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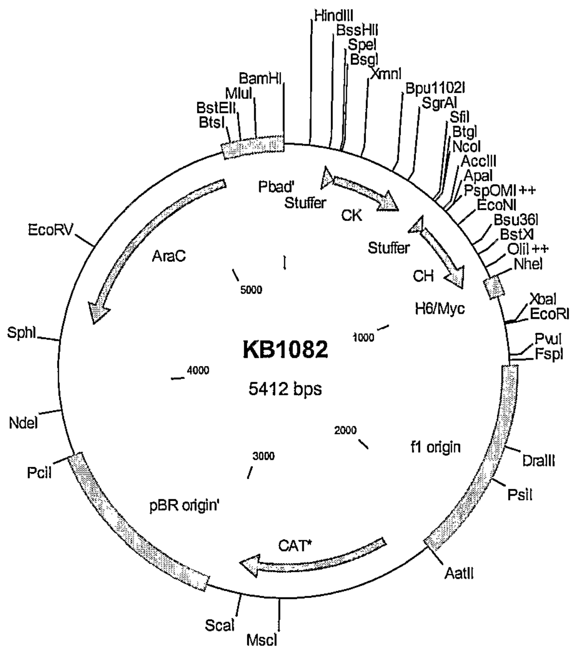
(54) Title: ANTIBODY SPECIFICITY TRANSFER USING MINIMAL ESSENTIAL BINDING DETERMINANTS



(57) Abstract: The present invention provides methods of making antibodies having the binding specificity of a reference antibody. Antibodies generated by the methods of the inventions have at least one minimal essential binding specificity determinant from a heavy chain or light chain CDR3 from the reference antibody. The method can be used, e.g., in humanization procedures. The invention also provides libraries and antibodies made in accordance with the methods.

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Antibody Specificity Transfer Using Minimal Essential Binding Determinants

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. provisional application no. 60/537,364, filed January 20, 2004 and U.S. provisional application no. 60/545,216, filed February 23, 2004, each of which applications is herein incorporated by reference.

BACKGROUND OF THE INVENTIONW

[0002] Many molecular targets suitable for antibody-mediated disease therapy have been validated with the use of non-human antibody reagents, and this process will continue for many of the new therapeutic targets which are expected to emerge from the human genome in the coming years. As a target becomes validated for therapy, the antibodies, typically murine, used to validate the target become leads for the development of biologic drugs. However, for many therapeutic applications the efficacy and safety of non-human antibodies are compromised because of their immunogenicity in patients. Thus, before such antibodies can be approved for therapeutic use they must be replaced with human counterparts having equivalent bioactivity, or they must be "humanized" in some way to eliminate or minimize immunogenicity in humans.

[0003] Established methods for the isolation of antigen-specific human antibodies include the screening of hybridomas from mice that are transgenic for the human immunoglobulin loci (*e.g.*, Jakobavits, 1998, *Adv Drug Deliv Rev.* 31:33-42), and *in vitro* methods in which recombinant libraries of human antibody fragments displayed on and encoded in filamentous bacteriophage (*e.g.*, McCafferty *et al.*, 1990, *Nature* 348:552-554), yeast cells (*e.g.*, Boder and Wittrup, 1997, *Nat Biotechnol* 15:553-557), and ribosomes (*e.g.*, Hanes and Pluckthun, 1997, *Proc Natl Acad Sci U S A* 94:4937-4942) are panned against immobilized antigen. These methods have yielded many useful human antibodies. However, for many non-human antibodies with desirable therapeutic properties, human antibodies with equivalent bioactivities have not been isolated using these methods.

[0004] Mice transgenic for human immunoglobulin loci generally do not express the full complement of human diversity, and affinity maturation is less efficient. Thus, the success rates for desired affinities and specificities tend to be lower than with conventional mice. The principal limitations of the display technologies stem from biased expression of antibody repertoires, and the specificity and affinity limitations of naïve repertoires. Antigen-binding antibodies from naïve libraries typically require additional affinity maturation, which with current *in vitro* methods is an arduous and uncertain process and moreover, may introduce immunogenic epitopes into the antibody.

[0005] The most widely used methods for minimizing the immunogenicity of non-human antibodies while retaining as much of the original specificity and affinity as possible involve grafting the CDRs of the non-human antibody onto human frameworks typically selected for their structural homology to the non-human framework (Jones *et al.*, 1986, *Nature* 321:522-5; US Patent 5,225,539). Originally these methods resulted in drastic losses of affinity. However, it was then shown that some of the affinity could be recovered by restoring the non-human residues at key positions in the framework that are required to maintain the canonical structures of the non-human CDRs 1 and 2 (Bajorath et al., 1995, *J Biol Chem* 270:22081-4; Martin et al., 1991, *Methods Enzymol.* 203:121-53; Al-Lazikani, 1997, *J Mol Biol* 273:927-48). Recovering the native conformations of CDR3s is a much more uncertain enterprise because their structures are more variable. Determining which non-human residues to restore to recover functional CDR3 conformation is thus largely a matter of modeling where possible combined with trial and error. As a result, in many cases the full affinity of the original non-human antibody is not recovered. Exemplary methods for humanization of antibodies by CDR grafting are disclosed, for example, in U.S. Patent No. 6,180,370.

[0006] To mitigate the shortcomings of the traditional CDR-grafting approaches, various hybrid selection approaches have been tried, in which portions of the non-human antibody have been combined with libraries of complementary human antibody sequences in successive rounds of selection for antigen binding, in the course of which most of the non-human sequences are gradually replaced with human sequences. These approaches have generally not fared better than CDR-grafting, however. For example, in the chain-shuffling technique (Marks, et al., 1992, *Biotechnology* 10:779-83) one chain of the non-human antibody is combined with a naïve human repertoire of the other chain on the rationale that the affinity of the non-human chain will be sufficient to constrain the selection of a human partner to the same epitope on the antigen. Selected human partners are then used to guide selection of human counterparts for the remaining non-human chains.

[0007] Other methodologies include chain replacement techniques where the non-human CDR3s were retained and only the remainder of the V-regions, including the frameworks and CDRs 1 and 2, were individually replaced in steps performed sequentially (*e.g.*, U.S. Patent Application No. 20030166871; Rader, *et al.*, *Proc Natl Acad Sci USA* 95:8910-15, 1998; Steinberger, *et al., J. Biol. Chem.* 275:36073-36078, 2000; Rader, et al., J. Biol. Chem. 275:13668-13676, 2000). However, this strategy still has the drawback that selectable human V-regions must be compatible not just with the non-human CDR3 but with the non-human companion V-region as well. This inter-species compatibility imposes a high demand for structural homology in the selected human V-regions, such that those most homologous to the non-human V-regions are generally selected. In this regard, the result is quite similar to that of the CDR grafting approach, except that CDRs 1 and 2 are initially human in the selected V-regions.

[0008] Existing methods for the isolation of human antibodies with required bioactivities for therapeutic use, or for "humanizing" non-human antibodies for therapeutic use thus have many limitations, as noted above. Primarily, these limitations relate to the retention or inclusion of nonhuman sequences in order to maintain binding affinity. Thus, there is a need for efficient humanization methods that minimize nonhuman sequences and thereby minimize introduction of immunogenic epitopes. The current invention addresses this need.

BRIEF SUMMARY OF THE INVENTION

[0009] The invention provides methods of creating antibodies with the binding specificity of a reference antibody for a target antigen by transferring the minimal essential binding specificity determinants from the reference antibody. This can be accomplished by transferring the minimal essential binding specificity in the context of transferring a D segment, or a CDR3, or a CDR3-FR4, or any other CDR3-FR4 fragment that comprises the minimal essential binding specificity determinant. Antibodies created using these methods retain the binding specificity, and often affinity, of the reference antibody with minimal divergence from germline sequences. Accordingly, such antibodies, *e.g.*, humanized antibodies, should be less immunogenic than those derived from prior art technologies.

[0010] In one aspect, the invention provides a method of making an antibody having a binding specificity of a reference antibody, the method comprising:

a) joining a heavy chain CDR3 binding specificity determinant (BSD) from the reference antibody to a diverse population of human VH segments thereby creating a library of human VH regions having the reference antibody heavy chain CDR3 BSD;

b) joining a light chain CDR3 BSD from the reference antibody to a diverse population of human VL segments, thereby creating a library of human VL regions having the reference antibody light chain CDR3 BSD;

c) combining the libraries of step a and step b to create an antibody library comprising members where a member has one VH comprising the reference antibody heavy chain CDR3 BSD and one VL comprising the reference antibody light chain CDR3 BSD; and

d) isolating a member of the library of step c that binds the same antigen as the reference antibody. In one embodiment, the diverse population of human VH segments is human germline. The diverse population of human VL segments can also be human germline, or near human germline. Thus both the diverse population of VH segment and the diverse population of VL segments can be human germline. In some embodiments, *e.g.,*, embodiments, in which a selected antibody has a germline VH and/or VL segment, the method further comprises mutagenizing one or both CDR3s from an antibody selected in step (d) and selecting an antibody that has a higher affinity for the target antigen than antibody selected in step (d).

[0011] The diverse population of human VH segments and/or the diverse populations of human VL segments can also be from one V segment subclass.

[0012] In some embodiments, at least one of the CDR3 BSDs from the reference antibody is a CDR3-FR4 segment from the reference antibody.

[0013] An FR4 can be a human germline FR4. In some embodiments, the human FR4 is a library of diverse human FR4 sequences.

[0014] In some embodiments of the invention, the J segment is a human antibody J segment.

[0015] The BSD from the reference antibody can be a heavy or light chain CDR3 from the reference antibody. In some embodiments, the BSD for both the heavy and light chain is the CDR3 from the reference antibody.

[0016] The heavy chain BSD can also be the D segment from the reference antibody. In other embodiments, the heavy and/or light chain BSD can be the minimal essential binding specificity determinant from the reference antibody.

[0017] In some embodiments, the step of isolating a member of the library of step (c) comprises a screening step to identify a member of the library that binds to the antigen with the same or higher affinity than the reference antibody.

[0018] For the methods of the invention, a reference antibody can be any antibody, but is typically a non-human antibody.

[0019] The step of combining the libraries can comprise expressing the library of human VH regions and the library of human VL regions on a single expression vector using separate promoters, or using a single promoter to drive expression of the VH and VL regions. Alternatively, the step of combining the libraries can comprise expressing the library of human VH regions and the library of human VL regions using two expression vectors, one to express each library.

[0020] In the methods of the invention, the antibody library of step c) can comprise antibodies where an antibody is an IgG, an Fv, an Fab, an Fab', an F(ab')2, a single chain Fv, or an IgG with a deletion of one or more domains.

[0021] The step of isolating the members of the library can comprise various screening methodologies including using a colony lift binding assay or screening using display technology, such as bacteriophage display, yeast cell display, bacterial cell display, ribosome display, and mammalian cell display. In one embodiment, screening is performed by screening pools of library members.

[0022] In some embodiments, the method further comprises:
e) combining heavy chain V regions from a plurality of members selected in accordance with (d) with a library of human VL regions having the reference antibody light chain CDR3 BSD; and f) selecting a member that binds to the same antigen as the reference antibody.

[0023] In other embodiments, the method further comprises:
e) combining light chain V regions from a plurality of members selected in accordance with (d) with a library of human VH regions having the reference antibody heavy chain CDR3 BSD; and f) selecting a member that binds to the same antigen as the reference antibody.

[0024] The method can also comprise:

e) combining heavy chain V regions from a plurality of members selected in accordance with (d) with light chain V regions from a plurality of members selected in accordance with (d); and f) selecting a member that binds to the same antigen as the reference antibody.

[0025] The invention additionally provides a method of making an antibody having a binding specificity of a reference antibody, comprising:

a) joining a CDR3 BSD from the heavy chain of the reference antibody to a diverse population of human VH segments, thereby creating a population comprising diverse VH regions having a BSD from the reference antibody heavy chain CDR3;

b) combining the population of step a with a VL comprising a human germline subclass V segment joined to a light chain CDR3 BSD from the reference antibody to create an antibody library; and

c) isolating a member of the library of step c that binds the same antigen the reference antibody. At least one of the CDR3 BSDs from the reference antibody can be a minimal essential binding specificity determinant, or a D segment from the reference antibody (for the heavy chain), or a CDR3. In some embodiments, the diverse population of human VH regions comprises germline VH segments. In other embodiments, the J segment region of the VL of step (b) can also be a human germline sequence.

[0026] The invention also provides a method of making an antibody having a binding specificity of a reference antibody, the method comprising:

a) joining a CDR3 BSD from the light chain of the reference antibody to a diverse population of human VL segments, thereby creating a population comprising diverse VL regions having a BSD from the reference antibody light chain CDR3;

b) combining the population of step a with aVH comprising a human germline subclass V segment joined to a CDR3 BSD from the reference antibody to create an antibody library; and c) isolating a member of the library of step c that binds the same antigen the reference antibody. The BSDs from the reference antibody can be a minimal essential binding specificity determinant, or a D segment (where the BSD is from the heavy chain), or a CDR3. In some embodiments, the diverse population of human VL regions can comprise germline VL segments. In additional embodiments, the J segment region of the VH comprising the human subclass V segment joined to the CDR3 BSD is a human germline sequence.

[0027] The invention also provides a method of making an antibody having a binding specificity of a reference antibody, the method comprising:

a) joining a minimal essential binding specificity determinant from the light chain CDR3 of the reference antibody to a diverse population of human VL segments, thereby creating a population comprising diverse VL regions having a reference antibody light chain CDR3 BSD;

b) combining the population of step (a) with a VH region from the reference antibody to create an antibody library; and

c) isolating a member of the library of step c that binds the same antigen as the reference antibody. In some embodiments, the diverse population of human VL segments in (a) are germline.

[0028] The method can further comprise:

d) joining a BSD from the heavy chain CDR3 from the reference antibody to a diverse population of human VH segments, thereby creating a population comprising diverse VH regions having the reference antibody heavy chain CDR3 BSD;

e) providing a population of VL regions from the antibody isolated in (c);

f) combining the population of step (d) and step (e) to create an antibody library; and

g) isolating a member of the library of step (f) that binds the same antigen as the reference antibody.

[0029] The invention further provies a method of making an antibody having a binding specificity of a reference antibody, the method comprising

a) joining a BSD from the heavy chain CDR3 of the reference antibody to a diverse population of human VH segments, thereby creating a population comprising diverse VH regions having the reference antibody heavy chain CDR3 BSD, wherein the BSD is selected from the group consisting of the minimal essential binding specificity determinant, the D segment, and the D segment-FR4;

b) combining the population of step a with a VL region from the reference antibody to produce an antibody library; and

c) isolating a member of the library of (b) that binds the same antigen as the reference antibody. The diverse population of human VH segments of (a) can, *e.g,* be germline.

[0030] In some embodiments, the method o further comprises:

d) joining a BSD from the CDR3 from the light chain of the reference antibody to a diverse population of human VL segments, thereby creating a population comprising diverse VL regions having the reference antibody light chain CDR3 BSD;

e) providing a population of VH regions from the antibody isolated in (d);

f) combining the population of step (d) and step (e) to create an antibody library; and

g) isolating a member of the library of (f) that binds the same antigen as the reference antibody.

[0031] In another aspect, the invention provides libraries. For example, such a library can comprise a plurality of nucleic acids that encode a diverse population of heavy chain V segments, wherein the V segments are not linked to a CDR3. The invention also provides a library comprising nucleic acids that encode a diverse population of light chain V segments, wherein the V segments are not linked to a CDR3. The V segments of either or both libraries can be, *e.g.,* human germline.

[0032] In another embodiment, the invention provides a library comprising a plurality of human antibody V-region pairs where a V-region pair comprises: i) an unselected heavy chain V-region comprising a human V segment and a heavy chain CDR3 from a reference antibody, and ii) an unselected light chain V-region comprising a human V segment and a light chain CDR3 from the reference antibody.

[0033] In other embodiments, the library is a library comprising nucleic acids encoding human antibody V-region pairs, where the VH and VL V segments are each linked to a MEBSD from a reference antibody of interest.

[0034] A library of the invention can also comprise nucleic acids encoding a plurality of VH or VL regions, wherein the VH or VL regions comprise V segments from one VH or VL subclass, wherein the V regions lack D and/or J segments. In one embodiment, the V segments of the VH regions are germline and/or the V segments of the VL regions are germline.

[0035] The invention also provides a library comprising a plurality of antibody V region pairs, wherein a pair comprises: i) a heavy-chain V region comprising a binding specificity determinant BSD from a heavy chain CDR3 from a reference antibody joined to a diversity of V segments, and ii) a light chain V region comprising a BSD from a light chain CDR3 from the reference antibody joined to a diversity of V segments, wherein at least one of the BSDs comprises less than the reference antibody CDR3.

[0036] In another embodiment, a library of the invention is a library comprising a plurality of VH regions comprising a BSD from a heavy chain CDR3 of a reference antibody joined to a diverse population of VH segments, with the proviso that the BSD is less that the reference antibody heavy chain CDR3.

[0037] In other embodiments, a library of the invention comprises a plurality of VL regions comprising aBSD from a light chain CDR3 of a reference antibody joined to a diverse population of VL segments, with the proviso that the BSD is less that the reference antibody light chain CDR3.

[0038] In another aspect, the invention provides antibodies. In some embodiments, such an antibody comprises a VH region comprising a human V segment, a D segment from a non-human reference antibody and a human J segment, *e.g.,* a germline J segment. The human V segment can also be a germline V segment.

[0039] In another embodiment, the invention provides an antibody comprising a VH region having a human germline V segment and a BSD from a heavy chain CDR3 from a reference antibody. The BSD can be, *e.g.,* a CDR3-FR4 from the reference antibody, a CDR3 from the reference antibody or a D segment from the reference antibody, or an MEBSD.

[0040] The invention also provides an antibody comprising a VL region having a human germline V segment and a BSD from a light chain CDR3 from a reference antibody. The BSD can be , *e.g.,* a CDR3-FR4 from the reference antibody, an MEBSD, or a CDR3 from the reference antibody.

[0041] In any of the methods, libraries, or antibodies of the invention, an antibody can be an IgG, an Fv, an Fab, an Fab', an F(ab')2, a single chain Fv, or an IgG with a deletion of one or more domains.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] Figure 1 shows a plasmid map of a vector for expression of Fab fragments from *E. coli.* Plasmid KB1082 contains an arabinose-inducible promoter for directing expression of a dicistronic message containing coding sequences for a kappa light chain and a heavy chain Fd fragment.

[0043] Figure 2 shows the complete nucleotide sequence of KB1082.

[0044] Figure 3 shows a vector for the expression of Fab' fragments in *E. coli*. Plasmid KB5000 has two IPTG-inducible tac promoters for expression of a human kappa chain and a human heavy chain Fd' fragment.

[0045] Figure 4 shows the complete sequence of KB5000.

[0046] Figure 5 shows the sequence of the M166 CDRH3 region showing the D- and J-segments.

[0047] Figure 6 shows the sequence of variable regions of human antibodies containing a minimal essential binding specificity domain (MEBSD) in CDR3 of the heavy chain from the murine anti-PcrV antibody M166 and a complete human J-region (JH6). The MEBSD is shown in bold and underlined.

