(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 May 2009 (07.05.2009)

PCT

(10) International Publication Number
WO 2009/058812 A1

(51) International Patent Classification:
C07K 16/06 (2006.01) *C07K 16/28* (2006.01)
C07K 1/18 (2006.01) *C07K 16/22* (2006.01)

(21) International Application Number: PCT/US2008/081516

(22) International Filing Date: 29 October 2008 (29.10.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/983,825 30 October 2007 (30.10.2007) US

(71) Applicant *(for all designated States except US)*: **GENENTECH, INC.** [US/US]; 1 DNA Way, South San Francisco, California 94080 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **LEBRETON, Benedicte Andree** [FR/US]; 1880 Vallejo Street #3, San Francisco, California 94123 (US). **O'CONNOR, Deborah Ann** [US/US]; 1641 Cedar Street, San Carlos, California 94070 (US). **SAFTA, Aurelia** [US/US]; 1655 North California Boulevard #453, Walnut Creek, California 94596 (US). **SHARMA, Mandakini** [IN/US]; 563 Montego Terrace, Sunnyvale, California 94089 (US).

(74) Agents: **PAZDERA, R. Minako** et al.; 1 DNA Way, MS 49, South San Francisco, California 94080 (US).

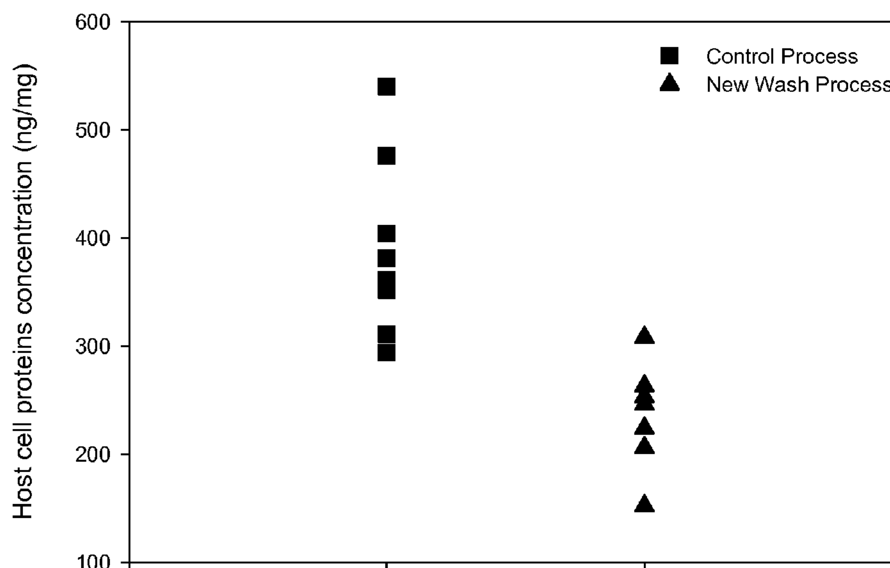
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: ANTIBODY PURIFICATION BY CATION EXCHANGE CHROMATOGRAPHY

FIG. 3 – Host Cell Proteins Removal for Rituximab Processes



(57) Abstract: A method for purifying an antibody by cation exchange chromatography is described in which a high pH wash step is used to remove of contaminants prior to eluting the desired antibody using an elution buffer with increased conductivity.

WO 2009/058812 A1

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

5

ANTIBODY PURIFICATION BY CATION EXCHANGE CHROMATOGRAPHY

Related Applications

This application claims the benefit of U.S. Provisional Patent Application No. 60/983825, filed 30 October 2007, the disclosure of which is hereby incorporated by
10 reference in its entirety for all purposes.

Background of the Invention

Field of the Invention

This invention relates generally to protein purification. In particular, the invention relates to a method for purifying antibody from a composition comprising the antibody
15 and at least one contaminant using cation exchange chromatography, wherein a high pH wash step is used to remove contaminants prior to eluting the desired antibody using an elution buffer with increased conductivity.

Description of the Related Art

20 The large-scale, economic purification of proteins is an increasingly important problem for the biotechnology industry. Generally, proteins are produced by cell culture, using either eukaryotic or prokaryotic cell lines engineered to produce the protein of interest by insertion of a recombinant plasmid containing the gene for that protein. Since the cells typically used are living organisms, they must be fed with a complex growth
25 medium, containing sugars, amino acids, and growth factors, usually supplied from preparations of animal serum. Separation of the desired protein from the mixture of compounds fed to the cells and from the by-products of the cells themselves to a purity sufficient for use as a human therapeutic poses a formidable challenge.

Procedures for purification of proteins from cell debris initially depend on the site
30 of expression of the protein. Some proteins can be cased to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate,
35 and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The

5 same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a 10 combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move 15 at different rates down a long column, achieving a physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through".

20 Ion exchange chromatography is a chromatographic technique that is commonly used for the purification of proteins. In ion exchange chromatography, charged patches on the surface of the solute are attracted by opposite charges attached to a chromatography matrix, provided the ionic strength of the surrounding buffer is low. Elution is generally achieved by increasing the ionic strength (*i.e.* conductivity) of the buffer to compete with 25 the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute is another way to achieve elution of the solute. The change in conductivity or pH may be gradual (gradient elution) or stepwise (step elution). In the past, these changes have been progressive; *i.e.*, the pH or conductivity is increased or decreased in a single direction

30 US Patent Nos. 6,339,142, 6,417,355, 6,489,447, and 7,074,404 (Basey *et al.*) describe ion exchange chromatography for purifying polypeptides. US Patent Nos. 6,127,526, 6,333,398, and 6,797,814 (Blank, G.) describe purifying proteins, such as anti-HER2 antibodies, by Protein A chromatography. Methods for purifying proteins, such as antibodies, by ion exchange chromatography are described in US Application Publication 35 No. 2004/0082047.

US Patent No. 5,110,913 refers to purifying an antibody in an aqueous solution by binding the antibody to an ion exchange resin at a first pH of 4.6, washing at a second pH

5 of 5.5, and eluting the antibody at pH 6.5, wherein the ionic strength of the solutions of these three steps remains constant. Zhang *et al.* refer to Q membrane, anion exchange chromatography of a human antibody (Zhang et al. "Q Membrane Chromatography Application for Human Antibody Purification Process," Poster presented at *BioProduction*, Oct. 26-27. Munich, Germany, 2004). Other publications concerning
10 protein purification include: Barnthouse *et al. J. Biotech.* 66: 125-136 (1998); Blank *et al. Bioseparation* 10: 65-71 (2001); Follman and Fahrner *J. Chromatog.* 1024: 79-85 (2004); Iyer *et al. BioPharm* 15(1):14-16, 18, 20, 53 (2002); US 2004/0082047A1; EP 333,574; EP 460,426 B1; EP 556,083; WO 89/05157; WO 92/22653; WO 93/06217; WO 95/22389; WO 96/33208; WO 96/40883; US 4,753,894; US 4,966,851; US 5,110,913;
15 US 5,112,951; US 5,115,101; US 5,118,796; US 5,169,774; US 5,196,323; US 5,256,769; US 5,279,823; US 5,429,746; US 5,451,662; US 5,525,338; US 5,677,171 ; US 6,005,081; US 6,054,561; US 6,127,526; US 6,267,958; US 6,339,142; US 6,417,335; US 6,489,447; Adachi *et al., Journal of Chromatography. A.* 763(1-2):57-63 (Feb 28, 1997); Gagnon, P., *Purification Tools for Monoclonal Antibodies,* Tucson:Validated Biosystems,
20 Inc., Chapter 4, pps. 57-86 (1996); Graf et al., *Bioseparation* 4(1):7-20 (Feb 1994); Mhatre *et al., Journal of Chromatography A* 707(2):225-231 (Jul 21, 1995); Neidhardt *et al., Journal of Chromatography* 590(2):255-261 (1992); P*rotein Purification Applications - A Practical Approach,* Harris and Angal, IRL Press pps. 151-156 (1995); Sofer *et al. Handbook of Process Chromatography: A Guide to Optimization, Scale-up, and*
25 *Validation*, San Diego:Academic Press pps. 65-80 (1997); Tishchenko *et al., Journal of Chromatography B* 706(1):157-166 (Feb 27, 1998).

Summary of the Invention

The invention herein concerns an improved method for cation exchange chromatography of antibodies in which a high pH wash step is used to remove
30 contaminants prior to eluting the desired antibody product. The process results, amongst other things, in improved removal of Chinese Hamster Ovary Proteins (CHOP) contaminants.

According to a first aspect, the invention provides a method for purifying an antibody from a composition comprising the antibody and at least one contaminant, which
35 method comprises the sequential steps of:

(a) loading the composition onto a cation exchange material wherein the composition is at a first pH;

5 (b) washing the cation exchange material with a first wash buffer at a pH which is greater than that of the composition in (a), wherein the pH of the first wash buffer is from about 6.8 to about 9.0;

(c) washing the cation exchange material with a second wash buffer at a pH which is less than that of the first wash buffer; and

10 (d) eluting the antibody from the cation exchange material with an elution buffer at a conductivity which is substantially greater than that of the second wash buffer.

Preferably the antibody binds human CD20, such as rituximab, or binds human vascular endothelial growth factor (VEGF), such as bevacizumab.

15 According to one preferred embodiment, the invention concerns a method for purifying an antibody that binds human CD20 from a composition comprising the antibody and one or more contaminants selected from the group consisting of Chinese Hamster Ovary Proteins (CHOP), leached protein A, DNA, and aggregated CD20 antibody, which method comprises the sequential steps of:

20 (a) loading the composition onto a cation exchange material wherein the composition is at a pH from about 4.0 to about 6.0;

(b) washing the cation exchange material with a first wash buffer at a pH from about 6.8 to about 9.0;

(c) washing the cation exchange material with a second wash buffer at a pH from 25 about 5.0 to about 6.0; and

(d) eluting the antibody from the cation exchange material using an elution buffer with a pH from about 5.0 to about 6.0 and a conductivity from about 10 to about 100mS/cm. Preferably the CD20 antibody is rituximab.

