

METHODS FOR MODULATING MONOCLONAL ANTIBODY CHARGE VARIANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/624,043, filed January 23, 2024, which is hereby incorporated by reference in its entirety.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

[0002] The present application contains a Sequence Listing, which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. The computer readable format copy of the Sequence Listing, which was created on January 21, 2025, is named 10571-WO01-SEC_ST26.xml and is 5,196 bytes in size.

FIELD OF THE INVENTION

[0003] The present invention relates to the field of biopharmaceutical manufacturing. In particular, the invention relates to methods for modulating the charge variant profile of a recombinantly produced monoclonal antibody through control of bioreactor media hold duration. The invention also relates to methods for increasing product titer and yield by adjusting bioreactor media hold durations.

BACKGROUND OF THE INVENTION

[0004] Recombinant production of a monoclonal antibody using engineered host cells typically results in a heterogenous mixture of molecular variants of the monoclonal antibody. Molecular variants that have properties comparable to those of the target monoclonal antibody are classified as product-related substances and are not considered to be impurities. In contrast, molecular variants that have properties differing from the target monoclonal antibody, particularly those that affect the efficacy and safety of the monoclonal antibody, are classified as product-related impurities. Such product-related impurities must be carefully monitored and controlled during the manufacturing process. Product-related impurities can include molecular variants with different charge profiles due to post-translational modifications of the expressed monoclonal antibody. Such charge variants can include both acidic variants and basic variants of the

monoclonal antibody that can be difficult to separate from the desired monoclonal antibody product during purification. Thus, control of the generation of product-related impurities, such as molecular charge variants, by manipulating aspects of the cell culture process is particularly useful and can reduce or eliminate some downstream purification steps. However, modification of cell culture parameters to reduce formation of molecular variants can negatively affect cell viability and overall yield of the cell culture. Accordingly, there is a need in the art for cell culture methods that reduce molecular variants of a monoclonal antibody, such as charge variants, while also maintaining or increasing yield of the recombinantly-produced monoclonal antibody.

SUMMARY OF THE INVENTION

[0005] The present invention is based, in part, on the development of cell culture methods that reduce the amount of charge variants of a monoclonal antibody as well as increase yield of the desired form of the monoclonal antibody. Accordingly, in certain embodiments, the present invention provides methods for reducing the amount of charge variants in a recombinant monoclonal antibody composition. In some embodiments, the methods comprise maintaining a cell culture medium in a bioreactor within a specified temperature range (i.e. media hold temperature) for a specified duration (i.e. media hold duration); inoculating the cell culture medium with mammalian cells expressing the monoclonal antibody; culturing the mammalian cells under conditions where the monoclonal antibody is expressed and secreted into the medium; and recovering the expressed monoclonal antibody from the cell culture medium to obtain the recombinant monoclonal antibody composition.

[0006] In some embodiments, the methods of the invention reduce acidic variants of the monoclonal antibody. Acidic variants can arise from various post-translational modifications of the antibody during cell culture production and such acidic variants can have different functional properties than the main isoform of the antibody. The methods of the invention can reduce the amount of acidic variants in a recombinant monoclonal antibody composition as compared to the amount of the acidic variants present in a composition of the recombinant monoclonal antibody produced from a process where the media hold duration is not limited (e.g. where the media hold duration exceeds 30 hours). For instance, in certain embodiments, the methods of the invention reduce the amount of acidic variants from about 1%-5%. Depending on the particular

recombinant monoclonal antibody produced, compositions obtained from the methods of the invention may comprise about 32% or less acidic variants of the monoclonal antibody, for example, between about 28% to about 32% acidic variants. In particular embodiments, the amount of acidic variants in the recombinant monoclonal antibody compositions is measured by cation exchange high performance liquid chromatography (CEX-HPLC). In some embodiments, the recombinant monoclonal antibody composition is harvested cell culture fluid. In other embodiments, the recombinant monoclonal antibody composition is an elution pool from a cation exchange chromatography material. In still other embodiments, the recombinant monoclonal antibody composition is drug substance.

[0007] In certain other embodiments, the present invention provides methods for increasing yield of a recombinant monoclonal antibody cell culture process. In some embodiments, the methods comprise maintaining a cell culture medium in a bioreactor within a specified temperature range (i.e. media hold temperature) for a specified duration (i.e. media hold duration); inoculating the cell culture medium with mammalian cells expressing the monoclonal antibody; and culturing the mammalian cells under conditions where the monoclonal antibody is expressed and secreted into the medium. In related embodiments, the methods further comprise recovering the expressed monoclonal antibody from the cell culture medium to obtain a recombinant monoclonal antibody composition. In some embodiments, the methods of the invention increase yield of the cell culture process as compared to the yield of a process with a media hold duration exceeding 30 hours. For example, in some embodiments, the methods of the invention increase yield of the process by at least 10%, such as from about 15% to about 40%. The methods of the invention can also increase product titer (e.g. antibody titer) of a cell culture process. In certain embodiments, the methods of the invention increase product titer (e.g. antibody titer) as compared to the product titer (e.g. antibody titer) of a process with a media hold duration exceeding 30 hours. Product titer (e.g. antibody titer) can be increased by the methods of the invention by at least 13%, at least 20%, at least 25%, or at least 30%.

[0008] In some embodiments of the methods of the invention, the media hold temperature is at least 30.0°C, for example between about 34.0°C to about 38.0°C or from about 35.5°C to about 36.5°C. In these and other embodiments, the media hold duration is less than 20 hours, such as from about 1 hour to about 18 hours prior to inoculation. In one embodiment, the media hold duration is about 6 hours to about 18 hours prior to inoculation. In another embodiment, the

media hold duration is about 12 hours to about 18 hours prior to inoculation. In certain embodiments of the methods of the invention, the cell culture medium is maintained in a bioreactor at a temperature of about 34.0°C to about 38.0°C for a duration of about 1 hour to about 18 hours prior to inoculation. In certain other embodiments of the methods of the invention, the cell culture medium is maintained in a bioreactor at a temperature of about 34.0°C to about 38.0°C for a duration of about 6 hours to about 18 hours prior to inoculation. In one embodiment of the methods of the invention, the cell culture medium is maintained in a bioreactor at a temperature of about 35.5°C to about 36.5°C (e.g. about 36.0°C) for a duration of about 12 hours to about 18 hours (e.g. about 12 hours) prior to inoculation. In certain embodiments of the methods of the invention, the media hold is performed in a production bioreactor, for example a production bioreactor having a volume of at least 500 liters. In one particular embodiment, the media hold is performed in a production bioreactor having a volume of at least 2,000 liters.

[0009] Any monoclonal antibody, including humanized antibodies or fully human antibodies, can be produced using the methods of the invention. In certain embodiments, the monoclonal antibody produced according to the methods of the invention is an IgG1, IgG2, IgG3, or IgG4 antibody. In some embodiments, the monoclonal antibody produced according to the methods of the invention is an IgG1 or IgG2 antibody. In particular embodiments, the monoclonal antibody produced according to the methods of the invention is adalimumab, bezarituzumab, bevacizumab, denosumab, eculizumab, erenumab, evolocumab, inebilizumab, infliximab, nivolumab, ordesekimab, panitumumab, pembrolizumab, rituximab, rocatinlimab, romosozumab, teprotumumab, tezepelumab, trastuzumab, or ustekinumab. In other particular embodiments, the monoclonal antibody produced according to the methods of the invention is bezarituzumab, denosumab, erenumab, evolocumab, inebilizumab, ordesekimab, panitumumab, rocatinlimab, romosozumab, teprotumumab, or tezepelumab. In certain other embodiments, the monoclonal antibody produced according to the methods of the invention is erenumab. Although the methods of the invention are particularly suitable for the recombinant production of monoclonal antibodies, the methods of the invention can also be used to produce other types of recombinant proteins, such as cytokines, growth factors, enzymes, hormones, muteins, fusion proteins, and multi-specific antigen binding proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] **Figure 1** shows a representative CEX-HPLC profile of an erenumab reference standard. The erenumab reference standard was analyzed by CEX-HPLC using a sodium phosphate pH 6.6 mobile phase with elution by a gradient of sodium chloride and detection at 280 nm absorbance.

[0011] **Figure 2** is a plot of the percentage of acidic peaks, which reflects the amount of acidic variants of erenumab, in drug substance versus the duration of the media hold in the production bioreactor. The media hold duration is the period between the time at which the media reaches a specific hold temperature and the inoculation start time. Limiting the media hold duration to 18 hours or less reduced the amount of acidic variants of erenumab.

[0012] **Figure 3** is a plot of the erenumab product titer (g/L) in harvested cell culture fluid versus the duration of the media hold in the production bioreactor (hours). The bivariate linear regression line is shown and the parameter estimates for the linear model are shown beneath the plot. Crosses represent data points from production runs at Manufacturing Site 1, whereas the circles represent data points from production runs at Manufacturing Site 2.

DETAILED DESCRIPTION

[0013] The present invention is based, in part, on the finding that reduction of the media hold duration in the production bioreactor not only reduces formation of charge variants of a monoclonal antibody but also increases the product yield of the recombinantly-produced monoclonal antibody. Thus, in some embodiments, the present invention provides methods for reducing the amount of charge variants in a recombinant monoclonal antibody composition comprising maintaining a cell culture medium in a bioreactor within a specified temperature range for a limited duration (e.g. less than 20 hours); inoculating the cell culture medium with mammalian cells expressing the monoclonal antibody; culturing the mammalian cells under conditions where the monoclonal antibody is expressed and secreted into the medium; and recovering the expressed monoclonal antibody from the cell culture medium to obtain the recombinant monoclonal antibody composition. In other embodiments, the present invention provides methods for increasing yield of a recombinant monoclonal antibody cell culture process comprising maintaining a cell culture medium in a bioreactor within a specified temperature range for a limited duration (e.g. less than 20 hours); inoculating the cell culture medium with mammalian cells expressing the monoclonal antibody; and culturing the mammalian cells under

conditions where the monoclonal antibody is expressed and secreted into the medium. In such embodiments, the methods may further comprise recovering the expressed monoclonal antibody from the cell culture medium to obtain a recombinant monoclonal antibody composition.

[0014] The methods of the invention are particularly useful for the production of recombinant proteins, particularly antibodies. The term “recombinant protein” refers to a heterologous protein produced by a host cell transformed with a nucleic acid encoding the protein when the host cell is cultivated in cell culture. The recombinant protein may contain a single polypeptide chain or multiple polypeptide chains. Recombinant proteins can include, but are not limited to, cytokines, growth factors, enzymes, hormones, muteins, fusion proteins, antibodies, antibody fragments, peptibodies, and multi-specific antigen binding proteins. In some embodiments, the recombinant protein is a fusion protein. A “fusion protein” is a protein that contains at least one polypeptide fused or linked to a heterologous polypeptide. Typically, a fusion protein is expressed from a fusion gene in which a nucleotide sequence encoding a polypeptide sequence from one protein is appended in frame with, and optionally separated by a linker from, a nucleotide sequence encoding a polypeptide sequence from a different protein. The fusion gene can then be expressed by a recombinant host cell to produce the fusion protein. The fusion protein may comprise a fragment from an immunoglobulin protein, such as an Fc region, fused or linked to a ligand polypeptide, a receptor polypeptide, a hormone, a cytokine, a growth factor, an enzyme, or other polypeptide that is not a component of an immunoglobulin.