[0048] Figure 7 shows the sequence of variable regions of human antibodies containing a MEBSD in CDR3 of the heavy chain from the murine anti-PcrV antibody M166 and a complete human J-region (JH3). The MEBSD is shown in bold and underlined.

[0049] Figure 8 shows the results of an exemplary ELISA analysis to detect binding to PcrV antigen.

[0050] Figure 9 shows sequences of V-regions of anti-PcrV antibodies with sequences close to human germ-line. Amino acid residues which differ from the closest human germ-line sequence in the V-segment or FR4 are underlined and the CDR3 sequences are marked in bold. The VH-segment of antibody F6 is identical to human germ-line sequence VH3-33, except for the first amino acid. The VL-segment of antibody 1F1 is identical to human germ-line VκIII L6.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0051] As used herein, an "antibody" refers to a protein functionally defined as a binding protein and structurally defined as comprising an amino acid sequence that is recognized by one of skill as being derived from the framework region of an immunoglobulin encoding gene of an animal producing antibodies. An antibody can consist of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

[0052] A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region (V) of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively.

[0053] Antibodies exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, papain digestion above the hinge produces a Fab. Pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'2, a dimer of Fab' which itself is a light chain joined to VH-CH1-hinge by one or more disulfide bonds. The F(ab)'2 may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the (Fab')2 dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (see, Fundamental Immunology, W.E. Paul, ed., Raven Press, N.Y. (1993), for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab or Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies. Antibodies of the invention include single chain antibodies (antibodies that exist as a single polypeptide chain), often single chain Fv antibodies (sFv or scFv) in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide. The single chain Fv antibody is a covalently linked VH-VL heterodimer which may be expressed from a nucleic acid including VH- and VL- encoding sequences either joined directly or joined by a peptide-encoding linker. Huston, *et al.* (1988) *Proc. Nat. Acad. Sci.* USA, 85: 5879-5883. While the VH and VL are connected to each as a single polypeptide chain, the VH and VL domains associate non-covalently. The first functional antibody molecules to be expressed on the surface of filamentous phage were single-chain Fv's (scFv), however, alternative expression strategies have also been successful. For example Fab molecules can be displayed on phage if one of the chains (heavy or light) is fused to g3 capsid protein and the complementary chain exported to the periplasm as a soluble molecule. The two chains can be encoded on the same or on different replicons, but the two antibody chains in each Fab molecule assemble post-translationally and the dimer is incorporated into the phage particle via linkage of one of the chains to g3p (*see, e.g.*, U.S. Patent No: 5733743). The scFv antibodies and a number of other structures converting the naturally aggregated, but chemically separated light and heavy polypeptide chains from an antibody V region into a molecule that folds into a three dimensional structure substantially similar to the structure of an antigen-binding site are known to those of skill in the art (*see e.g.*, U.S. Patent Nos. 5,091,513, 5,132,405, and 4,956,778). Antibodies of the invention include all those that have been displayed on phage (*e.g.*, scFv, Fv, Fab and disulfide linked Fv (Reiter *et a*l. (1995) *Protein Eng.* 8: 1323-1331). Antibodies can also include diabodies and miniMabs.

[0054] "V-region" as used herein refers to an antibody variable region comprising the segments of Framework 1 (FR1), CDR1, Framework 2 (FR2), CDR2, Framework 3 (FR3), CDR3 and Framework 4 (FR4).

[0055] The term "V-segment" as used herein is that part of a variable region that comprises the segments FR1, CDR1, FR2, CDR2, and FR3 and does not include CDR3 and Framework 4 (FR4).

[0056] A "D-segment" refers to the region of a heavy chain variable region (in this case, a CDR3 in the V-region) that is encoded by a D gene segment. Similarly, a "J-segment" refers to a region encoded by a J gene segment. These terms include various modifications, additions, deletions, and somatic mutations that can occur or be introduced during affinity maturation.

[0057] "Binding" refers to the adherence of molecules to one another, for example, enzymes to substrates, antibodies to antigens, DNA strands to their complementary strands. Binding occurs because the shape and chemical natures of parts of the molecules surfaces are complementary. A common metaphor is the "lock-and-key," used to describe how enzymes fit around their substrate.

[0058] The "binding specificity" of an antibody refers to the ability of an antibody to recognize an antigen to the exclusion of other antigens and is generally measured against nonspecific background binding. Typically, an antibody is considered specific when it binds to the target antigen at least 10 times above background binding.

[0059] A "binding specificity determinant" (BSD) as used in the context of this invention refers to a CDR3-FR4 region, or a portion of this region that mediates binding specificity. BSDs function as heavy chain and light chain pairs, *i.e.,* a BSD functions together with its cognate partner on a complementary chain of a reference antibody. A binding specificity determinant therefore can be a CDR3-FR4, a CDR3, a minimal essential binding specificity determinant of a CDR3, the D segment (with regard to a heavy chain region), or other regions of CDR3-FR4 that confer the binding specificity of a reference antibody.

[0060] A "minimal essential binding specificity determinant" (MEBSD) as used herein refers to any region smaller than the CDR3 that confers binding specificity when present in the V region of an antibody. The MEBSD functions in a pair together with a cognate partner on a complementary chain of a reference antibody.

[0061] "Complementarity-determining region" or "CDR" refers to the art-recognized term as exemplified by the Kabat and Chothia. CDRs are also generally known as hypervariable regions or hypervariable loops (Chothia and Lesk (1987) *J. Mol. Biol.* 196: 901; Chothia *et al.* (1989) *Nature* 342: 877; Kabat *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md.) (1987); and Tramontano *et al.* (1990) *J. Mol. Biol.* 215: 175). "Framework region" or "FR" refers to the region of the V domain that flank the CDRs. The positions of the CDRs and framework regions can be determined using various well known definitions in the art, *e.g.,* Kabat, Chothia, international ImMunoGeneTics database (IMGT), and AbM (*see, e.g.,* Johnson *et al., supra*; Chothia & Lesk, 1987, *J. Mol. Biol.* 196, 901-917; Chothia, *et al.*, 1989, *Nature* 342, 877-883; Chothia, *et al.*, 1992, *J. Mol. Biol.* 227, 799-817; Al-Lazikani *et al., J.Mol.Biol* 1997, 273(4)). Definitions of antigen combining sites are also described in the following: Ruiz *et al.*, IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res.*, 28, 219–221 (2000); and Lefranc,M.-P. IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res.* Jan 1;29(1):207-9 (2001); MacCallum *et al*, *J. Mol. Biol.*, 262 (5), 732-745 (1996); Martin *et al*, *Proc. Natl Acad. Sci.* USA, 86, 9268–9272 (1989); Martin, *et al*, *Methods Enzymol.*, 203, 121–153, (1991); Pedersen *et al*, *Immunomethods*, 1, 126, (1992); and Rees *et al*, In Sternberg M.J.E. (ed.), Protein Structure Prediction. Oxford University Press, Oxford, 141–172 1996).

[0062] "Antigen" refers to substances that are capable, under appropriate conditions, of inducing a specific immune response and of reacting with the products of that response, that is, with specific antibodies or specifically sensitized T-lymphocytes, or both. Antigens can be soluble substances, such as toxins and foreign proteins, or particulates, such as bacteria and tissue cells; however, only the portion of the protein or polysaccharide molecule known as the antigenic determinant (epitopes) combines with the antibody or a specific receptor on a lymphocyte. More broadly, the term “antigen” is used herein to refer to any substance to which an antibody binds, or for which antibodies are desired, regardless of whether the substance is immunogenic. For such antigens, antibodies can be identified by recombinant methods, independently of any immune response.

[0063] “Epitope” refers to that portion of an antigen or other macromolecule capable of forming a binding interaction that interacts with the variable region binding pocket of an antibody. Typically, such binding interaction is manifested as an intermolecular contact with one or more amino acid residues of a CDR.

[0064] “Target” is used here to refer to the molecule to which a reference antibody binds. Thus, “target” is often used herein synonymously with “antigen”.

[0065] A "reference antibody” as used here refers to an antibody for which the practitioner wants to obtain a variant with “improved” characteristics, *e.g.,* reduced immunogenicity, increased affinity, and the like. The reference antibody is the source of the pairs of variable region BSDs.

[0066] “Library” means a collection of nucleotides sequences, *e.g.*, DNA, encoding antibodies within clones; or a genetically diverse collection of antibody polypeptides.

[0067] “Repertoire library” refers to a library of genes encoding antibodies or antibody fragments such as Fab, scFv, Fd, LC, VH, or VL, which is obtained from the natural ensemble, or “repertoire”, of antibodies present in human donors, and obtained primarily from the cells of peripheral blood and spleen. Often, the human donors are “non-immune”, i.e., not presenting with symptoms of infection.

[0068] An “expression vector” is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

[0069] "Link" or "join" or "fuse" refers to functionally connecting polypeptide, including, without limitation, recombinant fusion of the coding sequences.

[0070] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length, including full-length proteins (*i.e.*, antigens), wherein the amino acid residues are linked by covalent peptide bonds.

[0071] "Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs).

[0072] Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or residues such as deoxyinosine residues. The term nucleic acid refers to gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

[0073] "Recombinant nucleic acid" refers to a nucleic acid in a form not normally found in nature. That is, a recombinant nucleic acid is flanked by a nucleotide sequence not naturally flanking the nucleic acid or has a sequence not normally found in nature. Recombinant nucleic acids can be originally formed *in vitro* by the manipulation of nucleic acid by restriction endonucleases, or alternatively using such techniques as polymerase chain reaction. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, *i.e.*, using the *in vivo* cellular machinery of the host cell rather than *in vitro* manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention.

[0074] "Recombinant polypeptide" refers to a polypeptide expressed from a recombinant nucleic acid, or a polypeptide that is chemically synthesized *in vitro*.

[0075] "Purified"or "isolated" means that the indicated nucleic acid or polypeptide is present in the substantial absence of other biological macromolecules, *e.g.*, polynucleotides, proteins, and the like. In one embodiment, the polynucleotide or polypeptide is purified such that it constitutes at least 95% by weight, more preferably at least 99.8% by weight, of the indicated biological macromolecules present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 1000 daltons, can be present).

INTRODUCTION

[0076] Current antibody humanization strategies retain substantial amounts of non-human sequence in the human sequence selection process (*e.g.,* either all six CDRs or at least one entire V-region is retained in the initial selection). Retention of binding specificity and affinity is therefore constrained to depend on the selected human sequences being highly homologous to the original non-human sequences. At the same time, however, considerable sequence divergence is required to make non-human proteins sufficiently human to minimize, immunogenicity in humans. The result is a compromise between retention of binding specificity and affinity and replacement of the non-human protein sequence with human, such that "humanized" antibodies are not fully human, and have typically lost affinity relative to the starting antibody.

[0077] The present invention is based on the discovery that neither the optimal antigen-binding conformation of the BSD pair, nor antigen binding by the other CDRs, requires significantly greater homology than the average homology of human V-regions to those of the non-human in question, even though the vast majority of human V-regions would not support the antigen-binding conformation of the $CDR3_2$, nor provide antigen binding by the other CDRs. Thus, the invention provides methods of transferring BSD pairs from a reference antibody to human V-segments, thereby creating humanized antibodies that have minimal potentially immunogenic sequences.

[0078] Although the invention is largely described in terms of applying the methods to humanizing non-human reference antibodies, it is understood that the methods can be employed in any situation where it is desirable to transfer BSDs from a reference antibody to non-reference antibody V-segments. For example in certain applications, *e.g.,* veterinary medicine applications, it may be desirable to transfer the BSDs of a reference antibody to V segments from other species. The reference antibody can be from any species, including mouse, rat, or rabbit, as well as sheep, horse, bovine, goat, camellids, or primates, or any other vertebrate that produced antibodies.

[0079] The present invention provide methods of humanizing antibodies where the resulting antibodies retain binding specificity and affinity while at the same time have most of the non-human sequences replaced with human sequences. This is accomplished by transferring a BSD pair from the reference antibody, *e.g.,* a CDR3 pair ($CDR3_2$). In antibodies that are affinity-matured, *e.g.,* the reference antibody, the heavy chain and light chain BSDs are in close contact with one another and are optimized for mutual stabilization of the combined antigen-binding conformation, hence, they form a unit, *i.e.*, a BSD pair. The antigen-binding conformation is, of course, dependent on the support of the underlying frameworks of the V-regions. When an affinity-matured BSD, *e.g.,* that of the reference antibody, is combined with the structural diversity and stability of the complete human repertoire of heavy chain or light chain V-segment pairs, scaffolds that fully support the optimal antigen-binding conformation of the BSD are readily identified with the aid of selection systems including, but not limited to, phage display, cell viability, colony lift binding assays (CLBA), or a variety of immunoassays, *e.g.,* ELISA assays.

[0080] Further, transfer of a BSD pair to diverse germline V-segments often result in selection of V-regions that that have affinities of greater than 50 nM. These selected V-regions can also be incorporated into the affinity maturation process of any antibody. V-segment libraries are relatively small without CDR3 repertoires, thus selection of human V-regions can also be combined with limited mutagenic diversification of one or both BSDs in libraries of searchable size for many conventional selection systems.

[0081] It should be emphasized that while the present invention minimizes the homology constraint on antibody humanization, it does not necessarily impose any selective pressure against homology. Thus, human V-segments with high homology to the non-human parent segments can still be selected by the present invention, *e.g.,* if they retain equal or higher affinity for the antigen.

[0082] The following sections will additionally describe V-region repertoire cloning, transfer of BSDs, generation of libraries, and screening methodologies.

V-segment Repertoire Cloning

[0083] The V-segment repertoire used in generating libraries to replace the heavy and/or light chain V-segment of the reference antibody can be from any source. The human repertoires can be generated, *e.g.*, by polymerase chain reaction (PCR) amplification using primers appropriate for the desired segments from cDNA obtained from peripheral blood or spleen, in which case the repertoires are expected to contain clones with somatic mutations. Alternatively, the repertoires can be obtained by amplification of genomic DNA from non-immune system cells in order to obtain germline-encoded sequences.

[0084] The human germline V- segment repertoire consists of 51 heavy chain V-regions, 40 κ light chain V-segments, and 31 λ light chain V-segments, making a total of 3,621 germline V-region pairs. In addition, there are stable allelic variants for most of these V-segments, but the contribution of these variants to the structural diversity of the germline repertoire is limited. The sequences of all human germ-line V-segment genes are known and can be accessed in the V-base database, provided by the MRC Centre for Protein Engineering, Cambridge, United Kingdom (*see,* also Chothia *et al.*, 1992, *J Mol Biol* 227:776-798; Tomlinson *et al.*, 1995, *EMBO J* 14:4628-4638; and Williams *et al.*, 1996, *J Mol Biol* 264:220-232). V-segment variants generated by somatic hypermutagenesis during the affinity maturation process may also make important contributions to the V-segment repertoire, since these mutations appear to be non-random, and may confer structural adjustments which facilitate high-affinity antigen specificity. While naïve antibodies are optimized for broad specificity and low affinity for maximum binding diversity, affinity matured antibodies may contain structural adaptations which favor the more rigid CDRs required for high-affinity antigen-specific binding (*e.g.*, Diaz and Klinman, 2000, *Immunol Res.* 21:89-102).

[0085] Human V-region repertoires, both germline and affinity-matured, can be recovered, *e.g.*, from peripheral blood lymphocytes (PBL), often pooled from multiple (*e.g.*, at least 10) healthy individuals, using conventional cDNA cloning methods (Sambrook and Russell, eds, *Molecular Cloning: A Laboratory Manual*, 3rd Ed, vols. 1-3, Cold Spring Harbor Laboratory Press, 2001). Insofar as the germline frequency distribution is not uniform in expressed sequences, it is prudent to capture at least 10^3 independent clones for each of the three V-region isotypes (VH, Vκ, and Vλ) to ensure optimal diversity of the repertoires. The PCR can be used to amplify V-region sequences during the cloning process. However, exponential amplification mechanisms are prone to random biases, and this may be compounded by the use of degenerate primers, which have variable priming efficiencies, resulting in a loss of diversity. Thus, when amplification is desired, it may be desirable, where possible, to use a primer-independent linear amplification method, such as *in vitro* transcription (Sambrook and Russell, eds, *Molecular Cloning: A Laboratory Manual*, 3rd Ed, vols. 1-3, Cold Spring Harbor Laboratory Press, 2001).

[0086] In one embodiment, mRNA is isolated from human PBLs or other lymphocyte-rich tissues using standard methods (*e.g.*, *Current Protocols in Molecular Biology*, Ausubel, ed. John Wiley & Sons, Inc. New York, 1997). The human V-region sequences are copied and cloned using standard PCR protocols, *e.g.*, as described in the Examples or using *in vitro* transcription -based protocols. For example, in an *in vitro* transcription protocol, immunoglobulin-encoding first-strand cDNA is copied from the mRNA template using a reverse transcriptase (RT) and primers which are complementary to the human heavy chain and light chain constant region genes, Cμ1 or Cγ1, Cκ, and Cλ. Cμ1 primers are required for the capture of naïve germline VH sequences, while Cγ1 primers allow the capture of affinity-matured VH domains. For the synthesis of second-strand cDNA, the second-strand primers may also contain a promoter sequence, such as that of bacteriophage T7, which when incorporated into the complementary strand, allows continuous linear amplification of the V-region sequences *in vitro* using T7 DNA-dependent RNA polymerase (Sambrook and Russell, eds, *Molecular Cloning: A Laboratory Manual*, 3rd Ed, vols. 1-3, Cold Spring Harbor Laboratory Press, 2001). If desired, the amplification process can be repeated by copying the RNA into double-stranded cDNA using complementary primers, and then repeating the *in vitro* transcription step. The *in vitro* transcription procedure is necessary to provide a sufficient quantity of nucleic acid for cloning the V-region library into a plasmid vector, which should be a standard expression vector such as one of the pBR322 derivatives. After transformation into *E. coli* cells, the size of the V-region library in independent clones can be determined and the quality of the library assessed by sequencing a sufficient number of clones (for example at least 30 of each isotype) to determine the proportion of open reading frames and the proportional representation of germline diversity. It is relatively easy to collect at least 10^4 independent clones of each isotype, which should ensure that all germline genes are amply represented with open reading frames.

[0087] For BSD-guided V-segment selection, the human CDR3s is eliminated from the V-region repertoires. This can be readily done, e.g., during the final cDNA synthesis step, before cloning by using degenerate primers which are collectively complementary to the carboxyl terminal sequences of Framework 3 in all germline V-region genes. The C-terminal sequences of Framework 3 are highly conserved, so that a restriction endonuclease site can be included in the primer to create a universal "sticky end" for in-frame ligation to any guiding CDR3-FR4 without altering any Framework 3 sequence.

