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(54) Title: METHODS AND MATERIALS FOR TARGETED MUTAGENESIS

(57) Abstract: The present disclosure relates to methods and materials for mutagenesis, including for the generation of novel or improved proteins and libraries or arrays of mutant proteins or nucleic acids encoding such mutant proteins.

TITLE

METHODS AND MATERIALS FOR TARGETED MUTAGENESIS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. § 119 of U.S. Provisional Application No. 61/018,113, filed December 31, 2007; U.S. Provisional Application No. 61/018,105, filed December 31, 2007; U.S. Provisional Application No. 61/018,101, filed December 31, 2007, the disclosures of which are herein incorporated by reference in their entirety.

FIELD

[0002] The present disclosure relates to methods and materials for mutagenesis, including for the generation of novel or improved proteins and libraries or arrays of mutant proteins or nucleic acids encoding such mutant proteins.

BACKGROUND

[0003] Mutagenesis is a powerful research tool whereby genetic information is deliberately changed in a stable manner (see, *e.g.*, Hutchinson *et al*. (1978) *J. Biol. Chem*. 253:6551; and Razin *et al*. (1978) *Proc. Natl. Acad. Sci. USA* 75:4268). Mutagenesis may be performed experimentally by employing the use of recombinant DNA technology and used to introduce specific mutations in a gene to study the effects on its encoded protein. By comparing the properties of a wild-type protein and the mutants generated, it may be possible to identify individual amino acids or domains of amino acids that may be important for the structural integrity and/or biochemical function of the protein (*e.g.*, binding and/or catalytic activity).

[0004] Methods of mutagenesis may be random (*e.g.*, error prone PCR) or deliberate (*e.g.*, site directed mutagenesis). A common feature of many methods of mutagenesis is the use of synthetic primers (*e.g.*, oligonucleotides) carrying desired changes in a nucleotide sequence at the site of mutagenesis. For example, saturation mutagenesis uses primers with one or more degenerate codons (*e.g.*, a NNN, a NNK or a NNS codon) that codes for all possible amino acid substitutions at one or more sites in the parent nucleic sequence. Such degenerate codons may code for 32 to 64 unique codons which collectively may encode 20 amino acids in a redundant fashion (*e.g.*, multiple codons for the same amino acid) and a stop codon. For example, given

the degeneracy of the genetic code some amino acid residues may be overrepresented (*e.g.*, Arg, Leu, and Ser). This technique depends on screening a large number of variants and may require multiple large libraries using phage or ribosomal display to explore the variants. Another technique, error prone PCR uses polymerase to introduce mutations at random positions in a parent nucleic acid sequence. This technique may introduce mutations outside of an area of interest (*e.g.*, a binding pocket such as a CDR) and necessitate backmutation to identify a productive mutation. Accordingly, methods for mutagenesis are desired that produce manageable libraries comprising targeted mutations at one or more positions in a parent nucleic acid sequence.

SUMMARY

[0005] The present disclosure relates to methods and materials for mutagenesis, including for the generation of novel or improved proteins and libraries or arrays of mutant proteins or nucleic acids encoding such mutant proteins. The present disclosure provides methods and materials for targeted mutagenesis of proteins, including by mutating one or more selected positions in a parent nucleic acid sequence. The proteins targeted for mutagenesis can be natural, synthetic or engineered proteins or variants (*e.g.*, mutants of such proteins). The proteins can include binding proteins such as antibodies or their binding fragments and ligands or receptors. The proteins can also include enzymes or catalytic molecules.

[0006] The present disclosure provides methods of mutagenesis of a parent nucleic acid sequence encoding a protein by obtaining one or more primers that each comprise at least one 2 to 12 fold degenerate codon, wherein the primers are complementary to a sequence in the parent nucleic acid sequence and wherein the primers code for amino acid mutations at each of one or more amino acid positions encoded by the parent nucleic acid sequence; and subjecting the parent nucleic acid sequence to replication or polymerase based amplification using the obtained primers, wherein replication or amplification of the parent nucleic acid sequence with the primers generates variant nucleic acid sequences and wherein the variant nucleic acid sequences comprise amino acid mutations at the one or more positions in the parent nucleic acid sequence with the exception of cysteine and methionine.

[0007] The present disclosure provides methods of mutagenesis of a parent nucleic acid encoding a protein to generate modified proteins by obtaining one or more primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each

primer comprises at least two oligonucleotide sequences that are complementary to a sequence in the parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid; and mutating the parent nucleic acid by replication or polymerase based amplification using the one or more obtained primers, wherein replication or amplification of the parent nucleic acid with the one or more primers generates mutated nucleic acids that encode modified proteins.

[0008] The present disclosure also provides libraries/arrays of mutated nucleic acid sequences generated by the methods of the present disclosure.

[0009] The present disclosure also provides methods for mutagenesis of a protein to obtain modified proteins with mutated amino acid sequences by identifying one or more amino acid positions in the protein for mutagenesis; substituting one or more of the identified amino acid residues in the protein with other amino acid residues excluding cysteine and methionine to generate a library or an array of modified proteins with mutated amino acid sequences; screening the library or array of modified proteins in an assay for a biological activity of the protein; and obtaining modified proteins having the biological activity of the protein.

[0010] The present disclosure also provides methods for generating an array of nucleic acids encoding modified proteins by obtaining a collection of nucleic acids encoding modified proteins containing amino acid mutations other than cysteine and methionine at amino acid residues of a parent protein sequence by mutagenesis of a nucleic acid encoding the protein sequence using primers that each comprise at least one 2 to 12 fold degenerate codon; sequencing the collection of nucleic acids encoding the modified proteins; and arranging each sequenced nucleic acid encoding a modified protein to generate an array of nucleic acid sequences each encoding a modified protein.

[0011] The present disclosure also provides methods for generating an array of nucleic acid sequences encoding modified proteins by preparing a plurality of nucleic acid sequences by mutagenesis that encode a plurality of modified proteins that vary from a parent protein sequence at one or more amino acid positions and contain one of eighteen different amino acids excluding cysteine and methionine at each position mutated from the parent protein sequence; and arranging each prepared nucleic acid sequence to generate an array of nucleic acid sequences each encoding a modified protein.

