Figure 9
CROSS REFERENCE TO RELATED APPLICATIONS

This non-provisional application filed under 37 CFR §1.53(b), claims the benefit under 35 USC §119(e) of U.S. Provisional Application Serial No. 61/485,249 filed on May 2011, which is incorporated by reference in entirety.

FIELD OF THE INVENTION

The present invention relates to methods of detecting and determining the amount of a human or humanized antibody of interest from an animal sample such as tissue, plasma or serum. The methods include affinity enrichment and protease digestion of the sample to produce one or more peptides unique and conserved to the framework region of a human or humanized antibody detected and quantified by mass spectrometry.

BACKGROUND

The analysis of plasma/serum samples generated from in vivo studies of therapeutic proteins is of interest in the biopharmaceutical industry. The conventional ELISA approach has been used for over 25 years and has several limitations. The ELISA require high quality custom reagents that can take several months to generate and the assay optimization can take an additional number of months. Thus, ELISA has a long assay development time which is a limitation in both the early discovery stage and the development stage of protein-based drugs (Murray et al (2001) J. Imm. Methods 255:41-56; Kirchner et al (2004) Clin. Pharmacokinetics 43(2):83-95). Suitable ELISA reagents and assay conditions may not be possible in some cases due to the highly custom binding requirements for each protein therapeutic. Another limitation of ELISA is that reagents may bind non-specifically with plasma/serum proteins; matrix interference is a common phenomenon. Protein quantification by mass spectrometry on the other hand is highly specific and therefore matrix interference is rare compared to ELISA. Development of ELISA assays can be labor-intensive and require complex, specific reagents. ELISA is also sensitive to matrix interferences and cross-reactivity of antibodies. ELISA measures analyte concentration indirectly using binding
properties. These many variables make ELISA methods of protein quantification challenging to develop and transfer to other laboratories with robust performance. On the basis of these differences, mass spectrometry is an orthogonal method to ELISA. Mass spectrometry methods of protein quantification, LC-MS/MS in particular, do not require custom reagents and generally yields faster assay development. In addition, Mass spectrometry is less subject to matrix interferences and provides generic assay conditions which are highly specific and can be multiplexed and automated. The high specificity of mass spectrometry measures analyte concentration using intrinsic physical chemical properties of the analyte, i.e. mass and fragmentation pattern. The robust format allows ready lab-to-lab transfer, a significant advantage for approved antibody therapies. A general methodology for quantifying proteins by mass spectrometry is trypsin digestion of the intact protein. The resulting peptides are analyzed by mass spectrometry by introducing corresponding stable isotope labeled internal standards at a fixed concentration.


Liquid chromatography-tandem mass spectrometry is a powerful tool for protein analysis and quantitation in very complex matrices like plasma/serum samples. Since peptides resulting from the digestion of the protein of interest and other plasma/serum proteins may have the same or similar nominal mass, the second dimension of MS fragmentation often provides a unique fragment of a peptide of interest. The combination of the specific parent peptide and the unique fragment ion is used to selectively monitor for the molecule to be quantified. Such approach is termed "Multiple reaction monitoring" (MRM), also referred to as Selected Reaction Monitoring (SRM), which is a commonly used mode for protein quantitation.

Electrospray ionization (ESI) provides for the atmospheric pressure ionization (API) of a liquid sample. The electrospray process creates highly-charged droplets that, under evaporation, create ions representative of the species contained in the solution. An ion-
sampling orifice of a mass spectrometer may be used to sample these gas phase ions for mass analysis. The response for an analyte measured by the mass spectrometer detector is dependent on the concentration of the analyte in the fluid and independent of the fluid flow rate.

5 SUMMARY

The invention provides a method of detecting human or humanized antibodies comprising the steps of:

(a) treating a biological sample with a digestive enzyme to form a digested antibody sample, wherein the biological sample is serum, plasma, tissue, or cells from an animal that has been treated with a human or humanized antibody; and

(b) analyzing the digested antibody sample by mass spectrometry to detect one or more human framework peptides.

In an exemplary embodiment, human framework peptides comprise one or more sequences selected from SEQ ID NOS. 1-8.

In an exemplary embodiment, the digestive enzyme is trypsin.

In an exemplary embodiment, the biological sample is contacted with an affinity capture media or chromatography adsorbent. An enriched biological sample is eluted then treated with the digestive enzyme.

In an exemplary embodiment, the concentration of digested antibody sample is measured.

An aspect of the invention are methods of protease digestion of the sample or immunoaffinity capture followed by protease digestion to produce one or more peptides unique to the framework region of a human or humanized antibody, i.e., not present in animal biological samples, detected and quantified by mass spectrometry (LC-MS/MS).

An embodiment of the invention is human or humanized antibodies conjugated to drug moieties where antibody-drug conjugates are measured by the methods of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the alignment of heavy chain amino acid sequences of a human 2H7 antibody ocrelizumab, Hu2H7 (SEQ ID NO:1) starting at residue 101, and five cynomolgus monkey anti-CD20 antibodies: CynoHC 1a D3 1 (SEQ ID NO: 12), CynoHC 1b E5 1 (SEQ ID NO: 13), Cyno HC 2a (SEQ ID NO: 14), CynoHC 2b E6 1 (SEQ ID NO: 15), CynoHC 3 (SEQ ID NO: 16). Framework signature peptides are identified (FSP 1-8) as underlined
which are unique to human 2H7 (hu 2H7) Mab and are not present in cynomolgus monkey IgG heavy chain, each bearing at least one amino acid difference in the sequences.

Figure 2 shows the heavy chain (SEQ ID NO: 17) and light chain (SEQ ID NO: 18) of trastuzumab (Herceptin®, Genentech Inc.; rhuMAbHER2, Anti p85HER2), a recombinant derived humanized monoclonal antibody, CAS Registry No. 180288-69-1.

Figure 3 shows the heavy chain (SEQ ID NO: 11) and light chain (SEQ ID NO: 19) of ocrelizumab, rhuMAb 2H7, PRO70769, a humanized anti-CD20 antibody, CAS Registry No. 637334-45-3.

Figure 4 shows the heavy chain (SEQ ID NO:20) and light chain (SEQ ID NO:21) of pertuzumab, rhuMAb 2C4, CAS Registry No. 380610-27-5. FSP2, FSP3, FSP8 are identified as underlined in heavy chain (SEQ ID NO:20).

Figure 5 shows the heavy chain (SEQ ID NO:22) and light chain (SEQ ID NO:23) of anti-PDL1, member of the extended CD28/CTLA-4 family of T cell regulators. FSP2, FSP4, FSP8 are identified as underlined in heavy chain (SEQ ID NO:22).

Figure 6 shows the heavy chain (SEQ ID NO:24) and light chain (SEQ ID NO:25) of anti-neuropilin-1, anti-NRPI, MRNP1685A. FSP2, FSP4, FSP8 are identified as underlined in heavy chain (SEQ ID NO:24).

Figure 7 shows the heavy chain (SEQ ID NO:26) and light chain (SEQ ID NO:27) of anti-MUC16, MMUC3333A/DMUC4064A. FSP2, FSP4, FSP8 are identified as underlined in heavy chain (SEQ ID NO:26).

Figure 8 shows the (SEQ ID NO:28) and light chain (SEQ ID NO:29) of rituximab, C2B8, MabThera, (Rituxan®, Genentech Inc., Biogen/Idec), CAS Registry No. 174722-31-7. FSP2, FSP4, FSP8 are identified as underlined in heavy chain (SEQ ID NO:28).

Figure 9 shows the generic steps for LC-MS/MS method to quantify a therapeutic antibody in animal plasma/serum using one or more framework signature peptides (FSP).

Figure 10 shows multiple reaction monitoring of trastuzumab digested by trypsin. Framework signature peptides FSP3 (12.5 mins), FSP8 (15 mins) and FSP5 (17 mins) are baseline separated and quantitated.

Figure 11 shows the MS/MS spectrum of FSP5 from affinity-captured then digested anti-MUC16 antibody-drug conjugate that had been spiked into plasma.

Figure 12 shows a calibration curve of FSP8 spiked at various concentrations from 1-1000 µg/mL into lithium Heparinised Cynomolgus monkey plasma prepared by the whole plasma digest/SPE approach.
Figure 13 shows LC-MS/MS chromatograms demonstrating the detection of FSP8 spiked into lithium heparinised cynomolgus monkey plasma at LLOQ = 1 µg/mL after whole plasma digest/SPE sample preparation.

Figure 14 shows a flow chart of steps for the LC-MS/MS method with affinity capture of the protein therapeutic and enzymatic digestion to generate Framework Signature Peptides (FSP) of mAb in animal plasma/serum.

Figure 15 shows a cartoon of capture of mAb from animal plasma/serum on streptavidin coated magnetic beads bound to a biotinylated capture probe or Protein A, G coated magnetic bead, followed by isolation by magnetic separation, digestion of the captured antibody and analysis by LC-MS/MS.

Figure 16 shows embodiments of Protein A, G coated magnetic bead (top) for generic capture of antibody and streptavidin coated magnetic beads bound to a biotinylated capture probe (bottom) for specific capture of antibody.

Figure 17a shows LC-MS/MS separation and detection of FSP8 at 1 µg/mL of trastuzumab antibody in rat plasma.

Figure 17b shows LC-MS/MS separation and detection of stable-isotope labelled (SIL) FSP8 internal standard.

Figure 18 shows the linearity of detection, plotting the ratio of FSP8 to stable-isotope labelled FSP8 internal standard versus concentration of trastuzumab (HERCEPTIN®) from 1-250 µg/mL in rat plasma.

Figure 19a shows a cartoon of the monoclonal antibody (mAb therapeutics) captured by binding to an immobilized extra-cellular domain (ECD) or anti-human IgG polyclonal antibody and detected with an anti-human IgG polyclonal antibody labeled with horse radish peroxidase (HRP) in an ELISA assay with electrochemiluminescent or colorimetric detection.

Figure 19b shows elements of the LC-MS/MS assay beginning with Protein A bead capture of an mAb therapeutic from a biological sample, trypsin digestion of the captured mAb therapeutic to form one or more framework signature peptides (FSP), e.g. FSP8, and LC/MS/MS detection of multiple reaction monitoring (MRM) to detect the transition of 938.0 (M, 2+) to 836.7 (y15, 2+).

Figure 20 shows the concordance between the LC-MS/MS assay shown in Figure 19b and the ELISA assay of Figure 19a based on individual pharmacokinetics (PK) of plasma/serum samples from rats dosed with trastuzumab, an anti-HER2 mAb.
Figure 21 shows the concordance between the LC-MS/MS assay shown in Figure 19b and the ELISA assay of Figure 19a based on individual pharmacokinetics (PK) of plasma/serum samples from rats dosed with 3A5, an anti-MUC 16 mAb.

Figure 22 shows the concordance between the LC-MS/MS assay shown in Figure 19b and the ELISA assay of Figure 19a based on individual pharmacokinetics (PK) of plasma/serum samples from rats dosed with an anti-mesothelin (Msln) mAb.

Figure 23 shows the concordance between the LC-MS/MS assay shown in Figure 19b and the ELISA assay of Figure 19a based on mean pharmacokinetics (PK) of plasma/serum samples from cynomolagus monkey dosed with 3A5 (MMUC1206A), an anti-MUC 16 mAb by measurement of antibody in the plasma over 28 days.

Figure 24 shows the concordance between the LC-MS/MS assay shown in Figure 19b and the ELISA assay of Figure 19a based on mean pharmacokinetics (PK) of plasma/serum samples from cynomolagus monkey dosed with an anti-mesothelin (Msln) mAb by measurement of antibody in the plasma over 40 days.

Figure 25 shows the concordance between the LC-MS/MS assay shown in Figure 19b and the ELISA assay of Figure 19a based on individual pharmacokinetics (PK) of plasma/serum samples from mice (A, B, C) dosed with an antibody-drug conjugate (ADC), anti-LY6E-MC-vc-PAB-MMAE, in mouse efficacy studies.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs, and are consistent with: Singleton et al, (1994) "Dictionary of Microbiology and Molecular Biology", 2nd Ed., J. Wiley & Sons, New York, NY; and Janeway, et al (2001) "Immunobiology", 5th Ed., Garland Publishing, New York. When trade names are used herein, the trade name product formulation, the generic drug, and the active pharmaceutical ingredient(s) of the trade name product are also included.

DEFINITIONS

The term "biological sample" is any component derived or separated from an animal and includes blood, plasma, serum, cells, urine, cerebrospinal fluid (CSF), milk, bronchial lavage, bone marrow, amniotic fluid, saliva, bile, vitreous, tears, or tissue.

The term "digestive enzyme" is an enzyme capable of cleaving or hydrolyzing peptides or proteins into fragments in either a specific or generic, random manner. A
digestive enzyme can form a digested antibody sample from an antibody where the antibody is a component of a biological sample. Digestive enzymes include proteases such as trypsin, papain, endoproteinase LysC, endoproteinase ArgC, staph aureus V8, chymotrypsin, Asp-N, Asn-C, pepsin, and endoproteinase GluC.

The term "antibody" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity.

An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')_2, diabodies; linear antibodies; single-chain antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments.

In certain embodiments, an antibody provided herein is an antibody fragment.

Antibody fragments include, but are not limited to, Fab, Fab', Fab'-SH, F(ab')_2, Fv, and scFv fragments, and other fragments described below. For a review of certain antibody fragments, see Hudson et al. Nat. Med. 9:129-134 (2003). For a review of scFv fragments, see, e.g., Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds., (Springer-Verlag, New York), pp. 269-315 (1994); WO 93/16185; US 5571894; US 5587458. For discussion of Fab and F(ab')_2 fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see US 5869046.


Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (US 6248516).

Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (e.g. E. coli or phage), as described herein.
The term "chimeric" antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc regions and variant Fc regions. In one embodiment, a human IgG heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

"Framework" or "FR" refers to constant domain residues other than hypervariable region (HVR) residues. The FR of a constant domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

The terms "full length antibody," "intact antibody," and "whole antibody" are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc region as defined herein.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

A "human consensus framework" is a framework region of an antibody which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat et al. Sequences of Proteins of Immunological Interest, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al., supra. In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al, supra.

A "humanized" antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain
embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A "humanized form" of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization.

A "chimeric" antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region (US 4816567; Morrison et al. (1984) Proc. Natl. Acad. Sci. USA, 81:685 1-6855). In a further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

In certain embodiments, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which HVRs, e.g., CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the HVR residues are derived), e.g., to restore or improve antibody specificity or affinity.


Human framework regions that may be used for humanization include but are not limited to: framework regions selected using the "best-fit" method (see, e.g., Sims et al. J. Immunol. 151:2296 (1993)); framework regions derived from the consensus sequence of


Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic mice, the endogenous immunoglobulin loci have generally been inactivated. For review of methods for obtaining human antibodies from transgenic animals, see Lonberg, Nat. Biotech. 23:1117-1125 (2005). See also, e.g., U.S. Patent Nos. 6,075, 181 and 6,150,584 describing XENOMOUSE™ technology; US 5770429 describing HUMAB® technology; US 704 1870 describing K-M MOUSE® technology, and US 2007/006 1900, describing VELOCIMOUSE® technology). Human variable regions from intact antibodies generated by such animals may be further modified, e.g., by combining with a different human constant region.