[0015] In certain embodiments, the recombinant protein to be produced according to the methods of the invention is an antibody or binding fragment thereof. As used herein, the term “antibody” generally refers to a tetrameric immunoglobulin protein comprising two light chain polypeptides (about 25 kDa each) and two heavy chain polypeptides (about 50-70 kDa each). The term “light chain” or “immunoglobulin light chain” refers to a polypeptide comprising, from amino terminus (N-terminus) to carboxyl terminus (C-terminus), a single immunoglobulin light chain variable region (VL) and a single immunoglobulin light chain constant domain (CL). The immunoglobulin light chain constant domain (CL) can be a human kappa (κ) or human lambda (λ) constant domain. The term “heavy chain” or “immunoglobulin heavy chain” refers to a polypeptide comprising, from amino terminus (N-terminus) to carboxyl terminus (C-terminus), a single immunoglobulin heavy chain variable region (VH), an immunoglobulin heavy chain constant domain 1 (CH1), an immunoglobulin hinge region, an immunoglobulin heavy chain

constant domain 2 (CH2), an immunoglobulin heavy chain constant domain 3 (CH3), and optionally an immunoglobulin heavy chain constant domain 4 (CH4). Heavy chains are classified as mu (μ), delta (Δ), gamma (γ), alpha (α), and epsilon (ϵ), and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. The IgG-class and IgA-class antibodies are further divided into subclasses, namely, IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2, respectively. The heavy chains in IgG, IgA, and IgD antibodies have three constant domains (CH1, CH2, and CH3), whereas the heavy chains in IgM and IgE antibodies have four constant domains (CH1, CH2, CH3, and CH4). The immunoglobulin heavy chain constant domains can be from any immunoglobulin isotype, including subtypes, with those from the IgG-class being preferred. The antibody chains are linked together via inter-polypeptide disulfide bonds between the CL domain and the CH1 domain (i.e. between the light and heavy chain) and between the hinge regions of the two antibody heavy chains.

[0016] Variable regions of immunoglobulin chains generally exhibit the same overall structure, comprising relatively conserved framework regions (FR) joined by three hypervariable regions, more often called “complementarity determining regions” or CDRs. The CDRs from the two chains of each heavy chain and light chain pair typically are aligned by the framework regions to form a structure that binds specifically to a specific epitope on the target protein. From N-terminus to C-terminus, naturally-occurring light and heavy chain variable regions both typically conform with the following order of these elements: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. A numbering system has been devised for assigning numbers to amino acids that occupy positions in each of these domains. This numbering system is defined in Kabat Sequences of Proteins of Immunological Interest (1987 and 1991, NIH, Bethesda, MD), or Chothia & Lesk, 1987, *J. Mol. Biol.* 196:901-917; Chothia *et al.*, 1989, *Nature* 342:878-883. The CDRs and FRs of a given antibody may be identified using this system. Other numbering systems for the amino acids in immunoglobulin chains include IMGT[®] (the international ImMunoGeneTics information system; Lefranc *et al.*, *Dev. Comp. Immunol.* 29:185-203; 2005) and AHO (Honegger and Pluckthun, *J. Mol. Biol.* 309(3):657-670; 2001).

[0017] An “antigen-binding fragment,” used interchangeably herein with “binding fragment” or “fragment,” is a portion of an antibody that lacks at least some of the amino acids present in a full-length heavy chain and/or light chain, but which is still capable of specifically binding to an antigen. An antigen-binding fragment includes, but is not limited to, a single-chain variable

fragment (scFv), a nanobody (e.g. VHH fragment), a Fab fragment, a Fab' fragment, a F(ab')₂ fragment, a Fv fragment, a Fd fragment, and a complementarity determining region (CDR) fragment, and can be derived from any mammalian source, such as human, mouse, rat, rabbit, or camelid. Antigen-binding fragments may compete for binding of a target antigen with an intact antibody and the fragments may be produced by the modification of intact antibodies (e.g. enzymatic or chemical cleavage) or synthesized *de novo* using recombinant DNA technologies or peptide synthesis. In some embodiments, the antigen-binding fragment comprises at least one CDR from an antibody that binds to the antigen, for example, the heavy chain CDR3 from an antibody that binds to the antigen. In other embodiments, the antigen-binding fragment comprises all three CDRs from the heavy chain of an antibody that binds to the antigen or all three CDRs from the light chain of an antibody that binds to the antigen. In still other embodiments, the antigen-binding fragment comprises all six CDRs from an antibody that binds to the antigen (three from the heavy chain and three from the light chain).

[0018] Papain digestion of antibodies produces two identical antigen-binding fragments, called “Fab” fragments, each with a single antigen-binding site, and a residual “Fc” fragment which contains all but the first domain of the immunoglobulin heavy chain constant region. The Fab fragment contains the variable domains from the light and heavy chains, as well as the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Thus, a “Fab fragment” is comprised of one immunoglobulin light chain (light chain variable region (VL) and constant region (CL)) and the CH1 domain and variable region (VH) of one immunoglobulin heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule. The “Fd fragment” comprises the VH and CH1 domains from an immunoglobulin heavy chain. The Fd fragment represents the heavy chain component of the Fab fragment. The “Fc fragment” or “Fc domain” of an immunoglobulin generally comprises two constant domains, a CH2 domain and a CH3 domain, and optionally comprises a CH4 domain.

[0019] A “Fab' fragment” is a Fab fragment having at the C-terminus of the CH1 domain one or more cysteine residues from the antibody hinge region.

[0020] A “F(ab')₂ fragment” is a bivalent fragment including two Fab' fragments linked by a disulfide bridge between the heavy chains at the hinge region.

[0021] The “Fv” fragment is the minimum fragment that contains a complete antigen recognition and binding site from an antibody. This fragment consists of a dimer of one immunoglobulin

heavy chain variable region (VH) and one immunoglobulin light chain variable region (VL) in tight, non-covalent association. It is in this configuration that the three CDRs of each variable region interact to define an antigen binding site on the surface of the VH-VL dimer. A single light chain or heavy chain variable region (or half of an Fv fragment comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site comprising both VH and VL.

[0022] A “single-chain variable fragment” or “scFv fragment” comprises the VH and VL regions of an antibody, wherein these regions are present in a single polypeptide chain, and optionally comprising a peptide linker between the VH and VL regions that enables the Fv to form the desired structure for antigen binding (*see e.g.*, Bird *et al.*, Science, Vol. 242:423-426, 1988; and Huston *et al.*, Proc. Natl. Acad. Sci. USA, Vol. 85:5879-5883, 1988).

[0023] A “nanobody” is the heavy chain variable region of a heavy-chain antibody. Such variable domains are the smallest fully functional antigen-binding fragment of such heavy-chain antibodies with a molecular mass of only 15 kDa. *See* Cortez-Retamozo *et al.*, *Cancer Research* 64:2853-57, 2004. Functional heavy-chain antibodies devoid of light chains are naturally occurring in certain species of animals, such as nurse sharks, wobbegong sharks and *Camelidae*, such as camels, dromedaries, alpacas and llamas. The antigen-binding site is reduced to a single domain, the VHH domain, in these animals. These antibodies form antigen-binding regions using only heavy chain variable regions, i.e., these functional antibodies are homodimers of heavy chains only having the structure H₂L₂ (referred to as “heavy-chain antibodies” or “HCAbs”). Camelized VHH reportedly recombines with IgG2 and IgG3 constant regions that contain hinge, CH2, and CH3 domains and lack a CH1 domain. Camelized VHH domains have been found to bind to antigen with high affinity (Desmyter *et al.*, J. Biol. Chem., Vol. 276:26285-90, 2001) and possess high stability in solution (Ewert *et al.*, Biochemistry, Vol. 41:3628-36, 2002). Methods for generating antibodies having camelized heavy chains are described in, for example, U.S. Patent Publication Nos. 2005/0136049 and 2005/0037421. Alternative scaffolds can be made from human variable-like domains that more closely match the shark V-NAR scaffold.

[0024] In some embodiments, the recombinant protein produced according to the methods of the invention is a monoclonal antibody. The term “monoclonal antibody” (or “mAb”) as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e.,

the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against an individual antigenic site or epitope, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different epitopes. Monoclonal antibodies may be produced using any technique known in the art, e.g., by immortalizing spleen cells harvested from an animal (e.g. a transgenic animal expressing human immunoglobulin genes) after completion of an immunization schedule.

[0025] In some embodiments, the antibody (e.g. monoclonal antibody) or binding fragment thereof is a humanized antibody or binding fragment thereof. A “humanized antibody” refers to an antibody in which regions (e.g. framework regions) have been modified to comprise corresponding regions from a human immunoglobulin. Generally, a humanized antibody can be produced from a monoclonal antibody raised initially in a non-human animal, such as a rodent or rabbit. Certain amino acid residues in this monoclonal antibody, typically from non-antigen recognizing portions of the antibody, are modified to be homologous to corresponding residues in a human antibody of corresponding isotype. Humanization can be performed, for example, using various methods by substituting at least a portion of a rodent or rabbit variable region for the corresponding regions of a human antibody (see, e.g., United States Patent Nos. 5,585,089 and 5,693,762; Jones *et al.*, Nature, Vol. 321:522-525, 1986; Riechmann *et al.*, Nature, Vol. 332:323-27, 1988; Verhoeyen *et al.*, Science, Vol. 239:1534-1536, 1988). The CDRs of light and heavy chain variable regions of antibodies generated in another species can be grafted to consensus human framework regions (FRs) or FRs from specific human germline genes. To create consensus human FRs, FRs from several human heavy chain or light chain amino acid sequences may be aligned to identify a consensus amino acid sequence.

[0026] In other embodiments, the antibody (e.g. monoclonal antibody) or binding fragment thereof is a fully human antibody or binding fragment thereof. A “fully human antibody” is an antibody that comprises variable and constant regions derived from or indicative of human germ line immunoglobulin sequences. Fully human antibodies can be produced by immunizing transgenic animals (usually mice or rats) that are capable of producing a repertoire of human antibodies in the absence of endogenous immunoglobulin production. In one example of such a method, transgenic animals are produced by incapacitating the endogenous immunoglobulin loci encoding the endogenous (e.g. rodent) heavy and light immunoglobulin chains therein, and

inserting into the animal genome large fragments of human genome DNA containing loci that encode human heavy and light chain proteins. Partially modified animals, which have less than the full complement of human immunoglobulin loci, are then cross-bred to obtain an animal having all of the desired immune system modifications. When administered an immunogen, these transgenic animals produce antibodies that are immunospecific for the immunogen but have human rather than rodent amino acid sequences, including the variable regions. For further details of such methods, *see*, for example, WO96/33735 and WO94/02602. One particular transgenic mouse line suitable for generation of fully human antibodies is the XenoMouse[®] transgenic mouse line described in U.S. Pat. Nos. 6,114,598; 6,162,963; 6,833,268; 7,049,426; 7,064,244; Green *et al.*, 1994, *Nature Genetics* 7:13-21; Mendez *et al.*, 1997, *Nature Genetics* 15:146-156; Green and Jakobovitis, 1998, *J. Ex. Med.*, 188:483-495; Green, 1999, *Journal of Immunological Methods* 231:11-23; Kellerman and Green, 2002, *Current Opinion in Biotechnology* 13, 593-597. Additional methods relating to transgenic mice for making human antibodies are described in United States Patent No. 5,545,807; No. 6,713,610; No. 6,673,986; No. 6,162,963; No. 5,939,598; No. 5,545,807; No. 6,300,129; No. 6,255,458; No. 5,877,397; No. 5,874,299 and No. 5,545,806; in PCT publications WO91/10741, WO90/04036, WO 94/02602, WO 96/30498, WO 98/24893 and in EP 546073B1 and EP 546073A1.