[0088] In other embodiments, the V-segment can be amplified by using suitable PCR primers, e.g., primers described in the Example section. Sets of oligonucleotide primers are designed to 5' and 3' regions of the V-segments for cloning the V-segment repertoire. Suitable primer sequences are known (*e.g.,* Welschof *et al., J. Immunological Methods* 179:203-214, 1995 and Little *et al., J. Immunological Methods*, 231:3-9, 1991]) or can be designed. An oligonucleotide or a set of oligonucleotides are used to construct CDR3-FR4 regions and are either linked to the V-segment sequences through an introduced restriction site or by overlap-extension PCR or by primer extension. For example, amplification primers at the 3' of the V-segment may be designed to sequences encoding amino acids 86-89 (according to the Kabat numbering system) for Vh; amino acids 81-86 for Vκ and 81-85 for Vλ and include a non-templated restriction site near the 5' end. The oligonucleotide encoding CDR3-FR4 may extend into the region encoding VYYCAR on the heavy chain (Kabat amino acids 89-94) or VYYC (Kabat residues 85-88) on the light chain and contain a restriction site compatible with that at the 3'end of the V-segment repertoire. Thus, a library of complete V-regions can be generated by ligation of the CDR3-FR4 oligonucleotide to the V-segment repertoire. The CDR3-FR4 sequence can also be joined to the V-segment by other methods known to those with skill in the art, such as overlap extension PCR or primer extension of cRNA synthesized from the V-segment repertoire. The sequences encoding complete V-regions are cloned into a suitable expression vector and can be fused to constant region sequences at this stage for expression as Fab, Fab' or other antibody fragments, whole IgG or fusion proteins used for display on a cell or virus.

[0089] Libraries for-guided V-region repertoires or V-region-guided BSD repertoires may be expressed in any cell type, including bacteriophage, yeast, bacteria, mammalian cells and the like. The library can be screened for antigen-binding activity by any of a number of assays, *e.g.,* high-throughput ELISA-based assays. Such libraries can also be expressed in display format, such as on bacteriophage, bacterial cells, yeast cells, mammalian cells or ribosomes, and screened for binders as described in the art.

[0090] A heavy or light chain V-segment library can be directed to only one V segment subclass or isotype. In such an embodiment, for example, where it is desired to select a human V segment isotype that most closely matches the V-segment isotype of the reference antibody, appropriate primers can be used to amplify only sequences corresponding to the desired isotype.

[0091] In some embodiments, the V-segments are germline. As noted above, there are 51 germ-line VH genes in humans and each of these can be recombined. There are 40 Vκ genes and 31 Vλ genes. The VH germ-line genes are sub-divided into 7 subclasses (VH1 – VH7) and the germ-line light chains are sub-divided into 16 sub-classes (Vκ1 – Vκ6 and Vλ1-Vλ10). Germ-line human V-gene sequences can be cloned from human genomic DNA by PCR or linear amplification methods in the same way that re-arranged and somatically mutated V-gene sequences are cloned from cDNA. For example, degenerate primers encoding all germline Framework 1 amino-terminal sequences (not including signal peptide leaders) and all Framework 3 carboxyl-terminal sequences can be used for ligation to CDR3. After cloning, selection for intact reading frames, sequence verification, and archiving, the repertoires can be used for assembly of combinatorial human V-region libraries for BSD pairs.

Transferring BSDs of the reference antibody

[0092] BSDs from the reference antibody are transferred to a library of V-segment sequences generated as described above. The BSDs can be incorporated into the expression vector before or after the population of V-segments is cloned into the expression vector. The BSD that is transferred can be a CDR3-FR4, a CDR3, a D segment (where the BSD is from the heavy chain), a MEBSD, or any other fragment of CDR3-FR4 that has binding specificity in combination with the complementing BSD from the other chain of the reference antibody. It is understood that when transferring a BSD from a reference antibody to a different V-region, the structure of the heavy or light chain V region is maintained in the resulting V-region. Thus, if the BSD from the reference antibody is a subregion of CDR3-FR4, the complete CDR3-FR4 structural length is maintained, *i.e.,* the remainder of the CDR3-FR4 residues that are not from the reference antibody are made up of other residues, typically human germline residues.

[0093] As noted, the BSD can include Framework 4 regions, *e.g.,* from the reference antibody, which are part of the J-segments, but which are highly conserved among mammals, and are important for CDR3 structure. These sequences can, for example, be amplified by PCR with primers containing restriction sites for in-frame ligation to Framework 3, and other unique restriction sites downstream from the carboxyl terminus of Framework 4, *e.g.,* for ligation to the C-region. Each CDR3-FR4 is then transferred into the appropriate sites of the V-region library construct. Alternatively, the desired sequence or mix of sequences for the CDR3-FR4 region can be synthesized as one continuous oligonucleotide or mix of oligonucelotides and can be joined to the V segment repertoire by primer extension using *in vitro* transcribed cRNA synthesized from the repertoire as a template for first-strand cDNA synthesis. Diversity can be introduced into a region, *e.g.,* CDR3 and/or FR4.

[0094] In other embodiments, the FR4 region can be a human FR4, *e.g.,* a germline FR4. In some embodiments, the libraries can comprise a diversity of FR4 sequences. The human FR4 sequences are typically introduced by PCR using appropriate primers to amplify the FR4 sequences to be incorporated into the library expression vector. The FR4 sequences can be introduced into a library to which the CDR3 or MEBSD has already been transferred, or can be introduced concurrently with the CDR3 or MEBSD. Diversity can be introduced into the FR4 using mixed oligonucleotides, or mutagenesis protocols as described for introducing diversity into a CDR3. The FR4 can be cloned from a library, *e.g.,* as a J segment from a human repertoire.

[0095] The BSD that is transferred can also be a reference antibody CDR3. Again, the transfer is performed via amplification methodology to amplify the desired sequence containing the CDR3 for incorporation into the expression vector at the appropriate site using known methodologies

Defining Minimum Essential Binding Specificity Determinants

[0096] The BSD can also be a sequence that is less than the complete CDR3, *e.g.* the D segment of a heavy chain CDR3 or a MEBSD. As appreciated by one of skill in the art, when the reference antibody BSD is less than a complete CDR3, a complete CDR3 still results in the antibody expression library, as the remaining CDR3 residues are incorporated into the construct. For example, appropriate oligonucleotides can be designed to incorporate human sequences, *e.g.,* germline J segments, to replace the CDR3 residues that are not part of the MEBSD.

[0097] The MEBSD is the region within a CDR3 sequence or a pair of CDR3s that is required to retain the binding specificity of the reference antibody when combined with human sequences that re-constitute the remainder of CDR3 and the rest of the V-region. The MEBSD can be defined empirically or can be predicted from structural considerations.

[0098] For empirical determination, methods such as alanine scanning mutagenesis can be performed on the CDR3 region of a reference antibody (Wells, *Proc. Natl Acad. Sci. USA* 93:1-6, 1996) in order to identify residues that play a role in binding to antigen. Additional analyses can include Comprehensive Scanning Mutagenesis, in which each residue of CDR3 is replaced, one-at-a-time, with each of the 19 alternative amino acids, rather than just replacement with alanine. Binding assays, *e.g,* colony-lift binding assays, can be used to screen libraries of such mutants to determine those mutants that retain binding specificity. Colonies that secrete antibody fragments with assay signals reduced by at least ten-fold relative to the reference antibody can be sequenced and the DNA sequences used to generate a database of amino acid positions in CDR3 that are important for retention of binding. The MEBSD can then be defined as the set of residues that do not tolerate single-site substitution, or which tolerate only conservative amino acid substitution.

[0099] An MEBSD can also be determined by deletion analysis in which progressively shorter sequences of a reference antibody CDR3 are evaluated for the ability to confer binding specificity and affinity. This is accomplished by substituting the CDR3 residues with progressively longer human sequences, *e.g.,* from a human germline J segment.

[0100] The MEBSD can also be deduced from structural considerations. For example, if the x-ray crystal structure is known, or if a model of the interaction of antibody and antigen is available, the MEBSD may be defined from the amino acids required to form suitable contact with the epitope and to retain the structure of the antigen-binding surface.

[0101] Alternatively, the MEBSD can be predicted from the primary structure of the CDR3. In V_H domains, for instance, the MEBSD can, in some antibodies, correspond to a D-segment (including any deletions or identifiable N-additions resulting from the re-arrangement and maturation of the reference antibody). In this case, the J-segment may be replaced by a cloned human J-segment or a repertoire of J-segments. The binding specificity of the modified reference V_H-domain with substituted J-segment may be determined in combination with a suitable complementary light-chain. This complementary chain can be the light chain of the reference antibody or can be a human light chain containing the CDR3 of the light chain of the reference antibody. Binding specificity can be determined by colony-lift binding assay or by another known assay methodology. If colonies secreting antigen-binding antibodies are not identified by this approach, additional sequences from the reference antibody CDR3 may be substituted for corresponding sequences in the J-segment and these additional mutants screened with the complementary light chain until a MEBSD is identified.

[0102] MEBSDs can similarly be identified in CDR3 of the light chain, in which case the complementary chain used in the screening assay comprises a V_H-domain. In this case the V_H domain may be derived from the reference antibody or may be a human V_H domain with the CDR3 from the reference antibody. As there is no D-segment in the light chain, the MEBSD can be deduced by scanning mutagenesis or by inspection of the sequence of CDR3 and substitution of those sequences in CDR3 encoded by the V-gene segment, or those sequences encoded by the J-segment. Screening for antigen binding, *e.g.,* by colony-lift binding assay, can be used to define which segment of the CDR3 constitutes the MEBSD.

[0103] Further, software programs such as JOINSOLVER™ Souto-Carneiro, et al., *J. Immunol.* 172 :6790-6802, 2004). can be used to analyze CDR3 of immunoglobulin gene to search for D germline sequences. The strategy of JOINSOLVER® is to search for D germline sequences flanking V_H and J_H germline genes. Additionally, it searches for P- and N-type additions in the V_HD and DJ_H junctions. The human D germline gene database employed includes all D segments from the IMGT databank as well as the reverse and DIR germline genes.

Expression of antibodies

[0104] Libraries of secreted antibodies or antibody fragments can be expressed in prokaryotic or eukaryotic microbial systems or in the cells of higher eukaryotes such as mammalian cells. The antibody library can be a library where the antibody is an IgG, an Fv, an Fab, an Fab', an $F(ab')_2$, a single chain Fv, an IgG with a deletion of one more domains, or any other antibody fragment that includes the V-region.

[0105] The antibodies can be displayed on the surface of a virus, cell, spore, virus-like particle, or on a ribosome. For this purpose, one or both chains of the antibody fragment are typically expressed as a fusion protein, for example as a fusion to a phage coat protein for display on the surface of filamentous phage. Alternatively, the antibodies of the antibody library can be secreted from a host cell.

[0106] The following provides an exemplary description using secretion systems to express the antibodies as Fab or Fab' fragments. It is readily apparent to those in the art, however, that the expression systems can be adapted for any library format. For this general example, a library of complete V-regions is constructed by ligation of oligonucleotides encoding CDR3-FR4 segments to the V-segment repertoire as described above. The amplified sequences encoding complete V-regions are cloned into a suitable expression vector and can be fused to constant region sequences at this stage for expression of Fab or Fab' molecules. The antibody fragments can be secreted from prokaryotic or eukaryotic cells including bacteria, yeast, plant cells and mammalian cells.

[0107] In one preferred method, the V-region libraries are expressed and secreted as assembled and functional Fab or Fab' fragments from a microbial host cell. Secreted fragments are then screened for antigen binding *e.g.*, by a filter screening assay or ELISA as described further below. An example of a suitable expression vector for secretion of antibody fragments from yeast is pESC (obtained from Stratagene), which contains two separate promoters for expression of the heavy and light chains of the antibody fragment. Vectors for secretion from *E. coli* may make use of dicistronic messages for the co-ordinate expression of heavy and light chains, as exemplified by plasmid KB1082, shown in Figure 1, or may use two separate transcription units for the two antibody chains, as exemplified by KB1150 shown in Figure 3. A signal peptide is advantageously fused to the N-terminus of the mature heavy and light chain coding sequences in order to facilitate secretion from the host cell. The sequence of the signal peptide, which is encoded as part of the expression plasmid, may be provided by a naturally occurring secretion signal appropriate for the host cell. For example, a yeast invertase signal peptide may be chosen for secretion from yeast cells. For *E. coli*, a number of suitable prokaryotic signal peptides are known in the art, including the PelB or OmpA signal sequences. Alternatively a non-natural synthetic signal peptide may be chosen. An example of a synthetic signal peptide, suitable for antibody libraries expressed in *E. coli*, is the non-natural signal sequence designated SP2 the amino acid sequence of: MGKKQLVVFALLLAFLSPAMA.

Library Screening

[0108] As explained, the invention is not limited to technologies where the antibody constructs are expressed in microbial cells. Other screening methodologies can also be employed. The following provides an example of library screening using a microbial expression system.

[0109] Filter screening methodologies have been described for detection of secreted antibodies specific for a particular antigen. In one format, the secreted antibody fragments are trapped on a membrane which is probed with soluble antigen (Skerra *et al* (1991) *Anal Biochem*. 196:151-5). In this case, bacteria harboring plasmid vectors that direct the secretion of Fab fragments into the bacterial periplasm are grown on a membrane or filter. The secreted fragments are allowed to diffuse to a second "capture" membrane coated with antibody which can bind the antibody fragments (eg anti-immunoglobulin antiserum) and the capture filter is probed with specific antigen. Antibody - enzyme conjugates can be used to detect antigen-binding antibody fragments on the capture membrane as a colored spot. The colonies are re-grown on the first membrane and the clone expressing the desired antibody fragment recovered.

[0110] Colony lift binding assays have also been described in which the antibodies are allowed to diffuse directly onto an antigen-coated membrane. Giovannoni et al have described such a protocol for the screening of single-chain antibody libraries (Giovannoni et al., *Nucleic Acids Research 2001, Vol. 29, No. 5 e27*).

[0111] Libraries of secreted antibody fragments can also be screened by ELISA, either using pools of multiple clones or screening of individual clones each secreting a unique antibody sequence. One such method for screening individual clones is described by Watkins et al (1997) Anal. Biochem. 253: 37-45. In this case, microtiter wells were coated with anti-Fab antibody to capture Fab fragments secreted directly in the wells. The Fab samples were then probed with soluble biotinylated antigen followed by detection with streptavidin-alkaline phosphatase conjugates.

[0112] In some embodiments of the present invention, screening systems are used that result in relatively low levels of expression. For example, when a colony lift binding assay (CLBA, Govannoni et al., 2001, *Nucleic Acids Research* 29(5):e27) is combined with an immuno-chemiluminescent labeling system, the sensitivity of the system, even for sub-micromolar K_d affinities, permits expression levels which are below the aggregation thresholds of most Fabs.

[0113] Exemplary protocols for CLBA are provided in the Examples section. Conditions for the CLBA can be optimized empirically. For example, the transcription inducer may be optimized to avoid over-expression or under-expression by experimentally determining the amount required for *e.g.*, 100% ten-fold-over-background detection by chemi-luminescence of the library when a universal antibody fragment-binder, *e.g.*, an anti-human Ig antibody, is used as the antigen on the filter. The stringency of selection can also be manipulated by adjusting the concentration of antigen on the filter. For example, the antigen concentration on which the antibody fragment to be humanized produces a minimal signal, *e.g.*, no more than 10-fold over background, may be determined and used for selection, so that antibodies with higher affinities and/or higher expression levels may be readily identified by the intensity of their signals. Expression levels may be determined in parallel by making replicate colony lifts and incubating them on filters coated with a universal antibody binder, such as an anti-human Ig antibody. The relative affinity for each colony is then determined as the ratio of its chemi-luminescent signal from the antigen filter to its signal from the antibody-binder filter, and the ratios can be compared to each other and to the same ratio for the parent non-human antibody to rank-order the selected antibodies according to affinity. Absolute affinities may then be determined by any of several methods, *e.g.*, surface plasmon resonance methods (SPA, Fägerstam et al., 1992, *J Chromatog* 597:397-410). Human Fc domains may be appended to selected Fabs for expression and production as full-length Ig, generally without loss of affinity.

[0114] In some cases, the selected human V-regions of the highest-affinity antibodies may not support sufficiently robust expression of a Fab or other derivative Ig for cost-effective production for intended applications such as therapeutics or diagnostics. In such cases, the expression data provided by the replicate filter in the CLBA may be used to identify the highest-affinity Fabs with the desired expression levels. The expression stringency of the assay may also be increased by using antigen densities on the filter which are restrictive for the parent antibody when expressed at higher levels than those actually used for the selection. Selected higher-expressing Fabs can be affinity-matured, if desired, by mutagenesis of the BSD paris, and selection by CLBA as described above and below.

[0115] Guided selection can also be used to replace one BSD at a time. If the original heavy chain CDR3 BSD is retained, then the guiding affinity will be high enough for guided selection of a human light chain CDR3 BSD, though the same is less likely to be true if the original light chain CDR3 BSD is retained for guided selection of a human heavy chain CDR3 BSD. The CDR3 BSD repertoires used for V-region-guided selection can be captured from mRNA from PBL or other immune tissues using degenerate primers complementary to all germline Framework 3 and Framework 4 sequences. Alternatively, the repertoires can be constructed synthetically by recombining the sequences for the germline D-segment and J-segment repertoires (Tomlinson et al., 1995, *EMBO J* 14:4628-4638) with a few random residues at the D-segment junctions to simulate N-addition.

Antibodies with Human Germline V-regions

[0116] Selected antibodies can deviate from the human germline V-region sequences at a number of positions that don't significantly contribute to the binding activity of the antibody. It is also possible that many of these alterations will induce an immune response in at least some humans, which may thereby compromise the efficacy of the antibody. As, the human germline V-region sequences should be the least immunogenic, the V-region sequences of the such selected antibodies can be converted to the human germline sequences and tested for retention of affinity.

[0117] In some embodiments, an antibody made in accordance with the invention has a V segment plus FR4 that is greater than 90% identical, often greater than 95% identical, and preferably identical to a human germline sequence. Such antibodies can be identified by comparing the V segment and FR4 sequence to know human germline sequences.

[0118] If some loss of affinity does occur upon conversion to the germline sequences, the affinity can be recovered by affinity maturation of the CDR3 sequences.

Affinity Maturation

[0119] BSD-guided V-region selection often produces antibodies of equal or even higher affinity than that of the reference antibody. However, it may be desirable to also employ affinity maturation techniques, either before or after the selection procedure. "Affinity matured" in the context of antibodies refers to an antibody that is derived from a reference antibody, binds to the same epitope as the reference antibody, and has a higher affinity for the antigen than that of the reference antibody. For example, affinity maturation may be performed on antibodies selected in accordance with the invention in which the heavy and/or light chain V-segments are germline.

[0120] To avoid immunogenicity, affinity maturation is typically performed focusing on the BSD pair. Efficient affinity maturation of the BSD can be accomplished, *e.g.,* using the method of Parsimonious Mutagenesis (PM, Balint and Larrick, 1993 *Gene* 137:109-118) to diversify the BSD pair, and then screening for higher affinity binders, *e.g.,* using a CLBA or other method.

[0121] In other embodiments, affinity maturation techniques can be performed using fragment complementation systems, *e.g.,* described in US Patent Applications 09/526,106 and 09/999,413; the competitive activation system (CompAct) described in US Patent Application 10/076,845; and the auto-inhibited β-lactamase reactivation systems (ReAct or RAIR) described in US Patent Applications 10/208,730 and 10/677,131.