Human antibodies may also be generated by isolating Fv clone variable domain sequences selected from human-derived phage display libraries. Such variable domain sequences may then be combined with a desired human constant domain. Techniques for selecting human antibodies from antibody libraries are described below.


In certain phage display methods, repertoires of VH and VL genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter et al., *Ann. Rev. Immunol.* 12: 433-455 (1994). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (e.g., from human) to provide a single source of antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths et al., *EMBO J* 12: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom and Winter, *J. Mol. Biol.* 227: 381-388 (1992). Human antibody phage libraries are described in US 5750373; US 2005/0079574; US 2005/01 19455; US 2005/0266000; US 2007/01 17126; US 2007/0160598; US 2007/0237764; US 2007/0292936; US 2009/0002360. Antibodies or antibody fragments isolated from human antibody libraries are considered human antibodies or human antibody fragments herein.
In certain embodiments, an antibody is a multispecific antibody, e.g. a bispecific antibody. Multispecific antibodies are monoclonal antibodies that have binding specificities for at least two different sites. In certain embodiments, one of the binding specificities is for one antigen and the other is for a second antigen. In certain embodiments, bispecific antibodies may bind to two different epitopes of the same antigen. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express an antigen. Bispecific antibodies can be prepared as full length antibodies or antibody fragments.


Engineered antibodies with three or more functional antigen binding sites, including "Octopus antibodies," are also included herein (see, e.g. US 2006/0025576A1).

The antibody or fragment herein also includes a "Dual Acting FAb" or "DAF" comprising an antigen binding site that binds to an antigen as well as another, different antigen (see, US 2008/0069820, for example).

**Antibody Variants**

In certain embodiments, amino acid sequence variants of the antibodies provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of an antibody may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding.
Antibodies include fusion proteins comprising an antibody and a protein, drug moiety, label, or some other group. Fusion proteins may be made by recombinant techniques, conjugation, or peptide synthesis, to optimize properties such as pharmacokinetics. The human or humanized antibody of the invention may also be a fusion protein comprising an albumin-binding peptide (ABP) sequence (Dennis et al. (2002) "Albumin Binding As A General Strategy For Improving The Pharmacokinetics Of Proteins" J Biol Chem. 277:35035-35043; WO 01/45746). Antibodies of the invention include fusion proteins with ABP sequences taught by: (i) Dennis et al (2002) J Biol Chem. 277:35035-35043 at Tables III and IV, page 35038; (ii) US 2004/0001827 at [0076]; and (iii) WO 01/45746 at pages 12-13, and all of which are incorporated herein by reference.

Substitution, Insertion, and Deletion Variants

In certain embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Conservative substitutions are shown in Table 1 under the heading of "conservative substitutions." More substantial changes are provided in Table 1 under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired activity, e.g., retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

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Amino acids may be grouped according to common side-chain properties:

1. hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
2. neutral hydrophilic: Cys, Ser, Thr, Asn, Gin;
3. acidic: Asp, Glu;
4. basic: His, Lys, Arg;
5. residues that influence chain orientation: Gly, Pro;
6. aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g., a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (e.g., improvements) in certain biological properties (e.g., increased affinity, reduced immunogenicity) relative to the parent antibody and/or will have substantially retained certain biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, e.g., using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant antibodies displayed on phage and screened for a particular biological activity (e.g., binding affinity).

Alterations (e.g., substitutions) maybe made in HVRs, e.g., to improve antibody affinity. Such alterations may be made in HVR "hotspots," i.e., residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (see, e.g.,
Chowdhury, Methods Mol. Biol. 207:179-196 (2008)), and/or SDRs (a-CDRs), with the resulting variant VH or VL being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, e.g., in Hoogenboom et al. in Methods in Molecular Biology 178:1-37 (O'Brien et al, ed., Human Press, Totowa, NJ, (2001).) In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (e.g., 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, e.g., using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind antigen. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may be outside of HVR "hotspots" or SDRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

A useful method for identification of residues or regions of an antibody that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) Science, 244:1081-1085. In this method, a residue or group of target residues (e.g., charged residues such as arg, asp, his, lys, and glu) are identified and replaced by a neutral or negatively charged amino acid (e.g., alanine or polyalanine) to determine whether the interaction of the antibody with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of
terminal insertions include an antibody with an N-terminal methionyl residue. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

**Glycosylation variants**

In certain embodiments, an antibody provided herein is altered to increase or decrease the extent to which the antibody is glycosylated. Addition or deletion of glycosylation sites to an antibody may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region (Wright et al. (1997) TIBTECH 15:26-32). The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the invention may be made in order to create antibody variants with certain improved properties.

In one embodiment, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antibody may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e. g. complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc region (Eu numbering of Fc region residues); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function (US 2003/0157108; US 2004/0093621). Examples of publications related to "defucosylated" or "fucose-deficient" antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/010704; US 2004/010282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; WO2002/031 140;

Antibodies variants are further provided with bisected oligosaccharides, e.g., in which a biantennary oligosaccharide attached to the Fc region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, e.g., in WO 2003/01 1878 (Jean-Mairet et al); US Patent No. 6,602,684 (Umana et al); and US 2005/0123546 (Umana et al). Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described (WO 1997/30087; WO 1998/58964; WO 1999/22764).

Fc region variants

In certain embodiments, one or more amino acid modifications may be introduced into the Fc region of an antibody provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence {e.g., a human IgG1, IgG2, IgG3 or IgG4 Fc region} comprising an amino acid modification {e.g. a substitution} at one or more amino acid positions.

In certain embodiments, the invention contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half life of the antibody in vivo is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. In vitro and/or in vivo cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol. 9:457-492 (1991). Non-limiting examples of in vitro assays to assess ADCC activity of a molecule of interest is described in US 5500362; Hellstrom, I. et al. (1986) Proc. Nat'l Acad. Sci. USA 83:7059-

Antibodies with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (US 6737056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US 7332581).

Certain antibody variants with improved or diminished binding to FcRs are described. (US 6737056; WO 2004/056312; Shields et al. (2001) J. Biol. Chem. 9(2): 6591-6604).

In certain embodiments, an antibody variant comprises an Fc region with one or more amino acid substitutions which improve ADCC, e.g., substitutions at positions 298, 333, and/or 334 of the Fc region (EU numbering of residues).

In some embodiments, alterations are made in the Fc region that result in altered (i.e., either improved or diminished) Clq binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in US Patent No. 6,194,551, WO 99/51642, and Iduesogic et al. (2000) J. Immunol. 164: 4178-4184.

Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al, J. Immunol. 24:249 (1994)), are described in US2005/0014934A1 (Hinton et al.). Those antibodies comprise an Fc region with one or
more substitutions therein which improve binding of the Fc region to FcRn. Such Fc variants include those with substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, e.g., substitution of Fc region residue 434 (US Patent No. 7,371,826). See also Duncan & Winter, Nature 322:738-40 (1988); US 5648260; US 5624821; and WO 94/29351 concerning other examples of Fc region variants.

Cysteine engineered antibody variants

In certain embodiments, it may be desirable to create cysteine engineered antibodies, e.g., "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an antibody-drug conjugate (ADC), also referred to as an immunoconjugate, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region. Cysteine engineered antibodies may be generated as described, e.g., in US 7521541.

Antibody Derivatives

In certain embodiments, an antibody provided herein may be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/proplylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof.

Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer is attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations.
including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

In another embodiment, conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the nonproteinaceous moiety is a carbon nanotube (Kam et al. (2005) Proc. Natl. Acad. Sci. USA 102: 11600-1 1605). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the nonproteinaceous moiety to a temperature at which cells proximal to the antibody-nonproteinaceous moiety are killed.

The term "hypervariable region" or "HVR," as used herein, refers to each of the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops ("hypervariable loops"). Generally, native four-chain antibodies comprise six HVRs; three in the VH (HI, H2, H3), and three in the VL (LI, L2, L3). HVRs generally comprise amino acid residues from the hypervariable loops and/or from the "complementarity determining regions" (CDRs), the latter being of highest sequence variability and/or involved in antigen recognition. Exemplary hypervariable loops occur at amino acid residues 26-32 (LI), 50-52 (L2), 91-96 (L3), 26-32 (HI), 53-55 (H2), and 96-101 (H3) (Chothia and Lesk, (1987) J. Mol. Biol. 196:901-917). Exemplary CDRs (CDR-L1, CDR-L2, CDR-L3, CDR-HI, CDR-H2, and CDR-H3) occur at amino acid residues 24-34 of LI, 50-56 of L2, 89-97 of L3, 31-35B of HI, 50-65 of H2, and 95-102 of H3 (Kabat et al, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991). With the exception of CDR1 in VH, CDRs generally comprise the amino acid residues that form the hypervariable loops. CDRs also comprise "specificity determining residues," or "SDRs," which are residues that contact antigen. SDRs are contained within regions of the CDRs called abbreviated-CDRs, or a-CDRs. Exemplary a-CDRs (a-CDR-L1, a-CDR-L2, a-CDR-L3, a-CDR-HI, a-CDR-H2, and a-CDR-H3) occur at amino acid residues 31-34 of LI, 50-55 of L2, 89-96 of L3, 31-35B of HI, 50-58 of H2, and 95-102 of H3 (Almagro and Fransson, (2008) Front. Biosci. 13:1619-1633). Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al, supra.

An "isolated" antibody is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse

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 and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, 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 the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

A "naked antibody" refers to an antibody that is not conjugated to a heterologous moiety (e.g., a cytotoxic moiety) or radiolabel. The naked antibody may be present in a pharmaceutical formulation.

"Native antibodies" refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning
the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNAStar) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU5 10087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y, where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). See for example, Kindt et al. Kuby
Immunology, 6th ed., W.H. Freeman and Co., page 91 (2007). A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively (Portolano et al. (1993) J. Immunol. 150:880-887; Clarkson et al. (1991) Nature 352:624-628).

"Tumor-associated antigens" (TAA) are known in the art, and can prepared for use in generating human or humanized antibodies using methods and information which are well known in the art. In attempts to discover effective cellular targets for cancer diagnosis and therapy, researchers have sought to identify transmembrane or otherwise tumor-associated polypeptides that are specifically expressed on the surface of one or more particular type(s) of cancer cell as compared to on one or more normal non-cancerous cell(s). Often, such tumor-associated polypeptides are more abundantly expressed on the surface of the cancer cells as compared to on the surface of the non-cancerous cells. The identification of such tumor-associated cell surface antigen polypeptides has given rise to the ability to specifically target cancer cells for destruction via antibody-based therapies.

Examples of TAA include, but are not limited to, TAA (l)-(36) listed below. For convenience, information relating to these antigens, all of which are known in the art, is listed below and includes names, alternative names, Genbank accession numbers and primary reference(s), following nucleic acid and protein sequence identification conventions of the National Center for Biotechnology Information (NCBI). Nucleic acid and protein sequences corresponding to TAA (l)-(36) are available in public databases such as GenBank. Tumor-associated antigens targeted by antibodies include all amino acid sequence variants and isoforms possessing at least about 70%, 80%, 85%, 90%, or 95% sequence identity relative to the sequences identified in the cited references, or which exhibit substantially the same biological properties or characteristics as a TAA having a sequence found in the cited references. For example, a TAA having a variant sequence generally is able to bind specifically to an antibody that binds specifically to the TAA with the corresponding sequence listed. The sequences and disclosure in the reference specifically recited herein are expressly incorporated by reference.

TUMOR-ASSOCIATED ANTIGENS (l)-(36):

(Page 38-39); WO2002/102235 (Claim 13; Page 296); WO2003/055443 (Page 91-92); WO2002/99122 (Example 2; Page 528-530); WO2003/029421 (Claim 6); WO2003/024392 (Claim 2; Fig 112); WO2002/98358 (Claim 1; Page 183); WO2002/54940 (Page 100-101); WO2002/59377 (Page 349-350); WO2002/30268 (Claim 27; Page 376); WO2001/48204 (Example; Fig 4); NP_001 194 bone morphogenetic protein receptor, type IB /pid=NP_001 194.1. Cross-references: MIM:603248; NP_001 194.1; AY065994


WO2004/032842 (Example IV); WO2003/042661 (Claim 12); WO2003/016475 (Claim 1);

WO2002/78524 (Example 2); WO2002/99074 (Claim 19; Page 127-129); WO2002/86443 (Claim 27; Pages 222, 393); WO2003/003906 (Claim 10; Page 293); WO2002/64798 (Claim 33; Page 93-95); WO2000/14228 (Claim 5; Page 133-136); US2003/224454 (Fig 3);

WO2003/025138 (Claim 12; Page 150); NP_003477 solute carrier family 7 (cationic amino acid transporter, y+system), member 5 /pid=NP_003477.3 - Homo sapiens; Cross-references: MIM:600182; NP_003477.3; NM_015923; NM_003486_1


EP1394274 (Example 11); WO2004/016225 (Claim 2); WO2003/042661 (Claim 12);

US2003/157089 (Example 5); US2003/185830 (Example 5); US2003/064397 (Fig 2);

WO2002/89747 (Example 5; Page 618-619); WO2003/022995 (Example 9; Fig 13A,

Example 53; Page 173, Example 2; Fig 2A); NP_036581 six transmembrane epithelial antigen of the prostate; Cross-references: MIM:604415; NP_036581.1; NM_012449_1

(4) 0772P (CA125, MUC16, Genbank accession no. AF361486); J. Biol. Chem. 276 (29):27371-27375 (2001)); WO2004/045553 (Claim 14); WO2002/92836 (Claim 6; Fig 12);

WO2002/83866 (Claim 15; Page 116-121); US2003/124140 (Example 16); Cross-references: GL34501467; AAK74120.3; AF361486_1

(5) MPF (MPF, MSLN, SMR, megakaryocyte potentiating factor, mesothelin,


WO2003/101283 (Claim 14); (WO2002/ 102235 (Claim 13; Page 287-288); WO2002/101075
(Claim 4; Page 308-309); WO2002/71928 (Page 320-321); WO94/10312 (Page 52-57); Cross-references: MIM:601051; NP_005814.2; NM_005823_1


(7) Sema 5b (FLJ10372, KIAA1445, Mm.42015, SEMA5B, SEMAG, Semaphorin 5b Hlog, sema domain, seven thrombospondin repeats (type I and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B, Genbank accession no. AB040878); Nagase T., et al (2000) DNA Res. 7 (2): 143-150; WO2004/000997 (Claim 1); WO2003/003984 (Claim 1); WO2002/06339 (Claim 1; Page 50); WO2001/88133 (Claim 1; Page 41-43, 48-58); WO2003/054152 (Claim 20); WO2003/101400 (Claim 11); Accession: Q9P283; EMBL; AB040878; BAA95969.1. Genew; HGNC: 10737

(8) PSCA hlg (2700050C12Rik, C530008O16Rik, RIKEN cDNA 2700050C12, RIKEN cDNA 2700050C 12 gene, Genbank accession no. AY358628); Ross et al (2002) Cancer Res. 62:2546-2553; US2003/129192 (Claim 2); US2004/044180 (Claim 12); US2004/044179 (Claim 11); US2003/096961 (Claim 11); US2003/232056 (Example 5); WO2003/105758 (Claim 12); US2003/206918 (Example 5); EP1347046 (Claim 1); WO2003/205148 (Claim 20); Cross-references: GL37182378; AAQ88991.1; AY358628J