[0027] Antibodies, multi-specific antigen-binding proteins, and fusion proteins that may be produced according to the methods of the invention may specifically bind to one or more target proteins including, but not limited to, CD2, CD3, CD4, CD8, CD11a, CD14, CD18, CD19, CD20, CD22, CD23, CD28, CD25, CD33, CD38, CD40, CD44, CD52, CD80 (B7.1), CD86 (B7.2), CD147, IL-1 α , IL-1 β , IL-4, IL-5, IL-8, IL-10, IL-13, IL-15, IL-17, IL-23, IL-2 receptor, IL-4 receptor, IL-6 receptor, IL-13 receptor, IL-18 receptor subunits, IL-31 receptor alpha subunit, angiopoietin (e.g. angiopoietin-1, angiopoietin-2, or angiopoietin-4), platelet derived growth factor receptor beta (PDGF- β), vascular endothelial growth factor (VEGF), transforming growth factors (TGF), including, among others, TGF- α and TGF- β , including TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, or TGF- β 5, epidermal growth factor (EGF) receptor, insulin-like growth factor 1 receptor (IGF-1R), VEGF receptor, HER2, FGF receptor, C1s complement, C3 complement, C5 complement, Beta-klotho, calcitonin gene-related peptide (CGRP), CGRP receptor, pituitary adenylate cyclase activating polypeptide (PACAP), pituitary adenylate cyclase activating polypeptide type 1 receptor (PAC1 receptor), IgE, tumor antigens, PD-1, PD-L1, integrin alpha 4

beta 7, the integrin VLA-4, B2 integrins, TRAIL receptors 1,2,3, and 4, RANK, RANK ligand, sclerostin, Dickkopf-1 (DKK-1), TLA1, tumor necrosis factor alpha (TNF- α), epithelial cell adhesion molecule (EpCAM), intercellular adhesion molecule-3 (ICAM-3), leukointegrin adhesin, the platelet glycoprotein gp IIb/IIIa, cardiac myosin heavy chain, proprotein convertase subtilisin/Kexin Type 9 (PCSK9), thymic stromal lymphopoietin (TSLP), parathyroid hormone, rNAPc2, MHC I, carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), CTLA-4 (which is a cytotoxic T lymphocyte-associated antigen), Fc- γ -I receptor, HLA-DR 10 beta, HLA-DR antigen, L-selectin, IPN- γ , respiratory syncytial virus, human immunodeficiency virus (HIV), hepatitis B virus (HBV), *Streptococcus mutans*, and *Staphylococcus aureus*.

[0028] An antibody or binding fragment thereof, multi-specific antigen-binding protein, or fusion protein “specifically binds” to a target antigen when it has a significantly higher binding affinity for, and consequently is capable of distinguishing, that antigen compared to its affinity for other unrelated proteins, under similar binding assay conditions. Antibodies or binding fragments thereof, multi-specific antigen-binding proteins, or fusion proteins that specifically bind an antigen may bind to that antigen with an equilibrium dissociation constant (K_D) of $\leq 1 \times 10^{-6}$ M. Antibodies or binding fragments thereof, multi-specific antigen-binding proteins, or fusion proteins specifically bind antigen with “high affinity” when the K_D is $\leq 1 \times 10^{-8}$ M. Binding affinity can be determined using a variety of techniques, including affinity ELISA, surface plasmon resonance (e.g., with a BIAcore[®] instrument), a Kinetic Exclusion Assay (KinExA) as described in Rathanaswami *et al.* Analytical Biochemistry, Vol. 373:52-60, 2008, and bio-layer interferometry, such as that described in Kumaraswamy *et al.*, Methods Mol. Biol., Vol. 1278:165-82, 2015 and employed in Octet[®] systems (Pall ForteBio).

[0029] The methods of the invention are particularly useful for the recombinant production of monoclonal antibodies. Any monoclonal antibody with any given antigen specificity, such as those described herein, can be produced according to the methods of the invention. In some embodiments, the monoclonal antibodies are humanized antibodies. In other embodiments, the monoclonal antibodies are fully human antibodies. In these and other embodiments, the monoclonal antibodies are IgG1, IgG2, IgG3, or IgG4 antibodies. In one embodiment, the monoclonal antibodies are IgG1 antibodies. In another embodiment, the monoclonal antibodies are IgG2 antibodies. Monoclonal antibodies or binding fragments thereof that can be produced according to the methods of the invention include, but are not limited to, abciximab,

adalimumab, adecatumumab, aducanumab, alemtuzumab, alirocumab, anifrolumab, ansuvimab, atezolizumab, avelumab, basiliximab, belimumab, bemarituzumab, benralizumab, bevacizumab, bezlotoxumab, bimekizumab, biosozumab, brodalumab, burosumab, camrelizumab, canakinumab, cemiplimab, cetuximab, conatumumab, crizanlizumab, daclizumab, daratumumab, denosumab, dinutuximab, dostarlimab, dupilumab, durvalumab, eculizumab, edrecolomab, efalizumab, elotuzumab, emapalumab, epratuzumab, eptinezumab, erenumab, evinacumab, evolocumab, fremanezumab, galcanezumab, galiximab, ganitumab, gemtuzumab, golimumab, guselkumab, ibalizumab, idarucizumab, inebilizumab, infliximab, ipilimumab, isatuximab, itolizumab, ixekizumab, lanadelumab, lecanemab, lerdelimumab, levilimab, lumiliximab, mapatumumab, margetuximab, mepolizumab, mogamulizumab, natalizumab, naxitamab, necitumumab, nemolizumab, netakimab, nimotuzumab, nivolumab, obinutuzumab, ocrelizumab, ofatumumab, olokizumab, omalizumab, ordesekimab, ormutivimab, palivizumab, panitumumab, pembrolizumab, penpulimab, pertuzumab, pexelizumab, ramucirumab, ranibizumab, ravulizumab, raxibacumab, regdanvimab, reslizumab, rilotumumab, risankizumab, rituximab, rocatinlimab, romosozumab, sarilumab, satralizumab, secukinumab, serplulimab, siltuximab, sintilimab, sotrovimab, sugemalimab, sutimlimab, tafasitamab, teprotumumab, tezepelumab, til-drakizumab, tislelizumab, tocilizumab, toripalimab, tralokinumab, trastuzumab, ustekinumab, vedolizumab, visilizumab, volociximab, zanolimumab, zalutumumab, zimberelimab, and biosimilars of any of the foregoing. In certain embodiments, the monoclonal antibody produced according to the methods of the invention is adalimumab, bemarituzumab, bevacizumab, denosumab, eculizumab, erenumab, evolocumab, inebilizumab, infliximab, nivolumab, ordesekimab, panitumumab, pembrolizumab, rituximab, rocatinlimab, romosozumab, teprotumumab, tezepelumab, trastuzumab, or ustekinumab. In certain other embodiments, the monoclonal antibody produced according to the methods of the invention is bemarituzumab, denosumab, erenumab, evolocumab, inebilizumab, ordesekimab, panitumumab, rocatinlimab, romosozumab, teprotumumab, or tezepelumab. In some embodiments, the monoclonal antibody produced according to the methods of the invention is erenumab.

[0030] Erenumab is an IgG2 antibody comprising a heavy chain variable region having the sequence of SEQ ID NO: 3 and a light chain variable region having the sequence of SEQ ID NO: 4. In one embodiment, erenumab comprises a heavy chain comprising the sequence of SEQ ID NO: 1 and a light chain comprising the sequence of SEQ ID NO: 2. In such embodiments,

erenumab is an antibody comprising two heavy chains and two light chains, wherein each of the heavy chains comprises the sequence of SEQ ID NO: 1 and each of the light chains comprises the sequence of SEQ ID NO: 2. When produced recombinantly, erenumab can undergo post-translational modifications at the termini of the heavy and light chains, such as removal of the C-terminal lysine residue at position 456 from the heavy chain and cyclization of the N-terminal glutamine residues in the light and heavy chains to pyroglutamate. Thus, the term erenumab can also refer to an IgG2 antibody that lacks the C-terminal lysine residue in one or both of the heavy chains and/or comprises a pyroglutamate residue as the N-terminal residue in place of the glutamine residue in one or both of the light chains and/or one or both of the heavy chains.

[0031] In certain embodiments, the present invention provides methods for reducing the amount of charge variants in a recombinant monoclonal antibody composition such as compositions of any of the monoclonal antibodies described herein. A charge variant refers to a variant of a monoclonal antibody that has a different charge profile resulting from, *inter alia*, post-translational modifications that directly alter the net charge of the antibody, induce conformational changes, or affect local charge distribution. Such post translational modifications can include deamidation of asparagine amino acids, isomerization of aspartic amino acids, glycation (e.g. at lysine residues), oxidation (e.g. oxidation of methionine, tryptophan, and histidine amino acids), sialylated glycosylation, fragmentation (e.g. truncation of light chain polypeptides, heavy chain polypeptides or both), and disulfide isoform variants which have altered arrangements of disulfide bonds between the two heavy chains or between the heavy and light chains. Charge variants can be separated from the main isoform of the monoclonal antibody and detected using ion exchange chromatography (cation exchange chromatography or anion exchange chromatography), reversed phase chromatography, hydrophobic interaction chromatography, isoelectric focusing gel electrophoresis, or capillary isoelectric focusing gel electrophoresis. Charge variants can have different functional properties than the main isoform of the monoclonal antibody, such as reduced antigen binding affinity, reduced potency, altered pharmacokinetic profile, and increased immunogenicity, and as such can be considered product-related impurities. *See e.g.*, Du *et al.*, MABs, Vol. 4(5): 578–585, 2012; Liu *et al.*, MABs, Vol. 6(5):1145–1154, 2014; and Vulto and Jaquez, Rheumatology, Vol. 56:iv14–iv-29, 2017.

[0032] Charge variants of a monoclonal antibody can be categorized into either acidic variants or basic variants. An acidic variant refers to a variant of a monoclonal antibody that has gained

negative charge or lost positive charge, or has an altered surface charge profile due to conformational changes, and thus has more acidic character relative to the main isoform of the monoclonal antibody. A basic variant refers to a variant of a monoclonal antibody that has gained a positive charge or lost negative charge, or has an altered surface charge profile due to conformational changes, and thus has more basic character relative to the main isoform of the monoclonal antibody. When analyzed by ion exchange chromatography, acidic variants and basic variants can be identified by their retention times relative to the main peak, which corresponds to the main isoform of the monoclonal antibody. For instance, acidic variants elute earlier than the main peak from cation exchange chromatography (CEX) – that is, acidic variants have retention times shorter than the retention time for the main peak in CEX. *See, e.g.*, Figure 1. Basic peaks elute later than the main peak from CEX – i.e. have longer retention times than the retention time for the main peak from CEX. *See, e.g.*, Figure 1. When using anion exchange chromatography (AEX), acidic variants elute later than the main peak and thus have retention times longer than the retention time for the main peak in AEX, whereas basic variants elute earlier than the main peak and thus have shorter retention times than the retention time for the main peak in AEX.