According to another preferred embodiment, the invention relates to a method for 30 purifying an antibody that binds human vascular endothelial growth factor (VEGF) from a composition comprising the antibody and one or more contaminants selected from the group consisting of a cell culture media component, garamycin, Chinese Hamster Ovary Proteins (CHOP), DNA, viral contaminant, and aggregated VEGF antibody, which method comprises the sequential steps of:

35 (a) loading the composition onto a cation exchange material wherein the composition is at a pH from about 4.0 to about 6.0;

(b) washing the cation exchange material with a first wash buffer at a pH from

5 about 6.8 to about 8.0;

(c) washing the cation exchange material with a second wash buffer at a pH from about 5.0 to about 6.0; and

(d) eluting the antibody from the cation exchange material using an elution buffer with a pH from about 5.0 to about 6.0 and a conductivity from about 10 to about 10 100mS/cm. Preferably, the VEGF antibody is bevacizumab.

The invention also concerns a composition comprising rituximab in a buffer comprising about 25mM HEPES, at a pH of about 7.8.

In addition, the invention provides a composition comprising bevacizumab in a buffer comprising about 25mM MOPS at a pH of about 7.0.

15

Brief Description of the Drawings

Figures 1A and 1B provide the amino acid sequences of the heavy chain (SEQ ID No. 1) and light chain (SEQ ID No. 2) of rituximab antibody. Each of the framework regions (FR1-4) and each of the CDR regions (CDR1-3) in each variable region are 20 identified, as are the human gamma 1 heavy chain constant sequence and human kappa light chain constant sequence. The variable heavy (VH) region is in SEQ ID No. 3. The variable light (VL) region is in SEQ ID No. 4. The sequence identifiers for the CDRs are: CDR H1 (SEQ ID No. 5), CDR H2 (SEQ ID No. 6), CDR H3 (SEQ ID No. 7), CDR L1 (SEQ ID No. 8), CDR L2 (SEQ ID No. 9), and CDR L3 (SEQ ID No. 10).

25 Figures 2A and 2B provide the amino acid sequences of the heavy chain (SEQ ID No. 11) and light chain (SEQ ID No. 12) of bevacizumab antibody. The end of each variable region is indicated with ‖. The variable heavy (VH) region is in SEQ ID No. 13. The variable light (VL) region is in SEQ ID No. 14. Each of the three CDRs in each variable region is underlined. The sequence identifiers for the CDRs are: CDR H1 (SEQ 30 ID No. 15), CDR H2 (SEQ ID No. 16), CDR H3 (SEQ ID No. 17), CDR L1 (SEQ ID No. 18), CDR L2 (SEQ ID No. 19), and CDR L3 (SEQ ID No. 20).

Figure 3 provides a side-by-side comparison of host cell proteins removal by the cation exchange chromatography process of the improved rituximab process compared to the original process. Superior CHOP removal was achieved with the new process.

35

5 Detailed Description of the Preferred Embodiment

Definitions:

Herein, numerical ranges or amounts prefaced by the term “about” expressly include the exact range or exact numerical amount.

The “composition” to be purified herein comprises the antibody of interest and 10 one or more contaminants. The composition may be “partially purified” (*i.e.* having been subjected to one or more purification steps) or may be obtained directly from a host cell or organism producing the antibody (*e.g.* the composition may comprise harvested cell culture fluid).

As used herein, "polypeptide" refers generally to peptides and proteins having 15 more than about ten amino acids. Preferably, the polypeptide is a mammalian protein, examples of which include: renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; 20 glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation 25 normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular 30 endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor 35 (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins (IGFBPs); CD proteins such as CD3,

5 CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), *e.g.*, M-CSF, GM-CSF, and G-CSF; interleukins (ILs), *e.g.*, IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a
10 portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments and/or variants of any of the above-listed polypeptides as well as antibodies, including antibody fragments, binding to any of the above-listed polypeptides. A
15 preferred polypeptide is an intact antibody or an antibody fragment that binds to human CD20, for example, rituximab; or an intact antibody or an antibody fragment that binds to human vascular endothelial growth factor (VEGF), for example bevacizumab.

A "contaminant" is a material that is different from the desired antibody product. The contaminant includes, without limitation: host cell materials, such as Chinese
20 Hamster Ovary Proteins (CHOP); leached protein A; nucleic acid; a variant, fragment, aggregate or derivative of the desired antibody; another polypeptide; endotoxin; viral contaminant; cell culture media component (*e.g.* garamycin; GENTAMYCIN®) etc.

The phrase "cation exchange material" refers to a solid phase that is negatively charged and has free cations for exchange with cations in an aqueous solution passed over
25 or through the solid phase. The charge may be provided by attaching one or more charged ligands to the solid phase, *e.g.* by covalent linking. Alternatively, or in addition, the charge may be an inherent property of the solid phase (*e.g.* as is the case for silica, which has an overall negative charge). Commercially available cation exchange materials include carboxy-methyl-cellulose, BAKERBOND ABX™, sulphopropyl (SP)
30 immobilized on agarose (*e.g.* SP-SEPHAROSE FAST FLOW™, SP-SEPHAROSE FAST FLOW XL™ or SP-SEPHAROSE HIGH PERFORMANCE™, from GE Healthcare), CAPTO S™ (GE Healthcare), FRACTOGEL-SO3™, FRACTOGEL-SE HICAP™, and FRACTOPREP™ (EMD Merck), sulphonyl immobilized on agarose (*e.g.* S-SEPHAROSE FAST FLOW™ from GE Healthcare), and SUPER SP™ (Tosoh
35 Biosciences). A preferred cation exchange material herein comprises cross-linked poly(styrene-divinylbenzene) flow-through particles (solid phase) coated with a

5 polyhydroxylated polymer functionalized with sulfopropyl groups (for example, POROS 50 HS® chromatography resin).

By "solid phase" is meant a non-aqueous matrix to which one or more charged ligands can adhere. The solid phase may be a purification column (including, without limitation, expanded bed and packed bed columns), a discontinuous phase of discrete 10 particles, a membrane, or filter etc. Examples of materials for forming the solid phase include polysaccharides (such as agarose and cellulose) and other mechanically stable matrices such as silica (*e.g.* controlled pore glass), poly(styrene-divinylbenzene), polyacrylamide, ceramic particles and derivatives of any of the above.

The term “load” herein refers to the composition loaded onto the cation exchange 15 material. Preferably, the cation exchange material is equilibrated with an equilibration buffer prior to loading the composition which is to be purified.

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in *Buffers. A Guide for the Preparation and 20 Use of Buffers in Biological Systems*, Gueffroy, D., Ed. Calbiochem Corporation (1975).

An “equilibration buffer" is a buffer that is used to equilibrate the cation exchange material, prior to loading the composition comprising the antibody of interest and one or more contaminants onto the cation exchange material. Preferably the pH of the equilibration buffer herein is in the range from about 5.0 to about 6.0, preferably about 25 5.5. Preferably, the conductivity of the equilibration buffer herein is in the range from about 1 to about 8mS/cm, preferably from about 4 to about 8mS/cm, and most preferably from about 5 to about 8mS/cm. Optionally, the equilibration buffer comprises a salt, such as NaCl, for example, in an amount from about 40mM to about 80mM, preferably about 60mM NaCl.

30 The term “wash buffer” is used herein to refer to the buffer that is passed over the cation exchange material following loading of a composition and prior to elution of the protein of interest. The wash buffer may serve to remove one or more contaminants from the cation exchange material, without substantial elution of the desired antibody product. According to the preferred embodiment of the invention herein a “first wash buffer” and a 35 “second wash buffer” are used.

Herein, the expression “first wash buffer” refers to a wash buffer having a pH increased relative to the pH of the load and/or equilibration buffer. The first wash buffer

may be used herein to elute one or more contaminants from the cation exchange material, without substantially eluting the antibody product of interest therefrom. The term “first” should not be interpreted as excluding the use of one or more additional wash or other buffers between the load and the first wash buffer. Preferably the pH of the first wash buffer herein is in the range from about 6.8 to about 9.0, preferably from about 7.0 to about 8.0, and most preferably pH about 7.0 or pH about 7.8. Preferably, the conductivity of the first wash buffer herein is in the range from about 0.01 to about 5mS/cm, preferably from about 0.1 to about 3mS/cm, and most preferably from about 0.2 to about 2mS/cm. Optionally, the first wash buffer is substantially free of a salt (such as NaCl) therein.

The expression “second wash buffer” for the purposes of this application refers to a wash buffer used after the first wash buffer to prepare the cation exchange material for elution of the antibody of interest. The term “second” should not be interpreted as excluding the use of one or more additional wash or other buffers between the first wash buffer and second wash buffer. Preferably the pH of the second wash buffer herein is in the range from about 5.0 to about 6.0, preferably about 5.5, and most preferably pH 5.5. Preferably, the conductivity of the second wash buffer herein is in the range from about 0.01 to about 5 mS/cm, preferably about 0.1 to about 3 mS/cm, and most preferably from about 0.5 to about 3.0mS/cm.