Affinity determination

[0122] Antibodies isolated from primary screens of secreted antibodies or selected from display technologies are subjected to further analysis in order to determine quantitative affinities for target antigen. Typically, the antibodies are expressed in soluble form for this purpose, which may necessitate re-formatting as a soluble fragment or as a whole IgG if the antibodies were originally isolated as fusion proteins from a surface display approach.

[0123] Affinities can be determined by a variety of competition binding studies requiring interaction of antibody in solution with native antigen, either in solution or on whole cells whole cells, and analysis of affinity from scatchard plots. Alternatively affinity may be determined on isolated antigen, for example in Enzyme-linked Immunosorbent Assays (ELISA) or by surface plasmon resonance analysis or numerous other immunoassays known in the art (*see, e.g.,* Harlow & Lane, Using Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1999). Harlow & Lane and similar procedure manuals also disclose techniques to map epitopes or alternatively, competition experiments, to determine whether an antibody binds to the same epitope as the donor antibody. Functional, *e.g., cell-based* assays can also used to demonstrate that the specificity and activity of the reference antibody is retained.

[0124] The first screening steps, *e.g..,* screens that analyze replacement of one exchange cassette where the remainder of the antibody sequences are reference antibody, an antibody that has a demonstrable affinity for the antibody is selected. The affinity may be lower than the reference antibody.

[0125] Antibodies of the invention are typically high affinity antibodies and may have dissociation constants in the range 50nM to 1 pM. Preferably the antibody has an affinity less than 10nM and most preferably less than 1 nM. Where the antibody has one or more germline V segments, the affinities are preferably less than 50 nM, often less than 20 nM, most preferably less than 1 nM. Similarly, an antibody that has been selected using the methods of the invention in which the D segment from the heavy chain of the reference antibody has been transferred, or in which one or more MEBSDs from the reference antibody CDR3s have been transferred, or in which one or both CDR3s from the reference antibody has been transferred, preferably have an affinity that is less than 50 nM, often less than 20 nM, preferably less than 1 nM.

[0126] The antibodies have affinities typically no more than 5-fold worse, often no more than 2-fold worse than the reference antibody and most preferably have higher affinity, *e.g.*, 2-fold, 5-fold, or higher, than the reference antibody.

General Methods

Nucleic Acids and Polypeptides

[0127] Expression methodology is well known to those of skill in the art. Recombinant polypeptides can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences by methods known in the art, in the proper reading frame, and expressing the product by methods known in the art (*see, e.g.*, Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York 1994; Sambrook and Russell, eds, Molecular Cloning: A Laboratory Manual, 3rd Ed, vols. 1-3, Cold Spring Harbor Laboratory Press, 2001; and Current Protocols in Molecular Biology, Ausubel, ed. John Wiley & Sons, Inc. New York, 1997).

[0128] Nucleic acids encoding the polypeptides of the invention can be obtained using routine techniques in the field of recombinant genetics (see, e.g., Sambrook and Russell, eds, Molecular Cloning: A Laboratory Manual, 3rd Ed, vols. 1-3, Cold Spring Harbor Laboratory Press, 2001; and Current Protocols in Molecular Biology, Ausubel, ed. John Wiley & Sons, Inc. New York, 1997).

[0129] Often, the nucleic acid sequences encoding the polypeptides to be expressed are amplified from cDNA or genomic DNA libraries using oligonucleotide primers. Amplification techniques can be used to amplify and isolate sequences from DNA or RNA (see, e.g., Dieffenbach & Dveksler, PCR Primers: A Laboratory Manual (1995)). Alternatively, overlapping oligonucleotides can be produced synthetically and joined to produce one or more domains.

[0130] Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.*, eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3: 81-94; (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86: 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.* (1989) *J. Clin. Chem.*, 35: 1826; Landegren *et al.*, (1988) *Science* 241: 1077-1080; Van Brunt (1990) *Biotechnology* 8: 291-294; Wu and Wallace (1989) *Gene* 4: 560; and Barringer *et al.* (1990) *Gene* 89: 117.

[0131] In some embodiments, it may be desirable to modify an antibody sequence of the invention. One of skill will recognize many ways of generating alterations in a given nucleic acid construct. Such well-known methods include site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells containing the nucleic acid to mutagenic agents or radiation, chemical synthesis of a desired oligonucleotide (*e.g.*, in conjunction with ligation and/or cloning to generate large nucleic acids) and other well-known techniques. *See, e.g.*, Giliman and Smith (1979) *Gene* 8:81-97, Roberts *et al.* (1987) *Nature* 328: 731-734.

[0132] In some embodiments, the recombinant nucleic acids encoding the polypeptides to be expressed are modified to provide preferred codons which enhance translation of the nucleic acid in a selected organism (*e.g.*, yeast preferred codons are substituted into a coding nucleic acid for expression in yeast).

Expression Cassettes And Host Cells

[0133] There are many expression systems for producing polypeptides that are well know to those of ordinary skill in the art. (*See, e.g.*, Gene Expression Systems, Fernandes and Hoeffler, Eds. Academic Press, 1999.) An extremely wide variety of promoters are available, and can be used in the expression vectors of the invention, depending on the particular application. Ordinarily, the promoter selected depends upon the cell in which the promoter is to be active. Other expression control sequences such as ribosome binding sites, transcription termination sites, enhancers, operators, and the like are also optionally included. Constructs that include one or more of these control sequences are termed “expression cassettes.” Accordingly, the nucleic acids that encode the joined polypeptides are incorporated for the desired level of expression in a desired host cell.

[0134] Expression control sequences that are suitable for use in a particular host cell are employed in the expression vectors. Commonly used prokaryotic control sequences, including promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta-lactamase (penicillinase) and lactose (*lac*) promoter systems (Change *et al.*, *Nature* (1977) 198: 1056), the tryptophan (*trp*) promoter system (Goeddel *et al.*, *Nucleic Acids Res.* (1980) 8: 4057), the *tac* promoter (DeBoer, *et al., Proc. Natl. Acad. Sci. U.S.A.* (1983) 80:21-25); and the lambda-derived P_L promoter and N-gene ribosome binding site (Shimatake *et al.*, *Nature* (1981) 292: 128). The particular promoter system is not critical to the invention, any available promoter that functions in prokaryotes can be used. Standard bacterial expression vectors include plasmids such as pBR322-based plasmids, *e.g.*, pBLUESCRIPT™, pSKF, pET23D, λ-phage derived vectors, p15A-based vectors (Rose, *Nucleic Acids Res.* (1988) 16:355 and 356) and fusion expression systems such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, *e.g.*, c-myc, HA-tag, 6-His tag, maltose binding protein, VSV-G tag, anti-DYKDDDDK tag, or any such tag, a large number of which are well known to those of skill in the art.

[0135] For expression of fusion polypeptides in prokaryotic cells other than *E. coli*, regulatory sequences for transcription and translation that function in the particular prokaryotic species are required. Such promoters can be obtained from genes that have been cloned from the species, or heterologous promoters can be used. For example, the hybrid *trp-lac* promoter functions in *Bacillus* in addition to *E. coli*. These and other suitable bacterial promoters are well known in the art and are described, *e.g.*, in Sambrook *et al.* and Ausubel *et al.* Bacterial expression systems for expressing the proteins of the invention are available in, *e.g., E. coli, Bacillus sp.,* and *Salmonella* (Palva *et al., Gene* 22:229-235 (1983); Mosbach *et al., Nature* 302:543-545 (1983). Kits for such expression systems are commercially available.

[0136] Similarly, for expression of the polypeptides of the invention in eukaryotic cells, transcription and translation sequences that function in the particular eukaryotic species are required. For example, eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. In yeast, vectors include pEFC, Yeast Integrating plasmids (*e.g.,* YIp5) and Yeast Replicating plasmids (the YRp series plasmids) and pGPD-2. Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, *e.g.*, SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the CMV promoter, SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0137] Either constitutive or regulated promoters can be used in the present invention. Regulated promoters can be advantageous because the concentration of heterologous protein in the host cell can be controlled. An inducible promoter is a promoter that directs expression of a gene where the level of expression is alterable by environmental or developmental factors such as, for example, temperature, pH, anaerobic or aerobic conditions, light, transcription factors and chemicals.

[0138] For *E. coli* and other bacterial host cells, inducible promoters are known to those of skill in the art. These include, for example, the *lac* promoter, the bacteriophage lambda P_L promoter, the hybrid *trp-lac* promoter (Amann *et al.* (1983) *Gene* 25: 167; de Boer *et al.* (1983) *Proc. Nat'l. Acad. Sci. USA* 80: 21), and the bacteriophage T7 promoter (Studier *et al.* (1986) *J. Mol. Biol.*; Tabor *et al.* (1985) *Proc. Nat'l. Acad. Sci. USA* 82: 1074-8). These promoters and their use are discussed in Sambrook *et al.*, *supra.*

[0139] Inducible promoters for other organisms are also well known to those of skill in the art. These include, for example, the metallothionein promoter, the heat shock promoter, as well as many others.

[0140] Translational coupling can be used to enhance expression. The strategy uses a short upstream open reading frame derived from a highly expressed gene native to the translational system, which is placed downstream of the promoter, and a ribosome binding site followed after a few amino acid codons by a termination codon. Just prior to the termination codon is a second ribosome binding site, and following the termination codon is a start codon for the initiation of translation. The system dissolves secondary structure in the RNA, allowing for the efficient initiation of translation. See Squires, *et. al.* (1988), *J. Biol. Chem.* 263: 16297-16302.

[0141] The construction of polynucleotide constructs generally requires the use of vectors able to replicate in host bacterial cells, or able to integrate into the genome of host bacterial cells. Such vectors are commonly used in the art. A plethora of kits are commercially available for the purification of plasmids from bacteria (for example, EasyPrepJ, FlexiPrepJ, from Pharmacia Biotech; StrataCleanJ, from Stratagene; and, QIAexpress Expression System, Qiagen). The isolated and purified plasmids can then be further manipulated to produce other plasmids, and used to transform cells.

[0142] The polypeptides of the invention can be expressed and displayed on a cell or phage surface, or can be secreted from a cell. A variety of host cells can be used, including *E. coli,* other bacterial hosts, noted above, yeast cells, insect cells, fungal cells, and various mammalian cells such as the COS, CHO and HeLa cells lines and myeloma cell lines.

[0143] Once expressed, the recombinant polypeptides can be purified according to standard procedures of the art, *e.g.*, using affinity columns, column chromatography, gel electrophoresis and the like (*see*, generally, R. Scopes, *Protein Purification*, Springer-Verlag, N.Y. (1982), Deutscher, *Methods in Enzymology Vol. 182: Guide to Protein Purification.*, Academic Press, Inc. N.Y. (1990)). Substantially pure compositions of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity are most preferred.

[0144] To facilitate purification of the polypeptides of the invention, the nucleic acids that encode the polypeptides can also include a coding sequence for an epitope or "tag" for which an affinity binding reagent is available. Examples of suitable epitopes include the myc and V-5 responder genes; expression vectors useful for recombinant production of fusion polypeptides having these epitopes are commercially available (*e.g.*, Invitrogen (Carlsbad CA) vectors pcDNA3.1/Myc-His and pcDNA3.1/V5-His are suitable for expression in mammalian cells). Additional expression vectors suitable for attaching a tag to the fusion proteins of the invention, and corresponding detection systems are known to those of skill in the art, and several are commercially available (*e.g.*, FLAG" (Kodak, Rochester NY). Another example of a suitable tag is a polyhistidine sequence, which is capable of binding to metal chelate affinity ligands. Suitable metal chelate affinity ligands that can serve as the binding moiety for a polyhistidine tag include nitrilo-tri-acetic acid (NTA) (Hochuli, E. (1990) "Purification of recombinant proteins with metal chelating adsorbents" In Genetic Engineering: Principles and Methods, J.K. Setlow, Ed., Plenum Press, NY; commercially available from Qiagen (Santa Clarita, CA)).

[0145] One of skill would recognize that modifications can be made to the protein domains without diminishing their biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of a domain into a polypeptide. Such modifications are well known to those of skill in the art and include, for example, the addition of codons at either terminus of the polynucleotide that encodes the binding domain to provide, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (*e.g.*, poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

[0146] Display technologies have also permitted the selection of monoclonal antibodies that are fully human or other animal, chimeric, synthetic, and/or semi-synthetic. Examples of such display technologies are phage display (examples are disclosed in U.S. Patent Nos. 5,821,047, 5,922,545, 5,403,484, 5,885,793, and 6,291,650) or yeast display (examples are disclosed in U.S. Patent No. 6,300,065).

[0147] *Antibody Libraries.* Naïve libraries and Immunized libraries. Naïve libraries are made from the B-lymphocytes of a suitable host which has not been challenged with any immunogen, nor which is exhibiting symptoms of infection or inflammation. Immunized libraries are made a from a mixture of B-cells and plasma cells obtained from a suitably "immunized" host, i.e., a host that has been challenged with an immunogen. In one embodiment, the mRNA from these cells is translated into cDNA using methods well known in the art (e.g., oligo-dT primers and reverse transcriptase). In an alternative embodiment, nucleic acids encoding antibodies from the host cells (mRNA or genomic DNA) are amplified by PCR with suitable primers. Primers for such antibody gene amplifications are well known in the art (e.g., U.S. Patent No. 6,096,551 and PCT Patent Application WO 00/70023A1 disclose such primers). In a hybrid embodiment, the mRNA from the host cells is synthesized into cDNA and these cDNAs are then amplified in a PCR reaction with antibody specific primers (e.g., U.S. Patent No. 6,319,690 discloses such a hybrid method). Alternatively, the repertoires may be cloned by conventional cDNA cloning technology (Sambrook and Russell, eds, *Molecular Cloning: A Laboratory Manual*, 3rd Ed, vols. 1-3, Cold Spring Harbor Laboratory Press, 2001), without using PCR.

[0148] The invention will be further understood by the following non-limiting examples.

EXAMPLES

Example 1. Construction of epitope-focused libraries of antibody fragments by *in vitro* transcription.

[0149] Messenger RNA encoding an antibody repertoire is isolated from Ig-producing cells of the human immune system. One unit of whole blood is drawn from a human donor and a buffy coat preparation is made using standard procedures. Peripheral blood mononuclear cells (PBMCs) are purified by Ficoll-Hypaque density centrifugation to enrich for Ig-producing B lymphocytes. Total RNA is purified from the PBMCs using a commercially available RNA purification kit (Qiagen RNeasy) used according to the manufacturer's specifications. mRNA is enriched from the total RNA using a commercially available RNA purification kit (Qiagen Oligotex mRNA kit) used according to the manufacturer's specifications. The mRNA from several donors of different ethnic backgrounds is pooled to increase the diversity of the final V segment repertoires. Additional diversity can be obtained from human spleen mRNA obtained from either a human donor or from a commercial source.

[0150] Sets of oligonucleotide primers were designed to 5' and 3' regions of the V regions for cloning the V region repertoire. Three primer sets are used to ensure that immunoglobulin variable regions are amplified exclusively from all other expressed mRNAs. Some primers in each set are degenerate at one or more positions in order to capture the sequence diversity present in the immunoglobulin genes. Most of the primer sequences described here have been previously published (Welschof et al., *J. Immunological Methods,* 179: 203-214 [1995] and Little et al., *J. Immunological Methods*, 231: 3-9 [1999]) and some have been modified for use in this work.

1st Primer Set:

Vkappa (Vκ): [P]GAAGACAGATGGTGCAGCCACAG

Vlambda (Vλ): [P]AGAGGASGGYGGGAACAGAGTGAC

Vheavy (Vh) IgG: [P]GACSGATGGGCCCTTGGTGGA

Vh IgM: [P]AAGGGTTGGGGCGGATGCACT

2nd Primer Set:

VκI:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGGAC ATCCAGWTGACCCAGTCTCC

VκII:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGGATGTTGTGATGACTCAGTCTCC

VκIII:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGGAAATTGTGWTGACRCAGTCTCC

VκIV:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGGATATTGTGATGACCCACACTCC

VκV:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGGAAACGACACTCACGCAGTCTCC

VκVI:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGGAAATTGTGCTGACTCAGTCTCC

Vλ1a:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGCAGTCTGTGCTGACTCAGCCACC

Vλ1b:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGCAGTCTGTGYTGACGCAGCCGCC

Vλ1c:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGCAGTCTGTCGTGACGCAGCCGCC

Vλ2:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGCARTCTGCCCTGACTCAGCCT

Vλ3a:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGTCC TATGWGCTGACTCAGCCACC

Vλ3b:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGTCT TCTGAGCTGACTCAGGACCC

Vλ4:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGCAC GTTATACTGACTCAACCGCC

Vλ5:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGCAG GCTGTGCTGACTCAGCCGTC

Vλ6:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGAAT TTTATGCTGACTCAGCCCCA

Vλ7 and Vλ8:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGCAG RCTGTGGTGACYCAGGAGCC

Vλ9:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCACCATGGGCGCGCTGCW GCCTGTGCTGACTCAGCCMCC

Vh1b andVh7:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCAGGCCCAGCCGGCCATG GCTCAGRTGCAGCTGGTGCARTCTGG

Vh1c:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCAGGCCCAGCCGGCCATG GCTSAGGTCCAGCTGGTRCAGTCTGG

Vh2:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCAGGCCCAGCCGGCCATG GCTCAGRTCACCTTGAAGGAGTCTGG

Vh3b:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCAGGCCCAGCCGGCCATGGCTSAGGTGCAGCTGGTGGAGTCTGG

Vh3c:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCAGGCCCAGCCGGCCATGGCTGAGGTGCAGCTGGTGGAGWCYGG

Vh4b:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCAGGCCCAGCCGGCCATGGCTCAGGTGCAGCTACAGCAGTGGGG

Vh4c:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCAGGCCCAGCCGGCCATGGCTCAGSTGCAGCTGCAGGAGTCSGG

Vh5:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCAGGCCCAGCCGGCCATGGCTGARGTGCAGCTGGTGCAGTCTGG

Vh6:

CATGTGTAATACGACTCACTATAGGGAGTCATACATCAGGCCCAGCCGGCCATGGCTCAGGTACAGCTGCAGCAGTCAGG

3rd Primer Set:

Jκ1 and Jκ2 and Jκ4: TATAGCGGCCGCACTAGTTCGTTTGATRTCCASCTTGGTCC

Jκ3: TATAGCGGCCGCAGTAGTTCGTTTGATATCCACTTTGGTCC

Jκ5: TATAGCGGCCGCACTAGTTCGTTTAATCTCCAGTCGTGTCC

Jλ1: TATAGCGGCCGCCCTAGGCTGCCYAAGGACGGTGACCTTGGTCC

Jλ2 and Jλ3: TATAGCGGCCGCCCTAGGCTGCCYAAGGACGGTCAGCTTGGTCC

Jλ7: TATAGCGGCCGCCCTAGGCTGCCYGAGGACGGTCAGCTGGGTGC

JH1 and JH2: GCGGATGCACTTCCGGAGGAGACGGTGACCAGGGTGCC

JH3: GCGGATGCACTTCCGGAAGAGACGGTGACCATTGTCCC

JH4 and JH5: GCGGATGCACTTCCGGAGGAGACGGTGACCAGGGTTCC

JH6: GCGGATGCACTTCCGGAGGAGACGGTGACCGTGGTCCC

[0151] The primer sets are designed to be pooled and to be used in individual reactions for Vh, Vκ or Vλ. The first primer (for Vκ or Vλ) or primer set (for Vh IgM and Vh IgG) is annealed to the 'constant' region of the immunoglobulin-encoding mRNAs. Each primer of the first primer set is phosphorylated at the 5' end for subsequent digestion with lambda exonuclease. First strand cDNA is synthesized by reverse transcriptase using standard procedures. The mRNA is digested from the first-strand cDNA with a cocktail of RNase H and RNase A. A second primer set is annealed to N-terminal end of the V regions of the first strand cDNA. Each primer in the second set contains a T7 RNA polymerase promoter, a restriction site not (or rarely) present in the cDNA repertoire and a region complementary to the cDNA (positions 1-8 for Vh, Vκ and Vλ according to the Kabat numbering scheme). Second strand cDNA is synthesized by standard procedures. Lambda exonuclease is used to degrade the first strand cDNA. A third primer set is annealed to the C-terminal end of the V region (in the same orientation but nested upstream of the first primer set) of the second strand cDNA. Each primer in the third set contains restriction site not (or rarely) present in the cDNA repertoire and a region complementary to the cDNA (positions 107-114 for Vh, positions 101 to 108 for Vκ and positions 101 to 108 for Vλ according to the Kabat numbering scheme). The annealed third set primers are extended by DNA polymerase, and the antisense strand cDNA is synthesized.