[0012] The present disclosure also provides libraries/arrays of mutated nucleic acid sequences generated by the method of the present disclosure.

[0013] The present disclosure also provides methods for generating an array of clones comprising nucleic acids encoding modified proteins by preparing a plurality of nucleic acids by mutagenesis that encode a plurality of modified proteins that vary from a parent protein sequence at one or more amino acid positions and contain one of eighteen different amino acids excluding cysteine and methionine at each position varied from the parent protein sequence; transfecting the prepared nucleic acids prepared into host cells and selecting clones comprising the transfected nucleic acids; and arranging each selected clone to generate an array of clones with each arrayed clone capable of expressing a modified protein.

[0014] The present disclosure also provides methods of producing a nucleic acid library with an equal representation of non-redundant amino acid changes at an amino acid position encoded by a parent nucleic acids by providing a set of primers that each comprise at least one degenerate codon, wherein each primer comprises at least two oligonucleotide sequence that are complementary to a sequence in the parent nucleic acid and code for an amino acid mutation with the exception of cysteine and methionine at one amino acid position encoded by the parent nucleic acid, wherein the primers code for an equal representation of non-redundant amino acid changes at the one position; hybridizing a primer from the set to the parent nucleic acid; replicating or amplifying the parent nucleic acid molecule with the primer to generate nucleic acids that code for amino acid changes at the one position; repeating the hybridizing and replicating steps with each remaining primer from the set; pooling the nucleic acids produced with each primer; and obtaining a library of nucleic acids coding for an equal representation of amino acid changes at the one position.

[0015] The present disclosure also provides methods for obtaining a nucleic acid sequence with an improvement in comparison to a parent nucleic acid sequence with respect to at least one molecular or biological property of interest by obtaining a set of primers that each comprise at least one 2 to 12 fold degenerate codon that does not code for cysteine and methionine, wherein the primers are complementary to a sequence in the parent nucleic acid sequence and wherein the primers code for non-redundant amino acid mutations at one amino acid position encoded by the parent nucleic acid sequence; mutating the parent nucleic acid sequence by replication or polymerase based amplification using the obtained set of primers to generate variant nucleic acid sequences; producing a library or array of variant nucleic acid sequences

from (b) coding for amino acid mutations at the one position in the parent nucleic acid sequence; and screening the library or array of variant nucleic acid sequences to identify nucleic acid sequences that have a desirable improvement in comparison with the parent nucleic acid sequence with respect to at least one molecular or biological property of interest.

[0016] The present disclosure also provides methods of making modified proteins with mutated amino acid sequences by modifying the amino acid sequence of a protein to produce amino acid mutations at an amino acid residue in the protein to generate a library or an array of modified proteins with mutated amino acid sequences, wherein the amino acid mutations exclude cysteine and methionine; and selecting modified proteins from the library or the array that have a biological activity of an unmodified protein.

[0017] The present disclosure also provides methods for selecting modified proteins with mutated amino acid sequences by obtaining a library or an array of modified proteins comprising amino acid mutations at one amino acid residues in an amino acid sequence of a protein, wherein the amino acid mutations exclude cysteine and methionine; assaying the modified proteins for a biological activity of an unmodified protein; and selecting the modified proteins that have a biological activity of the unmodified protein.

[0018] The present disclosure also provides a set of primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid.

[0019] The present disclosure also provides a kit for mutagenesis of an amino acid residue encoded by a parent nucleic acid comprising a set of primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid.

[0020] The present disclosure also provides libraries/arrays comprising variants of a protein sequence, wherein the variants each comprise an amino acid mutation at one amino acid position in the protein sequence of a parent protein and wherein the amino acid mutations are not cysteine or methionine.

[0021] The present disclosure also provides methods for obtaining a nucleic acid sequence with an improvement in comparison to a parent nucleic acid sequence with respect to at least one molecular or biological property of interest by mutating the parent nucleic acid by polymerase based amplification using one or more primers that each comprise at least one 2 to 12 fold degenerate codon to generate mutated nucleic acid sequences, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in the parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid; sequencing the mutated nucleic acid sequences; arranging each sequenced mutated nucleic acid sequence comprising one amino acid mutation to generate an array of mutated nucleic acid sequences; and screening the array of variant nucleic acid sequences to identify nucleic acid sequences that have a desirable improvement in comparison with the parent nucleic acid sequence with respect to at least one molecular or biological property of interest.

[0022] In some embodiments, the amino acid mutations are selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at each position.

[0023] In some embodiments, the primers code for eighteen amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

[0024] In some embodiments, three primers that each comprise at least one 2 to 12 fold degenerate codon are obtained. In some embodiments, seven primers that each comprise at least one 2 to 12 fold degenerate codon are obtained. In some embodiments, the degenerate codons are selected from the group consisting of: NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T). In some embodiments, the degenerate codons are selected from the group consisting of: ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G).

[0025] In some embodiments, the primers code for basic amino acid mutations at the one amino acid position encoded by the parent nucleic acid. In some embodiments, one primer that comprises at least one 2 to 12 fold degenerate codon is obtained. In some embodiments, the one primer comprises a degenerate codon which codes for arginine and lysine. In some embodiments, the degenerate codon is represented by ARG (where, R=A/G).

[0026] In some embodiments, the primers code for polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid. In some embodiments, two primers that comprise at least one 2 to 12 fold degenerate codon is obtained. In some embodiments, the two primers comprise degenerate codons that collectively code for serine, threonine, asparagine and tyrosine. In some embodiments, the degenerate codons are represented by WMC (where, W=A/T; M=A/C) and CAS (where S=C/G).