“Elution buffer" is used to elute the antibody of interest from the solid phase. Herein, the elution buffer has a substantially increased conductivity relative to that of the second wash buffer, such that the desired antibody product is eluted from the cation exchange material. Preferably, the conductivity of the elution buffer is substantially greater than that of the load and of each of the preceding buffers, namely of the equilibration buffer, first wash buffer, and second wash buffer. By “substantially greater” conductivity is meant, for example, that the buffer has a conductivity which is at least 2, 3, 4, 5 or 6 conductivity units (mS/cm) greater than that of the composition or buffer to which it is being compared. In one embodiment, the pH of the elution buffer is substantially the same as that of the equilibration and/or second wash buffer. Preferably the pH of the elution buffer herein is in the range from about 5.0 to about 6.0, preferably about 5.5, and most preferably pH 5.5. Preferably, the conductivity of the elution buffer herein is in the range from about 10mS/cm to about 100mS/cm, preferably from about 12mS/cm to about 30mS/cm, and most preferably from about 12 to about 20mS/cm. Increased conductivity may be achieved by the addition of a salt, such as sodium chloride,

5 sodium acetate, potassium chloride to the elution buffer. Preferably, the elution buffer comprises from about 100 to about 300mM NaCl, preferably from about 150mM to about 200mM NaCl, for example about 175mM NaCl or about 160mM NaCl.

A "regeneration buffer" may be used to regenerate the cation exchange material such that it can be re-used. The regeneration buffer has a conductivity and/or pH as 10 required to remove substantially all contaminants and the antibody of interest from the cation exchange material.

The term "conductivity" refers to the ability of an aqueous solution to conduct an electric current between two electrodes. In solution, the current flows by ion transport. Therefore, with an increasing amount of ions present in the aqueous solution, the solution 15 will have a higher conductivity. The basic unit of measure for conductivity is the Siemen (or mho), mho (mS/cm), and can be measured using a conductivity meter, such as various models of Orion conductivity meters. Since electrolytic conductivity is the capacity of ions in a solution to carry electrical current, the conductivity of a solution may be altered by changing the concentration of ions therein. For example, the concentration of a 20 buffering agent and/or the concentration of a salt (*e.g.* sodium chloride, sodium acetate, or potassium chloride) in the solution may be altered in order to achieve the desired conductivity. Preferably, the salt concentration of the various buffers is modified to achieve the desired conductivity.

By "purifying" an antibody from a composition comprising the antibody and one 25 or more contaminants is meant increasing the degree of purity of the antibody in the composition by removing (completely or partially) at least one contaminant from the composition. A "purification step" may be part of an overall purification process resulting in a "homogeneous" composition. "Homogeneous" is used herein to refer to a composition comprising at least about 70% by weight of the antibody of interest, based on 30 total weight of the composition, preferably at least about 80% by weight, more preferably at least about 90% by weight, even more preferably at least about 95% by weight.

By "binding" a molecule to a cation exchange material is meant exposing the molecule to the cation exchange material under appropriate conditions (pH and/or conductivity) such that the molecule is reversibly immobilized in or on the cation 35 exchange material by virtue of ionic interactions between the molecule and a charged group or charged groups of the cation exchange material.

5 By "washing" the cation exchange material is meant passing an appropriate buffer through or over the cation exchange material.

By "eluting" a molecule (*e.g.* antibody or contaminant) from a cation exchange material is meant to remove the molecule therefrom.

In preferred embodiments of the invention, the antibody to be purified herein is a 10 recombinant antibody. A "recombinant antibody" is one which has been produced in a host cell which has been transformed or transfected with nucleic acid encoding the antibody, or produces the antibody as a result of homologous recombination. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing nucleic acid into a cell. Following transformation or transfection, the nucleic 15 acid may integrate into the host cell genome, or may exist as an extrachromosomal element. The "host cell" includes a cell in *in vitro* cell culture as well as a cell within a host animal. Methods for recombinant production of polypeptides are described in US Patent No. 5,534,615, expressly incorporated herein by reference, for example.

A "variant" or "amino acid sequence variant" of a starting polypeptide is a 20 polypeptide that comprises an amino acid sequence different from that of the starting polypeptide. Generally, a variant will possess at least 80% sequence identity, preferably at least 90% sequence identity, more preferably at least 95% sequence identity, and most preferably at least 98% sequence identity with the native polypeptide. Percentage sequence identity is determined, for example, by the Fitch *et al.*, *Proc. Natl. Acad. Sci.* 25 *USA* 80:1382-1386 (1983), version of the algorithm described by Needleman *et al.*, *J. Mol. Biol.* 48:443-453 (1970), after aligning the sequences to provide for maximum homology. Amino acid sequence variants of a polypeptide may be prepared by introducing appropriate nucleotide changes into DNA encoding the polypeptide, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions 30 into and/or substitutions of, residues within the amino acid sequence of the polypeptide of interest. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processing of the polypeptide, such as by changing the number or position of glycosylation sites. Other post-translational 35 modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, threonyl or tyrosyl residues, methylation of the α-amino groups of lysine, arginine and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular

5 Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)). Methods for generating amino acid sequence variants of polypeptides are described in US Patent No. 5,534,615, expressly incorporated herein by reference, for example.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal 10 antibodies, multispecific antibodies (*e.g.,* bispecific antibodies), and antibody fragments so long as they exhibit the desired binding specificity.

The antibody herein is directed against an “antigen” of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that 15 mammal. However, antibodies directed against non-polypeptide antigens (such as tumor-associated glycolipid antigens; see US Patent 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (*e.g.* receptor) or ligand such as a growth factor. Exemplary antigens include those polypeptides discussed above. Preferred molecular targets for antibodies encompassed by the present invention include 20 CD polypeptides such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor (HER1), HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and av/b3 integrin including either a or b subunits thereof (*e.g.* anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 25 receptor; obesity (OB) receptor; *mpl* receptor; CTLA-4; polypeptide C etc. Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (*e.g.* the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as 30 the immunogen. Such cells can be derived from a natural source (*e.g.* cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

Examples of antibodies to be purified herein include, but are not limited to: HER2 antibodies including trastuzumab (HERCEPTIN®) (Carter *et al., Proc. Natl. Acad. Sci.* 35 *USA*, 89:4285-4289 (1992), U.S. Patent No. 5,725,856) and pertuzumab (OMNITARG™) (WO01/00245); CD20 antibodies (see below); IL-8 antibodies (St John *et al., Chest*, 103:932 (1993), and International Publication No. WO 95/23865); VEGF or VEGF

5 receptor antibodies including humanized and/or affinity matured VEGF antibodies such as the humanized VEGF antibody huA4.6.1 bevacizumab (AVASTIN®) and ranibizumab (LUCENTIS®) (Kim *et al.*, *Growth Factors*, 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published October 15, 1998); PSCA antibodies (WO01/40309); CD11a antibodies including efalizumab (RAPTIVA®) (US Patent No.
10 5,622,700, WO 98/23761, Steppe *et al., Transplant Intl.* 4:3-7 (1991), and Hourmant *et al., Transplantation* 58:377-380 (1994)); antibodies that bind IgE including omalizumab (XOLAIR®) (Presta *et al., J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181;US Patent No. 5,714,338, issued February 3, 1998 or US Patent No. 5,091,313, issued February 25, 1992, WO 93/04173 published March 4, 1993,
15 or International Application No. PCT/US98/13410 filed June 30, 1998, US Patent No. 5,714,338); CD18 antibodies (US Patent No. 5,622,700, issued April 22, 1997, or as in WO 97/26912, published July 31, 1997); Apo-2 receptor antibody antibodies (WO 98/51793 published November 19, 1998); Tissue Factor (TF) antibodies (European Patent No. 0 420 937 B1 granted November 9, 1994); α_4-α_7 integrin antibodies (WO 98/06248
20 published February 19, 1998); EGFR antibodies (*e.g.* chimerized or humanized 225 antibody, cetuximab, ERBUTIX®as in WO 96/40210 published December 19, 1996); CD3 antibodies such as OKT3 (US Patent No. 4,515,893 issued May 7, 1985); CD25 or Tac antibodies such as CHI-621 (SIMULECT®) and ZENAPAX® (See US Patent No. 5,693,762 issued December 2, 1997); CD4 antibodies such as the cM-7412 antibody
25 (Choy *et al. Arthritis Rheum* 39(1):52-56 (1996)); CD52 antibodies such as CAMPATH-1H (ILEX/Berlex) (Riechmann *et al. Nature* 332:323-337 (1988)); Fc receptor antibodies such as the M22 antibody directed against FcγRI as in Graziano *et al. J. Immunol.* 155(10):4996-5002 (1995); carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey *et al. Cancer Res.* 55(23Suppl): 5935s-5945s (1995)); antibodies directed
30 against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani *et al. Cancer Res.* 55(23): 5852s-5856s (1995); and Richman *et al. Cancer Res.* 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton *et al. Eur J. Immunol.* 26(1):1-9 (1996)); CD38 antibodies, *e.g.* AT 13/5 (Ellis *et al. J. Immunol.* 155(2):925-937 (1995)); CD33 antibodies such as Hu M195 (Jurcic *et al.*
35 *Cancer Res* 55(23 Suppl):5908s-5910s (1995)) and CMA-676 or CDP771; EpCAM antibodies such as 17-1A (PANOREX®); GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); RSV antibodies such as MEDI-493 (SYNAGIS®); CMV