[0152] The double stranded cDNA is added to an *in vitro* transcription reaction that includes NTPs and T7 RNA polymerase. The T7 promoter appended to the second primer set drives the synthesis of sense strand cRNA. The fold-amplification is estimated to be >500. To obtain enough cDNA for subsequent cloning, the cRNA is converted to ds cDNA by priming first and second strand cDNA synthesis with primer sets three and two, respectively, using well-known procedures for synthesis. The resulting ds cDNA is added to an IVT reaction in order to synthesize additional cRNA. The additional amplification step can be repeated as many times as necessary to generate a sufficient quantity of cRNA for cloning, usually about 500 ng to 1 μg.

[0153] When a sufficient quantity of cRNA has been synthesized, it is converted to ds cDNA by routine procedures and cut with the restriction enzymes for which sites have been appended by inclusion in the primer sequence. The restriction enzyme sites for Vh are *Sfi* I or *Nco* I and *Bsp* EI, for Vk *Bss* HII and *Spe* I, and for Vl *Bss* HII and *Avr* II. The restricted cDNA repertoire is ligated into an appropriate cloning vector and transformed into *E. coli*. We estimate that $\geq 10^7$ transformants for VH and $\geq 10^6$ transformants for VL are sufficient to represent the variable region diversity in one individual. Typically, the immunoglobulin repertoire from several individuals is combined into a single library.

[0154] Human antibodies with the specificity of the mouse Mab 166 (US Patent number 6,827,935), against an epitope on *Pseudomonas aeruginosa* PcrV protein, were generated as follows. A V segment consists of the region from FR1 to FR3 (positions 1 to 94 for Vh, positions 1 to 88 for Vκ and positions 1 to 88 for Vl using the Kabat numbering scheme) and lacks a CDR3 and FR4. The M166 reference antibody CDR3-FR4 regions for both the Vh and the Vκ chains was appended to the V segment library as described below.

[0155] A library of V segments (BA19) was derived from the V region cRNA. An antisense primer was designed to Kabat positions 86 to 89 for Vh, Kabat positions 80 to 84 for Vκ and Kabat positions 81 to 85 for Vλ. The primer sequences are shown below. In order to capture as many gene variants as possible, degeneracy is added to primer positions in which the germ-line repertoire varies.

Vh:
CACAGTAGTATACGGCCGTGTC
CACAGTAGTATACRGCNGTGTC
CACAGTAGTATACGGCCGTCTC

Vκ:
CAAATGTATACTGCMAMATCTTCAG
TTCAAATGTATACTGCAATATCTTCAG
TTCAAATGTATACYCCRACATCCTCAG
TTCAAATGTATACTGCAGCATCTTCAG

Vλ:
TTGTAAAGATATCRGCYTCRTCYHYNC
GTAAAGATATCRGCCTCRTCBTYHG

[0156] A non-templated *Bst*1107I (for Vh and Vκ) or *Eco* RV (for Vλ) restriction site is appended to the 5' end of the primer for subsequent cloning of the V segment repertoire. The primer is hybridized to the cRNA population and is extended during first strand cDNA synthesis. The second strand cDNA is synthesized using standard protocols.

[0157] The Vh segment cDNA is restricted with *Sfi* I and *Bst*1107 I and is cloned upstream of the CDR3-FR4 region of the M166 reference Vh chain to create recombinant Vh regions in which the V segment repertoire is attached to a single CDR3-FR4 region. The sequence of the CDR3-FR4 region of the M166 Vh chains, modified to include a *Bst*1107 I restriction site at its 5' end and a *Bsp*E I site at its 3' end, is shown below.

M166 Vh CDR3-FR4:
GTATACTACTGTGCCAGAAATAGAGGGGATATTTACTATGATTTCACTTATGCCA
TGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCCGGA

[0158] The Vκ segment cDNA is restricted with *Bss* HII and *Bst*1107 I and is cloned upstream of the CDR3-FR4 region of the M166 reference Vκ chain to create recombinant Vκ regions in which the V segment repertoire is attached to a single CDR3-FR4 region. The sequence of the CDR3-FR4 region of the M166 Vκ chains, modified to include a *Bst*1107 I restriction site at its 5' end and a *Spe* I site at its 3' end, is shown below.

M166 Vκ CDR3-FR4:
GTATACTACTGTCAACATTTTTGGAGTACTCCGTACACGTTCGGAGGGGGGACCA
AGCTGGAAATAAAACGAACTAGT

[0159] To construct the library BA46, the CDR3-FR4 region was appended to the V segment libraries by primer extension. An antisense oligonucleotide containing the sequence of CDR3-FR4 region of the M166 reference antibody Vh chain was synthesized by standard procedures. In addition to the CDR3-FR4 region, nucleotides encoding the highly conserved YYCAR sequence in human FR3 (Kabat positions 90 to 94) were included at the 3' end of the oligonucleotide and a restriction site for cloning, *Bsp*E I, was added to the 5' end. The sequence of the oligonucleotide is shown below.

M166 Vh CDR3-FR4:
TAGATCCGGAGGAGACGGTGACTGAGGTTCCTTGACCCCAGTAGTCCATGGCAT
AAGTGAAATCATAGTAAATATCCCCTCTATTTCTGGCACAGTAATA

[0160] The oligonucleotide was hybridized to the Vh region library cRNA previously described. First strand synthesis was accomplished with reverse transcriptase using standard procedures. The template cRNA was removed with a cocktail of RNase H and RNase A. The second strand cDNA was synthesized using standard procedures.

[0161] The Vκ segment library was appended to the M166 CDR3-FR4 region in a similar fashion. An antisense oligonucleotide containing the sequence of CDR3-FR4 region of the M166 reference antibody Vκ chain was synthesized by standard procedures. In addition to the CDR3-FR4 region, nucleotides encoding the highly conserved YYC sequence in human FR3 (Kabat positions 86 to 88) was included at the 3' end of the oligonucleotide and a restriction site for cloning, *Spe* I, was added to the 5' end. The sequence of the oligonucleotide is shown below. The oligonucleotide is degenerate at three positions in order to capture the sequence diversity present in the germ-line immunoglobulin mRNA

M166 Vκ CDR3-FR4:
CGAATTGAACTAGTTCGTTTATTTCCAGCTTGGTCCCCCCTCCGAACGTGTACGG
AGTACTCCAAAAATGTTGRCARTARTA

[0162] The oligonucleotide was hybridized to the Vκ region library cRNA previously described. First strand synthesis was accomplished with reverse transcriptase using standard procedures. The template cRNA was removed with a cocktail of RNase H and RNase A. The second strand cDNA was synthesized using standard procedures.

[0163] V region libraries created by either of the above methods are cloned into a Fab expression vector for generating secreted assembled Fab fragments to be screened for antigen binding. The full, in-frame Vh region library is restricted with *Sfi* I and *Bsp* EI and is inserted into a Fab expression vector such as KB1082, which is shown in Figure 1. The complete nucleotide sequence is shown in Figure 2. The full Vκ region is restricted with *Bss*H II and *Spe* I and is inserted into the same vector that contains the Vh region library. Alternatively, a similar vector can be constructed that contains a Clambda instead of a Ckappa constant region and can be used for the expression of Vλ V-regions. The Fab expression vector comprises an antibiotic resistance gene for selection in *E. coli,* a dicistronic expression cassette driven by an inducible promoter (such as pBAD), VH and VL constant regions and cloning sites for the VH region and VL region repertoires.

[0164] Fabs expressed from plasmid KB1082 were screened by a colony-lift binding assay (CLBA) as described in Examples 5 and 6 or pools of colonies were screened and subsequently de-convoluted using an antigen-binding ELISA as described in Example 6.

Example 2. Construction of epitope-focused libraries of antibody fragments by PCR.

[0165] For PCR-mediated amplification and cloning of antibody repertoires, two primer sets are used. The sense primer-set anneals to the N-terminal region (positions 1-8 for Vh, Vκ and Vλ according to the Kabat numbering scheme) of the V segment and contains a restriction site(s) appended to the 5' end (in this case *Sfi* I and *Nco* I) suitable for use in cloning into a plasmid vector. The anti-sense primer-set anneals to the C-terminal end of framework three (FR3; positions 86 to 90 for Vh, positions 80 to 86 for Vκ and positions 81 to 86 for Vλ according to the Kabat numbering scheme). All of the antisense primers include an invariant nucleotide sequence for the YYC peptide (Kabat positions 90 to 92 for Vh, 86 to 88 for Vκ and Vλ. Each antisense primer includes a restriction site appended to the 5' end (in this case *Sal* I) suitable for use in cloning into a plasmid vector. Some primers in each set are degenerate at one or more positions in order to capture the sequence diversity present in the germ-line immunoglobulin mRNA.

Sense Primer Sets:

Vκ:

VκI: CAGCCGGCCATGGCCGCGCTGGACATCCAGWTGACCCAGTCTCC

VκII: CAGCCGGCCATGGCCGCGCTGGATGTTGTGATGACTCAGTCTCC

VκIII: CAGCCGGCCATGGCCGCGCTGGAAATTGTGWTGACRCAGTCTCC

VκIV: CAGCCGGCCATGGCCGCGCTGGATATTGTGATGACCCAGTCTCC

VκV: CAGCCGGCCATGGCCGCGCTGGAAACGACACTCACGCAGTCTCC

VκVI: CAGCCGGCCATGGCCGCGCTGGAAATTGTGCTGACTCAGTCTCC

Vλ:

Vλ1a: CATGTATCAGCGCGCTGCAGTCTGTGCTGACTCAGCCACC

Vλ1b: CATGTATCAGCGCGCTGCAGTCTGTGYTGACGCAGCCGCC

Vλ1c: CATGTATCAGCGCGCTGCAGTCTGTCGTGACGCAGCCGCC

Vλ2: CATGTATCAGCGCGCTGCARTCTGCCCTGACTCAGCCT

Vλ3a: CATGTATCAGCGCGCTGTCCTATGWGCTGACTCAGCCACC

Vλ3b: CATGTATCAGCGCGCTGTCTTCTGAGCTGACTCAGGACCC

Vλ4: CATGTATCAGCGCGCTGCACGTTATACTGACTCAACCGCC

Vλ5: CATGTATCAGCGCGCTGCAGGCTGTGCTGACTCAGCCGTC

Vλ6: CATGTATCAGCGCGCTGAATTTTATGCTGACTCAGCCCCA

Vλ7 and Vl8: CATGTATCAGCGCGCTGCAGRCTGTGGTGACYCAGGAGCC

Vλ9: CATGTATCAGCGCGCTGCWGCCTGTGCTGACTCAGCCMCC

Vλ10: CATGTATCAGCGCGCTGCAGGCAGGGCTGACTCAGCCACC

Vh:

Vh1b and Vh7: GCCCAGCCGGCCATGGCTCAGRTGCAGCTGGTGCARTCTGG

Vh1c: GCCCAGCCGGCCATGGCTSAGGTCCAGCTGGTRCAGTCTGG

Vh2: GCCCAGCCGGCCATGGCTCAGRTCACCTTGAAGGAGTCTGG

Vh3b: GCCCAGCCGGCCATGGCTSAGGTGCAGCTGGTGGAGTCTGG

Vh3c: GCCCAGCCGGCCATGGCTGAGGTGCAGCTGGTGGAGWCYGG

Vh4b: GCCCAGCCGGCCATGGCTCAGGTGCAGCTACAGCAGTGGGG

Vh4c: GCCCAGCCGGCCATGGCTCAGSTGCAGCTGCAGGAGTCSGG

Vh5: GCCCAGCCGGCCATGGCTGARGTGCAGCTGGTGCAGTCTGG

Vh6: GCCCAGCCGGCCATGGCTCAGGTACAGCTGCAGCAGTCAGG

[0166] Antisense Primer Sets:

Vκ:

VκI: CAGATAATGTCGACTGGCAGTAGTAAGTTGCAAAATCTTCAG

CAGATAATGTCGACTGGCAGTAGTATGTTGCAAYATCTTCAG

VκII: CAGATAATGTCGACTGGCAGTAGTAAACYCCRACATCCTCAG

VκIII: CAGATAATGTCGACTGGCAGTAGTAMACTGCAAAATCTTCAG

VκIV: CAGATAATGTCGACTGGCAGTAGTAAACAGCCACATCTTCAG

VκV: CAGATAATGTCGACTGGCAGAAGTAGTATGCAGCATCCTCAG

VκVI: CAGATAATGTCGACTGGCAGTAGTAYGTTGCAGCATCTTCAG

Vλ:

Vλ1, Vλ2, Vλ3, Vλ4, Vλ5, Vλ6, Vλ7 and Vλ110:

CAGATAATGTCGACTGGCAGTAGTARTCRGCCTCRTCCTC

Vλ11 and Vλ3: CAGATAATGTCGACTGGCAGTAGTAGTCRGCCTCRTCYCC

Vλ4c: CAGATAATGTCGACTGGCAGTGGTACTCAGCCTCATCGTC

Vλ8: CAGATAATGTCGACTGGCAGTAGTAATCAGATTCATCATC

Vλ9: CAGATAATGTCGACTGGCAGTGGTAGTCACTCTCATCCTC

Vh:

Vh1, Vh3, Vh4 and Vh6: CAGATAATGTCGACGTGCGCAGTAGTACACRGCYGTGTC

Vh2: CAGATAATGTCGACGTGCGCAGTAGTAYGTGGCTGTGTC

Vh5: CAGATAATGTCGACGTGCGCAGTAGTACATGGCGGTGTC

Vh7: CAGATAATGTCGACGTGCGCAGTAGTACACGGCAGTGTC

[0167] First-strand cDNA is prepared from the mRNA of peripheral blood lymphocytes or spleen with a polydT primer and reverse transcriptase according to established procedures. A cocktail of RNase H and RNase A is used to remove the mRNA from the first strand cDNA. After purification to remove the primer and dNTPs, the first strand cDNA is used as a template for PCR.

[0168] PCR reactions are assembled with up- and downstream primers such that members of a particular V segment subclass (e.g., Vh2 excluding Vh1, Vh3, Vh4, Vh5, Vh6 and Vh7) will be amplified exclusively. Fifty ul reactions are assembled containing 50 nM sense primer, 50 nM antisense primer, ~50 ng first-strand cDNA, 100 uM of each dNTP, buffer and Taq polymerase. The reactions are cycled with the following parameters: 95° C for 5 min - [94° C for 10 sec; 55° C for 1 min; 72° C for 30 sec]$_{25\text{-}35}$ - 72° C for 5 min. The PCR products are purified away from the primers and nucleotides by passage through a DNA purification column (Qiagen Qiaquick) used according to the manufacturer's specifications.

[0169] The full VH or VL repertoires, comprising all of the VH or VL subclasses, can be reconstituted by mixing the desired ratios of the subclass specific PCR products. The desired ratio of each subclass can reflect the incidence of each subclass in the germ-line, can mirror subclass usage *in vivo* (for example see Sheets et al. *Proc. Natl. Acad. Sci.* 95: 6157-6162 [1998])), or can be an arbitrary ratio. The PCR products are restricted with *Nco*I and *Sal*I and cloned into a plasmid vector. In a preferred embodiment, the plasmid vector has a T7 RNA polymerase promoter immediately upstream of the V segment insert in order to drive the synthesis of single-stranded cRNA representing the V segment repertoire.

[0170] The V segment repertoire can be attached to a CDR3-FR4 region by one of many schemes that include ligation of restricted DNA, overlap extension PCR or primer-directed cDNA synthesis.

[0171] In a preferred embodiment, an oligonucleotide corresponding to CDR3-FR4 of the reference antibody is synthesized by standard procedures and is appended to the V segment library. An antisense oligonucleotide containing the M166 Vh CDR3-FR4 sequence was synthesized and its sequence is shown below. A single amino acid change was made in FR4 to make it identical to the FR4 sequence encoded by the human germ-line JH6-segment. A sequence complementary to the YYCAR region of the V segment library (Kabat positions 90 to 94) is included at the 3' end of the oligonucleotide. Additionally, a *Bsp* EI restriction site useful for cloning is appended to the 5' end of the oligonucleotide.

M166 Vh CDR3-Human FR4 oligonucleotide:
CTGTTCCGGAGCTGACGGTGACTGTGGTTCCTTGACCCCAGTAATCCATCGCATAGGTGAAATCATAGTAAATATCACCACGGTTACGTGCGCAGTAGTA

[0172] The oligonucleotide is annealed to the 3' ends of the Vh segment cRNA repertoire via the nucleotide sequence coding for the YYCAR peptide and is extended by reverse transcriptase using standard protocols. A cocktail of RNase A and RNase H is used to degrade the template cRNA and second strand cDNA is synthesized according to established procedures.

[0173] An antisense oligonucleotide containing the M166 Vκ CDR3-FR4 sequence was synthesized and is shown below. A single amino acid change was made in FR4 to make it identical to the FR4 sequence encoded by human germ-line Jκ2. A sequence complementary to the YYC region of the V segment library (Kabat positions 86 to 88) is included at the 3' end of the oligonucleotide. Additionally, a *Spe* I restriction site useful for cloning is appended to the 5' end of the oligonucleotide.

M166 Vκ CDR3-Human FR4 oligonucleotide:
ATTGAACTAGTTCGTTTTATTTCCAGCTTGGTCCCCTGTCCGAACGTGTACGGAGTACTCCAAAAATGCTGGCAGTAGTA

[0174] The oligonucleotide is annealed to the 3' ends of the Vκ segment cRNA repertoire via the nucleotide sequence coding for the YYC peptide and is extended by reverse transcriptase using standard protocols. A cocktail of RNase A and RNase H is used to degrade the template cRNA and second strand cDNA is synthesized according to established procedures.