[0027] In some embodiments, the primers code for acidic amino acid mutations at the one amino acid position encoded by the parent nucleic acid. In some embodiments, one primer that comprises at least one 2 to 12 fold degenerate codon is obtained. In some embodiments, the one primer comprises a degenerate codon that codes for glutamic acid and aspartic acid. In some embodiments, the degenerate codon is represented by GAS (where S=C/G).

[0028] In some embodiments, the primers code for non-polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid. In some embodiments, three primers that comprise at least one 2 to 12 fold degenerate codon are obtained. In some embodiments, the three primers comprise degenerate codons that collectively code for glutamic acid and aspartic acid. In some embodiments, the degenerate codons are represented by NTC (where, N=A/G/C/T), KGG (where, K=G/T), and SCG (where S=C/G).

[0029] In some embodiments, the parent nucleic acid encodes a binding molecule. In some embodiments, the binding molecule is an antibody or fragment thereof.

[0030] In some embodiments the methods may further comprise selecting the one or more positions in the parent nucleic acid sequence for mutation. In some embodiments the methods may further comprise transforming the mutated nucleic acid sequences into competent cells.

[0031] In some embodiments, the step of substituting is performed with one or more primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid and code for an amino acid substitution with the exception of cysteine and methionine at one amino acid position encoded by the parent nucleic acid. In some embodiments, the step of modifying is performed with one or more primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary

to a sequence in a parent nucleic acid and code for an amino acid substitution with the exception of cysteine and methionine at one amino acid position encoded by the parent nucleic acid.

[0032] In some embodiments, the biological property of interest is binding.

[0033] In some embodiments, modified proteins are selected that have increased activity as compared to the unmodified protein. In some embodiments, modified proteins are selected that have decreased activity as compared to the unmodified protein. In some embodiments, modified proteins are selected that have equal activity as compared to the unmodified protein.

[0034] Additional features and advantages are described herein, and will be apparent from, the following Detailed Description and the Figures.

BRIEF DESCRIPTION OF THE FIGURES

[0035] The Figures relate to exemplary proteins, including those useful for mutagenesis according to methods and materials disclosed herein.

[0036] Figure 1 is a generalized schematic map of an exemplary antibody combining site as described herein, looking downward onto the "top" surface of a variable domain comprising a light chain variable region and a heavy chain variable region. It shows the six CDR loops (L1, L2, L3, H1, H2, H3) which are spatially located directly above the three-dimensional structure of the evolutionarily-conserved framework underneath. As shown and discussed herein, this map provides roughly approximate higher-order structural information, which is not available from the linear primary sequence alone, such as the identity of potential nearest neighbors in the space-filling model of a generic variable domain. Specific features of the murine ING1 monoclonal antibody have been added to this map, so that it can also call attention to localized domains of the antibody's combining site containing clusters of high-conspicuousness positions as described herein, which are likely to be in contact with sidechains on the antigen. In particular, each amino-acid position in the murine ING1 antibody is represented on this map by a white rectangle containing a group of symbols. The letter and number at the bottom-left of each rectangle (*e.g.*, "H 98" in CDR-loop H3) is the Kabat-position number of the amino-acid residue on the antibody molecule within either chain (L=light, H=heavy). The small upper-case letter (*e.g.*, "B") at the bottom-right is a designation for the residue's proximity as described herein (C=Contacting, P=Peripheral, S=Supporting, I=Interfacial) relative to the antibody's binding site (shown on the “prox” line in Figures 2A-2D). The large upper-case letter

(*e.g.*, "A") at the upper-left is the amino-acid code for the residue's sidechain (line "murING1" in Figures 2A-2D). The large single digit at the upper right (*e.g.*, "3") in some rectangles is the non-zero conspicuousness-value as described herein of affinity enhancement for the sidechain (line "cspc" in Figures 2A-2D), calculated in reference to the appropriate human consensus sequence for light chain (hK2) or heavy chain (hH 1). Rectangles with no such value reflect a conspicuousness of zero.

[0037] Figures 2A-2D: Alignments of sequences in the light chain and heavy chain, with lines (*e.g.*, prox, cspc) relating to affinity enhancement and lines relating to human engineering (*e.g.*, risk) are shown. In each set of lines, the top ones apply the present disclosure to the murine ING1 antibody (2A-2B), and the bottom ones relate the present disclosure to the general principles of human engineering (Studnicka *et al*., Protein Engineering, 7(6):805-814 (1994); U.S. Patent No. 5,766,886). Each set of lines shows the Kabat position numbers (pos), the general classification of proximity groups for each position of every antibody (prox), the murine ING1 monoclonal antibody sequence to be affinity-enhanced (murINGI), the conspicuousness value as described herein of each position for affinity-enhancement when the murine ING1 antibody is compared to murine consensus sequences (cspc), several murine consensus sequences to which ING1 is compared (mK2 or mH2a), the human ING1 residues which are introduced during the HUMAN ENGINEERING™ process (humING1), the degree of disconnection of the sidechain from the antibody's combining site (disc) as described herein, the degree of outward-orientation of the sidechain on the antibody's surface (outw) as described herein, the degree of risk for human engineering (risk), and the Kabat position numbers (pos) (2A-2B). Similarly, Figures 2C and 2D are alignments of sequences in the light chain and heavy chain of IL-1 antibody (also referred to as cA5 and/or XPA23), with lines (*e.g.*, prox, cspc) relating to affinity enhancement and lines relating to human engineering (*e.g.*, risk).

[0038] Figures 3A-3D are mutual alignments of consensus sequences (Kabat *et al*. (1991) (eds), Sequences of Proteins of Immunological Interest, 5th ed.) for major murine and human subgroups of the light chain and heavy chain. Each alignment relates them to the proximity groups as described herein for each position (prox), and the Kabat position numbers (pos).

[0039] Figure 4 shows a chart of the numerical components which can be added together to calculate each amino acid's affinity-enhancement conspicuousness value, including the components for changes in class-and-charge, for changes in

physical size due to somatic mutation, and for repeated identical mutations at the same position in multiple homologous antibodies.