5 antibodies such as PROTOVIR®; HIV antibodies such as PRO542; hepatitis antibodies such as the Hep B antibody OSTAVIR®; CA 125 antibody OvaRex; idiotypic GD3 epitope antibody BEC2; αvβ3 antibody (*e.g.* VITAXIN®; Medimmune); human renal cell carcinoman antibody such as ch-G250; ING-1; anti-human 17-1An antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoman antibody R24
10 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); human leukocyte antigen (HLA) antibody such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1); CD37 antibody such as TRU 016 (Trubion); IL-21 antibody (Zymogenetics/Novo Nordisk); anti-B cell antibody (Impheron); B cell targeting MAb (Immunogen/Aventis); 1D09C3 (Morphosys/GPC); LymphoRad 131 (HGS); Lym-1
15 antibody, such as Lym -1Y-90 (USC) or anti-Lym-1 Oncolym (USC/Peregrine); LIF 226 (Enhanced Lifesci.); BAFF antibody (*e.g.*, WO 03/33658); BAFF receptor antibody (see *e.g.*, WO 02/24909); BR3 antibody; Blys antibody such as belimumab; LYMPHOSTAT - B™; ISF 154 (UCSD/Roche/Tragen); gomilixima (Idec 152; Biogen Idec); IL-6 receptor antibody such as atlizumab (ACTEMRA™; Chugai/Roche); IL-15 antibody such as
20 HuMax-Il-15 (Genmab/Amgen); chemokine receptor antibody, such as a CCR2 antibody (*e.g.* MLN1202; Millieneum); anti-complement antibody, such as C5 antibody (*e.g.* eculizumab, 5G1.1; Alexion); oral formulation of human immunoglobulin (*e.g.* IgPO; Protein Therapeutics); IL-12 antibody such as ABT-874 (CAT/Abbott); Teneliximab (BMS-224818; BMS); CD40 antibodies, including S2C6 and humanized variants thereof
25 (WO00/75348) and TNX 100 (Chiron/Tanox); TNF-α antibodies including cA2 or infliximab (REMICADE®), CDP571, MAK-195, adalimumab (HUMIRA™), pegylated TNF-α antibody fragment such as CDP-870 (Celltech), D2E7 (Knoll), anti-TNF-α polyclonal antibody (*e.g.* PassTNF; Verigen); CD22 antibodies such as LL2 or epratuzumab (LYMPHOCIDE®; Immunomedics), including epratuzumab Y-90 and
30 epratzumab I-131, Abiogen's CD22 antibody (Abiogen, Italy), CMC 544 (Wyeth/Celltech), combotox (UT Soutwestern), BL22 (NIH), and LympoScan Tc99 (Immunomedics). Preferably, the antibody that is purified herein is a naked, intact antibody which binds to human CD20, or a naked, intact antibody which binds to human VEGF.

35 The human "CD20" antigen, or "CD20," is an about 35-kDa, non-glycosylated phosphoprotein found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs. CD20 is present on both normal B cells as well as malignant B cells,

5 but is not expressed on stem cells. Other names for CD20 in the literature include "B-lymphocyte-restricted antigen" and "Bp35". The CD20 antigen is described in Clark *et al., Proc. Natl. Acad. Sci.* (USA) 82:1766 (1985), for example.

A "CD20 antibody antagonist" herein is an antibody that, upon binding to CD20 on B cells, destroys or depletes B cells in a subject and/or interferes with one or more B-
10 cell functions, *e.g.*, by reducing or preventing a humoral response elicited by the B cell. The antibody antagonist preferably is able to deplete B cells (*i.e.*, reduce circulating B-cell levels) in a subject treated therewith. Such depletion may be achieved via various mechanisms such antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC), inhibition of B-cell proliferation and/or
15 induction of B-cell death (*e.g.*, via apoptosis).

As used herein, "B cell depletion" refers to a reduction in B cell levels in an animal or human generally after drug or antibody treatment, as compared to the level before treatment. B cell depletion can be partial or complete. B cell levels are measurable using well known techniques such as those described in Reff *et al.*, *Blood* 83: 435-445
20 (1994), or US Patent No. 5,736,137 (Anderson *et al.*). By way of example, a mammal (*e.g.* a normal primate) may be treated with various dosages of the antibody or immunoadhesin, and peripheral B-cell concentrations may be determined, *e.g.* by a FACS method that counts B cells.

Examples of CD20 antibodies include: "C2B8," which is now called "rituximab"
25 ("RITUXAN®") (US Patent No. 5,736,137); the yttrium-[90]-labelled 2B8 murine antibody designated "Y2B8" or "Ibritumomab Tiuxetan" (ZEVALIN®) commercially available from IDEC Pharmaceuticals, Inc. (US Patent No. 5,736,137; 2B8 deposited with ATCC under accession no. HB11388 on June 22, 1993); murine IgG2a "B1," also called "Tositumomab," optionally labelled with ^{131}I to generate the "131I-B1" or "iodine I131
30 tositumomab" antibody (BEXXAR™) commercially available from Corixa (see, also, US Patent No. 5,595,721); murine monoclonal antibody "1F5" (Press *et al. Blood* 69(2):584-591 (1987) and variants thereof including "framework patched" or humanized 1F5 (WO 2003/002607, Leung, S.; ATCC deposit HB-96450); murine 2H7 and chimeric 2H7 antibody (US Patent No. 5,677,180); humanized 2H7 (WO 2004/056312, Lowman *et al.*,
35 and as set forth below); 2F2 (HuMax-CD20), a fully human, high-affinity antibody targeted at the CD20 molecule in the cell membrane of B-cells (Genmab, Denmark; see, for example, Glennie and van de Winkel, *Drug Discovery Today* 8: 503-510 (2003) and

5 Cragg *et al., Blood* 101: 1045-1052 (2003); WO 2004/035607; US2004/0167319); the human monoclonal antibodies set forth in WO 2004/035607 and US2004/0167319 (Teeling *et al.*); the antibodies having complex N-glycoside-linked sugar chains bound to the Fc region described in US 2004/0093621 (Shitara *et al.*); monoclonal antibodies and antigen-binding fragments binding to CD20 (WO 2005/000901, Tedder *et al.*) such as
10 HB20-3, HB20-4, HB20-25, and MB20-11; CD20 binding molecules such as the AME series of antibodies, *e.g.*, AME 33 antibodies as set forth in WO 2004/103404 and US2005/0025764 (Watkins *et al.*, Eli Lilly/Applied Molecular Evolution, AME); CD20 binding molecules such as those described in US 2005/0025764 (Watkins *et al.*); A20 antibody or variants thereof such as chimeric or humanized A20 antibody (cA20, hA20,
15 respectively) or IMMU-106 (US 2003/0219433, Immunomedics); CD20-binding antibodies, including epitope-depleted Leu-16, 1H4, or 2B8, optionally conjugated with IL-2, as in US 2005/0069545A1 and WO 2005/16969 (Carr *et al.*); bispecific antibody that binds CD22 and CD20, for example, hLL2xhA20 (WO2005/14618, Chang *et al.*); monoclonal antibodies L27, G28-2, 93-1B3, B-C1 or NU-B2 available from the
20 International Leukocyte Typing Workshop (Valentine *et al.*, In: *Leukocyte Typing* III (McMichael, Ed., p. 440, Oxford University Press (1987)); 1H4 (Haisma *et al. Blood* 92:184 (1998)); anti-CD20 auristatin E conjugate (Seattle Genetics); anti-CD20-IL2 (EMD/Biovation/City of Hope); anti-CD20 MAb therapy (EpiCyte); anti-CD20 antibody TRU 015 (Trubion). The preferred CD20 antibodies herein are chimeric, humanized, or
25 human CD20 antibodies, more preferably rituximab, humanized 2H7, 2F2 (Hu-Max-CD20) human CD20 antibody (Genmab), and humanized A20 or IMMUN-106 antibody (Immunomedics).

For the purposes herein, the terms "rituximab," "RITUXAN®," and "C2B8" herein refer to a recombinant chimeric antibody which binds to the human CD20 antigen
30 as described in US Patent No. 5,736,137, Anderson et al. Such antibody preferably comprises a heavy chain comprising CDR H1 (SEQ ID No. 5), CDR H2 (SEQ ID No. 6), CDR H3 (SEQ ID No. 7), and a light chain, wherein the light chain preferably comprises CDR L1 (SEQ ID No. 8), CDR L2 (SEQ ID No. 9), and CDR L3 (SEQ ID No. 10); preferably the heavy chain comprises a variable heavy (VH) region comprising SEQ ID
35 No. 3 and a variable light (VL) region comprising SEQ ID No. 4; and most preferably comprises a heavy chain comprising SEQ ID No. 1 (with or without a C-terminal lysine residue), and a light chain, wherein the light chain preferably comprises SEQ ID No. 2.

The terms expressly include variant forms such as described in Moorhouse *et al. J. Pharm Biomed. Anal.* 16:593-603 (1997).

The term "human VEGF" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors.

The present invention provides anti-VEGF antagonistic antibodies which are capable of inhibiting one or more of the biological activities of VEGF, for example, its mitogenic or angiogenic activity. Antagonists of VEGF act by interfering with the binding of VEGF to a cellular receptor, by incapacitating or killing cells which have been activated by VEGF, or by interfering with vascular endothelial cell activation after VEGF binding to a cellular receptor. All such points of intervention by a VEGF antagonist shall be considered equivalent for purposes of this invention.

For the purposes herein, the terms “bevacizumab,” “AVASTIN®,” “F(ab)-12,” and “rhuMAb VEGF” herein refer to a recombinant humanized monoclonal antibody which binds human vascular endothelial growth factor (VEGF) antigen (rhuMAb VEGF) as described in US Patent No. 7,169,901, Presta et al. Such antibody preferably comprises a heavy chain comprising CDR H1 (SEQ ID No. 15), CDR H2 (SEQ ID No. 16), CDR H3 (SEQ ID No. 17), and a light chain, wherein the light chain preferably comprises CDR L1 (SEQ ID No. 18), CDR L2 (SEQ ID No. 19), and CDR L3 (SEQ ID No. 20); most preferably the heavy chain comprises a variable heavy (VH) region comprising SEQ ID No. 13 and a variable light (VL) region comprising SEQ ID No. 14; and preferably comprises a heavy chain comprising SEQ ID No. 11 (with or without a C-terminal lysine residue), and a light chain, wherein the light chain preferably comprises SEQ ID No. 12. The terms expressly include variant forms that form during production of the recombinant antibody product.

