementation of process changes as a product moves through development and commercialization. Process changes for monoclonal antibody products are the inevitable result of the tradeoff between the time required to complete full process development and optimization of the downstream process and the pressure to initiate first in human clinical trials. Often it may be prudent to wait until after Phase 2 trials are completed to complete final process optimization so that there is some certainty of the product's clinical efficacy. The platform approach to purification development described in Section 4 of this chapter can help minimize the risk that optimization of the downstream process later in development will adversely impact the critical quality attributes of the product leading to increased requirement for additional clinical trials, which is why many companies have now developed downstream process platforms.

The use of design space, as described in Chapter 3, can be highly advantageous in managing changes to or changes that impact downstream processing. Process design space describes the multi-dimensional range that a process can operate in to ensure final product quality.96 For products approved under a QbD filing, changes made to the process within the design space may not require regulatory approval or extensive validation. Knowledge gained from similar platforms can be applied to the concept of process design space as well, which can facilitate ongoing improvements.

Under pressure to move a new monoclonal antibody product candidate as rapidly as possible into human clinical trials, companies often initiate downstream process development prior to full optimization of the upstream cell culture process. As the cell culture process is optimized, the cell density and product titer in the production bioreactor increase, often resulting in an increase in the amount of cell lysis and host cell protein impurities found in the bioreactor harvest. In addition, changes in media composition may significantly impact impurity profiles as well as performance of the downstream process steps. Finally, the level of high molecular weight aggregates can often dramatically increase at high monoclonal antibody titers, sometimes to levels which may be difficult to reduce to acceptable levels. Therefore, downstream process development must plan for continual improvements and changes in the upstream process throughout product development and the purification process must be continually refined to accommodate these changes.

In addition to the inevitable improvements and changes in the upstream process throughout product development, other changes that occur later in development and that may impact or involve the downstream process include the following:

- Increases in production scale, to support increased product requirement during clinical development and to support sales growth or introduction of new clinical indications for the product following approval
- Transferring the product to one or more different manufacturing facilities
- Changes in availability or quality of raw materials or media

• Responses to problems that arise in routine production When evaluating process changes in the downstream process for a monoclonal antibody product, the interrelationship between various process parameters and unit operations must always be considered. For example, the pH and ionic strength of the equilibration buffer of a chromatography column may have opposite effects on the capacity and separation performance of that chromatography step. To this end, Quality Risk Management may be employed as part of a comprehensive change management program. Iterative, phase appropriate risk assessments should be used to assess and control any changes that impact downstream processing and to determine appropriate risk mitigation.97 These interactions should be carefully studied in statistically designed experimental matrices to define each process parameter within the context of the other parameters for a specific unit operation. The process risk assessment and statistical design of experiment characterization studies, discussed in Chapter 3, are essential tools in evaluating performance parameters and unit operations.

9. The Future of Downstream Processing

A number of interesting technical and engineering developments are being made in biopharmaceutical manufacturing that may affect the design of recovery and purification processes in the near to long term.

Advancements in Chromatography Media and Other Adsorbers

Chromatography media and filtration membrane devices used for protein purification have been in use for decades and are generally well-refined products. However, advancements continue to be made on a number of fronts, which may result in more efficient production of antibody products.

Chromatography media manufacturers continually work to develop improved products. Specifically, advancements have been made in increased binding capacity, better resistance to higher pressure and better mass transport kinetics allowing for faster flow rates and, therefore, reduced process cycle time, and improved chemical stability and durability, particularly toward harsh cleaning conditions.21 These improvements tend to come in waves, particularly because vendors must continue to support older chromatography products used in the manufacture of previously developed monoclonal antibody products.

Another area of product development is in different surface chemistries and selectivity. In recent years, a wide range of different approaches has been developed for either antibody capture ("Protein A alternatives") or for more selective intermediate purification steps. Many of these new approaches involved so-called mixed mode ligands, combining ionic, hydrophobic and other binding mechanisms in ligands that can impart substantial selectivity gains over single mode media. Another approach involves highly selective protein or peptide ligands, developed using refined screening and selection technologies and manufactured using synthetic or recombinant processes.

As mentioned above, membrane-base anion exchange cartridges have recently been introduced for highly selective and rapid flow-through polishing.^{98,99} These devices also work well in single-use manufacturing approaches that are becoming more widely adopted by manufacturers. As described above, membrane adsorber technology is still advancing, and newer, high capacity membranes as well as porous "monolith" media continue to be introduced to the market.

Filtration devices are also undergoing continual evolution. Improved designs can produce significant increases in flux (flow rate per unit membrane area), resulting in more economical operation.

Continuous Processing and Other Alternative Manufacturing Approaches

In addition to new chromatography media and singleuse bioprocessing systems, there are several processengineering approaches under development that may impact biopharmaceutical manufacturing in the relatively near future. These include expanded or fluidized beds as alternatives to traditional fixed bed chromatography columns100 and the use of multiple columns in a continuous or semi-continuous chromatography operation.^{101, 102}

The advantage to expanded bed chromatography is that a monoclonal antibody product can be directly captured from the bioreactor product feedstream, effectively combining the removal of cells and cell debris from the bioreactor harvest with the initial capture chromatography step into a single unit operation. Expanded bed technology uses chromatography media with a dense inorganic core and relatively large particle size so that when a feedstream is pumped upward through the bed at a sufficiently high flow rate, the bed will expand or fluidize, allowing both liquid and particles such as cells or debris to flow freely around the media particles and through the bed without plugging. Once the chromatography media has been loaded to an acceptable level, it may be washed, eluted, cleaned and re-equilibrated similarly to a conventional column. This approach has been used for many years in other industries (including food/dairy and antibiotic production), but the complexity and challenges involved have so far prevented widespread use in the production of biopharmaceuticals. However, with the rapid growth of monoclonal antibody production, an increasing emphasis on efficiency and economics, and the adoption of platform approaches for monoclonal antibody purification, a number of vendors are developing and launching new products in this field.

The use of multiple, relatively small chromatography columns in a continuous or semi-continuous manner can significantly increase the efficiency of any chromatography media and improve buffer utilization by breaking a single large chromatography column into several smaller columns (typically 312), connected by a system of valves and plumbing designed to enable different flow streams to be introduced into and collected from each column at different times in an operational cycle. Normally several columns are connected in series during the loading phase of such a process so that the first column in the series is fully saturated with product (since any product that leaks through will be collected by the columns downstream), resulting typically in a 3060% reduction in the amount of expensive media and buffers required for a given throughput¹⁰³. Following loading, each individual column is washed, eluted, and cleaned similarly to a single column operation. Once an individual column has completed a cycle of loading, washing, eluting, and cleaning, the column is again loaded with the column feedstream and the process repeats itself. Like expanded bed chromatography, this multicolumn approach, sometimes called simulated moving bed or SMB chromatography, has been used for decades in the food, chemical, and wastewater treatment industries at the multi-ton scale and has proven to be very reliable and robust. Recently several vendors have begun to produce systems scaled and designed for use in production of biopharmaceuticals, particularly monoclonal antibodies.^{100, 101}

Non-Chromatographic Techniques

In addition to new technologies based on chromatographic separations, non-chromatographic techniques are also being explored to reduce costs and provide alternatives to conventional purification approaches for biopharmaceutical manufacturing. Among these, precipitation and crystallization techniques continue to be areas of active research and interest.

Research into the use of crystallization in antibody purification is still at an early stage. Recently, Zang et al published a case study showing the development of crystallization conditions for an IgG4 antibody. High yield and purification were obtained when crystallizing from purified solutions at high concentration; however, the ability to apply these conditions to complex and variable cell culture supernatant solutions remains a challenge.¹⁰⁴

The use of precipitation methods is more advanced, with several groups evaluating low pH precipitation in cell culture bioreactors pre-clarification as well as PEG precipitation during downstream processing.¹⁰⁵ Gronke et al presented a case study describing the use of continuous precipitation of antibodies using PEG and zinc chloride in a process that provided a good combination of yield and purification for a high titer antibody product.¹⁰⁶

While non-chromatographic techniques are still early in development, they represent powerful alternative approaches for purification process development that may have viable applications in certain situations.

References

- Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Center for Veterinary Medicine (US). Guidance for industry: process validation: general principles and practices. Revision 1 [Internet]. Washington, DC: Department of Health and Human Services; Jan 2011 [cited 2016 Nov 22]. 22 p. Available from: http://www.fda.gov/downloads/Drugs/Guidances/ UCM070336.pdf.
- World Health Organization (CH). WHO study group on cell substrates for production of biologicals [Internet]. Geneva (CH): World Health Organization; 2007 Jun 11-12 [cited 2016 Nov 21]. 30 p. Available from: www.who.int/biologicals/publications/meetings/ areas/vaccines/cells/Cells.FINAL.MtgRep.IK.26_Sep_07.pdf.
- 3 Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research (US). Guidance for industry: characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications. [Internet]. Washington, DC: Department of Health and Human Services; Feb 2010 [cited 2016 Nov 21]. 50 p. Available from: http://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/vaccines/ucm202439.pdf.
- 4 Castillo FJ, et al. Hybridoma stability. In: Brown F, Lubiniecki AS, editors. Genetic stability and recombinant product consistency. Basel (CH): S. Karger AG; 1994. p. 55-64. (Developments in biological standardization; vol. 83).
- 5 Weidner J. Validation considerations for disposable components and systems. Presented at North Carolina Biotechnology Center, Bioprocessing and Process Development Group. 2004 Nov 18; Research Triangle Park, NC.
- **6** Gagnon P. The Protein A paradigm: can it be improved? Can it be replaced? Presented at International Business Communications Life Sciences' 18th International Antibody Development and Production Conference; 2007 Feb 28-Mar 2; Carlsbad, CA.
- 7 Bee JS, et al. Response of a concentrated monoclonal antibody formulation to high shear. Biotechnol Bioeng. 2009 Aug 1;103(5):936-43.
- 8 Cordoba-Rodriguez RV. Aggregates in MAbs and recombinant therapeutic proteins: a regulatory perspective. BioPharm Int. 2008 Nov;21(11):44-53.
- 9 Rosenberg A. FDA perspective on immunogenicity testing-a risk based analysis. Presented at International Business Communications Life Sciences' Sixth International Well Characterized Biologicals Conference; 2003 May 8-9; Bethesda, MD.
- 10 Salfeld JG. Isotype selection in antibody engineering. Nat Biotechnol. 2007;25(12):1369-72.
- 11 Raju TS. Terminal sugars of Fc glycans influence antibody effector functions of IgGs. Curr Opin Immunol. 2008 Aug;20(4):471-8.
- 12 Berkowitz SA, et al. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. Nat Rev Drug Discov. 2012 Jul;11(7):527-40.
- 13 Li H, et al. Optimization of humanized IgGs in glycoengineered Pichia pastoris. Nat Biotechnol. 2006 Feb;24(2):210-5.
- 14 Weikert S. Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins. Nat Biotechnol. 1999 Nov;17(11):1116-21.
- 15 Wheelwright S. Strategies for process design: definitions and basis for design. In: Protein purification design and scale up of downstream processing. New York (NY): John Wiley & Sons, Inc.; 1993 Nov. p. 10-2.

- 16 Levine HL, et al. Efficient, flexible facilities for the 21st century. BioProcess Int. 2012;10(Suppl 11):20–30.
- 17 Sinclair A, Monge M. Disposable cost contributions: a sensitivity analysis. BioPharm Int. 2009 Apr;22(4):28-32.
- 18 Jones SD, Ransohoff TC. Single-use products for bioproduction-available options for cell culture and downstream processing. Amer Pharm Rev. 2011 May-Jun;14(4):12-20.
- 19 Shukla AA, Kandula JR. Harvest and recovery of monoclonal antibodies from large-scale mammalian cell culture. BioPharm Int. 2008 May;21(5):34-45.
- 20 Russell E, et al. Harvest of a therapeutic protein product from high cell density fermentation broths: principles and case study. In Shukla, AA, et al., editors. Process scale bioseparations for the biopharmaceutical industry. London (GB): CRC, Taylor & Francis; 2007. p. 1-58. (Biotechnology and bioprocessing series; vol. 31).
- 21 Riske FJ, et al. The use of chitosan as a flocculant in mammalian cell culture dramatically improves clarification throughput without adversely impacting monoclonal antibody recovery. J Biotech. 2007 Mar:128(4):813-23.
- 22 Curling J, Gottschalk U. Process chromatography: five decades of innovation. BioPharm Int. 2007 Oct;20(10):70-94.
- 23 GE Healthcare. MabSelect SuRe Data file 11-0011-65 AC [Internet]. Buckinghamshire (UK): GE Healthcare UK Ltd; 2011 Jun [cited 2016 Nov 22]. 6 p. Available from: https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1341321346285/litdoc11001165_20161013190615.pdf.
- 24 Trexler-Schmidt M, et al. Purification strategies to process 5 g/L of monoclonal antibodies. BioPharm Int. 2009 Mar;22(Suppl):8-15.
- 25 Farid SS. Process economics of industrial monoclonal antibody manufacture. J Chromatogr B Analyt Technol Biomed Sci. 2007 Mar;848(1):8–18.
- 26 Kelley B. Industrialization of mAb production technology-The bioprocessing industry at a crossroads. mAbs 2009 Sep-Oct;1(5):443-52.
- 27 Sofer G, Chirica LC. Downstream processing: improving productivity in downstream processing. BioPharm Int. 2006 Nov;19(11):48-55.
- 28 Jones SCB, Smith MP. Evaluation of an alkali stable Protein A matrix versus Protein A Sepharose Fast Flow and considerations on process scaleup to 20,000L. Presented at the Third International Symposium on Downstream Processing of Genetically Engineered Antibodies and Related Molecules; 2004 Oct 3-5; Nice, France.
- 29 Hubscher T. Making the leap from discrete resin lifetime validation protocols to continuous process verification. Presented at IBC Life Sciences' Biopharmaceutical Development and Production Conference; 2013 Feb 26-Mar 1; Huntington Beach, CA.
- **30** Korneyeva M, et al. Enveloped virus inactivation by caprylate: a robust alternative to solvent-detergent treatment in plasma derived intermediates. Biologicals. 2002 Jun;30(2):153-62.
- 31 Kim IS, et al. Dry-heat treatment process for enhancing viral safety of an antihemophilic factor VIII concentrate prepared from human plasma. J Microbiol Biotechnol. 2008 May;18(5):997-1003.
- **32** Sauerbrei A, Wutzler P. Testing thermal resistance of viruses. Arch Virol. 2009;154(1):115-9.

- 33 Grieb T, et al. Effective use of gamma irradiation for pathogen inactivation of monoclonal antibody preparations. Biologicals. 2002 Sep;30(3):207-16.
- 34 Schleh M, et al. Susceptibility of mouse minute virus to inactivation by heat in two cell culture media types. Biotechnol Prog. 2009 May-Jun;25(3):854-60.
- 35 Bailey MR, et al. Evaluation of microfluidics reactor technology on the kinetics of virus inactivation. Biotechnol Bioeng. 2008 Apr 15;99(6):1384-91.
- 36 Gagnon P. Polishing methods for monoclonal IgG purification. In: Shukla A, Etzel M, Gadam S, editors. Process scale bioseparations for the biopharmaceutical industry. Boca Raton (FL): Taylor and Francis Group, CRC Press; 2006. p. 491-505.
- 37 Ransohoff T. The use of disposable technologies in biopharmaceutical purification processes. Presented at the 2012 American Association of Pharmaceutical Scientists Annual Meeting and Exposition; 2012 Oct 14-18; Chicago, IL.
- 38 Lacki KM, et al. Development and scale-up of a downstream process for a monoclonal antibody: a case study. Presented at the Society for Biological Engineering's Second International Conference on Accelerating Biopharmaceutical Development; 2009 Mar 9-12; Coronado, CA.
- **39** Ransohoff T. Advances in disposable-format downstream processing technologies. Presented at International Business Communications BioProcess International Conference and Exhibition Pre-Conference Workshops; 2008 Sep 22; Anaheim, CA.
- 40 Robinson J. Continuous ion exchange for influenza vaccine recovery. Presented at International Business Communications BioProcess International Conference; 2009 Oct 14; Raleigh, NC.
- 41 Kelley B, et al. Weak partitioning chromatography for anion exchange purification of monoclonal antibodies. Biotechnol Bioeng. 2008 Oct;101(3):553-66.
- 42 Johansson HJ. Protein A and multi-modal chromatography, a two-step process for purification of monoclonal antibodies. Presented at the American Chemical Society's 229th National Meeting; 2005 Mar 13-17; San Diego, CA.
- **43** Gagnon P. Dissection of the separation mechanisms and enhancement of aggregate removal by charged-hydrophobic mixed-mode chromatography. Presented at the Sixth International Symposium on HIC and RPC; 2009 Mar 16-19; Napa, CA.
- 44 Boschetti E. Antibody separation by hydrophobic charge induction chromatography. Trends Biotechnol. 2002 Aug;20(8):333-7.
- 45 Gagnon P. Purification tools for monoclonal antibodies. Tucson (AZ): Validated Biosystems, Inc; 1996. Chapter 5, Hydroxyapatite chromatography; p. 87-100.
- **46** Bio-Rad Laboratories. CHT ceramic hydroxyapatite, instruction manual [Internet]. Hercules (CA): Bio-Rad Laboratories; [cited 2016 Nov 22]. 15 p. Available from: http://www.bio-rad.com/cmc_upload/0/000/039/227/Lit-611d.pdf.
- 47 Glynn J, et al. The development and application of a monoclonal antibody purification platform. BioPharm Int. 2009 Mar;22(Suppl):16-20.
- 48 Cheryan M. Ultrafiltration and microfiltration handbook. Lancaster (PA): Technomic Publishing Co; 1998. 527 p.
- 49 Shukla AA, et al. Downstream processing of monoclonal antibodies application of platform approaches. J Chromatogr B Analyt Technol Biomed Sci. 2007 Mar;848(1):28-39.

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- 50 Low D, et al. Future of antibody purification. J Chromatogr B Analyt Technol Biomed Sci. 2007 Mar;848(1):48-63.
- 51 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Development and manufacture of drug substances Q11 Step 4 [Internet]. Geneva (CH): International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2012 May; [cited 2016 Nov 22]. 30 p. Available from: http://www.ich. org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q11/Q11_Step_4.pdf.
- 52 Arunakumari A, et al. Advances in non-Protein A purification processes for human monoclonal antibodies. BioPharm Int. 2009 Mar;22(Suppl):22-6.
- 53 Ferreira GM, et al. Chromatography: A two-column process to purify antibodies without Protein A. BioPharm Int. 2007 May;20(5):32-43.
- 54 Gagnon P, et al. Nonspecific interactions of chromatin with immunoglobulin G and protein A, and their impact on purification performance. J Chromatogr A. 2014 May 2;1340:68-78.
- 55 Lee J, et al. Principles and applications of steric exclusion chromatography. J Chromatogr A. 2012 Dec 28;1270:162-70.
- 56 Nian R, et al. Void exclusion of antibodies by grafted-ligand porous particle anion exchangers. J Chromatogr A. 2013 Mar 22;1282:127-32.
- 57 Lacki KM, et al. High throughput process development: determination of dynamic binding capacity using a microtiter plates filled with chromatography resins. Presented at the American Chemical Society's 234th National Meeting; 2007 Aug 19-23; Boston, MA.
- 58 Coffman JL, et al. High-throughput screening of chromatographic separations: I. Method development and column modeling. Biotechnol Bioeng. 2008 Jul 1;100(4):605-18.
- 59 Chhatre S, Titchener-Hooker NJ. Predicting process scale chromatographic performance using micro scale-down devices. Presented at University College of London's Innovative Manufacturing Research Center, steering group meeting; 2008 Sep 17; London, United Kingdom.
- 60 Tecan [Internet]. Mannedorf (CH): Tecan Trading, AG; c2016. Freedom Evo® Overview; c2016 [cited 2016 Nov 22]; [about 3 screens]. Available from: http://lifesciences.tecan.com/products/liquid_handling_and_automation/freedom_evo_series?ads_cmpid=141213619&ads_adid=8163541939&ads_matchtype=p&ads_network=g&ads_creative=151738277219&utm_term=freedom%20evo&ads_targetid=kwd-12698639929&utm_campaign=&utm_source=adwords&utm_medium=ppc&ttv=2&gclid=CPzJrqeLvdACFU5XDQodjDsOAA.
- 61 Kelley BD, et al. High-throughput screening of chromatographic separations: IV. Ion-exchange. Biotechnol Bioeng. 2008 Aug 1;100(5):950-63.
- **62** Kelley BD, et al. Weak partitioning chromatography for anion exchange purification of monoclonal antibodies. Biotechnol Bioeng. 2008 Oct 15;101(3):553-66.
- 63 Lacki KM, et al. Accelerated development of a downstream purification process for production of monoclonal antibodies: a case study. Presented at the 13th Recovery of Biological Products Conference; 2008 Jun 22-27; Quebec City, Canada.
- **64** Kramarczyk JF, et al. High-throughput screening of chromatographic separations: II. Hydrophobic interaction. Biotechnol Bioeng. 2008 Jul 1;100(4):707-20.
- 65 Rege K, et al. High-throughput process development for recombinant protein purification. Biotechnol Bioeng. 2006 Mar 5;93(4):618-30.

- 66 Linden T. Untersuchungen zum inneren transport bei der proteinadsorption an porose medien mittels konfokaler laser-rester-mikroskopie [Investigations of internal transport during protein adsorption on porous media using confocal laser scanning microscopy] [dissertation]. Dusseldorf (DE): Heinrich-Heine University Dusseldorf; 2001 Dec. Section 3.7.3, Einkomponentenadsorption monoklonaler antikorper oder BSA [Single component adsorption of monoclonal antibodies or BSA]; p. 87-8. German.
- 67 Bergander T, et al. High-throughput process development: determination of dynamic binding capacity using microtiter filter plates filled with chromatography resin. Biotechnol Prog. 2008 May-Jun;24(3):632-9.
- **68** Wenger MD, et al. An automated microscale chromatographic purification of virus-like particles as a strategy for process development. Biotechnol Appl Biochem. 2007 Jun;47(Pt 2):131-9.
- 69 Bhambure R, et al. High-throughput process development for biopharmaceutical drug substances. Trends Biotechnol. 2011 Mar;29(3):127-35.
- 70 Rodrigo G, Nilsson-Valimaa K. Developing a MAb aggregate removal step by high throughput process development. 2010 Apr;23(4):45-61.
- 71 High-Throughput Process Development Conference. Extended reports from the second international conference on high-throughput process development; Avignon, France, 4-7 June 2012 [Internet]. Buckinghamshire (UK): GE Healthcare UK Ltd; 2012 Nov [cited 2016 Nov 22]. 52 p. Available from: https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1354036544405/ litdoc29034193_20161015172308.pdf.
- 72 Chollangi S, et al. Accelerating purification process development of an early phase MAb with high-throughput automation. Part 2. Bioprocess Int. 2014 Apr;12(4):32-41.
- **73** Chollangi S, et al. Accelerating purification process development of an early phase MAb with high-throughput automation. Part 1. Bioprocess Int. 2014 Mar;12(3):48-52.
- 74 Wierling PS, et al. High-throughput screening of packed-bed chromatography coupled with SELDI-TOF MS analysis: monoclonal antibodies versus host cell protein. Biotechnol Bioeng. 2007 Oct 1;98(2):440-50.
- 75 Oelmeier SA, et al. Application of an aqueous two-phase systems high-throughput screening method to evaluate mAb HCP separation. Biotechnol Bioeng. 2011 Jan;108(1):69-81.
- **76** The Lacki KM, et al. Development and scale-up of a downstream process for a monoclonal antibody: a case study. Presented at the Society for Biological Engineering's Second International Conference on Accelerating Biopharmaceutical Development; 2009 Mar 9-12; Coronado, CA.
- 77 Johansson HJ, et al. Rational development of cleaning-in-place protocols for affinity media. Presented at the American Chemical Society's 236th National Meeting & Exposition; 2008 Aug 17-21; Philadelphia, PA.
- **78** Sinclair A, Monge M. Biomanufacturing for the 21st century: designing a concept facility based on single-use systems. Bioprocess Int. 2004 Oct;2(9):26-32.
- 79 Bee JS, et al. Response of a concentrated monoclonal antibody formulation to high shear. Biotechnol Bioeng. 2009 Aug 1;103(5):936-43.
- **80** Jaspe J, Hagen SJ. Do protein molecules unfold in a simple shear flow? Biophys J. 2006 Nov 1;91(9):3415-24.
- 81 Smith MP. Strategies for the purification of high titre, high volume mammalian cell culture batches. Presented at International Business Communications BioProcess International European Conference and Exhibition; 2005 Apr 11-14 Berlin, Germany.

- 82 Antoniou C. Methods and guidelines for use of buffer concentrates in antibody manufacturing. Presented at the International Business Communications 20th International Antibody Production Conference, 2008 Mar 13; San Diego, CA.
- 83 van Reis R, Zydney A. Bioprocess membrane technology. J Membrane Sci. 2007 Jul;297(1-2):16-50.
- 84 Badmington F, et al. Vmax testing for practical microfiltration train scale-up in biopharmaceutical manufacturing. Pharm Technol. 1995 Sep;19:64-76.
- 85 Daniels C, et al. Linear scale-up of ultrafiltration of high viscosity process streams. In Shukla, AA, et al., editors. Process scale bioseparations for the biopharmaceutical industry. London (GB): CRC, Taylor & Francis; 2007. p. 523-39. (Biotechnology and bioprocessing series; vol. 31).
- **86** van Reis R, et al. Linear scale ultrafiltration. Biotechnol Bioeng. 1997 Sep 5;55(5):737-46.
- 87 Hagel L, et al. Handbook of process chromatography: development, manufacturing, validation and economics. Second ed. Amsterdam (NL): Elsevier Ltd., Academic Press; c2008. 382 p.
- 88 Kidal S, Jensen OE. Using volumetric flow to scale up chromatographic processes. BioPharm Int. 2006 Mar;19(3):34-45.
- 89 Lightfoot EN, et al. Refining the description of protein chromatography. J Chromatogr A. 1997 Feb;760:139-49.
- 90 Stickel JJ, Fotopoulos A. Pressure-flow relationships for packed beds of compressible chromatography media at laboratory and production scale. Biotechnol Prog. 2001 Jul-Aug;17(4):744-51.
- **91** Keener RN, et al. Toward a robust model of packing and scale-up for chromatographic beds. 1. Mechanical compression. Biotechnol Prog. 2004 Jul-Aug;20(4):1146-58.
- 92 Keener RN, et al. Toward a robust model of packing and scale-up for chromatographic beds. 2. Flow packing. Biotechnol Prog. 2004 Jul-Aug;20(4):1159-68.
- **93** Keener RN, et al. Advancement in the modeling of pressure-flow for the guidance of development and scale-up of commercial-scale biopharmaceutical chromatography. J Chromatogr A. 2008 May 9;1190(1-2):127-40.
- 94 McCue JT, et al. Application of a two-dimensional model for predicting the pressure-flow and compression properties during column packing scale-up. J Chromatogr A. 2007 Mar 23;1145(1-2):89-101.
- **95** McCue JT, et al. Modeling the effects of column packing quality and residence time changes on protein monomer/aggregate separation. J Chromatogr A. 2009 Jun 12;1216(24):4895-901.
- 96 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Pharmaceutical Development Q8(R2) Step 4 [Internet]. Geneva (CH): International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2005 Nov 9 [cited 2016 Nov 22]. 28 p. Available from: http://www.ich.org/ fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q8_R1/Step4/ Q8_R2_Guideline.pdf.

- 97 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Quality risk management Q9 Step 4 [Internet]. Geneva (CH): International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2005 Nov 9 [cited 2016 Nov 22]. 23 p. Available from: http://www.ich.org/ fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q9/Step4/ Q9_Guideline.pdf.
- **98** Walter JK. Strategies and considerations for advanced economy in downstream processing of biopharmaceutical proteins. In: Subramanian G. editor. Bioseparation and bioprocessing: a handbook. Vol. 2. Berlin (DE): Wiley-VCH; 1998. p. 447-60.
- 99 Martin J. Case study orthogonal membrane technologies for viral and DNA clearance. Presented at the Society of Chemical Industry's Membrane Chromatography Conference; 2004 Nov; London, United Kingdom.
- 100 Noel R, et al. Industrial processing of immunoglobulins: applicability for monoclonal antibody manufacturing. BioProcess Int. 2007 May;5(5):58-61.
- 101 Bisschops M, Ransohoff T. BioSMB™: a new disposable chromatography process. Presented at the American Chemical Society's 236th National Meeting, Division of Biochemical Technology (BIOT); 2008 Aug 17-21; Philadelphia, PA.
- 102 Lacki KM, Bryntesson LM. Protein A periodic counter-current chromatography for continuous antibody purification. Presented at the American Chemical Society's 228th National Meeting; 2004 Mar 29-Apr 1; Anaheim, CA.
- 103 Müller-Späth T, et al. Increasing capacity utilization in Protein A chromatography. Biopharm Int. 2013 Oct;26(10):33-8.
- 104 Zang Y, et al. Towards protein crystallization as a process step in downstream processing of therapeutic antibodies: screening and optimization at microbatch scale. PLOS One. 2011 Sep;6(9):e25282.
- 105 Kuczewski M, et al. PEG precipitation: A powerful tool for monoclonal antibody purification. Biopharm Int. 2010 Mar;23(Suppl):20-8.
- **106** Gronke R, et al. Design and optimization of a continuous, scalable precipitation process. Presented at IBC's Biopharmaceutical Development and Production Conference; 2014 Mar 24-27; San Diego, CA.



CHAPTER 8:

Formulation Development and Stability

Μ

onoclonal antibodies, like all protein therapeutics, degrade with time. The goal of formulation development is to identify a matrix of buffers, stabilizers, and other excipients, which maintain the high quality of the monoclonal antibody and retain the product's activity and structure during storage. Formulation details for currently marketed therapeutic monoclonal antibody products as of October 31, 2016 are presented in Table 8.1.

Table 8.1. Formulation Details for Currently Marketed Therapeutic Monoclonal Antibody Products ^a					
Product Name (INN Name)	Presentation ^b	Concentration °	Buffer and Excipients ^d		
Abthrax (raxibacumab)	Liquid	50 mg/mL	Sodium Citrate, Glycine, Sucrose, Polysorbate 80, pH 6.5		
Actemra (tocilizumab)	Liquid	20 mg/mL	Sodium Phosphate, Sucrose, Polysorbate 80, pH 6.5		
	Liquid ^e /Syringe	180 mg/mL	Histidine, Arginine, Methionine, Polysorbate 80, pH 6.0		
Adcetris (brentuximab vedotin)	Lyophilized	5 mg/mL	Sodium Citrate, Trehalose, Polysorbate 80, pH 6.6		
AlprolIX (Factor IX Fcfusion protein, eftrenonacog alfa)	Lyophilized	8.6 mg/mL ^f	Histidine, Sucrose, Mannitol, Sodium Chloride, Polysorbate 20 g		
Amjevita (adalimumab-atto)	Liquid [®] /Autoinjector/ Syringe	50 mg/mL	Acetic Acid, Sucrose, Polysorbate 80, pH 5.2		
Anthim (obiltoxaximab)	Liquid	100 mg/mL	Histidine, Sorbitol, Polysorbate 80, pH 5.5		
Arcalyst (rilonacept)	Lyophilized	80 mg/mL	Arginine, Histidine, Glycine, Sucrose, Polyethylene Glycol 3350, pH 6.26.8		
Arzerra (ofatumumab)	Liquid	20 mg/mL	Sodium Acetate, Arginine, Sodium Chloride, Edetate Disodium, Polysorbate 80, pH 5.5		
Avastin (bevacizumab)	Liquid	25 mg/mL	Sodium Phosphate, Trehalose, Polysorbate 20, pH 6.2		
Benepali (etanercept)	Liquid ^e /Pen/Syringe	50 mg/mL	Sodium Chloride, Sodium Phosphate, Sucrose, pH 5.96.5		
Benlysta (belimumab)	Lyophilized	80 mg/mL	Sodium Citrate, Sucrose, Polysorbate 80, pH 6.5		
Blincyto (blinatumomab)	Lyophilized	12.5 mcg/mL	Lysine, Trehalose, Polysorbate 80, pH 7.0		
Cimzia (certolizumab pegol)	Liquid e/Syringe	200 mg/mL	Sodium Acetate, Sodium Chloride, pH 4.7		
	Lyophilized h	200 mg/mL	Lactic Acid, Sucrose, Polysorbate i, pH 5.2		

Table 8.1. Formulation Details for Currently Marketed Therapeutic Monoclonal Antibody Products ^a					
Product Name (INN Name)	Presentation ^b	Concentration °	Buffer and Excipients ^d		
Cinqair (reslizumab)	Liquid	10 mg/mL	Acetic Acid, Sodium Acetate, Sucrose, pH 5.5		
Cosentyx (secukinumab)	Liquid °/Pen/Syringe	150 mg/mL	Trehalose, Histidine, Methionine, Polysorbate 80, pH 5.8		
	Lyophilized	150 mg/mL	Sucrose, Histidine, Polysorbate 80, pH 5.8		
Cyramza (ramucirumab)	Liquid	10 mg/mL	Histidine, Glycine, Sodium Chloride, Polysorbate 80, pH 6.0		
Darzalex (daratumumab)	Liquid	20 mg/mL	Mannitol, Sodium Acetate, Sodium Chloride, Polysorbate 20, pH 5.5		
Eloctate (Factor VIII Fc-fusion protein)	Lyophilized	0.1 mg/mL ⁱ	Histidine, Sucrose, Sodium Chloride, Calcium Chloride, Polysorbate 80 g		
Empliciti (elotuzumab)	Lyophilized	25 mg/mL	Sodium Citrate, Sucrose, Polysorbate 80 ^g		
Enbrel (etanercept)	Liquid º/Pen/Syringe	50 mg/mL	Sodium Phosphate, Arginine, Sucrose, Sodium Chloride, pH 6.16.5		
	Lyophilized	10 mg/mL ^k 50 ml/mL ^k	Tromethamine, Mannitol, Sucrose, pH 7.1-7.7		
	Lyophilized	25 mg/mL	Tromethamine, Mannitol, Sucrose, Benzyl Alcohol (US) I, pH 7.1-7.7		
Entyvio (vedolizumab)	Lyophilized	60 mg/mL	Arginine, Histidine, Sucrose, Polysorbate 80, pH 6.3		
Erbitux (cetuximab)	Liquid	2 mg/mL ^m	Sodium Phosphate, Sodium Chloride, pH 7.0-7.4		
	Liquid	5 mg/mL ^k	Sodium Citrate, Glycine, Sodium Chloride, Polysorbate 80, pH 7.07.4		
Erelzi (etanercept-szzs)	Liquid °/Pen	50 mg/mL	Sodium Chloride, Sucrose, Lysine, pH 6.16.5		
Eylea (aflibercept)	Liquid/Syringe ⁿ	40 mg/mL	Sodium Phosphate, Sucrose, Sodium Chloride, Polysorbate 20, pH 6.2		
Flixabi (infliximab)	Lyophilized	10 mg/mL	Sucrose, Sodium Phosphate, Polysorbate 80 g		
Gazyva (obinutuzumab)	Liquid	25 mg/mL	Histidine, Trehalose, Poloxamer 188, pH 6.0		
Herceptin (trastuzumab)	Liquid o/Cartridge	120 mg/mL	Histidine, Methionine, Trehalose, rhHyaluronidase p, Polysorbate 20, pH 6.0		
	Lyophilized	21 mg/mL	Histidine, Trehalose, Polysorbate 20, pH 6.0		
Humira (adalimumab)	Liquid/Pen/Syringe	50 mg/mL	Sodium Citrate, Sodium Phosphate, Mannitol, Sodium Chloride, Polysorbate 80, pH 5.2		
	Liquid °/Pen ⁿ / Syringe ^h	100 mg/mL	Mannitol, Polysorbate 80, pH 5.2		
llaris (canakinumab)	Lyophilized	150 mg/mL	Histidine, Sucrose, Polysorbate 80, pH 6.2-6.8		
Inflectra (infliximabdyyb, infliximab)	Lyophilized	10 mg/mL	Sodium Phosphate, Sucrose, Polysorbate 80, pH 7.2		
Kadcyla (adotrastuzumab emtansine)	Lyophilized	20 mg/mL	Sodium Succinate, Sucrose, Polysorbate 20, pH 5.0		
Keytruda (pembrolizumab)	Liquid ^h	25 mg/mL	Histidine, Sucrose, Polysorbate 80, pH 5.5		
	Lyophilized	25 mg/mL	Histidine, Sucrose, Polysorbate 80, pH 5.5		
Lartruvo (olaratumab)	Liquid	10 mg/mL	Glycine, Histidine, Mannitol, Sodium Chloride, Polysorbate 20, pH 5.25.8		