[0040] Figure 5 shows PCR mutagenesis of CDR3 utilizing the CDR-H3 oligonucleotide H3-3NP2 (SEQ ID NO: 267): 5'-GCTACATATTTCTGTGCAAGATTTG GCTCTKGGGTGGACTACTGGGGTCAAGG-3', which introduces an amino acid substitution into CDR3, and the reverse primer NotI-R (SEQ ID NO: 285): 5'-AGCGGCCGCACAAGATTTGGGCTCAACTCTC-3', which incorporates the *Not*I restriction site into the PCR product.

[0041] Figure 6 depicts the plasmid map of the pXOMA-gIII-Fab vector. The vector is 5,202 base pairs in length and has *Asc*I and *Not*I restriction sites flanking the heavy chain encoding sequences, and *Hind*III and *Asc*I restriction sites flanking the light chain encoding sequences. The heavy chain encoding sequences are fused to pIII encoding sequences in the vector. The pXOMA-Fab vector is similar but lacks the pIII encoding sequences.

[0042] Figure 7 depicts the strategy for creating the light chain combination variants.

[0043] Figure 8 depicts the strategy for creating the heavy chain combination variants.

[0044] Figure 9A-9B shows CDR1, CDR2 and CDR3 as identified by the Kabat, Chothia and IMGT numbering scheme for ING-1 (9A) and XPA23 (9B).

[0045] Figure 10A-10D depict a continuous numbering scheme for the heavy and light chain of XPA23 (10A and 10B, respectively). Consecutive numbering from position 1 in the light chain continues in the heavy chain such that position 1 in the heavy chain is also assigned number 108 since the light chain sequence ends at number 107. Boxed residues indicate CDRs identified by the IMGT method. Figures 10C and 10D show a continuous numbering scheme for the heavy and light chain of ING-1 (10C and 10D, respectively).

[0046] Figure 11: Periplasmic extracts of clones containing one of the eighteen preferred amino acid mutations at Heavy Chain contacting positions in ING-1 were tested on Biacore for improved off-rate (see example 7). Clones with greater than 1.9-fold decrease in off-rate are listed.

[0047] Figure 12: Periplasmic extracts of clones containing one of the eighteen preferred amino acid mutations at Light Chain contacting positions in ING-1 were tested on Biacore for improved off-rate (see example 7). Clones with greater than 1.9-fold decrease in off-rate are listed.

[0048] Figure 13: Periplasmic extracts of clones containing one of the eighteen preferred amino acid mutations at Heavy Chain contacting positions in XPA23 were tested on Biacore for improved off-rate (see example 7). Clones with greater than 1.9-fold decrease in off-rate are listed.

[0049] Figure 14: Periplasmic extracts of clones containing one of the eighteen preferred amino acid mutations at Light Chain contacting positions in XPA23 were tested on Biacore for improved off-rate (see example 7). Clones with greater than 1.9-fold decrease in off-rate are listed.

[0050] Figure 15A-15D depicts two modified IgGs with an A102F or 102G substitution that were prepared and evaluated by Biacore with improved affinity (15B-15C, respectively) as compared to the parental (15A) ING-1 antibody. 15D shows the affinity determination kinetics for both the modified and parental ING-1 antibodies.

[0051] Figure 16A-16C are sensogram profiles depicting ING-1 light chain binding to Ep-Cam.

[0052] Figure 17 depicts modified ING-1 antibodies each comprising two or more heavy chain mutations as compared to the parental antibody. Combinations of heavy chain mutations yield affinity improvements up to 25-fold over the parental ING-1 antibody. Affinity improvements are driven largely by improvements in k_{off}.

[0053] Figure 18 shows amino acid substitutions at position 32 in the light chain variable region of XPA23. Generally the substitutions at position 30 decreased kd of the antibody-antigen interaction compared to the parental antibody.

[0054] Figure 19 shows amino acid substitutions at position 30 in the light chain variable region of XPA23. Generally the substitutions at position 30 resulted in a comparable kd of the antibody-antigen interaction compared to the parental antibody.

[0055] Figure 20 shows amino acid substitutions at position 45 in the heavy chain variable region of XPA23. Generally the substitutions improved kd of the antibody-antigen interaction at this position compared to the parental antibody.

DETAILED DESCRIPTION

[0056] The present disclosure provides methods and materials for targeted mutagenesis of one or more selected positions in a parent nucleic acid sequence (*e.g.*, a nucleotide sequence coding for an antibody or binding fragment thereof such as an IgG, Fab or ScFv). When the parent nucleic acid sequence encodes an antibody variable region, preferred positions for selection and mutagenesis are those encoding one of more CDR amino acid residues. Particularly preferred is mutation of each of the

CDR residues in a heavy and/or light chain variable region. Techniques for site-directed mutagenesis of a nucleotide sequence rely on using degenerate codons including, for example, NNK or NNS. However, these degenerate codons may code for an overrepresentation of one or more amino acid residues which may result in the production of a large nucleotide library that does not contain all possible amino acid substitutions at a position of interest. The present disclosure provides novel degenerate codons that encode for an equal representation of eighteen amino acid residues including a stop codon. Notably, the degenerate codons may not code for cysteine and/or methionine. Additionally, the novel degenerate codons each collectively code for eighteen amino acid residues eliminating any redundancy which may result in an over-representation of one or more amino acid residues. As a result, the degenerate codons of the present disclosure allow for the generation of smaller, focused libraries that contain eighteen amino acid substitutions at a position of interest.

[0057] The present disclosure provides methods of mutagenesis of a parent nucleic acid sequence encoding a protein (*e.g.*, an antibody or binding fragment thereof such as an IgG, Fab or scFv) by obtaining one or more primers that each comprise at least one 2 to 12 fold degenerate codon (*e.g.*, NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T), ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G)), wherein the primers are complementary to a sequence in the parent nucleic acid sequence and wherein the primers code for amino acid mutations at each of one or more amino acid positions encoded by the parent nucleic acid sequence; and subjecting the parent nucleic acid sequence to replication or polymerase based amplification using the obtained primers, wherein replication or amplification of the parent nucleic acid sequence with the primers generates variant nucleic acid sequences and wherein the variant nucleic acid sequences comprise amino acid mutations at the one or more positions in the parent nucleic acid sequence with the exception of cysteine and methionine. Optionally, two or more mutations may be combined by recombinant DNA techniques into a single mutated protein.