(10) MSG783 (RNF124, hypothetical protein FLJ20315, Genbank accession no. NM_017763); WO2003/104275 (Claim 1); WO2004/046342 (Example 2); WO2003/042661 (Claim 12); WO2003/083074 (Claim 14; Page 61); WO2003/018621 (Claim 1); WO2003/024392 (Claim 2; Fig 93); WO2001/66689 (Example 6); Cross-references: LocusID:54894; NP_060233.2; NM_017763_1

(11) STEAP2 (HGNC_8639, IPCA-1, PCANAP1, STAMP1, STEAP2, STMP, prostate cancer associated gene 1, prostate cancer associated protein 1, six transmembrane epithelial antigen of prostate 2, six transmembrane prostate protein, Genbank accession no. AF455138); Lab. Invest. 82 (11): 1573-1582 (2002)); WO2003/087306; US2003/064397 (Claim 1; Fig 1); WO2002/72596 (Claim 13; Page 54-55); WO2001/72962 (Claim 1; Fig 4B); WO2003/104270 (Claim 11); WO2003/104270 (Claim 16); US2004/005598 (Claim 22); WO2003/042661 (Claim 12); US2003/060612 (Claim 12; Fig 10); WO2002/26822 (Claim 23; Fig 2); WO2002/16429 (Claim 12; Fig 10); Cross-references: GL22655488; AAN04080.1; AF455138J


(Example 2); WO2004/027049 (Fig II); WO2004/009622; WO2003/081210; WO2003/089904 (Claim 9); WO2003/016475 (Claim 1); US2003/I 18592; WO2003/008537 (Claim 1); WO2003/055439 (Claim 29; Fig 1A-B); WO2003/025228 (Claim 37; Fig 5C); WO2002/22636 (Example 13; Page 95-107); WO2002/12341 (Claim 68; Fig 7); WO2002/13847 (Page 71-74); WO2002/14503 (Page 114-117); WO2001/53463 (Claim 2; Page 41-46); WO2001/41787 (Page 15); WO2000/44899 (Claim 52; Fig 7); WO2000/20579 (Claim 3; Fig 2); US5869445 (Claim 3; Col 31-38);WO9630514 (Claim 2; Page 56-61); EP1439393 (Claim 7); WO2004/043361 (Claim 7); WO2004/022709; WO2001/00244 (Example 3; Fig 4); Accession: P04626; EMBL; M11767; AAA35808.1. EMBL; M11761; AAA35808.1


(19) MDP (DPEP1, Genbank accession no. BC017023); Proc. Natl. Acad. Sci. U.S.A. 99 (26):16899-16903 (2002)); WO2003/016475 (Claim 1); WO2002/64798 (Claim 33; Page 85-87); JP05003790 (Fig 6-8); W099/46284 (Fig 9); Cross-references: MIM: 179780; AAH17023.1; BC017023 _1


Fig 52); US2003/1 19126 (Claim 1); US2003/1 19121 (Claim 1; Fig 52); US2003/1 19129 (Claim 1); US2003/1 19130 (Claim 1); US2003/1 19128 (Claim 1; Fig 52); US2003/1 19125 (Claim 1); WO2003/0 16475 (Claim 1); WO2002/02634 (Claim 1)

(22) EphB2R (DRT, ERK, Hek5, EPHT3, Tyro5, Genbank accession no.

NM_004442); Chan, J. and Watt, V.M., Oncogene 6 (6), 1057-1061 (1991) Oncogene 10 (5): 897-905 (1995), Annu. Rev. Neurosci. 21:309-345 (1998), Int. Rev. Cytol. 196:177-244 (2000)); WO2003042661 (Claim 12); WO200053216 (Claim 1; Page 41); WO2004065576 (Claim 1); WO2004020583 (Claim 9); WO2003004529 (Page 128-132); WO200053216 (Claim 1; Page 42); Cross-references: MIM:600997; NP_004433.2; NM_004442_1

(23) ASLG659 (B7h, Genbank accession no. AX092328); US2004/0101899 (Claim 2); WO2003 104399 (Claim 11); WO2004000221 (Fig 3); US2003/165504 (Claim 1); US2003/124140 (Example 2); US2003/065143 (Fig 60); WO2002/102235 (Claim 13; Page 299); US2003/091580 (Example 2); WO2002/10187 (Claim 6; Fig 10); WO2001/94641 (Claim 12; Fig 7b); WO2002/02624 (Claim 13; Fig 1A-1B); US2002/034749 (Claim 54; Page 45-46); WO2002/06317 (Example 2; Page 320-321, Claim 34; Page 321-322); WO2002/71928 (Page 468-469); WO2002/02587 (Example 1; Fig 1); WO2001/40269 (Example 3; Pages 190-192); WO2000/36107 (Example 2; Page 205-207); WO2004/053079 (Claim 12); WO2003/004989 (Claim 1); WO2002/71928 (Page 233-234, 452-453); WO 01/16318

(24) PSCA (Prostate stem cell antigen precursor, Genbank accession no. AJ297436); Reiter R.E., et al. Proc. Natl. Acad. Sci. U.S.A. 95, 1735-1740, 1998; Gu Z., et al. Oncogene 19, 1288-1296, 2000; Biochem. Biophys. Res. Commun. (2000) 275(3):783-788; WO2004/022709; EP1394274 (Example 11); US2004/018553 (Claim 17); WO2003/008537 (Claim 1); WO2002/81646 (Claim 1; Page 164); WO2003/003906 (Claim 10; Page 288); WO2001/40309 (Example 1; Fig 17); US2001/055751 (Example 1; Fig 1b); WO2000/32752 (Claim 18; Fig 1); WO98/51805 (Claim 17; Page 97); WO98/51824 (Claim 10; Page 94); WO98/40403 (Claim 2; Fig IB); Accession: 043653; EMBL; AF043498; AAC39607.1

(25) GEDA (Genbank accession No. AY260763); AAP 14954 lipoma HMGIC fusion-partner-like protein /pid=AAP14954.1 - Homo sapiens (human); WO2003/054152 (Claim 20); WO2003/000842 (Claim 1); WO2003/023013 (Example 3, Claim 20); US2003/194704 (Claim 45); Cross-references: GL30102449; AAP14954.1; AY260763_1

(26) BAFF-R (B cell -activating factor receptor, BLyS receptor 3, BR3, Genbank accession No. AF1 16456); BAFF receptor /pid=NP_443 177.1 - Homo sapiens: Thompson, J.S., et al Science 293 (5537), 2108-211 1 (2001); WO2004/058309; WO2004/01 161 1;
WO2003/045422 (Example; Page 32-33); WO2003/014294 (Claim 35; Fig 6B); WO2003/035846 (Claim 70; Page 615-616); WO2002/94852 (Col 136-137); WO2002/38766 (Claim 3; Page 133); WO2002/24909 (Example 3; Fig 3); Cross-references: MIM:606269; NP_443177.1; NM_052945.1; AF132600

(27) CD22 (B-cell receptor CD22-B isoform, BL-CAM, Lyb-8, Lyb8, SIGLEC-2, FLJ22814, Genbank accession No. AK026467); Wilson et al (1991) J. Exp. Med. 173:137-146; WO2003/072036 (Claim 1; Fig 1); Cross-references: MIM:107266; NP_001774_1; NM_001771_1


(29) CXCR5 (Burkitt's lymphoma receptor 1, a G protein-coupled receptor that is activated by the CXCL13 chemokine, functions in lymphocyte migration and humoral defense, plays a role in HIV-2 infection and perhaps development of AIDS, lymphoma, myeloma, and leukemia); 372 aa, pi: 8.54 MW: 41959 TM: 7 [P] Gene Chromosome: 11q23.3, Genbank accession No. NP_001707.1; WO2004/040000; WO2004/015426; US2003/105292 (Example 2); US6555339 (Example 2); WO2002/61087 (Fig 1); WO2001/57188 (Claim 20, page 269); WO2001/72830 (pages 12-13); WO2000/22129 (Example 1, pages 152-153, Example 2, pages 254-256); W099/28468 (claim 1, page 38); US5440021 (Example 2, col 49-52); W094/28931 (pages 56-58); W092/17407 (claim 7, Fig 5); Dobner et al (1992) Eur. J. Immunol. 22:2795-2799; Barella et al (1995) Biochem. J. 309:773-779


(34) FcRHI (Fc receptor- like protein 1, a putative receptor for the immunoglobulin Fc domain that contains C2 type Ig-like and ITAM domains, may have a role in B-lymphocyte differentiation); 429 aa, pi: 5.28, MW: 46925 TM: 1 [P] Gene Chromosome: lq21-lq22, Genbank accession No. NP_443170.1); WO2003/077836; WO2001/38490 (claim 6, Fig 18E-1-18-E-2); Davis et al (2001) Proc. Natl. Acad. Sci USA 98(17):9772-9777; WO2003/089624 (claim 8); EP1347046 (claim 1); WO2003/089624 (claim 7)

(35) IRTA2 (FcPvH5, Immunoglobulin superfamily receptor translocation associated 2, a putative immunoreceptor with possible roles in B cell development and lymphomagenesis; deregulation of the gene by translocation occurs in some B cell malignancies); 977 aa, pi: 6.88, MW: 106468, TM: 1 [P] Gene Chromosome: lq21, Genbank

Recombinant Methods and Compositions

Antibodies may be produced using recombinant methods and compositions, e.g., as described in US 4816567. In one embodiment, isolated nucleic acid encoding an antibody described herein is provided. Such nucleic acid may encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the antibody (e.g., the light and/or heavy chains of the antibody). In a further embodiment, one or more vectors (e.g., expression vectors) comprising such nucleic acid are provided. In a further embodiment, a host cell comprising such nucleic acid is provided. In one such embodiment, a host cell comprises (e.g., has been transformed with): (1) a vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and an amino acid sequence comprising the VH of the antibody, or (2) a first vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and a second vector comprising a nucleic acid that encodes an amino acid sequence comprising the VH of the antibody. In one embodiment, the host cell is eukaryotic, e.g., a Chinese Hamster Ovary (CHO) cell or lymphoid cell (e.g., Y0, NS0, Sp20 cell). In one embodiment, a method of making an antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the antibody, as provided above, under conditions...
suitable for expression of the antibody, and optionally recovering the antibody from the host cell (or host cell culture medium).

For recombinant production of an antibody, nucleic acid encoding an antibody, e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such nucleic acid may be readily isolated and sequenced 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).

Suitable host cells for cloning or expression of antibody-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, antibodies may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed. For expression of antibody fragments and polypeptides in bacteria, see, e.g., US 5648237; US 5789199; US 5840523; Charlton, Methods in Molecular Biology, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 245-254, describing expression of antibody fragments in E. coli.) After expression, the antibody may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized," resulting in the production of an antibody with a partially or fully human glycosylation pattern (Gerngross, (2004) Nat. Biotech. 22:1409-1414; Li et al. (2006) Nat. Biotech. 24:210-215).

Suitable host cells for the expression of glycosylated antibody are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of Spodoptera frugiperda cells.

Plant cell cultures can also be utilized as hosts (US 5959177; US 6040498; US 6420548; US 7125978; US 6417429, describing PLANTIBODIES™ technology for producing antibodies in transgenic plants).

Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CVI line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293 cells as described, e.g., in Graham et al. (1977, J. Gen Virol. 36:59); baby hamster kidney cells (BHK); mouse Sertoli cells (TM4 cells as described, e.g., in Mather, (1980) Biol. Reprod. 23:243-251); monkey kidney cells (CVI); African green monkey kidney
cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK; buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells, as described, e.g., in Mather et al. (1982) Annals N.Y. Acad. Sci. 383:44-68; MRC 5 cells; and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including DHFR CHO cells (Urlaub et al. (1980) Proc. Natl. Acad. Sci. USA 77:4216); and myeloma cell lines such as Y0, NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, e.g., Yazaki and Wu, Methods in Molecular Biology, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ), pp. 255-268 (2003).

Assays

Antibodies may be identified, screened for, or characterized for their physical/chemical properties and/or biological activities by various assays known in the art. In one aspect, an antibody of the invention is tested for its antigen binding activity, e.g., by known methods such as ELISA, Western blot, etc. In another aspect, competition assays may be used to identify an antibody that competes with another known antibody for binding to antigen. In certain embodiments, such a competing antibody binds to the same epitope (e.g., a linear or a conformational epitope) that is bound by the known antibody. Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris (1996) "Epitope Mapping Protocols," in Methods in Molecular Biology, Vol. 66 (Humana Press, Totowa, NJ).

In an exemplary competition assay, immobilized antigen is incubated in a solution comprising a first labeled antibody that binds to antigen (e.g., HER2 or CD20) and a second unlabeled antibody that is being tested for its ability to compete with the first antibody for binding to antigen. The second antibody may be present in a hybridoma supernatant. As a control, immobilized antigen is incubated in a solution comprising the first labeled antibody but not the second unlabeled antibody. After incubation under conditions permissive for binding of the first antibody to antigen, excess unbound antibody is removed, and the amount of label associated with immobilized antigen is measured. If the amount of label associated with immobilized antigen is substantially reduced in the test sample relative to the control sample, then that indicates that the second antibody is competing with the first antibody for binding to antigen. See Harlow and Lane (1988) Antibodies: A Laboratory Manual ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
In one aspect, assays are provided for identifying antibodies thereof having biological activity. Biological activity may include, e.g., tumor inhibition.

In certain embodiments, antibodies of the methods of the invention are useful for detecting the presence of an antigen in a biological sample. The term "detecting" as used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological sample comprises blood, plasma, serum, cells, urine, vitreous, tears, or tissue. In certain embodiments, the method comprises contacting the biological sample with an antibody as described herein under conditions permissive for binding of the antibody to the antigen, and detecting whether a complex is formed between the antibody and antigen. Such method may be an *in vitro* or *in vivo* method. In one embodiment, an antibody is used to select subjects eligible for therapy with an antibody, e.g., where the expressed antigen protein is a biomarker for selection of patients. Exemplary disorders that may be diagnosed using an antibody of the invention include cancer and immune disorders.

Labeled and conjugated antibodies are utilized in certain embodiments of the methods of the invention. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes $^{32}$P, $^{14}$C, $^{125}$I, $^{3}$H, and $^{131}$I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (US 4737456), luciferin, 2,3-dihydropthalalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Drug moieties which effect cell-killing may be covalently attached to antibodies through a linker unit to form antibody-drug conjugates for targeted cell-killing therapeutic effects. An exemplary embodiment of an antibody-drug conjugate (ADC) compound comprises an antibody (Ab) which targets a tumor cell, and cytotoxic or cytostatic drug moiety (D), and a linker moiety (L) that attaches Ab to D. The antibody is attached through the one or more amino acid residues, such as lysine and cysteine, by the linker moiety (L) to D; the composition having Formula I:

\[
\text{I}
\]
Ab-(L-D)p

where p is 1 to about 20, or from about 2 to about 5. The number of drug moieties which may be conjugated via a reactive linker moiety to an antibody molecule may be limited by the number of free cysteine residues, which are introduced by the methods described herein.