Table 8.1. Formulation Details for Currently Marketed Therapeutic Monoclonal Antibody Products ^a						
Product Name (INN Name)	Presentation ^b	Concentration °	Buffer and Excipients ^d			
Lemtrada (alemtuzumab)	Liquid	10 mg/mL	Sodium Phosphate, Potassium Phosphate, Edetate Disodium, Sodium Chloride, Potassium Chloride, Polysorbate 80, pH 7.0-7.4			
Lucentis (ranibizumab)	Liquid	6 mg/mL ™ 10 mg/mL	Histidine, Trehalose, Polysorbate 20, pH 5.5			
	Liquid °/Syringe ⁿ	10 mg/mL	Histidine, Trehalose, Polysorbate 20, pH 5.5			
Nplate (romiplostim)	Lyophilized	0.5 mg/mL	Histidine, Mannitol, Sucrose, Polysorbate 20, pH 5.0			
Nucala (mepolizumab)	Lyophilized	100 mg/mL	Sodium Phosphate, Sucrose, Polysorbate 80, pH 7.0			
Nulojix (belatacept)	Lyophilized	25 mg/mL	Sodium Phosphate, Sucrose, Sodium Chloride, pH 7.2-7.8			
Opdivo (nivolumab)	Liquid	10 mg/mL	Sodium Citrate, Sodium Chloride, Mannitol, Pentetic Acid, Polysorbate 80, pH 6.0			
Orencia (abatacept)	Liquid e/Pen [®] /Syringe	125 mg/mL	Sodium Phosphate, Sucrose, Poloxamer 188, pH 6.8-7.2			
	Lyophilized	25 mg/mL	Sodium Phosphate, Maltose, Sodium Chloride, pH 7.2-7.8			
Perjeta (pertuzumab)	Lyophilized	30 mg/mL	Histidine, Sucrose, Polysorbate 20, pH 6.0			
Portrazza (necitumumab)	Liquid	16 mg/mL	Glycine, Mannitol, Sodium Chloride, Sodium Citrate, Polysorbate 80, pH 6.0			
Praluent (alirocumab)	Liquid º/Pen/Syringe	75 mg/mL 150 mg/mL	Histidine, Sucrose, Polysorbate 20, pH 6.0.			
Praxbind (idarucizumab)	Liquid	50 mg/mL	Sodium Acetate, Sorbitol, Polysorbate 20, pH 5.35.7			
Prolia (denosumab)	Liquid	60 mg/mL	Sodium Acetate, Sorbitol, pH 5.2			
	Liquid ^e /Syringe	60 mg/mL	Sodium Acetate, Sorbitol, Polysorbate 20, pH 5.2			
Remicade (infliximab)	Lyophilized	10 mg/mL	Sodium Phosphate, Sucrose, Polysorbate 80, pH 7.2			
Removab (catumaxomab)	Liquid ^e /Syringe	0.1 mg/mL	Sodium Citrate, Polysorbate 80, pH 5.6			
Remsima (infliximab)	Lyophilized	10 mg/mL	Sodium Phosphate, Sucrose, Polysorbate 80, pH 7.2			
ReoPro (abciximab)	Liquid	2 mg/mL	Sodium Phosphate, Sodium Chloride, Polysorbate 80, pH 7.2			
Repatha (evolocumab)	Liquid ^e /Syringe/ Autoinjector	140 mg/mL	Proline, Sodium Acetate, Polysorbate 80, pH 5.0			
Rituxan (rituximab)	Liquid	10 mg/mL	Sodium Citrate, Sodium Chloride, Polysorbate 80, pH 6.5			
	Liquid	120 mg/mL ^k	Histidine, Methionine, Trehalose, rhHyaluronidase ^p , Polysorbate 20, pH 6.5			
Simponi/Simponi Aria	Liquid ^h	12.5 mg/mL	Histidine, Sorbitol, Polysorbate 80, pH 5.5			
(golimumab)	Liquid ^e /Pen/Syringe	100 mg/mL	Histidine, Sorbitol, Polysorbate 80, pH 5.5			
Simulect (basiliximab)	Lyophilized	4 mg/mL	Sodium Phosphate, Glycine, Sucrose, Mannitol, Sodium Chloride, pH 6.5			
Soliris (eculizumab)	Liquid	10 mg/mL	Sodium Phosphate, Sodium Chloride, Polysorbate 80, pH 7.0			
Stelara (ustekinumab)	Liquid/Syringe	90 mg/mL	Histidine, Sucrose, Polysorbate 80, pH 5.7-6.3			
Strensiq (asfotase alfa)	Liquid	40 mg/mL 100 mg/mL	Sodium Chloride, Sodium Phosphate, pH 7.4			
Sylvant (siltuximab)	Lyophilized	20 mg/mL	Histidine, Sucrose, Polysorbate 80, pH 5.2			

Table 8.1. Formulation Details for Currently Marketed Therapeutic Monoclonal Antibody Products ^a					
Product Name (INN Name)	Presentation ^b	Concentration °	Buffer and Excipients ^d		
Synagis (palivizumab)	Liquid	100 mg/mL	Histidine, Glycine, Chloride (US) ª, pH 6.0		
	Lyophilized "	100 mg/mL	Histidine, Glycine, Mannitol ^g		
Taltz (ixekizumab)	Liquid °/Syringe/ Autoinjector	80 mg/mL	Sodium Chloride, Sodium Citrate, Polysorbate 80, pH 5.36.1		
Tecentriq (atezolizumab)	Liquid	60 mg/mL	Acetic Acid, Histidine, Sucrose, Polysorbate 20, pH 5.8		
Trulicity (dulaglutide)	Liquid ^e /Pen/ Syringe/	1.5 mg/ml 3 mg/mL	Sodium Citrate, Citric Acid, Mannitol, Polysorbate 80 g		
Tysabri (natalizumab)	Liquid	20 mg/mL	Sodium Phosphate, Sodium Chloride, Polysorbate 80, pH 6.1		
Unituxin (dinutuximab)	Liquid	3.5 mg/mL	Histidine, Sodium Chloride, Polysorbate 20, pH 6.8		
Vectibix (panitumumab)	Liquid	20 mg/mL	Sodium Acetate, Sodium Chloride, pH 5.6-6.0		
Xgeva (denosumab)	Liquid	70 mg/mL	Sodium Acetate, Sorbitol, pH 5.2		
Xolair (omalizumab)	Liquid ^e ⁿ /Syringe	150 mg/mL	Arginine, Histidine, Polysorbate 20, pH 5.5-6.5		
	Lyophilized	125 mg/mL	Histidine, Sucrose, Polysorbate 20, pH 5.5-6.5		
Yervoy (ipilimumab)	Liquid	5 mg/mL	Tromethamine, Mannitol, Sodium Chloride, Pentetic Acid, Polysorbate 80, pH 7.0		
Zaltrap (zivaflibercept)	Liquid	25 mg/mL	Sodium Citrate, Sodium Phosphate, Sucrose, Sodium Chloride, Polysorbate 20, pH 6.2		
Zevalin (Y90ibritumomab tiuxetan)	Liquid	1.6 mg/mL	Sodium Phosphate, Potassium Phosphate, Sodium Acetate, Human Serum Albumin, Pentetic Acid, Sodium Chloride, Potassium Chloride, pH 7.1		
Zinbryta (daclizumab (highyield))	Liquid ^e /Pen ⁿ / Syringe ^h	150 mg/mL	Sodium Chloride, Sodium Succinate, Succinic Acid, Polysorbate 80, pH 6.0		
Zinplava (bezlotoxumab)	Liquid	25 mg/mL	Citric Acid, Sodium Chloride, Sodium Citrate, Pentetic Acid, Polysorbate 80, pH 6.0		

- ^a Information gathered from product package inserts, EMA public assessment reports, and US FDA product approval information reviews. Data presented for all products available for commercial sale as of October 31, 2016.
- ^b All products presented in glass vial unless otherwise noted. Products available in syringes, pens, or autoinjectors are presented as pre-filled liquids in these container/closure systems.
- For lyophilized products, concentration after reconstitution with appropriate diluent according to package insert.
- ^d Excipients and buffers in various formulations may be present in various salt forms or enantiomers; see package inserts for details.
- Product in solution at given concentration not available in glass vial format; available in listed formats only
- ^f IU/mL converted to mg/mL using a product specific average specific activity of 69.5 IU/mg.
- ^g pH not published.
- ^h Although product approved in EU and US, product format only available in US.

- ⁱ Specific type of polysorbate used in formulation not specified.
- ^j IU/mL converted to mg/mL using a product specific average specific activity of 9,231 IU/mg.
- ^k Although product approved in EU and US, product concentration(s) only available in EU.
- ¹ Although product approved in EU and US, excipient only listed on US package insert.
- ^m Although product approved in EU and US, product concentration(s) only available in US.
- ⁿ Although product approved in EU and US, product format only available in EU.
- Although product approved in EU and US, product format(s), concentration(s) and formulation(s) only available in EU.
- ^p Recombinant human hyaluronidase.
- ^q Although product approved in EU and US, excipient only listed on EU package insert, counter ion not specified.

Briefly, the general structure of an IgG molecule can be depicted as a Y consisting of two heavy chains and two light chains as shown in Figure 8.1.¹ Monoclonal antibodies have been shown to exhibit variability at several locations in the molecule resulting from conversion of the N-terminal glutamic acid residue of the heavy chain to pyro-glutamic acid, deamidation of various asparagine or glutamine residues in either the heavy or light chain, oxidation of methionine residues, variation of the extent of glycosylation and/or the structure of the glycosylation at the several potential glycosylation sites within the antibody, and removal of the C-terminal lysine residue of the heavy chain by proteolysis. The impact on the molecular structure may be subtle, such as isomerization of a single aspartic acid residue, but this difference may have a significant impact on the potency of the monoclonal antibody drug product.² Characterization of the monoclonal antibody requires the use of multiple and varied analytical techniques discussed in detail in Chapter 4.

other, Kozlowski and Swann have calculated that there are over 9,600 theoretically different individual variants possible in a typical monoclonal antibody product for a potential of nearly 108 forms of the antibody.1 It is not currently known what impact each of these variants has on the efficacy or safety of a monoclonal antibody, and some of these variants may not be present in any particular monoclonal therapeutic. However, some of the possible variants are known to affect the biological activity of proteins in general. Variations in glycosylation and the glycoform structure are known to impact the biological activity³ and the thermal stability of the antibody.⁴ With this level of variability inherent in the monoclonal antibody, determination of the degradation pathways, which should be done as part of the pre-formulation work, can be quite complicated.

1. Degradation Pathways

In contrast to traditional small molecule pharmaceuticals,

Figure 8.1 Structure of a Monoclonal Antibody

The general structure of a monoclonal antibody is shown along with the most common sites of variability and/or degradation. Reprinted from Reference 1 with the author's permission.



The use of the phrase monoclonal antibody implies a single amino acid sequence as well as a unique set of posttranslational modifications as well as secondary and tertiary structure from molecule to molecule than is actually seen in practice in monoclonal antibody products. Taking into account all of the potential sources of variability in an IgG molecule and considering that there are two light and two heavy chains each of which may vary slightly from the



the degradation of monoclonal antibody products is not easily determined from the structure of the product. Degradation of a monoclonal antibody can result from either the chemical degradation of specific amino acid residues in the monoclonal antibody, physical degradation caused by the loss of tertiary structure, or the covalent or non-covalent aggregation of monoclonal antibody monomers to form aggregates in solution. In developing a monoclonal antibody product formulation, it is therefore important to minimize the impact of inherent environmental factors, such as airliquid interfaces, interaction of the monoclonal antibody with buffer salts, and other components of the product formulation, as well as intermolecular interactions, which can lead to degradation of the monoclonal antibody product.

The various types of degradation to which monoclonal antibodies are susceptible and the variety of analytical methods

used to detect them are summarized in Table 8.2. For chemical degradation pathways involving specific amino acid side chains, the amino acids potentially susceptible to each type of degradation is also listed in Table 8.2. In some cases, more than one type of degradation may occur at the same amino acid and some types of degradation are more likely to occur at specific amino acid sequences than others. A brief description of each type of chemical degradation is provided below.

Table 8.2 Potential	Degradation	Pathways o	f Monoclonal	Antibody	Products	and	Analytical
Methods to Detect	Them						

Type of Degradation	Susceptible Amino Acid	Suitable Analytical Methodology
Oxidation	Methionine, some Tryptophan and Histidine	Reverse phase HPLC, Peptide mapping
Deamidation	Asparagine, some Glutamine	lon exchange HPLC, Isoelectric focusing (IEF), Quantitative assay of isoaspartic acid
Disulfide Bond rearrangement	Cysteine and Cystine	Peptide mapping, Quantitative analysis of free sulfhydryl groups
β -elimination with rearrangement	Aspartic Acid	Peptide mapping
Hydrolysis	Aspartic Acid	Size exclusion (SEC) HPLC, SDS-PAGE, Mass spectrometry (MS)
Cross-linking	Cysteine, some Lysine and Glutamic Acid	SDS-PAGE, SEC HPLC, Multi-angle light scattering (MALS), Analytical ultracentrifugation (AUC), MS
Loss of tertiary structure	N/A	Circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), Intrinsic and extrinsic tryptophan fluorescence, Potency
Aggregation	N/A	SDS-PAGE, SEC HPLC, MALS, AUC, MS
Precipitation	N/A	Visual observation, MALS, AUC
Adsorption	N/A	Protein Concentration, Surface Extraction
Details of these analytical methods ar	e included in Chapter 4	

Oxidation

Oxidation, either auto-oxidation or metal catalyzed, commonly occurs at exposed methionine residues as shown in Figure 8.2. Oxidation of tryptophan and histidine may also occur, but is much less likely. Proteins containing, His, Met, Cys, Tyr, and Trp amino acids can all be damaged by a number of reactive oxygen species. These reactive side chains may oxidize at any stage of monoclonal antibody production.⁵

Figure 8.2 Mechanism of Methionine Oxidation



Controlling oxidation, which can be impacted by intrinsic and extrinsic factors, is an important element of monoclonal antibody production. Intrinsic factors tend to be related to the overall structure of the protein, while extrinsic factors may include pH and buffer type.⁵ Approaches for controlling oxidation vary. Oxidation can be prevented by limiting a protein's exposure to oxygen. Agitation of a liquid formulation, which increases the level of dissolved oxygen, can lead to increased oxidation.⁵

Deamidation

Deamidation is a common degradation pathway for peptides and proteins. The general mechanism of deamidation of asparagine and, to a lesser extent, glutamine residues in monoclonal antibodies, is shown in Figure 8.3. At pH \leq 4, the succinimide intermediate formed by hydrolysis of the amide group of the side chain is predominantly converted to yield aspartic acid. However, when the pH of the solution is greater than 6, the succinimide intermediate can be hydrolyzed to form either aspartic acid or isoaspartic acid.6 The ratio of isoaspartic acid to aspartic acid formed depends on both the solution pH and the surrounding amino acid structure.

Figure 8.3 Mechanism of Deamidation of Asparagine Residues



Deamidation can be the cause of process-related impurities and degradation products as well as increased immunogenicity.⁵ It is capable of causing multiple kinds of chemical instability in monoclonal antibodies. Deamidation can be controlled most effectively by controlling the pH and rates of deamidation may be slowed by changing the conformation of the protein.⁵

Disulfide Bond Rearrangement

The many disulfide bonds (cystine) in a monoclonal antibody are important in controlling the three-dimensional shape of the molecule as well as in keeping all four protein chains (two heavy and two light chains) together. Reduction of any disulfide bond results in the production of two free sulfhydryl groups (one on each of the cysteine residues originally linked by the disulfide bond, see Figure 8.4). These free sulfhydryl groups may re-form the original disulfide bond or may form a disulfide bond with other cysteine residues elsewhere in the monoclonal antibody, resulting in a disulfide rearrangement of the molecule. In some cases, the new disulfide bonds can be formed between free sulfhydryl groups of cysteine residues on two different antibody molecules resulting in dimers or other aggregated forms. The impact of this disulfide rearrangement on the antibody activity depends on the location of the rearrangement and the structural alteration that occurs.⁵

Figure 8.4 Disulfide Rearrangement



Source: BPTC

Beta-elimination with Rearrangement

Alkaline conditions can result in a beta-elimination reaction at a cysteine, serine or threonine in the antibody (Figure 8.5). The abstraction of a beta hydrogen by hydroxide ions leads to the formation of an unstable anion, which may further react to cleave the original peptide bond generating two peptide fragments from the original protein sequence or may result in racemization of the amino acid by re-addition of a proton in a non-stereochemical manner resulting in both the L- and D- forms in the protein sequence. The impact of this degradation will vary from monoclonal antibody to monoclonal antibody but should be carefully monitored since changes, even those which occur remotely from the monoclonal antibody binding site, may result in altered bioactivity.

Figure 8.5 Mechanism of β-Elimination and Rearrangement or Hydrolysis







Hydrolysis

Hydrolysis of the peptide chain may occur during degradation of the peptide bond between aspartic acid residues and the neighboring amino acid residue (X) on either the N or C terminal side of the aspartic acid. The aspartic acid–X bond, and to a lesser extent, the X-aspartic acid bond, is known to be labile under acidic conditions. Cleavage of these bonds will result in the cleavage of the protein sequence into two new peptides. Hydrolysis of peptide bonds in the monoclonal antibody sequence may also occur under basic conditions if the monoclonal antibody structure is such that certain amino acid side chains are properly positioned to catalyze the aspartic acid–X cleavage, (see Figure 8.6). Figure 8.6 Hydrolysis of Asp-Gly Peptide Bonds



Cross-linking

In addition to the cross-linking resulting from disulfide scrambling discussed above, chemical cross-linking may occur between acid and basic side chains of amino acids. Crosslinking between the acidic side chain of glutamic acid and the basic side chain of lysine, forming an amide bond, is known to occur in collagen where it contributes to the mechanical strength of the molecule.⁷ These cross-links may be inter- or intra-molecular. Formation of inter-molecular cross-links may result in formation of non-reversible dimers or higher-order aggregates, which may retain biological activity, but may be more-rapidly cleared from the circulation.

Loss of tertiary structure

This loss of structure may be a decrease in helical structure or β -sheet content of the monoclonal antibody or it may be an increase in either structured or non-structured regions. Unfolding or refolding of the monoclonal antibody by either of these mechanisms is often the initial step in further degradation of the protein as it may expose amino acids and sequence structures normally protected from the surrounding environment. These exposed amino acids can undergo chemical reactions described above or form new or different hydrogen bonds resulting in further loss of three-dimensional structure.

Aggregation

Aggregation may be viewed as either a chemical or physical degradation mechanism and often involves a combination of both as shown in Figure 8.7. The aggregation pathway generally begins with the partial unfolding of the monoclonal antibody structure that can expose amino acids, which can then undergo either chemical cross-linking or form new hydrogen bonds either intramolecularly within a single monoclonal antibody molecule, which were not present in the originally properly folded antibody, or intermolecularly between different antibody molecules. The formation of new intramolecular hydrogen bonds may result in unstable conformation of the protein, which can then further unfold, exposing more amino acid residues and leading to additional destabilization of the antibody structure and ultimately result in precipitation of the antibody from solution. Since the formation of intermolecular hydrogen bonds requires the interaction of two monoclonal antibody molecules in solution, the tendency for monoclonal antibodies to form intermolecular hydrogen bonds and aggregate is higher at higher protein concentrations, making this potentially a significant concern in today's high concentration monoclonal antibody formulations.

Figure 8.7. Aggregation Pathways for Monoclonal Antibody Products



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Aside from the non-covalent aggregation described above, covalent aggregates may also form as a result of the formation of intermolecular disulfide bonds or as a result of the formation of a peptide-like bond between the acidic side chain of certain glutamate residues and the eamino group of nearby lysine residues. Disulfide aggregates of monoclonal antibodies may not be detected by reducing SDSPAGE analysis since the treatment of the protein with a reducing agent prior to electrophoresis will reduce the disulfide bond and separate the two covalently bound antibody molecules. Cross-linking between lysine and glutamine residues is not reversible so these covalent aggregates will still appear as higher molecular weight forms when analyzed by reducing SDS-PAGE.

For aggregate testing of high-concentration antibody formulations, existing analytical methods may need to be adapted.8 Size-exclusion HPLC of high-concentration formulations without the need for dilution may be facilitated by changing the UV detection wavelength from 215 to 235 nm, for example.⁹

The presence of sub-visible particles in parenteral products, including monoclonal antibodies, is of increasing concern to regulatory agencies due to their potential for causing unintended immunogenicity problems and the difficulty in detecting them.¹⁰ These subvisible particles, defined as particles larger than 0.1 μ m, but too small to be visible to the unaided eye (<100 μ m), may form either during product manufacture or over time as initially soluble aggregates associate in solution. Of particular concern are particles less than 10 μ m in size, which are not well detected by current analytical methods used to monitor degradation of monoclonal antibody products.¹¹

Adsorption

Proteins may adsorb to hydrophobic surfaces, a problem of special concern and significance when the protein is present in low concentration (<1 mg/mL) since a larger percentage of protein is lost to surfaces. Since most monoclonal antibodies are formulated at relatively high concentrations, ≥ 2 mg/mL (see Table 8.1) this loss to surfaces is not as great a concern for antibodies as for other recombinant protein therapeutics. Still the antibodies may adsorb to the glass or stopper surface, especially after loss of three-dimensional structure. It is often difficult to detect this phenomenon as it may result in the loss of only a small amount of protein

initially, though the adsorbed protein may act to catalyze increased protein unfolding over time.

A key role of the formulation is to control the nature and amount of antibody aggregation.⁵ As discussed in Chapter 4, analytical methods for measuring all types and sizes of aggregate are required and will be an important tool for assessing the suitability of any formulation.

2. Determining Degradation Pathways for a Monoclonal Antibody Product

For each monoclonal antibody it is necessary to know which kinds of degradation the molecule undergoes as well as the kinds of environmental stress that leads to the degradation. This knowledge provides guidance on which formulation attributes are most important to control. These formulation attributes will be added to the Quality by Design (QbD) Control Strategy, as discussed in ICH Q11.¹² This information is also important to the analytical development scientist developing stability-indicating methods. In order to determine which degradation pathways are most relevant for a specific antibody, one or more forced degradation studies are performed. In a forced degradation study the antibody is subjected to varied physico-chemical conditions and the impact of these environmental stresses on the structure and activity of the antibody is evaluated over time. The stress conditions are usually more extreme than the molecule would commonly experience. These forced degradation studies should examine the degradation of the antibody due to oxidizing conditions, extremes of pH and temperature, multiple freezing and thawing cycles, agitation, and extended exposure to light. The degradation products produced in a forced degradation study may never be observed in a monoclonal antibody drug stored under standard conditions even over long periods of time. Prior to submitting a BLA, forced degradation studies should be performed on both the monoclonal antibody drug substance and drug product. However, in early development the forced degradation studies are typically performed only on the drug substance. The range of forced degradation studies typically performed on a monoclonal antibody product is shown in the matrix of studies outlined in Table 8.3.

Analytical methods used in a forced degradation study during early development of a monoclonal antibody are unlikely to be validated and may include methods, such as analytical ultracentrifugation, which will not be validated. The use of good scientific practices and suitable controls during the analysis of forced degradation samples is particularly important to provide assurance of the reliability of results. The analyses performed as part of a forced degradation study should be chosen to provide a complete picture of all kinds of degradants. Analytical methods typically employed for the characterization of a monoclonal antibody product in a forced degradation study are listed in Table 8.4.

Table 8.3. Example of a Forced Degradation Matrix for a Monoclonal Antibody Product											
Stress Conditions	Time \rightarrow	2 Hr	4 Hr	8 Hr	1 Day	2 Days	5 Days	7 Days	14 Days	21 Days	28 Days
Control		•	•	•	•	•	•	•	•	•	•
рН	12	•	•	•	•	•	•	•	•		
	10	•	•	•	•	•	•	•	•	•	•
	9	•	•	•	•	•	•	•	•	•	•
	8	•	•	•	•	•	•	•	•	•	•
	7	•	•	•	•	•	•	•	•	•	•
	6	•	•	•	•	•	•	•	•	•	•
	5	•	•	•	•	•	•	•	•	•	•
Temperature	-70°C				•	•	•	•	•	•	•
	-20°C				•	•	•	•	•	•	•
	5°C				•	•	•	•	•	•	•
	25°C		•	•	•	•	•	•	•	•	•
	40°C	•	•	•	•	•	•	•	•	•	
	50°C	•	•	•	•	•	•	•	•		
Oxidation	1% H2O2	•	•	•	•	•	•	•			
	2% H2O2	•	•	•	•	•	•				
	10% H2O2	•	•	•	•	•					
Agitation					•			•	•	•	
Freeze/Thaw	Test sample at	fter 1, 3, an	d 5 freeze/t	haw cycles							
Light Exposure	Single test poi	nt following	g exposure t	o 1.2 M lux-	hr of artific	ial daylight	1				

Table 8.4. Typica	I Analytical Methods Used In				
Analytical Method Information Obtained					
Visual Inspection	Color and clarity of the solution Absence of Visible Particles				
рН	Confirm solution pH				
UV Absorbance	Antibody concentration				
SDS-PAGE, reduced	Molecular weight, hydrolysis and some covalent aggregates				
SDS-PAGE, non-reduced	Hydrolysis and aggregation				
Isoelectric Focusing	Charge heterogeneity				
Peptide Mapping	Primary structure, oxidation and deamidation				
SEC HPLC	Aggregation and hydrolysis				
RP-HPLC	Oxidation				
IEC-HPLC	Charge heterogeneity				
Bioassay	Potency				
Receptor Binding	Potency				
Mass Spectroscopy	Molecular weight				
Light Scattering	Aggregation and molecular weight				
Analytical Ultracentrifugation	Molecular weight, hydrolysis and aggregation				
Circular Dichroism	Three-dimensional structure				
FTIR	Three-dimensional structure				
UV Fluorescence	Three-dimensional structure				

Each forced degradation study should be performed according to a detailed written protocol and executed using good scientific principles. Minimally, the protocol should be approved by members of the development and quality departments and all changes must be documented and approved prior to implementation. There should be sufficient detail to allow a reviewer to understand the design of the study, including the details of sample preparation, sample handling and conditions to be examined. Whenever possible, aliquots of the forced degradation samples should be saved for reanalysis in the future or for use in the qualification of new characterization methods developed after the study was performed.Forced degradation studies performed early in development may use one or more different batches of drug substance. These may include development lot(s), lots used for toxicity testing or the first reference standard. Forced degradation should utilize batches, which are produced in a manner as similar as possible to the process utilized to manufacture clinical trial material. This will decrease the need to repeat forced degradation studies.

Once the formulation and container/closure system are finalized an additional forced degradation study of the drug product is commonly performed to determine if there is any impact of the container/closure system on the degradation pathways of the drug product. The conditions examined in these later studies are usually limited to those stress conditions to which the drug product may be exposed to such as temperature extremes, agitation, and light exposure. Regulatory expectations for the light exposure conditions are described in ICH Q1B.¹³ However, ICH Q5C, which addresses stability of biotechnology and biological drugs, states that the photostability conditions should be determined on a case by case basis as is the case for most forced degradation conditions.¹⁴ Photostability studies may be performed on the drug substance earlier in development to determine if there is any light sensitivity of the monoclonal antibody.

3. Excipients Used in Monoclonal Antibody Product Formulation

A variety of excipients are used in monoclonal antibody product formulations to provide control of pH and tonicity of the solution, stabilize the monoclonal antibody structure, reduce degradation and aggregation of the product, and prevent surface denaturation or adsorption of the product to container/closure surfaces. A lyophilized formulation may need additional excipients to protect the protein from damage during freezing and to stabilize the cake structure after lyophilization. Examples of excipients used in the formulation of monoclonal antibody products include buffers, salts, amino acids, surfactants, and sugars.²

Excipients used in the formulation of monoclonal antibody products, as with other pharmaceutical products, should meet the appropriate USP (or other regional) compendial standards whenever possible. To further simplify the formulation of monoclonal antibody products, many companies prefer to use only those buffers and excipients included in the FDA Inactive Ingredient Database¹⁵ or those that comply with the standards for the manufacture of pharmaceutical grade excipients set by the International Pharmaceutical Excipient Council (IPEC).¹⁶

Excipients used in the formulation of monoclonal antibody products must be tested and released as any other raw material or component used in GMP manufacturing of a monoclonal antibody product. Impurity testing of excipients must conform to the ICH Q3C,¹⁷ and, in particular, the level of any residual solvents present in the excipients must be carefully controlled and limited.

pH Control

The most suitable pH for the formulation of a specific monoclonal antibody product will be that which minimizes pH-dependent degradation, such as isomerization at acidic pH or disulfide scrambling at basic pH, as well as minimizing aggregation and deamidation, which can occur at both acidic and basic pH. Since most monoclonal antibodies are more stable at neutral to slightly acidic pH, all currently approved monoclonal antibody drug products are formulated in the pH range between 5.0 and 7.8. This is also consistent with the common practice of keeping the pH of a parenteral drug at pH near neutrality to minimize injection site responses. Formulations far from neutral pH are more likely to result in an adverse response; however not all monoclonal antibody products are dosed by direct bolus injection. Monoclonal antibodies dosed by infusion are diluted prior to administration so that the pH of the infusion solution may be adjusted to neutrality prior to administration with the appropriate choice of diluent, allowing the drug product to be formulated at a more extreme pH, if necessary. The rate of both deamidation and isomerization of proteins, especially at asparagine residues, is affected by the pH and some amino acid bonds are labile at low pH.18

The typical buffers used to control pH in antibody formulations are generally used in the concentration range of 10100 mM.¹⁹ Selection of a buffer early in development is often based on an examining a limited number of product characteristics such as maintenance of activity and lack of precipitation. During formulation development as more product characteristics are investigated, it may be apparent that a different buffer is superior for maintaining product quality and potency. Commonly used buffers for recombinant proteins formulation are shown in Table 8.5 along with the pH range where they are most effective.

Choosing a suitable buffer for maintaining the pH of a monoclonal antibody product formulation should also include consideration of additional stabilizing activities of the salt. In some cases, amino acids, such as glycine or histidine are used to provide additional buffering capacity and stabilization of the protein.²⁰ Histidine in particular has been used frequently as a buffer for monoclonal antibody formulations in the pH range between 5 and 7.

Table 8.5. Commonly Used Buffers inMonoclonal Antibody Formulations				
Buffer	pH Range			
Acetate	4.0 - 6.0			
Histidine	5.0 - 7.0			
Citrate	5.5 – 7.5			
Phosphate	6.0 - 8.0			

A review of the formulations of fifty currently approved monoclonal antibody products (see Table 8.1) shows that histidine and phosphate are now used in the majority of commercial monoclonal antibody products for the maintenance of pH. 46% percent of the monoclonal antibodies use histidine as the main buffering component, while phosphate is in only 33% of the formulations. In the first editions of this report 65% of the formulations used phosphate for maintenance of drug product pH. This change illustrates the shift away from the prevalence of phosphate buffer in monoclonal antibody formulations in light of the known tendency for the pH of phosphate buffers to drift during freezing and other storage conditions.^{21,22,23}

Osmolality Control

Control of osmolality of the formulation is also important to ensure that the product delivered to the patient is isotonic (i.e., approximately 300 mOsm/kg). As with pH if the drug product will not be delivered directly, but will be diluted for infusion delivery, control of osmolality is a lesser concern since the diluents, frequently physiologic saline will help to control osmolality. A common approach to controlling osmolality in monoclonal antibody parenteral drug products is to include sodium chloride in the formulation in an amount that creates an isotonic formulation after consideration of all other excipients, which is why formulations with few other excipients have sodium chloride present at a concentration of approximately 150 mM. This intermediate ionic strength has been found useful for modulating high viscosity and opalescence that result from intermolecular protein-protein interactions in high concentration antibody formulations.²⁴

Cryoprotectants

Cryoprotectants stabilize a monoclonal antibody during freezing whether it is for long term storage as a frozen liquid or prior to lyophilization. The result of multiple freeze thaw forced degradation experiments can provide an indication of the need for this type of protectant, which includes non-reducing amorphous disaccharides such as sucrose or trehalose, polyols such as glycerol or mannitol and certain amino acids such as histidine. These cryoprotectants stabilize the monoclonal antibody product by forming a hydration sphere around the protein and, in the lyophilized state, by forming a glassy matrix which provides protection of the monoclonal antibody during storage.^{25, 26} Utilization of cryoprotectants early in formulation development is advised since most monoclonal antibody drug products are frozen as the bulk drug substance, final form or as part of lyophilization.

Lyoprotectants and Bulking Agents

A lyophilized formulation may need additional excipients to protect the protein against damage from freezing and to stabilize the cake structure after lyophilization.¹⁹ Common lyoprotectants include mannitol, disaccharides, and amino acids such as glycine, histidine and arginine. Lyoprotectants are required to protect the antibody against irreversible loss of activity when the water of hydration is removed during lyophilization by replacing the hydration sphere.²⁷ Bulking agents are used to stabilize the 'cake' by increasing the mechanical structure of the lyophilizate. The need for bulking agents is not as critical for monoclonal antibody drug products as for many other recombinant protein therapeutics due to the higher protein concentration in the antibody therapeutics.

Surfactants

As with many proteins, monoclonal antibodies in solution are likely to interact with the surface of the container closure or the air-liquid interface.²⁸ While the higher concentrations of protein (>5 mg/mL) in many monoclonal formulations helps to maintain the protein in solution through protein/ protein interaction, protein interaction with the hydrophobic glass surface of the vial may lead to surface denaturation of the protein resulting in aggregation, precipitation, and/or loss of activity. Determining whether a particular monoclonal antibody formulation should include a surfactant is commonly done using a shipping simulation in which the vial is shaken in a horizontal orientation to maximize the airliquid interface. The shaken solution(s) can then be analyzed for particulate, aggregation and total protein concentration in the presence and absence of surfactant.

The most commonly employed surfactants for monoclonal antibody product formulations are non-ionic surfactants, which do not disrupt the three-dimensional structure of the antibody as ionic surfactants can.²⁹ Non-ionic surfactants such as those derived from polyethoxylated sorbitan and oleic acid (i.e., polysorbate) or block copolymers of polyoxypropylene and polyoxyethylene (i.e., poloxomer) are most frequently used in monoclonal antibody product formulations. Polysorbate surfactants, commercially available as Tween, are classified by the number of polyoxyethylene groups in the polymer and the type of fatty acid associated with the polyoxyethylene sorbitan part of the molecule. Tween 20 and Tween 80, two polysorbates commonly used in monoclonal antibody product formulations, each contain 20 polyoxyethylene groups linked to sorbitan monolaurate, but have very different critical micellar concentrations (0.08 mM and 0.00600.012 mM, respectively). Poloxamers are similarly named based on the lengths of the different polymer blocks in poloxamers making a specific poloxamer surfactant. For the commercially available poloxamer surfactants, known as Pluronic surfactants, the surfactants are coded with a letter to define their physical form at room temperature (L = liquid, P = paste, F = flake (solid)) followed by two or three digits. The first digit (or first two digits in a three-digit number) multiplied by 300 gives the approximate molecular mass of the polyoxypropylene component of the surfactant and the second digit (third in a three-digit number) multiplied by 10 corresponds to the percentage of polyoxyethylene in the surfactant. For example, Pluronic F68, commonly used in monoclonal antibody product formulations, is a solid surfactant at room temperature, which contains polyoxypropylene of molecular mass of 1,800 g/mol and has an 80% polyoxyethylene content. Nonionic surfactants bind to the air-liquid interface and other hydrophobic surfaces (possibly including the surface of the monoclonal antibody product) thereby preventing surface stress-induced denaturation as well as interaction between aggregation-prone hydrophobic areas on the surface of the protein. Historically, the polysorbate surfactants contained a significant amount of peroxide impurities, which could cause the oxidation of proteins in solution. However, low peroxide and non-animal derived surfactants are now available, reducing the likelihood of this potential problem.

Chelating Agents

Metal ions present in protein solutions can catalyze the oxidation of methionine residues in the product. Reduction or removal of the metal ions from a monoclonal antibody product is generally done during the purification of the product so that the final drug substance contains very low levels of metal ions. However, if complete removal of metal ions from the monoclonal antibody product is not possible or if the monoclonal antibody product is particularly sensitive to oxidation, the addition of a chelating agent such as ethylenediaminetetraacetic acid (EDTA) may provide increased stability of the product. If citric acid is used as the buffering agent in a monoclonal antibody formulation, it too can chelate metal ions. However, since monoclonal antibody products are generally not sensitive to metal ioncatalyzed oxidation, chelating agents are not commonly included in formulations for these products.³⁰

Preservatives

The use of preservatives in monoclonal antibody formulations is not common since these products are generally marketed in single-use presentations. An antimicrobial preservative in a single-use vial of a parenteral product is not necessary and may lead to more difficulties such as new degradants or decreased stability of the antibody. If a preservative is added to a monoclonal antibody formulation, regulatory agencies will require a sound scientific reason for adding a preservative to a sterile product.

Other Excipients

In addition to the excipients listed above, the monoclonal

antibody may be formulated by adding a carrier protein such as albumin.³¹ Albumin has historically been used as a stabilizer in protein formulations and appears in the formulation for many biologic products. However, its use has diminished in recent years due in part to concerns related to its source from human plasma. Now that an animal product free form is available, its consideration as a potential excipient in protein or antibody formulations may be renewed.³²

Viscosity, solubility, aggregation and opalescence have historically limited the development of high-concentration antibody formulations.8 To facilitate the subcutaneous distribution of injection volumes of several milliliters over a greater area and enable painless administration, recombinant human hyaluronidase (rHuPH20) has recently been included in liquid subcutaneous presentations of some monoclonal antibodies. Using the Enhanze[™] Technology developed by Halozyme Therapeutics, Inc., rHuPH20 reversibly breaks down the gel-like hyaluronan barrier in the tissues between cells under the skin.33 rHuPH20-containing subcutaneous presentations of Herceptin SC and MabThera SC (Rituxan) have been approved in Europe in 2013 and 2014, respectively. ^{33, 34, 35}

Amino acid blends have been designed to stabilize proteins in high concentration formulations, minimizing aggregation, reducing viscosity and improving syringeability.³⁶ Excelse[™] Technology developed by Excelse Bio is using this approach to improve the formulation and delivery of injectable biotechnology products with concentrations up to 300 mg/mL.³⁷

4. Formulation Development

As seen in Table 8.1, there is great diversity in the formulation of the currently approved monoclonal antibody products, resulting from the differences in properties of different monoclonal antibody products, different approaches to formulation development between companies, the different indications for which monoclonal antibody products are approved, and the fact that some specific monoclonal antibody formulations are covered by patents, preventing others from using the same combination of ingredients in their formulations.^{30, 38}

Formulation development should begin as early as possible in the development of a monoclonal antibody product to reduce the risk of stability issues during clinical testing and to simplify the manufacture and storage of the product. During the initial phases of formulation development, often referred to as pre-formulation, data related to the stability of the monoclonal antibody product (e.g., aggregate levels, purity) over a wide range of solution conditions and after exposure to various stresses (forced degradation) is collected. These data provide critical information not only for development of a stable formulation, but also for development of the purification process, analytical methods, and manufacturing process for the monoclonal antibody product. Preformulation and stability analysis are increasingly being incorporated in the developability assessment of discoverystage drug candidates prior to initiation of the CMC activities in preparation for IND submission.^{39,40} The target product profile (TPP), discussed in Chapter 3, for the monoclonal antibody product should serve as a guide for the initial pre-formulation activities. For most monoclonal antibody products, the TPP may describe the ideal product formulation as a presentation of the drug product in a form that requires minimal manipulation, is easily administered, and is stable for up to two years under reasonable storage conditions.