[0058] The present disclosure provides methods of mutagenesis of a parent nucleic acid encoding a protein (*e.g.*, an antibody or binding fragment thereof such as an IgG, Fab or scFv) to generate modified proteins by obtaining one or more primers that each comprise at least one 2 to 12 fold degenerate codon (*e.g.*, NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where

B=C/G/T, D=A/G/T), ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G)), wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in the parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid; and mutating the parent nucleic acid by replication or polymerase based amplification using the one or more obtained primers, wherein replication or amplification of the parent nucleic acid with the one or more primers generates mutated nucleic acids that encode modified proteins.

[0059] The present disclosure also provides libraries/arrays of mutated nucleic acid sequences generated by the methods of the present disclosure.

[0060] The present disclosure also provides methods for mutagenesis of a protein (*e.g.*, an antibody or binding fragment thereof such as an IgG, Fab or scFv) to obtain modified proteins with mutated amino acid sequences by identifying one or more amino acid positions in the protein for mutagenesis; substituting one or more of the identified amino acid residues in the protein with other amino acid residues excluding cysteine and methionine to generate a library or an array of modified proteins with mutated amino acid sequences; screening the library or array of modified proteins in an assay for a biological activity of the protein; and obtaining modified proteins having the biological activity of the protein.

[0061] The present disclosure also provides methods for generating an array of nucleic acids encoding modified proteins (*e.g.*, an antibody or binding fragment thereof such as an IgG, Fab or scFv) by obtaining a collection of nucleic acids encoding modified proteins containing amino acid mutations other than cysteine and methionine at amino acid residues of a parent protein sequence by mutagenesis of a nucleic acid encoding the protein sequence using primers that each comprise at least one 2 to 12 fold degenerate codon (*e.g.*, NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T), ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G)); sequencing the collection of nucleic acids encoding the modified proteins; and arranging each sequenced nucleic acid encoding a modified protein to generate an array of nucleic acid sequences each encoding a modified protein.

[0062] The present disclosure also provides methods for generating an array of nucleic acid sequences encoding modified proteins (*e.g.*, an antibody or binding

fragment thereof such as an IgG, Fab or scFv) by preparing a plurality of nucleic acid sequences by mutagenesis that encode a plurality of modified proteins that vary from a parent protein sequence at one or more amino acid positions and contain one of eighteen different amino acids excluding cysteine and methionine at each position mutated from the parent protein sequence; and arranging each prepared nucleic acid sequence to generate an array of nucleic acid sequences each encoding a modified protein.

[0063] The present disclosure also provides libraries/arrays of mutated nucleic acid sequences generated by the method of the present disclosure.

[0064] The present disclosure also provides methods for generating an array of clones comprising nucleic acids encoding modified proteins (*e.g.*, an antibody or binding fragment thereof such as an IgG, Fab or scFv) by preparing a plurality of nucleic acids by mutagenesis that encode a plurality of modified proteins that vary from a parent protein sequence at one or more amino acid positions and contain one of eighteen different amino acids excluding cysteine and methionine at each position varied from the parent protein sequence; transfecting the prepared nucleic acids prepared into host cells and selecting clones comprising the transfected nucleic acids; and arranging each selected clone to generate an array of clones with each arrayed clone capable of expressing a modified protein.

[0065] The present disclosure also provides methods of producing a nucleic acid library with an equal representation of non-redundant amino acid changes at an amino acid position encoded by a parent nucleic acids by providing a set of primers that each comprise at least one degenerate codon (*e.g.*, NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T), ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G)), wherein each primer comprises at least two oligonucleotide sequence that are complementary to a sequence in the parent nucleic acid and code for an amino acid mutation with the exception of cysteine and methionine at one amino acid position encoded by the parent nucleic acid, wherein the primers code for an equal representation of non-redundant amino acid changes at the one position; hybridizing a primer from the set to the parent nucleic acid; replicating or amplifying the parent nucleic acid molecule with the primer to generate nucleic acids that code for amino acid changes at the one position; repeating the hybridizing and replicating steps with each remaining primer from the set; pooling the nucleic acids produced with each

primer; and obtaining a library of nucleic acids coding for an equal representation of amino acid changes at the one position.

[0066] The present disclosure also provides methods for obtaining a nucleic acid sequence with an improvement in comparison to a parent nucleic acid sequence with respect to at least one molecular or biological property of interest by obtaining a set of primers that each comprise at least one 2 to 12 fold degenerate codon (*e.g.*, NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T), ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G)) that does not code for cysteine and methionine, wherein the primers are complementary to a sequence in the parent nucleic acid sequence and wherein the primers code for non-redundant amino acid mutations at one amino acid position encoded by the parent nucleic acid sequence; mutating the parent nucleic acid sequence by replication or polymerase based amplification using the obtained set of primers to generate variant nucleic acid sequences; producing a library or array of variant nucleic acid sequences from (b) coding for amino acid mutations at the one position in the parent nucleic acid sequence; and screening the library or array of variant nucleic acid sequences to identify nucleic acid sequences that have a desirable improvement in comparison with the parent nucleic acid sequence with respect to at least one molecular or biological property of interest.

[0067] The present disclosure also provides methods of making modified proteins (*e.g.*, an antibody or binding fragment thereof such as an IgG, Fab or scFv) with mutated amino acid sequences by modifying the amino acid sequence of a protein to produce amino acid mutations at an amino acid residue in the protein to generate a library or an array of modified proteins with mutated amino acid sequences, wherein the amino acid mutations exclude cysteine and methionine; and selecting modified proteins from the library or the array that have a biological activity of an unmodified protein.