The drug moiety (D) of an antibody-drug conjugate (ADC) includes any compound, moiety or group that has a cytotoxic or cytostatic effect. Drug moieties may impart their cytotoxic and cytostatic effects by mechanisms including but not limited to tubulin binding, DNA binding or intercalation, and inhibition of RNA polymerase, protein synthesis, and topoisomerase. Some cytotoxic drugs tend to be inactive or less active when conjugated to large antibodies or protein receptor ligands. Exemplary drug moieties include, but are not limited to, a maytansinoid, dolastatin, auristatin, calicheamicin, pyrrolobenzodiazepine (PBD), PNU-159682, anthracycline, duocarmycin, vinca alkaloid, taxane, trichothecene, CC1065, duocarmycin, camptothecin, elinafide, and stereoisomers, isosteres, analogs or derivatives thereof, and including the derivatives of these drugs that have cytotoxic activity.

Antibody-drug conjugates (ADC) are targeted anti-cancer therapeutics designed to reduce nonspecific toxicities and increase efficacy relative to conventional small molecule and antibody cancer chemotherapy. They employ the powerful targeting ability of monoclonal antibodies to specifically deliver highly potent, conjugated small molecule therapeutics to a cancer cell. To evaluate properties such as pharmacokinetics and toxicity of these antibody-drug conjugates, it is useful to be able to characterize and quantitate them from plasma, urine, and other biological samples. Methods to detect and screen antibody-drug conjugates by Immunoaffinity membrane (IAM) capture and mass spectrometry have been disclosed (US 2005/0232929), including bead-based affinity capture methods (US 2009/0286258).

METHODS OF MEASURING HUMAN AND HUMANIZED ANTIBODIES IN BIOLOGICAL SAMPLES

One aspect of the invention is a reproducible, efficient and economic generic LC-MS/MS-based method for quantification of various human and humanized monoclonal antibody (MAb) therapeutics with a common antibody scaffold structure in cynomolgus monkey and rat plasma and tissue samples, and potentially other non-human species, from preclinical studies. Digestion of antibodies gives peptides from the conserved framework...
region which are unique to administered human or humanized therapeutic antibodies, and not found in endogenous monkey and rat proteins.

Figure 9 shows the generic LC-MS/MS method to quantify a therapeutic antibody in animal plasma/serum using one or more framework signature peptides (FSP).

Methods of the invention include a generic approach of quantifying human or humanized antibodies comprising the steps of:

(a) treating a biological sample with a digestive enzyme to form a digested antibody sample, wherein the serum or plasma sample is from an animal that has been treated with a human or humanized antibody; and

(b) analyzing the digested antibody sample by mass spectrometry to detect and measure the concentration of one or more common human framework peptides, wherein the human framework peptides comprise one or more sequences selected from SEQ ID NOS. 1-8.

In one exemplary embodiment, the digestive enzyme is trypsin. Alternative proteases may generate additional framework peptides besides those having SEQ ID NOS. 1-8. Any specific enzyme could be used, such as endoproteinase LysC, endoproteinase ArgC, staph aureus V8, and endoproteinase GluC. Non-specific proteases such as papain may be used. The peptides generated for quantitation may have different sequences than with trypsin, but the concept of using a generic framework peptide present in human and not present in animal antibodies is the same.

In another embodiment, the method further comprises contacting the digested antibody sample with an affinity capture media or solid-phase extraction (SPE) sample cleanup and eluting an enriched digested antibody sample.

In another embodiment, the method includes affinity capture the antibody comprising one or more human framework peptides having sequences selected from SEQ ID NOS. 1-8, followed by digestion. The affinity capture method may be achieved by ECD (extra-cellular domain antigen binding), anti-ID capture or Protein A or G.

In another embodiment, the biological sample is serum, plasma, tissue or cell line derived from a non-human mammal.

In an exemplary demonstration of the method, the antibody is anti-HER2 trastuzumab (HERCEPTIN®, Genentech, Inc.). Other antibodies were subjected to the methods of the invention. Antibodies which have been trypsin digested from non-clinical plasma and analyzed include anti-MUC16, anti-MSLN (mesothelin), anti-Steapl, anti-CD20 (2H7 and
rituximab), anti-HER3 (2C4, pertuzumab), anti-NRPI, anti-PDLI, anti-LRP6, anti-B7-H4, anti-GFRA1 7C9, anti-NRGl, and anti-LY6E.

In another embodiment, the antibody sample was analyzed with an immunoprecipitation (IP) affinity capture by bead-supported Protein A/G, followed by on-bead digestion and analysis.

FRAMEWORK SIGNATURE PEPTIDES

The framework regions of human antibodies are largely conserved. Figure 1 shows the sequence alignment of heavy chain amino acid sequences of a human 2H7 antibody ocrelizumab (SEQ ID NO: 11) and five cynomolgus monkey anti-CD20 antibodies: CynoHC 1a D3 1 (SEQ ID NO: 12), CynoHC 1b E5 1 (SEQ ID NO: 13), Cyno HC 2a (SEQ ID NO: 14), CynoHC 2b E6 1 (SEQ ID NO: 15), CynoHC 3 (SEQ ID NO: 16). Framework signature peptides are identified (FSP 1-8) which are unique to human 2H7 (hu 2H7) Mab and are not present in cynomolgus monkey IgG heavy chain, each bearing at least one amino acid difference in the sequences. Based on the available sequence information, framework signature peptides (FSP 1-8) are unique only to the human antibody and not to the cynomolgus IgG variants. The framework signature peptides (FSP 1-8) of Table 2 are also present in endogenous IgGl and in some cases in IgG2, IgG3, and IgG4. These peptides are also common among other human or humanized therapeutic antibodies and human IgGs.
8 framework signature peptides (FSPs) with residues unique (bold) to human IgG

Trastuzumab (HERCEPTIN®, Genentech) was used as a model reference standard and spiked into cynomolgus monkey and rat plasma, followed by direct whole plasma digestion with or without SPE preconcentration or immunoprecipitation by Protein A/G magnetic beads and a subsequent on-bead digestion prior to LC-MS/MS analysis, a working calibration range was established at 1-1000 µg/mL in both plasma matrices. Specificity was also tested and confirmed with both negative control blank plasma and spiked plasma samples.

Trastuzumab (HERCEPTIN®, huMAb4D5-8, rhuMAb HER2, Genentech) is a recombinant DNA-derived humanized, IgGl kappa, monoclonal antibody version of the murine HER2 antibody which selectively binds with high affinity in a cell-based assay (Kd = 5 nM) to the extracellular domain of the human epidermal growth factor receptor2 protein, HER2 (ErbB2) (US 5821337; US 6054297; US 6407213; US 6639055; Coussens L, et al (1985) Science 230:1132-9; Slamon DJ, et al (1989) Science 244:707-12). Trastuzumab contains human framework regions with the complementarity-determining regions of a murine antibody (4D5) that binds to HER2. Trastuzumab binds to the HER2 antigen and thus inhibits the growth of cancerous cells. Trastuzumab has been shown, in both in vitro assays

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### Table 2. Heavy chain Framework Signature Peptides (FSP 1-8) from Hu MAb

<table>
<thead>
<tr>
<th>FSP</th>
<th>Sequence</th>
<th>MW</th>
<th>SEQ ID NO:</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSP1</td>
<td>GPSVFPLAPSSK</td>
<td>1185.6</td>
<td>1</td>
</tr>
<tr>
<td>FSP2</td>
<td>STSGGTAALGCLVK</td>
<td>1263.6</td>
<td>2</td>
</tr>
<tr>
<td>FSP3</td>
<td>TPEVTCDVVDVSHEDPEVK</td>
<td>2081.01</td>
<td>3</td>
</tr>
<tr>
<td>FSP4</td>
<td>FNWYVDGVEVHNAK</td>
<td>1676.8</td>
<td>4</td>
</tr>
<tr>
<td>FSP5</td>
<td>VVSVLTVLHQDWLNGK</td>
<td>1807.0</td>
<td>5</td>
</tr>
<tr>
<td>FSP6</td>
<td>ALPAPIEK</td>
<td>837.5</td>
<td>6</td>
</tr>
<tr>
<td>FSP7</td>
<td>GFYPSDIAVEWESNGQPPNYK</td>
<td>2543.1</td>
<td>7</td>
</tr>
<tr>
<td>FSP8</td>
<td>TTPPVLDSDGSFFLYSK</td>
<td>1872.9</td>
<td>8</td>
</tr>
</tbody>
</table>
and in animals, to inhibit the proliferation of human tumor cells that overexpress HER2
approved in 1998 for the treatment of patients with ErbB2-overexpressing metastatic breast

Stable isotope-labeled (SIL) analogs of FSP1-8 (SEQ ID NOS:i-8) can be used as in situ ("spiked in") internal standards. Stable isotope labels typical include $^{13}$C, $^{15}$N, and $^2$H. The internal standards can be incorporated into one or more amino acid residues of the peptide sequence. The internal standards can be introduced into the sample before or after digestion, and function to compensate variations occurring during the LC-MS/MS analysis (e.g., changes in autosampler performance, LC separation and MS responses). For example, a stable isotope labeled version of FSP8 was prepared where the lysine between phenylalanine (F) and tyrosine (Y) was labeled with $^{13}$C and $^{15}$N.

Based on their MS characteristics, FSP8 was empirically chosen due to its most intense signal response as the primary peptide for quantification. In addition, three other peptides, FSP4, FSP5, and FSP3, were monitored for qualitative confirmation. Each of these four peptides could be used as surrogate to quantify the mAb in animal biological matrices. Their corresponding SIL ISs were used in the assay (Table 3). FSP6 can be used only in animal matrices other than cynomolgus monkey since a co-eluting interference background was detected in cynomolgus monkey plasma. Relatively weaker ionization was observed for FSP1, FSP5 and FSP2.

<table>
<thead>
<tr>
<th>Internal Standard</th>
<th>Amino Acid Sequence</th>
<th>SEQ ID NO:</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSP8 IS</td>
<td>TTPVLDSDGSFFL* ($^{13}$C$_6$, $^{15}$N$_1$) YSK</td>
<td>8</td>
</tr>
</tbody>
</table>
HUMAN AND HUMANIZED ANTIBODIES

Figure 2 shows the heavy chain (SEQ ID NO: 17) and light chain (SEQ ID NO: 18) of trastuzumab (Herceptin®, Genentech Inc.; rhuMABHER2, Anti pl85HER2), a recombinant derived humanized monoclonal antibody, CAS Registry No. 180288-69-1.

Figure 3 shows the heavy chain (SEQ ID NO: 11) and light chain (SEQ ID NO: 19) of ocrelizumab, rhuMab 2H7, PRO70769, a humanized anti-CD20 antibody, CAS Registry No. 637334-45-3.

Figure 4 shows the heavy chain (SEQ ID NO: 20) and light chain (SEQ ID NO: 21) of pertuzumab, rhuMab 2C4, CAS Registry No. 380610-27-5. FSP2, FSP3, FSP8 are identified as underlined in heavy chain (SEQ ID NO:20).

Figure 5 shows the heavy chain (SEQ ID NO: 22) and light chain (SEQ ID NO: 23) of anti-PDL1, member of the extended CD28/CTLA-4 family of T cell regulators. FSP2, FSP4, FSP8 are identified as underlined in heavy chain (SEQ ID NO:22).

Figure 6 shows the heavy chain (SEQ ID NO: 24) and light chain (SEQ ID NO: 25) of anti-neuropilin-1, anti-NRPl, MNRPI685A. FSP2, FSP4, FSP8 are identified as underlined in heavy chain (SEQ ID NO:24). Anti-NRPl is a recombinant, phage-derived, human monoclonal antibody that specifically targets neuropilin-1 (NRPl), a multi-domain receptor known to bind a variety of ligands, including members of the VEGF family. Anti-NRPl has demonstrated efficacy in combination with anti-VEGF in mouse xenograft models and strong nonlinear pharmacokinetics across a wide dose range in preclinical models. It is currently being evaluated in Phase I studies as a single agent and in combination with bevacizumab with or without paclitaxel.

Figure 7 shows the heavy chain (SEQ ID NO: 26) and light chain (SEQ ID NO: 27) of anti-MUC16, DMUC4064A. FSP2, FSP4, FSP8 are identified as underlined in heavy chain (SEQ ID NO:26).
Figure 8 shows the heavy chain (SEQ ID NO: 28) and light chain (SEQ ID NO: 19) of rituximab, C2B8, MabThera, (Rituxan®, Genentech Inc., Biogen/Idec). FSP2, FSP4, FSP8 are identified as underlined in heavy chain (SEQ ID NO:28). Rituximab (RITUXAN®, Genentech/Biogen Idec; MABTHERA®, Roche, REDITUX®, CAS Reg. No. 174722-31-7) is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in US 5736137. Rituximab is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20-positive, B-cell NHL. Rituximab binds to cell surface CD-20 and results in B-cell depletion (Cartron et al (2002) Blood 99: 754-758; Idusogie et al (2000) J. Immunol. 164: 4178-4184; Grillo-Lopez AJ, et al (1999) Semin Oncol; 26:66-73; US 5736137). RITUXAN (US 5677180; US 5736137) is the most widely used monoclonal antibody in hematopoietic malignancies and is established in widespread clinical practice. RITUXAN first received FDA approval in 1997 for the treatment of relapsed or refractory, low-grade or follicular, CD20-positive, B-cell non-Hodgkin's lymphoma (NHL). It was also approved in the European Union under the trade name MabThera® in June 1998. In February 2006, RITUXAN also received FDA approval in combination with methotrexate to reduce signs and symptoms in adult patients with moderately-to-severely-active rheumatoid arthritis who have had an inadequate response to one or more TNF antagonist therapies. The amino acid sequence of rituximab antibody (also designated C2B8) and exemplary methods for its production via recombinant expression in Chinese Hamster Ovary (CHO) cells are disclosed in US Patent No. 5736137.

SAMPLE PREPARATION

Figure 15 shows a cartoon of capture of mAb from animal plasma/serum on streptavidin coated magnetic beads bound to a biotinylated capture probe or Protein A, G coated magnetic bead, followed by isolation by magnetic separation, digestion of the captured antibody and analysis by LC-MS/MS.

Figure 16 shows embodiments of Protein A, G coated magnetic bead (top) for generic capture of antibody and streptavidin coated magnetic beads bound to a biotinylated capture probe (bottom) for specific capture of antibody.

The potential of immunoprecipitation to efficiently and reproducibly isolate the target monoclonal antibody (MAb) with Protein A was evaluated in two formats: (a) Protein A coupled to magnetic beads and (b) Protein A coated on a 96-well Micro-titer plate. In a preliminary test, a Protein A Micro-titer plate was too capacity limited compared to Protein A
magnetic beads in capturing the total applied load of endogenous IgGs along with the target Mab, particularly from monkey plasma. On-bead digestion using Protein A magnetic beads was selected for evaluation for isolation of FSP from both lithium/heparin treated Cynomolgus monkey and Sprague-Dawley rat plasma. Whole plasma digestion followed by solid phase extraction (SPE) was also tested and found to be less effective in removing the interference from background noise.

SEPARATION AND ANALYSIS OF BIOLOGICAL SAMPLES

Two approaches to assess FSP specificity, detection sensitivity, and reproducibility were investigated: (1) whole plasma digest/SPE (solid phase extraction), and (2) immunoprecipitation (IP).