Formulation development is intricately linked to both analytical and process development activities. Due to the interconnected nature of formulation development, and the need for real-time stability data in regulatory submissions, it is helpful to develop formulations that will remain consistent over the course of the monoclonal antibody product lifecycle whenever possible.

Monoclonal antibody products are often initially formulated in phosphate buffered saline (PBS) for pre-clinical and early human clinical studies and stored frozen at 20°C or lower to minimize product degradation. This formulation is often used in early stages of development due to its simplicity and use of excipients, which are known to be safe in man. While such a formulation has been successfully used for several monoclonal antibody products, the use of PBS, especially for frozen solutions, is not always optimal for long term stability and storage of monoclonal antibody products since freezing PBS may result in a shift in pH of up to 3 pH units, which may have deleterious effects on the monoclonal antibody, including loss of activity.^{21, 22, 23, 41} As an alternative to PBS, many companies have developed a platform formulation that comprises an intermediate protein concentration of 520 mg/mL, a neutral buffering system such as histidine, citrate, or phosphate, sodium chloride to control tonicity, a sugar or sugar alcohol as a cryoprotectant, and a non-ionic surfactant to prevent surface denaturation and aggregation. Although each monoclonal antibody product is different, most monoclonal antibodies are reasonably stable in this formulation so that such a platform approach can work well for rapidly developing an acceptable formulation for early-stage development of monoclonal antibody products.⁴²

To be suitable for use, all of the excipients in a monoclonal antibody formulation must combine to form a stable and active product without causing any adverse reactions in patients. Since changes in a single component in a formulation may affect how one or more of the other components perform, it is important to study the interactions between all potential excipients before finalizing a formulation. If the number of components in the formulation is limited to one or two, a series of experiments could be conducted in which each component is varied, keeping the other components constant. Such a "one at a time" method in which only a single parameter is changed in each experiment requires a total of 2n experiments where "n" equals the number of different components being evaluated to fully examine the effect of each component on the suitability of the formulation. However, since most monoclonal antibody product formulations contain a number of excipients, there are usually too many variables to study all interactions in a simple "one at a time" approach. In this case, a Design of Experiments (DOE) approach is particularly useful.^{43,44} DOE is a multivariate approach that provides a statistically valid method for examining the effect of multiple formulation components and their interactions on the structure and stability of a monoclonal antibody product in a relatively small number of experiments. For example, the effects of varying four different components of a potential formulation, namely the presence or absence of 150 mM sodium chloride to the buffer, the inclusion of either mannitol or trehalose in the formulation, a buffer pH of 6.5 or 7.5, and the presence or absence of Polysorbate 80, could be studied in a DOE using sixteen different experiments as outlined in Table 8.6 to study the impact of each formulation component and any interactions between
them. A "one at a time" approach to studying these same formulation variables would also require sixteen different experiments but provide little information on interactions between components. If, however, adding a fifth component to the formulation (such as the presence or absence of an additional excipient or two different salt concentrations rather than just one) were to be evaluated, the number of experiments required to study all the interactions using a "one at a time" approach would double to thirty-two while the DOE approach still requires only sixteen experiments to examine the impact of each excipient and any interactions.

Table 8.6. Example of Design of ExperimentsStudy Investigating Four or Five Componentsof a Potential Monoclonal Antibody ProductFormulation

Expt. No	±150 mM NaCl	Sugar	рH	±Polysorbate 80	Fifth Excipient
1	+	Mannitol	6.5	-	-
2	+	Mannitol	6.5	+	+
3	+	Mannitol	7.5	-	+
4	+	Mannitol	7.5	+	-
5	+	Trehalose	6.5	-	+
6	+	Trehalose	6.5	+	-
7	+	Trehalose	7.5	-	-
8	+	Trehalose	7.5	+	+
9	-	Mannitol	6.5	-	+
10	-	Mannitol	6.5	+	-
11	-	Mannitol	7.5	-	-
12	-	Mannitol	7.5	+	+
13	-	Trehalose	6.5	-	-
14	-	Trehalose	6.5	+	+
15	-	Trehalose	7.5	-	+
16	-	Trehalose	7.5	+	-

As in other process development activities, the DOE approach can be coupled with high throughput screening methods to examine the impact of a wide range of excipients and other components on several key product characteristics during formulation development.^{45,46,47} The analytical methods applied in the early stages of high throughput screening are biophysical, such as size exclusion chromatography, mass spectrometry, etc., which are more readily automated and can provide significant structural information.

Identifying the buffer conditions that maximize the structural stability is crucial during formulation development. Since the structural stability of a protein is susceptible to different chemical and physical conditions, the use of several complementary techniques can be expected to provide the best answers. Stability measurements that rely on temperature or chemical [urea or guanidine hydrochloride (GuHCl)] denaturation have been the preferred approaches in research laboratories, and together provide a thorough evaluation of protein stability. Automated chemical denaturation has been introduced as a tool in the optimization of formulation conditions for biologics, complementing the role of thermal denaturation for this purpose.⁴⁸

Once a few formulations have been identified by high throughput screening additional biological analyses should be performed to determine the impact of the formulations on potency.

Liquid versus Lyophilized Formulations

When developing a suitable formulation for a monoclonal antibody product, consideration must be given to whether the product will be presented in a liquid or lyophilized form. Of the currently approved monoclonal antibody products listed in Table 8.1, forty three are presented as liquid formulations, while twenty products are presented in a lyophilized formulation and eight are presented in both liquid and lyophilized formulation. A review of the distribution of liquid vs. lyophilized formulations approved as a function of year of approval of the monoclonal antibody product shows a trend (61%) towards the liquid presentation (see Figure 8.8). The decision of liquid or lyophilized presentation is dependent on the suitability of the presentation for the specific monoclonal antibody drug product.



Figure 8.8 Liquid and Lyophilized Formulations for Currently Marketed Therapeutic Monoclonal Antibody Products

Source: BPTC

Year of Formulation Approval

Liquid formulations are usually easier and quicker to develop since they contain fewer components than a lyophilized formulation and do not require as much formulation optimization or any lyophilization cycle optimization during development. As discussed above many companies have simplified formulation development during early product development, by adopting a platform liquid formulation. This formulation may also facilitate development of a lyophilized presentation, if preferred, as the platform formulation already includes a cryoprotectant so that lyophilization may not require significant changes in excipients.

Achieving acceptable long term stability or sufficient protein concentration in a liquid formulation may be difficult. Therefore, many commercial antibody products are produced in a lyophilized format, which may provide greater stability.⁴⁹ Stabilizing the appearance of the lyophilized cake as well as the structure and activity of the antibody frequently requires the inclusion of one or more excipients to provide a more open structure and bulk to the lyophilized cake as well as a more uniform distribution of the protein throughout the cake. Some common excipients used to provide stability during the freezing and drying process and to provide bulk to the final cake include non-reducing sugars such as sucrose and trehalose, sugar alcohols such as mannitol or sorbitol, and a variety of amino acids such as glycine or arginine.⁵⁰

Container/Closure Systems for Monoclonal Antibody Products

The considerations for the commercial drug product container and closure require both marketing and scientific input. This selection will depend on the indication and target market, route of administration, and product format (i.e., liquid or lyophilized). For liquids (and lyophilizates after reconstitution) the container closure must maintain seal integrity and adsorption to the glass vial should be minimized. For lyophilizates the presence of moisture, especially in the autoclaved stopper, as well as the ingress of moisture or oxygen during storage are the key parameters to evaluate.

While most monoclonal antibody products are currently formulated as either liquid or lyophilized products in a glass vial sealed with a rubber stopper, other dosage forms and container/closure systems are being actively investigated as alternatives that may provide more stable or easier to use products. Formulating a monoclonal antibody drug product to make self-administration easier may lead to increased use of pens or pre-filled syringes. For example, Enbrel is now available as a pre-filled syringe in addition to the glass vial preparation originally developed for this product. Prefilled syringes (PFS) have multiple benefits as a delivery system, including, convenience, reduced dosing errors, and minimized risk of contamination.51 There are, however, drawbacks to PFS, mainly related to protein stability resulting from container closure issues. Interestingly, aside from the change in the container/closure system, the formulation of Enbrel was changed from a lyophilized preparation in sodium phosphate buffer, pH 7.17.8, mannitol, sucrose, tromethamine, and benzyl alcohol, to a liquid format in sodium phosphate buffer at a lower pH (6.16.5) containing sucrose, sodium chloride, and arginine (see Table 8.1).

Silicone is often used to coat the stoppers or syringe plungers to ensure consistent insertion of these closures into the drug product containers during manufacturing. Some drug product manufacturers coat the glass vial to minimize adsorption to the hydrophobic surface. However, regulators have become increasingly concerned about the possibility of silicone leaching into the drug product and producing particles.⁵² New developments in drug delivery technology continue to expand the range of available container closure systems and materials.

Often a standard container closure is chosen for early clinical studies, such as a Teflon coated butyl rubber stopper and a type I glass vial, and no further studies of the suitability are done until late in development when a commercial container-closure system is selected. Later in development it will be necessary to perform additional work to demonstrate compatibility of the formulation with the container-closure system, including evaluation of potential extractables and leachables. As mentioned previously, a forced degradation study with the drug product will be required as well to examine any impact of the container closure on the stability of the monoclonal antibody drug.

Glass Delamination

Glass delamination is a well-defined phenomenon in which the interior of a glass vial can flake off.⁵³ This can be a serious issue, particularly in the absence of visible flakes in the drug. The human eye can see 100 micron and larger glass flakes in solution, while inspections of filled vials can typically see 50 micron flakes.⁵⁴ Glass delamination can be a result of product variation that should be understood and controlled sufficiently so that it does not impact product quality.⁵⁵ There are multiple factors that contribute to glass delamination.

Glass delamination can be caused by flame cutting and bottom forming during the manufacture of tubing glass vials.^{54, 56} During this process high heat and high temperature gradients can cause alkali gasses to condense on the inside wall of the vial causing surface disruption.^{54, 56} This stress causes cracking, which results in flakes.

New Antibody Drug Product Formats

Spray dried formulations may provide the same benefits of lyophilized products but have the advantage of being less expensive to manufacture and the spray drying process avoids the potentially damaging freezing operations required for lyophilization. For many years, the development of an acceptable spray drying method for monoclonal antibody products has remained an elusive target.^{57, 58} More recently, substantial progress has been made in the manufacture of high-concentration monoclonal antibody formulations via spray drying and their stable suspension at 200 to 400 mg/ mL in a non-aqueous suspension vehicle with viscosity below 20 centipoise, enabling subcutaneous administration with thin walled needles.^{59,60}

Recent years have seen an increased focus on selfadministered delivery systems, such as PFS outlined above. Self-administration offers the advantages of increased patient compliance, greater ease of use, reduction of pain, and reduced risk of dosing error as compared to intravenous (IV), intramuscular (IM), subcutaneous (SC), or intradermal (ID) injections. PFS and auto-injectors have been available for some time now, offering easier to use products that remain more stable over time. These, however, are still limited by many of the drawbacks as IV and SC injection, including risk of infection and pain.⁶¹ Injection, whether self-administered or not, is the primary delivery system for monoclonal antibodies.

The complex structure of therapeutic monoclonal antibodies, combined with their size, and the need for high doses have made developing delivery methods other than parenteral administration difficult. In general, oral delivery would be the preferred method among both patients and clinicians as this could reduce discomfort and increase patient compliance. Oral administration is limited by gastrointestinal degradation, low bioavailability, slow uptake, and irritation.61 Some progress has been made in developing therapeutic proteins that can pass into the intestinal tract without being digested, but this is as of yet not a viable option for monoclonal antibody delivery. The nasal and pulmonary routes of administration have been examined, and some have reached the market, but they are limited by many of the same drawbacks as oral administration, including gastrointestinal degradation and low bioavailability, making them less than viable options.⁶²

Transdermal delivery systems (TDDS) have emerged has a potential alternative to other routes of administration. TDDS offers many advantages compared to others methods of delivery including, less degradation compared to oral, nasal, or pulmonary delivery; a more controlled delivery rate; easy removal in the event of adverse effects; little to no pain; and added convenience.^{62,63} TDDS patches are well known for delivering a constant dose of medication over a long period of time, conveniently and painlessly. TDDS potentially offers greater control over dosing and improved shelf life and storage resulting from dry formulations.⁶¹

Human skin consists of three layers, the stratum corneum, epidermis, and dermis. The stratum corneum, the outermost layer of the skin, is made up of dead tissue and acts as a barrier against anything but small, potent, and moderately lipophilic molecules.⁶² The epidermis presents the second barrier and consists of living cells, but no blood vessels. This presents an obstacle in delivering high molecular weight monoclonal antibodies through the skin. Multiple approaches of breaching the skin barrier have been explored including the use of chemical enhancers, electric fields, ultrasound, jet injectors, and thermal methods.^{61,62,63}

Liquid jet injection, which eliminates the need for a needle, uses a high-pressure jet to inject a highly viscous formulation in a similar manner as an SC injection. Microneedles are micron-sized needles, a hybrid of a traditional passive transdermal patch and an active injection, capable of penetrating the stratum corneum and epidermis and reaching the dermis, which contains vascular and lymph capillary networks. There are three primary types of microneedles: small needles through which the formulation is injected into the skin; metallic and/or silastic microneedles that are coated with the drug; and metallic and/or silastic microneedles that create conduits in the skin that the drug is applied to following removal of the microneedles.^{61,63}

5. Stability Studies for Monoclonal Antibody Products

Product stability is a critical product attribute, which must be demonstrated and controlled to assure that the product administered to patients during clinical trials or following market approval is as potent as the label claim and contains no harmful degradants. Stability of monoclonal antibody drug products and drug substances must be adequately demonstrated to the regulatory authorities and is specifically expected in any regulatory filing including the initial IND or equivalent that is filed prior to initiation of first-in-human trials.

Appropriately designed stability studies should examine the degradation of both the monoclonal antibody product drug substance and drug product in their respective container/ closure systems at the proposed and accelerated storage conditions over a period of time typically from 12 to 36 months. These studies should be conducted after the completion of forced degradation studies for the determination of the antibody degradation pathways for the monoclonal antibody product. For drug substance stability studies, a scaledown model of the container-closure system is often used to minimize material requirements for the stability studies. Combining the data from all of these stabilities studies allows the manufacturer to propose retest or expiry dating for the drug substance and product. Stability studies should also be conducted on any intermediate held during production as well as for the monoclonal antibody reference standard(s) used for testing of the monoclonal antibody product. This requirement to formally study the stability of the reference standard is a significant difference from the requirements for small molecule therapeutics and is sometimes overlooked by companies developing monoclonal antibody products.64

Stability Data Required for Regulatory Submissions

Only limited stability data on the drug substance and drug product must be included in a regulatory submission to support the initiation of Phase 1 human clinical trials.⁶⁵ While "... stability data are required in all phases of the IND to demonstrate that the new drug substance and drug product are within acceptable chemical and physical limits for the planned duration of the proposed clinical investigation, if very short-term tests are proposed, the supporting stability data can be correspondingly limited...⁷⁶⁶ meaning that it is only necessary to show that the drug substance is stable for the period of time in which it is stored between completion of drug substance manufacturing and the initiation of drug product manufacturing and to demonstrate that the drug product is stable for the duration of the clinical trial.

In an initial regulatory submission for initiation of Phase 1 clinical trials (e.g., IND, IMPD, or equivalent), stability data derived from laboratory studies on development lots and those produced under non-GMP conditions can be included to support the stability of the monoclonal antibody product during the initial human clinical trials. Data from freeze-thaw studies should also be included, especially if the drug substance is stored frozen prior to the manufacture of the drug product or if the drug product is stored frozen. The IND should typically include at least one-month stability data for the drug product to be used in the clinical trial along with a protocol and commitment by the sponsor to continue the stability studies throughout the duration of the clinical trials or beyond.

Despite the need for only limited stability data to support first-in-human clinical trials, stability studies on both the drug substance and drug product are required to support later stage clinical trials and ultimately commercialization of the monoclonal antibody product. These studies may be performed at temperatures both above and below the intended storage temperature of the drug substance and drug product to collect information on the longterm storage of the monoclonal antibody product and to explore potential degradation pathways for the product. ICH Q1A provides detailed guidance on the design of stability studies, however, this guidance67, and the related guidance documents on the interpretation of stability data68, were written specifically for small molecules where the degradation can be appropriately modeled by the Arrhenius equation. Monoclonal antibody products, as with most biologics, generally do not degrade in a similarly straightforward manner so that ICH Q5C, which specifically relates to biologic products, states "conditions [in ICH Q1A] may not be appropriate for biotechnological/biological products."14

When the clinical studies for a monoclonal antibody product have been completed and the marketing authorization application is being prepared, the BLA or equivalent regulatory submission must include data from formal stability studies on at least three primary batches of the antibody drug substance and drug product. The batches should be manufactured, at a minimum, at pilot scale by the same method of manufacture and processes that will be used for commercial batches and a commitment made to place the first three manufacturing scale lots on stability.¹⁴ The overall quality of the batches of drug substance placed on formal stability studies should be representative of the quality of the material that will be manufactured at commercial scale and intended for commercial sale. Based on the data collected from these stability studies, the drug product is given an expiry period after which the product is no longer usable or sellable. Similarly, an expiry period is also established for drug substances. However, for drug substances, a retest period can be established, where the drug substance's expiry date can be extended through periodic testing, provided the re-test results are acceptable and the drug substance remains within specification.

Design of Stability Studies

Stability studies of a monoclonal antibody product must be performed according to written protocols which have been reviewed and approved by, at a minimum, the development and quality departments and must be performed under cGMP, though some analytical methods may not be validated prior to initiating human clinical studies. Any changes to a stability protocol once the stability study has begun must be made under a formal change control system and the risks associated with the changes identified and evaluated. Stability protocols should be prepared and studies conducted.

The analytical methods included in a stability study should include those methods used for the testing and release of the drug substance at the time it is manufactured as well as any additional analytical methods that adequately detect specific degradation products for the monoclonal antibody product. Additional tests may also be included to assist in the characterization of the monoclonal antibody product and to gain further knowledge of the protein's behavior during long-term storage. It is not necessary to include measurement of those product parameters that are not expected to change with time, such as residual host cell DNA or some process related impurities, in the stability study. As with forced degradation studies discussed above, those analytical methods used in the stability study, which are also used for product release testing, should be validated while other analytical methods used in the stability study need only be qualified for their use in the study.

Drug Substance Stability Studies

There is an expectation that stability studies of the monoclonal antibody drug substance performed during development of the product will be conducted at a minimum of at least two temperatures, namely, the proposed storage temperature of the drug substance and an accelerated temperature. For most monoclonal antibody products, the proposed storage temperature for the drug substance will be either frozen at either -20°C or -80°C or refrigerated at 5°C. Regardless of the intended storage temperature, accelerated stability studies are often performed at 25°C, with additional studies performed at 5°C if the drug substance will be stored frozen. While the degradation at the higher temperature cannot substitute for real-time storage data, the absence of degradation during storage at the accelerated temperature could provide strong support for extending the retest (or expiry date) beyond the current real-time data available.

While stability data during process and formulation development can provide meaningful information regarding the stability of the drug substance and stability testing of non-GMP engineering (scale-up) batches or development batches from the representative process can be used to support claims of drug substance stability, a formal stability program for material produced using the final manufacturing process and intended for clinical use must be initiated as soon as a representative batch of drug substance is available. Data from accelerated stability studies and freeze/thaw studies can be used to evaluate the impact of short-term excursions outside the intended storage conditions for the drug substance that might occur during shipping or further processing of the material. When designing stability studies, a sufficient number of samples should be set aside for the study to allow analyses to be repeated if necessary. In practice, this usually means that approximately twice the number of samples required for all analytical tests should be set aside for the study.

The frequency of testing the drug substance in formal, GMP stability studies should follow the recommendations in ICH Q1A.⁶⁷ For drug substances with a proposed re-test period of 12 months or more, the frequency of testing at the intended long term storage temperature should normally be every three months over the first year, every six months for the second year, and annually thereafter through the proposed re-test period. Testing is often also included at one month, especially early in product development, to reduce risk of product failing before being used.

At the accelerated storage condition, a minimum of three time points, including the initial and final time points (e.g., 0, 3, and 6 months), from a six-month study is recommended by regulators. Based on development experience, if the results from accelerated studies are likely to fail specification, increased testing should be conducted either by adding samples at the final time point or by including a fourth, earlier time point (e.g., 1 month as shown in Table 8.7) in the study design. When testing at an intermediate storage condition is necessary due to observation of significant changes at the accelerated storage condition, a minimum of four time points, including the initial and final time points (e.g., 0, 6, 9, 12 months), from a 12-month study is recommended.

The outline of a typical stability study for a monoclonal antibody drug substance to support early stage clinical development is shown in Table 8.7. Since the stability of the product is unknown at this early stage and the product may be stored either refrigerated or frozen, three storage temperatures are included in this study design.

Table 8.7. Typical Stability Study Design for a Monoclonal Antibody Drug Substance to Support Early Stage Clinical Development										
Test	Storage	Typical Specification	Initial	Months on Stability						
	Temperature			1	3	6	9	12	18	
Visual Inspection	-20°C	Clear, colorless solution; essentially free of visible	•	•	•	•	•	•	•	
	5°C			•	•	•	•	•	•	
	25°C			•	•	•				
рН	-20°C	As at release, e.g., 7.2 ± 0.2	•	•	•	•	•	•	•	
	5°C			•	•	•	•	•	•	
	25°C			•	•	•				
Protein Concentration	-20°C	As at release, e.g., 5.5 ± 0.5	•	•	•	•	•	•	•	
	5°C			•	•	•	•	•	•	
	25°C			•	•	•				
Potency	-20°C	75-125% of initial value	•	•	•	•	•	•	•	
	5°C			•	•	•	•	•	•	
	25°C			•	•	•				
SDS-PAGE,	-20°C	Two major bands of molecular weight corresponding to	•	•	•	•	•	•	•	
Teuuceu	5°C			•	•	•	•	•	•	
	25°C			•	•	•				
	-20°C	Antibody heavy and light chains comprise \geq 95% of the	•	•	•	•	•	•	•	
	5°C	וטנמו גנמוודע מודמ		•	•	•	•	•	•	
	25°C			•	•	•				

Test Storage		Typical Specification		Months on Stability						
	Temperature			1	3	6	9	12	18	
Size Exclusion	-20°C	Monomer peak represents ≥95% of the total peak area in	•	•	•	•	•	•	•	
Ginomatography	5°C	- the chromatogram		•	•	•	•	•	•	
	25°C			•	•	•				
	-20°C	Total high molecular weight peaks represent ≤5% of the	•	•	•	•	•	•	•	
	5°C	 total peak area in the chromatogram 		•	•	•	•	•	•	
	25°C			•	•	•				
	-20°C	Total low molecular weight peaks represent \leq 5% of the	•	•	•	•	•	•	•	
	5°C	 total peak area in the chromatogram 		•	•	•	•	•	•	
	25°C			•	•	•				
lon Exchange	-20°C	Retention time of the main peaks correspond to those seen in the Reference Standard		•	•	•	•	•		
Chromatography	5°C			•	•	•	•	•	•	
	25°C			•	•	•				
	-20°C	Main peaks comprise \geq 95% of the total peak area in the	•	•	•	•	•	•	•	
	5°C	- chromatogram		•	•	•	•	•	•	
	25°C			•	•	•				
Bioburden	-20°C	As at release, e.g., ≤ 10 cfu per 100 mL	•					•		
	5°C							•		
	25°C					•				
Endotoxin	-20°C	As at release, e.g., ≤ 1 EU/mL	•					•		
	5°C							•		
	25°C					•				

Table 9.7 Tunical Stability Study Design for a Monoclonal Antibody Drug Substance

Frequently, the stability testing of a monoclonal antibody drug substance covers up to 18 months storage, as shown in the example in Table 8.7. However, stability testing for this length of time is not required as long as the drug substance stability program covers the anticipated time that the material will be stored prior to being used to manufacture

drug product. Nevertheless, it is good business practice to extend the early drug substance stability program to cover as long a storage period as possible to enable greater flexibility in scheduling drug substance and drug product production in the future. On the other hand, if changes are anticipated in the manufacturing process or the drug substance

formulation later in development, the stability studies must be repeated so that stability studies of extended duration for monoclonal antibody drug substances early in development may not be needed. At commercialization, the shelf life of a monoclonal antibody drug substance at refrigerator temperature is typical 18 months to three years.

During the manufacture of the monoclonal antibody drug substance, there may be several points in the manufacturing process where the partially purified monoclonal antibody is held either awaiting the results of some in-process assay, as an intermediate pool during process steps that involve long processing times or extensive cycling, or as a hold point in the manufacturing process. For each such intermediate, it is critical to establish the stability of the intermediate during storage. The design of stability studies for these process intermediates is similar to that of the drug substance stability studies. However, since most process intermediates are generally stored for only for few days or weeks, the studies necessary to demonstrate stability of the intermediate can be relatively short.

Drug Product Stability Testing

The general outline of a two-year stability study for an early stage monoclonal antibody drug product is shown in Table 8.8. The design of this stability study is very similar to that for a monoclonal antibody drug substance with the exception that sterility and particulate matter testing must be included in the drug product stability study, although FDA has recently proposed the use of container closure integrity testing for drug product during stability studies.⁶⁹ The final design of a drug product stability study will vary slightly depending on whether the drug product is stored as a liquid or lyophilized product and a typical drug product stability study is also usually longer in duration than a drug substance stability study to enable accurate assessment of the stability of the product administered to patients in clinical trials. The frequency of testing in a monoclonal antibody drug product stability study should be the same as used for drug substance during the first 12 months of the study; sampling frequency may then be reduced later in the study so that samples are tested at 18 months, 24 months, and yearly thereafter.

 Table 8.8. Typical Stability Study Design for a Monoclonal Antibody Drug Product

 to Support Early Stage Clinical Development

Test	Storage Typical Specification		Initial	Mon	Months on Stability						
	Temperature			1	3	6	9	12	18	24	
Visual Inspection	-20°C	White to off-white cake	•	•	•	•	•	•	•	•	
Lyophilized products	5°C			•	•	•	•	•	•	•	
	25°C			•	•	•					
Visual Inspection, Liquid products or reconstituted lyophilized products	-20°C	Clear, colorless solution; essentially free of visible particles	•	•	•	•	•	•	•	•	
	5°C			•	•	•	•	•	•	•	
	25°C			•	•	•					
рН	-20°C	As at release, e.g., 7.2 ± 0.2	•	•	•	•	•	•	•	•	
	5°C			•	•	•	•	•	•	•	
	25°C			•	•	•					
Residual Moisture	-20°C	1-2%	•	•	•	•	•	•	•	•	
(Lyophilized products only)	5°C			•	•	•	•	•	•	•	
	25°C			•	•	•					

Table 8.8. Typical to Support Early S	Stability Stud Stage Clinical	y Design for a Monoclona Development	al Antibo	ody D	rug P	rodu	ct			
Test	Storage	Typical Specification	Initial	Mon	ths on	Stabil	ity			
	Temperature			1	3	6	9	12	18	24
Protein Concentration	-20°C	As at release, e.g., 5.5 ± 0.5	•	•	•	•	•	•	•	•
	5°C	-		•	•	•	•	•	•	•
	25°C	-			•	•				
Potency	-20°C	75-125% of initial value	•	•	•	•	•	•	•	•
	5°C	-		•	•	•	•	•	•	•
	25°C	-		•	•	•				
SDS-PAGE, reduced	-20°C	Two major bands corresponding	•	•	•	•	•	•	•	•
	5°C	to reference material		•	•	•	•	•	•	•
	25°C	-		•	•	•				
	-20°C	Heavy and light chains comprise	•	•	•	•	•	•	•	•
	5°C	≥95% of total band area		•	•	•	•	•	•	•
	25°C	-		•	•	•				
Size Exclusion	-20°C	Monomer is ≥95% of total peak	•	•	•	•	•	•	•	•
Chromatography	5°C	area		•	•	•	•	•	•	•
	25°C			•	•	•				-
	-20°C	Total high molecular weight	•	•	•	•	•	•	•	•
	5°C	- peaks ≤5% of local peak area		•	•	•	•	•	•	•
	25°C	-		•	•	•				
	-20°C	Total low molecular weight peaks	•	•	•	•	•	•	•	•
	5°C	≤5% of total peak area		•	•	•	•	•	•	•
	25°C			•	•	•				
lon Exchange	-20°C	Retention time of main peaks	•	•	•	•	•	•		
Chromatography	5°C	corresponds to reference material		•	•	•	•	•	•	•
	25°C			•	•	•				
	-20°C	Main peaks comprise ≥95% of	•	•	•	•	•	•	•	•
	5°C	† peak area		•	•	•	•	•	•	•
	25°C			•	•	•				

Table 8.8. Typical Stability Study Design for a Monoclonal Antibody Drug Product to Support Early Stage Clinical Development											
Test	Storage	Typical Specification	Initial	Months on Stability							
	Temperature			1	3	6	9	12	18	24	
Sterility	-20°C	Sterile	•					•		•	
	5°C							•		•	
	25°C					•					
Endotoxin	-20°C	≤1 EU/mL	•					•		•	
	5°C							•		•	
	25°C					•					
Particulate Matter	-20°C	As in USP<788>	•					•		•	
	5°C							•		•	
	25°C					•					

References

- 1 Kozlowski S, Swan P. Current and future issues in the manufacturing and development of monoclonal antibodies. Adv Drug Delivery Rev. 2006 Nov;58(5-6):707-22.
- 2 Wang W, et al. Antibody structure, instability, and formulation. J PharmSci. 2007 Jan;96(1):1-26.
- 3 Kanda Y, et al. Comparison of biological activity among nonfucosylated therapeutic lgG1 antibodies with three different N-linked Fc oligosaccharides: the high-mannose, hybrid, and complex types. Glycobiology. 2006 Jan;17(1):104-18.
- 4 Wen J, et al. Effect of carbohydrate on thermal stability of antibodies. Am Pharm Rev. 2008 Sep;11(6):98-104.
- 5 Manning MC, et al. Stability of protein pharmaceuticals: an update. Pharmaceutical Research. 2010 Apr: 27(4): p. 544-575.
- 6 Banga AK. Therapeutic peptides and proteins formulation, processing and delivery systems. Second ed. Boca Raton (FL): CRC Press, Taylor and Francis Group; c2006. Chapter 3, Stability of therapeutic peptides and proteins; p. 67-90.
- 7 Avery NC, Bailey AJ. Restraining cross-links responsible for the mechanical properties of collagen fibers: natural and artificial. In: Frantzl P, editor. Collagen structure and mechanics. New York: Springer Science and Business Media LLC; 2008. p. 81-110.
- 8 Goswami S, et al. Developments and Challenges for mAb-Based Therapeutics. Antibodies. 2013, 2, 452-500; doi:10.3390/antib2030452
- 9 Molloy S, et al. Optimized UV detection of high-concentration antibody formulations using high-throughput SE-HPLC. J Pharm Sci. 2015 Feb;104(2):508-14. doi: 10.1002/jps.24240. Epub 2014 Nov 12.
- 10 Carpenter J, et al. Overlooking sub-visible particles in therapeutic protein products: gaps that may compromise product quality. J Pharm Sci. 2009 Apr;98(4):1201-5.
- 11 United States Pharmacopeial Convention. The United States Pharmacopeia–National Formulary (USP32–NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008 <788> Particulate matter in injections p. 310-2.
- 12 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Development and Manufacture of Drug Substances (Chemical Entities and Biotechnological/Biological Entities Q11[Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2012 May 1 [cited 2015 Jul 15] 30 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ ICH_Products/Guidelines/Quality/Q11/Q11_Step_4.pdf
- 13 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Stability testing: Photostability testing of new drug substances Q1B Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1996 Nov 6 [cited 2015 Jul 15]. 12 p. Available from: http:// www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1B/Step4/Q1B_Guideline.pdf.
- 14 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Quality of biotechnological products: Stability testing of biotechnological/biological products Q5C Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1995 Nov 30 [cited 15 Jul 2015]. 10 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5C/Step4/Q5C_Guideline.pdf.

- 15 Inactive Ingredients Database [Internet]. Silver Spring (MD): U.S. Food and Drug Administration, Center for Drug Evaluation and Research. [date unknown]-[cited 2015 Jul 15]. Available from: http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm.
- 16 International Pharmaceutical Excipients Council of the Americas. IPEC-Americas, Inc. [Internet]. Arlington (VA): IPEC-Americas; [cited 2015 Jul 15]. Available from: http://www.ipecamericas.org.
- 17 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Impurities: Guideline for residual solvents Q3C(R5) Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2011 Feb 4 [cited 2015 Jul 15]. 29 p. Available from: http://www.ich.org/ fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3C/Step4/Q3C_R5_Step4.pdf.
- 18 Allen G. Determination of peptide sequences. In: Work TS, Burdon RH, editors. Laboratory techniques in biochemistry and molecular biology. Vol. 9, Sequencing of proteins and peptides. Second ed. Amsterdam: Elsevier Science Publishers B.V.; c1989. p. 207-93.
- 19 Sellars SP, Yuh-Fun M. Principles of biopharmaceutical protein formulation: an overview. In: Smales CM, James DC, editors. Therapeutic proteins: methods and protocols. Vol. 308, Methods in molecular biology. Totowa (NJ): Humana Press Inc; 2005. p. 243-64.
- 20 Pikal-Cleland KA, et al. Effect of glycine on pH changes and protein stability during freeze-thawing in phosphate buffer systems. J Pharm Sci. 2002 Sep;91(9):1969-79.
- 21 Gomez G, et al. Effect of initial buffer composition on pH changes during far-from-equilibrium freezing of sodium phosphate buffer solutions. Pharm Res. 2001 Jan;18(1):90-7.
- 22 Zheng JY, Janis LJ. Influence of pH, buffer species, and storage temperature on physicochemical stability of a humanized monoclonal antibody LA298. Int J Pharm. 2006 Feb 3;308(1-2):46-51.
- 23 Kuelto L, et al. Effects of solution conditions, processing parameters, and container materials on aggregation of a monoclonal antibody during freeze-thawing. J Pharm Sci. 2008 May;97(5):1801-12.
- 24 Salinas BA, et al. Understanding and modulating opalescence and viscosity in a monoclonal antibody formulation. J Pharm Sci. 2010 Jan;99(1):82-93. doi: 10.1002/jps.21797.
- 25 Jovanovic N, et al. Distinct effects of sucrose and trehalose on protein stability during supercritical fluid drying and freeze-drying. Eur J Pharm Sci. 2006 Mar;27(4):336-45.
- 26 Lee J, Timasheff S. The stabilization of proteins by sucrose. J Biol Chem. 1981 Jul 25;256(14):7193-201.
- 27 Akers MJ, et al. Formulation development of protein dosage forms. In: Nail SL, Akers MJ, editors. Development and manufacture of proteins. Vol. 14, Pharmaceutical Biotechnology. New York: Kluwer Academic/Plenum Publishers; c2002. p. 47-128.
- 28 Levine HL, et al. The use of surface tension measurements in the design of antibody-based product formulations. J Parenter Sci Technol. 1991 May-Jun;45(3):160-5.
- 29 Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int J Pharm. 1999 Aug 20;185(2):129-88.
- 30 Lam XM, et al, inventors; Genentech Inc., assignee. Antibody formulation. United States patent US 6,171,586. 2001 Jan 9.

- **31** Tarelli E, et al. Recombinant human albumin as a stabilizer for biological materials and for the preparation of international reference reagents. Biologicals. 1998 Dec;26(4):331-46.
- 32 Bosse D, et al. Phase I comparability of recombinant human albumin and human serum albumin. J. Clin Pharmacol. 2005 Jan;45(1):57-67.
- 33 Halozyme Therapeutics. Halozyme announces Roche receives marketing authorization for Herceptin SC in European Union. News Release, San Diego, CA, 2013 Sep 2. Available from: http://www.halozyme.com/Investors/News-Releases/2013/default.aspx.
- 34 Hoffmann-La Roche. Roche's new time-saving subcutaneous formulation of MabThera approved in Europe for the treatment of common forms of non-Hodgkin Lymphoma. Media Release, Basel, Switzerland, 2014 Mar 28. Available from: http://www.roche.com/med-cor-2014-03-28-e.pdf.
- 35 Halozyme Therapeutics. Halozyme announces Roche marketing authorization for MabThera SC for patients with common forms of Non-Hodgkin Lymphoma in European Union. News Release, San Diego, CA, 2014 Mar 28. Available from: http://www.halozyme.com/Investors/News-Releases/2014/default.aspx.
- 36 Chang BS, inventor. Protein formulations containing amino acids. United States patent application 20140127227 A1. 2014 May 8.
- 37 Excelse Bio, Thousand Oaks, CA, www.excelsebio.com.
- 38 Lam XM, et al, inventors; Genentech Inc., assignee. Antibody formulation. United States patent US 6,991,790. 2006 Jan 31.
- 39 Lorenz T, et al. Developability Assessment of Biologics by Integrated Biologics Profiling. Am Pharm Rev. 2014 Jul-Aug;17(5): 71-75.
- 40 Jarasch A, et al. Developability assessment during the selection of novel therapeutic antibodies. J Pharm Sci. 2015 Mar 26. doi: 10.1002/ jps.24430.
- 41 Williams-Smith DL, et al. Changes in apparent pH on freezing aqueous buffer solutions and their relevance to biochemical electron-paramagneticresonance spectroscopy. Biochem J. 1977 Dec 1;167(3):593-600.
- 42 Warne N. From clone to clinic the art and the science of an optimum development strategy. Presented at International Business Communications Life Sciences' Early to Late Stage Bioprocess Development Summit; 2008 Oct 20-22; Boston, MA.
- 43 Montgomery DC. Design and analysis of experiments. Seventh ed. Hoboken (NJ): John Wiley and Sons; c2009. 680 p.
- 44 Chen B, et al. Strategies for rapid development of liquid and lyophilized antibody formulations, an integrated design. BioProcess Int. 2004 Jan;2(1):48-52.
- 45 Kras E. Monoclonal antibody development: A case study utilizing temperature stress and agitation-induced forced degradation techniques to identify and optimize a liquid drug product formulation for an IgG1 monoclonal antibody Presented at Cambridge Health Institute's PepTalk; 2009 Jan 11-16; San Diego, CA.
- 46 Simler R, et al. Maximizing data collection and analysis during preformulation of biotherapeutics. BioProcess Int. 2008 Nov;6(10):38-45.
- 47 Das T. Early stage protein formulation development and use of high throughput screening methods. Presented at the American Association of Pharmaceutical Scientists' National Biotechnology Conference; 2007 Jun 24-27; San Diego, CA.