[0068] The present disclosure also provides methods for selecting modified proteins (*e.g.*, an antibody or binding fragment thereof such as an IgG, Fab or scFv) with mutated amino acid sequences by obtaining a library or an array of modified proteins comprising amino acid mutations at one amino acid residues in an amino acid sequence of a protein, wherein the amino acid mutations exclude cysteine and methionine; assaying the modified proteins for a biological activity of an unmodified

protein; and selecting the modified proteins that have a biological activity of the unmodified protein.

[0069] The present disclosure also provides a set of primers that each comprise at least one 2 to 12 fold degenerate codon (*e.g.*, NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T), ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G)), wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid.

[0070] The present disclosure also provides a kit for mutagenesis of an amino acid residue encoded by a parent nucleic acid comprising a set of primers that each comprise at least one 2 to 12 fold degenerate codon (*e.g.*, NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T), ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G)), wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid.

[0071] The present disclosure also provides libraries/arrays comprising variants of a protein sequence, wherein the variants each comprise an amino acid mutation at one amino acid position in the protein sequence of a parent protein and wherein the amino acid mutations are not cysteine or methionine.

Selection of a Defined Region or Amino Acid Residue for Mutagenesis

[0072] A region(s) or a specific amino acid residue(s) in a protein may be subjected to the methods of mutagenesis as described herein. A region of a protein for mutagenesis may be deduced from comparing the region(s) to what is known from the study of other proteins, and may be aided by modeling information. For example, the region may be one that has a role in a functional site including, for example, in binding, catalysis, or another function. Regions involved in binding may include, for example, a hypervariable region or complementarity determining region (CDR) of an antigen binding molecule.

[0073] In an exemplary method to select amino acid residues in an antibody or binding fragment thereof for mutation, the amino acid residues may be assigned to a proximity group. For example, each amino acid in an antibody heavy and/or light chain variable region may be assigned to one of the following unique groups, which includes, contacting (C), peripheral (P), supporting (S), interfacial (I), or distant (D) residues, as shown, for example, on the "prox" lines of Figures 2A, 2B, 2C, 2D, 3A, and/or 3B. For example, each of the more-than-200 amino-acid positions in an antibody's variable light chain and heavy chain has been designated as a member of one of these five novel groups. The "prox" line as shown in Figure 3A or 3B is useful for any variable region sequence, irrespective of the present disclosure provides methods for mutagenesis of one or more defined region(s) within a protein. The region(s) may be important to a protein's structure or function. These region(s) can be deduced, for example, from what structural and/or functional aspects are known or specific amino acid sequence, such that residues can be selected as candidates for change (*e.g.*, any and/or all contacting (C) residues). Additionally or alternatively, methods are provided that identify the presence of conspicuous amino-acid residues which may be candidates for change. Conspicuous amino acid changes may differ in charge or size or chemical functionality from the corresponding residues in the selected sequence (*e.g.,* consensus or germline sequence) and represent positions where amino acid changes may enhance affinity.

[0074] Exemplary methods for characterization of amino acid residues in an antibody binding domain may include: a determination of each amino acid residue's proximity group as designated on the "prox" line of Figures 2A, 2B, 2C, 2D, 3A and / or 3B and additionally or alternatively a determination of each amino acid residue's conspicuousness as calculated by the methods provided in the present disclosure.

A. Determination of Proximity Groups

[0075] The characterization process may determine the proximity group for each amino-acid position simply by inspecting the corresponding symbol ("CPSI.:") on the "prox" lines as shown, for example, in Figures 2A, 2B, 2C and/or 2D. In some embodiments, the antibody's light-chain and/or heavy-chain sequences are aligned with appropriate sequences (*e.g.*, such as consensus or germline sequences) and also with the "prox" lines of the present methods (Figures 2A, 2B, 2C and/or 2D),

[0076] Each position in the light chain and heavy chain has been assigned to one of five novel groups designated as contacting (C), peripheral (P), supporting (S),

interfacial (I), or distant (D) on the "prox" lines, for example, of Figures 2A, 2B, 2C, 2D, 3A, and/or 3B according to the methods disclosed herein. These Figures (*e.g.,* 2A, 2B, 2C, 2D, 3A and/or 3B) contain a disc line to reflect disconnection from any significant effect upon an antibody's binding site, and an outw line to reflect outward-orientation on an antibody's surface.

[0077] Table 1 shows five proximity groups, as well as a novel designation of disconnection (as shown on a "disc" line, for example, in Figures 2A, 2B, 2C, 2D, 3A and/or 3B) and outward-orientation (shown as an "outw" line, for example, in Figures 2A, 2B, 2C, 2D, 3A and/or 3B) as defined for each group. The number of positions of each type of proximity group for an exemplary antibody (*e.g.*, ING-1, as described herein) in a light chain, a heavy chain, and both chains together are shown in Table 2.

Table 1.

Proximity	Abbr	Disc/Outw				
Contacting	C	-/+	-/o			
Peripheral	P	o/+	o/o			
Supporting	S	-/-	o/-			
Interfacial	I	-/=	o/=	+/=		
Distant	•	+/+	+/o	+/ -	p	c

Table 2.

Proximity	L	H	L+H
Contacting	16	21	37
Peripheral	3	8	11
Supporting	14	16	30
Interfacial	9	10	19
Distant	70	63	133

[0078] Without being bound by a theory of the invention, it has been hypothesized that amino acid residues designated as contacting (C) are located within the combining site (see, *e.g.*, "-" on the "disc" line of Figures 2A, 2B, 2C and/or 2D), and their sidechains are mostly outward-oriented (see, *e.g.*, "+" or "o" on the outw line). It has been further hypothesized that these are generally surface-exposed residues in the CDR loops themselves, so their sidechains are very favorably situated for making direct contact with corresponding residues on a binding partner.