The term "monoclonal antibody" 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 a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed

5 against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with 10 the present invention may be made by the hybridoma method first described by Kohler *et al., Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). In a further embodiment, "monoclonal antibodies" can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al., Nature*, 348:552-554 (1990). Clackson *et al., Nature*, 352:624-628 15 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al., Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large 20 phage libraries (Waterhouse *et al., Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies. Alternatively, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous 25 immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits 30 *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993); and Duchosal *et al. Nature* 355:258 (1992).

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or 35 homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from

5 another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

The term “hypervariable region” when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable 10 region comprises amino acid residues from a “complementarity determining region” or “CDR” (*i.e.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Polypeptides of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from 15 a “hypervariable loop” (*i.e.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

20 "Humanized" forms of non-human (*e.g.,* murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit 25 or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized 30 antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human 35 immunoglobulin.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-

5 called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al., J. Immunol.*, 151:2296 (1993); Chothia *et al., J. Mol. Biol.*, 196:901 (1987)).

10 Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al., Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al., J. Immnol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity 15 for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer 20 programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be 25 selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

"Antibody fragments" comprise a portion of a full length antibody, generally the 30 antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, 35 Morimoto *et al.*, *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al., Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated

5 from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, Bio/Technology 10:163-167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, $F(ab')_2$ fragments can be isolated directly from 10 recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185. "Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. 15 Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding 20 sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; 25 WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression “linear antibodies” when used throughout this application refers to the antibodies described in Zapata *et al. Polypeptide Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or 30 monospecific.

“Multispecific antibodies” have binding specificities for at least two different epitopes, where the epitopes are usually from different antigens. While such molecules normally will only bind two antigens (*i.e.* bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression 35 when used herein. Examples of BsAbs include those with one arm directed against a

5 tumor cell antigen and the other arm directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-p185^{HER2}/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-p185^{HER2}, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-
10 CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell ahesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A
15 chain, anti-interferon-α(IFN-α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA);
20 BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (*e.g.* FcγRI, or FcγRIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-FcγR/anti-HIV; BsAbs for tumor detection *in vitro* or *in vivo* such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-
25 p185^{HER2}/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-HRP/anti-FITC, anti-CEA/anti-β-galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be
30 prepared as full length antibodies or antibody fragments (*e.g.* F(ab')$_2$ bispecific antibodies).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* 147: 60 (1991).

A “naked antibody” for the purposes herein is an antibody that is not conjugated to
35 a cytotoxic moiety or radiolabel.

5 An “intact antibody” herein is one which comprises two antigen binding regions, and an Fc region. Preferably, the intact antibody has a functional Fc region.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

10 A "disorder" is any condition that would benefit from treatment with the antibody purified as described herein. This includes both chronic and acute disorders and diseases and those pathological conditions which predispose the mammal to the disorder in question.

The word "label" when used herein refers to a detectable compound or
15 composition which is conjugated directly or indirectly to the antibody. The label may be itself be detectable (*e.g.,* radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or
20 prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.* At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small-molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

25

Modes for Carrying Out the Invention

The invention herein provides methods for purifying an antibody from a composition (*e.g.* an aqueous solution) comprising the antibody and one or more contaminants. The composition is generally one resulting from the recombinant
30 production of the antibody, but may be that resulting from production of the antibody by peptide synthesis (or other synthetic means) or the antibody may be purified from a native source of the antibody. Preferably the antibody binds human CD20 antigen, such as rituximab, or binds human VEGF antigen, such as bevacizumab.

Recombinant Production of Antibodies

35 For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the antibody is readily isolated and sequenced

5 using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence
10 (*e.g.* as described in US Patent 5,534,615, specifically incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryotic cells. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*,
15 *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537),
20 and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic
25 host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K . thermotolerans*, and *K. marxianus; yarrowia* (EP
30 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *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*.

Suitable host cells for the expression of glycosylated antibody are derived from
35 multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes*

5 *albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton,
10 corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines include, but are not limited to, monkey kidney CV1 cells transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney cells (293 or
15 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); 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
20 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 liver 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; FS4 cells; and human hepatoma cells (Hep G2). Often, CHO cells
25 are preferred for the expression of antibodies, and may be advantageously used to produce the antibodies purified in accordance with the present invention.

Host cells are transformed with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes
30 encoding the desired sequences.

The host cells used to produce the antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition,
35 any of the media described in Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as

5 culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as garamycin; GENTAMYCIN®), trace elements (defined as inorganic compounds
10 usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

15 When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (*e.g.* resulting from homogenization), is removed, for example, by centrifugation or ultrafiltration. Where the antibody is secreted into the medium, supernatants from such
20 expression systems may be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

The Cation Exchange Chromatography Method of the Invention

25 In the preferred embodiment of the invention, the composition to be subjected to the purification method herein is a recombinantly produced antibody, preferably an intact antibody, expressed by a Chinese Hamster Ovary (CHO) recombinant host cell culture. Optionally, the composition has been subjected to at least one purification step prior to cation exchange chromatography. The composition contains the antibody of interest and
30 one or more contaminants, such as Chinese Hamster Ovary Proteins (CHOP); leached protein A; nucleic acid; a variant, fragment, aggregate or derivative of the desired antibody; another polypeptide; endotoxin; viral contaminant; cell culture media component (*e.g.* garamycin; GENTAMYCIN®), etc.

Examples of additional purification procedures which may be performed prior to,
35 during, or following the cation exchange chromatography method include fractionation on a hydrophobic interaction chromatography (*e.g.* on PHENYL-SEPHAROSE™), ethanol precipitation, isoelectric focusing, Reverse Phase HPLC, chromatography on silica,

5 chromatography on HEPARIN SEPHAROSE^{TM}, anion exchange chromatography, further cation exchange chromatography, mixed-mode ion exchange, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, hydroxyapatite chromatography, gel electrophoresis, dialysis, hydrophic charge induction chromatography, and affinity chromatography (*e.g.* using protein A, protein G, an antibody, or a specific substrate, 10 ligand or antigen as the capture reagent).

According to the present invention, the cation exchange purification scheme typically includes the following steps performed sequentially: (1) equilibration of the cation exchange material; (2) loading the composition to be purified onto the cation exchange material, (3) a first wash step; (4) a second wash step, and (5) elution of the 15 antibody of interest.

By including at least two wash steps in the cation exchange purification scheme, at least the first of which is conducted at high pH (about pH 6.8 or greater), the efficacy of purification can be significantly improved. In particular, performing the first wash step using a wash buffer with a pH in the range from about 6.8 to about 9.0 (*e.g.* from about 20 7.0 to 8.0), such as, for example, about pH 7.8 or about pH 7.0, contaminants as described above are removed more efficiently than using the conventional lower pH range of about 5.0 to about 5.5. As a result, the host cell protein content of the composition comprising the antibody eluted from the cation exchange material is typically less than about 200 ppm, which is below the approximately 500 ppm level achieved using one wash step at a 25 pH of about 5 to 5.5.

In the preferred embodiment of the invention, the cation exchange material comprises cross-linked poly(styrene-divinylbenzene) flow-through particles (solid phase) coated with a polyhydroxylated polymer functionalized with sulfopropyl groups, for example, a POROS 50 HS® column available from Applied Biosystems.

30 Usually, an equilibration buffer is passed over or through the cation exchange material prior to loading the composition comprising the antibody of interest and one or more contaminants onto the material. In the preferred embodiment of the invention, the equilibration buffer has a pH from about 5.0 to about 6.0, for example about pH 5.5. One exemplary equilibration buffer comprises 19mM MES, 60mM NaCl, pH 5.50. Another 35 exemplary equilibration buffer comprises 23mM MES, 60mM NaCl, pH 5.50.

Following equilibration, an aqueous solution comprising the antibody of interest and one or more contaminants is loaded onto the cation exchange material. Optionally,

5 the pH of the load is in the range from about 4.0 to about 6.0, for example about pH 5.0 or about pH 5.5. In a preferred embodiment, a conditioned product pool from a prior purification step is loaded. In one embodiment, a Protein A pool from a prior Protein A chromatography purification, pH 5.0 is loaded on the cation exchange material. In another embodiment, a conditioned Q-SEPHAROSE® pool, pH 5.5 is loaded onto the 10 cation exchange material. Exemplary load densities are in the range from about 10 to about 100 g/L resin, preferably from about 10 to about 60g/L resin, most preferably from about 15 to about 45 g/L resin. The antibody of interest is bound to the cation exchange material as a result of this loading step.

After loading, the cation exchange material is washed in a first wash step with a 15 first wash buffer. During the wash process, wash buffer is passed over the cation exchange material. The composition of the wash buffer is typically chosen to elute as many contaminants as possible from the resin without eluting a substantial amount of the antibody of interest. The pH of the first wash buffer is generally higher than that of the equilibration buffer and/or of the loaded composition, for example about 2 to about 3 pH 20 units higher. Preferably the pH of the first wash buffer is in the range from about pH 6.8 to about 9.0, preferably from about pH 6.8 to about 8.0, for example about pH 7.8 or about pH 7.0. Examples of buffers which buffer in this pH range include, but are not limited to HEPES, MES, sodium acetate, TRIS/HCl, Triethanolamine hydrochloride/NaOH, Bicine/HCl, Tricine/HCl etc. The preferred first wash buffer 25 comprises or consists of: (1) 25mM HEPES, pH 7.8 or (2) 25mM MOPS, pH 7.0.

In this regard, the present invention provides a composition comprising a recombinant chimeric CD20 antibody, such as rituximab, in 25mM HEPES, pH 7.8. The invention also provides a recombinant humanized VEGF antibody, such as bevacizumab, in 25mM MOPS, pH 7.0. Such compositions are useful, among other things, as 30 intermediate compositions used in the purification of these products.