[0033] In some embodiments, the methods of the invention reduce the amount of acidic variants in a recombinant monoclonal antibody composition. As described in Example 1, limiting the duration of the media hold in the production bioreactor reduced the amount of acidic variants of a monoclonal antibody generated during expression of the antibody. Thus, in certain embodiments, the methods of the invention reduce the amount of acidic variants in a recombinant monoclonal antibody composition compared to the amount of the acidic variants present in a composition of the recombinant monoclonal antibody produced from a process where the media hold duration is not limited (i.e. the media hold duration in the production bioreactor is longer than 30 hours). For instance, the methods of the invention may reduce the amount of acidic variants of the monoclonal antibody by about 10%, about 9%, about 8%, about 7%, about 6%, about 5%, about 4.5%, about 4%, about 3.5%, about 3%, about 2.5%, about 2%, about 1.5%, or about 1% as compared to the amount of the acidic variants produced from a process in which the duration of the media hold in the production bioreactor is longer than 30 hours. In some embodiments, the methods of the invention reduce the amount of acidic variants of the monoclonal antibody from about 1% to about 5%. In other embodiments, the methods of

the invention reduce the amount of acidic variants of the monoclonal antibody from about 2% to about 4%. In one embodiment, the methods of the invention reduce the amount of acidic variants of the monoclonal antibody by about 3%. In another embodiment, the methods of the invention reduce the amount of acidic variants of the monoclonal antibody by about 2%. In still another embodiment, the methods of the invention reduce the amount of acidic variants of the monoclonal antibody by about 1%.

[0034] Recombinant monoclonal antibody compositions produced according to the methods of the invention will generally have amounts of acidic variants of about 35% or less. For example, the amount of acidic variants in the compositions can be 34% or less, 33% or less, 32% or less, 31% or less, 30% or less, or 29% or less. In one embodiment, the amount of acidic variants in the monoclonal antibody compositions produced according to the methods of the invention is 32% or less. In another embodiment, the amount of acidic variants in the monoclonal antibody compositions produced according to the methods of the invention is 30% or less. In some embodiments, the amount of acidic variants in the monoclonal antibody compositions can be from about 28% to about 32%. In other embodiments, the amount of acidic variants in the monoclonal antibody compositions can be from about 29.5% to about 31.5%.

[0035] The amount of acidic variants in the monoclonal antibody compositions produced according to the methods of the invention can be determined by any method that separates proteins based on charge characteristics, such as any of the methods described above for detecting these variants. In certain embodiments, the amount of acidic variants in the monoclonal antibody compositions is determined by ion exchange chromatography. In one particular embodiment, the amount of acidic variants in the monoclonal antibody compositions is determined by cation exchange high performance liquid chromatography (CEX-HPLC), such as the method described in Example 1. The amount of acidic variants in the monoclonal antibody compositions can be determined from the peak area percentage of the acidic peaks in a CEX-HPLC chromatogram. The acidic peaks are those peaks with a peak height above the limit of detection that have retention times shorter than the retention time for the main peak. The basic peaks are those peaks with a peak height above the limit of detection that have retention times longer than the retention time for the main peak. The peak area percentage for the desired component (e.g. acidic peaks, main peak, or basic peaks) can be calculated by dividing the peak area for the desired component (e.g. acidic peaks, main peak, or basic peaks) by the total

integrated peak area and multiplying the result by 100. In certain embodiments, the CEX-HPLC method is conducted as described in Example 1.

[0036] In certain other embodiments, the present invention provides methods for increasing yield of a recombinant monoclonal antibody cell culture process. The term “product yield,” used interchangeably with “protein yield” or “yield,” refers to the amount of the desired form of the recombinant protein expressed by cultured cells (e.g. in grams or kilograms). Product yield can also be expressed as the mass ratio of the main isoform of the recombinant protein to the total amount of protein expressed by the cells. The methods of the invention increase yield of a recombinant monoclonal antibody process by reducing the production of undesired forms of the antibody, such as acidic variants, as well as by increasing the product titer. “Product titer” refers to the amount of the recombinant protein produced per volume of culture medium (e.g. in grams/liter). The amount of protein in a cell culture system can be measured by various methods known to those of skill in the art, including liquid chromatography with UV detection at 280 nm, immunoassay (e.g. enzyme-linked immunosorbent assay), and affinity chromatography.

[0037] As described in Example 2, the duration of the media hold in the production bioreactor significantly impacted the product titer and overall yield for a recombinant monoclonal antibody. An increase in product titer and yield was observed when the media hold duration was limited. Accordingly, in some embodiments, the methods of the invention increase yield of a recombinant monoclonal antibody cell culture process as compared to the yield of a process with a media hold duration in the production bioreactor exceeding 30 hours. In other embodiments, the methods of the invention increase yield of a recombinant monoclonal antibody cell culture process as compared to the yield of a process with a media hold duration in the production bioreactor exceeding 20 hours. In some such embodiments, the product yield of the process can be increased by at least 5%, for example from about 5% to about 70%, from about 10% to about 60%, from about 15% to about 50%, from about 10% to about 20%, or from about 15% to about 40%. In certain embodiments, the methods of the invention increase the product yield by at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, or at least 60%. In one embodiment, the methods of the invention increase the product yield by at least 10%. In another embodiment, the methods of the invention increase the product yield by at least 15%. In still another embodiment, the methods of the invention increase the product yield by at least 20%.

[0038] In some embodiments, the methods of the invention may be used to increase product titer (e.g. titer of the monoclonal antibody) of a recombinant protein cell culture process. In some such embodiments, the methods of the invention increase product titer (e.g. antibody titer) as compared to the product titer (e.g. antibody titer) of a process with a media hold duration in the production bioreactor exceeding 30 hours. In other such embodiments, the methods of the invention increase product titer (e.g. antibody titer) as compared to the product titer (e.g. antibody titer) of a process with a media hold duration in the production bioreactor exceeding 20 hours. In certain embodiments, the product titer (e.g. antibody titer) of the process can be increased by at least 5%, at least 8%, at least 10%, at least 13%, at least 15%, at least 18%, at least 20%, at least 23%, at least 25%, at least 28%, at least 30%, at least 33%, at least 35%, at least 38%, at least 40%, at least 43%, at least 45%, at least 48%, at least 50%, at least 53%, at least 55%, at least 58%, or at least 60%. In some embodiments, the product titer (e.g. antibody titer) of the process can be increased from about 5% to about 40%, from about 10% to about 30%, from about 13% to about 25%, or from about 20% to about 25%. In one embodiment, the product titer (e.g. antibody titer) of the process can be increased by at least 13%. In another embodiment, the product titer (e.g. antibody titer) of the process can be increased by at least 20%. In yet another embodiment, the product titer (e.g. antibody titer) of the process can be increased by at least 25%. In still another embodiment, the product titer (e.g. antibody titer) of the process can be increased by at least 30%.

[0039] The methods of the invention entail the use of a specific media hold step in the production bioreactor prior to inoculating the production bioreactor with cells expressing the recombinant protein (e.g. recombinant monoclonal antibody). A media hold refers to a process step in which cell culture medium is held in a cell culture vessel (e.g. bioreactors, tanks, single-use bags) or storage container within a specified temperature range prior to using the cell culture medium to culture cells. In some cases, the temperature range employed in the media hold is similar to the temperature range to be used for the cell culture. The “media hold duration” or “media hold time” refers to the time period between the media hold start time at which time the media is at a temperature within the specified range and the time the bioreactor is inoculated with cells. Accordingly, the methods of the invention comprise maintaining a cell culture medium in a bioreactor at a specified temperature range for a limited period of time; inoculating the cell culture medium with mammalian cells expressing the recombinant protein (e.g. recombinant

monoclonal antibody); culturing the mammalian cells under conditions where the protein (e.g. monoclonal antibody) is expressed and optionally secreted into the medium; and optionally recovering the expressed protein (e.g. monoclonal antibody) from the cell culture medium to obtain a recombinant protein (e.g. recombinant monoclonal antibody) composition.

[0040] In some embodiments of the methods of the invention, the temperature of the media hold is at least 30.0°C. For example the media hold temperature may be from about 30.0°C to about 38.0°C, from about 32.0°C to about 36.0°C, from about 34.0°C to about 38.0°C, from about 35.0°C to about 37.0°C, or from about 35.5°C to about 36.5°C. In one particular embodiment of the methods of the invention, the media hold temperature is from about 34.0°C to about 38.0°C. In another particular embodiment of the methods of the invention, the media hold temperature is from about 35.5°C to about 36.5°C. In another particular embodiment of the methods of the invention, the media hold temperature is about 36.0°C.

[0041] In certain embodiments of the methods of the invention, the media hold duration is less than 20 hours. For instance, the media hold duration can be from about 1 hour to about 18 hours, from about 6 hours to about 18 hours, from about 12 hours to about 18 hours, from about 5 hours to about 15 hours, from about 8 hours to about 14 hours, or from about 15 hours to about 18 hours. In one embodiment, the media hold duration is about 1 hour to about 18 hours. In another embodiment, the media hold duration is about 6 hours to about 18 hours. In yet another embodiment, the media hold duration is about 12 hours to about 18 hours. In still another embodiment, the media hold duration is about 12 hours.

[0042] In some embodiments of the methods of the invention, the methods comprise maintaining a cell culture medium in a bioreactor at a temperature of about 34.0°C to about 38.0°C for a duration of about 1 hour to about 18 hours prior to inoculation. In other embodiments, the methods comprise maintaining a cell culture medium in a bioreactor at a temperature of about 35.5°C to about 36.5°C for a duration of about 1 hour to about 18 hours prior to inoculation. In certain embodiments, the methods comprise maintaining a cell culture medium in a bioreactor at a temperature of about 35.5°C to about 36.5°C for a duration of about 6 hours to about 18 hours prior to inoculation. In certain other embodiments, the methods comprise maintaining a cell culture medium in a bioreactor at a temperature of about 35.5°C to about 36.5°C for a duration of about 12 hours to about 18 hours prior to inoculation. In one embodiment, the methods comprise maintaining a cell culture medium in a bioreactor at a temperature of about 34.0°C to about

38.0°C for a duration of about 6 hours to about 18 hours prior to inoculation. In another embodiment, the methods comprise maintaining a cell culture medium in a bioreactor at a temperature of about 34.0°C to about 38.0°C for a duration of about 12 hours to about 18 hours prior to inoculation. In one particular embodiment, the methods comprise maintaining a cell culture medium in a bioreactor at a temperature of about 36.0°C for a duration of about 1 hour to about 18 hours prior to inoculation. In another particular embodiment, the methods comprise maintaining a cell culture medium in a bioreactor at a temperature of about 36.0°C for a duration of about 12 hours prior to inoculation. The bioreactor in any of the foregoing embodiments is preferably a production bioreactor.