Monkey (Cynomolgus) and rat plasma sample (n = 10 lots each species) were evaluated to assess lot-to-lot specificity, potential interference effects, and reproducibility. Along with a blank plasma control, blank plasma samples were fortified, i.e. "spiked", with 20 µg/ml of trastuzumab. A set of calibrator trastuzumab samples ranging from 1-1000 µg/ml trastuzumab were prepared in pooled plasma matrix and run in parallel with individual monkey and rat plasma samples.

Figure 12 shows a calibration curve of trastuzumab (using FSP8 as surrogate) spiked at various concentrations from 1-1000 µg/mL into lithium heparin Cynomolgus monkey plasma prepared by the whole plasma digest/SPE approach. Stable isotope-labeled peptide internal standards (ISs) were prepared in 20% acetonitrile to make the working internal standard solution containing the appropriate concentrations.

Figure 14 shows the steps for immunoprecipitation (IP) of monoclonal antibodies and generating corresponding framework signature peptides (FSPs) in animal plasma or serum. Protocols are defined in Examples 1-4.

Animal samples were prepared by immunoprecipitation (IP) with Protein A paramagnetic beads (Millipore) followed by trypsin digestion. To a 96-well conical-bottom microtiter plate (VWR Scientific), an aliquot of 25 µL diluted plasma or serum [1:2 v/v dilution with loading buffer (SN1) of 0.1:5.0:3.0:0.2:91.7:0.1 Tween 20 / Trizma hydrochloride (1 M) / sodium chloride (5 M) / EDTA (0.5 M) / water / BSA, v/v/v/v/v/w] was placed, together with 125 µL loading buffer. An aliquot of 25 µL of Protein A magnetic beads, pre-washed and re-suspended in loading buffer, was added to each well. The mixture was then incubated at room temperature (RT) for 120 minutes under constant mixing to allow the capture of mAbs. The supernatant was discarded and the beads were washed thoroughly.
in washing buffer (SN2) of 5.0:3.0:0.2:91.8 Trizma hydrochloride (1 M) / sodium chloride (5 M) / EDTA (0.5 M) / water, v/v/v/v). The wash and magnetic separation were performed using a microplate washer (BioTek, VT, USA) and a 96-well flat magnet (Biotek), respectively. The wash/separation process could also be conducted with a KingFisher 96 magnetic particle processor (Thermo Scientific).

Following immunoprecipitation (IP), a 25 µL aliquot of working IS solution was spiked into each well except blanks, where 25 µL of 20% acetonitrile were added instead. Aliquots of 75 µL RapiGest solution (0.05:37.5:10 RapiGest powder/50 mM ammonium bicarbonate/Acetonitrile, w/v/v) and 10 µL of 0.1 M DTT were added. The plate was covered by an adhesive sealing film (VWR Scientific) and shook gently for approximately 1 minute, followed by incubation at 60 °C for 60 minutes. A volume of 25 µL iodoacetic acid (0.1 M) was added, and the plate was covered by an aluminum foil and incubated at room temperature for approximately 30 minutes protected from light. An aliquot of 10 µL trypsin solution (0.250 mg/mL) was added to each well, and the plate was subsequently incubated at 37 °C for approximately 90 minutes. Digestion was terminated by adding 15 µL of 2 M HC1 to each well and incubating the plate at 37 °C for 30 minutes. Samples were then transferred by Tomtec (CT, USA) to a Multiscreen HTS filter plate (0.45 µm, Millipore) placed on top of a 96-well, conical-bottom collection plate and centrifuged for 5 minutes at 3000 rpm to collect the filtrate. The collection plate was sealed, and a volume of 20 µL filtrate was injected directly onto LC-MS/MS.

ELISA and LC-MS/MS

The ELISA (Figure 19a) and LC-MS/MS (Figure 19b) were compared in measuring total antibody in rat plasma after a single dose of antibody. Figure 19a shows a cartoon of the monoclonal antibody (mAb therapeutics) captured by binding to an immobilized extracellular domain (ECD) or anti-human IgG polyclonal antibody, and detected with an anti-human IgG polyclonal antibody labeled with horse radish peroxidase (HRP) in an ELISA assay with electrochemiluminescent, colorometric, or chromophoric substrate detection.

Liquid chromatographic separation was carried out by either an HP Agilent 1100 Series LC binary system or a Shimadzu LC-IOADvp binary system with a reversed phase BioSuite C18 PA-A column (2.1 x 50 mm, 3 µm, Waters) operated at a flow rate of 200 µL/min. The column was maintained at 50 °C by a column heater (Analytical Sales and Products, NJ, USA). Mobile phase consisted of A : 0.1% formic acid in water and B : 0.1% formic acid in Acetonitrile/methanol (75:25, v/v). The gradient condition was maintained at
5% B for 0.4 min, ramped to 40% B in 3.4 min, further increased to 95% B in 1 min, kept at 95% B for 0.5 min before brought back to 5% B in 0.1 min. It was then kept at 5% for 0.5 min before being ramped back up to 95% B in 0.1 min, and maintained at the level for 0.5 min to reduce potential carryover. Finally, the gradient was returned to 5% B in 0.1 min and re-equilibrated at 5% B for 0.9 min. The total LC run time was 7.5 min. Samples were injected using a CTC HTS PAL autosampler (LEAP Technologies, NC, USA) with an injector loop of 20 µL. The autosampler wash 1 was Acetonitrile/isopropanol/trifluoroethanol/methanol/water/formic acid (60:15:15:5:5:0.2, by volume) and wash 2 was water/Acetonitrile/formic acid (95:5:0.2, by volume).

A Sciex API 4000® triple quadrupole mass spectrometer (AB Sciex, CA, USA) equipped with a turbo ionspray was used for quantitation. The MS instrument was operated in the positive ion mode with the source temperature set at 500 °C and the ionspray voltage at 5000 V. Gas parameters were set with the curtain gas at 25, the nebulizer gas at 45 and the auxiliary gas at 40. A collision gas of 10 was used. Details of the multiple reaction monitoring (MRM) transitions for the signature peptides and their corresponding internal standards are listed in Table 4. The dwell time was set at 50 ms for each MRM transition, and the same entrance potential of 10 V was applied. Both Q1 and Q3 resolutions were set at unit. Quantitation was performed using Intelliquan based on the peak area.

<table>
<thead>
<tr>
<th>Surrogate Peptide</th>
<th>Peptide Sequence</th>
<th>Q1 (m/z)</th>
<th>Q3 (m/z)</th>
<th>DP</th>
<th>CE</th>
<th>CXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSP8</td>
<td>TTPVLDSDGSFFLYSK</td>
<td>938.0 (2+)</td>
<td>836.7 (y15, 2+)</td>
<td>90</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>FSP4</td>
<td>VVSVLTVLHQDWLNGK</td>
<td>603.5 (3+)</td>
<td>712.8 (y12, 2+)</td>
<td>60</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>FSP5</td>
<td>FNWYVDGVEVHNAK</td>
<td>560.2 (3+)</td>
<td>709.3 (y12, 2+)</td>
<td>95</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>FSP3 IS</td>
<td>TPEVTeVVVDVSHEDEPVK</td>
<td>714.0 (3+)</td>
<td>472.2 (y4)</td>
<td>75</td>
<td>38</td>
<td>12</td>
</tr>
<tr>
<td>FSP8 IS</td>
<td>TTPVLDSDGSFL*YSK</td>
<td>941.5 (2+)</td>
<td>840.0 (y15, 2+)</td>
<td>90</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>FSP4 IS</td>
<td>VVSVLTVLHQDWL*NGK</td>
<td>605.8 (3+)</td>
<td>716.3 (y12, 2+)</td>
<td>60</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>FSP5 IS</td>
<td>FNWYVDGVE*HNAK</td>
<td>562.2 (3+)</td>
<td>712.3 (y12, 2+)</td>
<td>95</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>FSP3 IS</td>
<td>TPEVTeVVVDVSHEDEP*K</td>
<td>716.0 (3+)</td>
<td>478.2 (y4)</td>
<td>75</td>
<td>38</td>
<td>12</td>
</tr>
</tbody>
</table>

Lower case "c" indicates the cysteine residue that has been alkylated with iodoacetamide
Method qualification was performed using trastuzumab as a model. Calibration standards were prepared by spiking trastuzumab into cynomolgus monkey plasma or Sprague-Dawley rat plasma at 1.00, 1.75, 3.00, 10.0, 25.0, 75.0, 200 and 250 µg/mL. Quality controls (QCs) were prepared at 1.00 (LLOQ), 2.50 (LQC), 15.0 (MQC) and 190 µg/mL (UQC) of trastuzumab in plasma. In addition, a dilution QC of 1000 µg/mL original concentration with 10x dilution factor was included. The intra-assay QCs were prepared in 6 replicates and Dilution QC was prepared in 3 replicates. The data of the method qualification is reported in Table 5. Data indicates that the LC-MS/MS assay has good precision and accuracy with values within predefined acceptance criteria.

Table 5  Method qualification of precision and accuracy for trastuzumab in rat plasma

<table>
<thead>
<tr>
<th>QC</th>
<th>LLOQ 1.00</th>
<th>LQC 2.50</th>
<th>MQC 15.0</th>
<th>UQC 190</th>
<th>Dil10 QC 1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.01</td>
<td>2.42</td>
<td>14.2</td>
<td>206</td>
<td>996</td>
</tr>
<tr>
<td>2</td>
<td>1.07</td>
<td>2.48</td>
<td>15.1</td>
<td>203</td>
<td>984</td>
</tr>
<tr>
<td>3</td>
<td>1.03</td>
<td>2.43</td>
<td>14.7</td>
<td>191</td>
<td>1050</td>
</tr>
<tr>
<td>4</td>
<td>1.05</td>
<td>2.51</td>
<td>15.3</td>
<td>187</td>
<td>n/a</td>
</tr>
<tr>
<td>5</td>
<td>1.12</td>
<td>2.76</td>
<td>14.8</td>
<td>186</td>
<td>n/a</td>
</tr>
<tr>
<td>6</td>
<td>1.08</td>
<td>2.40</td>
<td>14.5</td>
<td>183</td>
<td>n/a</td>
</tr>
<tr>
<td>Mean</td>
<td>1.06</td>
<td>2.50</td>
<td>14.8</td>
<td>193</td>
<td>1010</td>
</tr>
<tr>
<td>SD</td>
<td>0.0375</td>
<td>0.135</td>
<td>0.391</td>
<td>9.61</td>
<td>34.3</td>
</tr>
<tr>
<td>% CV</td>
<td>3.5</td>
<td>5.4</td>
<td>2.7</td>
<td>5.0</td>
<td>3.4</td>
</tr>
<tr>
<td>% Theoretical</td>
<td>106.0</td>
<td>100.0</td>
<td>98.5</td>
<td>101.4</td>
<td>100.9</td>
</tr>
<tr>
<td>% Bias</td>
<td>6.0</td>
<td>0.0</td>
<td>-1.5</td>
<td>1.4</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Table 5 shows detection of 1 µg/mL (LLOQ) of trastuzumab antibody in rat plasma using FSP8 as surrogate, with stable-isotope labelled FSP8 internal standard (Figure 17b) detected at the same retention time. Samples were prepared according to the protocol in Example 4. Good linearity was demonstrated from 1-250 µg/mL of trastuzumab in rat plasma in Figure 18.
Figure 20 shows the individual concentration time profiles of rats (1A, IB, 1C) dosed with a 2 mg/kg bolus of trastuzumab, an anti-HER2 mAb. Plasma samples over 28 hours post-dose were analyzed by the LC-MS/MS (Figure 19b) and ELISA (Figure 19a) assays. Good concordance was observed between the two assay methods. The PK parameters from Figure 20 are:

<table>
<thead>
<tr>
<th>Assay</th>
<th>CL (mL/d/kg)</th>
<th>V0 (mL/kg)</th>
<th>T1/2 (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>6.45</td>
<td>41.6</td>
<td>9.77</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>7.04</td>
<td>43.7</td>
<td>11.0</td>
</tr>
</tbody>
</table>

*PK parameters based on n=2 due to probable ATA (No SD included)*

Figure 21 shows the individual concentration time profiles of rats (2D, 2E, 2F) dosed with a 2 mg/kg bolus of 3A5, an anti-MUC 16 mAb. Plasma samples over 28 hours post-dose were analyzed by the LC-MS/MS (Figure 19b) and ELISA (Figure 19a) assays. Good concordance was observed between the two methods. The PK parameters from Figure 21 are:

<table>
<thead>
<tr>
<th>Assay</th>
<th>CL (mL/d/kg)</th>
<th>V0 (mL/kg)</th>
<th>T1/2 (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>8.25 ± 4.03</td>
<td>38.0 ± 1.83</td>
<td>8.61 ± 3.77</td>
</tr>
<tr>
<td>LC-MS</td>
<td>8.14 ± 3.09</td>
<td>42.6 ± 15.1</td>
<td>8.09 ± 3.26</td>
</tr>
</tbody>
</table>

Figure 22 shows the results of rats (3G, 3H, 3I) dosed with a 2 mg/kg bolus of an anti-mesothelin (Msln) mAb. Plasma samples over 28 hours were analyzed post-dose by the LC-MS/MS (Figure 19b) and ELISA (Figure 19a) assays. Good concordance was observed between the two methods. The PK parameters from Figure 22 are:
Figure 23 shows the concordance between the LC-MS/MS assay shown in Figure 19b and the ELISA assay of Figure 19a based on mean pharmacokinetics (PK) of plasma/serum samples from cynomolgus monkey dosed with 3A5, an anti-MUC 16 mAb by measurement of antibody in the blood over 28 days. The PK parameters from Figure 23 are:

<table>
<thead>
<tr>
<th>Assay</th>
<th>CL (mL/d/kg)</th>
<th>VO (mL/kg)</th>
<th>TI/2 (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>5.38 ± 0.975</td>
<td>50.2 ± 2.10</td>
<td>11.6 ± 4.37</td>
</tr>
<tr>
<td>LCMS</td>
<td>6.51 ± 0.896</td>
<td>47.1 ± 3.30</td>
<td>11.8 ± 1.94</td>
</tr>
</tbody>
</table>

All values are Mean ± standard deviation (SD)

Figure 24 shows the concordance between the LC-MS/MS assay shown in Figure 19b and the ELISA assay of Figure 19a based on mean pharmacokinetics (PK) of plasma/serum samples from cynomolgus monkey dosed with an anti-mesothelin (Msln) mAb by measurement of antibody in the blood over 42 days. The PK parameters from Figure 24 are:

<table>
<thead>
<tr>
<th>Assay</th>
<th>CL (mL/d/kg)</th>
<th>C_max (µg/mL)</th>
<th>TI/2 (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>7.86 ± 2.75</td>
<td>32.0 ± 2.25</td>
<td>7.97 ± 2.72</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>7.63 ± 2.64</td>
<td>31.6 ± 1.46</td>
<td>6.99 ± 2.97</td>
</tr>
</tbody>
</table>

All values are Mean ± standard deviation (SD)

Figure 25 shows the concordance between the LC-MS/MS assay shown in Figure 19b and the ELISA assay of Figure 19a based on individual pharmacokinetics (PK) of plasma/serum samples from mice (A, B, C) dosed with an antibody-drug conjugate, (ADC), anti-LY6E-MC-vc-PAB-MMAE, which has the structure:
where \( \text{Ab} \) is an anti-LY6E antibody linked through a cysteine amino acid to the
maleimidocaproyl (MC) group of the linker, and by \( p \) is the number of drug moieties
(MMAE) per antibody in an ADC molecule. The range of \( p \) in a typical mixture of ADC is
about 0 to about 20, or from 0 to about 8. Where \( p \) is 0, a certain amount of naked,
unconjugated antibody may be present. The average drug loading per antibody may be about
2 to about 5, or about 3 to about 4. Thus a typical preparation of an antibody-drug conjugate
(ADC) is a heterogeneous mixture of species with antibodies conjugated with some number
of drug moieties, such as MMAE. The linker also includes a valine-citrulline (Val-Cit)
dipeptide unit susceptible to cathepsin recognition and the para-aminobenzyloxymethyl
(PAB) unit (US 7659241; US 7498298; Doronina et al. (2006) Bioconjugate Chem. 17:1 14-

The drug moiety MMAE (vedotin, \((S)-N-(3R,45,55)-1-((S\,2R)-3-((15,2 R)-1-
hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)piperidine-1-yl)-3-
methoxy-5-methyl-1-oxoheptan-4-yl-N,3-dimethyl-2-((5)-3-methyl-2-
(methylamino)butanamido)butanamide, CAS Reg. No. 474645-27-7) is a
monomethylauristatin analog of dolastatin (US 5635483; US 5780588) linked through its N-
terminus to the antibody. MMAE has the structure:

\[
\text{MMAE}
\]

The PK profiles and parameters from the two assay methods (LC-MS/MS and
ELISA) from the antibody dosing experiment of Figures 23-28 show high concordance
indicating that quantification of mAbs in animals, including cynomolgus monkeys, rats and
mice, by a single generic LC-MS/MS assay using common framework signature peptides as
surrogates is feasible and with robust performance. The single MS-based approach is capable
of generate reliable PK data that otherwise needs multiple ELISA assays to achieve. In
addition, the MS approach doesn't require any custom reagents, which can help accelerate the
method development process and make the PK evaluation available at the early stages of drug development.