The invention herein generally entails at least one further, or a second, wash step using a second wash buffer. The pH of the second wash buffer preferably is lower than that of the first wash buffer, for example from about 2 to about 3 pH units lower. So, for example, the pH of the second wash buffer may be in the range from about pH 5.0 to 35 about pH 6.0. Preferably, the pH of the second wash buffer is about 5.5. Examples of buffers which buffer in this pH range include, but are not limited to, MES, acetic acid/sodium acetate or NaOH, NaH_2PO_3/Na_2HPO_4, Bis.Tris/HCl. MES, pH 5.5 is the

5 preferred buffer for the second wash. In one embodiment, the second wash buffer comprises or consists of: 19mM MES, 10mM NaCl, pH 5.50. In another embodiment, the second wash buffer comprises or consists of 23mM MES, 10mM NaCl, pH 5.50.

While additional wash steps may be employed, preferably only a first and second wash step are performed, prior to eluting the desired antibody. Contaminants such as 10 those discussed above are removed from the cation exchange material during the first and/or second wash step. Preferably, the first wash step removes most of the contaminants.

Following the wash step(s) noted above, the desired antibody is eluted from the cation exchange material. Elution of the antibody may be achieved by increasing the 15 conductivity or ionic strength. Desirably, the conductivity of the elution buffer is greater than about 10mS/cm. Increased conductivity may be achieved by including a relatively high salt concentration in the elution buffer. Exemplary salts for this purpose include, without limitation, sodium acetate, sodium chloride (NaCl), and potassium chloride (KCl). In one embodiment, the elution buffer comprises from about 100 to about 300mM 20 NaCl. The elution buffer generally will have approximately the same pH as the second wash buffer. A preferred elution buffer comprises: 19mM MES, 160mM NaCl, pH 5.5. Another preferred elution buffer comprises: 23mM MES, 175mM NaCl, pH 5.5. Elution preferably involves step elution (as opposed to gradient elution).

While the elution step is optionally followed by a regeneration step, such is not 25 necessary according to the preferred embodiment of the invention.

While additional steps are contemplated, preferably the cation exchange purification method herein consists of only the following steps: equilibration (*e.g.* using equilibration buffer pH about 5.5), loading a composition comprising antibody and contaminant(s) (*e.g.* where pH of the loaded composition is about 5.0 or about 5.5), first 30 wash step for eluting contaminants (*e.g.* using first wash buffer pH about 7.8 or first wash buffer pH about 7.0), second wash step (*e.g.* using second wash buffer pH about 5.5), and elution (*e.g.* using elution buffer pH about 5.5, and increased conductivity relative to each of the earlier steps for eluting antibody).

The antibody preparation obtained according to the cation exchange 35 chromatography method herein may be subjected to additional purification steps, if necessary. Exemplary further purification steps have been discussed above.

5 Optionally, the antibody is conjugated to one or more heterologous molecules as desired. The heterologous molecule may, for example, be one which increases the serum half-life of the antibody (*e.g.* polyethylene glycol, PEG), or it may be a label (*e.g.* an enzyme, fluorescent label and/or radionuclide), or a cytotoxic molecule (*e.g.* a toxin, chemotherapeutic drug, or radioactive isotope etc).

10 A therapeutic formulation comprising the antibody, optionally conjugated with a heterologous molecule, may be prepared by mixing the antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. "Pharmaceutically acceptable" carriers, 15 excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as 20 methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including 25 glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.,* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The active ingredients may also be entrapped in microcapsule prepared, for 30 example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 35 16th edition, Osol, A. Ed. (1980).

The formulation to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

5 Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody variant, which matrices are in the form of shaped articles, *e.g.,* films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), 10 polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

15 The antibody purified as disclosed herein or the composition comprising the antibody and a pharmaceutically acceptable carrier is then used for various diagnostic, therapeutic or other uses known for such antibodies and compositions. For example, the antibody may be used to treat a disorder in a mammal by administering a therapeutically effective amount of the antibody to the mammal. In the case of a CD20 antibody such as 20 rituximab it can be used to deplete B-cells, treat lymphoma (for example Non-Hodgkin's Lymphoma, NHL), or leukemia (for example Chronic Lymphocytic Leukemia, CLL) as well as autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), lupus etc. For an antibody that binds to VEGF, such as bevacizumab, it can be used to inhibit angiogenesis, treat cancer, and treat macular degeneration, etc.

25 The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLE 1: PURIFICATION OF A CD20 ANTIBODY

30

This example describes an improved cation exchange chromatography process for purifying a CD20 antibody, rituximab. Rituximab is used for therapy of NHL, CLL, RA, MS, etc. The structure of the Rituximab molecule is disclosed in 5,736,137, Anderson *et al.,* (expressly incorporated herein by reference) as well as Figs. 1A-1B herein. Rituximab 35 is commercially available from Genentech, Inc.

Cation-exchange chromatography is used to further reduce the levels of CHOP, DNA, leached protein A, garamycin (GENTAMYCIN®), Rituximab aggregates, and

5 potential viruses. Rituximab binds to the column under the load conditions. The column is then washed, eluted, regenerated/sanitized, and stored until the next use. Multiple cycles may be used to process an entire batch of affinity pool. The cation-exchange pool may be held at room temperature up to 30○C for up to 3 days or at 5○C for up to 7 days.

The cation-exchange resin (POROS 50 HS®, Applied Biosystems) is packed in a
10 column to a bed height of 17-33 cm. Before the affinity pool is loaded, the cation-exchange column is purged of storage solution with equilibration buffer. After equilibration, the affinity pool is loaded onto the column. The product binds to the column under these conditions. The column is then washed with wash 1 buffer, followed by wash 2 buffer. Rituximab is eluted from the column using a high-ionic-strength
15 elution buffer.

A comparison of the conditions for the process of the present invention compared to the original (control) process is provided in the following table.

Table 1: Comparison of Buffers for Rituximab Cation Exchange Chromatography Processes

Phase	Buffer composition (original process)	Buffer composition (exemplified process)
Pre-equilibration	20mM MES, 500mM NaCl, pH 5.50	None
Equilibration	20mM MES, 60mM NaCl, pH 5.50	19mM MES, 60mM NaCl, pH 5.50
Load	Conditioned Protein A pool, pH 5.00, Load density ≤ 50g/L resin	Conditioned Protein A pool, pH 5.00, Load density ≤ 50g/L resin
Wash 1	20mM MES, 60mM NaCl, pH 5.50	25mM HEPES, pH 7.80
Wash 2	None	19mM MES, 10mM NaCl, pH 5.50
Elution	20mM MES, 160mM NaCl,	19mM MES, 160mM NaCl,

	pH 5.50	pH 5.50
Regeneration	20mM MES, 500mM NaCl, pH 5.50	None
Sanitization	0.5 N NaOH	0.5 N NaOH
Storage	0.1 N NaOH	0.1 N NaOH

5

The desired pH, conductivity and molarity ranges for the load and buffers in the rituximab process are provided in the following table.

Table 2: Preferred pH, Conductivity and Molarity Ranges for Rituximab Process

Buffer	**Buffer Composition**	**Target pH**	**Preferred Buffer Molarity Range**	**Preferred Buffer pH Range**	**Allowable Conductivity Range for Buffers**
Equilibration	19mM MES, 60mM NaCl	5.5	14 – 23mM MES 50 – 70 mM NaCl	5.0 – 6.0	5.0 – 7.2 mS/cm
Load	Conditioned Protein A Pool	5.0	NA	4.5 – 5.5	2.5 – 5.5 mS/cm
Wash 1	25mM HEPES	7.8	15-35 mM HEPES	7.5 – 8.1	0.5 – 1.5 mS/cm
Wash 2	19mM MES 10mM NaCl	5.5	14 – 23mM MES 5 – 15 mM NaCl	5.0 – 6.0	0.6 – 2.2 mS/cm
Elution	19 mM MES 160 mM NaCl	5.5	14 – 23 mM MES 140 -180 mM NaCl	5.3 – 5.7	13.4 – 17.2 mS/cm
Sanitization	0.5N NaOH	NA	NA	NA	NA
Storage	0.1N NaOH	NA	NA	NA	NA

10 * Conductivity values measured with temperature compensation based on a temperature of 20○C and an alpha value of 1.77.

5 The exemplified process for Rituximab purification enhanced the robustness of host cell protein removal by enabling higher removal of host cell proteins in the wash phases, resulting in lower levels of host cell proteins in the product pool (elution pool) and facilitating the removal of the impurities in the subsequent downstream step. Fig. 3 illustrates the advantages of the present process in terms of host cell proteins removal.

10

EXAMPLE 2: PURIFICATION OF A VEGF ANTIBODY

This example describes a cation exchange chromatography process for purifying a recombinant humanized vascular endothelial growth factor antibody (rhuMAb VEGF), bevacizumab. The structure of the bevacizumab molecule is disclosed in US Patent 7,169,901, Presta *et al.,* expressly incorporated herein by reference. See also Figs. 2A-2B
15 herein. Bevacizumab is commercially available from Genentech, Inc.

This example summarizes the development studies performed on the cation exchange step for an improved bevacizumab purification process. Three cation exchange resins were evaluated in these studies: CM SEPHAROSE FAST FLOW®, SP SEPHAROSE FAST FLOW® and POROS 50HS®. The cation exchange purification
20 processes using these three resins were evaluated with respect to: process performance (impurities removal, retrovirus removal, and step yield), product quality, process robustness and process fit at all current manufacturing sites. Based on the data generated in these studies, POROS 50HS® showed superior process performance and robustness and was selected as the cation exchange resin for the improved purification process.
25 Cation exchange chromatography is the final chromatography step in the purification process. It serves to remove cell culture media components (garamycin), host cell derived impurities (CHOP, and DNA) and aggregated forms of bevacizumab. It also functions as a viral removal step.