- **48** Freire E, et al. Chemical denaturation as a tool in the formulation optimization of biologics. Drug Discov Today. 2013 Oct;18(19-20):1007-13. doi: 10.1016/j.drudis.2013.06.005. Epub 2013 Jun 21.
- 49 Daugherty AL, Mrsny, RJ. Formulation and delivery issues for monoclonal antibody therapeutics. Adv Drug Deliv Rev. 2006 Aug 7;58(5-6):686-706.
- 50 Meyer JD, et al. Impact of bulking agents on the stability of a lyophilized monoclonal antibody. Eur J Pharm Sci. 2009 Aug 12;38(1):29-38.
- 51 Badkar A, et al. Development of Biotechnology Products in Pre-filled Syringes: Technical Considerations and Approaches. AAPS PharmSciTech. 2011 May: 1-9.
- 52 Sharma DK, et al. Silicone microdroplets in protein formulations. Pharm Tech. 2009 Apr;33(4):74-9.
- 53 Iacocca RG. Glass Delamination: A Pharmaceutical Perspective. Presented at RX 360 Symposium on Glass Delamination; 2011 May 25; Arlington, VA.
- 54 Kraus D. Tubular Glass. Presented at Rx360 Delamination Symposium; 2011 May 25 Arlington, VA.
- 55 Evans K. Rx360: Glass Delamination Scientific Symposium: Audit and Inspection Findings. Presented at RX360 Delamination Symposium; May 25, 2011; Arlington, VA.
- 56 Guadagnino E. Product Contact and Glass Interactions. Presented at RX360 Delamination Symposium; May 25, 2011; Arlington, VA.
- 57 Andya JD, et al. The effect of formulation excipients on protein stability and aerosol performance of spray-dried powders of a recombinant humanized anti-IgE monoclonal antibody. Pharm Res. 1999 Mar;16(3):350-8.
- 58 Maury M, et al. Spray-drying of proteins: effects of sorbitol and trehalose on aggregation and FT-IR amide I spectrum of an immunoglobulin G. Eur J Pharm Biopharm. 2005 Feb;59(2):251-61.
- 59 Armstrong AJ, et al, inventors; Genentech Inc., assignee. High-concentration monoclonal antibody formulations. United States patent application 20130309226 A1. 2013 Nov 21.
- **60** Gikanga B, et al. Manufacturing of High-Concentration Monoclonal Antibody Formulations via Spray Drying—the Road to Manufacturing Scale. PDA J Pharm Sci Technol. 2015 Jan-Feb;69(1):59-73. doi: 10.5731/pdajpst.2015.01003.
- 61 Ford R. Transdermal Delivery of Macromolecules using Macroflux Technology. Presented at NCCAVS-Thin Film Users Group. June 17, 2009.
- 62 Kalluri H, Banga AK. Transdermal Delivery of Proteins. PharmaSciTech. 2011 March: 12(1): 431-441.
- 63 Fukashima K et al. Two Layered Dissolving Microneedles for Percutaneous Delivery of Peptide Protein Drugs in Rats. Pharma Res. 2011: 28: 7-21.
- 64 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Specifications: test procedures and acceptance criteria for biotechnological/biological products Q6B Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1999 Mar 10 [cited 2015 Jul 20]. 20 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf.

- 65 Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Office of Regulatory Affairs (US). Guidance for industry: cGMP for phase 1 investigational drugs [Internet]. Washington, DC: Department of Health and Human Services; 2008 Jul [cited 2015 Jul 20]. 20 p. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ UCM070273.pdf.
- 66 Investigational New Drug Application, 21 CFR Section 312 (1987).
- 67 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Stability testing of new drug substances and products Q1A(R2) Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2003 Feb 6 [cited 2015 Jul 20]. 24 p. Available from: http://www.ich. org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2__Guideline.pdf.
- 68 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Evaluation for stability data Q1E Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2003 Feb 6 [cited 2015 Jul 20]. 19 p. Available from: http://www.ich.org/fileadmin/Public_Web_ Site/ICH_Products/Guidelines/Quality/Q1E/Step4/Q1E_Guideline.pdf.
- **69** Food and Drug Administration, Center for Biologics Evaluation and Research, Center for Drug Evaluation and Research, Center for Devices and Radiological Health, Center for Veterinary Medicine. Guidance for industry: container and closure system integrity testing in lieu of sterility testing as a component of the stability protocol for sterile products [Internet]. Washington, DC: Department of Health and Human Services; 2008 Feb [cited 2015 Jul 20]. 9 p. Available from: http://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM146076.pdf.

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CHAPTER 9:

Drug Product Manufacturing

he manufacture of a monoclonal antibody drug product from the associated drug substance involves three critical steps, formulation, sterilization, and aseptic filling of the product (shown schematically in Figure 9.1). In the first step, formulation, the protein concentration is adjusted to the desired level for storage and administration, and excipients, such as buffering agents, stabilizers, or cryoprotectants, are added to the bulk drug substance solution to ensure the stability of the product in the final container. For most monoclonal antibody products, especially during the early stages of development, formulation of the product with its final excipients is often incorporated into the drug substance manufacturing process so that no additional formulation is required in the manufacture of the drug product.

Figure 9.1. Steps in the Manufacture of a Monoclonal Antibody Drug Product



Following formulation of the monoclonal antibody, the formulated bulk drug substance is pooled, diluted and sterilized by filtration to remove all bioburden from the solution and produce a sterile product prior to filling. This sterile bulk drug product is then aseptically filled into suitable sterile and depyrogenated containers and either stoppered and sealed with an appropriate closure for liquid products or lyophilized under aseptic conditions in the final container and then stoppered and sealed with an appropriate closure for lyophilized products. For most monoclonal antibody products, the drug product is filled into a presterilized vial or syringe, although other containers, such as IV bags, are occasionally used as discussed in Chapter 8.

1. Formulated Bulk Drug Substance

The development of suitable formulations for monoclonal antibody drug products is described in detail in Chapter 8. Many monoclonal antibody drug products are formulated as sterile liquids in their final form but some are lyophilized in order to improve stability and extend the shelf life of the product. These lyophilized preparations are often supplied with a vial or prefilled syringe containing a suitable diluent for reconstitution. This diluent is typically sterile WFI but can also contain additional buffering agents or stabilizers. The diversity of monoclonal antibody product formulations can be seen in the list of product formulations for all currently marketed monoclonal antibody products in Chapter 8. Monoclonal antibody products are usually formulated in simple buffered salt solutions, often with the addition of a non-ionic surfactant (e.g., polysorbate 80 or 20) to prevent aggregation. For lyophilized products, appropriate bulking agents and/or cryoprotectants (e.g., sucrose, mannitol) are often added as well. For currently approved monoclonal antibody products, the volume of liquid drug product filled into the final container varies from 0.25 mL to 50 mL depending on the container, the intended human dose and the antibody concentration in the product. For reconstitution of lyophilized monoclonal antibody drug products, the volume of sterile WFI or buffer used depends on the intended human dose, route of administration and required final antibody concentration.

Most monoclonal antibody products are formulated at the end of the drug substance manufacturing so that no additional formulation is required during manufacture of the drug product. However, in some cases the drug substance may be stored in a different solution or at a different concentration than the drug product. In these cases, formulation of the drug substance is required during drug product manufacturing and may include dilution of a concentrated bulk drug substance to the final desired antibody concentration, diafiltration of the drug substance into a different buffer, or addition of stabilizers, bulking agents, or cryoprotectants. The need for reformulation of a monoclonal antibody drug substance during drug product manufacturing will depend on the properties of the specific monoclonal antibody, its stability in the final drug product formulation, and the capabilities of the drug product manufacturing facility.

Monoclonal antibody bulk drug substances are usually stored in either stainless steel or single use, disposable containers, shown to be compatible with the drug substance solution and storage conditions. Often the drug substance is stored in multiple containers and the containers are pooled into one vessel as the first step in drug product manufacturing. Generally, formulation of monoclonal antibody drug products is done in the same types of stainless steel or disposable containers as are used for drug substance storage. For both drug substance storage and drug product manufacturing, appropriate compatibility studies must be conducted to ensure that no unacceptable materials are leached into the product and that any undesirable reactions, e.g., aggregate formation, as a result of contact of the product with the container, are minimized.

Since drug product manufacturing typically does not include any product purification steps, it is important that all potential impurities and contaminants are removed during drug substance manufacture and that the formulated bulk drug substance is stable. Precautions should be taken during drug product manufacture so that the process and equipment do not cause any negative impact on the product quality, especially increases in the level of aggregation of the product. Because of the potential immunogenicity that can result from protein aggregates,¹ formation of aggregates due to agitation at the air-liquid interface during sterile filtration or aseptic filling operations or temperature fluctuations, such as the thawing of bulk drug substances that are stored frozen or the freezing of the drug product during lyophilization, must be minimized.

2. Sterile Filtration

Following formulation of the drug substance, the formulated bulk drug substance solution must be sterilized and dispensed into appropriate containers for use. Due to the fragile nature of proteins and their general instability to heat or irradiation, sterilization of monoclonal antibody products is achieved by filtration of the antibody solution through a pre-sterilized sterilizing-grade filter immediately before the filling process. A sterilizing filter must be capable of removing all microorganisms, including bacteria, yeasts, and fungi, from the process fluid while not leaching or shedding any material into the product filtrate or binding or removing the monoclonal antibody product from the formulated bulk drug substance. Sterilizing-grade filters are generally constructed of biocompatible membranes with a pore size rating of $0.22 \,\mu\text{m}$ or less,² which are rated based on the ability of the filter membrane to retain microorganisms of size represented by specified strains and not by determination of an average pore size and statement of distribution of sizes.³

Since filtration of the formulated bulk drug substance

through a sterilizinggrade filter will not remove viruses, endotoxin, or any other adventitious agents smaller than the pore size of the filter, it is important that these potential impurities be removed or significantly reduced during the drug substance manufacturing process and that safeguards are taken not to reintroduce them during drug product manufacturing. To ensure that no such adventitious agents are introduced into the drug product, all drug product manufacturing operations, especially those done under aseptic conditions, are done under strictly controlled environmental conditions, and routine environmental monitoring, including monitoring of all manufacturing personnel, are conducted during all aseptic manipulations.

The bioburden of the formulated bulk drug substance is determined just prior to filtration. Normally, the specifications for the monoclonal antibody bulk drug substance will specify that the bioburden of the product be relatively low, typically ≤ 10 CFU/mL, and any formulation operations or other manipulations of the drug substance, including compounding, or pooling of bulk solutions, is done in a clean environment to minimize potential microbial contamination. The processing times of the bulk drug substance through formulation and sterile filtration should be minimized in order to prevent any potential increase in bioburden prior to filtration. Increased bioburden in the drug substance can negatively impact product quality and lead to increased endotoxin levels in the product.

Following sterile filtration, the sterile bulk drug substance should be tested in accordance with the relevant pharmacopeia sterility guideline (e.g., USP <71> Sterility Test⁴) to confirm that the bulk product is sterile prior to aseptic filling. Sterility testing of the sterile bulk drug substance should include appropriate tests for bacteriostasis and fungistasis to confirm that the product itself does not inhibit the actual sterility test. Typically, a sample of the sterile bulk drug substance from the initial volume of product passing through the sterile filter is collected and used to perform this test.

3. Container/Closure Systems

The regulatory guidances on the manufacture of drug products emphasize the requirements for container/closure systems of suitable quality. The nature of the materials used to manufacture these components can have a direct effect upon the stability of the product. Injectable products must be filled into containers that will not add to or delete anything from the product, and the closure system must be capable of maintaining a hermetic seal to prevent both the entry of oxygen into the product and contamination by adventitious agents. If the product is to be lyophilized, then the closure must also prevent the entry of water vapor into the container.

Containers

A variety of container/closure systems are used for monoclonal antibody drug products, with many products presented in multiple formats. Glass vials and pre-filled syringes are the most commonly used containers for these products although alternative container/closure systems are also becoming popular.

Vials

Monoclonal antibodies, like most biopharmaceuticals, are commonly filled into clear glass vials, especially for early clinical studies. The glass vial is the least expensive, simplest, and most convenient container available for parenteral products. The equipment for filling vials is less complicated than for alternative container systems, such as syringes, and aseptic filling of glass vials is readily available at contract manufacturers.

Glass vials used for monoclonal antibody products are almost always of USP Type I (ASTM Type I, Class A) quality or equivalent. As specified by the USP,⁵ Type I glass or borosilicate glass, contains high concentrations of boric oxide, aluminum oxide, and alkali and/or alkaline earth oxides. Type I glass is heat-resistant, chemically inert, and typically has a low level of extractable substances.

Occasionally, Type II glass may be used for a monoclonal antibody product, but this is rare. Type II glass is a chemically treated form of soda-lime glass, a silica glass containing alkali metal oxides.⁵ Soda-lime glass has a moderate hydrolytic resistance due to the chemical composition of the glass itself and is classified as Type III glass.⁵ When the inner surface of a Type III glass container is treated to improve its hydrolytic resistance, the glass becomes Type II glass. Type III glass is not recommended for use with monoclonal antibodies.

Syringes

For monoclonal antibody products where a fixed volume is

administered by direct injection, prefilled syringes (PFS) are becoming an increasingly common. As of November 1, 2014, approximately 25% of the marketed therapeutic monoclonal antibody products include a PFS packaged product (See Chapter 8). Compared to typical vial systems, PFS have more components in their container closure systems including barrel, plunger, needle and needle sheath cover.

The use of prefilled syringes has expanded significantly in the past several years, and the rate of adoption is expected to continue to be strong. There are multiple drivers for this expansion including minimizing drug waste and improving administration convenience, which translates to better patient compliance. Prefilled syringes accommodate volumes that typically range from 0.25 to 5.0 mL, and therefore, are best suited for products administered by subcutaneous or intramuscular injection.

For PFS, the syringe barrels made of either glass or plastic may be used. As with vials, glass syringes are non-reactive and generally comprised of Type I glass so are readily accepted by the regulatory authorities. Plastic syringes have several benefits over glass by providing improved robustness against breakage and lighter weight. Plastic materials used for PFS are mainly of two types, cyclopoly-olefin polymer or cyclo-olefin copolymer. These cyclic olefin copolymers and polymers have excellent transparency, good moisture barrier properties, are chemically clean with very low extractables, break resistant, have low protein surface adsorption, are compatible with wide pH range solutions, have good dimensional tolerance and with high flexibility in design. There are limitations to the plastic syringe system, such as the risk of scratching during machining, reduced moisture and oxygen barriers and limited high temperature resistance.

Pen injectors and auto-injectors, used in combination with PFS, are particularly common for self-administered insulin injections and other hormone replacement therapies, but are also used in the home-based monoclonal antibody treatment of major therapeutic areas like autoimmune disease. Continued innovations in container closures include advances in PFS/ cartridge technologies, dual chamber devices, electronicallyenabled delivery devices and needle-free injection technologies. Blow-fill-seal technology is also gaining interest as an alternative to glass based containers. This technology combines aseptically forming a plastic container, filling and sealing the container in a continuous process that reduces or eliminates many concerns associated with traditional glass vials, including the risk of injuries to treatment providers and patients, the potential for glass particulate contamination and accidental breakage in transit, and subsequent product wastage. A unique advance in the blow-fill-seal technology is ADVASEPTTM, where a stopper is inserted during the blow-fillseal process to create a glass-free stoppered vial.⁶

Closures

Vials used for monoclonal antibody drug products are closed by elastomeric stoppers usually made of a synthetic rubber compound that may be treated/coated to reduce non-specific protein adsorption, leaching of rubber components into the drug product, or for ease of handling. In the case of PFS, a plunger serves as the primary closure. In order to achieve a nearly effortless injection process, it is necessary to apply a silicone oil coating on the inside of a glass barrel to provide lubricity between the plunger and the glass barrel. Surface silicone oil has been known to cause protein aggregation. Alternatives to silicone have been developed and include fluoropolymer films like FluorTec® barrier film, and to be acceptable for monoclonal antibody products, the closures must be compatible with the drug product and have low levelf of extractablef, leachablef, and volatile substances. Stoppers/ plungers must also have low moisture and gas transmission rates and in the case of vial/stopper combination, must have a high resistance to coring or fragmentation on needle penetration to prevent contamination with particulates when the vial contents are withdrawn for administration. In addition, stoppers used to seal vials of lyophilized products must have low moisture absorption from the washing and autoclaving processes used to clean and sterilize the stopper.

The most common rubber formula used in stoppers for parenteral use is a butyl rubber, which may also be coated with a layer of a fluorinated compound such as polytetrafluoroethylene (Teflon) on the surfaces that may contact the contents of the container. This fluorinated layer will further reduce the extractables from the stopper, minimize the passage of gas or water vapor into or out of the vial, and also minimize non-specific binding of the monoclonal antibody product to the stopper.

Rubber preparations used for stoppers have evolved considerably over the past twenty years as manufacturers

have strived to develop more stable, inert, and compatible rubbers for this purpose. A comparison of typical rubber formulations used for stoppers prior to 1980 with those typically used today is shown in Table 9.1.⁷ The relatively simple rubber formulation used today attests to the simplification and improvements in stopper rubber formulation that has been achieved in the last twenty years.

Table 9.1. Improvements in Rubber StopperFormulations

Before 1980		Today	
Halobutyl Aluminum Silicate Crepe Calcium Carbonate Phenolic Resin Isobutene Silicone Oil Zinc Oxide Titanium Dioxide Antioxidant Stearic Acid Color	40% 23% 16% 3% 2% 2% 2% 1% 1% 1% 1% <1%	Halobutyl Magnesium Silicate Magnesium Oxide Titanium Dioxide Sulfur Color Other	51% 40% 3% 2% <1% <1% <2%

The rubber stopper is sealed into the vial by the application of an aluminum ring seal, which is crimped over the rim of the vial neck. Such seals usually carry a colored plastic disk, which is a flip-off top covering the area of the stopper through which the needle will pass when the contents are withdrawn. Once removed, this disc cannot be replaced, thus providing an indication of the integrity of the closure.

Container/Closure Preparation

The preparation of the container/closure components for drug product manufacturing is a critical process consisting of thoroughly washing and then sterilizing the components. They are washed and given a final rinse with WFI prior to sterilization. After washing/rinsing, glass containers are depyrogenated by dry heat at high temperatures up to 250°C in order to destroy any residual bacterial endotoxins and sterilize the vials. In today's drug product manufacturing facilities, this is usually done batch-wise in an oven or continuously by passing the clean container on a conveyor belt through a tunnel where they are sterilized by radiant heaters and then cooled under HEPA-filtered air. The conveyor delivers the container directly into Class 100 (ISO Class 5)¹⁰ filling machine enclosure so that they are not exposed to the outside atmosphere upon exiting the tunnel.

In order to facilitate the handling of rubber stoppers by the filling and stoppering machines used in aseptic manufacturing, the stoppers are often treated with silicone oil after washing and prior to sterilization.8 This silicone treatment aids the stopper insertion process, but may lead to the leaching of silicone into the final product, so it must be carefully controlled. An improved stopper preparation method, which applies a cross-linked silicone coating to the stoppers, provides the benefits of easy handling without the high risk of silicon leaching seen with the silicone oil treatment.7 The silicone coating can be selectively applied to the bottom and/or top surfaces of the stopper as desired. Coating the top surfaces of the stopper is often done for stoppers used in lyophilization as this process will help prevent the stoppers from sticking to the underside of the lyophilizer shelves during the closure process. Following washing and silicone treatment, stoppers are sterilized by autoclaving, usually at 121°C, before use in drug product manufacturing.

Preparation of syringes for filling is slightly different from the process used for vials. Rubber components are washed and siliconized in a similar manner as for vials, but are then sterilized by gamma irradiation. Syringe barrels are washed and then siliconized to enable the plunger to glide smoothly within the syringe body. The siliconized syringe is then typically sterilized by ethylene-oxide gassing or irradiation.

4. Aseptic Filling

Filling the drug product or any associated reconstitution diluent or placebo into the final container must be done under tightly controlled conditions described in guidance documents published by the US and European regulatory authorities.^{2,9} These guidance documents specify that the filling and stoppering of open drug product containers must be done under ISO Class 5¹⁰ or equivalent conditions. The ISO specifications for a Grade A (ISO Class 4/5) environment specify that the air supplied to such an area contains no more than 3,500 particles of 0.5 µm in diameter per cubic meter, no particles greater than 5 µm in diameter, and no viable microorganisms.9 There are various ways to achieve these environmental requirements. Conventional clean rooms are the most common technology employed to achieve ISO specifications for aseptic filling lines, but this technology is no longer considered state of the art. There are two newer

technologies used in aseptic manufacturing that ensure higher levels of contamination-free product, the isolator and the restricted access barrier system, commonly referred to as RABS.^{11,12} Isolators have been installed in hundreds of filling lines and enable full isolation of the machinery from the environment resulting in the highest aseptic quality. However, such units require more complex procedures making them more effective in monolines that run the same product continuously, especially products requiring containment such as antibody drug conjugates. RABS is a newer technology relative to isolators, and has seen rapid growth and adoption in aseptic manufacturing operations. RABS combines the high aseptic safety of an isolator with the flexibility of a conventional cleanroom. It is more commonly used in a multi-product CMO environment due to higher changeover speed and process flexibility. A significant technology trend for aseptic processing is the integration of small isolators with robotics, which may enable sterility assurance of isolators with improved operational flexibility and changeover times.

Because isolators create fully enclosed environments, these units can be installed in Grade D (ISO Class 8) rooms; whereas, clean rooms and RABS must operate within grade A/B (ISO Class 4/5) rooms. Irrespective of the surrounding room classification, all operators working in these areas wear appropriate non-shedding, sterilized clothing and are properly trained in aseptic procedures.^{2,9}

It is also important to note that cGMP compliance for the manufacture of drug substance is phase appropriate as development moves from Phase 1 clinical trials to commercial manufacturing;¹³ however, there is no such phase appropriate approach to cGMP compliance for aseptic drug product manufacturing. Therefore, all equipment and processes used in aseptic drug product manufacturing must be fully validated, even if the development, manufacture, and testing of the product is at a relatively early stage of development.

5. Lyophilization

Because water can have a dramatic effect on protein stability, it can be difficult to achieve acceptable stability of a monoclonal antibody drug product with a liquid formulation. Lyophilization can often help to stabilize the drug product during shipping and storage at refrigerated or ambient temperatures.^{14, 15} Less than half of the monoclonal antibody products on the market as of October 31, 2016 are presented as lyophilized drug products.

Prior to lyophilization, the drug product is formulated, sterile filtered, and filled into glass vials or into one of the chambers of a dual chamber syringe. Following filling, the containers are partially stoppered, placed into trays, and then transferred to the lyophilizer under aseptic conditions. To maintain sterility of the product, the transfer of the containers from the filling line to the lyophilizer is done in an ISO Class 5 environment.

The lyophilization process itself begins with the freezing of the monoclonal antibody drug product containers. During freezing, the shelf temperature of the lyophilizer is lowered well below the eutectic point of the monoclonal antibody drug product solution so that ice crystals are formed causing the unfrozen fraction of the formulation to become increasingly concentrated. Freezing continues until water and all solutes in the drug product solution are completely frozen.

As part of the freezing process, an annealing step, or temperature cycling, is often included to help avoid uncontrolled moisture release by removing a fraction of the excipient from the amorphous phase and thereby increasing the collapse temperature of the product. In the annealing process, the temperature of the lyophilizer shelf is cycled up and down in temperature to increase the size of ice crystals in the container as an aid to lyophilization. However, care should be taken during the annealing phase to ensure that the crystals are not too large to prevent the formation of in-homogeneities in the lyophilization cake, which can result in an abnormal volume, or appearance of the cake following lyophilization.

Once the drug product solution is completely frozen and any annealing completed, the lyophilizer chamber is evacuated and the frozen solution is dried by sublimation of the ice from the product. This sublimation is driven by the extent of the vacuum in the lyophilizer chamber and the temperature of the shelves. As sublimation proceeds, the shelf temperature is slowly increased to drive off water vapor as it sublimes from the frozen solution. In order to ensure a uniform, dry cake, it is important that the drying temperature be kept below the collapse and eutectic melting temperatures of amorphous and crystalline solutes, respectively. Above the melting temperature, the melting of the crystalline solutes can lead to collapse of the cake structure and an unacceptable appearance of the final product. At the end of the primary drying stage, the final residual amounts of moisture left in the drug product are removed by secondary drying, a process in which the vacuum is slowly released and the shelf temperature increased to ambient or higher temperatures. The temperature and extent of secondary drying will influence the moisture content of the final product.

After the lyophilization process is complete, the chamber is slowly filled with an inert sterile gas, such as argon or nitrogen, to completely break the vacuum and bring the containers back to atmospheric pressure. Back-filling the containers with an inert gas will aid in product stability by preventing oxidation. Once the chamber has returned to atmospheric pressure, the stoppers are fully seated in the vials by slowly lowering the shelf to push the stoppers into the vials. Prefilled syringes with dual chambers can have the contents of one chamber lyophilized and the second chamber filled with diluent following the lyophilization process.

Lyophilization generally requires a significant development effort to find appropriate conditions to obtain a final drug product with an acceptable process cycle time, moisture level and appearance. Cycle times can vary widely from 24 hours to longer than 96 hours; however these longer cycle times are very expensive to operate so most companies invest in additional lyophilization formulation development to achieve a shorter cycle time. The size of the lyophilizer also will dictate the batch size in terms of the number of vials, i.e., large vials will result in smaller sized batches relative to smaller vials. As a result, it is important that the lyophilization cycle be well defined and that the product temperature, as well as the shelf temperature and pressure, be controlled over the defined time periods of the lyophilization cycle. The specific cycle used to freeze dry a monoclonal antibody drug product will vary with the product formulation and the specific equipment used for lyophilization. However, it is important that the cycle yield a firm, goodlooking cake with a moisture content typically no greater than 5%, which is important for reconstitution time and solubility. The moisture content of the drug product is a specification for product release, expiration dating, and stability and should be established based on worst-case data.

Alternative strategies for stabilizing monoclonal antibody products are currently being investigated, although

none have been adopted in a commercial setting. Like lyophilization, freeze-spray drying relies on the sublimation of ice for removal of water from the product. It involves spraying the protein solution through atomizing nozzles into a freezing medium such as liquid nitrogen. The protein and excipients are rapidly frozen and the resulting material collected on precooled trays. The ice is then sublimated under vacuum. Compared to conventional freeze drying, spray freeze drying results in porous particles that exhibit favorable aerodynamic properties for pulmonary delivery.

Unlike lyophilization and freeze-spray drying, spray drying and vacuum drying do not depend on sublimation of ice for removal of water, but rely instead on the evaporation of liquid water. In the case of spray drying, this is accomplished via a very brief (millisecond) exposure of microdroplets of the protein solution to elevated temperatures (801200 C) in a drying chamber. Preservation of activity and reduction of aggregation of the protein is provided through a combination of evaporative drying of the droplets and the use of an excipient matrix that usually contains a mix of glassy carriers such as sucrose, trehalose, and arginine. Unlike lyophlization, spray drying is a continuous process and so scale increases can be accomplished with longer processing times and not through the required use of larger equipment. Aseptic spray drying options are available, but are not commonplace. In addition, the need for subsequent aseptic solids filling for parenterally administered products, and the need to handle powdered materials under aseptic conditions must also be considered with this option. While spray drying of a monoclonal antibody product has not yet been implemented commercially, pulmonary delivery of spray-dried recombinant insulin has been commercialized.

With vacuum drying, the protein solution is brought to rapid, controlled boiling under highly -controlled conditions of reduced atmospheric pressure in specially modified freeze dryers. As with all of the other techniques described, stabilization of the product and reduction of protein aggregation is provided by the stabilizing excipients such as amino acids and disaccharides. Unlike spray drying, vacuum drying can be accomplished directly in vials following aseptic filling. As stated, the use of these alternative methods has not yet been widely adopted for monoclonal antibodies in a commercial setting. However, pulmonary delivery of spray-dried insulin has been commercialized.

6. Storage and Labeling

Drug product is usually stored unlabeled in sealed trays or boxes until all QC release testing has been performed and the product is released for further processing. Once released, containers are individually labeled, packaged into cartons of one or more units depending on the intended use of the product. Cartons are then packaged into larger boxes containing multiple cartons for shipment to a clinical site or distributor. To prevent mislabeling, there must be written procedures designed to ensure that the correct labels are used and to fully account for all labeling and packaging materials.¹⁶

Most monoclonal antibody drug products are stored refrigerated at 28°C so that the shelf life dating of the product starts on the date the product is released by the Quality Assurance department. Some monoclonal antibody drug products are stored frozen at -20°C or -70°C during early development if a robust formulation has not yet been developed. In these cases, the filled drug product should be frozen as soon as possible after filling and the shelf life of the product is measured from the date of freezing. The shelf life of a monoclonal antibody product and its proper storage temperature are determined through stability testing as described in Chapter 8.

7. Release Testing

A monoclonal antibody drug product must pass a series of release tests for identity, potency, purity, and strength, similar to those used for the bulk drug substance. As with most biologic products, many of the biochemical tests related to the structure, purity, and integrity of the monoclonal antibody product itself are conducted on the drug substance and not repeated for the drug product. As a result, the biochemical tests for a monoclonal antibody drug product are typically limited to a subset of those used to test the bulk drug substance. All tests that will be used in a stability study are typically included in the drug product release tests. In addition, the drug product specifications will also include specifications for the number of insoluble particulates present, the volume filled into the vial for liquid products or residual moisture for lyophilized products, bacterial endotoxin, and specific tests for excipients included in the drug product formulation. While all lots of monoclonal antibody drug product must be tested for sterility, the destructive nature of this test limits the number of units from each lot that can be tested to a relatively small number.⁴

A typical set of specifications and release tests for a monoclonal antibody product are shown in Table 9.2. These tests and the corresponding specifications may vary depending on the exact nature of the product, the intended clinical indication, and dose. In addition, specifications for monoclonal antibody products for early stage clinical trials are generally broader than those for Phase 3 clinical trials or commercialization. Product specifications are typically finalized and established based on the product dose, compendial limits, and process history.

Table 3.2. Typical monocional Antibody brug i roduct specifications						
Test	Test Method	Typical Specification				
Appearance	Visual inspection	For liquid products, a specification of "Clear, colorless solution essentially free of visible particles" or similar is common. For lyophilized products, the specification is typically "White to off-white powder" or similar. For lyophilized products, a second appearance test for the contents after reconstitution is added. This specification is often "Clear, colorless solution with no visible particles" or similar.				
Moisture content ^a	Karl Fischer	Residual moisture content will vary from product to product but is typically ${\leq}5\%$				
Reconstitution time ^a	Measure time for cake to dissolve using calibrated stopwatch	Specification will vary from product to product but is typically \leq 5 min				

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	Table 9.2.	Typical	Monoclonal	Antibody	Drug	Product S	specifications

Table 9.2. T	Table 9.2. Typical Monoclonal Antibody Drug Product Specifications						
Test	Test Method	Typical Specification					
pHb	Potentiometric method according to appropriate compendial method	Target pH \pm 0.2 pH units					
Osmolality	Osmometer	Target 270 to 320 mmol/kg					
Identity ^b	SDS-PAGE or HPLC	Conforms to standard					
Purity⁵	Aggregate content determined by SEC HPLC	≥95% monomer; ≤5% aggregate/fragment					
Strength ^b	Protein concentration determined by UV absorbance at 280 nm and extinction coefficient	Target concentration $\pm 10\%$					
Potency ^b	Specific method will vary depending on characteristics of the monoclonal antibody product. Typically, a binding assay of some sort is used.	Specification will vary depending on method, but generally: Report EC 50 Result (Mean EC50 of the test sample is within 50 to 150% of the mean reference standard value)					
Excipient concentration ^b	Specific method will vary depending on excipient measured	Target concentration $\pm 10\%$					
Particulate Matter ^{b, c}	Tested by light obscuration using the appropriate compendial method	≤6,000 particles/vial of size ≥10 μm; ≤600 particles/vial of size ≥25 μm					
Sterility	Determined by the appropriate compendial method	Sterile					
Bacterial Endotoxin	Turbidimetric or gel clot method according to the appropriate compendial method	Limit set based on dose, compendial limits, and process history. Typically endotoxin specifications are less than 1 EU/mg protein					

^a For lyophilized products only.

^b Performed after reconstitution for lyophilized products.

^c Alternatively, microscope methods may be used. If so, the release specification will be different.

Under the original 21CFR610.11 regulations for manufacture and testing of biologics, a general safety test (GST) was required for all biologic products to ensure the safety, purity, and potency of the product. However, in 2003, FDA acknowledged that the GST may not be relevant or necessary for certain biologic products such as monoclonal antibodies and amended the regulations to allow companies to file for an exemption from the GST requirements provided they submit suitable information to demonstrate that appropriate production controls and quality assurance safeguards are in place. Today, this exemption is granted almost universally for monoclonal antibody products and is generally not included in the drug product release specifications.¹⁷

8. Validation of Drug Product Manufacturing Process

Drug product manufacturing must be validated from the early stages of development, starting with the production of materials for Phase 1 human clinical trials. Failure of the drug product manufacturing process to produce a sterile, non-pyrogenic product poses a critical risk to patients. Thus, regulatory authorities worldwide expect that those procedures that directly impact sterility and other safety concerns be validated even for the earliest clinical trials.

References

- 1 Vazquez-Rey M, Lang DA. Aggregates in monoclonal antibody manufacturing processes. Biotechnol Bioeng. 2011 Jul;108(7):1494-508.
- Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Office of Regulatory Affairs (US). Guidance for industry: sterile drug products produced by aseptic processing current good manufacturing practice [Internet]. Washington, DC: Department of Health and Human Services; Sep 2004 [cited 2016 Nov 22]. 63 p. Available from: http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070342.pdf.
- 3 United States Pharmacopeial Convention. The United States Pharmacopeia–National Formulary (USP32-NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008. <1211> Sterilization and sterility assurance of compendial articles; p. 720-5.
- 4 United States Pharmacopeial Convention. The United States Pharmacopeia–National Formulary (USP32-NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008. <71> Sterility tests; p. 80-6. Updated in USP32-NF27 Suppl(1):3494.
- 5 United States Pharmacopeial Convention. The United States Pharmacopeia–National Formulary (USP32-NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008. <660> Containers glass; p. 243-6. Updated in USP32-NF27 Suppl(1):3919.
- 6 Shah D. Compatibility assessment of model monoclonal antibody formulation in glass and blow fill seal (BFS) plastic vial delivery formats. Presented at the American Association of Pharmaceutical Scientists' National Biotechnology Conference; 2014 May 19-21; San Diego, CA.
- 7 Schafers M. Considerations for the selection of elastomer closures for lyophilized products. Presented at the International Society of Lyophilization and Freeze Drying and the Pharmaceutical and Healthcare Sciences Society's Fourth International Conference on Lyophilization and Freeze Drying; 2007 Oct 17-19; Dublin, Ireland.
- 8 Parenteral Drug Association, Lubrication of Packaging Components Task Force. Siliconization of parenteral drug packaging components, technical report no. 12. J Parenter Sci Technol. 1988 Jul-Aug;42(4S):S1-13.
- 9 European Commission (BE). EudraLex volume 4: EU guidelines to good manufacturing practice, medicinal products for human and veterinary use. Annex 1: manufacture of sterile medicinal products (corrected version) [Internet]. Brussels: European Commission; 2008 Nov 25 [cited 2016 Nov 22]. 16 p. Available from: http://ec.europa.eu/health/files/eudralex/vol-4/2008_11_25_gmp-an1_en.pdf.
- 10 International Organization for Standardization (CH). Cleanrooms and associated controlled environments -- Part 1: classification of air cleanliness [Internet]. Geneva: International Organization for Standardization; 2015. (ISO 14644-1:2015). [10 screens] [cited 2016 Nov 22]. Available from: https://www.iso.org/obp/ui/#iso:std:53394:en.
- 11 Fruergaard CE. Trends in sterile manufacturing technology. Presented at the International Society for Pharmaceutical Engineering Thailand Affiliate's Annual Meeting. 2013 Jul 17-19; Bangkok, Thailand.
- 12 Lysfjord J. ISPE Definition-Restricted access barrier systems (RABS) for aseptic processing, Pharm Eng. 2005 Nov-Dec; 25(6): 93 p.
- 13 Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Office of Regulatory Affairs (US). Guidance for industry cGMP for phase 1 investigational drugs [Internet]. Washington, DC: Department of Health and Human Services; 2008 Jul [cited 2014 Dec 2]. 20 p. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/ Guidances/ucm070273.pdf.

- 14 Wang BS, Pikal MJ. Stabilization of lyophilized pharmaceuticals by process optimization-challenges and opportunities. Am Pharm Rev. 2012 Sep-Oct;15(6):[11 p.].
- 15 Patel SM, Pikal MJ. Emerging freeze-drying process development and scale-up issues. AAPS PharmSciTech. 2001 Mar;12(1):372-8.
- 16 Packaging and Labeling Operations, 21 CFR Part 211 Section 130 (2005).
- 17 Revision to the general safety requirements for biological products. Federal Register 2003 Mar 4;68(42):10157-60.