[0043] In some embodiments, the pH of the culture medium is maintained within a certain range during the media hold. For example, the pH of the culture medium during the media hold can be from about 6.70 to about 7.20, from about 6.80 to about 7.10, from about 6.85 to about 7.05, or from about 6.90 to about 7.00. In one embodiment, the cell culture medium is maintained in the bioreactor at a pH of about 6.85 to about 7.05 prior to inoculation. In another embodiment, the cell culture medium is maintained in the bioreactor at a pH of about 6.90 to about 7.00 prior to inoculation. In still another embodiment, the cell culture medium is maintained in the bioreactor at a pH of about 6.95 prior to inoculation.

[0044] Following the media hold, the methods of the invention comprise inoculating the cell culture medium in the production bioreactor with mammalian cells expressing the recombinant protein (e.g. recombinant monoclonal antibody). To generate mammalian cell lines engineered to express the recombinant protein of interest, one or more nucleic acids encoding the recombinant protein (or components thereof in the case of multi-chain proteins like antibodies) is initially inserted into one or more expression vectors. The term “expression vector” or “expression construct” as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid control sequences necessary for the expression of the operably linked coding sequence in a particular host cell, e.g. a mammalian host cell. Vectors can include viral vectors, non-episomal mammalian vectors, plasmids and other non-viral vectors. An expression vector can include sequences that affect or control transcription, translation, and, if introns are present, affect RNA splicing of a coding region operably linked thereto. “Operably linked” means that the components to which the term is applied are in a relationship that allows them to carry out their inherent functions. For example, a control

sequence, e.g., a promoter, in a vector that is “operably linked” to a protein coding sequence is arranged such that normal activity of the control sequence leads to transcription of the protein coding sequence resulting in recombinant expression of the encoded protein. Nucleic acid control sequences useful in expression vectors for expression in mammalian cells include promoters, enhancers, and termination and polyadenylation signals. A secretory signal peptide sequence can also, optionally, be encoded by the expression vector, operably linked to the coding sequence of interest, so that the expressed protein can be secreted by the recombinant host cell, for more facile isolation of the recombinant protein from the cell, if desired. Vectors may also include one or more selectable marker genes to facilitate selection of host cells into which the vectors have been introduced. In some embodiments, vectors are used that employ protein-fragment complementation assays using protein reporters, such as dihydrofolate reductase (see, for example, U.S. Pat. No. 6,270,964). Suitable mammalian expression vectors are known in the art and are also commercially available.

[0045] Typically, vectors used in any of the host cells will contain sequences for plasmid maintenance and for cloning and expression of exogenous nucleotide sequences. Such sequences will typically include one or more of the following nucleotide sequences: a promoter, one or more enhancer sequences, an origin of replication, transcriptional and translational control sequences, a transcriptional termination sequence, a complete intron sequence containing a donor and acceptor splice site, a native or heterologous signal peptide sequence (leader sequence or signal peptide) for polypeptide secretion, a ribosome binding site, a polyadenylation sequence, a polylinker region for inserting the polynucleotide encoding the polypeptide to be expressed, and a selectable marker element. Vectors may be constructed from a starting vector such as a commercially available vector, and additional elements may be individually obtained and ligated into the vector.

[0046] Vector components may be homologous (i.e., from the same species and/or strain as the host cell), heterologous (i.e., from a species other than the host cell species or strain), hybrid (i.e., a combination of flanking sequences from more than one source), synthetic or native. The sequences of components useful in the vectors may be obtained by methods well known in the art, such as those previously identified by mapping and/or by restriction endonuclease digestion. In addition, they can be obtained by polymerase chain reaction (PCR) and/or by screening a genomic library with suitable probes.

[0047] A ribosome-binding site is usually necessary for translation initiation of mRNA and is characterized by a Shine-Dalgarno sequence (prokaryotes) or a Kozak sequence (eukaryotes). The element is typically located 3' to the promoter and 5' to the coding sequence of the polypeptide to be expressed.

[0048] An origin of replication aids in the amplification of the vector in a host cell. They may be included as part of commercially available prokaryotic vectors and may also be chemically synthesized based on a known sequence and ligated into the vector. Various viral origins (e.g., SV40, polyoma, adenovirus, vesicular stomatitis virus (VSV), or papillomaviruses such as HPV or BPV) are useful for cloning vectors in mammalian cells.

[0049] Expression and cloning vectors used in the methods of the invention will typically contain a promoter that is recognized by the host organism and operably linked to the polynucleotide encoding the polypeptide. Promoters are non-transcribed sequences located upstream (i.e., 5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control transcription of the structural gene. Promoters are conventionally grouped into one of two classes: inducible promoters and constitutive promoters. Inducible promoters initiate increased levels of transcription from polynucleotides under their control in response to some change in culture conditions, such as the presence or absence of a nutrient or a change in temperature. Constitutive promoters, on the other hand, uniformly transcribe a gene to which they are operably linked, that is, with little or no control over gene expression. A large number of promoters, recognized by a variety of potential host cells, are well known. A suitable promoter is operably linked to the polynucleotide encoding a recombinant protein by removing the promoter from the source nucleic acid by restriction enzyme digestion and inserting the desired promoter sequence into the vector. Suitable promoters for use with mammalian host cells are well known and include, but are not limited to, those obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, retroviruses, hepatitis-B virus and most preferably Simian Virus 40 (SV40). Other suitable mammalian promoters include heterologous mammalian promoters, for example, heat-shock promoters and the actin promoter.

[0050] An enhancer sequence may be inserted into the vector to increase transcription of a polynucleotide encoding a recombinant protein by higher eukaryotes. Enhancers are cis-acting elements of nucleic acid, usually about 10-300 bp in length, that act on the promoter to increase

transcription. Enhancers are relatively orientation and position independent, having been found at positions both 5' and 3' to the transcription unit. Several enhancer sequences available from mammalian genes are known (e.g., globin, elastase, albumin, alpha-feto-protein and insulin). Typically, however, an enhancer from a virus is used. The SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers known in the art are exemplary enhancing elements for the activation of eukaryotic promoters. While an enhancer may be positioned in the vector either 5' or 3' to a coding sequence, it is typically located at a site 5' from the promoter.

[0051] A sequence encoding an appropriate native or heterologous signal peptide sequence (leader sequence or signal peptide) can be incorporated into an expression vector, to promote extracellular secretion of the recombinant protein. The choice of signal peptide or leader depends on the type of host cells in which the recombinant protein is to be produced, and a heterologous signal sequence can replace the native signal sequence. Examples of signal peptides that are functional in mammalian host cells include the signal sequence for interleukin-7 (IL-7) described in US Patent No. 4,965,195; the signal sequence for interleukin-2 receptor described in Cosman et al., 1984, *Nature* 312:768; the interleukin-4 receptor signal peptide described in EP Patent No. 0367566; the type I interleukin-1 receptor signal peptide described in U.S. Patent No. 4,968,607; and the type II interleukin-1 receptor signal peptide described in EP Patent No. 0460846. Other suitable signal peptides, such as those from human germline immunoglobulin genes, are known to those of skill in the art and can be incorporated into expression vectors.

[0052] A transcription termination sequence is typically located 3' to the end of a polypeptide coding region and serves to terminate transcription. Usually, a transcription termination sequence in prokaryotic cells is a G-C rich fragment followed by a poly-T sequence. While the sequence is easily cloned from a library or even purchased commercially as part of a vector, it can also be readily synthesized using methods for nucleic acid synthesis known to those of skill in the art.

[0053] Exemplary transcriptional and translational control sequences for mammalian host cell expression vectors can be excised from viral genomes. Commonly used promoter and enhancer sequences are derived from polyoma virus, adenovirus 2, simian virus 40 (SV40), and human cytomegalovirus (CMV). For example, the human CMV promoter/enhancer of immediate early gene 1 may be used. *See e.g.* Patterson et al. (1994), *Applied Microbiol. Biotechnol.* 40:691-98. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late

promoter, enhancer, splice, and polyadenylation sites can be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment, which can also contain a viral origin of replication (Fiers et al. (1978), *Nature* 273:113; Kaufman (1990), *Meth. in Enzymol.* 185:487-511). Smaller or larger SV40 fragments can also be used, provided the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the SV40 viral origin of replication site is included.

[0054] A selectable marker gene encoding a protein necessary for the survival and growth of a host cell grown in a selective culture medium can be incorporated into expression vectors to identify and select host cells that have incorporated the expression vector for the recombinant protein. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, tetracycline, or kanamycin for prokaryotic host cells; (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex or defined media. Specific selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, and the tetracycline resistance gene. Advantageously, a neomycin resistance gene may also be used for selection in both prokaryotic and eukaryotic host cells.

[0055] Other selectable genes may be used to amplify the gene that will be expressed.

Amplification is the process wherein genes that are required for production of a protein critical for growth or cell survival are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of suitable selectable markers for mammalian cells include glutamine synthase (GS)/methionine sulfoximine (MSX) system, dihydrofolate reductase (DHFR), and promoterless thymidine kinase genes. Mammalian cell transformants are placed under selection pressure wherein only the transformants are uniquely adapted to survive by virtue of the selectable gene present in the vector. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the medium is successively increased, thereby leading to the amplification of both the selectable gene and the DNA that encodes a protein of interest. As a result, increased quantities of a polypeptide of interest are synthesized from the amplified DNA.

[0056] After the expression vector(s) has been constructed and the one or more nucleic acid molecules encoding the recombinant protein (or components thereof in the case of multi-chain proteins like antibodies) has been inserted into the proper site(s) of the vector or vectors, the

completed vector(s) may be inserted into a suitable host cell for amplification and/or polypeptide expression. In certain embodiments, mammalian host cells are preferred for use in the methods of the invention. The transformation of an expression vector into a selected host cell may be accomplished by well-known methods including transfection, transduction, infection, calcium phosphate co-precipitation, electroporation, microinjection, lipofection, DEAE-dextran mediated transfection, or other known techniques. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan and are set forth in manuals and other technical publications, for example, in Sambrook *et al.* *Molecular Cloning; A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (2001), and Ausubel *et al.* (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989).

[0057] As used herein, the term “transformation” refers to a change in a cell’s genetic characteristics, and a cell is considered to have been transformed when it has been modified to contain new DNA or RNA. For example, a cell is transformed where it is genetically modified from its native state by introducing new genetic material via transfection, transduction, or other techniques. Following transfection or transduction, the transforming DNA can recombine with that of the cell by physically integrating into a chromosome of the cell or can be maintained transiently as an episomal element without being replicated or can replicate independently as a plasmid. A cell is considered to have been “stably transformed” when the transforming DNA is replicated with the division of the cell.

[0058] As used herein, the term “transfection” refers to the uptake of foreign or exogenous DNA by a cell. A number of transfection techniques are well known in the art and are disclosed herein. *See, e.g.*, Graham *et al.*, 1973, *Virology* 52:456; Sambrook *et al.*, 2001, *Molecular Cloning: A Laboratory Manual*, *supra*; Davis *et al.*, 1986, *Basic Methods in Molecular Biology*, Elsevier; Chu *et al.*, 1981, *Gene* 13:197. As used herein, the term “transduction” refers to the process whereby foreign DNA is introduced into a cell via viral vector. *See Jones et al.*, (1998). *Genetics: principles and analysis*. Boston: Jones & Bartlett Publ.