DATA ANALYSIS

Calibration curves were established in the immunoprecipitation approach for:

5 DWYIHWWVR (SEQ ID NO:9), FSP3, FSP4, FSP5, FSP8 (all for both monkey and rat) and NQVSLTCLVK (SEQ ID NO: 10) (rat only), and in the Whole Plasma Digest/SPE Approach for: FSP4, FSP5, FSP8 (all for both monkey and rat). Specificity data for blank and spiked plasma samples were established. Area ratios and corresponding CV’s (coefficient of variation) for different peptides with respect to FSP8 (candidate for primary quantification) have been provided as a gauge of digestion reproducibility. A quadratic (1/curv.e, 2 weighted) regression was used to fit the data.

In the whole plasma digest/SPE approach to treatment of monkey plasma samples (Example 3b), FSP8 is the most sensitive peptide. Figure 13 shows LC-MS/MS chromatograms demonstrating the detection of FSP8 spiked into lithium heparinised cynomolgus monkey plasma at LLOQ (lower limit of quantitation) = 1 μg/mL after whole plasma digest/SPE sample preparation. Peaks at 4.47 and 5.20 minutes are in the blank. FSP8 has a retention time at 4.66 minutes. The only other peptides quantifiable at low concentrations are FSP4 and FSP5. However, their signal to noise ratio (s/n) at the 1 μg/mL-LLOQ level is less than (<) 5. As expected, peptide NQVSLTCLVK (SEQ ID NO: 10) showed high endogenous background levels in all monkey plasma. Nine of the ten individual lots tested had similar and acceptable specificity. Lot# 5 had an unusual background level for all quantifiable peptides. The pattern is suggestive that it might be contaminated with some human plasma. Overall, FSP (i.e. spiked) samples showed good accuracy and precision for all lots for FSP8, the potential signature peptide for primary quantitation (exception: FSP7 with a 27% CV). Peak area ratios of FSP4 and FSP5 compared to FSP8 showed CV’s less than 20% across different samples and sample types.

In the whole plasma digest/SPE approach to treatment of rat plasma samples (Example 3b), FSP8 is the most intense peptide. The other peptides quantifiable at low level concentrations are FSP4 and FSP5. However, the s/n for these peptides at the LLOQ level is less than 5. The calibration curves showed a split nature, possibly due to signal suppression of later injected samples. None of the individual lots tested, had quantifiable background peaks for all peptides. Due to the split curve, FSP samples showed highly variable accuracy...
and precision values. Peak area ratios of FSP4 and FSP5 with respect to FSP8 showed CV's greater than 20% in most cases, again pointing out the assay variability.

In the immunoprecipitation approach to treatment of monkey plasma samples (Example 4), FSP8 is the most sensitive, i.e. intense, peptide. All other peptides are also quantifiable at low level concentrations. The s/n for all other peptides at the LLOQ level is greater than 5 (except FSP2). As expected, peptide NQVSLTCLVK (SEQ ID NO: 10) showed background levels for all monkey plasma samples. The relationship between concentration and response showed some nonlinearity at high concentrations, possibility due to saturation of the Protein A magnetic beads and/or from competition with endogenous cynomolgus monkey IgGs. Of the 10 individual lots tested, the samples from Lot# 5 had background peaks for all quantifiable peptides at a similar level, except for the variable region peptide DTYIHWVR (SEQ ID NO: 9). Overall, the FSP samples showed good accuracy and precision for all lots for FSP8, (including Lot#5 if the background concentration in the blank is taken into account). Peak area ratios of almost all peptides showed CV's less than 15% across different samples and sample types (except FSP5 with a CV for CALS about 23%).

In the immunoprecipitation (IP) approach to treatment of rat plasma samples (Example 4), FSP8 is the most intense peptide. All other peptides are also quantifiable at low level concentrations. The s/n for all other peptides at the LLOQ level is greater than 5 (except FSP2). The relationship between concentration and response showed better linearity that the monkey immunoprecipitation (IP) data, possibility due to less competition from lower affinity endogenous IgGs in Rat plasma. None of the individual lots tested, had quantifiable background peaks for all peptides. Overall, FSP samples showed good accuracy and precision for all lots for FSP8. Peak area ratios of almost all peptides showed CV's less than 20% across different samples and sample types (except FSP5 and FSP2 with a CV for CALS greater than (> 24%).

The immunoprecipitation (IP) extraction with Protein A magnetic beads showed better sensitivity and reproducibility than whole plasma digest/SPE extraction. The IP approach was faster and "cleaner" than the whole matrix digest/SPE approach.

**EXAMPLES**

Example 1 Simple Whole Plasma Digestion and Extraction procedure

1. Aliquot 10 μL of (sodium citrate) plasma into a lo-bind plate
2. Add 95 µL of a 10 mM DTT solution in 50 mM ammonium bicarbonate
3. Mix and incubate at 60 °C for 60 minutes
4. Add 20 µL of 100 mM iodoacetamide (IAA) in 50 mM ammonium bicarbonate
5. Incubate in the dark at room temperature for 30 minutes
6. Leave in the light for 20 minutes
7. Add 15 µL of a 500 µg/mL trypsin solution in 50 mM ammonium bicarbonate
8. Incubate overnight at 37 °C
9. Add 15 µL of a 1% formic acid solution. Mix and centrifuge.
10. Analyze by LC-MS/MS (20 µL injection, at 0.7 mL/min UPLC)

**Example 2** Solid-phase extraction protocol for MAX/WCX microElution® Plate (Waters Corp.)
1. Mix 600 µL of serum/plasma digest with 100 µL of 8% H3PO4
2. Condition MAX/WCX µE[μioη SPE plate (Waters Corp., Milford MA) with 200 µL ofMeOH
3. Equilibrate MAX/WCX µE[μioη SPE plate with 200 µL of H2O
4. Load diluted serum/plasma digest on to the MAX/WCX µE[μioη SPE plate (2 x 350 µL aliquots). Apply just enough vacuum after each addition to allow the sample to pass through the bed in a drop-wise fashion.
5. Wash with 200µL of 5% NH4OH
6. Wash with 200µL of 20% ACN (acetonitrile)
7. Elute under moderate vacuum with (2 x 25 µL) of 75:25:1 acetonitrile / water / TFA, v/v/v into a 96-position, 2.0 mL, square-well, conical-bottom, polypropylene plate.
8. Add 200 µL of water and seal the plate with a purple mat seal and vortex for approximately 30 s.
9. Inject 25 µL on the 4000 QTRAP® LC/MS/MS System (AB Sciex, Foster City, CA, USA)

**Example 3a** Whole Plasma Digest Procedure #1
1. Aliquot 10 µL of plasma into a lo-bind plate
2. Add 95 µL of a 10 mM DTT solution in 50 mM ammonium bicarbonate
3. Mix and incubate at 60°C for 60 minutes
4. Add 20 µL of 100 mM iodoacetamide in 50 mM ammonium bicarbonate
5. Incubate in the dark at room temperature for 30 minutes
6. Leave in the light for 20 minutes
7. Add 15 µL of 500 µg/mL trypsin in 50 mM ammonium bicarbonate
8. Incubate overnight at 37°C
9. Add 15 µL of 1% formic acid solution. Mix and centrifuge.
10. Analyze by LC-MS/MS

Example 3b  Whole Plasma Digest/SPE (Solid Phase Extraction) - Protocol #2
1. Mix 25 µL of plasma sample with 100 µL of RapiGest™ (Waters Corp., Milford MA) SF surfactant solution (0.05:40:10 RapiGest™/Ammonium Acetate, 50 mM / ACN, w/v/v). Add an additional 400 µL of RapiGest™ diluent (80:20 Ammonium Acetate, 50 mM / ACN, v/v).
2. Add 10 µL of DTT (1 M). Incubate at 60°C for about 1 hour.
3. Add 25 µL of IAA (1M). Incubate at RT for about 0.5 hours protected from light.
4. Add 20 µg of trypsin. Incubate at 37°C for about 16 hours.
5. Add another dose of 20 µg of trypsin. Incubate at 37°C for about 4 hours.
6. Add 50 µL of 6M HCl. Incubate at 37°C for about 0.5 hours.
7. Subject 500 µL of whole plasma digest to SPE using Oasis® MAX µEluion plate ((Waters Corp., Milford MA)).
8. Mix 500 µL of plasma digest with 100 µL of 8% H3P04
9. Condition Oasis® MAX µEluion SPE plate with 200 µL of MeOH
10. Equilibrate Oasis® MAX µEluion SPE plate with 200 µL of H2O
11. Load diluted plasma digest on to the Oasis® MAX µEluion SPE plate (2 x 300 µL aliquots). Apply just enough vacuum to allow the sample to pass through the bed in a drop-wise fashion.
12. Wash with 200µL of 5% NH40H
13. Wash with 200µL of 20% acetonitrile
14. Elute under moderate vacuum with (2 x 25 µL) of 75:25:1 acetonitrile / water / TFA, v/v/v
15. Add 200 µL of water and seal the plate. Vortex for approximately 30 s.
16. Final volume of SPE eluate is approximately 250 µL. Directly inject 25 µL of this extract.
Example 4  Immunoprecipitation Protocol

1. Gently mix the Protein A bead suspension so that all the beads are uniformly suspended.

2. Pipette the required volume of suspended beads in a polypropylene tube. With the help of an external magnet, separate the beads from the storage buffer, and gently remove the storage buffer with a pipette without disturbing the beads. (note: Calculate the required bead volume based on a 25-µL bead volume required per well).

3. Add a volume of SN1 buffer to the polypropylene tube equal to the initial bead volume. Vortex briefly so that the beads are re-suspended in SN1 buffer.

4. Again with the help of an external magnet, separate the beads from SN1 buffer, and gently remove the SN1 buffer without disturbing the beads.

5. Repeat steps 3-4 two additional times.

6. After washing the beads, again add a volume of SN1 buffer equal to the initial bead volume. Vortex briefly so that the beads are re-suspended in SN1 buffer. The washed bead solution is to be prepared fresh on the day of use. It should be stored at 2-4 °C if not used within an hour of washing.

7. Vortex the plasma sample and aliquot 25-µL in a microcentrifuge tube.

8. Dilute the plasma sample with 50 µL of SN1 buffer. Vortex briefly.

9. Aliquot 25 µL of the diluted plasma sample in a 96-well microtiter plate.

10. Add 125 µL of SN1 buffer to each well.

11. Add 25 µL of the washed Protein A beads (from step 6) to each well. Ensure that the beads are well suspended in solution prior to adding.

12. Cover the plate with an adhesive sealing film, and shake gently on a titer plate shaker for approximately two hours at room temperature.

13. Using an external magnet and a plate washer, separate the magnetic beads and discard the unbound proteins in the supernatant. Wash the beads three times with SN2 buffer using the plate washer. Ensure that the beads are well suspended in solution prior to each wash step by shaking on a titer plate shaker.

14. Add 25 µL of working internal solution to each well except blanks without internal standard. Add 25 µL of working internal standard diluent to wells containing blanks without internal standard.

15. Add 75 µL of RapiGest solution to each well.

16. Add 10 µL of 0.1 M DTT to each well. Cover the plate with an adhesive sealing film and shake gently on a titer plate shaker for approximately 1 minute.
17. Incubate the plate at 60 °C in a preheated oven for approximately one hour.
18. Add 25 µL of 0.1 M IAA to each well. Cover the plate with an adhesive sealing film and shake gently on a titer plate shaker for approximately 1 minute. These steps should be performed protected from light. Cover the plate with an aluminum foil and incubate at room temperature for approximately 30 minutes.
19. Add 10 µL of trypsin solution to each well. Cover the plate with an adhesive sealing film, and shake gently on a titer plate shaker for approximately 1 minute.
20. Incubate the plate at 37 °C in a preheated incubator for approximately 90 minutes.
21. Add 15 µL of 2 M HCl to each well. Cover the plate with an adhesive sealing film and shake gently on a titer plate shaker for approximately 1 minute.
22. Incubate the plate at 37 °C in a preheated incubator for 30 min.
23. Shake the plate gently on a titer plate shaker for approximately 1 minute. Using the Tomtec, transfer the solution from each well to a Multiscreen HTS Filter Plate placed on top of a 96-well, conical-bottom collection plate.
24. Centrifuge the Multiscreen HTS Filter Plate / 96-well conical-bottom collection plate combination for 5 min at 3000 rpm to collect the filtrate in the 96-well, conical-bottom collection plate.
25. Seal the 96- conical-bottom collection plate with a yellow injection mat and inject directly.

SN1 Buffer: 0.1:5.0:3.0:0.2:91.7:0.1 Tween 20 / Trizma Hydrochloride (1 M) / Sodium Chloride (5 M) / EDTA (0.5 M) / Water / Bovine Serum Albumin, v/v/v/v/w. This solution is prepared by combining 1 mL of Tween 20, 50.0 mL of 1M Trizma Hydrochloride, 30 mL of 5 M sodium chloride solution, 2 mL of 0.5 M EDTA and 1.00 g of bovine serum albumin in a 1 L volumetric flask. The volume is then made up with water and mixed well to dissolve the bovine serum albumin. It is stored in a closed container at 2-8 °C for up to one month.