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CHAPTER 10:

Comparability

uring development of a monoclonal antibody product, changes in the manufacturing process are inevitable. The amount of material required for evaluation of safety and efficacy will increase at each stage of development, so changes in production scale or facility to meet the increasing material requirements are often made throughout development. In addition, full process development and characterization are rarely completed prior to the initial Phase 1 clinical trials but should be completed prior to initiating Phase 3 clinical trials. Therefore, process improvements and changes are normally made concurrent with Phase 1 and 2 clinical development of a monoclonal antibody product. In addition, changes in formulation are often made after the initial Phase 1 first in human clinical trials for use in later stage clinical trials and commercialization. While these changes are considered normal in an antibody development program, there is a regulatory requirement to insure that the product resulting from a new process (post-change product) is sufficiently comparable to the product used earlier in development (pre-change product).¹ If process changes result in a product that is significantly different from the pre-change product, safety and efficacy data from the toxicology and early clinical trials may not be applicable so that these studies may need to be repeated with product manufactured by the new process. A major goal of process development is to optimize and scaleup a manufacturing process so that the post-change product is comparable to the pre-change product, minimizing the need to repeat the time-consuming and expensive non-clinical and/or clinical trials.

Throughout development of a monoclonal antibody product, maintaining comparable safety and efficacy of the product as process changes or scaleup occurs is of primary importance. For changes made very early in development, the focus of any comparability analysis will be primarily on assessing those changes in the product that impact safety. These changes include changes in the type of levels of process-related impurities and contaminants, changes that may impact product half-life or distribution in the patient, or changes that significantly alter biological properties of the product such as target binding. For changes made following clinical assessment of efficacy during Phase 2, the focus should be on both safety and efficacy.² When the mechanism of action of the monoclonal antibody product is well understood, evaluating the impact of product changes on clinical efficacy is easier than for products where the mechanism is poorly understood. For example, the activity of the monoclonal antibody is governed by its ability to selectively bind to the intended therapeutic target in the body and, in most cases, to activate other components of the immune system such as antibody dependent cell-mediated cytotoxicity.³ Antigen binding function resides in the complementarity determining region (CDR) of the variable region of both the heavy and light chains, so molecular changes in the CDRs are more likely to impact product efficacy than changes outside of the CDRs. For monoclonal antibodies of the IgG1 subtype, and to some extent also IgG2 effector functions, which activate other immune system components can be a significant component of the antibody's efficacy

Process changes made in the drug substance manufacturing process have the potential to impact product quality and heterogeneity since these are determined primarily in the bioreactor while product purity is largely governed by the downstream process. Changes in formulation and drug product manufacturing also have the potential to impact product quality and performance, particularly with respect to stability and product aggregate levels. Therefore, any significant change in a monoclonal antibody manufacturing process requires an evaluation of the potential impact of these changes on the safety and efficacy of the post-change product.

1. What is Comparability?

During development of a monoclonal antibody product, changes may be made in the manufacturing process to increase product yield or purity or improve the robustness and reproducibility of the manufacturing process. Changes may also be made in the scale of manufacturing or the facility used to produce the product. Such changes are permitted under the current regulations for biopharmaceutical products, however, the companies developing monoclonal antibody products are required to demonstrate comparability between the prechange and post-change product whenever such process, scale, or facility changes have the potential to result in significant changes to the product.^{4, 5, 6} The purpose of this evaluation is to determine whether prior pre-clinical or clinical studies performed with the pre-change product are still relevant to the post-change product by assessing any differences in quality that may impact efficacy and safety. Ideally, the post-change product will be comparable and the clinical development program can continue without interruption. However, if major differences are seen that are likely to impact safety or efficacy, additional non-clinical or clinical bridging studies or additional process modifications may be needed to demonstrate comparability. If comparability between the pre- and postchange products cannot be demonstrated, a more complex development decision is required, including such options as developing the post-change product as a new product or not implementing the new process at all.

To be considered comparable, pre-change and post-change product do not need to be identical, however, the pre- and post-change products must not differ significantly in safety, identity, purity, and potency, especially in any way that would increase toxicity or immunogenicity or decrease efficacy of the product. As defined in ICH Q5E comparable is "a conclusion that products have highly similar quality attributes before and after manufacturing process changes and that no adverse impact on the safety or efficacy, including immunogenicity, of the drug product occurred ..."⁶

Comparability of pre- and post-change product may be assessed by only biochemical and biophysical testing or may require additional animal and human studies depending on the nature of the process change and the resulting impact on the product.

For analytical testing, an increasing array of analytical methods is available to evaluate the comparability of monoclonal antibody products following process changes. While the number of potential variants present in a monoclonal antibody product is extensive (see Figure 10.1), it is important to determine which features contribute to overall antibody function, stability, and safety and to design suitable comparability protocols to evaluate potential changes in these variants rather than attempting to measure every possible change in the product structure that might occur. For example, in most cases evaluated to date, changes in the amount of cleavage of the C-terminal lysine residues have shown little impact on product function, whereas oxidation of critical amino acid residues within the CDR is likely to reduce target binding and overall product efficacy.^{7,8} By contrast, some post-translational changes, such as changes in glycan structure, can impact effector function and half-life and thereby impact the pharmacokinetic or pharmacodynamic profile of the product. If major differences in glycosylation are observed pre- and post-change, it will be difficult to assess the impact of changes in this quality attribute with in vitro assays only. Effector function is strongly dependent on the glycan structure that is formed at a single N-linked glycosylation site on each of the two heavy chains.9 This structure can be sensitive to changes in the cell culture conditions since changes in the glycan composition are known to increase or decrease function. For example, higher levels of sialylated glycan structures can reduce functionality, whereas addition of galactose or removal of fucose from the glycan is known to improve ADCC activity.^{10, 11, 12} Significant biochemical changes in the CDR or glycan structure are therefore likely to impact safety or efficacy and might require additional clinical evaluation to determine the extent of the impact.

Figure 10.1. Potential Sources of Monoclonal Antibody

Heterogeneity sites represented on the structure of the complete monoclonal antibody consisting of two light and two heavy chains. The symbols indicate the points along the amino acid chains that are subject to reactions or post translational modifications.



Adapted with permission from Reference 13

One critical attribute of monoclonal antibody products that is always important for comparability is similar function so that any assessment of comparability of monoclonal antibody products must include a measure of bioactivity (potency). To demonstrate comparable bioactivity for a pre- and post-change product, a cell or animal based potency method is usually required; the use of surrogate analytical methods such as an ELISA assay may not be sufficient.

Another key regulatory and technical consideration in evaluating the impact of process changes on product quality is the type and levels of impurities in the pre-change and postchange product. In general, a product can still be considered comparable if the level of a process-related impurity is reduced by the process change. However, if a new, previously unseen, product-related impurity appears at significant levels in the post-change product or if the levels of previously seen impurities increases, then the product will most likely require additional safety evaluation in animals and possibly humans to determine whether it is comparable. This is because the new product-related impurity has not previously been tested in patients or evaluated in toxicity studies, so its impact on the safety or immunogenicity of the product is unknown¹⁴.

2. Regulatory Requirements for Submission of Comparability Information

Several different guidances have been issued by regulatory agencies worldwide providing recommendations for managing process changes, including when and how to submit information regarding these changes to the regulatory authorities.^{1, 5, 6} However, these guidances apply predominantly to post-approval changes for previously approved products. The FDA guidance document does not mention process changes for products in clinical development,5 while the ICH guidance provides minimal information regarding changes during development.6 The EMEA has issued the most comprehensive guidance for managing process changes for products that are in clinical development, but this guidance is also predominantly focused on post approval process change management.¹

For monoclonal antibody products that are already approved for commercialization in the United States, companies are required to inform FDA of any manufacturing changes. The timing of submission of information regarding the process change and the need for pre-approval of the change prior to distribution of the post-change product will vary depending on the significance of the change.¹⁵ For the simplest of changes where there is very minimal potential to adversely affect the quality of the product, changes may be implemented by the Sponsor as soon as they are developed and validated and the resulting product released for distribution without prior regulatory approval. These changes are summarized in the Sponsor's annual report at the end of the year in which the change is implemented.

For those changes that are still relatively simple and where there is moderate potential for the change to impact the product quality, a CBE supplement is required. With a CBE supplement, FDA has determined that, based on experience with a particular type of change, the post-change product may be distributed immediately upon receipt of the supplement by FDA. Alternatively, with a CBE-30 supplement, the Sponsor must wait at least 30 days after receipt of the submission by FDA for any questions or response from the agency. If FDA has no questions about the submission or the process change, the product may then be released for distribution.

For those changes, which have a high potential to impact the quality of the product or to result in a post-change product that is not comparable to the pre-change product, a PAS is required. Changes requiring a PAS include such major changes as a change in the Master Cell Bank or significant changes to the purification process for the product. The requirements for notification of other regulatory agencies worldwide of process changes is similar to that in the United States although different agencies refer to the submissions required differently. The different regulatory submissions required worldwide to support process changes are summarized in Table 10.1.¹⁶

While the published guidances are more specifically applicable to post-approval process changes, regulatory agencies expect Sponsors to demonstrate that the product used at all stages of clinical development has comparable biochemical properties.1 This enables establishment of a direct link between the early stage safety and efficacy results of non-clinical and Phase 1 or 2 studies with the presumed safety and efficacy of the product intended for use in pivotal Phase 3 clinical trials. To meet this requirement, small comparability assessments throughout development will be performed as the process is optimized and scaled-up, although these studies are often limited to comparisons of just one batch of pre- and post-change product since many development programs do not require large numbers of batches to meet the early clinical demands for product development. Despite the expectation that product remain comparable throughout development, the regulatory authorities understand and expect that process changes will be made during development.

Table 10.1. Regulatory Submissions Worldwide Supporting Process Changes						
United States	Canada European Union		Japan			
PAS	Level 1: Supplemental New Drug Submission (sNDS)	Type II Variation	Partial Supplement Application			
CBE-30	Level 2: Notifiable Change	Type II Variation Type I Variation (currently restricted to Drugs only)	Subsequent notification			
Annual Report	Level 3: Notice of Change	N/A	N/A			
N/A ^a	Level 4	N/A	Documentation within quality systems			

^a = Not applicable

Changes made during Phase 1 or Phase 2 clinical development of a product are typically summarized in an amendment to the appropriate regulatory submission (e.g., IND or IMPD) for the product. This amendment will include a description of the process change and all available characterization data to demonstrate comparability of the pre- and post-change product. There is no requirement for Sponsors to submit a comparability protocol prior to conducting the comparability exercise for these changes. In fact, a formal comparability protocol may not even be prepared at this early stage, although it is considered good practice to design a protocol to ensure the appropriate testing is performed to demonstrate comparability, especially for significant process changes.

When approaching Phase 3 clinical trials, if major process changes are made, they should be discussed with FDA as part of a Type B meeting, such as the End-of-Phase 2 meeting. At this point, the changes would already have been implemented and product manufactured so the discussion with the regulatory authorities will focus on a review of the comparability data rather than pre-approval by the agency. Where process changes are introduced after the Phase 3 trial material has been manufactured and no additional clinical studies using this post-change material are planned to support the marketing authorization, the comparability exercise should be as comprehensive and thorough as one conducted for a post-approval change. In these cases, it is often advisable to discuss the process changes with the regulatory authorities prior to implementing them although it is not required. The BLA or MAA submission for the product will include a discussion of the process change during Phase 3 clinical trials and the comparability data available to support the change.

3. Timing and Risk Assessment

During initial monoclonal antibody product development, the pressure to develop a cell line and manufacturing process quickly to enable initiation of first-in-human safety and efficacy testing often leads companies to accelerate development through use of broadly applicable but nonoptimized platform processes, "good-enough" production cell lines, and simple formulations. While safety of the product at all stages is critical and should never be compromised, these short-cuts do lead to requirements for process improvements later in development of the product to improve process economics, meet later stage clinical and commercial demands, and enable a fully robust and reliable manufacturing process. Any steps taken in early development to shorten the time to the clinic at the expense of a fully optimized process will carry some risk that the product from a process that is optimized later in development will not be comparable and will require additional cost and time to repeat some non-clinical and/or clinical development activities. Individual companies must evaluate their risk tolerance compared to timeline pressure and determine what risks are acceptable early in development.

Comparability is evaluated anytime there is a significant process change, but the level and requirements for comparability assessment will depend on the stage of development, the known characteristics of the specific product, and the nature of the process change. While ICH Q5E briefly describes the expectations for comparability assessment for products in early development, the primary focus of the guidance is on post-approval changes.⁶ There are no regulatory requirements to perform a comparability assessment if non-clinical and GLP toxicology studies have not yet been performed since these studies will be performed using product from the most current process. Similarly, if minor process changes are introduced following animal toxicity studies but prior to GMP manufacture of clinical trial materials, comparability testing may not be required. However, all regulatory agencies expect companies to perform appropriate comparability studies to evaluate the impact of any significant process change that occurs following production of material for animal toxicology testing.

Under normal development conditions, companies rarely make changes to the manufacturing process between toxicology and Phase 1 clinical trials. In many cases, the first production run at scale is used to perform the toxicology evaluation and the subsequent run(s) support the clinical trials. The first production run may be performed without full batch record review by Quality Assurance, but no significant changes are expected to be introduced between this initial scale-up run and the first cGMP run. Process changes that often do occur in early development include scale up, facility change either from an in-house pilot plant to a CMO or to a different in-house facility, and changes in storage containers to accommodate available storage conditions. Later in clinical development, process improvements continue to be made while the clinical trial is ongoing, so changes in the production media, feed strategies, column elution conditions, or other process operations are the types of changes that are often implemented prior to Phase 3 and that require more extensive comparability analysis. Finally, because comparability assessment can be expensive, some companies choose to implement multiple process changes simultaneously so that they can be assessed by a single comparability study.

Process changes range from minor, where the risk of not producing a comparable product is minimal, to major, where there is some risk of failure to produce a comparable product. Minor changes include a different raw material supplier, assuming the material is the same and a sufficient incoming raw material testing program is in place, or a change in the method of preparation of a buffer used in downstream processing, assuming that the final buffer composition is unchanged. On the opposite end of the spectrum are changes in the production cell line or a change in one of the chromatography resins used in the downstream process, which would be considered major changes.

To determine if a comparability program is needed, and if so, to determine how extensive the program should be, a risk assessment, as outlined in ICH Q9 should be conducted.¹⁷ Evaluating the risk of a process change (or series of changes) is easier if the relationship between product characteristics and quality and the manufacturing process is known. However, this is almost never the case early in product development and may even be difficult to determine later in the product life cycle. The risk assessment should include a review of all process development data available as well as review of batch records from manufacturing campaigns executed to date, in-process monitoring and testing results, product release testing, and stability data. During this review, the range of product attributes seen throughout development and manufacturing is evaluated and used to determine the acceptable range for each characteristic or property of the monoclonal antibody product and whether it is likely to be impacted by the proposed process change. Although the risk assessment should focus on the specific antibody and its characteristics, some general guidelines for assessing the risk of process changes and the likely requirements for comparability testing are shown in Table 10.2.

Requirements in Early Development					
Process Change	Comparability Risk	Typical Comparability Program			
Facility transfer	Low	Release testing including potency, structural characterization, accelerated stability			
Facility transfer with minor process changes	Low-Medium	All tests for facility transfer plus antigen binding affinity assessment, ADCC or other functional assays (if relevant)			
Changes in bioreactor or purification operations	Medium	All of the above, with potential PK and/or PD studies in animals			
Cell line change	Medium to High	All of the above, potentially GLP toxicology studies, and human bridging studies			

Table 10.2. Risk Assessment and Comparability

4. Comparability Protocols

To initiate a comparability study, a well-defined, detailed plan to assess the effects of process changes on the product is assembled based on known product properties, availability of pre-change product, process development data, and the proposed process change. Companies are not required to submit such comparability protocols for assessment of process changes early in development (typically prior to Phase 2 clinical trials), but they may choose to do so in later stages of development to ensure regulatory review and acceptance before execution. The protocol should describe the process changes being evaluated and the proposed analytical and stability studies that will be performed to demonstrate comparability. All proposed studies should include pre-defined acceptance criteria, which, if met, will show that the product is comparable, and if not met, will result in the initiation of a new set of studies. A sample decision tree, developed by BioProcess Technology Consultants, outlining the testing requirements for comparability and the consequences of passing or failing each test on the overall product development is shown in Figure 10.2.




Source: BPTC

For process changes made early in development, a representative batch of pre-change product, including process intermediates if available, is compared to one or more batches of the post-change product. Ideally, the representative batch of pre-change product is a batch that was used in GLP toxicology and/or human clinical testing. For changes made later in development, such as between Phase 2 and Phase 3 clinical trials, multiple batches of post-change product should be compared to all batches of pre-change product to allow for statistical analysis of the comparability data. However, due to the fact that the number of number of batches of both pre- and post-change product that may have been produced may be limited, it is often difficult to perform a full statistical analysis of any analytical data making the demonstration of comparability more difficult later in development when regulatory agencies expect this statistical analysis. In these

cases, the data from full scale manufacturing batches can be supplemented with data from process development and scale-down models of the process to generate sufficient statistical data to demonstrate comparability.

The comparability protocol should contain detailed information on the pre-change process with a side-by-side comparison to the proposed post-change process and a justification for the process change. For example, if the scale of production of the post-change product is larger than the pre-change process, more cells will be required for inoculation of the larger production bioreactor. To generate sufficient cells for inoculation of the new bioreactor, the post-change process will either include an additional intermediate bioreactor for expansion of the cells prior to the production bioreactor or will include multiple seed trains at the original scale that are then combined to make the larger inoculum. Either of these approaches for inoculum preparation are acceptable and easy to justify, however, the use of a new intermediate bioreactor may result in a greater number of population doublings for the entire culture, which is more likely to have an impact on the product than the use of multiple smaller scale inoculum. Likewise, increasing the size of a chromatography column to accommodate a larger scale is readily justified but may have a significant impact on the product quality or purity if the scale-up requires changes in the scalable column parameters or operating conditions (e.g., residence time). More complex changes such as changes in the chromatography process or media feed strategy require additional justification with support from process development data. This data, if available, should be included as background in the comparability protocol.

Depending on the process change being implemented, the comparability study may not need to evaluate the entire process to demonstrate comparability. For example, if a change is being made to the chromatography media in the final polishing step in the manufacturing process with all other steps remaining the same, the comparability protocol should focus on that particular chromatography step and on the subsequent step(s) in the process. Conversely, if the changes are limited to the upstream, cell culture section of the process, the comparability study may compare material produced at full scale through harvesting the bioreactor and purified using a qualified scale-down model of the downstream process.

To facilitate setting acceptance criteria for each analytical test that will be performed on the post-change material, it is useful to review process development reports and data showing the historical ranges of results for each method. Of particular importance are the analytical results for product batches that were used in either animal toxicology studies or human clinical trials. Finally, the background information in the comparability protocol should include a discussion of the known or anticipated characteristics of the product pre- and post-change and any data or information on the impact of the proposed changes on these characteristics. Specific knowledge of product quality attributes can be used to justify the selection of analytical methods and the acceptance criteria for each method. In the absence of knowledge about these quality attributes, more extensive product testing may be required to evaluate comparability.

The comparability protocol should also include a full description of the analytical tests that will be performed, the samples that will be analyzed including in-process samples as well as final purified bulk drug substance from different batches, and the acceptance criteria for tests (numerical limits, ranges, or other criterion) on which comparability will be based. The toolbox of tests deployed to assess comparability should be carefully chosen to maximize the potential for detecting relevant differences in the pre- and post-change product. The list of tests to be employed in a comparability protocol, along with a brief description of each test method and any statistical analyses that will be performed should be included in the protocol. Analytical methods should have sufficiently sensitive to detect subtle changes in the quality of the post-change product relative to the pre-change product and the ability of each test used to measure changes in the product should be assessed prior to its use in a comparability protocol. Any of the analytical methods that are used as release tests for previous batches manufactured using the pre-change process may be included as part of the comparability program as well as additional methods used to characterize the product. In some cases, it may also be necessary to develop new analytical methods specifically to support comparability if the process change results from the introduction of a new raw material that may end up in the final product or if existing analytical methods are not sufficient to fully determine the impact of the product change on the post-change product. A list of analytical methods and their corresponding specifications often used for release testing of a monoclonal antibody product that may be included in a comparability protocol is shown in Table 10.3.

Table 10.3. Typical Monoclonal Antibody Product Release Tests Used in Comparability Protocols			
Parameter	Test	Example Limit or Range for Comparability	Recommended Comparison Samples
General	Appearance	Clear, colorless to slightly yellow solution (dependent on protein)	Historical lot release data
	рН	Target pH \pm 0.3	Historical lot release data
	Osmolality	Target osmolality \pm 10%	Historical lot release data

Table 10.3. Typical Monoclonal Antibody Product Release Tests Used in Comparability Protocols				
Parameter	Test	Example Limit or Range for Comparability	Recommended Comparison Samples	
Quantity	Protein concentration (UV)	$10.0 \text{ mg/mL} \pm 1.0 \text{ mg/mL}$	Historical lot release data	
Identity	IEF	Comparable to the reference standard	Side-by-side using frozen stored samples	
	Tryptic peptide map (HPLC)	Comparable to the reference standard	Side-by-side using frozen stored samples	
	Mapping of oligosaccharides	Comparable to the reference standard	Side-by-side using frozen stored samples	
Purity	HP-SEC	Monomer ≥95.0%	Side-by-side using frozen stored samples	
	CGE, reduced CGE, non-reduced	Sum of LC and HC: ³ 95.0% Main band: ³ 90.0%	Side-by-side using frozen stored samples	
	Cation exchange chromatography	Target purity $\pm 10\%$ Distribution of isoforms comparable to reference standard	Side-by-side using frozen stored samples	
	HCP ELISA	≤20 ng/mg protein	Historical lot release data	
	DNA	≤10 pg/mg protein	Historical lot release data	
	Protein A ELISA	≤50 ng/mg protein	Historical lot release data	
Potency	Binding activity (ELISA)	60-140% of the reference standard	Side-by-side using frozen stored samples	
	Cell-based bioassay	60-140% of the reference standard	Side-by-side using frozen stored samples	
Safety	Bacterial Endotoxins (LAL)	≤0.25 EU/mg	Historical lot release data	
	Bioburden	≤10 CFU/100 mL	Historical lot release data	

The acceptance criteria for methods that are used routinely for product release should be the same as the release specification for the product or, if no specific release specification has been established, the acceptance criteria can be set based on the actual process history. Upper limit levels are normally set for process-related impurities, whereas product related quality attributes will normally have ranges for the acceptance criteria. For certain tests, a comparison of historical data for the pre-change product to actual test results for the post-change product may be sufficient. These tests include such tests as appearance, pH, osmolality, protein concentration, host cell protein, host cell DNA, residual Protein A, endotoxin and bioburden. For more complicated tests, side-by-side analysis is generally performed if sufficient pre-change product is available. Retain samples from the pre-change process that have been frozen and are known to be stable are re-analyzed side-by-side with samples from the new process, both of which should be compared to a suitable reference standard. In cases where the pre-change drug substance is unavailable

due to stability reasons or lack of retained sample, the prechange drug product may be substituted provided that the intended methods can be performed on the formulated drug product. The tests that require side-by-side analysis include isoelectric focusing, gel electrophoresis, peptide mapping, glycan content, glycan mapping, size exclusion and ion exchange chromatography, and potency. For the analytical methods listed in Table 10.3, the recommended comparison (historical data vs. side by side comparison) is also indicated. Depending on the stage of development these analytical test methods may or may not be fully validated, however, for the comparability protocol, analytical methods should, at a minimum, be qualified and scientifically sound to measure and obtain trustworthy results. This analytical testing may be performed in a company's quality control laboratory under GMP or for products in early development the comparability analysis may be performed in a research laboratory under controlled, documented conditions using good scientific practice.¹⁸ To complement the data from standard release

testing, additional characterization tests may be conducted to more closely examine product changes in the product that may impact safety or efficacy or to obtain a more detailed assessment of the molecular changes that occur in postchange product.¹⁹ These analytical methods are not generally validated, but should be qualified to ensure confidence in the test results. The characterization tests listed in Table 10.4 are used to assess primary, secondary, and higher order structure, charge variants, glycosylation, oxidation, and purity, tend to be more complicated than the analytical methods used for routine product release. Because these tests are not used for routine product release, it is likely that there will only be limited historical data available to use in a comparison of pre- and postchange product. Therefore, side-by-side analysis of post-change product with pre-change product (if available) in these tests is preferable.

Table 10.4. Characterization Tests used inMonoclonal Antibody Product ComparabilityProtocols

Parameter	Test
Primary structure	Molecular mass of heavy and light chains by ESI-Q TOF-MS Molecular mass of intact protein by MALDI-TOF-MS Confirmation of primary structure by peptide mapping with LC-MS
Higher order structure	Far-UV Circular Dichroism (CD) spectroscopy Near-UV Circular Dichroism (CD) spectroscopy FT-IR spectroscopy Differential Scanning Calorimetry Intrinsic Tryptophan Fluorescence
Charge Variants: C-terminal Lysine	ESI-Q-TOF-MS
Charge Variants: Deamidation	lon exchange chromatography or IEF after CpB treatment
Glycosylation	Aglycosylation by ESI-Q-TOF-MS Oligosaccharide structure distribution by ESI-Q- TOF-MS Oligosaccharide structure distribution by mapping oligosaccharides
Oxidation	Quantitation of oxidized variants by ESI-Q-TOF-MS
Purity	SDS-PAGE silver staining, reduced and non-reduced
Aggregates	Analytical ultracentrifugation (AUC), field flow fractionation (FFF), or dynamic light scattering

Assessing stability of the post-change product is an important component of the comparability program, especially if the change has the known potential to impact the stability profile of the product. Historical stability data from the pre-change product can be used if the analytical methods and storage conditions will be the same for the current analysis of the post-change product. The stability plan should include both accelerated and real time conditions and can include stress studies, if appropriate. If the postchange antibody product exhibits greater stability in the real-time storage conditions compared to the pre-change product, this is considered acceptable and the products will still be considered comparable. In addition to evaluating stability of the purified drug substance, the stability of the formulated drug product manufactured using post-change drug substance should also be assessed. It is often s difficult to predict the impact of changes in the drug substance manufacturing process on drug product and differences in stability between pre- and post-change product may only show up in final drug product. A decision tree showing the expected comparability program depending on the results may also be included to describe what results could trigger evaluation in animal or human testing. In this case, any data supporting assumptions about product performance in vivo should be included and used to justify the proposed decision tree. For example, assessing the impact of a manufacturing change on immunogenicity requires an understanding of the relationship between process, product, and immunogenicity in previous clinical experience of the product or of related products (i.e., other antibodies). Such data may be available from process development, other similar antibody products, industry knowledge or literature. If it is known what product characteristics induce immunogenicity, then the comparability protocol can be designed to look for alterations in those characteristics post-change. It is difficult to provide a generic assessment of manufacturing changes that are likely to affect immunogenicity until it is known how the process impacts the product. Classes of products may however show similarities in what affects immunogenicity. For example, aggregation is thought to play a major role in immunogenicity of antibodies, so tight limits and effective analytical methods to measure aggregation are essential as a component of a monoclonal antibody comparability program.

Ideally the comparability protocol should be drafted prior to manufacturing product using the new process and should specify all samples including process intermediates that will be evaluated in the comparability program. A table of the batches, including the type of batch (pilot or cGMP), lot number, date of manufacture for pre-change batches, and manufacturer's name and location should be included in the protocol. These batches can be run as cGMP but will be 'at risk' until the formal comparability program has been completed. The results will either demonstrate comparability enabling continued clinical development or will not show comparability and will trigger additional in vivo testing. Having the protocol drafted prospectively allows for appropriate sampling to occur at the specific process steps being evaluated and allows sufficient final purified bulk antibody to be allocated to the comparability program. If the comparability study is not conducted using a prospective protocol, inadequate sampling is likely to be the result. Process intermediates and final purified antibody samples from the original manufacturing process will be needed as well, so incorporating a sampling and retention plan for the early production batches helps ensure that the necessary material is available when the inevitable process changes are implemented later in development. Failure to retain process intermediates and final purified antibody is a frequent mistake of many companies developing their first antibody products, so it is strongly recommended that companies save appropriate, representative samples from the original manufacturing batches and store them appropriately. The consequence of insufficient supply of pre-or post-change product and in-process samples is a potentially weaker comparability program and outcome than would otherwise have been possible if sufficient samples were taken during manufacturing.

5. Examples of Process Changes to Monoclonal Antibody Products

As noted above, regulatory authorities expect that the manufacturing process for a monoclonal antibody product entering Phase 3 will be a mature, well-defined process and that the identical process and scale used for preparation of Phase 3 will be the same as that used for commercial supply of the product. However, this is frequently not the case and process changes in late stages of clinical development and even post-approval have been introduced in several monoclonal antibody products. Some of these changes have come at the request of regulatory authorities,²⁰ but, more commonly these changes are the result of process improvements leading to higher yields, scale-up, addition of new manufacturing capacity, improved purity, or

improvements in formulation or delivery system. The most frequent manufacturing changes for approved monoclonal antibody products to date have been for changes in scale and/or manufacturing facilities although more and more frequently these changes are for process changes as well.

- Multiple manufacturing sites for one product are becoming increasingly common due to high product demand and increasing interest in ensuring security of supply.² Perhaps the most extreme example of multiple manufacturing sites for a monoclonal antibody product is Enbrel which is currently produced in six facilities in the United State and Europe.²¹ Examples of changes that have been introduced post- approval to monoclonal antibody products include the following:
- Cell line changes after completing Phase 3 clinical development (Zenapax²²)
- Manufacturing process changes (Enbrel,²³ Herceptin,^{24,25} Rituximab/MabThera^{26,27})
- Changes in manufacturing scale (Enbrel,²³ Raptiva,²⁸ Rituximab/MabThera,^{26,27} Synagis^{29,30})

Changes to or addition of manufacturing facilities (Enbrel,²³ Herceptin,^{24, 25} Raptiva,²⁸ Rituximab/MabThera,^{26, 27} Synagis^{29, 30}).

The following two examples of process changes for approved products are representative of the types of process changes made for approved monoclonal antibody product manufacturing processes and the comparability exercise required to gain approval of the new processes.

Synagis

Synagis is an approved humanized monoclonal antibody for treatment of RSV manufactured and marketed by Astra Zeneca (Medimmune). The primary mode of action of Synagis is to bind to a viral protein and inhibit virus binding to and infection of host cells. During clinical development of the product, material produced using the same manufacturing process but at scales of 20 L, 45 L, 100 L, and 200 L was used to treat patients. Each time the process was scaled up, comparability of the pre-change and post-change product was evaluated and biochemical comparability was demonstrated at all scales.30 The primary analytical methods used to evaluate the impact of process scale up on product quality and comparability were antigen binding ELISA, monosaccharide composition by reverse phase HPLC (RP-HPLC), N-linked-oligosaccharide content measurement, molecular weight determination by MALDI-TOF, and determination of aggregate levels by size exclusion HPLC (SEC-HPLC). Charge separation methods such as SDS-PAGE, capillary electrophoresis, and IEF were also used to provide further confirmation of comparability of the product used at different stages of development.

Following clinical development, the Synagis manufacturing process was transferred to a second manufacturing facility and the process was further scaled up to a final production scale of 10,000 L. The large data set from the comparability analysis of the product manufactured at different scales throughout development facilitated the setting of scientifically justified acceptance criteria for comparability of material produced at the commercial scale compared to material used in the pivotal clinical trials. Further, the clinical experience obtained with product produced at different scales provided data to support a range of acceptable specifications for the product quality attributes that provided a safe and efficacious product. The primary impact of process scale up and facility transfer was on the distribution of glycoforms in the product, however, based on the accumulated data, the product at the larger scale was accepted as comparable to the product manufactured at the smaller scales. This case study shows that extensive biochemical analysis of product manufactured during development is essential to define the critical quality attributes of a monoclonal antibody product and the acceptable release specifications for the product. In addition, it shows the importance of a dataset for correlating of process parameters with product quality, enabling more rapid process transfer and scale up to meet commercial demand.

Rituxan

Another example of comparability following process changes is seen with Roche's (Genentech's) successful monoclonal antibody product Rituxan, used to treat non-Hodgkin's lymphoma and other diseases.²⁷ The mode of action of Rituxan includes binding to the antigen CD20 on B cells and activating other immune system functions such as ADCC and CDC. Therefore, unlike Synagis, the activity of Rituxan is highly dependent on the ability to interact with and activate other components of the immune system, an ability that normally resides within the glycan structure on the Fc region of antibodies. During development, Rituxan manufacturing was scaled-up from 2,500 L to 12,000 L, the process was transferred from the initial pilot plant used to produce material to a new manufacturing facility, and the supplier of a component of the cell culture media was changed. Following these changes, a comparability study was performed in which slight changes in both the N-terminal and C-terminal sequences were observed, as well as minor changes in the glycosylation profile. However, the postchange product retained the full functionality of the prechange product, particularly its ability to activate the immune system, supporting the finding that the pre-change and postproducts were comparable. This case study highlights the fact that comparability does not mean identical, but that there is equivalent function and biochemical similarity between the pre- and post-change products.

Conformational Comparability

The level of analytical sophistication used to demonstrate comparability has increased with the explosion of biosimilar development. Recently developed Protein Conformational Array (PCA) ELISAs enable a detailed structural comparison of monoclonal antibody products, including innovator and biosimilar products, by measuring surface differences with an epitope array of more than 30 polyclonal antibodies in order to assess conformational differences and impurities as low as 0.1%.^{31,32,33} Interestingly, no significant differences were detected in the case of a biosimilar to Herceptin (trastuzumab). A biosimilar to Avastin (bevacizumab) showed an increase of signal across the epitope panel reflective of some unfolding, as well as new exposure of certain epitopes at a level of 0.1-0.2%. By comparison, bioassays had not indicated a difference for this bevacizumab biosimilar, which underwent further development. In the case of a biosimilar to Humira (adalimumab), three tested biosimilar batches showed three different conformational impurity profiles. Only one biosimilar batch showed good conformational similarity to the three identical batches of the innovator product. The other two biosimilar batches showed 0.1-0.2% new epitope exposure. Powerful tools such as PCA will enable both innovators and biosimilar developers to characterize their monoclonal antibodies and detect subtle changes that otherwise may not have been detected by bioassays or other analytical technologies currently available.

References

- European Medicines Agency, Committee for Proprietary Medicinal Products (GB). Guideline on comparability of biotechnology-derived medicinal products after a change in the manufacturing process [Internet]. London: The European Medicines Agency; 2007 Jul 19 [cited 2016 Nov 22]. 10 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003935.pdf.
- 2 Chirino AJ, Mire-Sluis A. Characterizing biological products and assessing comparability following manufacturing changes. Nat Biotechnol. 2004 Nov;22(11):1383-91.
- 3 Congy-Jolivet N, et al. Recombinant therapeutic monoclonal antibodies: mechanisms of action in relation to structural and functional duality. Crit Rev Oncol Hematol. 2007 Dec;64(3):226-33.
- 4 U.S. Food and Drug Administration [Internet]. Silver Spring (MD): U.S. Food and Drug Administration; c2009. Demonstration of comparability of human biological products, including therapeutic biotechnology-derived products; [updated 2005 Jul 6]; [cited 2016 Nov 22]; [about 10 screens]. Available from: http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122879.htm.
- 5 Food and Drug Administration, Center for Biologics Evaluation and Research, Center for Drug Evaluation and Research, Center for Veterinary Medicine (US). Guidance for industry comparability protocols – protein drug products and biological products –chemistry, manufacturing, and controls information-Draft [Internet]. Washington, DC: Department of Health and Human Services; 2016 Apr [cited 2016 Nov 22]. 24 p. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM496611.pdf.
- 6 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Comparability of biotechnological/biological products subject to changes in their manufacturing process Q5E Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2004 Nov 18 [cited 2016 Nov 22]. 16 p. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5E/Step4/Q5E_Guideline.pdf.
- 7 Harris RJ. Processing of C-terminal lysine and arginine residues of proteins isolated from mammalian cell culture. J Chromatogr A. 1995 Jun;705(1):129-34.
- 8 Lam XM, et al. Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2. J Pharm Sci. 1997 Nov;86(11):1250-5.
- 9 Fernandes D. Demonstrating comparability of antibody glycosylation during biomanufacturing. Eur Biopharm Rev. 2005 Aug;Summer:106-10.
- 10 Scallon BJ, et al. Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. Mol Immunol. 2007 Mar;44(7):1524-34.
- 11 Shinkawa T, et al. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human lgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem. 2003 Jan;278(5):3466-73.
- 12 Swann PG, et al. Considerations for the development of therapeutic monoclonal antibodies. Curr Opin Immunol. 2008 Aug;20(4):493-9.
- 13 Kozlowski S, Swann P. Current and future issues in the manufacturing and development of monoclonal antibodies. Adv Drug Delivery Rev. 2006 Aug;58(5-6):707-22.
- 14 Koren E, et al. Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. J Immunol Methods. 2008 Apr;333(1-2):1-9.

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- 15 Leininger E. Changes and comparability: what is needed for regulatory approval, demonstrating biocomparability to expedite process approval and change. Presented at International Business Communications Life Sciences' Eighth International Well Characterized Biologicals Conference; 2003 Nov 12-14; Vienna, VA.
- 16 Taticek R, Fallon E. Regulatory considerations in performing post-approval comparability: an industry perspective. Presented at International Business Communications Life Sciences' Meeting on Antibody Development and Production; 2009 Mar 2-6; Carlsbad, CA.
- 17 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Quality Risk Management Q9 Step 4 [Internet]. Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2005 Nov 9 [cited 2016 Nov 22]. 23 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ ICH_Products/Guidelines/Quality/Q9/Step4/Q9_Guideline.pdf.
- 18 Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Office of Regulatory Affairs (US). Guidance for industry cGMP for phase 1 investigational drugs [Internet]. Washington, DC: Department of Health and Human Services; 2008 Jul [cited 2016 Nov 22]. 20 p. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070273.pdf.
- **19** Blumel M. A case study on comparability of a monoclonal antibody during early stage development. Presented at the Technische Universität Braunschweig, Institut für Pharmazeutische Chemie [The Technical University of Braunschweig, Institute of Pharmaceutical Chemistry]; 2008 Oct 11; Braunschweig, Germany.
- 20 McCormick D. Small changes, big effects in biological manufacturing. Pharm Technol. 2004 Nov;11:16.
- 21 Paulino A. Enbrel (eternacept) lifecycle: multiple-site manufacturing and the challenge of maintaining comparability. Presented at International Business Communications Life Sciences' Antibody Production and Downstream Processing Conference; 2005 Mar 8-11; San Diego, CA.
- 22 European Medicines Agency (GB). Zenapax: scientific discussion [Internet]. London: The European Medicines Agency; c2005 [cited 2016 Nov 22]. 16 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000198/WC500057572.pdf.
- 23 Food and Drug Administration, Center for Biologics Evaluation and Research (US). CMC review of BLA 980286, Immunex's TNFR:Fc for RA [Internet]. Rockville (MD): Department of Health and Human Services; 1998 Jun 12 [cited 2016 Nov 22]. 26 p. Available from: http://www.fda. gov/downloads/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/ ucm088695.pdf.
- 24 Kuhne W. Site transfer and process validation in case of Herceptin API. Dev Biol (Basel). 2003;113:47-50.
- 25 European Medicines Agency (GB). Herceptin: scientific discussion [Internet]. London: The European Medicines Agency; c2005 [cited 2016 Nov 22]. 42 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000278/WC500049816.pdf.
- 26 European Medicines Agency (GB). Mabthera: scientific discussion [Internet]. London: The European Medicines Agency; c2005 [cited 2016 Nov 22]. 29 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000165/WC500025817.pdf.
- 27 Garnick RL. The product development continuum: the evolution and application of the concept of comparability. Dev Biol (Basel). 2002;109:3-8.
- 28 European Medicines Agency (GB). Raptiva: scientific discussion [Internet]. London: The European Medicines Agency; c2004 [cited 2016 Nov 22]. 37 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000542/WC500057849.pdf.