[0079] Without being bound by a theory of the invention, it has been hypothesized that amino acid residues designated as peripheral (P) are slightly disconnected from the binding site (see, *e.g.*, "o" on the "disc" line), and their sidechains are mostly outward-oriented (see, *e.g.*, "+" or "o" on the outw line). Many of

these are framework residues with variable orientation, which are located at curves or twists in the protein chain not too far from CDR loops. Although they may normally not make direct contact with a binding partner, they may possibly make contact if a particular binding partner is bound preferentially toward one side of the binding site instead of being centered.

[0080] Without being bound by a theory of the invention, it has been hypothesized that amino acid residues designated as supporting (S) are either directly within or close to the combining site (see, *e.g.*, "-" or "o" on the "disc" line), and their sidechains are inward-oriented (see, *e.g.*, "-" on the outw line). It has been further hypothesized that many of these residues are buried in the Vernier-zone platform directly underneath a combining site, so that their nonpolar sidechains are able to act as conformation-stabilizing "anchors" for CDR loops which rest on top of them.

[0081] Without being bound by a theory of the invention, it has been hypothesized that amino acid residues designated as interfacial (I) may be located anywhere in relation to the binding site (see, *e.g.*, "+" or "o" or "-" on the "disc" line), but their sidechains form the interface between the light and heavy subunits of the variable domain (see, *e.g.*, "=" on the outw line). It has been further hypothesized that amino acid changes of these residues may cause the two subunits to pivot or rotate relative to one another along their shared hydrophobic interfacial surface, producing strong allosteric effects upon an entire binding site, for example, all six CDR loops may be forced to change their conformation in response.

[0082] Without being bound by a theory of the invention, it has been hypothesized that amino acid residues designated as distant (D) are of two different types, with those of the first type being disconnected from a combining site and its targeted epitope (see, *e.g.*, "+" on the "disc" line), and their sidechains may have any orientation except interfacial (see, *e.g.*, "+" or "o" or "-" but not "=" on the outw line). It is further hypothesized that amino acid changes at these positions generally will have little or no effect on enhanced affinity to a binding partner.

B. Determination of Conspicuousness

[0083] In some embodiments, alternatively or additionally with determination of the proximity groups by inspection of the “prox” lines, the characterization process may involve a calculation of the conspicuousness value for each amino-acid position. The conspicuousness value of a sidechain at a particular antibody position is hypothesized to represent the degree to which it appears strikingly different or unusually outstanding

in comparison with selected sequences (*e.g.*, a consensus or germline sequence). Without being bound by a theory of the invention, this value indicates the likelihood that this particular residue may be a somatic mutation which was necessary to confer binding partner specific affinity upon an antibody. Consequently, the conspicuousness value also correlates with the hypothesis that a new engineered amino acid substitution at or near this position could possibly lead to forming or strengthening a bond with a residue on a binding partner surface.

[0084] Conspicuousness values are calculated by comparing each sidechain of a candidate antibody with the corresponding sidechain of an appropriate consensus or germline sequence, for example, from a mutual alignment. For example, numerical values for conspicuousness can be calculated readily for each amino-acid position in a given antibody, according to the following formula: add 1 point for each three units of difference in size (*e.g.*, divide the absolute value of the size-difference by 3 and drop the decimal without rounding); add 1 point for a shift from one sidechain class to another; add 1 point for each unit (absolute value) of difference in charge, and add 1 point for nonidentity (*see*, *e.g.*, Figure 4).

[0085] For example, where a single antibody sequence is aligned or compared with a single consensus or germline sequence, there is one "pair" of sequences being compared. The conspicuousness value for each amino-acid position in the alignment or comparison is the sum of the points for chemical function and physical size and nonidentity at that position. Where more than two sequences are aligned or compared together at the same time, each of the antibody sequences may form a separate "pair" with each of the consensus or germline sequences. The conspicuousness values are calculated as described (*e.g.*, sum of function and size and nonidentity) for each pair of sequences being aligned or compared, and then the overall conspicuousness value for each amino acid position in the whole alignment is the sum of the values obtained from each pair at that position, while also adding in a value for repeated identical mutations.

[0086] It is hypothesized that nonidentity simply marks an amino-acid position as minimally conspicuous if it displays any kind of difference when compared with a corresponding consensus or germline position. Even a conservative mutation (*e.g.*, from leucine to isoleucine or valine) may suggest a possible bond with a binding partner, especially if a slight change of size or shape was necessary to fine-tune steric relationships between the two molecules.

[0087] An exemplary calculation of conspicuousness is illustrated as follows. Four monoclonal antibodies to the same epitope were isolated, and portions of their

heavy chains were mutually aligned with a germline sequence, between Kabat positions 25 and 57 [Mendez *et al.*, Nature Genetics, 15:146-152 (1997)] (*see*, Table 3). Since this alignment contains more than two sequences, each of the four antibody sequences can separately form a "pair" with the one germline sequence. Thus, conspicuousness values are calculated separately for each of the four pairs, and then totaled at each amino-acid position, while also adding in the additional values for repeated identical mutations.

Table 3.

```
prox:PSSCSCCCCSISI.I.:...I.ISSCSCCCCCC
 pos:     30      _   40         50
germ:GSISSGGYYWSWIRQHPGKGLEWIGYIYYSGST
mAb1:    N   D                    S     N
mAb2:        D    T                      N
mAb3:   v    D         p           HL    N
mAb4:    N   D                DC
```

[0088] Three repetitions are shown in Table 3, at positions 28 and 31 and 56. In each of these cases, an identical amino acid (N or D) has appeared at the same location in more than one independently isolated antibody. Accordingly, as described herein, these positions are given very high conspicuousness in the affinity enhancement process. An additional 2 points are added for each repetition of an identical amino acid at a given position (*e.g.*, four D's amount to three repetitions of the first D, so it is worth 3x2=6 points).

[0089] In an example, at position 50, the first pair (germ:mAb 1) gets 3 points (Y to S = 2 for size + 0 for class + 0 for charge + 1 for nonidentity), the second pair (germ:mAb2) gets 0 points (unmutated Y = 0+0+0+0), the third pair (germ:mAb3) gets 3 points (Y to H = 0 for size + 1 for class + 1 for charge + 1 for nonidentity), and the fourth pair (germ:mAb4) gets 0 points (unmutated Y = 0+0+0+0). The total conspicuousness for position 50 is the sum (3+0+3+0) of these, plus 0 extra points for no repeated identical mutations, which finally gives 6.