SN2 Buffer: 5.0:3.0:0.2:91.8 Trizma Hydrochloride (1 M) / Sodium Chloride (5 M) / EDTA (0.5 M) / Water, v/v/v/v. This solution is prepared by combining 50.0 mL of 1M Trizma Hydrochloride, 30 mL of 5 M sodium chloride solution and 2 mL of 0.5 M EDTA in a 1 L volumetric flask. The volume is then made up with water and mixed well. It is stored in a closed container at 2-8 °C for up to one month.
Exemplary HPLC Method #1:

<table>
<thead>
<tr>
<th>Autosampler</th>
<th>Acquity BSM</th>
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<tbody>
<tr>
<td>Strong wash</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Weak wash</td>
<td>Acetonitrile:water 10:90 v/v</td>
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<tr>
<td>Injection mode</td>
<td>Full loop</td>
</tr>
<tr>
<td>LC system</td>
<td>Acquity BSM</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.7 mL/min</td>
</tr>
<tr>
<td>Analytical Column</td>
<td>100 x 2.1mm i.d. Waters Acquity Phenyl</td>
</tr>
<tr>
<td>Column temperature</td>
<td>Nominally +60°C</td>
</tr>
<tr>
<td>Run Time</td>
<td>7.5 minutes</td>
</tr>
<tr>
<td>Mobile phase A</td>
<td>Acetonitrile containing 0.2% (v/v) formic acid</td>
</tr>
<tr>
<td>Mobile phase B</td>
<td>Water containing 0.2% (v/v) formic acid</td>
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</tbody>
</table>

Gradient Profile

<table>
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<tr>
<th>Time (mins)</th>
<th>%A</th>
<th>%B</th>
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</thead>
<tbody>
<tr>
<td>Initial</td>
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<td>85.0</td>
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<tr>
<td>3.00</td>
<td>17.5</td>
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<td>72.5</td>
</tr>
<tr>
<td>6.10</td>
<td>95.0</td>
<td>5.00</td>
</tr>
<tr>
<td>6.90</td>
<td>95.0</td>
<td>5.00</td>
</tr>
<tr>
<td>7.00</td>
<td>15.0</td>
<td>85.0</td>
</tr>
<tr>
<td>7.50</td>
<td>15.0</td>
<td>85.0</td>
</tr>
</tbody>
</table>

Exemplary HPLC Method #2

Column: Waters BioSuite C18 PA-A, 3μη, 2.1 x 50 mm (Part# 188002425)

Mobile Phase A: 0.1:100 Formic Acid / Water, v/v

Mobile Phase B: 0.1:75:25 Formic Acid / Acetonitrile / Methanol, v/v/v

Flow Rate: 0.20 mL/min

Gradient:

<table>
<thead>
<tr>
<th>Time</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>95.0</td>
<td>5.0</td>
</tr>
<tr>
<td>0.50</td>
<td>95.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. The disclosures of all patent and scientific literature cited herein are expressly incorporated in their entirety by reference.
We claim:

1. A method of detecting human or humanized antibodies comprising the steps of:
   
   (a) treating a biological sample comprising a human or humanized antibody with a digestive enzyme to form a digested antibody sample, wherein the biological sample is serum, plasma, tissue, or cells from an animal that has been treated with a human or humanized antibody; and

   (b) analyzing the digested antibody sample by mass spectrometry to detect one or more human framework peptides, wherein the human framework peptides comprise one or more sequences selected from SEQ ID NOS. 1-8:

<table>
<thead>
<tr>
<th>Peptide</th>
<th>SEQ ID NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPSVPFLAPSSK</td>
<td>NO:1</td>
</tr>
<tr>
<td>STSGTAAALGCLVK</td>
<td>NO:2</td>
</tr>
<tr>
<td>TPEVTCVVVDVSHDEPEVK</td>
<td>NO:3</td>
</tr>
<tr>
<td>FNWYVDBGVEVHK</td>
<td>NO:4</td>
</tr>
<tr>
<td>VVSVLTVLHQLDGLNK</td>
<td>NO:5</td>
</tr>
<tr>
<td>ALPAPIEK</td>
<td>NO:6</td>
</tr>
<tr>
<td>GFYPSDIAVESNGQPENNYK</td>
<td>NO:7</td>
</tr>
<tr>
<td>TTPPVLDSDGSFLYK</td>
<td>NO:8</td>
</tr>
</tbody>
</table>

2. The method of claim 1 wherein the digestive enzyme is trypsin.

3. The method of claim 1 further comprising contacting the digested antibody sample with an affinity capture media or chromatography adsorbent and eluting an enriched digested antibody sample.

4. The method of claim 1 further comprising contacting the biological sample with an affinity capture media or chromatography adsorbent and eluting an enriched biological sample then treating the enriched biological sample with the digestive enzyme.

5. The method of claims 3 or 4 wherein the affinity capture media is bead-supported Protein A/G.
6. The method of claims 3 or 4 wherein the chromatography adsorbent is a solid-phase extraction (SPE) adsorbent.

7. The method of claim 1 wherein the biological sample is serum or plasma.

8. The method of claim 1 wherein the concentration of digested antibody sample is measured.

9. The method of claim 1 wherein the human or humanized antibody binds to one or more tumor-associated antigens or cell-surface receptors selected from (1)-(36):
   (1) BMPR1B (bone morphogenetic protein receptor-type IB);
   (2) E16 (LAT1, SLC7A5);
   (3) STEAP1 (six transmembrane epithelial antigen of prostate);
   (4) 0772P (CA125, MUC16);
   (5) MPF (MPF, MSLN, SMR, megakaryocyte potentiating factor, mesothelin);
   (6) Napi3b (NAPI-3B, NPTIib, SLC34A2, solute carrier family 34 (sodium phosphate), member 2, type II sodium-dependent phosphate transporter 3b);
   (7) Sema 5b (FLJ10372, KIAA1445, Mm.42015, SEMA5B, SEMAG, Semaphorin 5b Hlog, sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B);
   (8) PSCA hlg;
   (9) ETBR (Endothelin type B receptor);
   (10) MSG783 (RNF124, hypothetical protein FLJ20315);
   (11) STEAP2 (HGNC_8639, IPCA-1, PCANAP1, STAMP1, STEAP2, STMP, prostate cancer associated gene 1, prostate cancer associated protein 1, six transmembrane epithelial antigen of prostate 2, six transmembrane prostate protein);
   (12) TrpM4 (BR22450, FLJ20041, TRPM4, TRPM4B, transient receptor potential cation channel, subfamily M, member 4);
   (13) CRIPTO (CR, CR1, CRGF, CRIPTO, TDGFl, teratocarcinoma-derived growth factor);
   (14) CD21 (CR2 (Complement receptor 2) or C3DR (C3d/Epstein Barr virus receptor) or Hs 73792);
   (15) CD79b (CD79B, CD79b, IGb (immunoglobulin-associated beta), B29);
   (16) FcRH2 (IFGP4, IRTA4, SPAP1A (SH2 domain containing phosphatase anchor protein la), SPAP1B, SPAP1C);
   (17) HER2 (ErbB2);
(18) NCA;
(19) MDP;
(20) IL20Ra;
(21) Brevican;
(22) EphB2R;
(23) ASLG659;
(24) PSCA;
(25) GEDA;
(26) BAFF-R (B cell -activating factor receptor, BLyS receptor 3, BR3);
(27) CD22 (B-cell receptor CD22-B isoform);
(28) CD79a (CD79A, CD79a, immunoglobulin-associated alpha);
(29) CXCR5 (Burkitt's lymphoma receptor 1);
(30) HLA-DOB (Beta subunit of MHC class II molecule (Ia antigen));
(31) P2X5 (Purinergic receptor P2X ligand-gated ion channel 5);
(32) CD72 (B-cell differentiation antigen CD72, Lyb-2);
(33) LY64 (Lymphocyte antigen 64 (RP105), type I membrane protein of the leucine rich repeat (LRR) family);
(34) FcRH1 (Fc receptor-like protein 1);
(35) IRTA2 (FcRH5, Immunoglobulin superfamily receptor translocation associated 2); and
(36) TENB2 (putative transmembrane proteoglycan).

10. The method of claim 1 wherein the human or humanized antibody is selected from trastuzumab, ocrelizumab, pertuzumab, anti-PDL1, anti-neuropilin-1, anti-MUC16, rituximab, anti-mesothelin, and anti-LY6E.

11. The method of claim 1 wherein the human or humanized antibody is conjugated to a drug moiety.

12. The method of claim 11 wherein the drug moiety is selected from a maytansinoid, dolastatin, auristatin, calicheamicin, pyrrolobenzodiazepine (PBD), PNU-159682, anthracycline, duocarmycin, vinca alkaloid, taxane, trichothecene, CC1065, duocarmycin, camptothecin, elinafide, and stereoisomers, isosteres, analogs or derivatives thereof.
| CytoHC 1a D3 1 | 1 | ASTKGPSVFPLAPSSR STSESTAALGCL | 28 |
| CytoHC 1b E5 1 | 1 | ASTKGPSVFPLAPSSR STSESTAALGCL | 28 |
| Cyto HC 2a | 1 | ASTKGPSVFPLAPSSR STSQAALGCL | 28 |
| CytnoHC 2b E6 1 | 1 | ASTKGPSVFPLAPSSR STSQAALGCL | 28 |
| CytoHC 3 | 1 | ASTKGPSVFPLAPSSR STSESTAALGCL | 28 |
| Hu2H7 HC | 101 | YYSNSYWYFDVWGQGTLVTSSASTSKGSVFPLAPSSK STSGTAALGCL | 150 |

(CDR)

| CytoHC 1a D3 1 | 29 | VKDYFPEPVTVSWSNSGLTSGVHTFPAPLQSSGLYLSSVTVPSSSLGT | 78 |
| CytoHC 1b E5 1 | 29 | VKDYFPEPVTVSWSNSGLTSGVHTFPAPLQSSGLYLSSVTVPSSSLGT | 78 |
| Cyto HC 2a | 29 | VKDYFPEPVTVSWSNSGALTSGVHTFPAPLQSSGLYLSSVTVPSSSLGT | 78 |
| CytnoHC 2b E6 1 | 29 | VKDYFPEPVTVSWSNSGALTSGVHTFPAPLQSSGLYLSSVTVPSSSLGT | 78 |
| CytoHC 3 | 29 | VKDYFPEPVTVSWSNSGALTSGVHTFPAPLQSSGLYLSSVTVPSSSLGT | 78 |
| Hu2H7 HC | 151 | VKDYFPEPVTVSWSNSGALTSGVHTFPAPLQSSGLYLSSVTVPSSSLGT | 200 |

(UPPER HINGE)

| CytoHC 1a D3 1 | 79 | QTYVCNVNHKFSNTKVDKREVIEKTCHGSGKPTCPCPAPELLGGPSVFL | 128 |
| CytoHC 1b E5 1 | 79 | QTYVCNVNHKFSNTKVDKREVIEKTCHGSGKPTCPCPAPELLGGPSVFL | 128 |
| Cyto HC 2a | 79 | QTYVCNVHHPSNTKVDKTVGLPCR--------STCCPCAPELLGGPSVFL | 121 |
| CytnoHC 2b E6 1 | 79 | QTYVCNVHHPSNTKVDKTVGLPCR--------STCCPCAPELLGGPSVFL | 121 |
| CytoHC 3 | 79 | QTYVCNVHHPSNTKVDKREVETPP--------CCPCAPELLGGPSVFL | 120 |
| Hu2H7 HC | 201 | QTYICNVNHKFSNTKVDKKVEFKTCDKT----HTCPPCAPELLGGPSVFL | 247 |

| CytoHC 1a D3 1 | 129 | FPPKPKDLMISRTPEVTCVVDVSDQEDPDVK FNWVYNGAEVHAQTKFR | 178 |
| CytoHC 1b E5 1 | 129 | FPPKPKDLMISRTPEVTCVVDVSDQEDPDVK FNWVYNGAEVHAQTKFR | 178 |
| Cyto HC 2a | 122 | FPPKPKDLMISRTPEVTCVVDVSDQEDPDVK FNWVYNGAEVHAQTKFR | 171 |
| CytnoHC 2b E6 1 | 122 | FPPKPKDLMISRTPEVTCVVDVSDQEDPDVK FNWVYNGAEVHAQTKFR | 171 |
| CytoHC 3 | 121 | FPPKPKDLMISRTPEVTCVVDVSDQEDPDVK FNWVYNGAEVHAQTKFR | 170 |
| Hu2H7 HC | 248 | FPPKPKDLMISRTPEVTCVVDVSDHEDPEVK FNWVYVGVEVHNAKTCKPR | 297 |

**Figure 1**
<table>
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<tr>
<th></th>
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<th>FSP6</th>
<th>FSP7</th>
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<td>228 EEQFNSTRYSVSRTVTHQDRLNGKEYTCKVSNNKALPAPRQKTVSSTKQG</td>
<td>278 PREPQVYTLPPREELTKQNVSLSLCLIKGFPYPSDIVVEWASNGQPENTYK</td>
<td>328 TTPPVDSDGSGYFLYSLTVDKSRWQGNGVFCVSCVMHEALHNHTQKLSL</td>
</tr>
<tr>
<td>CynoHC 1b E5 1</td>
<td>179 EEQFNSTRYRVSVLTVTHTDRLNGKEYTCKVSNNKALPAPRQKTVSSTKQG</td>
<td>228 EEQFNSTRYRVSVLTVTHTDRLNGKEYTCKVSNNKALPAPRQKTVSSTKQG</td>
<td>278 PREPQVYTLPPREELTKQNVSLSLCLIKGFPYPSDIVVEWASNGQPENTYK</td>
<td>328 TTPPVDSDGSGYFLYSLTVDKSRWQGNGVFCVSCVMHEALHNHTQKLSL</td>
</tr>
<tr>
<td>Cyno HC 2a</td>
<td>172 ETQYNSTYRVSVLTVTHTDRLNGKEYTCKVSNNKALPAPRQKTVSSTKQG</td>
<td>221 EEQFNSTRYRVSVLTVTHTDRLNGKEYTCKVSNNKALPAPRQKTVSSTKQG</td>
<td>271 PREPQVYTLPPREELTKQNVSLSLCLIKGFPYPSDIVVEWASNGQPENTYK</td>
<td>321 TTPPVDSDGSGYFLYSLTVDKSRWQGNGVFCVSCVMHEALHNHTQKLSL</td>
</tr>
<tr>
<td>CynoHC 2b E6 1</td>
<td>172 EEQFNSTRYRVSVLTVTHTDRLNGKEYTCKVSNNKALPAPRQKTVSSTKQG</td>
<td>221 EEQFNSTRYRVSVLTVTHTDRLNGKEYTCKVSNNKALPAPRQKTVSSTKQG</td>
<td>271 PREPQVYTLPPREELTKQNVSLSLCLIKGFPYPSDIVVEWASNGQPENTYK</td>
<td>321 TTPPVDSDGSGYFLYSLTVDKSRWQGNGVFCVSCVMHEALHNHTQKLSL</td>
</tr>
<tr>
<td>CynoHC 3</td>
<td>171 EEQFNSTRYRVSVLTVTHTDRLNGKEYTCKVSNNKALPAPRQKTVSSTKQG</td>
<td>220 EEQFNSTRYRVSVLTVTHTDRLNGKEYTCKVSNNKALPAPRQKTVSSTKQG</td>
<td>270 PREPQVYTLPPREELTKQNVSLSLCLIKGFPYPSDIVVEWASNGQPENTYK</td>
<td>320 TTPPVDSDGSGYFLYSLTVDKSRWQGNGVFCVSCVMHEALHNHTQKLSL</td>
</tr>
<tr>
<td>Hu2H7 HC</td>
<td>298 EEQFNSTRYRVSVLTVTHTDRLNGKEYTCKVSNNKALPAPRQKTVSSTKQG</td>
<td>347 EEQFNSTRYRVSVLTVTHTDRLNGKEYTCKVSNNKALPAPRQKTVSSTKQG</td>
<td>397 PREPQVYTLPPREELTKQNVSLSLCLIKGFPYPSDIVVEWASNGQPENTYK</td>
<td>447 TTPPVDSDGSGYFLYSLTVDKSRWQGNGVFCVSCVMHEALHNHTQKLSL</td>
</tr>
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</table>