[0175] The resulting Vh and Vk region cDNAs are restricted with enzymes for which sites have been appended to each primer or primer set. The Vh and Vκ regions are cloned into an appropriate vector for expression of Fab' fragments in *E. coli*, such as KB1150 described below (Figure 3 and Figure 4). The Fab' library constructed in KB1150 was designated BA110.

[0176] The KB1150 vector is comprised of the following elements. 1. A chloramphenicol resistance gene for selection in *E. coli.* 2. A monocistronic cassette for VH-CH driven by the pTac promoter that contains a synthetic peptide leader sequence for secretion of the polypeptide chain into the media. 3. A monocistronic cassette for VL-CL driven by the pTac promoter that contains a synthetic peptide leader sequence for secretion of the polypeptide chain into the media. The vector KB1150 contains the Cκ constant region, however, a vector with the Cλ constant region can be constructed for the expression of Vλ V-regions. 4. An IgG1 hinge region for promoting Fab' production in *E. coli.* 5. A Myc peptide tag for detection and purification. 6. Suitable restriction sites for inserting the VH and VL regions. 7. The lac Iq gene which acts to repress the pTac promoter in the absence of the inducer IPTG.

[0177] Antibody Fab expression vector KB1150 was derived from pGEX-4T-1 (Amersham Biosciences), in which the ampicillin resistance gene is replaced by a chloramphenicol resistance gene. It contains two separate pTac expression cassettes separated by a T7 terminator. The upstream pTac promoter is used to express the light chain and the downstream pTac promoter is used to express the heavy chain. The VL-CL cassette is preceded by a novel secretion signal peptide SP2: MGKKQLVVFALLLAFLSPAMA. The VH-CH cassette is also preceded by the secretion signal peptide SP2. The VH-CH contains the hinge region of IgG 1 (THTCPPCPA) and a Myc tag peptide (GAAEQKLISEEDLN) at the end of heavy chain C region. The lac Iq gene represses the pTac promoter in the absence of the inducer IPTG.

[0178] Antibody fragments secreted from plasmid KB1150 are screened by CLBA or ELISA as described in Examples 5 and 6.

<u>Example 3. Identification of human anti-PcrV antibodies containing a MEBSD from a murine antibody CDRH3 region.</u>

[0179] The HCDR3 typically consists of amino acids from the D region, the J region and N-additions (amino acids encoded by nucleotides added during *in vivo* recombination). The LCDR3 consists of amino acids from the V region, J region and N-additions. The Minimal Essential Binding Specificity Determinant (MEBSD) can be all or part of a CDR3. Libraries of antibodies can be constructed by attaching V segment libraries onto the MEBSD derived from an HCDR3 or an LCDR3.

[0180] In order to determine whether a D-segment with associated N-additions could define a MEBSD, the CDRH3 region of murine antibody M166 was analyzed to identify the D-segment and any associated N-additions (see Figure 5). The M166 HCDR3 was compared to all identified murine germ-line D segments. The closest sequence similarity for the D region is to murine D segment D-SP2.2. The N additions are represented by two amino acids upstream and two amino acids downstream of the D region. The M166 J region is most similar to the human JH6 and JH3 variants.

[0181] V region repertoires combining the putative M166 MEBSD and a human JH6 or JH3 sequence were constructed in a Fab' expression vector and the Fab's were tested for binding to PcrV in a colony-lift binding assay. Antisense primers encoding the germ-line JH6 or JH3 region were synthesized and are shown below. The first four tyrosines (Y) of human JH6 were not included in the Antisense Primer JH6 as there is no amino-acid in these positions in the M166 CDRH3 region. One tyrosine was added to the human JH3 of Antisense Primer JH3 in order to preserve the length of the M166 CDR3. A sense primer for the Vh3 subclass family was also prepared.

Antisense Primer JH6:
CTGTTCCGGAGCTGACGGTGACTGTGGTTCCTTGACCCCAGACATCCATGCCATA
GGTGAAATC

Antisense Primer JH3:
CTGTTCCGGAGCTGACGGTGACCATTGTTCCTTGACCCCAAATATCGAACGCATA
GGTGAAATC

Sense Primer Vh3b: GCCCAGCCGGCCATGGCTSAGGTGCAGCTGGTGGAGTCTGG

[0182] A Vh region library containing the M166 CDR3-FR4 region was PCR-amplified with an N-terminal sense primer for the Vh3 family and either Antisense Primer JH6 or Antisense Primer JH3. After amplification, most Fab' molecules in each library contained the FR4 amino acid sequence encoded by the antisense primers. Some of the Vh regions were Vh1 instead of Vh3, due to cross-hybridization of the Vh3 5' primer to Vh1 segment ends..

[0183] The Vh region repertoire was restricted with *Nco* I and *Bsp* EI and cloned into the Fab' expression vector KB1150. The VL repertoire comprised pre-selected human Vk chains (appended to the M166 CDR3-FR4 region) that were known to bind PcrV in an ELISA assay when paired with a compatible Vh region. Approximately 3500 members of the resulting Fab' libraries were tested in a CLBA assay in which 20 uM IPTG was used to induce Fab' expression (as described in Example 6).

[0184] Of the ~3500 cloned Fab's that contain the BSD and a FR4 containing human JH6 sequence, 34 were positive in the CLBA assay, indicating that these Fab' clones could bind the PcrV antigen. Individual clones were isolated and soluble Fab' was enriched from the growth media. Each of the purified Fab's was positive in an ELISA specific for PcrV antigen. The amino acid sequence of the Vh and Vk regions from two of the positive clones is shown in Figure 6. The MEBSD sequence, comprising the M166 Vh D-region with N-additions, is marked.

[0185] Remarkably, both antibody BA130-5-E10 and BA130-1-1D have VH segments which are identical to human germ-line sequence over the entire region spanning FR1-CDR1-FR2-CDR2-FR3. The germ-line gene Vh1-69 is used in antibody BA130-1-1D and germ-line gene Vh1-02 is used in BA130-5-E10. In both antibodies, the J-segment derived sequences are also human germ-line, JH6. The MEBSD required for specificity of binding to the PcrV antigen is provided by a short sequence within CDRH3, comprised of the D-segment and N-additions from the murine M166 antibody. The heavy chains in each case are paired with human light chains containing CDRL3 sequences from M166.

[0186] A Vh library containing human JH3 sequences was prepared and screened in a similar fashion. Approximately 1500 cloned Fab's that contain the Vh MEBSD and a FR4 containing human JH3 sequence, 45 were positive in the CLBA assay for PcrV binding. Individual clones were isolated and soluble Fab' was enriched from the growth media. Each of the purified Fab's was positive in an ELISA assay with the antigen PcrV. The amino acid sequence of the Vh and Vk regions from two of the positive clones is shown in Figure 7. The M166 Vh MEBSD-region is marked.

<u>Example 4. Focused Vh and Vκ libraries</u>

[0187] The V segment repertoire can be restricted to one subclass of VH or VL before the CDR3-FR4 is appended. Sub-class specific primer sets are used in a PCR reaction with first strand cDNA from an immunoglobulin repertoire. The PCR products are restricted with the appropriate enzymes and cloned into the Fab' expression vector KB1150.

[0188] The murine M166 VH reference chain was compared to all human germ-line VH segments and has the highest degree of similarity to the human VH3 subclass. The M166 VL region is most similar to members of the VκI human subclass.

[0189] The M166 HCDR3-FR4 was appended to a VH3 subclass segment cRNA library by primer extension; the full VH region was cloned into a Fab' expression vector. The M166 LCDR3-FR4 was appended to a VκI subclass segment cRNA library by primer extension; the full VL region was cloned into the Fab' expression vector that contained the VH3 region library. Eight thousand Fab' expressing clones were assayed by CLBA with PcrV as the target antigen (as described in Example 6) using 10 μM IPTG to induce Fab' expression. Twenty four Fab' clones were positive, indicating that they could specifically bind the PcrV antigen. The clones were isolated and soluble Fab' was purified from the growth media. The individual Fab's were tested in an ELISA with PcrV as the target antigen. All of the selected Fab's bound PcrV.

[0190] In each case the Vh chain of the selected Fabs was confirmed to be from the Vh3 subclass and the Vκ chain from the VκI subclass. The frequency of Fab's detected in the CLBA assay was 0.3% with the libraries restricted to Vh3 and VκI compared with a frequency of 0.1% found with the libraries containing a complete representation of human Vh and Vλ sub-classes. This indicates that focused libraries can be used effectively to enhance the frequency of identification of antigen-specific antibodies for further analysis.

Example 5. Colony Lift Binding Assay (CLBA) (General Methods)

[0191] Colony lift binding assays for the screening of single-chain antibody libraries have been described (Giovannoni et al., *Nucleic Acids Research 2001, Vol. 29, No. 5 e27*). Libraries of human antibody Fab or Fab' fragments secreted from *E. coli* and released into the medium can be screened in a similar manner.

Plating of bacterial expressed antibody fragment library

[0192] Antibody libraries are transformed into a suitable bacterial host such as the E. coli strain TOP10. The transformed culture is plated onto 2YT agar (Becton, Dickinson Difco™ 2xYT yeast extract tryptone medium) containing the appropriate antibiotic (chloramphenicol at 34μg/ml). The plating efficiency is adjusted so the resulting bacterial colonies are discreet but dense enough to maximize the area of the plate. Various sizes of plate are used depending on the number of clonal colonies to be screened. Thus, at optimal density a 10cm diameter plate contains 4000 colonies, a 15cm diameter plate contains 10000 colonies and a 25cm square plate contains 50,000 colonies.

Coating of capture-filter with antigen

[0193] Nitrocellulose filters (Schleicher & Schuell BA85) of diameter 8.2cm, 13.2cm or 20cm square are pre-coated with antigen in Phosphate Buffered Saline (PBS) at an empirically determined concentration (usually between 0.5 and 20ug/ml). The volume of coating solution depends upon the filter size. 4ml, 8ml or 20ml can be used for the various filter sizes listed above. Filters are placed face down in a pool of the antigen and capillary action evenly distributes the antigen. The filters are coated for 2-3 hours at 33°C with occasional agitation. The filters are then rinsed once with excess PBS and blocked with a 5% solution of non-fat dry milk in PBS for an additional 2 hours at 25°C with agitation. The filters are then drained and rinsed once in PBS supplemented with 0.1% Tween 20 (PBST) and twice in excess 2YT liquid media supplemented with antibiotic selection and transcriptional inducer (e.g. chloramphenicol and IPTG). After allowing the filters to drain, they are placed on a 2YT-agar plate supplemented with the same concentration of antibiotic and inducer (the expression plate).

Lifting of colonies to the capture filter

[0194] Un-coated, dry nitrocellulose membrane is placed face-down on the plates of colonies containing the antibody-fragment library. Once the filters are visibly wet (~20sec) and in one movement, the filters are lifted and placed colony side up onto the coated filter which is already on the expression plate. A sterile needle is used pierce the filters in a pattern which will allow alignment.

Expression of antibody fragments

[0195] The expression plate with the nitrocellulose filter sandwich is placed at 33°C for 12-16 hours. During this time the antibody fragments are secreted and diffuse through the first nitrocellulose membrane to the second, antigen-coated membrane. If the antibody fragment from a given bacterial colony has antigen binding potential, it is retained on the antigen filter and is subsequently detected.

Detection of antibody fragments

[0196] After the 12-16 hour expression period the colony filter is removed from the expression plate and stored at 4°C on a 2YT-agar plate with antibiotic selection but no transcriptional inducer.

[0197] The antigen coated filter is removed and washed three times (5 minute washes) in excess PBST followed by blocking with a 5% solution of non-fat dry milk in PBST for 1.5 hours at 25°C. The antibody fragments retained on the antigen filter are then detected by first incubating with one of the following alternative primary antibodies: Goat anti-human Kappa-HRP conjugate (US Biological); 9E10 monoclonal SC-40 (Santa Cruz Biotech); or Penta-His monoclonal (Qiagen Inc.) For 9E10 and Penta-His antibodies, an appropriate secondary peroxidase-conjugated secondary antibody is used to reveal binding. After four 10-minute washes, the filters are incubated in peroxidase substrate solution (ECL plus, Amersham Biosciences) and used to expose light-sensitive photographic film. Alternatively, antibodies conjugated with fluorescent labels may be used. In this case a flatbed excitation scanner such as the Typhoon (Amersham Biosciences) or FX-Pro (Biorad) can be used to visualize the positive spots.

Picking of positive colonies

[0198] Using a light box for back illumination, the pattern of spots on the photographic film is aligned with the colony filter (this filter can be removed from the 2YT-agar plate and placed on a plastic transparency for this process). The colonies that give a positive signal are picked and used to inoculate a 2YT liquid mini-culture. Bacteria from the primary screen are then replated at a lower density and picked for subsequent analysis to ensure that a clonal population is expanded.

<u>Example 6. Screen for anti-PcrV antibodies using CLBA</u>

[0199] Recombinant PcrV, cloned as a fusion protein in frame with an amino terminal glutathione S-transferase (GST) purification tag, has been described previously (Frank et al (2002) J. Infectious Diseases 186: 64-73). The PcrV coding sequence is cloned in the expression vector pGEX 2TK (Amersham) to generate the GST-PcrV fusion protein.

[0200] GST-PcrV fusion protein was expressed from *E. coli* (BL21) transformed with pGEX 2TK-PcrV and purified as follows. 4 liter liquid culture batches of *E. coli* expressing GST-PcrV were grown in 2YT to an optical density of 0.6 at 600nm before induction of protein expression with 0.5 mM IPTG and a further 3 hours growth. The bacterial cells were pelleted by centrifugation and lysed in a solution of Bug Buster (Novagen) supplemented with 1U/ml rLysozyme (Novagen) and a protease inhibitor cocktail (Sigma-Aldrich) diluted to the manufacturer's instructions. After clearing the lysate by centrifugation and filtration it was past over a glutathione sepharose column (GSTrap FF, Amersham biosciences), washed and the pure GST-PcrV was eluted in 10mM Glutathione. The antigen was desalted back into PBS and used to coat nitrocellulose filters for CLBA at concentrations of 2– 20 μg/ml. CLBA was carried out as in Example 5.

[0201] For libraries expressed in KB1082, libraries were plated on 2YT expression plates containing chloramphenicol [34μg/ml] and arabinose [0.002%]. Cells were induced for 16 hours and antibody fragments binding to GST-PcrV on the antigen-coated filter were detected using a goat anti-human kappa antibody - Horseradish peroxidase conjugate (US Biological) at a dilution of 1/5000 in PBST. + After 4x 15 minute washes and the application of ECL Plus (Amersham biosciences), the filters were used to expose auto radiographic film (Hyperfilm from Amersham biosciences).

[0202] Nitrocellulose filters were initially coated with GST-PcrV at a concentration of 20 μg/ml. Two independent library screens were completed. For library BA19 15,000 colonies were plated and screened with 12 colonies giving a positive signal indicating the presence of a Fab with PcrV binding potential. For Library BA46 >50,000 colonies were screened resulting in >200 positive signals. The positive colonies from both libraries were picked and plated at lower density. Individual clones were grown in liquid culture and Fab expression into the growth media was induced. Those clones that gave a strong signal in subsequent ELISA assays for GST-PcrV binding were further analyzed in dilution ELISA and Biacore analysis as described in examples 7 and 8.

[0203] Subsequent screens on lower antigen densities (2 μg/ml coating concentration) gave fewer positive clones but ELISA and Biacore analysis showed the higher stringency screen resulted in higher affinity Fab fragments. The stringency of the screens can thus be set to select antibody fragments with equal or higher affinity than the murine parental antibody.

[0204] For libraries expressed in KB1150, expression plates were prepared with chloramphenicol [34 μg/ml] and either had no inducer or contained 10-20 μM IPTG. Colonies on the lift filter were cultured on the expression plate for 16 hours and antibodies binding to GST-PcrV were detected using goat anti-kappa-HRP conjugate (US Biological) as above.

[0205] As with the positive clones from libraries expressed in KB1082, those resulting from screens in KB1150 were picked and plated at lower density.

Example 7. Detection of human anti-PcrV antibodies by ELISA

[0206] Positive colonies from the CLBA were streaked on a 2YT-agar plate containing relevant antibiotics but no transcriptional induction. 6-8 colonies from each streak were individually inoculated in 2YT liquid culture in duplicate deep 96-well titer plates. One replica plate was grown for 16 hours, supplemented with glycerol to 15% and stored at -80°C as a glycerol stock. The other replica plate was grown at 33°C in a shaking incubator until an optical density of 0.5-0.8 at 600nm was achieved at which point antibody fragment expression was induced using 0.01% arabinose for those Fabs expressed in KB1082 or 0.5M IPTG for KB1150. A further 16 hours growth resulted in accumulation of antibody fragments in the growth medium.

[0207] ELISA plates (Costar EIA / RIA) were coated with 100 ng/well GST-PcrV in PBS by incubating them at 4°C for 16 hours and blocked for 1 hour with a 5% solution of non-fat dry milk in PBS 0.1% Tween 20 (PBST). Samples of media were cleared of cell debris by centrifugation and applied to the ELISA plate for 1hour at 33°C. After washing with PBST, antibody fragments binding to the antigen were detected with either anti-peptide tag (9E10, Santa Cruz biotech), at a dilution of 1/1000 in PBST followed by Goat anti-mouse polyclonal – HRP conjugate (Dakocytomation) at a dilution of 1/1000 in PBST, or goat anti-human kappa-HRP conjugate (US Biological) at a dilution of 1/1000 in PBST. Antibody binding was revealed using the peroxidase substrate Tetramethyl benzidine (TMB) (100 μl / well), and the reaction was stopped with the addition of 100ul 2N H_2SO_4 and read by a standard plate-reader.

[0208] The alignment of the positive ELISA signals with the replicate glycerol stocks allowed for the picking of one of the 6-8 cultures for each original CLBA clone. Selected antigen-binding clones were purified for determination of antibody-binding affinity by surface plasmon resonance (Example 8).

[0209] In some experiments, ELISA was used for primary screening of pools of up to 20 bacterial colonies obtained from the antibody library.

[0210] 380 pools, each containing an estimated 13 bacterial colonies/well from Library BA19 were cultured overnight in 96-well microtiter plates under inducing conditions (0.02% arabinose). Culture supernatants were screened by ELISA for antibody fragments binding to GST-PcrV. Antigen-binding antibody fragments were detected using mouse anti-Penta-His (Qiagen) diluted 1:100 in PBST and revealed using HRP-conjugated goat anti-mouse antibody (Dakocytomation) 1: 500 in PBST and TMB substrate. A single positive well was identified which showed strong binding to antigen. Cells from this pool were obtained from a replica glycerol plate and grown at low density on 2YT agar. 24 sub-clones were screened by antigen-binding ELISA and two clones were identified with high affinity for PcrV antigen, both of which expressed antibody fragments of identical sequence.