[0059] The term “host cell” as used herein refers to a cell that has been transformed, or is capable of being transformed, with a nucleic acid and thereby expresses a gene of interest. The term includes the progeny of the parent cell, whether or not the progeny is identical in morphology or in genetic make-up to the original parent cell, so long as the gene of interest is

present. A host cell that comprises a nucleic acid encoding a recombinant protein, preferably operably linked to at least one expression control sequence (e.g. promoter or enhancer), is a “recombinant host cell.” A host cell, when cultured under appropriate conditions, synthesizes the recombinant protein that can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted). The selection of an appropriate host cell will depend upon various factors, such as desired expression levels, polypeptide modifications that are desirable or necessary for activity (such as glycosylation or phosphorylation) and ease of folding into a biologically active molecule. In certain embodiments of the methods of the invention, the host cell is a mammalian host cell.

[0060] Mammalian cell lines suitable as hosts for recombinant protein expression are well known in the art and include, but are not limited to, immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to Chinese hamster ovary (CHO) cells, including CHOK1 cells (ATCC CCL61), DXB-11, DG-44, and Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77: 4216, 1980); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, (Graham et al., J. Gen Virol. 36: 59, 1977); baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23: 243-251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human hepatoma cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y Acad. Sci. 383: 44-68, 1982); MRC 5 cells or FS4 cells; mammalian myeloma cells, and a number of other cell lines. CHO cells are preferred mammalian host cells in some embodiments of the methods of the invention.

[0061] Although mammalian host cells are preferred in some embodiments, other types of hosts cells can be used in the methods of the invention. Exemplary host cells include prokaryote, yeast, or higher eukaryote cells. Prokaryotic host cells include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacillus*, such as *B. subtilis* and *B.*

licheniformis, *Pseudomonas*, and *Streptomyces*. Eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for recombinant polypeptides. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Pichia*, e.g. *P. pastoris*, *Schizosaccharomyces pombe*; *Kluyveromyces*, *Yarrowia*, *Candida*, *Trichoderma reesia*, *Neurospora crassa*, *Schwanniomyces*, such as *Schwanniomyces occidentalis*; and filamentous fungi, such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

[0062] In some embodiments of the methods of the invention, the cell culture medium in the production bioreactor is inoculated with the host cells, preferably mammalian cells, expressing the recombinant protein (e.g. recombinant monoclonal antibody) at a density of at least 40×10^5 cells/mL, at least 45×10^5 cells/mL, at least 50×10^5 cells/mL, at least 55×10^5 cells/mL, at least 60×10^5 cells/mL, at least 65×10^5 cells/mL, at least 70×10^5 cells/mL, at least 75×10^5 cells/mL, at least 80×10^5 cells/mL, or at least 85×10^5 cells/mL. In certain embodiments, the cell culture medium in the production bioreactor is inoculated with the host cells (e.g. mammalian cells) at a density from about 40×10^5 cells/mL to about 60×10^5 cells/mL. In certain other embodiments, the cell culture medium in the production bioreactor is inoculated with the host cells (e.g. mammalian cells) at a density from about 45×10^5 cells/mL to about 55×10^5 cells/mL. In one embodiment, the cell culture medium in the production bioreactor is inoculated with the host cells (e.g. mammalian cells) at a density of at least 40×10^5 cells/mL. In another embodiment, the cell culture medium in the production bioreactor is inoculated with the host cells (e.g. mammalian cells) at a density of at least 50×10^5 cells/mL.

[0063] Following inoculation of the cell culture medium in the production bioreactor with the host cells (e.g. mammalian cells), the methods of the invention comprise culturing the host cells (e.g. mammalian cells) under conditions where the recombinant protein (e.g. recombinant monoclonal antibody) is expressed and optionally secreted into the medium. The term “culture” or “culturing” refers to the growth and propagation of cells outside of a multicellular organism or tissue. Host cells may be cultured in suspension or in an adherent form, attached to a solid substrate. Cell cultures can be established in fluidized bed bioreactors, hollow fiber bioreactors, roller bottles, shake flasks, or stirred tank bioreactors, with or without microcarriers. In some embodiments, the transformed mammalian cells, such as transformed CHO cells, may be

cultured in production bioreactors at a small scale, for example, at a volume of 5 liters or less, 3 liters or less, or 1 liter or less. In other embodiments, the transformed mammalian cells (e.g. transformed CHO cells) are cultured in production bioreactors with a capacity of at least 500 liters, at least 1,000 liters, at least 2,000 liters, at least 5,000 liters, at least 10,000 liters, or at least 15,000 liters. Such production cell cultures may be maintained for several weeks and even months, during which the cells produce the desired recombinant protein. In some embodiments of the methods of the invention, the bioreactor has a volume of at least 500 liters. In other embodiments of the methods of the invention, the bioreactor has a volume of at least 2,000 liters.

[0064] Suitable culture conditions, including temperature, dissolved oxygen content, agitation rate, and the like, for mammalian cells are known in the art and may vary by the phase or stage of the cell culture. The “growth phase” of a cell culture refers to the period of exponential cell growth (i.e. the log phase) where cells are generally rapidly dividing. During the growth phase, cells are cultured in a cell culture medium containing the necessary nutrients and additives under conditions (generally at about a temperature of 25°-40°C, in a humidified, controlled atmosphere) such that optimal growth is achieved for the particular cell line. Cells are typically maintained in the growth phase for a period of between one and eight days, e.g., between three to seven days, e.g., seven days. The length of the growth phase for a particular cell line can be determined by a person of ordinary skill in the art and will generally be the period of time sufficient to allow the particular cells to reproduce to a viable cell density within a range of about 20% -80% of the maximal possible viable cell density if the culture was maintained under the growth conditions. A “production phase” of a cell culture refers to the period of time during which logarithmic cell growth has ended and recombinant protein production is predominant. During the production phase, the medium is generally supplemented to support continued recombinant protein production.

[0065] In certain embodiments of the methods of the invention, the culture conditions may be adjusted to facilitate the transition from the growth phase of the cell culture to the production phase. For instance, a growth phase of the cell culture may occur at a higher temperature than a production phase of the cell culture. In some embodiments, a growth phase may occur at a first temperature from about 35.0°C to about 38.0°C, and a production phase may occur at a second temperature from about 29.0°C to about 37.0°C, optionally from about 30.0°C to about 36.0°C or from about 30.0°C to about 34.0°C. In one embodiment, a shift in temperature from a range

of about 35.0°C to about 37.0°C to a temperature range of about 31.0°C to about 33.0°C may be employed to facilitate the transition from the growth phase of the culture to the production phase. In another embodiment, a shift in temperature from a range of about 35.5°C to about 36.5°C to a temperature range of about 32.0°C to about 33.0°C may be employed to facilitate the transition from the growth phase of the culture to the production phase. Chemical inducers of protein production, such as, for example, caffeine, butyrate, and hexamethylene bisacetamide (HMBA), may be added at the same time as, before, and/or after a temperature shift, or in place of a temperature shift. If inducers are added after a temperature shift, they can be added from one hour to five days after the temperature shift, optionally from one to two days after the temperature shift.

[0066] Cell culture medium, as the term is used herein, refers to a solution containing nutrients sufficient to sustain growth and survival of a host cell during *in vitro* cell culture. Typically, cell culture media contains a buffer, salts, energy source, amino acids, vitamins and trace essential elements. Any media capable of supporting growth of the appropriate host cell in culture can be used. Cell culture media, which may be further supplemented with other components to maximize cell growth, cell viability, and/or recombinant protein production in a particular cultured host cell, are commercially available and include RPMI-1640 Medium, RPMI-1641 Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium Eagle, F-12K Medium, Ham's F12 Medium, Iscove's Modified Dulbecco's Medium, McCoy's 5A Medium, Leibovitz's L-15 Medium, and serum-free media such as EX-CELL™ 300 Series, among others, which can be obtained from the American Type Culture Collection or SAFC Biosciences, as well as other vendors. Cell culture media can be serum-free, protein-free, growth factor-free, and/or peptone-free media. Cell culture media may also be enriched by the addition of nutrients or other supplements, which may be used at greater than usual, recommended concentrations. In certain embodiments, the culture medium used in the methods of the invention is a chemically defined medium, which refers to a cell culture medium in which all of the components have known chemical structures and concentrations. Chemically defined media are typically serum-free and do not contain hydrolysates or animal-derived components.

[0067] Various media formulations can be used during the life of the culture, for example, to facilitate the transition from one stage (e.g., the growth stage or phase) to another (e.g., the production stage or phase) and/or to optimize conditions during cell culture (e.g. concentrated

media provided during a perfusion culture). A growth medium formulation can be used to promote cell growth and minimize protein expression. A production medium formulation can be used to promote production of the recombinant protein of interest and maintenance of the cells, with minimal new cell growth). A feed media, typically a media containing more concentrated components such as nutrients and amino acids, which are consumed during the course of the production phase of the cell culture may be used to supplement and maintain an active culture, particularly a culture operated in fed batch, semi-perfusion, or perfusion mode. Such a concentrated feed medium can contain most of the components of the cell culture medium at, for example, about 5×, 6×, 7×, 8×, 9×, 10×, 12×, 14×, 16×, 20×, 30×, 50×, 100×, 200×, 400×, 600×, 800×, or even about 1000× of their normal amount.

[0068] In the methods of the invention, the host cells (e.g. mammalian cells) can be cultured in a batch, fed batch, or perfusion culture. A “batch culture” refers to a method of culturing cells in which all the components required to establish the cell culture, including the transformed host cells, culture medium, and nutrients, are provided to the culture vessel at the beginning of the culturing process and no supplementation of the culture occurs. A batch culture is typically stopped at some point and the cells and/or components in the medium are harvested and recovered recombinant protein optionally purified. A “fed-batch culture” refers to a method of culturing cells in which additional components or nutrients (e.g. feed medium) are provided to the culture at one or more discrete times subsequent to the beginning of the culture process. A fed-batch culture is typically stopped at some point and the cells and/or components in the medium are harvested and the recombinant protein optionally purified. A “perfusion culture” refers to a method of culturing cells in which additional components or nutrients (e.g. feed medium) are provided continuously or semi-continuously to the culture subsequent to the beginning of the culture process. A portion of the cells and/or components in the medium are typically removed on a continuous or semi-continuous basis in a perfusion culture. In certain embodiments of the methods of the invention, the transformed host cells (e.g. transformed mammalian cells) are cultured in a perfusion culture.

[0069] In the methods of the invention, the host cells (e.g. mammalian cells) are cultured for a defined period of time during which the recombinant protein (e.g. recombinant monoclonal antibody) is expressed and optionally secreted by the cells. This period of time (i.e. the duration of the production phase of the cell culture) is at least 3 days, at least 7 days, at least 10 days, or at

least 15 days. In certain embodiments, the duration of the production phase of the cell culture is about 7 days to about 28 days, about 10 days to about 30 days, about 7 days to about 14 days, about 10 days to about 18 days, about 3 days to about 15 days, about 5 days to about 8 days, about 12 days to about 15 days, about 12 days to about 18 days, or about 15 days to about 21 days. In some embodiments, the duration of the production phase of the cell culture is 7 days, 8 days, 9 days, 12 days, 15 days, 18 days, or 21 days.