- 29 European Medicines Agency (GB). Synagis: scientific discussion [Internet]. London: The European Medicines Agency; c2004 [cited 2016 Nov 22]. 19 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000257/WC500056731.pdf.
- **30** Schenerman MA, et al. Comparability testing of a humanized monoclonal antibody (Synagis) to support cell line stability, process validation, and scale-up for manufacturing. Biologicals. 1999 Sep;27(3):203-15.
- **31** Wang X, et al. Development of antibody arrays for monoclonal antibody higher order structure analysis. Front Pharmacol. 2013 Aug;4:103.
- 32 Wang X, et al. Higher order structure comparability: case studies of biosimilar monoclonal antibodies. BioProcess Int. 2014 Jun;12(6):32-37.
- 33 Davies M, et al. mAb higher order structure analysis with protein conformational array ELISA. Br J Pharm Res. 2015 Jul;7(6):401-12.



CHAPTER 11:

Process Validation

alidation is a scientifically rigorous and well-documented exercise, which demonstrates that a process or piece of equipment consistently does what it is intended to do. For monoclonal antibodies, the manufacturing process must produce a product with consistent and reproducible characteristics. To achieve this, the ability of the manufacturing process to remove potential contaminants and impurities, including endotoxins, viruses, cell membranes, nucleic acids, and proteins, media components, process chemicals, ligands leached from chromatography media, and modifications or inactive forms of the product itself must be well designed and validated.

The goal of process validation is to demonstrate that a manufacturing process, when operated within established limits, generates a product that routinely and reliably meets its required quality standards. The principles of process validation were initially established in a 1987 FDA guidance document, which defined process validation as "establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes."¹ The requirements for process validation have since been adopted worldwide with similar definitions appearing in guidance documents and the cGMP regulations promulgated by European regulatory agencies, ICH, and WHO.

When the 1987 FDA guidance was published, process validation consisted of a series of activities conducted primarily during the later stages of product development in preparation for filing a BLA and eventual commercialization of the product with minimal validation activities taking place during early stages of product development. The exception to this was a need to demonstrate adequate virus clearance (removal and inactivation) by the drug substance manufacturing process and validation of aseptic processes used to manufacture the sterile drug product. Until recently, process validation activities were primarily conducted during Phase 3 clinical trials and included the identification of critical process parameters (CPP), range studies on these parameters to determine the point at which the process fails to yield acceptable product, and a series of fullscale conformance lots (usually three) in qualified equipment under cGMP conditions. CPPs were defined as those independent process inputs or variables related to each individual unit operation in a manufacturing process that directly affected the quality of the product.

Equipment qualification involved confirming and documenting that the design, installation, operation, and performance of the manufacturing equipment were capable of satisfying the process requirements. Along with equipment qualification, the analytical methods used for in-process testing and final product release were validated prior to initiation of the fullscale conformance lots. After the approval of the conformance lots, the validated process could not be materially modified without performing revalidation to confirm that the process was still in control and still produced a product of acceptable quality.

Since 1987, the concepts of validation in general, and process validation in particular, have evolved so that process validation is now viewed as a continuum of activities rather than a series of discrete events performed once and rarely repeated. Process validation is now viewed as encompassing not only a full demonstration of process consistency and process understanding, but also ongoing verification to ensure the process remains within its qualified design space and product consistently meets all specifications. As a result, regulatory authorities now expect companies to develop unique validation protocols suited to the individual organization based not on conformance to a fixed set of guidelines but rather designed using a risk-based approach, which identifies and controls potential risks within the manufacturing process. This risk-based approach to overall product development and validation was outlined in 2004 by FDA in its GMP Initiative² and reinforced in 2005 with the approval of ICH Q9, formalizing the requirements of quality risk management for the pharmaceutical industry.³

As further defined in FDA's 2011 Guidance for Industry on process validation4 and EMA's 2012 process validation guideline,5 validation must now take a continuous lifecycle approach, acknowledging the need for change and improvement in manufacturing processes, which is in alignment with the QbD approach discussed in Chapter 3. In its 2011 guidance, FDA defined three stages of validation that together encompass the validation of a manufacturing process across the lifecycle of the product. These stages, summarized in Figure 11.1, include Process Design, Process Qualification, and Continued Process Verification. FDA places significant emphasis on the process design activities of Stage 1, including the definition of critical quality attributes (CQA), process characterization, and establishment of parameter criticality. Stage 2 of process validation encompasses Process Performance Qualification (PPQ) activities analogous to the consistency or conformance runs previous used as the basis for process validation.

However, unlike the previous conformance runs, which were viewed as a one-time requirement, PPQ is seen as an ongoing demonstration of process consistency. This leads to the Continued Process Verification of Stage 3, which encompasses ongoing process monitoring, management review, and annual reporting requirements for commercial products. Stage 3 of process validation is viewed as part of a feedback loop for continued process verification and control strategy evaluation during the lifecycle of the product and its manufacturing process. This process verification and control can include process analytical technology (PAT). As defined in the FDA GMP Initiative,² PAT should allow manufacturers to use reliable and consistent real-time process control methods such as in-line monitoring, feedback control of CPPs, and other data analysis and modeling methodologies, to continuously monitor and control a manufacturing process. However, despite continued interest in PAT by both industry and regulatory authorities, progress continues to be slow in this area due to the complexity of monoclonal antibodies and the lack of reliable in-line analytical technologies for its implementation.

1. Quality Risk Management

To meet the regulatory requirement that commercial pharmaceutical manufacturing processes be "validated with a high degree of assurance,"⁷ regulatory authorities now consider a systematic risk analysis and management program to be a critical component of validation.8 A quality risk management program, shown in Figure 11.2, encompasses risk assessment, risk control, and risk review, with risk assessment being the most critical aspect for process validation. Risk assessment should be based on sound science, process characterization information, and data collected from both scale-down models of the manufacturing process and actual batches of product produced during clinical development and scale-up. These data should include information regarding the source and quality of all materials used in a manufacturing process and the impact of each material or procedure in the manufacturing process on the overall quality, efficacy, and safety of the final product.



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Figure 11.2. Overview of Quality Risk Management A comprehensive quality risk management program should encompass three critical areas – risk assessment, risk control, and risk review. Adapted with permission from Reference 3.



Process risks should be assessed throughout the product lifecycle, starting with process design and continuing through ongoing assessment of commercial manufacturing operations. At the outset of process development, a risk assessment should be used as part of the process of defining the product CQAs. The risk assessments commonly used to evaluate criticality include risk ranking and Preliminary Hazard Analysis.⁹ Later risk assessments will include the Process Risk Assessment, which is conducted using FMEA, FMECA, or HACCP. Risk assessments should be conducted at phase appropriate intervals and any time that changes are anticipated or made to the manufacturing process. Risk assessments can, and should, be both formal and informal, depending on the situation and need. As the product matures and additional process knowledge is gained from increased process history, the risk assessment and analysis will increase in comprehensiveness so that the potential impact of subtle changes in the manufacturing process on product quality can be determined. For example, a range of factors including cellular metabolism, metabolic flux and the efficiency of the glycosylation process can alter the glycosylation of recombinant proteins. Since changes in the glycosylation of a monoclonal antibody product can have a significant impact on the pharmacokinetics and efficacy of the product, it is important to assess the risk of variations in the operating parameters for the production bioreactor and their effect on product glycosylation.¹⁰ This is especially true since subtle variations of nominally identical bioreactor operating parameters, such as pH, can alter glycosylation.¹¹ The impact of certain manufacturing parameters on glycosylation of a monoclonal antibody product may be difficult to determine early in the product lifecycle due to the limited number of batches produced during clinical development and the lack of clinically relevant data.

As part of the evaluation of the potential risks associated with raw materials, process equipment, and manufacturing processes on the quality of a monoclonal antibody product, the criticality of these risks should also be determined and methods or policies designed to eliminate, reduce, or control the risks. A quality risk management program will define which operating parameters must be controlled during a manufacturing process and enable prioritization of the development of controlled processes.

In alignment with QbD, quality risk management acknowledges that it is not possible to achieve control of a product's quality by final product testing alone, but that the CQAs of a product should be identified using appropriate risk assessments and confirmed during process development and early stage manufacturing of the product. These CQAs should then be maintained throughout the product lifecycle by careful control and monitoring of those CPPs that may affect them. By establishing the CQAs for a product, defining the acceptable ranges for each CPP to achieve these CQAs, and then carefully controlling the CPPs during manufacturing, a design space for each process step can be defined, which encompasses the acceptable operating ranges of all CPPs. This allows a manufacturing process to be optimized or otherwise changed within the design space without requiring re-validation of the manufacturing process. This approach to process development and validation encourages innovation and enables process changes to be implemented with minimum regulatory delay and expense.

An additional tool useful in conducting an initial risk assessment is the Ishikawa or fishbone diagram, which can be used to identify all of the possible causes for a given effect. Such an analysis is helpful, for example, in evaluating how different process parameters might impact certain process attributes. In the A-Mab Case Study, a fishbone diagram was used to identify equipment design, control parameters, processing conditions, and starting materials for a production bioreactor and its seed reactor that might pose a significant risk to the quality attributes of a monoclonal antibody product.⁹ This analysis, shown in Figure 11.3, helped to assess the potential impact of each process parameter on product yield or viability and turbidity of the culture at harvest, soluble aggregates, variability in glycosylation, deamidation, and levels of host cell protein or DNA.

Risk Assessment Tools

ICH Q⁹ recommends the use of such standard risk analysis tools as FMEA/FMECA and HACCP as a means of quantifying the risk associated with each step in a manufacturing process and determining critical process parameters.³ Additionally, risk ranking and Process hazard

Figure 11.3. An example of an Ishikawa or Fishbone Diagram

analysis (PHA) can be used for determining the CQAs.⁹ Individual risk assessment techniques are best used in a complementary manner to eliminate knowledge gaps.

Prior to initiating any risk assessment, the scope must be defined, the risk assessment tool selected, an appropriate team selected, and the potential decisions to be made based on the assessment clearly stated. Defining the scope of the risk assessment will help in selecting the proper team configuration. Risk assessment teams should be made up of all of the individuals required to bring the necessary expertise to the assessment and may include representatives from validation, process development, quality, and manufacturing.⁸

A simple but effective approach to risk analysis is provided by Katz and Campbell.¹² In their approach, shown in Figure 11.4, a manufacturing process is broken down to its constituent unit operations and the specific parameters of each operation are analyzed to determine whether that parameter poses a risk to the identity, strength, quality, purity, or potency of a product. Since the purpose of each unit operation is to deliver or protect some aspect(s) of the target product profile (TPP), the identification and management of those process parameters that impact the CQAs of a product constitutes the control strategy for that particular unit operation.



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Figure 11.4. Uni (adapted with permis	t Operati o ssion from Re	on-based eference 12)	Approach to	Risk Assess	ment		
Unit Op		Attribute at Risk					
			Identity	Strength	Quality	Purity	Potency
Significant Variable	EM	EM-1				\checkmark	
		EM-2		\checkmark			
		EM-3			\checkmark		
	MA	MA-1	√				
		MA-2					√
		MA-3				\checkmark	
		MA-4					
	QC	QC-1		√		\checkmark	
		QC-2			\checkmark		
		QC-3	√			\checkmark	
		QC-4					
		QC-4					

Risk Ranking

Risk ranking is used to assess the product quality attributes and determine which of these must be controlled as CQAs. Risk ranking evaluates quality attributes based on their potential to impact the patient multiplied by the level of confidence in the knowledge used to determine the impact. The impact is scored by evaluating known or potential effects on safety and/or efficacy.⁹ The uncertainty is scored by leveraging prior knowledge elements. Scoring criteria for each category should be established using a numerical system that is commensurate with the criteria for each category. The numerical scale used is arbitrary provided that it gives more weight to the impact score.

Risk ranking does not take into consideration the detectability or controllability of a failure and as a result

the criticality score will not change as product and process knowledge evolve. The criticality score will, however, change as understanding of the product increases. Risk ranking should be used during the initial assessment of product quality attributes and reevaluated over the course of the product lifecycle at phase appropriate intervals.

An example of the type of risk analysis and ranking that can be used to assess the impact of raw materials or process parameters on product quality attributes and the assignment of CQAs is provided by Boychyn and Hart in assessing the risk of adventitious agent contamination of raw materials used in cell culture media.¹³ This assessment concluded that the risk of contamination was highest for animal-derived raw materials that were a potential food source for rodents, raw materials, which were not highly purified prior to use in making the media, or raw materials that represented greater than 10% of the volume of the media with the highest risk raw materials representing orders of magnitude greater risk than potential lower risks identified. As a result of this analysis, cell culture media containing the highest risk raw materials were subjected to viral inactivation processes before the media is used in product manufacturing. A similar analysis by Kiss concluded that the highest impact risk mitigation strategy was to provide an efficacious virus barrier at the point of use in the manufacturing facility.¹⁴

Preliminary Hazard Analysis

PHA can be used to rank quality attributes based on the severity and probability of failure. This risk assessment tool leverages prior knowledge to identify future risks to the patient.³ The severity score considers risks to safety and/or efficacy based on prior knowledge elements. The probability score is based on the chances of a quality attribute having an impact on safety and/or efficacy by going outside of the currently established ranges.9 The severity and probability scores are multiplied to calculate the risk priority number (RPN), which allows the quality attributes to be ranked.

Failure Modes Effects Analysis and Failure Modes Effects

Criticality Analysis

FMEA is a methodology for identifying potential failure modes for a product or process, to assess the risk associated with those failure modes and to classify the severity of failures on the product or process. This analysis allows the ranking of potential failure modes and the identification of corrective actions to address the most serious concerns. Failure modes are any errors or defects in a process, or product, especially those that affect the safety or efficacy of a product.

FMEA considers three factors in evaluating the effect of a failure. FMEA evaluates a failure mode based on the impact of failure (severity), the likelihood of failure (occurrence), and the detectability of failure (detection).¹⁵ Severity, occurrence, and detection are assigned a score from 1 to 10 using a predetermined scale for each category. The resulting scores are multiplied to calculate the RPN, which allows the failure mode to be ranked. The RPN allows for the prioritization of risks and the evaluation of risk mitigation.³ FMEA is best suited for the evaluation of equipment and manufacturing processes and is frequently used as part

of a PRA to identify parameters for further screening or evaluation in process characterization studies.

Hazard Analysis Critical Control Point

HACCP is a systematic preventive approach to product safety that addresses hazards as a means of prevention rather than finished product inspection. HACCP has been used for years in the food industry¹⁶, and can be applied to monoclonal antibody product development and manufacturing as a means of identifying the points in a process at which specified CQAs may be controlled (critical control points), the limits of control available and monitoring requirements, as well as the required corrective actions. For most monoclonal antibody product manufacturing processes, FMEA is generally used to determine risks associated with the manufacturing process. However, for certain manufacturing unit operations or processes where environmental controls may be more critical than process controls, HACCP may be more appropriate than FMEA. This is because HACCP focuses on Critical Control Points as a means of prevention or elimination of hazards and risk while FMEA focuses on the potential effects of any identified failure mode. For example, a HACCP analysis may be better suited than FMEA analysis for determining risks associated with a filling process for a monoclonal antibody conjugated to a toxic compound due to the heavy reliance of the manufacturing process on environmental and manufacturing controls to ensure patient and operator safety as well as product quality.

2. Lifecycle Process Validation

Following the introduction of QbD and quality risk management (QRM), process validation has evolved from the traditional "fixed-point" concept of a manufacturing process being fixed through a series of conformance batches (usually three) following process development to a "lifecycle" approach. This approach should enable more continuous improvement of manufacturing processes as well as ensuring a reliable and robust manufacturing process at the time of BLA submission. In this modernized approach, manufacturing processes are continually reviewed during routine manufacture to ensure that adverse trends are identified and corrected before the product fails to meet its final specifications. The new process validation guidelines promote designing quality into the product rather than simply testing it in at the end. The lifecycle approach to process validation, defined in the FDA January, 2011 guidance,⁴ specifies that traditional process validation, typically relying on three consecutive successful full-scale conformance runs, should be replaced by a deliberate design process, commercial process qualification, and ongoing review of processes with increased use of continuous process monitoring.

The relationship between the various phases of clinical development and commercialization of a monoclonal antibody product and the three stages of process validation (Process Design, Process Qualification, and Process Verification) is shown in Figure 11.5. As knowledge regarding the safety and efficacy of a product increases during the clinical development of that product, so too does the knowledge of its manufacturing process. As emphasized above, process validation is now viewed as an ongoing activity throughout the lifecycle of a product with the CQAs of the product and CPPs of the manufacturing process initially defined during Stage 1 and 2 of process validation and then continuously monitored and verified during Stage 3. This requirement for continued process verification remains throughout the commercial life of the product.

Stage 1: Process Design

During process design, the manufacturing process is developed as outlined earlier in this report, characterized, and then scaledup to commercial levels. During Stage 1, the product's CQAs should be identified and the critical and key process parameters for the manufacturing process defined.¹⁷

Since CPPs must be maintained or controlled within their specified ranges in order to demonstrate process robustness and suitability, acceptable operating ranges for these parameters should be established during the process design stage. As described below, much of the process design and process development work can be done using scaled-down process models and the high throughput development techniques outlined throughout this report. The use of statistical Design of Experiments (DOE) to study the interaction of different process parameters using multivariate experiments is recommended by the FDA process validation guidance4 and is discussed in detail below.

Process design during Stage 1 encompasses laboratory activities for process development, process characterization, and establishing a commercial process control strategy. Key prerequisites for this activity include sufficient product characterization data to establish CQAs for the product, and sufficient scale-up/scale-down data to ensure that laboratory models used in process characterization are representative of full-scale manufacturing performance.

During Stage 1, a standardized approach to process design should be taken so that all unit operations, analytical methods, and product specifications are carefully scrutinized and properly developed. This includes the classification of process parameters as critical or noncritical. A CPP is "a process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired product quality."18 Process parameters can be classified as either critical or non-critical through a careful risk assessment process as outlined in Figure 11.6. This analysis first takes into consideration the potential for deviation in a particular process parameter to impact the product CQAs, followed by risk assessment of the likelihood of the control strategy for the parameter failing to keep the parameter within its specified range.

Figure 11.5. Relationship between the Phases of Product Development and the Process Validation Lifecycle Each stage of process validation can be mapped to the overall clinical development of a monoclonal antibody product as shown here. The process design activities for Stage 1 are typically completed during early stages of product development while Stage 2 is generally completed during Phase 3 clinical trials. Stage 3 activities begin with the filing of the BLA for a monoclonal antibody product and continue throughout the lifecyle of the product.



In certain cases, it may be desirable to categorize process parameters beyond simply critical and non-critical, adding the further definition of non-critical parameters as "key" or "non-key" in accordance with the definitions established by the PDA.6 These additional classifications, while not an absolute regulatory requirement, can be helpful during routine manufacturing to determine acceptable responses to process deviations or excursions. Non-CPPs may be divided into two discrete categories, key and non-key. Non-CPPs that do not impact product quality, but may impact process performance, such as yield, are classified as key process parameters. Non-key process parameters are those that have no impact on process performance or product quality.6 CPP, key, and non-key do not represent a continuum of criticality. While the designation of a process parameter as CPP or non-CPP is based on a continuum of risk, this decision is binary.

Figure 11.6. Risk Assessment for Classifying Process Parameter Criticality

Process parameters can be classified as critical or non-critical depending on their impact on the CQAs of a monoclonal antibody product. Non-critical parameters can be further classified as key and non-key depending on whether they impact process performance or not. Classification of all process parameters is an essential element of process validation and should be based on a thorough risk assessment. (repreinted with permission from Reference 6)



There is no universal definition for categorization of process parameters as CPP or non-CPP and as such these categorizations are not necessarily recognized by global regulatory authorities.^{6, 19} Regulatory authorities generally discourage the use of key and non-key parameters in regulatory submissions. However, it is possible to define categories of process parameter criticality to meet individual program requirements. The A-Mab Case Study provides an example of how criticality rankings can be customized. In the A-Mab Case Study, critical process parameters were classified as either CPP or well-controlled CPP. Non-critical process parameters were designated non-CPP or general process parameter. This process acknowledges that, although criticality assignment is binary, the potential impact of a process parameter can vary depending on a variety of factors, including the controllability of an individual process parameter.9 A criticality assignment process with greater granularity can facilitate better decisions regarding controls for process parameters.

A list of activities typically performed during Process Design is provided in Table 11.1 along with the deliverable used to document completion of the activity and its outcome.

Design Activities			
Activity	Deliverable		
Characterize the process and define control ranges: • Define process control ranges • Define parameter criticality • Establish scale down models of the manufacturing process	 Critical Controlled Parameters Report or Process Control Parameters report Process development (unit operations) report Scale down model report 		
Establish in-process and release specifications: • Define CQAs • Structure – Function elucidation	• Product Specification Justification of Specifications Report/CQA report		
Scale-up manufacturing process and gain manufacturing experience: • Engineering/scale-up batches • GMP manufacturing	 Tech transfer report Master Batch Record 		

Careful planning and forward thinking during Stage 1 are essential to a successful validation program. The lifecycle

Table 11.1. Typical Stage 1 ProcessDesign Activities

validation approach requires a strong foundation as quality must be built in from the start.

Stage 2: Process Qualification

Process qualification, as defined by the FDA process validation guidance, shares many of the same features as traditional fixed-point process validation approach used by companies prior to the issuance of the guidance. The main difference is in how the acceptance criteria that define suitability for market registration are set. Process qualification includes an evaluation of the process design defined in Stage 1 to ensure that the manufacturing process is capable of reliably producing a product that meets all release criteria during routine commercial manufacturing. During Stage 2, the defined, scaled-up manufacturing process is run at commercial scale by trained staff under full cGMP conditions using prequalified equipment in the proposed commercial manufacturing plant. Complete process qualification of the manufacture of a monoclonal antibody product will include the validation of the performance of process chemicals and raw materials used in each unit operation, qualification of all supporting facilities and utilities necessary for the manufacturing process, qualification of all process equipment, validation of each individual unit operation, and validation of the entire process as it is intended to be operated at commercial scale.

Prior to the actual performance of Process Qualification, a series of related activities, outlined in Table 11.2 must be completed to ensure the accuracy of the Process Qualification. These activities include the validation of in-process and release testing methods, scale-up of the manufacturing process, and validation of related equipment and processes.

Table 11.2. Typical Stage 2 1100055 Qualification Activities			
Activity	Deliverable		
Completed prior to execution of Performance Qualification Runs			
Implement Process Control Strategy	 Master Batch Record In-Process and Release Specifications Raw Material Specifications 		
Complete Utilities and Equipment Qualification	• Equipment IQ/OQ/PQ that meets process requirements		
Full Scale Manufacturing Runs	Completed Manufacturing Batch Records		
Validate Commercial Testing Methods	 Validation Reports for non-compendial in-process testing and product release methods Qualified assays can be used for characterization testing during PPQ 		
Sterile filtration membrane validation	• Required for any step claiming sterility – report validating compatibility of membranes with the process solution		
Container Closure Validation	Required for any container/closure claiming sterility		
Facility GMP Review	• Review facility and equipment design/qualification for regulatory compliance		
Performance Qualification			
Execute Performance Qualification runs	PPQ Protocol and Report		

Table 11.2. Typical Stage 2 Process Qualification Activities

Table 11.2. Typical Stage 2 Process Qualification Activities		
Activity	Deliverable	
Completed prior to or concurrent with Performance Qualification Runs		
Leachable Extractable Characterization	• Process leachable/extractables report. Toxicology assessment may need to be performed for compounds identified	
Cleaning Validation	Cleaning Validation protocol and report	
Membrane and Resin Reuse Lifetime Study	Column and membrane lifetime study protocols and reports	
Completed after completion of the Performance Qualification Runs		
Stability Assessment	GMP stability study for drug substance and drug product	
Shipping Qualification	• Evaluation of the impact of shipping conditions on drug substance, drug product, and finished goods	

Each batch of monoclonal antibody product produced during process qualification is tested using validated inprocess and final product test methods to confirm that the product meets pre-set specifications and in-process acceptance criteria. Additional process characterization methods and analyses are also expected during this stage to fully characterize and qualify the process. The process controls, including the use of analytical test methods used for both in-process testing and final product release must be sufficient to confirm that each CPP is held within its pre-approved range and that the final product meets all release specifications. The combination of process design studies performed during Stage 1 and process qualification performed during Stage 2 should confirm that the manufacturing processes are reliable and reproducible and adequately control all of the product's CQAs. Assuming this is the case, the process is "validated" and the product may be released for commercial use.

Stage 3: Process Verification

Following completion of Stage 1 and Stage 2 routine product manufacturing should be monitored using the validated in-process and final product test methods to ensure that the manufacturing process remains in control and that the product continues to meet all CQAs. The particular strategy for continuous process verification in Stage 3 should be dictated by information gathered during Stage 2 of process validation.²⁰ The intent of this continued process verification is to monitor the process throughout the product lifecycle, demonstrating continued control of the manufacturing process. Since changes may occur in the testing protocols or the analytical methods used during the product lifecycle, it is important that these revised test methods be appropriately validated and that results of these new methods correlated with those obtained previously using the original test methods.

While the FDA process validation guidance does not specify the extent of sampling and testing necessary to ensure adequate process control, it does recommend that monitoring and sampling of process parameters and quality attributes be continued until sufficient data are available to estimate the extent of variability of the manufacturing process. FDA recommends that testing programs be designed by someone with sufficient training and knowledge in statistics to ensure that the monitoring plan meets regulatory expectations and that the overall monitoring plan, including a description of how data trending and all other calculations will be performed, be fully described in the Stage 3 validation protocol.²¹

The purpose of continued process verification is to establish the appropriate levels and frequency of routine sampling and monitoring for a particular product and process to meet the cGMP requirement of "statistically appropriate and representative levels."21 During Stage 3, production data should be collected on an on-going basis and appropriate alert and action limits set. Since the number of batches of monoclonal antibody product produced prior to completion of process qualification (Stage 2) is likely to be small, the amount of sampling and inprocess testing required during routine commercial manufacturing may be greater in the early years of commercialization than later in the product lifecycle. The data collected should be sufficient to provide strong statistical evidence that all critical process parameters are being held within their acceptable ranges and that there are no trends among any of the CQAs towards out of specification results. As commercial manufacturing progresses, the extent of testing may decrease as increased confidence in process capability and reproducibility is confirmed. Once sufficient data are available to establish the statistically meaningful extent of process variability, the monitoring program can be adjusted accordingly.

Continuous process verification strategies will vary from process to process but typically involves additional process sampling and monitoring outside of parameters routinely recorded in the Master Batch Record. Based on the testing results, control ranges for certain operating parameters may be adjusted over time and some routine testing may be eliminated after sufficient manufacturing experience is obtained. The requirements for extensive inprocess testing and process monitoring during Stage 3 is more stringent than the simple trending review of routine production performance on an annual basis that has been required by regulatory authorities in the past. Once process robustness has been established, some of the extra in-process testing and process monitoring conducted during validation may be discontinued with appropriate justification.

In addition to ongoing process verification, new scientific discoveries or developments in the field of bioprocessing may occasionally require that the CQAs and CPPs for a monoclonal antibody product be revisited and modified, as needed or appropriate. For example, the discovery of new viruses that may infect mammalian cells (e.g. porcine circovirus) or the discovery of a previously unidentified contaminant or risk factor (e.g. glass delamination recently observed in glass vials) may require adjustments to certain CQAs and/or CPPs for a particular product. In such cases, process characterization and qualification (Stage 1 and Stage 2) should be repeated with a focus on the impact of the new area of concern on product quality and safety. Should changes to the manufacturing process be required to address these new discoveries or developments, these changes should be documented and implemented through approved change control procedures, including appropriate notification of the regulatory authorities.

3. Defining Critical Process Parameters

As with other products, the CQAs of a monoclonal antibody product will be those physical, chemical, biological, and microbiological properties and characteristics that must be controlled within an appropriate range to ensure the desired product quality. For monoclonal antibody products, the CQAs would be those parameters that affect product purity, strength, or stability, particularly post-translational modifications such as glycosylation and heterogeneity resulting from the presence of various glycoforms. Productrelated impurity levels (e.g., aggregated or clipped forms) and other process-related impurities may affect product safety or efficacy and may be included in the CQAs for a monoclonal antibody product. The CQAs of a monoclonal antibody product will always include product potency and immunogenicity. A key element of QbD and the new process validation standards is that these CQAs can be linked to certain CPPs in the manufacturing process. These critical operational parameters can be identified during the earlier stages of process design by an initial risk analysis but additional CPPs may be identified at any time during the product lifecycle as a result of continuous process monitoring. Besides its impact on the CQAs, the ability to control a process parameter within its intended range is a significant factor in defining its criticality, especially in the manufacture of monoclonal antibody products.

To control the CPP for a manufacturing process, it is important to have a clear understanding of the desired settings and ranges for each parameter. During process development, three nested ranges of relevance may be established for each process parameter. The widest range is the "proven acceptable range" (PAR) within which the product produced always meets its desired release specifications and CQAs. Outside the PAR, the process will fail and the product may not meet its desired CQAs. Establishing the PAR is sometimes referred to as "testing to the edge of failure" and is normally done during process development. Embedded within the PAR is the regulatory or validated range, that is, the range for which the parameter is tested during validation studies. The PAR also represents the parameter range included in a product registration application (e.g., BLA). Further embedded within the regulatory range is the normal operating range, which is the range for the parameter specified in the master batch record and expected to be used for routine commercial production of the monoclonal antibody product.^{22, 23} The pyramiding of ranges for operating parameters, illustrated in Figure 11.7, helps to ensure that the regulatory range for each parameter is wider than the routine operating range to allow for minor process variations beyond the operating range and to prevent failure of the unit operation or overall process. In addition, establishing a regulatory range that is within the proven acceptable range ensures that the process is not operating at the edge of failure and is therefore robust and less likely to fail.

Figure 11.7. Defining Operating Parameter Ranges Within a manufacutring operation and reglatory filing, several operating ranges for each process parameter may be defined. By pyramiding these ranges so that the regulatory ranges are wider than the routine operating range, minor process variations can be accepted in routine manufacturing without the need to reject a specific batch. Adapted from Reference 23.



To establish which of the many process parameters in a monoclonal antibody manufacturing process are critical, a risk analysis of each unit operation based on data collected during development and the potential impact of failure to control a specific parameter within its acceptable range on the CQAs of the product should be conducted. This analysis will help refine the acceptable ranges of each parameter and minimize the potential for process variability and failure. Many process parameters in a monoclonal antibody manufacturing process will have wide acceptable ranges so that it is not necessary to establish what the acceptable range truly is, as long as an operating range is defined within this broad range. These parameters are not likely to be critical. On the other hand, if the PAR for a specific process parameter is narrow, it is likely that this parameter is critical to meeting the CQAs of the product. In such a case, the validated range should be established so that it approaches the edge of failure at the boundaries of the acceptable range, but remains safely away from the edge of failure. For example, both the temperature and pH of the cell culture medium in a bioreactor may have the potential to impact product quality, but the acceptable range for temperature may be relatively broad while the acceptable pH range may be much tighter and represent a much higher risk for product failure resulting from a process excursion outside this range.

4. Scaleddown Models for Monoclonal Antibody Product Validation

Many of the data used to justify and validate operating parameters for a monoclonal antibody product manufacturing process are generated using scaled-down models of the process that mimic the performance of the full-scale system as closely as possible. To help ensure consistency between the scaleddown models and the fullscale manufacturing process all of the components and materials of construction of the product-contact surfaces should be the same in the model and full-scale systems. Feed streams from the full-scale manufacturing process can be used for the small-scale studies.

Scaleddown models are used in process development and validation to prevent contamination of full-scale production systems with infectious or hazardous materials used in clearance studies, to perform process characterization studies that would be difficult or expensive to perform on full-scale systems, to conduct lifetime studies for unit operations, to perform investigative cleaning protocols, and to validate operating ranges. Small-scale models are also an essential tool for conducting statistically based design of experiments to investigate the full range of all process parameters and to study the interactions between different parameters on the quality of the product produced. These DOE studies are essential in defining a design space and establishing robustness of a manufacturing process.

Such models can range in scale from the micro-reactors and small chromatography columns to smallscale laboratory bioreactors, larger chromatography columns, and ultrafiltration systems. Regardless of the scale, each scaleddown model used in the validation of a monoclonal antibody manufacturing process should be scientifically sound and its use should be supported by experimental data demonstrating the similarity of performance between the model and the full-scale process. In designing scaleddown models, it is also important that all significant process parameters be maintained constant. For example, in designing scaleddown models for viral clearance studies, ICH Q5A(R1), Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin, recommends that "the level of purification of the scaled-down version should represent as closely as possible the production procedure. For chromatographic equipment, column bed-height, linear flow-rate, flow-rate to bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein, salt, and product should all be shown to be representative of commercial-scale manufacturing."24

Some operational parameters or unit operations may be too complex to study easily in scaleddown systems. For example, the mixing behavior and shear forces generated in largescale bioreactors can be difficult to simulate adequately in smaller tanks with different agitator conformations. In such cases, the characterization data should be obtained at scale with appropriate engineering studies, often supplemented with data from simulation or computer modeling. The use of advanced simulation techniques, such as computer simulation of reactor dynamics using computational fluid dynamics, should simulate the actual manufacturing unit operations in relevant aspects as closely as possible and the performance of the model and the manufacturing operation should be compared using appropriate performance metrics. The performance of the scaled-down model, however, need not be identical to the manufacturing operation in all aspects, provided the CPP being studied or modeled can be adequately investigated.

5. Validation of Monoclonal Antibody Drug Substance Manufacturing

Monoclonal antibody products are produced predominantly in mammalian cell culture using appropriate host cell systems. In these processes, cells from a qualified cell bank are expanded in culture until sufficient numbers are obtained for the desired production scale. The monoclonal antibody product is continuously secreted into the culture medium where it accumulates and, at the end of a typical fed-batch bioreactor process, the cells are separated from the conditioned culture medium prior to purification. As discussed in Chapter 7, the purification process for a monoclonal antibody product is normally divided into four major steps, including initial product capture, intermediate purification, virus inactivation and removal (viral clearance), and final purification or polishing.

Once a suitable manufacturing process has been developed, it is important to quantify the cause and effect relationships of the input parameters for each unit operation in the process to the outputs of the process, i.e., the quality of the final product. Process validation of the manufacturing process will then entail assessing each of the manufacturing operations separately and in combination to demonstrate that the process can reliably and reproducibly produce the desired monoclonal antibody product. The following discussion is intended to provide examples of how to validate a monoclonal antibody manufacturing process. While not all inclusive of every validation study required for each monoclonal antibody product, this discussion should assist the reader in designing or implementing an appropriate validation program.

As discussed above, process characterization is a critical component of process validation and should be sufficient to fully understand the effect of process inputs (i.e., operating parameters) on process outputs (i.e., performance parameters). The process characterization should provide an identification of CPPs and key performance indicators, especially the acceptable ranges for all parameters determined from a risk analysis to be of a sufficiently high risk of affecting the CQAs or process performance parameters.²⁵ During process validation, these acceptable ranges are shown to provide sufficient control of the manufacturing process to ensure consistent performance of this process at the commercial scale and to demonstrate process robustness.

Validation of Upstream Processes

Some representative operating parameters, which may be considered CPPs in the upstream process for production of a monoclonal antibody product, are listed in Table 11.3. For those parameters that are shown to be critical, the acceptance criteria for process studies demonstrating the consistency and reproducibility of the crude monoclonal antibody product in the bioreactor harvest might include a minimum cell viability at harvest (e.g., \geq 70%), product titer of greater than a specified amount (e.g., \leq 3.0 g/L), a specified oligosaccharide (glycoform) content (e.g., GAL0: 6070%, GAL1: 2030%, GAL2: \leq 10%, and NeuAc: <1%), or a maximum aggregate level (e.g., \leq 5%).

Table 11.3. Potential Cell Culture CriticalProcess Parameters		
Process Parameters	Typical Operating Ranges	
Temperature pH Dissolved oxygen Agitation speed Seed density Basal medium strength Feed quantity Antifoam Initial medium osmolality	30 – 39°C 6.7 – 7.5 10 – 80% 190 – 210 rpm 2 – 8 x 105 cells/ml 0.8 – 1.2 x nominal 0.5 – 2.0 x nominal 0 – 100 ppm 260 – 320 m0smol/Kg H20	

An example of the validation of the CPPs for a cell culture process for a monoclonal antibody product was reported by Moran, et al.²⁶ In this study, the structure and functional activity of a monoclonal antibody produced at the outer limits of numerical ranges of fed-batch culture control parameters such as pH and temperature were examined using a half-factorial experimental design incorporating half of the thirty two possible combinations of five selected control parameters at high and low levels. Analytical characterization of the monoclonal antibody produced in each of the experimental conditions and statistical analysis of the data collected demonstrated that the purified product was identical throughout the set of experimental conditions and also to the reference standard for the product. Glycosylation analysis confirmed that the distribution of glycoforms of the antibody was not affected by the varying process control conditions of the fed-batch cultures when varied over the appropriate ranges.