[0211] ELISA assays were also used to determine relative binding affinities of purified antibody fragments expressed from bacteria. His-tagged antibody fragments were purified using Ni-sepharose as follows. One liter liquid cultures of *E. coli* expressing the antibody fragments were grown to an optical density of 0.6 at 600nm before induction with the addition of arabinose to a final concentration 0.01%. The cultures were grown for a further 3 hours at 33°C prior to harvesting the cells by centrifugation. The cells were fractionated and the periplasmic fractions retained as follows. The bacterial cell pellet from a 1 liter culture was resuspended in 10ml of TES buffer (0.2M Tris pH8.0, 17.12% sucrose and 0.5mM EDTA) and incubated at 4°C for 15 minutes. After the addition of 12.5ml of TES / H_2O at a ratio of 1 / 4 the cell mixture was incubated at 4°C for a further 15 minutes. The cells were pelleted by centrifugation at 7000rcf for 15 minutes and the supernatant was kept. The pellet was then resuspended in 10ml TES supplemented with 15mM Mg_2SO_4 and incubated at 4°C for 10 minutes followed by repelleting. The supernatants were pooled, dialyzed against PBS and antibody fragments were purified on Ni-NTA (Invitrogen) according to the manufacturer's instructions. Fab' fragments without a C-terminal tag were purified by Protein G affinity purification using HiTrap Protein G HP columns (Amersham Biosciences). Antibody fragments were checked for purity by SDS-PAGE and staining with Coomassie Blue. Antibody fragment concentrations were determined by densitometry of the Coomassie-stained gel in comparison with a bovine serum albumin (BSA) standard, using a ChemiDoc XRS (Bio-Rad Laboratories, Inc.). The variable regions (VH and VL) of the Mouse Mab 166 were cloned, expressed and purified from *E. coli* in this way. The Murine Fab was then used as a standard in the following ELISA and Biacore assays. Dilution ELISAs on purified antibody fragment samples were run with the same basic procedure described above for screening bacterial medium samples. Fabs were diluted to the same starting concentration in 100μl of PBS. A two fold dilution series across a 96-well microtiter plate was set up for each Fab in duplicate. This series was then applied to the pre-coated and blocked ELISA plate. After washing in PBST the bound Fab was detected as described above. This assay allowed for the affinity ranking of the Fabs prior to Biacore analysis. Examples of the data generated by this assay are shown in Figure 8.

Example 8. Analysis of affinities of anti-PcrV antibodies by surface plasmon resonance

[0212] Binding kinetics were analyzed by surface plasmon resonance using a Biacore 3000 analyzer (Pharmacia). The GST-PcrV antigen was coated onto the sensor chip at up to three different densities (20-300 RU). Immobilization was done on a CM4 sensor chip using standard amine coupling chemistry. The running buffer was 10 mM HEPES, 150 mM NaCl, 0.005% P20, 3 mM EDTA, and 0.2 mg/ml BSA (pH 7.4). Fab samples were applied to up to 3 different GST-PcrV density chips in duplicate. Bound complexes were regenerated with a 12 second pulse of 1/200 dilution of phosphoric acid. The mean binding response data from the different density surfaces were globally fit to determine the binding constants shown in the table below. The variable regions (VH and VL) from Mab 166 were also cloned and expressed from E. coli as a chimeric Fab fragment. Selected human Fab fragments could thus be compared with the starting murine Fab for binding kinetics (Murine Fab M166).

Table 1. *Kinetics of binding of Fab fragments to recombinant PcrV antigen determined by surface plasmon resonance analysis. Data represent the means of three determinations.*

Fab	*ka* (M-1s-1)	*kd* (s-1)	*KD* (nM)
M166 (murine)	2.49E+5	2.6E-4	1.1
A10	3.3E+5	7.4E-04	2.2
F6	1.44E+5	2.55E-3	17.7
1F1	7.91E+5	8.87E-4	1.13
1A8	1.60E+5	1.35E-4	0.726
BA89	3.4E+5	5.8E-5	0.174
BA90	3.4E+5	5.3E-5	0.160

[0213] Human Fabs were isolated by CLBA and ELISA screening with different binding kinetics. Several Fabs had affinities comparable to the affinity of the murine reference Fab and Fab1A8 had a significantly higher affinity and a significantly lower dissociation rate than Mab166 Fab (Table 1).

[0214] BA89 and BA90 are derivatives of Fab-1A8 generated using parsimonious mutagenesis according to previously described methods (Balint and Larrick *Gene* 137:109-18, 1993) in order to generate single amino-acid mutations in CDRH3 and CDRL3. Mutant derivatives were screened by CLBA at high stringency. The affinities of these two antibodies are higher and the dissociation rates are lower than 1A8 (Table 1).

Table 2: *Comparison of V-region sequences with the closest human germ-line sequences. The amino-acid sequences of the V-regions of each antibody were compared to the database of human germ-line sequences and the percent identity to the closest human germline is shown for each V-region excluding the CDR3 sequences.*

Clone	**Vh versus:**	**% Identity to Human Germline**	**Vκ versus:**	**% Identity to Human Germline**
M166 (murine)	**VH3-33**	**63**	**VκI A20**	**71**
A10	VH3-30.3	93	VκIII L6	98
F6	VH3-33	98	VκIII A27	92
1F1	VH3-30.3	93	VκIII L6	99
1A8	VH3-30.3	93	VκI L12	87
BA89	VH3-30.3	94	VκI L12	88
BA90	VH3-30.3	94	VκI L12	88

[0215] This analysis demonstrates that high affinity anti-PcrV antibodies can be isolated using the CLBA. The sequence of each of the anti-PcrV antibodies was compared with the database of human germ-line sequences and the percent of amino-acids identical to those of the closest human germ-line sequence is shown in Table 2. Each of the antibodies shows significantly higher homology with human germ-line sequences than the M166 murine reference antibody in both the VH and VL regions. Indeed, the complete V-regions for each antibody show a high degree of sequence identity with a germ-line human chain. (The CDR3 sequences were excluded from this analysis as they contain the binding specificity determinants for these antibodies). The sequences of the V-regions of two of the antibodies, F6 and 1F1 are shown in Figure 9. F6 has a VH-segment which is completely identical to a human germ-line V-segment (VH3-33). 1F1 has a VL-segment which is identical to germ-line VκIII L6. Thus, the methods described here have succeeded in identifying high affinity antibodies to PcrV with a high degree of homology to human germ-line antibody sequences. In some cases, at least one of the V-regions has a V-segment which is completely identical to a germ-line sequence.

Example 9. Antagonism of Type III secretion system by human Fabs to PcrV

[0216] The *Pseudomonas* Type III secretion system (TTSS) mediates the direct translocation of *Pseudomonas* exotoxins from the bacteria to host cells with which it comes into contact. Hence *Pseudomonas* strains expressing exotoxins show potent cytotoxic activity towards all mammalian cell types.

[0217] An exotoxin-dependent cytotoxicity assay was established using the mouse myeloma cell line P3-X63-Ag8 as the target. 2 x10^5 cells were infected with *P. aeruginosa* strain PA103 at an MOI of 10. After 3 hours, cells were stained with Propidium Iodide and the proportion of permeabilized cells was quantified by flow cytometry. At this time point approximately 50% of cells were stained with Propidium Iodide. Mab 166 has been shown to block TTSS-mediated exotoxin secretion and prevent cell-killing by *Pseudomonas* strain PA103 (Frank et al 2002. J. Infect. Disease 186:64). For antibody inhibition experiments, human Fab fragments or Mab166 whole IgG were incubated together with X63 cells and *Pseudomonas* PA103 as described above. In this assay, Fab-1A8 and murine Mab 166 IgG both showed effective inhibition of Pseudomonas-mediated cytotoxicity. The IC_{50} for Fab-1A8 was determined to be 68 nM $\pm$ 1.1 nM, compared with an IC_{50} of 93 nM $\pm$ 1.1 nM for Mab 166. (Data represent mean $\pm$ standard error of means, determined from 3 independent assays, expressed as concentration of antibody binding sites).

[0218] This indicates that Fab1A8 retains the biological activity of the Mab 166 reference antibody, demonstrating potent antagonist activity against TTSS-mediated cytotoxicity. The higher potency of Fab 1A8 compared with Mab166 in the cell-based assay is consistent with its higher affinity for recombinant PcrV antigen determined by surface plasmon resonance analysis.

Example 10. Expression and secretion of human Fab fragments in yeast

[0219] For expression and secretion of Fab fragments from *S. cerevisiae*, a yeast invertase (SUC2) signal-peptide was chosen for fusion to the N-terminus of the mature protein sequence for both the heavy and light chains. The coding sequences were then introduced into a yeast expression vector, pESC-Trp (Stratagene) which has a trp selectable marker and insertion sites for two coding sequences under the control of galactose-regulated promoters.

[0220] The Fd fragment of human anti-PcrV antibody 1A8 was amplified by two-step nested PCR reactions from 1A8 plasmid DNA using overlapped PCR primers pr37/pr32 and pr33/pr34. The primers also serve to introduce sequences encoding a yeast invertase secretion signal upstream of the heavy chain coding sequence. The Fd sequence was amplified for 15 cycles with pr32 plus pr37. The PCR fragment was then gel-purified and amplified for 15 cycles with pr33 plus pr34 and re-purified. PCR fragments were digested with Bgl II plus Sac II and ligated into pESC-Trp cut with BamH I plus Sac II in the multiple cloning sites downstream from the Gal1 promoter to make pSC0021-3.

Primer 32:
CAGAAATCAATTTCTGTTCCATAGAACCACCGCCACCACAAGATTTGGGCTCAAC
TTTC

Primer 37:
CTTGTTCTTAGCTGGTTTTGCTGCCAAGATATCTGCTGAGGTGCAGCTGGTGGAG

Primer 33:
AACCCCAGATCTGTCGACCACCATGTTGTTACAAGCCTTCTTGTTCTTAGCTGGTT
TTGC

Primer 34:
GATCTTAGCTAGCCGCGGTTAGTTCAAATCCTCTTCAGAAATCAATTTCTGTTCCA
TAG

[0221] Primers (pr66/pr67 and pr68/pr67) were used to amplify the light chain of Fab-1A8 by nested PCR reaction. The primers also provide the yeast invertase secretion signal upstream of the light chain. The light chain was amplified for 15 cycles with pr66 plus pr67. The PCR fragment was then gel-purified and amplified for 15 cycles with pr68 plus pr67, and re-purified. PCR fragments were digested with EcoRI plus BamH I and ligated into pESC-

Trp cut with EcoRI plus Bgl II in the multiple cloning sites downstream from the Gal10 promoter to make pSC0017-2.

Primer 66:
CTTATTCCTGGCTGGTTTCGCTGCTAAGATCTCTGCTGACATCCAGTTGACCCAGTCTC

Primer 67:
CACTAGACATGGATCCATATGCTAACACTCTCCCCTGTTGAAGCTC

Primer 68:
TGAAAATTCGAATTCCACCATGTTATTGCAAGCTTTCTTATTCCTGGCTGGTTTCGC

[0222] To construct a double-gene vector for expression of both heavy and light chains, the 1.5 kb *Eco*RI - *Nhe*I fragment from pSC0021-3, containing the Fd coding sequence, was sub cloned into the light-chain expression vector pSC0017-2, digested with the same enzymes, to make pSC0019-1. In this vector, expression of the light chain is directed from the GAL10 promoter and the Fd chain is expressed from the GAL1 promoter. Expression of both chains is induced in media lacking galactose.

Detection of Fab-1A8 secreted into the medium

[0223] Yeast strain YPH499 was obtained from Stratagene and growth and transformation were carried out according to the manufacturer's instructions (pESC Yeast epitope-tagging vectors: Instruction Manual revision # 104002d; Stratagene). Briefly, the YPH499 cells were streaked from a glycerol stock onto a YPAD plate and incubated at 30 °C for two days until colonies appeared. Fresh competent cells were prepared from the YPH499 colonies and used for transformation with 1 μg of pSC0019-1 DNA. The transformation reactions were plated onto SD dropout plates and incubated at 30 °C for three days to select transformants.

[0224] For expression of antibody fragments, six colonies from each transformation were inoculated into 5 ml of SD dropout medium (which contains 2% glucose), and incubated with shaking (350 rpm) for overnight. The OD_{600nm} was determined the next day and sufficient cells were centrifuged and resuspended in SG dropout medium (which contains 2% galactose) to generate a culture with OD of 0.25. Expression of Fab fragment was induced by culture in SG dropout medium with shaking (350 rpm) at 30 °C for 16 hours. After induction, the cells were cleared from the media by centrifugation twice and the supernatants were collected for detection of secreted Fab fragment by ELISA and Western blot analysis.

[0225] Expression of assembled Fab was confirmed by Western blotting using detection with HRP-conjugated anti-human kappa antibody (US Biological). Antigen-binding ELISAs were carried out on supernatants from induced cultures and the binding to GST-PcrV antigen was detected with HRP-conjugated anti-human kappa antibody thus confirming secretion of active Fab into the medium.

[0226] For screening of libraries of human Fabs, the V-region sequences in pSC0019-1 are replaced by libraries of VH and VL sequences using standard recombinant DNA techniques. Fab fragments secreted from yeast transformants are detected by antigen-binding ELISA as described above, or by colony-lift binding assay.

<u>Example 11. Colony lift binding assay (CLBA) in Yeast</u>

[0227] The CLBA methodology for antibody secretion and detection in yeast is essentially the same as described for bacterial colonies in Example 5. Yeast cultures containing the vector pSC0019-1 DNA with either a PcrV-binding Fab or a negative control Fab were grown for 16 hours at 30°C in SD dropout minimal medium. The optical density of the cultures at 600nm was measured and 1000 cells of each culture were plated onto separate SD dropout agar plates (growth plates). Small discrete colonies were seen after 16hrs growth at 30°C. A nitrocellulose filter was coated with 20 μg/ml GST-PcrV and blocked with a 5% milk solution as described in example 5. The antigen coated filter was rinsed in SG dropout media prior to placing it on an SG dropout agar plate (expression plate). Colonies on the growth plate were lifted onto a nitrocellulose filter which was then placed on top of an antigen coated filter on the expression plate. The expression plate was then incubated for a further 16 hours at 30°C. The HRP-conjugated anti-human kappa antibody (US Biological) was used for detection of the antibody Fab fragments as described in Examples 5 and 6.

[0228] On exposure to radiographic film the antigen filter from the plate of Fab-1A8 colonies showed positive signals corresponding to colonies secreting anti-PcrV Fab while the negative control was blank. This indicates that the CLBA can be used to screen libraries of antibody fragments expressed and secreted from yeast.

[0229] All publications, accession numbers, and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

[0230] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of making an antibody having a binding specificity of a reference antibody, the method comprising

a) joining a heavy chain CDR3 binding specificity determinant (BSD) from the reference antibody to a diverse population of human V_H segments thereby creating a library of human V_H regions having the reference antibody heavy chain CDR3 BSD;

b) joining a light chain CDR3 BSD from the reference antibody to a diverse population of human V_L segments, thereby creating a library of human V_L regions having the reference antibody light chain CDR3 BSD;

c) combining the libraries of step a and step b to create an antibody library comprising members where a member has one V_H comprising the reference antibody heavy chain CDR3 BSD and one V_L comprising the reference antibody light chain CDR3 BSD; and

d) isolating a member of the library of step c that binds the same antigen as the reference antibody.

2. The method of claim 1, wherein the diverse population of human V_H segments or the diverse population of human V_L segment is human germline.

3. The method of claim 2, further comprising: mutagenizing at least one of the CDR3s of the antibody selected in (d); and selecting an antibody that has a higher affinity for the antigen than the antibody selected in (d).

4. The method of claim 1, wherein both the diverse population of V_H segments and the diverse population of V_L segment are human germline.

5. The method of claim 4, further comprising: mutagenizing at least one of the CDR3s of the antibody selected in (d); and selecting an antibody that has a higher affinity for the antigen than the antibody selected in (d)

6. The method of claim 1, wherein the diverse population of human V_H segments is from one V segment subclass.

7. The method of claim 1, wherein the diverse population of human V_L segments is from one V region subclass.

8. The method of claim 1, wherein at least one of the CDR3 BSDs from the reference antibody is a CDR3-FR4 segment from the reference antibody.

9. The method of claim 1, wherein the FR4 is a human germline FR4..

10. The method of claim 1, wherein the human FR4 is a library of diverse human FR4 sequences.

11. The method of claim 1, wherein the J segment is a human antibody J segment.

12. The method of claim 1, wherein at least one of the CDR3 BSDs from the reference antibody is the reference antibody CDR3..

13. The method of claim 1, wherein a heavy chain CDR3 BSD is the reference antibody heavy chain CDR3 and the light chain CDR3 BSD is the reference antibody light chain CDR3.

14. The method of claim 1, wherein the heavy chain CDR3 BSD from the reference antibody is the D segment.

15. The method of claim 1, wherein at least one of the CDR3 BSDs from the reference antibody is a minimal essential binding specificity determinant.

16. The method of claim 1, wherein the step of isolating a member of the library of step c comprises a screening step to identify a member of the library that binds to the antigen with the same or higher affinity than the reference antibody.

17. The method of claim 1, wherein the reference antibody is a nonhuman antibody.

18. The method of claim 1, wherein the step of combining the libraries comprises expressing the library of human V_H regions and the library of human V_L regions on a single expression vector.

19. The method of claim 18, wherein the V_H and library and the V_L library are expressed using separate promoters.

20. The method of claim 1, wherein the step of combining the libraries comprises expressing the library of human V_H regions and the library of human V_L regions on two expression vectors.

21. The method of claim 1, wherein the human antibody library of step c) comprises antibodies where an antibody is an IgG, an Fv, an Fab, an Fab', an $F(ab')_2$, a single chain Fv, or an IgG with a deletion of one or more domains.

22. The method of claim 1, wherein the step of isolating the members of the library comprises screening using a colony lift binding assay.

23. The method of claim 1, wherein the step of isolating the members of the library comprises screening using a display technology selected from the group consisting of bacteriophage display, yeast cell display, bacterial cell display, ribosome display, and mammalian cell display.

24. The method of claim 1, wherein the step of isolating the members of the library comprises screening pools of library members.

25. The method of claim 1, further comprising:

e) combining heavy chain V regions from a plurality of members selected in accordance with (d) with a library of human V_L regions having the reference antibody light chain CDR3 BSD and

f) selecting a member that binds to the same antigen as the reference antibody.

26. The method of claim 1, further comprising:

e) combining light chain V regions from a plurality of members selected in accordance with (d) with a library of human V_H regions having the reference antibody hevy chain CDR3 BSD; and

f) selecting a member that binds to the same antigen as the reference antibody.

27. The method of claim 1, further comprising:

e) combining heavy chain V regions from a plurality of members selected in accordance with (d) with light chain V regions from a plurality of members selected in accordance with (d); and

f) selecting a member that binds to the same antigen as the reference antibody.

28. A method of making an antibody having a binding specificity of a reference antibody, the method comprising

a) joining a CDR3 BSD from the heavy chain of the reference antibody to a diverse population of human V_H segments, thereby creating a population comprising diverse V_H regions having a BSD from the reference antibody heavy chain CDR3;

b) combining the population of step a with aV_L comprising a human germline subclass V segment joined to a light chain CDR3 BSD from the reference antibody to create an antibody library; and

c) isolating a member of the library of step c that binds the same antigen the reference antibody.