**Figure 1 (cont.)**
rhuMAbHER2, recombinant, humanized trastuzumab
Heavy Chain

EVQLVESGGGLVQPGGLSLRLSCAASGFTYKDYKHWVRQAPGKGLEWVARIYPTNGTRYADSV
    / KGRFTISADTSKNTAYLQMRLAEDTAVYGRGSGWGDGFYAMDYWGQGTLVTVSSASTKPS
    FSP1 / FSP2 / VFPLAPSSKstsGCTAALGCLVFDYFEPEPVTVSWSNGALTSGVHTFPAPVLGQSSGSLSVVT(V)PSS
    / SLGTQTYICVNVHKPSNTKVDKVEPKSCDKHTCPFPAPELLGSGSVFLFPPKDPDTLMISRTPE
    FSP3 / FSP4 / FSP5 / VTCVVVDVSHEDPEYKFWYVFDGEVEVHNAKTFRPREEQNYSTYKVSYSTLLHLDGWNLNGKEYK
    / FSP6 / / / FSP7
CKVSNKALPAPIETISKAKGQFREPQVYTLPPRSVEMTKQVSLCTLVKGFPYPSDIAVWEESNGQ
    / FSP8 /
FENNYKTPPPFDSDGSFFLYSKLTVDKSRWQQGVFSCVHSAHLHNHYTQKSLSLPAGK

Light Chain
D1QMTQSPSSLSASVGVDRVTITCRAQDVNTAVAHYQQKPGKAPKLLYASFLYSGVPSRFSGSR
SGTDFATLISLREDFATYCYQHYTPTPTFGQGTKEIKRTVAPSIFPPSDEQLKSGTASVC
LLLLNFYPREAKVQWKNVALQSGNSQESVTEQDSKDTYSLSTLTLKADYKHKVYACEVTH
QGLSSPVTKSFNRGEC

Figure 2
rhuMAb 2H7, 2H7, ocrelizumab
Heavy Chain

EVQILVESGGGLVQPGGLVRLSCASGTYTFTSYNMHWVRQAPGKGGLEWVGAIYPNGDTSYNQK
  /FKGRFTISVDSKNTLYLQMNSLRAEDTAVYCARVYYSSNYFVDWMQGTLVTVSSASTKG
   /FSP1 / FSP2 /PSVFPFLAPSSKSTSGRTAALGCLVKDYFPEPVTVSSSWGDSALTSGVHTPFAVLQQLSGLYSLLSVVTVP
      /SSSLGTQTYICNPHPSNTKVDKVEPLKKPDHTCPPLAPELLGGPSELLGFPLFPPKDKTLISRT
         /FSP3 / FSP4 / FSP5......../PEVTCVYDDVSHEDPEVKSNWYVDGVEVHNAKTTPREEQYNSTYRVSILTVLQDWLNGKEY
            / FSP6 / FSP7....KCKVSNKALPAPEKTISAKGQPREPQYTLPPSSREEMTKQVSLCLVKGFYPSDIAVEWESNG
                 / FSP8 /QPENNYKTTPVLDSDGSFFLYSKLTVDKSRWQGKGVSCSVMHEALHNYHTQKSLSLSPGK

Light Chain
DIQMTIQSPSSLSASVQTVSADRLTSWYQQKPGKAPKFLYAPSSLSAGPSVPSRGSGS
GTDFTLTISLQPEDFATYYCQOWSNPPTFQGKVEIKRTVAAFSVIFIPPSDEQNLGSTAVVCL
LNNFYPRAEKWQKVQTNALQSGNSQESVTEQDSDKSTYSLSSTLTLSKAYEHKVACEVTHQ
GLSSPVTKSFNREGE

Figure 3
Pertuzumab, rhuMAb 2C4, 2C4

Heavy Chain
EVQLVESGGGLVQPGSSLRSLCAASGFTFDYTMNVRQAPGKGLEWVADVNPNSSGLYQNYRGK
RFTLYDRSKNTLQMNLLRAEDTAVYYCARNLGPSFYFQWKQGTLVTVSSASTKPSVFLAP
SSKTSTSGTAALGCLVKDYFEPVFQTVSNWSGALTSGVHTFPAVLLQSSGLYSLSSVTVPSSSLGTQ
TYICNQNHKPSANTKVKVEPKSCDKTHCPCPCAPELLGGSVFLFRPFKDFDLMIISRTEPVCT
VVDVSHEDPEVKFNWYGDYVEHNAKTPFREEQNYSTYRVSLVTVLHQDWLNGKEYKCVSKAL
PAPIEKTISAKGQPREPGVTLPSREEMTKQVSLTCRVKGFYPSDIAWESNGPQENNYSTICK
PPVLDSDGSSFLYSLKTVKDRSWQQGNSVCSNHEALHNHYTQKSLSLFG (SEQ ID NO:20)

Light Chain
DIQMTIQSPSSLSASVGDRVTITCKASQDIKSIVGYAWQQPKGAASKLITYASRYTGVPSSSG
SGTDFTLTISQLPEDFAQYQCQYYIPYTFQGKTEIKRTVAAPSVFIPPSDEQLKSGTASV
VCLNNFYPREAKQVQKVNDALSQNSQESVTEQDSDKSTYSLSSTLTSKADYEHKVKYACEVTH
QLSSPFVTKSFNQEC (SEQ ID NO:21)

Figure 4

Anti-PDL1

Heavy Chain
EVQLVESGGGLVQPGSSLRSLCAASGFTFDYTMNVRQAPGKGLEWVAWISFYGGSTYADSVVG
RFTLSADTSKNTLYMQNLRLAEDTAVYYCARNWGPFDYNQGTLVTVSSASTKPSVFLAP
SSKTSTSGTAALGCLVKDYFEPVFQTVSNWSGALTSGVHTFPAVLLQSSGLYSLSSVTVPSSSLGTQ
TYICNQNHKPSANTKVKVEPKSCDKTHCPCPCAPELLGGSVFLFRPFKDFDLMIISRTEPVCTV
VVDVSHEDPEVKFNWYGDYVEHNAKTPFREEQNYSTYRVSLVTVLHQDWLNGKEYKCVSKAL
PAPIEKTISAKGQPREPGVTLPSREEMTKQVSLTCRVKGFYPSDIAWESNGPQENNYSTICK
PPVLDSDGSSFLYSLKTVKDRSWQQGNSVCSNHEALHNHYTQKSLSLFGK (SEQ ID NO:22)

Light Chain
DIQMTIQSPSSLSASVGDRVTITCRASQDVSTAVANYQQPKGAPKLITYASFLYGVSFPSRSUG
SGTDFTLTISQLPEDFAQYQCQYYIPYTFQGKTEIKRTVAAPSVFIPPSDEQLKSGTASV
VCLNNFYPREAKQVQKVNDALSQNSQESVTEQDSDKSTYSLSSTLTSKADYEHKVKYACEVTH
QLSSPFVTKSFNQEC (SEQ ID NO:23)

Figure 5
Anti_Neuropilin-a (anti-NRP1, MNRP1685A)

Heavy Chain
EVQLVESGGGLVQPSGSLSSCAASGFTSSYAMSNDWVRQAPGKGLEWVSQISPAGGYTNYADSVKG
RFTISADTSKNTAYLQMNLSRAEDATAVYCYARGELPYYRSMKVMDWQGGLVTSSASTKPSVFL
PLAPSSKSTSGTALGCLYFLPVEPVTVSNNSGALTSGVHTFPAVLGLSSLHSSVTTGVS\n
Light Chain
DQMTQPSASLSAASGDRVTITCRASQFYSSYLAWYQQKPQKAPKLILYGASSRAGVFPSRGSG
SGTDFTLTISSLQPEDFATYQYYCQLGSGPSFPGSTGEKVEIKRTVAAAASVFIFPSDEQLKGTASV
VCLLLNFYPREAKVQKVDNALQGNSQESVTEQDSDKSTYSLSSLTTLKSADYEHVKVAYCETV
HQLSSFVTKSFRNGEC (SEQ ID NO:24)

Figure 6

3A5 anti-MUC16

Heavy Chain
EVQLVESGGGLVQPSGSLSSCAASGFTSSYAMSNDWVRQAPGKGLEWVSQISPAGGYTNYADSVKG
RFTISADTSKNTAYLQMNLSRAEDATAVYCYARGELPYYRSMKVMDWQGGLVTSSASTKPSVFL
PLAPSSKSTSGTALGCLYFLPVEPVTVSNNSGALTSGVHTFPAVLGLSSLHSSVTTGVS\n
Light Chain
DQMTQPSASLSAASGDRVTITCRASQFYSSYLAWYQQKPQKAPKLILYGASSRAGVFPSRGSG
SGTDFTLTISSLQPEDFATYQYYCQLGSGPSFPGSTGEKVEIKRTVAAAASVFIFPSDEQLKGTASV
VCLLLNFYPREAKVQKVDNALQGNSQESVTEQDSDKSTYSLSSLTTLKSADYEHVKVAYCETV
HQLSSFVTKSFRNGEC (SEQ ID NO:26)

Figure 7
C2B8, anti-CD20, rituximab

Heavy Chain

QVQLQQPAGELVKPGASVKMSCKASGYTFTSVMHWVKQTPGRGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQSSLSTSEDSAVYYCARSTTYGGDWFNYWGANWAGTVSAASTKGPSVFPLAPSSKSTSGTAALGCLVKDYFEPFPVTVSWNSGALTSGVHTFPAVLQSSGLYSVQLSSVTVPSSSLGVTYYICNWNHKPSNTKVAVKFEKSCDKTHTCPPAPELGGPSVFLFPPSDFKDELTIIMISRTFEVTCVVDVSHDEPEVKFNWYVDGVEVHNAKTKPREEQYQQNYSTYRVVSVLTVHQQWDLWNGKEYKCKVKSNALKPAPIEKTISKAKGQPREPSQVYLTLPPSDELTKQVSLTLCEVKGFYPSDIAVEWESNQEPENYTTPPVLDSDGSFFLSKLTVDKSSRWQQGNYFCSVMHELHNYHTQKSLSLPGK (SEQ ID NO:28)

Light Chain

QIVLQSPAILSSAPGKEKVTMCRASSSATSYIHWFQQPAPGPSSIPWYATSNLASGVPRFSGSGSGTSYLTIISRVEAEADDAYCQWTSNPPTPFGGTGKLEIKRTVAAPSVFIFPSDEQLKSGTASVVELLNFYPREAKVQWVDNALQSGNSQESVTEQDSTDYSLSSTLTLSSKADYEKHKVEACEVTHQGLSSPVTKSFNRCGK (SEQ ID NO:29)

Figure 8
Intact protein

Trypsin

Peptide fragments

stable isotope labeled internal standards added

MS/MS – Q3 m/z

Peptide Q1 m/z

signature peptide

Stable isotope labeled internal standard (IS)

Relative Intensity

Time, min

Sig. peptide/IS Area

MAb Conc. (µg/mL)

Figure 9
FSP5: VVSVLTVLHQDWLNGK

Figure 11
Calibration Curve
Lithium Heparinised Cynomolgus Monkey Plasma
Whole Plasma Digest / SPE Approach
FSP8:    TTPVLDSDGSFFLYSK

Figure 12
Figure 14
Figure 15
Generic Capture

mAb

Protein A, G Bead

Magnet

Specific Capture

mAb

Biotin-Anti ID (or target antigen)

Streptavidin Coated Paramagnetic Bead

Magnet

Figure 16
Figure 18
Protein A bead capture / Trypsin digestion

Framework signature peptide (FSP8): TTPPVLDSDGSFFLYSK

MRM transition: 938.0 (M, 2+) → 836.7 (y15, 2+)

LC-MS/MS

Figure 19b
Anti-HER2 (trastuzumab)

**Figure 20**
Anti-MUC16

Figure 21
Anti-mesothelin

Figure 22
Figure 25
**INTERNATIONAL SEARCH REPORT**

**International application No**
PCT/US2012/037455

### A. CLASSIFICATION OF SUBJECT MATTER

**INV. G01N33/68**

**ADD.**

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)

G01N

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

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

**EPO-Internal**, BIOSIS, EMBASE, FSTA, PAJ, WPI Data, Sequence Search

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

Further documents are listed in the continuation of Box C.

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 application or patent but published on or after the international filing date
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Date of the actual completion of the international search

10 July 2012

Date of mailing of the international search report

17/07/2012

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Lunter, Pirn

Form PCT/A/210 (second sheet) (April 2005)
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<th>Category</th>
<th>Citation</th>
<th>Relevant to claim No.</th>
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<tbody>
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<td>A</td>
<td>OLIVIER HEUDI ET AL: &quot;Towards Absolute Quantification of Therapeutic Monoclonal Antibodies in Serum by LC-MS/MS Using Isotope-Labelled Antibody Standard and Protein Cleavage Isotope Dilution on Mass Spectrometry&quot;, ANALYTICAL CHEMISTRY, vol. 80, no. 11, 1 June 2008 (2008-06-01), pages 4200-4207, XP55031993, ISSN: 0003-2700, DOI: 10.1021/ac800205s the whole document</td>
<td>1-12</td>
</tr>
</tbody>
</table>
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).

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

see additional sheet

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable 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 report covers 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.

☒ 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: l-12 (partially)

A method of detecting human or humanized antibodies comprising detecting a human framework peptide which is SEQ ID NO. 1

2. claims: l-12 (partially)

A method of detecting human or humanized antibodies comprising detecting a human framework peptide which is SEQ ID NO. 2

3. claims: l-12 (partially)

A method of detecting human or humanized antibodies comprising detecting a human framework peptide which is SEQ ID NO. 3

4. claims: l-12 (partially)

A method of detecting human or humanized antibodies comprising detecting a human framework peptide which is SEQ ID NO. 4

5. claims: l-12 (partially)

A method of detecting human or humanized antibodies comprising detecting a human framework peptide which is SEQ ID NO. 5

6. claims: l-12 (partially)

A method of detecting human or humanized antibodies comprising detecting a human framework peptide which is SEQ ID NO. 6

7. claims: l-12 (partially)

A method of detecting human or humanized antibodies comprising detecting a human framework peptide which is SEQ ID NO. 7

8. claims: l-12 (partially)

A method of detecting human or humanized antibodies...
compri si ng detecti ng a human framework pepti de which i s SEQ
ID NO. 8
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<td>WO 2011042027 A2</td>
<td>14-04-2011</td>
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