In another study, Kunkel, et al. used steady-state continuous cultures to demonstrate the effect of the level of dissolved oxygen in a bioreactor culture on monoclonal antibody glycosylation.¹¹ In this study, significant increases in the relative amounts of agalactosyl chains and a reduction in the relative amounts of digalactosyl chains was observed at lower dissolved oxygen concentrations while the relative amounts of monogalactosyl chains remained unchanged at dissolved oxygen levels ranging from 10 to 100% of air saturation.

Li, et al. used a systematic approach for scale-down model development and characterization of a commercial culture process before process validation.²⁷ A scale-down model using 2 L bioreactors was developed based on a 2,000 L commercial scale process utilizing CHO cells. Profiles of cell growth, productivity, product quality, culture environments (pH, DO, pCO2), and level of metabolites (glucose, glutamine, lactate, ammonia) were compared between the two scales to qualify the scale-down model. The key operating parameters were then characterized in single-parameter ranging studies and an interaction study using this scale-down model. Appropriate operating ranges and acceptance criteria for certain key parameters were determined to ensure process performance consistency and robustness. The worst-case conditions for the manufacturing process were also identified through this interaction study.

In a more recent study, Horvath, et al. applied the principles of QbD to the characterization of a monoclonal antibody cell culture process using a multivariate approach to define the acceptable ranges for the CQA of product yield.²⁸ In this study, a variety of process parameters were tested over a wide range to identify the range in which an acceptable product yield was achieved. Looby, et al. also applied QbD principles to the development and implementation of a major manufacturing process improvement for a commercially distributed therapeutic protein produced in CHO culture.²⁹ A fed-batch production culture and a virus inactivation step, described as representative examples of upstream and downstream unit operations, were characterized. A systematic approach incorporating QbD principles was applied to both unit operations, involving risk assessment of potential process failure points, small-scale model qualification, design and execution of experiments, definition of operating parameter ranges and process validation acceptance criteria followed by manufacturingscale implementation and process validation. Statistical experimental designs were applied to the execution of process characterization studies evaluating the impact of operating parameters on product quality attributes and process performance parameters. Data from process characterization experiments were used to define the proven acceptable range and classification of operating parameters for each unit operation. Analysis of variance and Monte Carlo simulation methods were used to assess the appropriateness of process design spaces. Successful implementation and validation of the process in the manufacturing facility and the subsequent manufacture of hundreds of batches of this therapeutic protein verifies the approaches taken as a suitable model for the development, scale-up and operation of any biopharmaceutical manufacturing process.

Similarly, Rouiller, et al. also applied QbD to the characterization of the cell culture process for production of an Fc-fusion protein.³⁰ Using a detailed risk assessment of the process, different parameters for the process were evaluated for their possible impact on product quality (a CPP) and process performance (a KPP). Following this risk assessment, the critical and key process parameters were evaluated using a design of experiment approach. The data from these experiments were analyzed using a regression model to characterize the impact of each process parameter on quality attributes. From this study, pH and dissolved oxygen were determined to be the main process parameters having an impact on product titer, while those having the highest impact on process- and product-related impurities and variants were pH and culture duration. The models derived these from characterization studies were then used to define the cell culture process design space with limits to this design space being set in such a way as to ensure that the drug substance produced by the overall manufacturing process would consistently have the desired quality.

Abu-Absi, et al. described a methodology using FMEA to map the design space for a monoclonal antibody cell culture process.³¹ Following the risk assessment, an integrated study of the progressive shake flask and seed bioreactor steps included in the inoculum stage of the process was performed. The operating conditions for the seed bioreactor were studied in an integrated fashion with the production bioreactor using a two stage DOE methodology to enable optimization of operating conditions. A two level Resolution IV design, followed by a central composite design (CCD), allowed the identification of the edge of failure of the process and classification of the operational parameters as non-key, key or critical. In addition, the models generated from the data provide further insight into balancing productivity of the cell culture process with product quality considerations. Finally, process and product-related impurity clearance was evaluated by studies linking the upstream process with downstream purification. Production bioreactor parameters that directly influence antibody charge variants and glycosylation in CHO systems were identified.

As discussed above, process qualification (i.e., Stage 2 of process validation) is usually performed after the completion of process development and optimization of the manufacturing process so that no further significant changes in the process are expected. Much process qualification of upstream processes can be performed using qualified scale-down models. However, these small-scale process qualification studies must also be accompanied by additional process qualification at full-scale to demonstrate consistent process performance and product quality when all CPPs are controlled within their pre-defined acceptable ranges at commercial scale. These full-scale process qualification or consistency runs are performed in the equipment and facilities intended for commercial manufacturing of the monoclonal antibody product using fully validated equipment and systems. The exact number of consistency runs required for process validation of a manufacturing process is not specified in any recent regulations or regulatory guidances, however, Li, et al. state that three such runs are normally required for BLA approval in the United States, while the European regulatory authorities typically require five consecutive full-scale runs for marketing approval.³²

Validation of Downstream Processes

For the validation of chromatographic separations used in the downstream processing of monoclonal antibody products, it is important to demonstrate that when operated in a specified manner, each chromatography unit operation yields a product of consistent quality, which conforms to appropriate in-process or final product specifications. During process development, screening studies are conducted in which a large number of process inputs are studied in a systematic way to identify the inputs that have the most significant effects on the process outputs. Following optimization of these process operating parameters, robustness studies can be performed in which the CPPs are studied in a systematic way within their control limits to verify that the resulting process outputs are robust. The robustness studies should demonstrate that the process will not fail when carried out within the normal operation range of such potentially critical process parameters as buffer pH and ionic strength, gradient slope, amount of material applied per unit volume of packing material, temperature, flow rate, and system back pressure.

For each of the CPPs in a chromatographic purification step for monoclonal antibody products, the acceptance criteria for process validation studies should include the appropriate product quality parameters, such as purity, aggregate levels, levels of specific contaminants such as host cell protein, nucleic acids, leached Protein A, or bacterial endotoxin, as well as product potency and safety. Kelley, et al have provided an example of how various process parameters in an anion exchange weak partitioning chromatography (WPC-AEX) operation can impact the quality of a monoclonal antibody.³³ WPC can be used as an alternative to conventional ion exchange chromatography and/or hydrophobic interaction chromatography in the purification of monoclonal antibodies in a two-step purification process (Protein A, WPC-AEX) as an alternative to the more commonly used three-step purification process (see Chapter 7).

Kelley, et al. studied the effect of variations in the pH and counter ion concentration (e.g., chloride) used in the Protein A affinity chromatography step on the ability of the WPC-AEX column to remove impurities from the monoclonal antibody product and also the effect of variations in pH and counter ion concentration in the buffers used in the WPC-AEX chromatography step to similarly control the levels of impurities in the purified monoclonal antibody product. The results of this study indicated that the counter-ion concentration in the buffers used for washing and eluting the Protein A affinity chromatography column and the pH of the monoclonal antibody-containing solution loaded onto the WPC-AEX column were critical in removing impurities from the monoclonal antibody product to the level specified by the product CQA. Therefore, control of these CPP are important to ensure that the levels of impurities found in the monoclonal antibody product following WPC-AEX are acceptable and that the process is robust and reproducible.³³

Another example of the importance of process characterization in demonstrating process robustness can be seen in the preparation of very high concentrations of monoclonal antibody solutions for certain therapeutic applications. Ultrafiltration has been used to achieve concentrations of greater than 180 mg/mL.³⁴ In the development of such a concentration operation, transmembrane pressure is often a CPP that controls permeate flux, product and solute permeability, and product yield. Experiments on the effect of varying transmembrane pressure are therefore important in determining the most robust and optimal operating conditions for any ultrafiltration or diafiltration operation.³⁵ The acceptance criteria for validation studies of the robustness of such unit operations may include the yield of monoclonal antibody product, the final pH and/or conductivity of the retentate, and the restoration of the clean water flux of the membrane following cleaning.

The purification processes for monoclonal antibody products typically contain a number of unit operations designed to inactivate or remove viral, nucleic acid, immunogenic, and pyrogenic contaminants without affecting the potency and activity of the therapeutic product. In addition to contaminants that may have been present in the initial bioreactor harvest, other contaminants, such as reagents used during purification or ligands that may have leached from chromatography media (e.g., Protein A) used in the purification of the monoclonal antibody product, must also be removed from the product during downstream processing. Since the elimination or inactivation of some of these contaminants, such as host cell DNA and proteins and residual Protein A, can be measured by specific assays (e.g., radio-immunoassays, enzyme immunoassays, or protein blotting assays) directed toward the contaminants, end-point testing and clearance studies demonstrating the removal of specific contaminants are typically included in the process validation of a monoclonal antibody purification process.

Clearance of potential virus contamination or process reagents (e.g., methotrexate, Pluronic F68, insulin) that may be present at levels too low to assay directly, is generally determined in scaled-down models in which the particular contaminant of interest is added to the input feed stream on a small scale and the recovery of the contaminant is measured at each stage of the process step such as the column flow-through, product pool, and regeneration fractions using scaled-down columns. In these studies, the addition of the contaminant should be kept to a minimum so the concentration of the feed stream is not significantly changed and care should be taken to demonstrate that the addition of the contaminant does not significantly alter the behavior of product recovery. Mass balance calculations should be performed to demonstrate the consistency and robustness of each unit operation and to ensure that regeneration and cleaning of process equipment is complete.

Viral Clearance Validation

Effective removal of potential viruses is a critical objective of any monoclonal antibody recovery and purification process. However, the unusual nature of viruses dictates a special approach to validating the ability of the process to reproducibly achieve this key objective.

As process contaminants, viruses have two characteristics that make them a special case. The first is the tremendous diversity of potential viruses that could contaminate a process. Viruses fall into several general classes (lipidenveloped and non-enveloped, DNA and RNA), but there are thousands of identified "species," each of which undergoes constant and significant mutations under the pressure of the host immune systems. New viral threats are constantly being identified. Since the precise nature of the potential viral contaminants is highly variable with great diversity, it is not possible to actually measure the degree of overall viral contamination in either the bioreactor feedstream or the final product.

The other special characteristic of viruses is that they are capable of replication in the presence of host cells (in a process or an organism such as a patient). Thus in principle a single virus particle could cause disease if injected into a patient. Given the devastating effects of many viral diseases, it is imperative the level of clearance be very high with a large margin for safety.

Because of these special characteristics of viruses as contaminants, a unique approach is taken to ensure that no viral contamination is present in the final antibody drug product. The basic concept is to include specific steps in the process that are capable of a high degree of removal or clearance of viral activity, either through physical separation or inactivation of the biological activity of viruses through physical or chemical means. At least two (or more) steps are included in the process that are expected to have a high degree of robust viral clearance and that operate by fundamentally different mechanisms (i.e., they are "orthogonal" to each other), to ensure that any viruses not removed or inactivated by one clearance step will be caught by one of the other steps. The final aspect of the approach is to test or validate for viral clearance using a set of diverse model viruses on a scaled-down version of the downstream process clearance and inactivation steps.

Clearance is typically measured on a log scale (i.e., factors of ten of removal). Overall validated clearance levels are expected that provide an adequate margin of safety for the product, including clearance factors that are substantially above the measured level of endogenous retrovirus in the unprocessed bulk (which will be cell line and process dependent).^{24, 36} The validated log clearance determined for each of the steps can be added together to achieve the total level of clearance by the manufacturing process, provided that care is taken to ensure that different steps do not operate by the same mechanism. For example, the unprocessed bulk culture harvest from a production bioreactor using a typical CHO cell line may contain 107 viral particles per milliliter. If a dose-equivalent of the unprocessed bulk supernatant is 100 mL and an overall clearance factor of at least 4 logs above the measured virus in unprocessed bulk is desired, then the process would need to provide a total viral clearance of at least 13 logs to meet this requirement.

The process of validating viral clearance is complex. The first step is to identify the process steps in which significant clearance is likely to occur. Because viruses must be handled on a small scale in a controlled laboratory setting, the selected process steps must be very carefully scaled down so that the critical mechanism of clearance is accurately represented. Once an appropriate scale-down model has been developed, the ability of the process step to clear or inactivate certain model viruses. Typically, four or five viruses are chosen for validation of a commercial process24 while only two or three viruses are generally used for validation of a process prior to IND filing and initiation of clinical trials.³⁷ Viruses selected should represent a diversity of classes and similarity to known viral threats and should also be able to be grown reasonably well in the lab and assayed with high sensitivity. Appropriate assays for the viruses must be developed and validated to

operate in the process samples, with all the appropriate controls. Finally, the clearance validation runs are performed, in which the scaled-down process is run with feed streams spiked with the model viruses, and the product samples assayed for clearance.

One major challenge in process validation for viral clearance is that there are limits to the concentration of virus that can be spiked into the process feedstream and in the sensitivity of the assays used to measure viral activity. Because of this, the clearance that can be demonstrated for a single step is typically limited to three to six logs even though this particular step may be capable of greater clearance. Since this greater clearance cannot be quantitatively demonstrated, the process can only claim to clear what can be measured. As a result, multiple, orthogonal clearance steps are required to ensure process robustness and to ensure that sufficient virus clearance can be achieved and validated.

A wide range of different approaches for viral clearance have been developed. Some are effective only on certain classes of viruses while others are more broadly applicable. Viral clearance technologies also vary widely in their ability to remove or inactivate viruses without causing yield loss, due to removal or denaturation of the antibody product itself. In some cases, a step is introduced specifically for viral clearance, while in other cases a step used for something else (such as purification) can also be optimized to maximize viral clearance.

In a typical platform monoclonal antibody purification process as described in Chapter 7, there are two steps that usually provide broad and robust viral clearance. One is the Protein A affinity chromatography capture step and associated low pH hold. This step actually provides two different types of robust viral clearance. First, the highly selective Protein A binding of the monoclonal antibody can effectively separate viruses that do not bind to the column from the monoclonal antibody, which does, because the viruses will flow through the column and be washed away prior to elution of the monoclonal antibody. Secondly, the low pH elution and hold step can provide a high degree of virus inactivation for enveloped viruses and other viruses susceptible to low pH inactivation. The second key step for viral clearance in a standard platform monoclonal antibody purification process is a nanofiltration step, which is specifically introduced for viral clearance and is generally viewed by regulatory authorities as a very robust

viral clearance step when executed properly. These two steps (Protein A affinity chromatography/low pH inactivation and nanofiltration) provide an orthogonal approach to robust clearance with a very high degree of clearance for all virus types.

In addition to measuring viral clearance for the initial product capture step and nanofiltration, at least one additional step in the monoclonal antibody purification process is also typically included in viral clearance validation studies to ensure the highest level of clearance.

Validation of Chromatography Media Lifetime As part of the validation of chromatography unit operations used in monoclonal antibody purification, it is critical to determine and validate the number of purification cycles that can be processed through a particular chromatography column before the separation media packed in that column must be replaced. This activity should take into account the number of cycles the media is used in each batch and the number of batches to be manufactured in a given timeframe. Chromatography media lifetime is determined by a number of factors, including the chemical and physical stability of the media in the presence of the column feedstream, operating conditions for the chromatography step, and, perhaps most importantly, the amount of irreversible fouling that takes place on the media during each cycle. Chromatography media lifetime is greatly affected by the particular feedstream for each chromatography step, the specific media used, and the cleaning processes employed. As the chromatography media reach the end of their useful lifetime, the purity or yield of final product may be adversely affected, so determination of the effective lifetime is critical to ensuring product quality.

For all process steps using cycling, proposed chromatography media lifetimes should be determined and validated prior to running the process to those lifetimes in production. Since typical chromatography media lifetimes in modern processes often run into hundreds of cycles, it is usually not possible to validate the lifetime as part of clinical trial production or at the proposed commercial scale. Thus, chromatography media lifetime validation studies are typically conducted using a scaled-down model, taking care that the design of studies ensures that both the feedstream and operating conditions in the scaled-down process model provide an accurate simulation of the full-scale process. In some cases, chromatography media lifetime may also be confirmed using an open current validation protocol to assess media lifetime during commercial scale manufacturing.

Media lifetime validation studies can take a considerable period of time to perform and may lead to changes in the process (e.g. re-optimization of cleaning conditions) if the effective lifetime observed in the study is not what was expected.

6. Validation of Monoclonal Antibody Drug Product Manufacturing

Validation of the drug product manufacturing process for a monoclonal antibody product will include validation of all equipment and components used in drug product manufacturing, the sterile filtration process, and all other aseptic manipulations, including stoppering of vials, transfer vials to/from lyophilizers, and the lyophilization process for freeze-dried products. Validation of process equipment includes validation of the sterilization process for all equipment used in sterile filtration and aseptic processing of the bulk drug substance, including in-process hold tanks, filters, product contact parts of the filling and stoppering equipment, validation of the sterilization process for the final container and closure for the monoclonal antibody drug product, and validation of the integrity of the final container/closure system.

Validation of the Sterilization of Drug Product Equipment and Components

In order to demonstrate that equipment, such as autoclaves, dry heat ovens, and vial sterilizing tunnels, used to sterilize components for monoclonal antibody drug product manufacturing properly sterilize these components, a series of equipment qualification and process validation runs are performed. During the qualification stage, the equipment is shown to have been properly designed and installed, and that it operates according to its design specifications. These installation qualification tests are then followed by pressure and heat distribution studies in the sterilizing chamber to map the "cold" spots in the equipment and to demonstrate the correct functioning of control equipment.¹

During validation of the sterilization procedures for drug

product equipment and components, the sterilizers are filled with typical loads of equipment; containers; stoppers; and similar items that will be used in the drug product manufacturing processes and fitted with temperature probes and test strips containing live thermophilic bacteria, representing a biological challenge. Once a sufficient sterilization cycle has been developed, validation of this process must show that the autoclave can reliably and reproducibly achieve the desired temperature at all points in its chamber for all loads tested and hold this temperature for sufficient time to kill all test bacteria and achieve full sterilization of the load.

Dry heat sterilization in a hot-air oven or radiant-heated tunnel of empty glass containers is also intended to destroy any bacterial endotoxins (pyrogens) that may be present on the surfaces of the container. Therefore, the validation of this process will also include a challenge of the sterilization and depyrogenation procedure with vials containing a measured amount of endotoxin to demonstrate complete inactivation or destruction of the endotoxin.

Sterile Filtration Validation

Validation of the sterile filtration process for monoclonal antibody products includes scale-down filtration runs in which the filter is challenged with a solution of the monoclonal antibody drug substance spiked with a standardized suspension of live diminute bacteria. The standard challenge organism for this validation of sterile filtration operations is Brevundimonas (Pseudomonas) diminuta (ATCC 19146) although some companies use the mycoplasma Acholeplasma laidlawii, which is smaller than B. diminuta, as an alternate challenge microorganism. The FDA Guidance on Aseptic Processing requires that the filter be challenged with a suspension containing a minimum concentration of 107 viable bacteria per square centimeter of membrane surface and that the resulting filtrate be sterile.³⁸ Standard test conditions and procedures for this sterile filter validation are defined in USP <71>.39

The conditions under which the filter will yield a sterile filtrate should be tested using the actual formulated bulk drug substance on a small scale, since the character of the solution may affect the filter's performance. Filters from different manufacturers may have different performance characteristics, especially for the removal of mycoplasma or the non-specific binding of proteins. Therefore, it is important that the filters used for these small-scale validation studies be from the same vendor and made of the same materials of construction as the actual process-scale filters used for drug product manufacturing. Validation results from one brand or type of sterilizing grade filter may not be used to justify the use of another brand or type of filter; each specific filter used for sterile filtration of monoclonal antibody drug products must be separately validated.

During routine drug product manufacturing operations, the integrity of the actual filter used for the sterile filtration should be demonstrated both before and after the product filtration to demonstrate that its integrity has not been compromised.⁴⁰ In addition, the bioburden of the formulated bulk drug substance should be measured before the sterile filtration and sterility testing should be performed on samples of the sterile bulk drug solution after passing through the filter.

Validation of Aseptic Filling and Finishing Operations

Validation of the actual aseptic filling operation is done by simulating the manufacturing process using solutions of bacterial culture media in place of the actual drug substance solution. The bacterial culture media is prepared and processed using the same operations as for drug product manufacturing and vials are filled under normal operating conditions. Complete validation of aseptic processing should include a simulation of all aseptic liquid manipulations relating to the manufacture of the monoclonal antibody drug product, including such operations as aseptic sampling of the bulk product prior to filling, aseptic transfer of the product from one container to another, and in-process sampling or inspection of filled vials during the filling operation.41 When designing a media fill, it is important that the most accurate simulation model be used so that the batch size and duration of the aseptic process validation mimics as closely as possible actual production operations. For example, the duration of the media fill run should be at least as long as the duration of the actual aseptic processing operation and interventions, such as fill volume checks and other sampling which occur routinely during actual manufacturing are simulated during the media fill.

The number of vials typically filled during a media fill should be based on contamination risk for a given process

and sufficient to accurately simulate activities that are representative of the manufacturing process. For large scale manufacturing batches of greater than 5,000 vials per batch, media fills of 5,000 to 10,000 vials are typically performed.³⁸ For aseptic fills of less than 5,000 vials, as is often the case for early stage clinical trial materials, the media fill batch size should be equal to or greater than the actual number of vials expected in the drug product batch.

Following the sterile filtration and aseptic filling and stoppering of the vials during a media fill, the containers are incubated at two different temperatures to determine whether any are contaminated. The aseptic filling and finishing process is considered validated as a sterile operation if no contaminated units are observed.^{38,41}

When a manufacturing line for the manufacture of a monoclonal antibody drug product is initially qualified, individual media fills should be repeated a sufficient number of times to ensure that results are consistent and meaningful.³⁸ Typically, the initial process validation includes three consecutive successful media fills per shift. Following this initial validation, the media fills should be repeated at defined intervals, generally repeated twice a year per shift and process.⁴¹ In addition, the media fills should be repeated after any significant modification or deviation from the normal operating conditions for drug product manufacturing, including such things as the changing of HEPA filters in the production area or other facility or equipment changes, a change in the production batch size, or a change in the overall manufacturing process or number of shifts used for the aseptic operations.

If a contract manufacturer is used for drug product manufacturing for clinical trials, it is often not necessary to perform new media fills if aseptic process validation for the particular container/closure combination to be used for the manufacture of clinical trial materials has already been completed by the contract manufacturer. In these cases, only limited additional validation may be required.

For monoclonal antibody drug products that are lyophilized, the lyophilization equipment and process must also be validated. This validation will include demonstration that the lyophilization cycle reliably and reproducibly produces an acceptable product and that all procedures for handling and transferring vials to and from the lyophilizer do not compromise the quality of the final drug product. In order to validate all aseptic manipulations involved in preparing a lyophilized drug product, an expanded media fill is often used in which a group of some of the vials are filled and immediately stopped and sealed as for the validation of a liquid fill operation. These vials are tested for contamination as in the validation of a liquid fill operation. Another group of vials is then filled, transported to the lyophilizer as in the normal manufacturing operation, and stoppered and sealed without lyophilization. These sealed vials are then incubated and tested for contamination. By testing vials with and without transport to the lyophilizer, the filling operation and the transport and stoppering of the vials can be validated independently.

Validation of Container and Closure System Integrity

Products labeled as sterile are expected to be free from viable microbial contamination throughout the product's shelf life or dating period, and drug manufacturers are required to demonstrate that the container and closure system for their product is capable of maintaining the microbial barrier integrity.

Dye ingress⁴² and sterility tests have been routinely used to verify that containers/closures⁴³ maintain their integrity and products maintain their sterility, but these methods have limitations. The pharmaceutical industry has sought alternatives to dye ingress and sterility testing and several instrumentation-based technologies have emerged and have been proven superior for detection capabilities. These technologies include high voltage leak detection,⁴⁴ vacuum decay,⁴⁵ mass extraction,⁴⁶ and tracer gas detection.^{47, 48, 49} The use of these new technologies is increasing because of their multiple advantages.⁴³ However, when selecting a technology to validate container/closure integrity it should be kept in mind that all have limitations.⁵⁰ Therefore, the suitability of the technology for its intended use, the applicability of the technology to the specific drug product container/closure to be validated, the detection capability and effectiveness of a technology, and whether or not the technology is nondestructive in nature should be considered.

The 2008 FDA guidance on the use of container/closure integrity testing, in lieu of sterility testing, during stability testing of sterile product specifically discusses the need to include sterility testing as part of any stability protocol

for these products.⁴³ Therefore, stability protocols for monoclonal antibody products should always include confirmation of sterility throughout the proposed product shelf life. While container/closer testing can be used during a stability protocol as a means of demonstrating sterility during storage, such testing cannot replace sterility testing for product release. If container/closure testing is used during a stability protocol, a formal stability test should be conducted on each stability batch, at a minimum, at the start and completion of the stability study.

Validation of the Transport and Storage of Monoclonal Antibody Products

All pharmaceutical products, including monoclonal antibody products, must be appropriately packaged to prevent breakage or adulteration during shipment and storage as well as to ensure that the product is maintained within acceptable temperature limits when properly shipped and stored. PDA Technical Report³⁹, Guidance for Temperature-Controlled Medicinal Products: Maintaining the Quality of Temperature-Sensitive Medicinal Products through the Transportation Environment,⁵¹ provides an outline of the essential principles and practices for shipment of products that require controlled temperature during transit and also provides a design approach to the development of specialized packages and systems, which will protect temperature-sensitive products during transportation and storage.

According to the Guidelines on Good Distribution Practice of Medicinal Products for Human Use, regardless of the mode of transportation or storage, drug manufacturers are required to demonstrate that medicines have not been exposed to conditions that may compromise their quality and integrity.52 As with other aspects of drug product validation, the shipment and storage of monoclonal antibody products should be validated using a risk-based approach that takes into consideration the extremes that a product may experience during handling, shipment, and storage. In the recently published revision of its guidelines for qualification and validation of the medicinal products for human and veterinary use,⁵³ the EMA included a chapter on verification of transportation of these products. This guideline, along with the earlier guideline on distribution of medicinal products,⁵² specifies that the transport of samples, investigational, bulk and finished medicinal products must be done in accordance with the conditions defined in the Marketing Authorization

(storage conditions), product specification file (for investigational products) or by the manufacturer. To ensure that the conditions chosen are appropriate, a risk assessment should be performed, which is not limited to just the impact of temperature on the product, but also the impact of such factors as humidity, vibration, handling, delays during transportation, failure of data-loggers, topping of dry ice or liquid nitrogen. A manufacturer must demonstrate that product integrity and quality is maintained during transport and that the packaging used can adequately maintain the appropriate temperatures to ensure product stability. Validation of the shipping of monoclonal antibody products should address the many variables that can influence product integrity during the logistics process, including environmental extremes, packaging degradation, courier mishandling, import/export requirement delays, seasonal changes, and transit time.

Because of the many factors that may impact product integrity or stability during transportation, these routes should be clearly defined and static stability studies, such as those described in Chapter 8, should be augmented with "dynamic" or "stress" stability studies conducted in uncontrolled "real world" shipping studies or simulated in a laboratory environment.^{54, 55} Validation of the shipping of both bulk drug substance and drug product for a monoclonal antibody product should include not only validation of temperature maintenance and control, but also validation of humidity, product handling, failure of data-loggers, and requirements for topping a shipment with ice, dry ice, or liquid nitrogen to maintain temperature during extended shipments.⁵⁶

7. Process Validation Planning and Execution

Although it is not mandatory, regulatory agencies have come to expect that a Sponsor's approach to process validation will be described in a Validation Master Plan (VMP). The VMP documents a company's approach to process validation and also clarifies or defines responsibilities, general objectives, and procedures to be followed for validation. It may reference several protocols and procedures to be written in order to conduct the qualification of several different pieces of equipment and different processes. It may also specify schedules for validation activities and the allocation of resources needed to perform each validation study. A typical VMP for the manufacture of a monoclonal antibody bulk drug substance should contain, at a minimum, the information listed in Table 11.4.

Table 11.4. Sample VMP Table of Contents Introduction Scope of Plan Acceptance Criteria for all Protocols Project Schedule and Budget Resource Requirements - Documentation - Facilities - Personnel Document Format List of Individual Protocols Standard Operating Procedures to be Followed

Individual process validation protocols should describe in detail the procedures to be followed and should specify critical and key operational parameters and their respective ranges, data acceptance criteria, as well as procedures required to perform the validation, including the sampling plan, and the responsibilities of the various functional team members participating in the validation study. The protocol should also specify a sufficient number of replicate process runs to demonstrate reproducibility of the process and provide an accurate measure of variability among successive runs. The test conditions for each process validation run should encompass the upper and lower processing limits and circumstances, including those within standard operating procedures, which pose the greatest chance of process or product failure compared to ideal conditions; such conditions have become widely known as "worst case" conditions (sometimes referred to as "most appropriate challenge" conditions.) The new process validation guidance specifies that it is not necessary to employ the "test to failure" approach, only ensure that those conditions which pose the greatest risk of variation beyond acceptable limits or the greatest risk to the quality of the product should be adequately studied.¹ It is anticipated that in the future a Design Space will be generated for each critical process that encompasses all acceptable operating conditions.

At the conclusion of each process validation study, a final validation report should be prepared that documents the results of the validation study, including data from any qualification or production batch run as part of the protocol, a summary of protocol or batch nonconformances along with the investigation of the nonconformance and any conclusions or recommendations resulting from the investigations, and a summary of whether the acceptance criteria of the protocol have been met.

Aside from meeting the regulatory requirements for process validation, the VMP, validation protocols and final reports will serve as a repository of key development and process information, which can be used to support future process changes and improvements and support further development of the design space for the manufacturing process. In the near term, the incorporation of QbD and the new concepts of process verification are expected to be flexible as regulatory authorities fully define the requirements and expectations of these new initiatives and industry moves from the traditional fixed-point validation to a lifecycle approach to process validation. During this transition, it is expected that regulatory filings will incorporate blended elements of both approaches.

References

- Food and Drug Administration, Center for Biologics Evaluation and Research, and Center for Devices and Radiological Health (US). Guideline on general principles of process validation [Internet]. Washington, DC: Department of Health and Human Services; 1987 May [cited 2016 Nov 23]. [about 19 screens]. Available from: http://www.fda-consultant.com/provalid.html
- 2 Food and Drug Administration (US). Pharmaceutical cGMPs for the 21st century a risk-based approach. Final report [Internet]. Washington, DC: Department of Health and Human Services; 2004 Sep [cited 2016 Nov 23]. 32 p. Available from: http://www.fda.gov/downloads/Drugs/Develop-mentApprovalProcess/Manufacturing/QuestionsandAnswersonCurrentGoodManufacturingPracticescGMPforDrugs/UCM176374.pdf.
- 3 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Quality risk management Q9 Step 4 [Internet]. Geneva (CH): International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2005 Nov 9 [cited 2016 Nov 23]. 23 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ ICH_Products/Guidelines/Quality/Q9/Step4/Q9_Guideline.pdf.
- 4 Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Center for Veterinary Medicine (US). Guidance for industry: Process validation: General principles and practices. Revision 1 [Internet]. Washington, DC: Department of Health and Human Services; 2011 Jan [cited 2016 Nov 23]. 22 p. Available from: http://www.fda.gov/downloads/Drugs/Guidances/UCM070336.pdf.
- 5 European Medicines Agency, Committee for Medicinal Products for Human Use and the Committee for Medicinal Products for Veterinary Use. Guideline on process validation. Draft [Internet]. London (GB): The European Medicines Agency; 2012 Mar 29 [cited 2014 Nov 19]. 11 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/04/WC500125399.pdf.
- 6 Parenteral Drug Association. Process validation: a lifecycle approach. PDA J Pharm Sci Technol. 2013;67(2 Suppl TR 60):2-102.
- 7 Quality System Regulation, 21 CFR Part 820 (2013).
- 8 Sidor L, Lewus P. Using risk analysis in process validation. Biopharm Int. 2007 Feb;20(2):25-32.
- 9 CMC Bio Working Group. A-Mab: a case study in bioprocess development Version 2.1 [Internet]. Emeryville (CA): California Separation Science Society. 2009 Oct 30 [cited 2016 Nov 239]. Available from: http://www.casss.org/associations/9165/files/A-Mab_Case_Study_Version_2-1.pdf.
- 10 Burleigh SC, et al. Synergizing metabolic flux analysis and nucleotide sugar metabolism to understand the control of glycosylation of recombinant protein in CHO cells. BMC Biotechnol. 2011 Oct 18;11:95.
- 11 Kunkel JP, et al. Comparisons of the glycosylation of a monoclonal antibody produced under nominally identical cell culture conditions in two different bioreactors. Biotechnol Prog. 2000 May-Jun;16(3):462-70.
- 12 Katz P, Campbell C. FDA 2011 process validation guidance: process validation revisited. J GXP Compliance. 2012 Autumn;16(4):18-29.
- 13 Boychyn M, Hart R. Design of a viral contamination barrier for a serum-containing cell culture process. Presented at IBC Life Science's BioProcess International Conference and Exhibition; 2010 Sep 20-24; Providence, RI.
- 14 Kiss RD. Practicing safe cell culture: applied process designs for minimizing virus contamination risk. PDA J Pharm Sci Technol. 2011 Nov-Dec;65(6):715-29.

- 15 International Electrotechnical Commission (CH). IEC 60812. Analysis techniques for system reliability Procedure for failure mode and effects analysis (FMEA). Geneva (CH): International Electrotechnical Commission; 2006. (IEC 60812 ed 2.0:2006)
- 16 U.S. Food and Drug Administration [Internet]. Silver Spring (MD): U.S. Food and Drug Administration; [updated 2014 May 2]. Hazard analysis and critical control point principles and application guidelines; [updated 2014 Sep 19; cited 2016 Nov 23]; [about 29 screens]. Available from: http://www.fda.gov/food/guidanceregulation/haccp/ucm2006801.htm.
- 17 Nosal R, Shultz T. PQLI definition of criticality. J Pharm Innov. 2008 Jun;3(2):69-78.
- 18 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Pharmaceutical Development Q8(R2) Step 4 [Internet]. Geneva (CH): International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2005 Aug [cited 2016 Nov 23]. 28 p. Available from: http://www.ich.org/ fileadmin/Public Web Site/ICH Products/Guidelines/Quality/Q8 R1/Step4/Q8 R2 Guideline.pdf.
- 19 European Medicines Agency, U.S. Food and Drug Administration. EMA-FDA pilot program for parallel assessment of Quality by Design applications: lessons learnt and Q&A resulting from the first parallel assessment [Internet]. London (GB): The European Medicines Agency; 2013 Aug 13 [cited 2016 Nov 23]. 3 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Other/2013/08/WC500148215.pdf.
- 20 Joneckis C. Regulatory expectations for process validation. Presented at the California Separation Science Society's Well Characterized Biological Products (WCBP) Conference 2004; 2004 Jan 6-10; Washington, DC.
- 21 McNally GE. Process validation: a lifecycle approach [Internet]. Silver Spring (MD): U.S. Food and Drug Administration; 2011 May 6 [cited 2016 Nov 23]. 40 p. Available from: http://www.fda.gov/downloads/AboutFDA/CentersOffices/ CDER/UCM255585.pdf.
- 22 Chapman KG. The PAR approach to process validation. Pharm Technol. 1984 Dec;8(12):22-36.
- 23 Chapman KG, et al. Proposed validation standard VS-1. J Validation Technol. 2000 Feb;6(2):502-21.
- 24 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Expert Working Group (CH). Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin Q5A(R1) Step 4 [Internet]. Geneva (CH): International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 1999 Sep 23 [cited 2016 Nov 23]. 31 p. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5A_R1/Step4/Q5A_R1_Guideline.pdf.
- 25 Seely JE, Seely R. A rational, step-wise approach to process characterization. BioPharm Int. 2003 Aug;16(8):24-34.
- 26 Moran EB, et al. A systematic approach to the validation of process control parameters for monoclonal antibody production in fed-batch culture of a murine myeloma. Biotechnol Bioeng. 2000 Aug;69(3):242-55.
- 27 Li F, et al. A systematic approach for scale-down model development and characterization of commercial cell culture processes. Biotechnol Prog. 2006 May-Jun;22(3):696-703.
- 28 Horvath B, et al. Characterization of a monoclonal antibody cell culture production process using a quality by design approach. Mol Biotechnol. 2010 Jul;45(3):203-6.
- **29** Looby M, et al. Application of quality by design principles to the development and technology transfer of a major process improvement for the manufacture of a recombinant protein. Biotechnol Prog. 2011 Nov-Dec;27(6):1718-29.
- **30** Rouiller Y, et al. Application of quality by design to the characterization of the cell culture process of an Fc-fusion protein. Eur J Pharm Biopharm. 2012 Jun;81(2):426-37.
- 31 Abu-Absi SF, et al. Defining process design space for monoclonal antibody cell culture. Biotechnol Bioeng. 2010 Aug 15;106(6):894-905.
- 32 Li F, et al. Cell culture processes for monoclonal antibody production. MAbs. 2010 SepOct;2(5):466-79.
- 33 Kelley B, et al. Weak partitioning chromatography for anion exchange purification of monoclonal antibodies. Biotechnol Bioeng. 2008 Oct;101(3):553-66.
- 34 Luo R, et al. High concentration UF/DF of a monoclonal antibody. BioProcess Int. 2006 Feb;4(2):44-8.
- **35** Thompson RE, et al. Validation of recovery and purification processes. In: Agalloco J, Carleton FJ, editors. Validation of pharmaceutical processes. Third ed. New York: Informa Healthcare USA; 2007. p. 455-72.
- 36 European Medicines Agency, Committee for Proprietary Medicinal Products (GB). Note for guidance on virus validation studies: the design contribution and interpretation of studies validating the inactivation and removal of viruses [Internet]. London (GB): The European Medicines Agency; 1996 Feb 14 [cited 2016 Nov 23]. 14 p. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003684.pdf.
- 37 European Medicines Agency, Committee for Medicinal Products for Human Use and the Biologics Working Party (GB). Guideline on virus safety evaluation of biotechnological investigational medicinal products [Internet]. London: The European Medicines Agency; 2008 Jul 24 [cited 2016 Nov 23]. 9 p. Available from: http://www.ema.europa.eu/ docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003795.pdf.
- 38 Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Office of Regulatory Affairs (US). Guidance for industry: Sterile drug products produced by aseptic processing – current good manufacturing practice [Internet]. Washington, DC: Department of Health and Human Services; 2004 Sep [cited 2016 Nov 23]. 63 p. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070342.pdf.
- 39 United States Pharmacopeial Convention. The United States Pharmacopeia–National Formulary (USP32-NF27). Rockville (MD): The United States Pharmacopeial Convention; c2008. <71> Sterility tests; p. 80-6. Updated in USP32-NF27, Suppl(1):3494.
- 40 McBurnie L, Bardo B. Validation of sterile filtration. Pharm Technol. 2004 Oct;28(Suppl):S13-23.
- 41 Sheridan G. Aseptic process validation. Presented at the Irish Medicines Board GMP & Market Compliance Information Day; 2012 Sept 27; Dublin, Ireland.
- 42 Burrell LS, et al. Development of a dye ingress method to assess container-closure integrity: correlation to microbial ingress. PDA J Pharm Sci Technol. 2000 NovDec;54(6):449-55.