29. The method of claim 28, wherein at least one of the CDR3 BSDs from the reference antibody is a minimal essential binding specificity determinant.

30. The method of claim 28, wherein the CDR3 BSD from the heavy chain of the reference antibody is the D segment.

31. The method of claim 28, wherein at least one of the CDR3 BSDs from the reference antibody is CDR3.

32. The method of claim 28, wherein the diverse population of human V_H regions comprises germline V_H segments.

33. The method of claim 28, wherein the J segment region of the V_L is a human germline sequence.

34. A method of making an antibody having a binding specificity of a reference antibody, the method comprising

a) joining a CDR3 BSD from the light chain of the reference antibody to a diverse population of human V_L segments, thereby creating a population comprising diverse V_L regions having a BSD from the reference antibody light chain CDR3;

b) combining the population of step a with aV_H comprising a human germline subclass V segment joined to a CDR3 BSD from the reference antibody to create an antibody library; and

c) isolating a member of the library of step c that binds the same antigen the reference antibody.

35. The method of claim 34, wherein at least one of the CDR3 BSDs from the reference antibody is a minimal essential binding specificity determinant.

36. The method of claim 34, wherein the CDR3 BSD from the heavy chain of the reference antibody is the D segment.

37. The method of claim 34, wherein at least one of the CDR3 BSDs from the reference antibody is CDR3.

38. The method of claim 34, wherein the diverse population of human V_L regions comprises germline V_L segments.

39. The method of claim 34, wherein the J segment region of the V_H comprising the human subclass V segment joined to the CDR3 BSD is a human germline sequence.

40. A method of making an antibody having a binding specificity of a reference antibody, the method comprising

a) joining a minimal essential binding specificity determinant from the light chain CDR3 of the reference antibody to a diverse population of human V_L segments, thereby creating a population comprising diverse V_L regions having a reference antibody light chain CDR3 BSD;

b) combining the population of step a with a V_H region from the reference antibody to create an antibody library; and

c) isolating a member of the library of step c that binds the same antigen as the reference antibody.

41. The method of claim 40, further comprising:

d) joining a BSD from the heavy chain CDR3 from the reference antibody to a diverse population of human V_H segments, thereby creating a population comprising diverse V_H regions having the reference antibody heavy chain CDR3 BSD;

e) providing a population of V_L regions from the antibody isolated in (c);

f) combining the population of step (d) and step (e) to create an antibody library; and

g) isolating a member of the library of step (f) that binds the same antigen as the reference antibody.

42. The method of claim 40, wherein the diverse population of human V_L segments of (a) are germline.

43. The method of claim 42, further comprising mutagenizing at least one of the CDR3s of the antibody selected in (g); and selecting an antibody that has a higher affinity for the antigen than the antibody selected in (g).

44. A method of making an antibody having a binding specificity of a reference antibody, the method comprising

a) joining a BSD from the heavy chain CDR3 of the reference antibody to a diverse population of human V_H segments, thereby creating a population comprising diverse V_H regions having the reference antibody heavy chain CDR3 BSD, wherein the BSD is selected from the group consisting of the minimal essential binding specificity determinant, the D segment, and the D segment-FR4;

b) combining the population of step a with a V_L region from the reference antibody to produce and antibody library; and

c) isolating a member of the library of (b) that binds the same antigen as the reference antibody.

45. The method of claim 44, further comprising:

d) joining a BSD from the CDR3 from the light chain of the reference antibody to a diverse population of human V_L segments, thereby creating a population comprising diverse V_L regions having the reference antibody light chain CDR3 BSD;

e) providing a population of V_H regions from the antibody isolated in (d);

f) combining the population of step (d) and step (e) to create an antibody library; and

g) isolating a member of the library of (f) that binds the same antigen as the reference antibody.

46. The method of claim 44, wherein the diverse population of human V_H segments of (a) are germline.

47. The method of claim 46, further comprising mutagenizing at least one of the CDR3s of the antibody selected in (g); and selecting an antibody that has a higher affinity for the antigen than the antibody selected in (g).

48. A library comprising a plurality of nucleic acids that encode a diverse population of heavy chain V segments, wherein the V segments are not linked to a CDR3.

49. The library of claim 48, wherein the V segments are human germline.

50. A library comprising nucleic acids that encode a diverse population of light chain V segments, wherein the V segments are not linked to a CDR3.

51. The library of claim 50, wherein the V segments are human germline

52. A library comprising a plurality of human antibody V-region pairs where a V-region pair comprises: i) an unselected heavy chain V-region comprising a human V segment and a heavy chain CDR3 from a reference antibody, and ii) an unselected light chain V-region comprising a human V segment and a light chain CDR3 from the reference antibody.

53. A library comprising nucleic acids encoding human antibody V-region pairs, where the V_H and V_L V segments are each linked to an MEBSD from a reference antibody.

54. A library comprising nucleic acids encoding a plurality of V_H or V_L regions, wherein the V_H or V_L regions comprise V segments from one V_H or V_L subclass, wherein the V regions lack D and/or J segments.

55. The library of claim 54, wherein the V segments of the V_H regions are germline and/or the V segments of the V_L regions are germline.

56. A library comprising a plurality of antibody V region pairs, wherein a pair comprises: i) a heavy-chain V region comprising a binding specificity determinant BSD from a heavy chain CDR3 from a reference antibody joined to a diversity of V segments, and ii) a light chain V region comprising a BSD from a light chain CDR3 from the reference antibody joined to a diversity of V segments, wherein at least one of the BSDs comprises less than the reference antibody CDR3.

57. A library comprising a plurality of V_H regions comprising a BSD from a heavy chain CDR3 of a reference antibody joined to a diverse population of V_H segments, with the proviso that the BSD is less that the reference antibody heavy chain CDR3.

58. A library comprising a plurality of V_L regions comprising a BSD from a light chain CDR3 of a reference antibody joined to a diverse population of V_L segments, with the *proviso* that the BSD is less that the reference antibody light chain CDR3.

59. An antibody comprising a V_H region comprising a human V segment, a D segment from a non-human reference antibody and a human J segment.

60. The antibody of claim 59, wherein the human V segment is a germline V segment.

61. An antibody comprising a V_H region having a human germline V segment and a MEBSD from a heavy chain CDR3 from a reference antibody.

62. The antibody of claim 61, wherein the BSD is a CDR3-FR4 from the reference antibody.

63. The antibody of claim 61, wherein the BSD is a CDR3 from the reference antibody

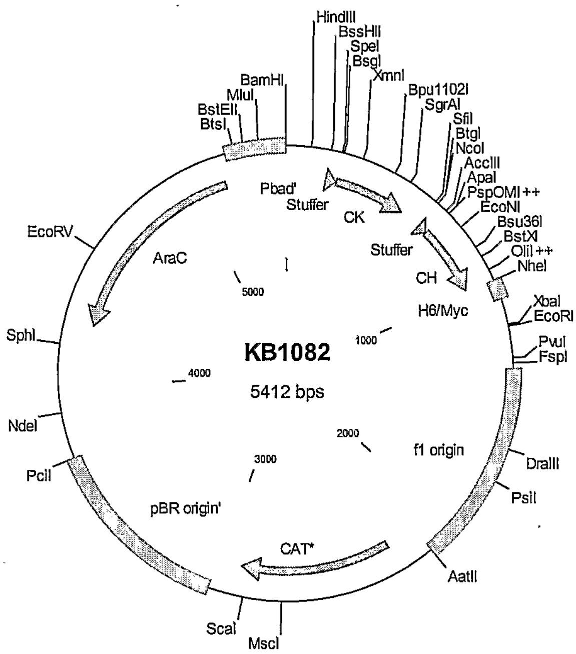
64. The antibody of claim 61, wherein the BSD is a D segment from the reference antibody.

65. An antibody comprising a V_L region having a human germline V segment and a BSD from a light chain CDR3 from a reference antibody.

66. The antibody of claim 65, wherein the BSD is a CDR3-FR4 from the reference antibody.

67. The antibody of claim 65, wherein the BSD is a CDR3 from the reference antibody.

Figure 1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EcoNI
Bsu36I
BstXI
OliI ++
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XbaI
EcoRI
PvuI
FspI
DraIII
PsiI
AatII
MscI
ScaI
PciI
NdeI
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Pbad'
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CK
Stuffer
CH
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AraC
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4000
2000
3000
f1 origin
pBR origin'
CAT*

Figure 2

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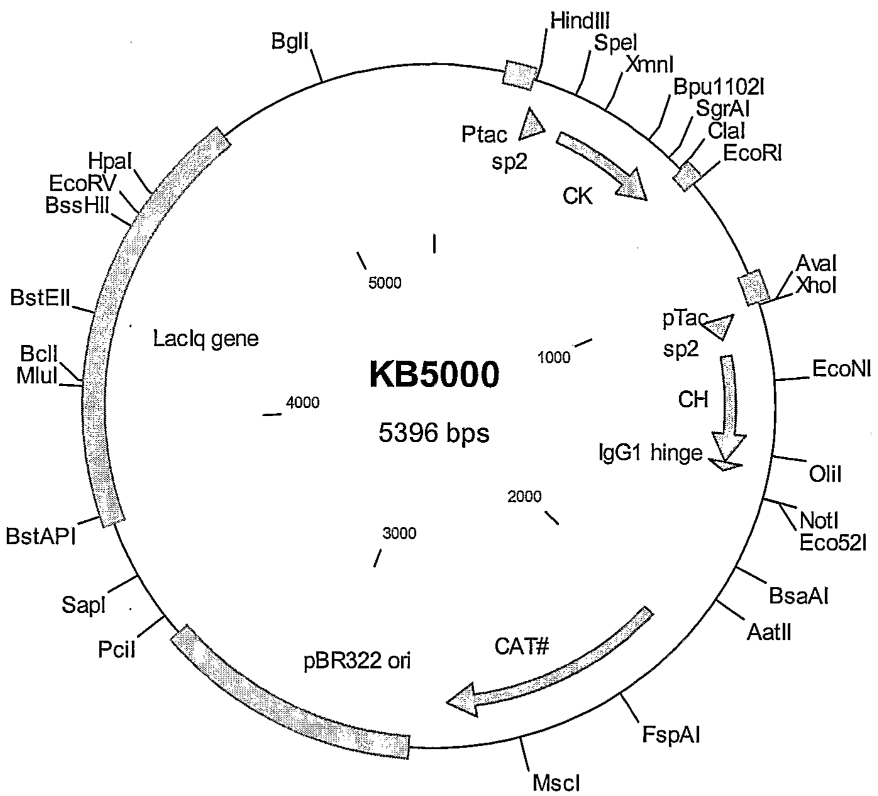
Figure 3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NotI
Eco52I
BsaAI
AatII
FspAI
MscI
PciI
SapI
BstAPI
BclI
MluI
BstEII
BssHII
EcoRV
HpaI
BglI
Ptac
sp2
CK
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CAT#
pBR322 ori
LacIq gene
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2000
3000
4000
5000

Figure 4

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Figure 5

	N-addition	D segment	N-addition	Jh-CDR3	Jh-Fr4	
Mab166	NRGD	IYYD	FT	YAMDY	WGQGTSVTVSS	
				YYYYYGMDV	WGQGTTVTVSS	JH6
				-AFDI	WGQGTMVTVSS	JH3

Figure 6

BA130-1-1D Vh: QVQLVESGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCAR**NRGDIYYDFT**YGMDVWGQGTTVTVSS

BA130-1-1D Vk: ALDIQMTQSPSSLSASVGDRVTITCRASQSISTYLNWYQQRPGKAPKLLIYAASRLLNGV PSRFSGSGSGTDFTLTISGLQPEDIATYYCQHFWSTPYTFGQGTKLEIK

BA130-5-E10 Vh: EVQLVESGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQAPGQGLEWMGWINPNSGGTNY AQKFQGWVTMTRDTSISTAYMELSRLRSDDTAVYYCAR**NRGDIYYDFT**YGMDVWGQGTTVTVSS

BA130-5-E10 Vk: ALDIQMTQSPSSLSASVGDRVTITCRASQSISTYLNWYQQRPGKAPKLLIYAASRLLNGV PSRFSGSGSGTDFTLTISGLQPEDIATYYCQHFWSTPYTFGQGTKLEIK

Figure 7

a) Antibody BA133-5-E6

BA133-5-E6 Vh: EVQLVESGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQGLEWMGWISAYNGNTNY AQKLQGRVTITTDTATRTTYMDLRSLRSDDTAVYYCAR**NRGDIYYDFTY**AFDIWGQGTMVTVSS

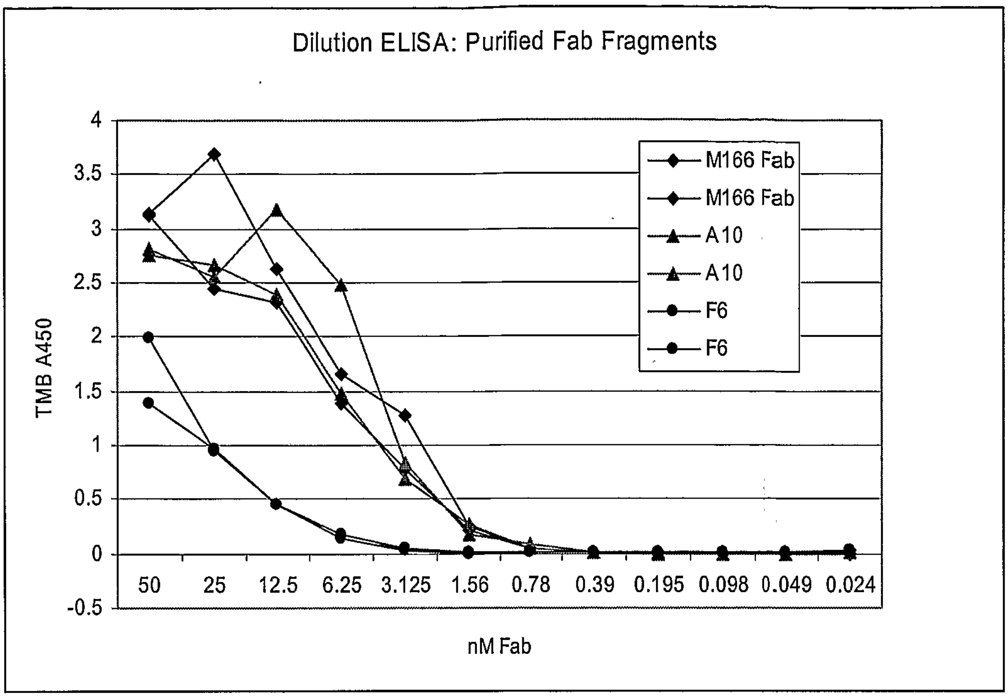
BA133-5-E6 Vk: ALDIQMTQSPSSLSASVGDRVTITCRASQSISTYLNWYQQRPGKAPKLLIYAASRLLNGV PSRFSGSGSGTDFTLTISGLQPEDIATYYCQHFWSTPYTFGQGTKLEIK

b) Antibody BA133-6-F5

BA133-6-F5 Vh: QVQLVESGPEVKKPGTSVKVSCKASGFTFTSSAMQWVRQARGQRLEWIGWIIVGSGNTNY AQKFQERVTITRDMSTSTAYMELSSLRAEDTAVYYCAR**NRGDIYYDFTY**AFDIWGQGTMVTVSS

BA133-6-F5 Vk: ALDIQMTQSPSSLSASVGDRVTITCRASQSISTYLNWYQQRPGKAPKLLIYAASRLLNGV PSRFSGSGSGTDFTLTISGLQPEDIATYYCQHFWSTPYTFGQGTKLEIK

Figure 8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TMB A450
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
50
25
12.5
6.25
3.125
1.56
0.78
0.39
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0.024
nM Fab
M166 Fab
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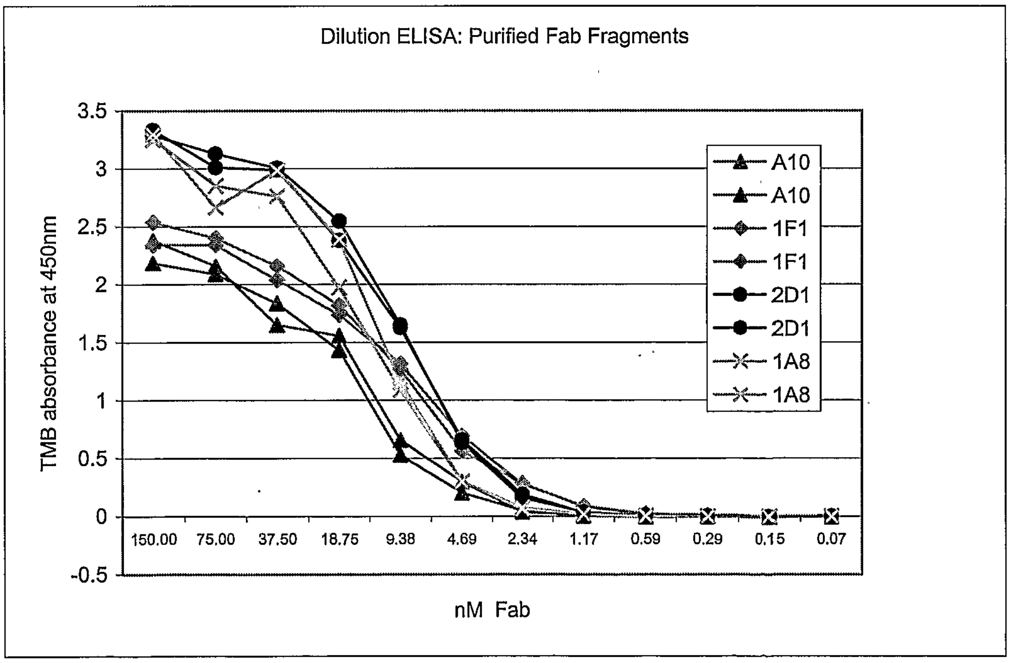
A10
A10
F6
F6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TMB absorbance at 450nm
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3
2.5
2
1.5
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0.5
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150.00
75.00
37.50
18.75
9.38
4.69
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0.59
0.29
0.15
0.07
nM Fab
A10
A10
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1F1
2D1
2D1
1A8
1A8

Figure 9

F6 VH: EVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVIWYDGSNKYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARN**RGDIYYDFTYAMDY**WGQGTSVTVSS

F6 VL: EIVLTQSPGTLSLSPGERATLSCTASQALISSTLAWYQQKPGQAPRLLIFGASSRATGTP DRFSGSGSGTDFTLTISRLEPEDFAVYYC**QHFWSTPYT**FGGGTKLEIK

1F1 VH: EVQLVESGGGVVQPGRSLRLSCAASGFTFSNYPMHWVRQAPGKGLEWVAVISYDGSEKWY ADSVKGRFTISRDNSKNTLYLEMNSLRPEDTAVYYCARN**RGDIYYDFTYAMDY**WGQGTSVTVSS

1F1 VL: EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPA RFSGSGSGTDFTLTISSLEPEDFAVYYC**QHFWSTPYT**FGGGTKLEIK