[0070] In certain embodiments of the methods of the invention, the methods further comprise recovering the expressed recombinant protein (e.g. recombinant monoclonal antibody) from the host cells (e.g. mammalian cells) or cell culture medium to obtain a recombinant protein (e.g. recombinant antibody) composition. If the recombinant protein is produced intracellularly (i.e. is not secreted by the host cell), as a first step, the host cells are lysed (e.g., by mechanical shear, osmotic shock, or enzymatic methods) and the particulate debris (e.g., host cells and lysed fragments), is removed, for example, by centrifugation, flocculation, acoustic wave separation, or filtration, including, for example, by microfiltration, ultrafiltration, tangential flow filtration, alternative tangential flow filtration, and depth filtration. In certain preferred embodiments, the recombinant protein (e.g. recombinant monoclonal antibody) is secreted into the culture medium by the host cell (e.g. mammalian host cell). In such embodiments, the recombinant protein can be separated from host cells through centrifugation or microfiltration, and optionally, subsequently concentrated through ultrafiltration. In some embodiments, the expressed recombinant protein (e.g. recombinant monoclonal antibody) is recovered from the cell culture medium by microfiltration. In these and other embodiments, the expressed recombinant protein (e.g. recombinant monoclonal antibody) is recovered from the cell culture medium by alternating tangential flow filtration.

[0071] In some embodiments of the methods of the invention, the recombinant protein (e.g. recombinant monoclonal antibody) recovered from the host cells or cell culture medium may be further purified or partially purified to remove cell culture media components, host cell proteins or nucleic acids, or other process or product-related impurities by one or more unit operations. The term “unit operation” refers to a functional step that is performed as part of a process of purifying a recombinant protein of interest. For example, a unit operation can include steps such as, but not limited to, capturing, purifying, polishing, viral inactivating, virus filtering, concentrating and/or formulating the recombinant protein of interest. Unit operations can be

designed to achieve a single objective or multiple objectives, such as capture and virus inactivating steps. Unit operations can also include holding or storing steps between processing steps. One of ordinary skill in the art can select the appropriate unit operation(s) for further purification of a recombinant protein based on the characteristics of the recombinant protein to be purified, the characteristics of host cell from which the recombinant protein is expressed, and the composition of the culture medium in which the host cells were grown.

[0072] A capture unit operation may include capture chromatography that makes use of resins and/or membranes containing agents that will bind to the recombinant protein of interest, for example affinity chromatography, size exclusion chromatography, ion exchange chromatography, hydrophobic interaction chromatography (HIC), immobilized metal affinity chromatography (IMAC), and the like. Such chromatographic materials are known in the art and are commercially available. For instance, if the recombinant protein is an antibody or contains components derived from an antibody (e.g. Fc domain), affinity chromatography using ligands such as Protein A, Protein G, Protein A/G, or Protein L may be employed as a capture chromatography unit operation to further purify the recombinant protein. In other embodiments, the recombinant protein of interest may comprise a polyhistidine tag at its amino or carboxyl terminus and subsequently purified using IMAC. Recombinant proteins can be engineered to include other purification tags, such as a FLAG® tag or c-myc epitope and subsequently purified by affinity chromatography using a specific antibody directed to such tag or epitope.

[0073] Unit operations to inactivate, reduce and/or eliminate viral contaminants may include filtration processes and/or adjusting solution conditions. One method for achieving viral inactivation is incubation at low pH (e.g., pH<4). A low pH viral inactivation operation can be followed with a neutralization unit operation that readjusts the virally inactivated solution to a pH more compatible with the requirements of the subsequent unit operations. A low pH viral inactivation operation may also be followed by filtration, such as depth filtration, to remove any resulting turbidity or precipitation. Adjusting the temperature or chemical composition (e.g. use of detergents) can also be used to achieve viral inactivation. Viral filtration can be performed using micro- or nano-filters, such as those available from Asahi Kasei (Plavona®) and EDM Millipore (VPro®).

[0074] A polishing unit operation may make use of various chromatographic methods for the purification of the protein of interest and clearance of contaminants and impurities. The polish

chromatography unit operation makes use of resins and/or membranes containing agents that can be used in either a “flow-through mode,” in which the protein of interest is contained in the eluent and the contaminants and impurities are bound to the chromatographic medium, or “bind and elute mode,” in which the protein of interest is bound to the chromatographic medium and eluted after the contaminants and impurities have flowed through or been washed off the chromatographic medium. Examples of such polish chromatography methods include, but are not limited to, ion exchange chromatography (IEX), such as anion exchange chromatography (AEX) and cation exchange chromatography (CEX); hydrophobic interaction chromatography (HIC); mixed modal or multimodal chromatography (MM), hydroxyapatite chromatography (HA); reverse phase chromatography, and size-exclusion chromatography (e.g. gel filtration).

[0075] Product concentration and buffer exchange of the recombinant protein of interest into a desired formulation buffer for bulk storage of the drug substance or drug product can be accomplished by ultrafiltration and diafiltration.

[0076] As described in detail herein, the methods of the invention reduce the amount of charge variants, particularly acidic variants, of a recombinant protein (e.g. recombinant monoclonal antibody) produced by a host cell during the cell culture process and thus obviate the need for downstream unit operations designed to specifically remove such charge variants. In certain embodiments, the recombinant protein composition (e.g. recombinant monoclonal antibody composition) is a harvested cell culture fluid. The term “harvested cell culture fluid” refers to a solution which has been processed by one or more operations to separate cells, cell debris, or other large particulates from the recombinant protein. Such operations, as described above, include, but are not limited to, flocculation, centrifugation, acoustic wave separation, and various forms of filtration (e.g. depth filtration, microfiltration, ultrafiltration, tangential flow filtration, and alternating tangential flow filtration). Harvested cell culture fluid includes cell culture lysates as well as cell culture supernatants. The harvested cell culture fluid may be further clarified to remove fine particulate matter and soluble aggregates by filtration with a membrane having a pore size between about 0.1 μm and about 0.5 μm , or more preferably a membrane having a pore size of about 0.22 μm . Thus, in some embodiments, the recombinant protein composition (e.g. recombinant monoclonal antibody composition) is a clarified harvested cell culture fluid. In other embodiments, the recombinant protein composition (e.g. recombinant monoclonal antibody composition) can be obtained from a purification unit operation, such as a

polish chromatography unit operation. In one such embodiment, the recombinant protein composition (e.g. recombinant monoclonal antibody composition) is an elution pool from a cation exchange chromatography material. In certain embodiments, the recombinant protein composition (e.g. recombinant monoclonal antibody composition) is drug substance. In certain other embodiments, the recombinant protein composition (e.g. recombinant monoclonal antibody composition) is drug product.

[0077] The following examples, including the experiments conducted and the results achieved, are provided for illustrative purposes only and are not to be construed as limiting the scope of the appended claims.

EXAMPLES

Example 1. Reduction of Acidic Variants of a Monoclonal Antibody by Limiting Media Hold Duration in the Production Bioreactor

[0078] Recombinant production of a monoclonal antibody often gives rise to variant forms of the antibody, some of which have undesirable characteristics. Molecular variants of an antibody that have properties that differ from the target antibody and affect the antibody's efficacy or safety profile are considered to be product-related impurities. To ensure consistent quality of a recombinantly-produced monoclonal antibody drug product, it is important to monitor and control the generation of product-related impurities during the cell culture process. This example describes the control of acidic variants of a monoclonal antibody by manipulating the duration of the media hold in the production bioreactor.

[0079] Erenumab is a fully human monoclonal antibody of the IgG2 subclass that specifically binds to the extracellular domain of the calcitonin gene-related peptide (CGRP) receptor. Erenumab consists of two heavy chains and two light chains of the lambda subclass. The amino acid sequences for the heavy chain and light chain of erenumab are set forth in SEQ ID NO: 1 and SEQ ID NO: 2, respectively. Nucleic acids encoding the heavy chain and light chain of erenumab were cloned into mammalian expression vectors and stably transformed into Chinese hamster ovary (CHO) cells.

[0080] After thawing, the erenumab-producing CHO cell line was cultured in a serum-free selective growth medium in a series of shake flasks and culture bags. Cultures were incubated at a temperature of 36.0 °C, 5.0% CO₂ and expanded until sufficient cell mass was obtained to

inoculate the N-2 and N-1 bioreactors. The N-1 bioreactor (working volume of about 500 L) was operated in batch mode for about 2 days followed by perfusion mode at about 0.5 bioreactor volumes/day for about 4 days with the following parameters: temperature at 36.0 °C, pH 7.00, dissolved oxygen (DO) at 48 mm Hg, and agitation at 112 RPM.

[0081] Prior to inoculation, a serum-free, chemically defined medium was incubated in the production (N) bioreactor (working volume of approximately 2,000 L) at a temperature between 35.5 °C to 36.5 °C and a pH between 6.85 and 7.05. To assess the impact of the duration of this media hold on product quality and cell culture performance, the duration of the media hold was varied between about 5 hours and about 50 hours. At the end of the media hold, the production bioreactor was seeded at an initial viable cell density (VCD) of about 50×10^5 cells/mL and run in perfusion mode from day 1 to day 15 using an alternating tangential flow (ATF) filtration system. From day 1 to day 15, the cell culture was continuously fed with a serum-free, chemically defined perfusion medium at an initial rate of 0.50 bioreactor volume/day that was increased to 1.0 bioreactor volume/day by day 5. The production bioreactor was operated at the following parameters: temperature at 36.0 °C initially and subsequently decreased to 32.5 °C at a pre-specific target VCD, pH 6.90, DO at 48 mm Hg, and agitation at 98 RPM. Glucose solution was fed to the bioreactor periodically to maintain a glucose concentration between 1 g/L to 8 g/L. The bioreactor was harvested by switching the filter in the ATF filtration system to a microfilter to allow erenumab to pass through the filter into the permeate and retain the cells and cell debris in the bioreactor. The permeate from the microfilter was collected to obtain the harvested cell culture fluid (HCCF). HCCF was subject to protein A affinity chromatography and two polish ion exchange chromatography steps followed by ultrafiltration/diafiltration to produce erenumab drug substance.

[0082] The amount and number of charge variants of erenumab in drug substance were determined using a cation exchange high performance liquid chromatography (CEX-HPLC) method. CEX-HPLC separates proteins based primarily on the heterogeneity of surface charge; however, separation may also be influenced by structural heterogeneity and other modifications that impact molecular interactions with the ion exchange resin. Peak elution in this method is a function of net surface charge with negatively charged species (i.e. acidic variants) eluting earlier and positively charged species (i.e. basic variants) eluting later. Samples of drug substance were loaded onto an analytical CEX-HPLC column (BioPro SP-F, 5 µm particle size, 4.6 mm × 100

mm, YMC America, Inc.). Mobile phase A contained 20 mM sodium phosphate at pH 6.6 and mobile phase B consisted of 20 mM sodium phosphate, 500 mM sodium chloride, at pH 6.6. Proteins were separated using a salt gradient generated with 5% to 12% mobile phase B from 0 min to 4 min, to 23% mobile phase B at 18 min, to 100% mobile phase B at 18.5 min to 20.5 min, and back to 5% mobile phase B at 21 min to 25 min. The eluent was monitored by UV absorbance at 280 nm. The column was operated at 28 °C and the mobile phase was applied to the column at a flow rate of 0.6 mL/min. The CEX-HPLC profile for erenumab contained three distinct regions, including acidic peaks (eluting from the column prior to the main peak), main peak, and basic peaks (eluting from the column after the main peak)(Figure 1). The amount of acidic variants of erenumab was determined by the percentage of acidic peaks, which was calculated as the sum of acidic peaks area/total integrated peak area x 100. Similarly, the amount of basic variants of erenumab was determined by the percentage of basic peaks, which was calculated as the sum of basic peaks area/total integrated peak area x 100. The amount of erenumab was determined by the percentage of main peak, which as calculated as the main peak area/total integrated peak area x 100.