The column is operated in a bind-and-elute mode and is performed at ambient
30 temperature. The column uses a cation exchange resin (POROS 50HS®). The resin consists of a porous, polystyrene-divinylbenzene bed support coupled with a negatively charged functional group. The column is removed from storage by washing with equilibration buffer. The viral filtered pool will be diluted with 0.3 volumes of water for injection (WFI) to meet the conductivity limit of ≤ 5.5 mS/cm. The viral filtered pool is
35 then loaded onto the equilibrated column. The product binds to the resin. After loading, the column is washed with a high pH buffer to flush the load material through the column

5 and remove CHOP impurities. The column is then washed with a low salt buffer to lower the pH and prepare the column for elution. Product is eluted using a step elution of high salt buffer with a maximum of 7 column volumes. After elution, the column and skid are sanitized with sanitization solution (0.5 N NaOH) prior to storage in storage solution (0.1 N NaOH) until its next use.

10 The following table provides a description of the conditions for the bevacizumab process of the invention herein.

Table 3 - Bevacizumab Process

Phase	Buffer / Solution	Process Parameter	Flow rate (cm/hr)
Bed Height (cm)	N/A	30	N/A
Equilibration	23mM MES / 60mM NaCl pH 5.5 , cond. 6.9 mS/cm	4 CV	100
Load	Conditioned Viral Filtered (VF) Pool pH 5.5 ± 0.2, cond. ≤ 5.5 mS/cm	15 – 45 g bevacizumab / L of resin	100
Wash 1	25mM MOPS, pH 7.0	3 CV	100
Wash 2	23mM MES / 10mM NaCl pH 5.5, cond.1.5 mS/cm	3 CV	100
Elution	23mM MES / 175 mM NaCl pH 5.5, cond. 18 mS/cm	7 CV	100
	Start Pooling	$OD_{280} \geq 0.5$	N/A
	End Pooling	$OD_{280} \leq 1.0$	N/A
Sanitization	0.5N NaOH	3 - 6 CV	50 - 100
Storage	0.1N NaOH	3 – 6 CV	50 - 100

15

The desired pH, conductivity and molarity ranges for the load and buffers in the bevacizumab process are provided in the following table.

5 **Table 4: Preferred pH, Conductivity and Molarity Ranges for Bevacizumab Process**

	Target buffer	**Target pH range**	**Target conductivity range**	**Preferred buffer molarity range**	**Preferred buffer pH range**
Equil.	23mM MES, 60mM NaCl	5.4 -5.60	6.1 - 7.7mS/cm	13-33mM MES 50-70mM NaCl	5.1-5.9
Load	VF pool diluted with water for injection (WFI)	5.3 -5.7	≤ 5.5 mS/cm	≤ 6.5mS/cm	5.2-5.8
Wash 1	25mM MOPS	6.9-7.1	0.2-1.2 mS/cm	15-35mM MOPs	6.6-7.4
Wash 2	23mM MES 10mM NaCl	5.4-5.6	1.2-1.8 mS/cm	13-33mM MES 5-20mM NaCl	5.1-5.9
Elution	23mM MES 175mM NaCl	5.45-5.55	17.5-18.5 mS/cm	13-33mM MES 160-190mM NaCl	5.4-5.6
Sanitization	0.5N NaOH	NA	50-60mS/cm (1:1 diluted)	0.5N NaCl	NA
Storage	0.1N NaOH	NA	17-27mS/cm	0.1N NaOH	NA

The present process was found to be superior to the original bevacizumab process
10 which used a first wash buffer pH 5.5. The new process herein was able to achieve pools

5 with lower CHOP levels, it achieved a higher step yield and was an overall more robust process to run in manufacturing.

5 WHAT IS CLAIMED IS:

1. A method for purifying an antibody from a composition comprising the antibody and at least one contaminant, which method comprises the sequential steps of:

(a) loading the composition onto a cation exchange material wherein the
10 composition is at a first pH;

(b) washing the cation exchange material with a first wash buffer at a pH which is greater than that of the composition in (a), wherein the pH of the first wash buffer is from about 6.8 to about 9.0;

(c) washing the cation exchange material with a second wash buffer at a pH which
15 is less than that of the first wash buffer; and

(d) eluting the antibody from the cation exchange material with an elution buffer at a conductivity which is substantially greater than that of the second wash buffer.

20 2. The method of claim 1 wherein the pH of the second wash buffer and the pH of the elution buffer are approximately the same.

3. The method of claim 1 wherein the antibody binds to human CD20.

25 4. The method of claim 1 wherein the antibody binds to human vascular endothelial growth factor (VEGF).

5. The method of claim 1 wherein the pH of the composition in (a) is from about 4.0 to about 6.0, the pH of the first wash buffer is from about 6.8 to about 8.0, the
30 pH of the second wash buffer is from about 5.0 to about 6.0, and the pH of the elution buffer is from about 5.0 to about 6.0.

6. The method of claim 1 wherein the conductivity of the elution buffer is from about 10mS/cm to about 100mS/cm.

35

7. The method of claim 1 wherein the elution buffer comprises about 100 to about 300mM NaCl.

5

8. The method of claim 1 wherein the cation exchange material comprises cross-linked poly(styrene-divinylbenzene) flow-through particles coated with a polyhydroxylated polymer functionalized with sulfopropyl groups.

10 9. The method of claim 1 wherein the contaminant is selected from the group consisting of Chinese Hamster Ovary Proteins (CHOP), leached protein A, DNA, aggregated antibody, cell culture media component, garamycin, and viral contaminant.

10. The method of claim 1 further comprising subjecting the composition
15 comprising the antibody to one or more further purification steps either before, during, or after steps (a) through (d) so as to obtain a homogeneous preparation of the antibody.

11. The method of claim 10 further comprising conjugating the purified antibody with a heterologous molecule.

20

12. The method of claim 10 or 11 further comprising preparing a pharmaceutical composition by combining the homogeneous preparation of the antibody or conjugated antibody with a pharmaceutically acceptable carrier.

25 13. A method for purifying an antibody that binds human CD20 from a composition comprising the antibody and one or more contaminants selected from the group consisting of Chinese Hamster Ovary Proteins (CHOP), leached protein A, DNA, and aggregated CD20 antibody, which method comprises the sequential steps of:

(a) loading the composition onto a cation exchange material wherein the
30 composition is at a pH from about 4.0 to about 6.0;

(b) washing the cation exchange material with a first wash buffer at a pH from about 6.8 to about 9.0;

(c) washing the cation exchange material with a second wash buffer at a pH from about 5.0 to about 6.0; and

35 (d) eluting the antibody from the cation exchange material using an elution buffer with a pH from about 5.0 to about 6.0 and a conductivity from about 10mS/cm to about 100mS/cm.

5

14. The method of claim 13 wherein the antibody is rituximab.

15. The method of claim 13 wherein the elution buffer comprises about 100 to about 300mM NaCl.

10

16. A method for purifying an antibody that binds human vascular endothelial growth factor (VEGF) from a composition comprising the antibody and one or more contaminants selected from the group consisting of a cell culture media component, garamycin, Chinese Hamster Ovary Proteins (CHOP), DNA, viral contaminant, and
15 aggregated VEGF antibody, which method comprises the sequential steps of:

(a) loading the composition onto a cation exchange material wherein the composition is at a pH from about 4.0 to about 6.0;

(b) washing the cation exchange material with a first wash buffer at a pH from about 6.8 to about 8.0;

20 (c) washing the cation exchange material with a second wash buffer at a pH from about 5.0 to about 6.0; and

(d) eluting the antibody from the cation exchange material using an elution buffer with a pH from about 5.0 to about 6.0 and a conductivity from about 10mS/cm to about 100mS/cm.

25

17. The method of claim 16 wherein the antibody is bevacizumab.

18. The method of claim 16 wherein the elution buffer comprises about 100 to about 300mM NaCl.

30

19. A composition comprising rituximab in a buffer comprising about 25mM HEPES, at a pH of about 7.8.

20. A composition comprising bevacizumab in a buffer comprising about 25mM
35 MOPS at a pH of about 7.0.

Fig. 1A – Rituximab Heavy Chain

```
       +1     FR1                       10                   15
       Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Pro Gly Ala
Ser

    20                  25                  30  31    CDR1    35
36
 Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asn Met His
Trp

       40  FR2              45               49  50       52  52A  53
54
 Val Lys Gln Thr Pro Gly Arg Gly Leu Glu Trp Ile Gly Ala Ile Tyr Pro Gly
Asn

 55         CDR2     60                  65  66  FR3        70
 Gly Asp Thr Ser Tyr Asn Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp
Lys

     75                  80      82  82A 82B 82C 83      85
 Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala
Val

 90                 94  95      CDR3        100 100A 100B 100C 100D 101 102  103
 Tyr Tyr Cys Ala Arg Ser Thr Tyr Tyr Gly Gly Asp Trp Tyr Phe Asn Val Trp
Gly

 105 FR4            110         113 114                      120
 Ala Gly Thr Thr Val Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val Phe
Pro
```

Human Gamma 1 Constant

```
130 133                         140
 Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu
Val

                     150             154 156 157 162
 Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser

 169 171                                 180 182
 Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser

 190                                     200 203 205                 210
 Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn

                                 220     222 225                 230 232 235
 His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Ala Glu Pro Lys Ser Cys Asp Lys

                     240         243 244                     250
 Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
```

```
 260                                     270
 Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr
```

Human Gamma 1 Constant - continued

```
     280                                     290     292
 Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val

 295 296 299 300                                     310             314 317
 Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser

         320                                     330
 Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys

             340                                     350                 355
 Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile

 357         360|361 363                             370
 Ser Lys Ala Lys|Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg

     378 381                                 390
 Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
Pro

         400     402 405         408 410 413                         420
 Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
Thr

                         428 430 433                         440
 Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
Val

                     450                                         460
 Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu
Ala

                             470                             478   Amino
Acid # (Kabat)
 Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys TER
```