- 43 Food and Drug Administration, Center for Biologics Evaluation and Research, Center for Drug Evaluation and Research, Center for Devices and Radiological Health and Center for Veterinary Medicine (US). Container and closure system integrity testing in lieu of sterility testing as a component of stability protocol for sterile products [Internet]. Washington, DC: Department of Health and Human Services; 2008 Feb [cited 2016 Nov 23]. 9 p. Available from: http://www.fda.gov/downloads/RegulatoryInformation/ Guidances/UCM146076.pdf.
- 44 Moll F, et al. Validation of a high voltage leak detector for use with pharmaceutical blow-fill-seal containers-a practical approach. PDA J Pharm Sci Technol 1998 SepOct;52(5):215-27.
- 45 Wolf H, et al. Vacuum decay container/closure integrity testing technology. Part 2. Comparison to dye ingress tests. PDA J Pharm Sci Technol 2009 SepOct;63(5):489-98.
- 46 Yoon SY, et al. Mass extraction container closure integrity physical testing method development for parenteral container closure systems. PDA J Pharm Sci Technol. 2012 Sep-Oct;66(5):403-19.
- 47 Kirsch LE, et al. Pharmaceutical container/closure integrity: I: Mass spectrometry-based helium leak rate detection for rubber-stoppered glass vials. PDA J Pharm Sci Technol 1997 SepOct;51(5):187-94.
- 48 Sudo H, et al. Development of a nondestructive leak testing method utilizing the head space analyzer for ampoule products containing ethanolbased solutions. PDA J Pharm Sci Technol. 2012 SepOct;66(5):434-44.
- **49** Zuleger B, et al. Container/closure integrity testing and the identification of a suitable vial/stopper combination for low-temperature storage at -80{degrees}C. PDA J Pharm Sci Technol. 2012 SepOct;66(5):453-65.
- 50 Li L. Container closure integrity testing method development and validation for prefilled syringes. Amer Pharm Rev. 2013 Jan-Feb;16(1):1-8.
- **51** Parenteral Drug Association. Guidance for temperature-controlled medicinal products: maintaining the quality of temperature-sensitive medicinal products through the transportation environment. PDA J Pharm Sci Technol. 2007;61(2 Suppl TR 39):2-19.
- 52 European Commission (BE). Guidelines of 5 November 2013 on Good Distribution Practice on medicinal products for human use. Official J EU. 2013 Nov 23; 56(C343):1-14.
- 53 European Commission (BE). EudraLex volume 4: EU guidelines to good manufacturing practice, medicinal products for human and veterinary use. Annex 15: qualification and validation [Internet]. Brussels (BE): European Commission; 2014 Feb 6 [cited 2016 Nov 23]. 17 p. Available from: http://ec.europa.eu/health/files/gmp/2014-02_pc_draft_gmp_annex.pdf.
- 54 Elliot MA, Halbert GW. Maintaining the cold chain shipping environment for Phase I clinical trial distribution. Int J Pharm. 2005 Aug 11;299(1-2):49-54.
- 55 Elliot MA, Halbert GW. Maintaining a frozen shipping environment for Phase I clinical trial distribution. Int J Pharm. 2008 Jan 4;346(1-2):89-92.
- **56** Ammann C. Stability studies needed to define the handling and transport conditions of sensitive pharmaceutical or biotechnological products. AAPS PharmaSciTech. 2011 Dec;12(4):1264-75.

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CHAPTER 12

Manufacturing Strategies

ne of the most critical challenges facing companies developing monoclonal antibody products that may be years from commercial launch, is securing sufficient and timely manufacturing capacity for the production of clinical trial materials, while planning for the eventual commercial manufacturing of the product following regulatory approval. Companies must choose from several different alternatives including building internal capabilities, acquiring existing manufacturing assets, outsourcing manufacturing to a CMO, or combinations of different aspects of all of these. Regardless of the manufacturing strategy adopted by a company, these decisions will involve significant capital resources, whether through the construction or acquisition of in-house manufacturing capacity, expenses associated with outsourcing, or a combination of both approaches. In addition, the opportunity cost of allocating funds away from other important initiatives, such as development of alternate products, may limit the ability of a company to develop a monoclonal antibody product from discovery through clinical trials and commercialization. Therefore, it is important to weigh all factors carefully to define a manufacturing strategy that best fits an organization's long-term goals and vision and that mitigates risk associated with untimely or insufficient manufacturing capacity to meet clinical trials needs and market demand for a new product. Traditionally, the design, construction, and start-up of an in-house manufacturing facility took as much as five years or more, at costs exceeding \$100 million.1 Today, however, a number of advances, including dramatic increases in product titer and yields, the development and availability of single-use technologies, and an increased focus on development of products for personalized medicine or small niche markets, are enabling companies to establish in-house manufacturing capabilities much faster and at lower capital investments.^{2,3} For companies who do choose to outsource manufacturing, there is no guarantee that an outsourced solution will be available when needed, or sufficiently cost effective and flexible to meet the needs of the company.

Since the technological risks associated with drug development efforts are high and the probability of a monoclonal antibody successfully completing clinical trials and reaching the market is less than 20%,^{4,5} accurately estimating capacity requirements years in advance is challenging. If a company builds too much in-house capacity, it will be left with an underutilized asset while trying to identify ways to recoup wasted investment dollars that could have been better utilized elsewhere. If they build too little capacity, the company may lose substantial product sales revenue and/or time in getting the product through the clinic due to delays in manufacturing. Given the risks (and opportunities) inherent in drug development, choosing the right manufacturing strategy to sufficiently mitigate risks, while remaining flexible enough to take full advantage of opportunities when they arise, presents a major and significant challenge to companies developing and commercializing therapeutic monoclonal antibody products.

1. Make versus Buy Strategies

A manufacturing strategy should focus on optimizing performance, flexibility, return on capital, and risk mitigation. When evaluating whether to build or acquire internal capabilities ("Make") or to outsource ("Buy"), the focus should be on optimizing internal company opportunities given that there is generally limited capital available for competing initiatives, such as manufacturing capacity versus R&D capacity.

This is not straightforward when both the opportunities and risks are moving targets. There is significant uncertainty when developing a manufacturing strategy for a pipeline of products extending a number of years into the future as shown in Figure 12.1. Companies cannot accurately predict how many products will be in the pipeline and at what stage of development each of the products will be over an extended period. The clinical plan success (or lack thereof), and business partnering deals affect the number of products in development. This, coupled with variability in manufacturing titer, process yields and scale, results in significant uncertainty when trying to balance pipeline demand with capacity supply.

Figure 12.1. Manufacturing Strategy Considerations

The large number of variables and the uncertainties of biopharmaceutical drug development make it difficult to accurately predict future manufacturing requirements for production of clinical trial material or commercial products. As a consequence, a variety of options can be considered depending on the risk tolerance of a company, its overall current and planned product pipeline, and its existing manufacturing experience and capabilities.



2. Key Issues Associated with Developing Manufacturing Strategies

New business and clinical developments can lead companies without a well-developed strategy to inadvertently establish a manufacturing strategy that relies too heavily on one end of the make versus buy spectrum or the other. To guide the strategic planning process and to minimize these fluctuations, a manufacturing strategy value proposition should be established that is consistent with the company's current and future views. This proposition would describe the current and future state of a company's manufacturing capabilities including access to proprietary knowledge and/ or technology, ability to innovate, operational excellence, risk management, and the ability to add value to the organization. The timing, costs, and benefits of achieving this future state should be included.

Capturing or leveraging this value in the future state requires developing strong capabilities through either in-house manufacturing or outsourcing. In-house manufacturing excellence should include superior process development, production and quality systems, continuous capability enhancement, ability to recruit and retain skilled staff, and effective translation of development results into manufacturing. Outsourcing excellence should include superior supplier selection, strong supply chain management, explicit performance driven contracts and quality agreements, sufficient in-house expertise to manage the supplier effectively, and rigorous supplier management programs.

Technology

As discussed in this report, advances in mammalian expression vectors, cell lines, and culture media, as well as improved cell culture process development approaches and targeted cell line engineering, have enabled development of high product titer cell culture processes prior to initial clinical trials.^{6,7} Coupled with the ongoing improvements in basic operating efficiencies and the use of single use bioreactors that reduce suite and equipment turnaround time, these advances have generally shifted the capacity bottleneck from bioreactor size to downstream processing capability. There are currently several service providers offering highly evolved, robust parental cell lines for cell line development, novel genetic elements in their expression systems, and process development methodology that together can result in bioreactor productivities of 5.0 g/L or greater.⁸ Companies with limited experience in industrial cell culture or that are new to biologics are unlikely to have developed expertise and resources in these areas, so it usually makes strategic sense to outsource early development activities to CMOs and technology companies rather than build internal capability. This is especially true if the number of products in a company's pipeline is limited. Although a highly productive cell culture process is not required to provide material for early clinical studies, the application of QbD principles to development of the initial production cell line and manufacturing process to obtain sufficient process information to enable successful scale up and production throughout clinical development is driving companies towards the development of the most productive cell lines and robust processes as early in development as possible. This paradigm shift is partially fueled by the growth of biosimilars, where achieving similarity to the innovator product is critical and the clinical timeline is too short to allow for major process changes. Even small companies developing innovative monoclonal antibody products should consider spending the time and effort upfront to develop robust cell lines and processes early in development to add value to their products and enhance their ability to find suitable development or commercialization partners. In addition to these long-term benefits, upstream processes with high expression levels will reduce the overall cost of manufacturing and shorten development timelines by reducing the number or scale of required batches to support clinical development.

Investment

Investment costs vary depending on what scale of capacity and/or facility is being built. Facility construction, expansion and/or acquisition and operating costs vary depending on the number of products and technology a facility is expected to support. These investment decisions are made using assumptions about pipeline size, timing and productivity, and, in order to support commercial launch, they must be made long before regulatory approval for distribution of any of the products.

The impact of new technology on the cost and timing for building manufacturing capacity can be seen in the increasing use of single-use technologies for monoclonal antibody production. Integrating these new technologies into a manufacturing process and facility can significantly reduce both the investment and operating costs of the facility, as well as the financial risk associated with the construction of such a facility.^{9, 10} By integrating these technologies and concepts, facilities for monoclonal antibody production require a smaller footprint than traditional facilities and may be deployed more rapidly in locations where clean-room and piping expertise may not be readily available.^{11, 12}

For example, Hodge has shown that a facility based on single-use bioreactors rather than traditional stainless steel bioreactors can be constructed up to twelve months faster than the conventional "all stainless steel" facility.13 Merck has recently shown that the capital cost of a facility with six stainless-steel 2,000 L bioreactors is approximately \$200 million compared to a capital cost of approximately \$70 million for the same facility using six 2,000 L single-use bioreactors.¹⁴ Overall, a facility incorporating single use technologies will require less infrastructure, such as utilities and space, compared to a traditional stainless-steel facility, in a dramatically smaller footprint, significantly reducing the capital cost of the facility.¹⁴ On the other hand, the operating costs of a facility using single-use technologies may be higher than a traditional all stainless facility due, in part, to the costs associated with the single-use components.

Risk

Product pipelines inevitably have attrition due to a number of factors such as unacceptable product safety or efficacy, low productivity resulting in unacceptable cost of manufacturing or unreasonable manufacturing scale, low or no efficacy, reprioritization of assets, and regulatory submission failure or delays that result in product candidates dropping out along the development cycle. There is also uncertainty associated with new product additions from business development activity, including product or company acquisitions. Another source of uncertainty derives from future product demand, which can significantly deviate, higher or lower, than projections after the product receives marketing approval. An approach to manage this risk could entail a blend of internal build and outsourcing to a CMO after marketing authorization is obtained and initial sales revenues are realized. Having a strategic plan in place allows faster execution of manufacturing options as new information provides more clarity as to the best options. For example, a production facility could be built in such a way as to allow relatively rapid build-out of additional capacity if needed, or CMO capacity can be identified and secured with

an option agreement in case approval is obtained and sales increase significantly above projections.

3. Options for Monoclonal Antibody Production

As noted, one of the most critical decisions affecting successful monoclonal antibody product development is the choice of how and where to manufacture product for pre-clinical and clinical testing. These choices include outsourcing production to a qualified CMO, building an internal manufacturing facility, or acquiring an appropriate existing manufacturing facility. The ultimate pathway chosen for a company's manufacturing strategy can dramatically influence the ability to rapidly supply product for clinical trials.

Outsourcing

Companies may choose to outsource monoclonal antibody development and manufacturing to enhance their business focus on core competencies, particularly if monoclonal antibody development and manufacturing is difficult to manage because of lack of resources, competency, or commitment within the organization. This approach also allows companies to leverage process development and manufacturing improvements that can be achieved elsewhere by organizations that focus solely on development and manufacturing of biopharmaceuticals. Other reasons to outsource include the need to reduce internal operating costs and to redeploy resources, as well as to avoid capital outlays.

There are several competent CMOs that can provide the depth of capability necessary to support an entire development program. There are also many CMOs that specialize in early-stage development programs and some that focus primarily on commercial products. If the pipeline is particularly large, it is wise to work with more than one CMO in order to spread the risk of product development and manufacturing across multiple entities; however, one should provide each CMO with sufficient business to gain a high level of attention within the CMO's organization. While some cost and timesaving can be achieved by placing all of the outsourced products with a single CMO, doing so carries a downside. The switching costs can be quite high from a current CMO to a completely new CMO. If a natural disaster, a force majeure event, a regulatory compliance problem or a change in ownership occurs at the CMO, necessitating a switch to a different CMO, development programs can be put at great risk if all of the products are

placed at a single CMO.

There are downsides to pursuing an outsourcing strategy. Outsourcing can delay development of internal manufacturing competencies if these are needed in the future. Capacity at a CMO may not be available or it may not be accessible at the time when it is needed. This can delay delivery of clinical trial materials material or even commercial product to meet market requirements. In addition, there are risks to product supply when using a CMO. For example, the Sponsor is ultimately responsible for the quality and cGMP compliance for its antibody product, and using a CMO does not absolve the sponsor of this responsibility. Outsourcing takes some control out of the hands of the Sponsor. Of course, other scenarios could negatively impact product supply from a CMO, such as serious compliance issues. The Sponsor also loses some control over the development process and may be locked into the some of the CMO's platform technologies and may not be able to drive the CMO to achieve the technological and quality goals the sponsor considers important within timeline and budget limitations. Finally, to the extent that the CMO uses proprietary technology, the ability to transfer processes to other manufacturers (including the sponsor company) must be carefully evaluated.

Facility Construction

Building a facility and a development and manufacturing organization to support a pipeline provides increased control and flexibility to advance a company's product pipeline through development. The facility can be designed to meet the most probable pipeline requirements including expansion space in a geographic location that is convenient and allows the company to recruit and retain talent without much difficulty. The design can incorporate singleuse technologies, particularly for a clinical production facility, to improve flexibility and reduce cost relative to a conventional stainless steel facility. Capacity is also an attractive asset to potential development partners who may not have adequate capacity themselves to develop and manufacture monoclonal antibodies.

Since pipeline projections have a tremendous amount of variability and uncertainty due to their long planning horizon, a new facility is often built based on projections of manufacturing needs five or more years in the future. If the actual demand, when realized, swings below or above projections, the facility could be too large or too small, resulting in extreme under-utilization (and wasted capital) or inability to meet demand (and the need to outsource). Even if a facility is located in a biotechnology hub, staffing a facility with enough experienced personnel will take time.

Acquisition of Existing Facilities

Acquiring a facility is an alternative to building a new facility and is an option of increasing relevance and importance as the industry matures. Although an acquired facility is unlikely to have all of the desired facility attributes, this option allows companies to obtain greater control over development resources and timing and to do so with less lead-time and generally less capital investment than is required to build a new facility. An acquired facility would most likely require some level of renovation and revalidation so that it would not be immediately available for operation. Renovations can be costly and time-consuming, which should be factored into any decision to acquire a facility. Renovation plans should be based on bioreactor scales that balance facility utilization with facility costs.

An acquisition approach also limits somewhat the geographic regions for operations since the majority of existing facilities are located in or around biotechnology hubs on the East and West coasts of the US and in Western Europe. In addition, the ability to acquire a suitable facility within the desired window of opportunity is very unpredictable. Nevertheless, it is advisable for companies to investigate acquisition options for suitability before committing to the build option.

4. Selecting a Contract Manufacturer

When outsourcing process development or manufacturing, selection of an appropriate CMO needs careful consideration. Organizations with strong quality assurance and quality control functions as well as excellent inspection histories are often top requirements when outsourcing for commercial production. For clinical manufacturing needs, prior experience with the production technologies to be used and the ability to deliver on time are often top considerations. For both commercial and clinical manufacturing, cost and geography, while important, are generally not critical requirements. A consistent, step-wise procedure for a CMO is recommended to ensure the best CMO is identified. The CMO selection process starts with defining a list of key requirements, which are prioritized and weighted. This list is then populated with the names of CMOs that are likely capable of meeting those requirements. However, evaluating and ranking the selection criteria for each CMO are not straightforward.

Consider that a typical CMO selection team is comprised of functional representatives from supply chain, development, quality control/analytical, quality assurance, manufacturing/ operations and CMC project leader. Each of these functions applies their respective criteria to the CMO selection process resulting, potentially, in divergent prioritization. Developing a consensus list of selection criteria can require several iterations.

Once criteria are established, a list of suitable CMOs is contacted to gauge interest and availability. Not all CMOs will be available to meet timing requirements, nor will all CMOs be able to meet project-specific technical requirements or be interested in pursuing every project opportunity. Once the CMO list has been narrowed down, confidential disclosure agreements are executed so that information can flow freely between parties. In parallel, a detailed RFP is prepared and distributed to the CMOs. The RFP should include, at a minimum, the information listed in Table 12.1 and will ideally outline all activities for which a time and cost estimate are required.¹⁵ Sufficient technical information should be provided in the RFP, such as detailed process descriptions, development reports, stability reports, and the like, to enable the CMO to gauge the maturity of the manufacturing process and aid in providing a more accurate proposal for the cost and time for various development and manufacturing activities. The RFP should be sent to all of the CMOs at the same time with the same deadline for responses. The deadline should be realistic as it is often better to extend the deadline than to receive incomplete or poor proposals from CMOs. If the CMOs do not provide their proposals in the requested format and within the requested time, the proposals cannot be easily compared. This is the first indication of the CMO's customer service, willingness and flexibility to follow instruction, and ability to deliver on time.

Table 12.1: Typical Contents of a Request forProposal for CMO Services		
Background and Objectives	Preparation of GMP Cell Banks	
Construction of Expression Vector, Cell Line, and Research Cell Bank	Purification Process Development and Scaleup	
Cell Culture Process Development and Scaleup	Production of Drug Substance	
Quality and Analytical Support Services	Project Time Estimates and Fee Schedule	
Documentation Required	Deadline for Submission of Proposals	
Qualifications	Appendices	
Scope of Services Requested		

For extensive development and manufacturing projects, five to six CMOs should be considered during the selection process and competitive bids obtained from a minimum of three CMOs. This will provide enough variation among the replies to differentiate between qualified CMOs, and, if it becomes apparent that a CMO will not be able to meet the project requirements, then there is still a competitive choice among the remaining CMOs.

Once proposals are received from each of the CMOs, they can be compared, analyzed and ranked. Formal technical site visits and quality audits generally follow the proposal process for the top contenders. Following these site visits, a lead CMO generally emerges. The rankings and ratings used initially can be updated to aid in the selection. It is also recommended to have a back-up CMO in the event the primary CMO negotiations run into a roadblock.

There are several essential elements to successful negotiations with CMOs. It is important to understand the CMO's business model and motivations during negotiations. Fundamentally, the CMO is providing infrastructure and capacity such as access to a cGMP facility, trained operators, and cGMP quality systems and procedures. When negotiating a development and manufacturing contract with a CMO, it is important to link payment for services to the performance of those services. Since the CMO is performing a service rather than producing an "off-the-shelf" product, it is important to focus on the specific tasks, and the associated costs and deliverables for these tasks, being performed by the CMO. A good and fair contract will have appropriately allocated risk between the parties.

The contract should detail mutual responsibilities (the who, what, where, when and how), standards for performance, procedures to remedy faulty performance, intellectual property ownership and rights to use process and methods, regulatory compliance, warranties and indemnification, and quality obligations, which should be defined in a separate quality agreement. If the CMO has proprietary technology that could enable faster entry into clinical trials or improved costs of goods and productivity, carefully consider all relevant intellectual property and associated restrictions, licenses and fees well in advance to understand the true cost of the outsourcing relationship.

Sponsor companies should budget adequately for the time and resources required to successfully manage an outsourced product. The identification of potential CMOs, the RFP process, due diligence efforts, site visits, selection and negotiations takes significant time and resources, as does the ongoing management of a CMO once selected. A company should plan for anywhere from an average of six to nine months for early stage products to twelve months or longer for Phase 3 or commercial products to complete the selection process before being able to begin the actual work at the CMO.

5. Construction of a Pilot Plant for Bulk Monoclonal Antibody Production

The role of a pilot plant in development and manufacturing is to enable process development at a scale closer to commercial scale. This enables the use of equipment and unit operations that more closely simulate the larger scale equipment and provide material for clinical trials as quickly and safely as possible. Occasionally, a pilot plant will also be used as a market launch facility for a new product. A simple monoclonal antibody pilot plant consists of an upstream cell culture train with a single downstream purification train.

The pilot plant may often include an area for aseptic filling of the bulk monoclonal antibody product into vials. This fill/finish suite can be very small utilizing manual filling techniques to fill up to 2,000 vials at a time or slightly larger using automated or semi-automated filling equipment to fill up to 50,000 vials per batch. Alternatively, drug product manufacturing can be outsourced to a CMO offering aseptic fill capabilities.

Having the pilot plant site situated close to the groups responsible for product development will often help facilitate continued process development, technology transfers, manufacturing trouble shooting and rapid production of clinical material. The pilot plant will be used to manufacture many different products over a number of years. During this time, technologies will change as will process definition parameters (e.g., final product titer in cell culture harvest). These challenges can best be met by having a flexible facility design with the proper equipment to handle multiple products.

The design of a monoclonal antibody pilot plant should reflect the product pipeline and manufacturing needs of an organization. In the past, some pilot facilities have been built based on unique manufacturing processes, resulting in facilities that are difficult to adapt to new products and new processes. Over the years, the increasing use of platform technologies and processes for the production of monoclonal antibody products has allowed pilot plants to be designed on more defined processes and flexible enough to be used for multiple products. The benefits of such a facility are lower cost, simplified start up (including commissioning and qualification), and reduced failure rates. For such pilot plants, a single set of production equipment, capable of being used for multiple products and installed on movable skids for increased operational flexibility provide a design and layout that is most beneficial to the end-users.¹⁶ The design of the pilot plant should also allow for evolving manufacturing processes and for future increases in product titers and yields. This is best accomplished through the use of simulation before finalizing the facility design.

Pilot Plant Design

Good facility design should provide for the movement of the product, equipment, personnel, raw materials, and waste through the facility while minimizing the cross interaction between personnel and process streams at different stages or purity.¹⁷ This is accomplished by unidirectional material, waste, and personnel flows; training personnel; controlling access and movement of personnel within the facility; and use of appropriate gowning procedures. Using unidirectional flow throughout the production area minimizes the potential for cross contamination within the facility design but still allows for flexibility if each segregated production area is accessed directly from a common clean corridor.

Given that most monoclonal antibody products today are produced using standard platform processes such as those described earlier, it is now possible to design and build a standardized production facility that can be readily adapted for different monoclonal antibody production processes.¹⁸ This standardized design consists of several different functional areas interconnected by a central spine or corridor to allow maximum flexibility and ease of future expansion. By incorporating single-use technologies and modular construction, the clean rooms for both bulk drug substance and final dosage manufacturing within the pilot facility can be readily constructed either in an existing or new building shell or as a freestanding building using modules specifically constructed for this purpose.

Bulk Drug Substance Manufacturing

With today's improved manufacturing processes, pilot facilities with single-use bioreactors up to 2,000 L can be used for production of clinical trial material as well as commercial launch for most monoclonal antibody products. Such a facility for the production of bulk monoclonal antibody will include adequate space for material staging and dispensing, media and buffer preparation and storage, upstream and downstream processing along with sufficient space for all support functions as well as appropriate airlocks and corridors. One such facility is shown in Figure 12.4. This modular facility, sized for a single 2,000 L singleuse production bioreactor is adoptable for bioreactors of different sizes or can be readily expanded to include multiple production bioreactors at the 2,000 L scale.

Figure 12.4. Pilot Plant for Production of Monoclonal Antibody Bulk Drug Substance (drawing courtesy of KeyPlants AB)

A conceptual design for a modular monoclonal antibody production facility is shown based on the maximum use of single-use and disposable technologies, which incorporates current best practices in engineering design and modern regulatory considerations.



By maximizing the use of single-use technologies for upstream processing and product storage, the overall footprint of the bulk monoclonal antibody pilot plant shown in Figure 12.4 is approximately 900 m². For comparison, if reusable stainless-steel equipment is used in the facility instead of single-use bioreactors, etc., the size of the facility increases to approximately 1,400 m². The smaller footprint of the single-use facility results primarily from the smaller utilities needed to operate the plant, further helping to reduce the overall cost of the facility.

The facility design for bulk monoclonal antibody production includes unidirectional flow of materials, product, waste, and personnel throughout the manufacturing area. In addition, separate downstream processing areas are provided for preand post-virus removal operations. Wherever possible, fully closed and contained processing is used, generally within a Class D (equivalent to Class 100,000 or ISO Class 8) environmental classification. Open processing areas, such as required for inoculum preparation, final purification, and bulk filling are designed to be Class C (equivalent to Class 10,000 or ISO Class 7) with specific open operations being performed in suitable Class A (equivalent to Class 100 or ISO Class 5) biosafety cabinets with laminar airflow. The facility also includes suitable staging areas for raw materials, consumables, and equipment and appropriate locker rooms and airlocks for personnel changing and entry and exit from the facility. To further ensure segregation within the facility, the design includes multiple air handlers. Using modular construction, the overall facility design can be readily modified to achieve appropriate segregation based on an appropriate risk assessment of the products to be produced, the details of the manufacturing process, and the risk for cross-contamination or contamination with adventitious agents.

Within the product processing area, there is a general U-shape design with product flow being unidirectional from one end of

the facility to the other. Media and buffer preparation areas are located in the center of the facility to allow the most possible adjacencies to processing areas. Wherever possible, buffers are stored in closed containers in controlled but unclassified space to minimize the environmental burden and lower the overall HVAC requirements for the facility.

The facility also includes optimized equipment positioning in order to minimize the tubing or piping needed for product and material transfer as well as a single access point for all production personnel and a surrounding clean corridor for easy access to all rooms.

Final Drug Product Manufacturing

The final drug product manufacturing facility is built using modular construction and comprises a core area for product formulation, vial filling, stoppering, capping, and loading onto trays. Space is also necessary for vial and stopper washing, sterilization, and depyrogenization and areas for material dispensing, washing and sterilization of parts. The module is equipped with appropriate internal air locks and corridors and independent HVAC units all on a single level. Including space for personnel gowning and de-gowning, lockers, etc. as well as ingress and egress airlocks, the drug product modular will have a total footprint of approximately 330 m². If lyophilization is required, this can be provided in the same module, adding approximately 60 m² to the total facility size.

For drug product manufacturing, a vial filling line with the capacity to fill six thousand 10 ml vials per hour or approximately 40,000 vials per day in a single shift is sufficient for most processes and clinical trial requirements. If it is necessary to fill larger batches of bulk product, the filling operation can be run over two or more shifts. For most processing, the aseptic fill/finish operation, coupled with preparation, inspection, labeling, etc., the output of a single 2,000 L bioreactor can be processed and ready to ship to clinical sites (pending QC testing and release) in approximately one week.

Cost and Timeline

A traditional monoclonal antibody pilot plant typically costs between \$2075 million to construct depending on the volume of capacity installed, the extent of singleuse technologies employed, and the level of automation utilized.¹⁹ The capital cost breakdown for the construction of such a pilot plant containing one 500 L production bioreactor and one downstream purification suite is shown in Figure 12.5. This cost breakdown assumes the use of single-use technologies in such areas as the inoculum train for cell culture, buffer preparation, and product collection.

Figure 12.5. Cost Breakdown for a Simple Monoclonal Antibody Pilot Plant

The capital costs for a simple monoclonal antibody pilot plant can be divided into six major categories shown here with costs for the building, pipework and HVAC, and equipment totalling approximately 75% of the total capital costs. Engineering costs, along with instrumentation, and facility validation encompass the remaining quarter of the costs.



The estimated operating costs for a pilot plant based on the standard platform processes described earlier in this report and incorporating either a single 1,000 L stainless-steel bioreactor or a 2,000 L single-use bioreactor are compared in Table 12.2. In the table, materials costs included the cost of cell culture media, buffer components, process water and WFI. Consumables include such items as filters used for preparing buffers and cell culture media and filtering product intermediates, disposable bags used for preparing and storing cell culture media, buffers, product intermediates, and final product, disposable bioreactors, ultrafiltration membranes used for ultrafiltration and/or diafiltration, and chromatography media. Labor costs are based on average labor rates in the United States for production operators, supervisors, and QA/QC staff in the New England area. Costs for maintenance, utility, and waste costs include those costs associated with maintaining and operating the facility in a GMP-ready mode.

Table 12.2. Operating Costs for Stainless Steel and Single-Use Facilities ^a					
Operating Costs	Stainless Steel Facility		Single-Use Facility		
	Annual	Annual Batch		Batch	
Quantity of Product Produced	105 Kg	7 Kg	105 Kg	7 Kg	
Materials	\$607,428	\$40,495	\$590,334	\$39,356	
Consumables	\$3,611,689	\$240,779	\$4,268,890	\$284,593	
Labor (Direct/Indirect)	\$17,133,065	\$1,142,204	\$12,011,414	\$800,761	
Maintenance/Utilities/Waste	\$2,268,686	\$151,246	\$1,454,109	\$96,941	
Total	\$23,620,868	\$1,574,725	\$18,324,748	\$1,221,650	
Operating Costs Per Gram	perating Costs Per Gram \$225/gram \$175/gram		·		
lotal Operating Costs Per Gram	I \$23,620,868 \$1,574,725 \$18,324,748 \$1,27 rating Costs Per Gram \$225/gram \$175/gram		\$1,221,650		

^a Based on a standard monoclonal antibody platform process with a product titer

of 5 g/L and overall purification yield of 70% and 15 production batches per year

Not unexpectedly, the cost of the disposable bioreactor and associated multiple single-use bags used for media and buffer preparation and storage as well as single-use bags used for product intermediate and final product storage increase the cost of consumables for the single-use facility compared to the stainless-steel facility. However, the approximately \$40,000 higher cost for consumables per batch in the singleuse facility is more than offset by the decreased costs for labor, maintenance, etc. so that the overall cost of production in the single-use facility is approximately 75% of the production cost in the stainless-steel facility.

In the past, the timeline for construction of a monoclonal antibody pilot facility could be as long as two or three years from the beginning of the conceptual design to initiation of manufacturing.²⁰ However, by incorporating the design features discussed above, coupled with engineering and construction best practices this timeline can be reduced to as little as 12 months.²⁰ A sample timeline for construction of a monoclonal antibody pilot plant as described above is shown in Figure 12.6.

Figure 12.6. Monoclonal Antibody Pilot Plant Construction Timeline

A high-level timeline for the construction of the pilot plant shown in Figure 4 is presented here. The overall project timeline shows completion and start-up of the pilot plant approximately one year from initiation of the project.



The significantly lower costs and shorter timelines possible today for the construction and operation of a manufacturing pilot plant make this alternative attractive once again for companies wishing to control the production of their product.

6. Manufacturing Strategies for Drug Product

The final steps in the manufacture of a monoclonal antibody product are the formulation, sterile filtration, aseptic filling, and, for some products, lyophilization, of the bulk drug substance into suitable containers for storage and distribution. These drug product manufacturing operations are usually performed in different facilities than those used for drug substance manufacture and, especially for products early in their stage, are often outsourced. For companies that choose to outsource monoclonal antibody drug product manufacturing, there are many CMOs that can provide the necessary support and have appropriate capabilities. In most cases, different contract manufacturers will be selected for drug substance and drug product manufacturing.

If a company chooses to outsource drug product manufacturing, the CMO selection process should be similar to that described above for identifying and selecting a CMO for drug substance manufacturing, including developing a list of key requirements and ranking CMOs against these ranking, and the use of the detailed RFP process. For those companies that choose to manufacture monoclonal antibody drug products themselves, it is necessary to construct a suitable facility for this aseptic manufacturing. For information on the design, construction, commissioning, and qualification of facilities for the manufacture of monoclonal antibody drug products, the reader is referred to the ISPE Sterile Manufacturing Facilities Baseline Guide.²¹

References

- 1 Kamarck ME, et al. Biotech manufacturing grows up. BioPharm Int. 2007 Oct;20(10):159-64.
- 2 Levine HL, et al. Efficient, flexible facilities for the 21st century. BioProcess Int. 2012;10(S11):20–30.
- 3 Witcher MF, Odum J. Biopharmaceutical manufacturing in the twenty-first century: the next generation manufacturing facility. Pharm. Eng. 2012 Mar-Apr;32(2):10–22.
- 4 Reichert JM, et al. Monoclonal antibody successes in the clinic. Nat Biotechnol. 2005 Sep;23(9):1073-8.
- 5 Nelson AL, et al. Development trends for human monoclonal antibody therapeutics. Nat Rev Drug Discov. 2010 Oct;9(10):767-74.
- 6 Wenger T. Challenges in Cell Line Development for NBEs and Biosimilars. Presented at IBC's Ninth Annual Cell Line Development and Engineering Conference; 2013 May 20-22; La Jolla CA.
- 7 Mermod N. CHO Genome Sequencing and Engineering to Improve Clone Selection and Expression. Presented at IBC's Ninth Annual Cell Line Development and Engineering Conference; 2013 May 20-22; La Jolla, CA.
- 8 DeMaria C. Higher titer clones in less time: An optimized workflow for recombinant CHO cell line development. Presented at IBC's Antibody Development and Production Conference; 2013 Feb 25-Mar 1; Huntington Beach, CA.
- 9 Sinclair A. How to evaluate the cost impact of using disposables in biomanufacturing. BioPharm Int. 2008 Jun;21(6):26-30.
- 10 Sinclair A, Monge M. Monoclonal antibody manufacturing, cost benefits of disposable systems. Bioprocess Int. 2011 Oct;9(9):12-7.
- 11 Rios M. Flexible manufacturing: evolving technologies combine to enable a new generation of processes and products. BioProcess Int. 2010 May;8(5):34-46.
- 12 Galliher P. Turnkey modular disposable vaccine production plants. Presented at the International Alliance for Biological Standardization's International Workshop: New Technologies, New Vaccines VIII; 2014 Mar 23-26; Wilmington, DE.
- 13 Hodge G. Using disposables to build a flexible manufacturing capacity. Presented at San Francisco Bay Area International Society Pharmaceutical Engineering's Vendor Night; 2008 Feb 28; San Francisco, CA.
- 14 BioProcess Online [Internet]. Erie (PA): VertMarkets, Inc; c1996-2014. Gladd T. Merck assesses single use: does it make sense at a large scale?; 2014 May 14 [cited 2016 Nov 23]; [about 8 screens]. Available from: http://www.bioprocessonline.com/doc/merck-assesses-single-use-does-it-make-sense-at-a-large-scale-0001.
- 15 Seymour P, et al. Successful CMO selection: CMC strategies for outsourcing biopharmaceutical product manufacturing. BioProcess Int. 2006 Sep:4(Suppl 5):26-9.
- 16 Kahn DS, Kyburz KH. Design of a biotech clinical trial manufacturing facility. Bioprocess J. 2007 Spring;6(1):25-8.
- 17 International Society for Pharmaceutical Engineering (US). Baseline pharmaceutical engineering guides for new and renovated facilities. Vol. 6, Biopharmaceuticals. Tampa (FL): International Society for Pharmaceutical Engineering; 2004 Jun. 196 p.

- 18 Levine HL, et al. Single-use technology and modular construction enabling biopharmaceutical facilities of the future. BioProcess Int. 2013 Jan;11(Suppl 4):40-5.
- 19 Walliser H. Large scale production of recombinant proteins: from micrograms to kilograms. Presented at the International Forum on Enabling Technologies in Systems Biology & Biologics Development; 2005 May 12-13; Beijing, China.
- 20 Levine HL. Economics of modular facility design and construction. Presented at Cambridge Healthtech Institute's 13th Annual PepTalk; 2014 Jan 13-17; Palm Springs, CA.
- 21 International Society for Pharmaceutical Engineering (US). Baseline pharmaceutical engineering guides for new and renovated facilities. Vol. 3, Sterile manufacturing facilities. Tampa (FL): International Society for Pharmaceutical Engineering; 1999 Jan. 166 p.

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