[0083] As shown in Figure 2, reducing the duration of the media hold in the production bioreactor lowered the amount of acidic variants of erenumab. Limiting the media hold duration to 18 hours or less reduced the amount of acidic variants by about 1% on average.

Example 2. Impact of Media Hold Duration on Product Yield and Titer of a Recombinant Monoclonal Antibody Cell Culture Process

[0084] This example describes the impact of media hold duration on various parameters of cell culture performance, such as product yield and product titer.

[0085] Two production runs for an erenumab cell culture process, in which the media hold duration in the production bioreactor was extended from ≤ 18 hours to ≤ 48 hours, were conducted. Significant reductions in product titer and cell viability were observed in these two runs as compared to historical runs in which the media hold duration was not extended. A multivariate data analysis was conducted assessing the impact of 65 different variables, including production bioreactor measurements of various parameters (e.g. pCO₂, glucose, viable cell density), media hold durations, and off-line measurements from the N-1 bioreactor, on product titer and cell viability. The media hold duration in the production bioreactor was negatively

correlated with product titer and cell viability and was the highest correlated input variable, suggesting that the media hold duration was contributing to the low cell culture performance in these two production runs.

[0086] To further evaluate the effect of media hold duration in the production bioreactor on product titer and yield, HCCF obtained from the production bioreactor under the conditions described in Example 1 from several different production runs at two different manufacturing sites, in which the media hold duration was varied between about 5 hours and about 50 hours, was assessed for erenumab product titer and yield. Product titer was determined using affinity Protein A Ultra High-Performance Liquid Chromatography (UHPLC) equipped with UV detection at 280 nm. Erenumab binds to the Protein A at neutral pH, is eluted at acidic pH and detected by UV absorbance at 280 nm. Mass yield was calculated according to the following equation:

$$\text{Mass Yield} = (\text{Final Day Bioreactor Mass} + \text{HCCF Pool Mass}) / \text{Bioreactor Weight}$$

Where:

$$\text{HCCF Pool Mass} = \text{HCCF Pool Titer} \times \text{HCCF Pool Weight} / \text{HCCF Pool Density}$$

$$\text{Packed Cell Volume (PCV) Adjusted Titer} = \text{Final Day Titer} - ((\text{Final Day Titer} \times \text{PCV}) / 100)$$

$$\text{Final Day Bioreactor Mass} = \text{PCV Adjusted Titer} \times \text{Bioreactor Weight}$$

[0087] The erenumab product titer in the HCCF was plotted as a function of media hold duration in the production bioreactor and a bivariate regression analysis was performed. *See Figure 3.* The media hold duration showed a statistically significant effect on product titer ($p < 0.0001$). As was found in the multivariate analysis of two production runs with extended media hold durations, a negative correlation between media hold duration in the production bioreactor and product titer was observed. A reduction of the media hold duration in the production bioreactor to 18 hours or less with an average of 12.2 hours resulted in an increase in erenumab product titer of about 4 g/L and a corresponding mass gain of ~3.1 kg (an approximate 15% increase in mass yield.).

[0088] All publications, patents, and patent applications discussed and cited herein are hereby incorporated by reference in their entireties. It is understood that the disclosed invention is not limited to the particular methodology, protocols and materials described as these can vary. It is also understood that the terminology used herein is for the purposes of describing particular embodiments only and is not intended to limit the scope of the appended claims.

[0089] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Table 1. Sequence Listing

SEQ ID NO:	Description	Sequence
1	Erenumab Heavy Chain	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSFGMHWVRQ APGKGLEWVAVISFDGSIKYSVDSVKGRFTISRDNKNTLF LQMNSLRAEDTAVYYCARDRLNYYDSSGYYHYKYYGMA VWGQGTTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSNFGTQYTCNVDHKPSNTKVDKTVKCCVECP APPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQ DWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTL PSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK TTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL HNHYTQKSLSLSPGK
2	Erenumab Light Chain	QSVLTQPPSVSAAPGQKVTISCSGSSSNIGNNYVSWYQQLP GTAPKLLIYDNNKRPSGIPDRFSGSKSGTSTTLGITGLQTGD EADYYCGTWDSRLSAVVFGGGTKLTVLGQPKANPTVTLFP PSSEELQANKATLVCLISDFYPGAVTVAWKADGSPVKAGV ETTKPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEG STVEKTVAPTECS
3	Erenumab Heavy Chain Variable Region	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSFGMHWVRQ APGKGLEWVAVISFDGSIKYSVDSVKGRFTISRDNKNTLF LQMNSLRAEDTAVYYCARDRLNYYDSSGYYHYKYYGMA VWGQGTTVTVSS
4	Erenumab Light Chain Variable Region	QSVLTQPPSVSAAPGQKVTISCSGSSSNIGNNYVSWYQQLP GTAPKLLIYDNNKRPSGIPDRFSGSKSGTSTTLGITGLQTGD EADYYCGTWDSRLSAVVFGGGTKLTVL

CLAIMS

What is claimed:

1. A method for reducing the amount of charge variants in a recombinant monoclonal antibody composition, the method comprising:
 - maintaining a cell culture medium in a bioreactor at a temperature of about 34.0°C to about 38.0°C for a duration of about 1 hour to about 18 hours;
 - inoculating the cell culture medium with mammalian cells expressing the monoclonal antibody;
 - culturing the mammalian cells under conditions where the monoclonal antibody is expressed and secreted into the medium; and
 - recovering the expressed monoclonal antibody from the cell culture medium to obtain the recombinant monoclonal antibody composition.
2. The method of claim 1, wherein the cell culture medium is maintained in the bioreactor at a temperature of about 35.5°C to about 36.5°C prior to inoculation.
3. The method of claim 1 or 2, wherein the cell culture medium is maintained in the bioreactor for a duration of about 6 hours to about 18 hours prior to inoculation.
4. The method of claim 1 or 2, wherein the cell culture medium is maintained in the bioreactor for a duration of about 12 hours to about 18 hours prior to inoculation.
5. The method of any one of claims 1 to 4, wherein the cell culture medium is maintained in the bioreactor at a temperature of about 36.0°C for a duration of about 12 hours prior to inoculation.
6. The method of any one of claims 1 to 5, wherein the cell culture medium is maintained in the bioreactor at a pH of about 6.85 to about 7.05 prior to inoculation.
7. The method of any one of claims 1 to 6, wherein the bioreactor has a volume of at least 500 liters.

8. The method of any one of claims 1 to 6, wherein the bioreactor has a volume of at least 2,000 liters.
9. The method of any one of claims 1 to 8, wherein the cell culture medium is inoculated with the mammalian cells at a density of at least 40×10^5 cells/mL.
10. The method of any one of claims 1 to 9, wherein the mammalian cells are cultured in a perfusion culture.
11. The method of any one of claims 1 to 10, wherein the expressed monoclonal antibody is recovered from the cell culture medium by microfiltration.
12. The method of any one of claims 1 to 11, wherein the mammalian cell is a CHO cell.
13. The method of any one of claims 1 to 12, wherein the recombinant monoclonal antibody composition is harvested cell culture fluid.
14. The method of any one of claims 1 to 13, wherein the charge variants are acidic variants.
15. The method of claim 14, wherein the recombinant monoclonal antibody composition comprises about 32% or less acidic variants of the monoclonal antibody.
16. The method of claim 14 or 15, wherein the amount of acidic variants is measured by cation exchange high performance liquid chromatography (CEX-HPLC).
17. The method of any one of claims 1 to 16, wherein the monoclonal antibody is an IgG1 or IgG2 antibody.
18. The method of any one of claims 1 to 17, wherein the monoclonal antibody is erenumab.

19. A method for increasing yield of a recombinant monoclonal antibody cell culture process, the method comprising:
- maintaining a cell culture medium in a bioreactor at a temperature of about 34.0°C to about 38.0°C for a duration of about 1 hour to about 18 hours;
 - inoculating the cell culture medium with mammalian cells expressing the monoclonal antibody; and
 - culturing the mammalian cells under conditions where the monoclonal antibody is expressed and secreted into the medium.
20. The method of claim 19, wherein the yield of the process is increased as compared to the yield of a process with a media hold duration exceeding 30 hours.
21. The method of claim 19 or 20, wherein the yield of the process is increased by at least 10%.
22. The method of claim 19 or 20, wherein the yield of the process is increased by at least 15%.
23. The method of any one of claims 19 to 22, wherein the cell culture medium is maintained in the bioreactor at a temperature of about 35.5°C to about 36.5°C prior to inoculation.
24. The method of any one of claims 19 to 23, wherein the cell culture medium is maintained in the bioreactor for a duration of about 6 hours to about 18 hours prior to inoculation.
25. The method of any one of claims 19 to 23, wherein the cell culture medium is maintained in the bioreactor for a duration of about 12 hours to about 18 hours prior to inoculation.
26. The method of any one of claims 19 to 25, wherein the cell culture medium is maintained in the bioreactor at a temperature of about 36.0°C for a duration of about 12 hours prior to inoculation.

27. The method of any one of claims 19 to 26, wherein the cell culture medium is maintained in the bioreactor at a pH of about 6.85 to about 7.05 prior to inoculation.
28. The method of any one of claims 19 to 27, wherein the bioreactor has a volume of at least 500 liters.
29. The method of any one of claims 19 to 27, wherein the bioreactor has a volume of at least 2,000 liters.
30. The method of any one of claims 19 to 29, wherein the cell culture medium is inoculated with the mammalian cells at a density of at least 40×10^5 cells/mL.
31. The method of any one of claims 19 to 30, wherein the mammalian cells are cultured in a perfusion culture.
32. The method of any one of claims 19 to 31, wherein the mammalian cell is a CHO cell.
33. The method of any one of claims 19 to 32, further comprising recovering the expressed monoclonal antibody from the cell culture medium to obtain a recombinant monoclonal antibody composition.
34. The method of claim 33, wherein the expressed monoclonal antibody is recovered from the cell culture medium by microfiltration.
35. The method of claim 33 or 34, wherein the recombinant monoclonal antibody composition is harvested cell culture fluid.
36. The method of any one of claims 33 to 35, wherein the recombinant monoclonal antibody composition comprises about 32% or less acidic variants of the monoclonal antibody.

37. The method of claim 36, wherein the amount of acidic variants is measured by cation exchange high performance liquid chromatography (CEX-HPLC).
38. The method of any one of claims 19 to 37, wherein the monoclonal antibody is an IgG1 or IgG2 antibody.
39. The method of any one of claims 19 to 38, wherein the monoclonal antibody is erenumab.