Fig. 1B - Rituximab Light Chain

```
                     | +1     FR1                           10
                     |Gln Ile Val Leu Ser Gln Ser Pro Ala Ile Leu Ser Ala Ser

                     20         23 | 24     CDR1     27/ 29  30            34
Pro Gly Glu Lys Val Thr Met Thr Cys |Arg Ala Ser Ser Ser Val Ser Tyr Ile His|

 35     FR2         40                    45                   49 | 50     CDR2
Trp Phe Gln Gln Lys Pro Gly Ser Ser Pro Lys Pro Trp Ile Tyr|Ala Thr Ser Asn

    55   56 | 57        60       FR3        65                      70
Leu Ala Ser|Gly Val Pro Val Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser

        75                    80                    85             88 |  89  90
Leu Thr Ile Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys|Gln Gln Trp

      CDR3  95      97 |98     100  FR4           105      107 |108     110
Thr Ser Asn Pro Pro Thr|Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys|Arg Thr Val

                                            120
Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
```

Human Kappa Constant

```
130                                       140
Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp

    150                                       160
Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp

        170                                       180
Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr

            190                                       200
Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val

                210             214             Amino Acid # (kabat)
Thr Lys Ser Phe Asn Arg Gly Glu Cys TER
```

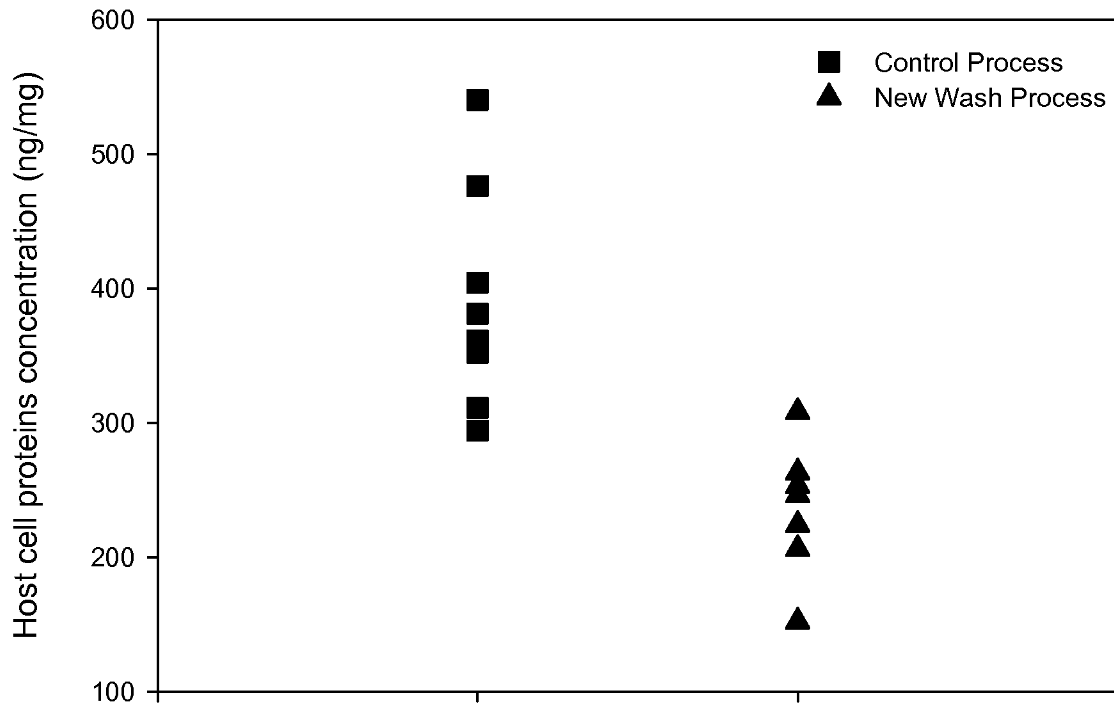
Fig. 2A - Bevacizumab Heavy Chain

EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGK
GLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLR
AEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS‖ASTKGP
SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS
VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV
YTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT
QKSLSLSPGK

Fig. 2B - Bevacizumab Light Chain

DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPK
VLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYS
TVPWTFGQGTKVEIKR‖TVAAPSVFIFPPSDEQLKSGTASVVCLLN
NFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLS
KADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG. 3 – Host Cell Proteins Removal for Rituximab Processes



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/081516

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/06 C07K1/18 C07K16/28 C07K16/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A X	WO 02/096457 A (NOVARTIS-ERFINDUNGEN VERWALTUNGSGESELLSCHAFT M.B.H.) 5 December 2002 (2002-12-05) page 24, paragraph 2	1-18 19,20
A X	WO 2004/001007 A (IDEC PHARMACEUTICALS CORPORATION) 31 December 2003 (2003-12-31) claims 1,5,6	1-18 19,20
A X	WO 2005/066139 A (MILLENNIUM PHARMACEUTICALS, INC.) 21 July 2005 (2005-07-21) paragraph [0609]	1-18 19,20

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 April 2009	14/04/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Nooij, Frans

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/081516

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6 339 142 B1 (BASEY ET AL.) 15 January 2002 (2002-01-15) cited in the application column 17, line 36 - line 43 column 17, line 47 - line 52 column 17, line 57 - line 61 column 17, line 62 - column 18, line 2 column 18, line 10 - line 13 column 18, line 17 - line 27 column 6, lines 38,44 figure 1	1-18
A	WO 99/57134 A (GENENTECH, INC.) 11 November 1999 (1999-11-11) example claims figures 1,2	1-18
A	WO 2004/024866 A (GENENTECH, INC.) 25 March 2004 (2004-03-25) examples	1-18
X	WO 2005/115453 A (GENENTECH INC.) 8 December 2005 (2005-12-08) example 7	19,20

International application No.
PCT/US2008/081516

INTERNATIONAL SEARCH REPORT

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-18

   A method for purifying an antibody from a composition comprising the antibody and at least contaminant, which method comprises the sequential steps of: (a) loading the composition onto a cation exchange material wherein the composition is at a first pH; (b) washing the cation exchange material with a first wash buffer at a pH which is greater than that of the composition in (a), wherein the pH of the first wash buffer is from about 6.8 to about 9.0; (c) washing the cation exchange material with a second wash buffer at a pH which is less than that of the first wash buffer; and (d) eluting the antibody from the cation exchange material with an elution buffer at a conductivity which is substantially greater than that of the second wash buffer.

   ---

2. claim: 19

   A composition comprising rituximab in a buffer comprising about 25 mM HEPES, at a pH of about 7.8.

   ---

3. claim: 20

   A composition comprising bevacizumab in a buffer comprising about 25 mM MOPS at a pH of about 7.0.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2008/081516

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 02096457	A	05-12-2002	BR	0209777	A	01-06-2004
			CA	2448345	A1	05-12-2002
			CN	1537015	A	13-10-2004
			EP	1397159	A2	17-03-2004
			JP	2004532262	T	21-10-2004
			US	2006127395	A1	15-06-2006
			US	2004170623	A1	02-09-2004
WO 2004001007	A	31-12-2003	AU	2003251592	A1	06-01-2004
			CA	2490423	A1	31-12-2003
			CN	1671741	A	21-09-2005
			EP	1551875	A2	13-07-2005
			JP	2005530845	T	13-10-2005
			NZ	537687	A	30-04-2008
WO 2005066139	A	21-07-2005	AR	047185	A1	11-01-2006
			AU	2005204060	A1	21-07-2005
			CA	2559888	A1	21-07-2005
			EP	1701944	A2	20-09-2006
			JP	2007517895	T	05-07-2007
			US	2006040968	A1	23-02-2006
US 6339142	B1	15-01-2002	US	2003078388	A1	24-04-2003
			US	6489447	B1	03-12-2002
			US	6417335	B1	09-07-2002
WO 9957134	A	11-11-1999	AT	321066	T	15-04-2006
			AT	370961	T	15-09-2007
			AU	760048	B2	08-05-2003
			AU	3877799	A	23-11-1999
			BR	9910332	A	25-09-2001
			CA	2329829	A1	11-11-1999
			CN	1299370	A	13-06-2001
			CN	1626547	A	15-06-2005
			CN	1626548	A	15-06-2005
			CN	1810292	A	02-08-2006
			CN	101333244	A	31-12-2008
			DE	69907439	D1	05-06-2003
			DE	69907439	T2	01-04-2004
			DE	69930424	T2	14-12-2006
			DE	69936946	T2	15-05-2008
			DK	1308456	T3	27-12-2007
			DK	1075488	T3	18-08-2003
			EP	1075488	A1	14-02-2001
			ES	2261589	T3	16-11-2006
			ES	2292682	T3	16-03-2008
			ES	2198913	T3	01-02-2004
			HK	1055307	A1	16-11-2007
			HK	1055308	A1	18-08-2006
			JP	2002513800	T	14-05-2002
			KR	20060081422	A	12-07-2006
			NZ	507557	A	31-10-2003
			PT	1308455	E	31-08-2006
			ZA	200005879	A	22-10-2001
WO 2004024866	A	25-03-2004	AU	2003265994	A1	30-04-2004
			CA	2496060	A1	25-03-2004
			EP	1543038	A2	22-06-2005

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2008/081516

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004024866 A		JP 2005538176 T	15-12-2005
WO 2005115453 A	08-12-2005	AR 049021 A1	21-06-2006
		AU 2005247303 A1	08-12-2005
		BR PI0509412 A	04-09-2007
		CA 2562243 A1	08-12-2005
		EP 1742660 A2	17-01-2007
		JP 2007532680 T	15-11-2007