[0090] In another example, at position 28, the first pair gets 1 point (S to N = 0+0+0+1), the second and third pairs get 0 points, and the fourth pair gets I point. Since the somatic mutation N appears at position 28 twice, it is repeated once, and thus gets 2 extra points. The total conspicuousness for position 28 is the sum (1+0+0+1), plus 2 points for one repetition, which finally gives 4.

[0091] In another example, at position 31, each of the four pairs gets 4 points (G to D = 1+1+1+1). Since the somatic mutation D appears at position 31 four times, it is repeated three times, and thus gets 3x2=6 extra points. The total conspicuousness for position 28 is the sum (4+4+4+4), plus 6 points for three repetitions, which finally gives 22.

[0092] The conspicuousness points can be calculated (one pair at a time and then summed) for positions 28, 31, and 50 in the antibody sequence provided in Table 2.

Degenerate Primers and Kits

[0093] The present disclosure provides primers and kits that may comprise one or more of the novel degenerate codons of the present disclosure. These degenerate codons that may be used to mutagenize a nucleotide sequence encoding a protein including, for example, an antibody such as an IgG, a Fab or a ScFv. The degenerate codons may code for an equal representation of eighteen amino acid substitutions including, for example, alanine (Ala, A), arginine (Arg, R), asparagine (Asn, N), aspartic acid (Asp, D), glutamine (Gln, Q), glutamic acid (Glu, E), glycine (Gly, G), histidine (His, H), isoleucine (Ile, I), leucine (Leu, L), lysine (Lys, K), phenylalanine (Phe, F), proline (Pro, P), serine (Ser, S), threonine (Thr, T), tryptophan (Trp, W), tyrosine (Tyr, Y) or valine (Val, V). The degenerate codons of the present disclosure do not code for cysteine and/or methionine. A degenerate primer may be between 2-fold degenerate (*e.g.*, comprise 2 oligonucleotide sequences that collectively code for 2 different amino acid residues at the same position) and 12-fold degenerate (*e.g.*, comprise 12 oligonucleotide sequence that collectively code for 12 different amino acid residues at the same position).

[0094] A set of primers is provided that comprise a set of primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid. In some embodiments, the primer set codes for eighteen amino acid changes at each of one or more positions in the parent nucleic acid. In some embodiments, the set of primers each comprise a degenerate codon which collectively code for alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and

valine at each position. A set of primers may comprise 2 or more primers (*e.g.*, 3 or 7 primers) and contains a number of oligonucleotides equal to the sum of the degeneracy of each primer in the set. For example, a set of primers that comprises a 2-fold and a 4-fold degenerate primer contains six oligonucleotide sequences.

[0095] Kits are also provided for mutagenesis of a position in a parent nucleic acid that comprise a set of primers a set of primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid.

[0096] A set of three degenerate codons may be used to mutagenize an amino acid position encoded by a parent nucleotide sequence. These degenerate codons may include, for example, NHT or NHC (where N=A/G/C/T, H=A/C/T), which codes for phenylalanine/serine/tyrosine/leucine/proline/histidine/isoleucine/threonine/ asparagine/valine/alanine/aspartic acid, VAG or VAA (where V=A/C/G), which codes for glutamine/lysine/glutamic acid, and BGG or DGG (where B=C/G/T, D=A/G/T), which codes for tryptophan/arginine/glycine. Alternatively, a set of seven degenerate codons may be used to mutagenize one or more selected positions in a parent nucleotide sequence. These degenerate codons may include, for example, ARG (where R=A/G), which codes for arginine/lysine, WMC (where W=A/T and M=A/C), which codes for serine/threonine/asparagine/tyrosine, CAS (where S=C/G), which codes for histidine/glutamine, GAS (where S=C/G), which codes for glutamic acid/aspartic acid, NTC (where N=A/G/C/T), which codes for leucine/phenylalanine/isoleucine/valine, KGG (where K=G/T), which codes for tryptophan/glycine, and SCG (where S=C/G), which codes for proline/alanine.

[0097] Alternate degenerate codons may be used to mutagenize one or more selected positions in a parent nucleotide sequence by modifying the degenerate codons described above. For example, ARG may be replaced with ARA, WMC may be replaced with WMT, CAS may be replaced with CAK (K=G,T), CAM (M=A or C), or CAW (W=A or T), NTC with NTT, SCG with SCA, SCC, or SCT. In addition, the primer listed as NTC or NTT may be replaced with two primers MTC, KTC (or MTT/KTT; MTC/KTT; MTT/KTC); STC, WTC (or STT/WTT; STT/WTC; STC/WTT); RTC, YTC (or RTT/YTT; RTC/YTT, RTT/YTC).

Methods for Targeted Mutagenesis

[0098] The methods of the present disclosure may be used to mutagenize a nucleic acid sequence coding for a protein including, for example, an antibody or binding fragment thereof (*e.g.*, an IgG, Fab or scFv). When the parent nucleic acid sequence encodes an antibody variable region, preferred positions for selection and mutagenesis are those encoding one of more CDR amino acid residues. Particularly preferred is mutation of each of the CDR residues in a heavy and/or light chain variable region. Methods for mutagenesis of the present disclosure may selectively target one or more regions of a protein including, for example, one or more amino acid residues. The region(s) mutagenized by the methods of the present disclosure may comprise a functional domain of a protein such as a binding or catalytic domain. For example, the region may be a hypervariable region (*e.g.*, complementarity-determining region or CDR) of an immunoglobulin, the catalytic site of an enzyme, or a binding domain.

[0099] The CDRs (*e.g.*, LCDR1, LCDR2 and LCDR3 for the light chain and HCDR1, HCDR2 and HCDR3 for the heavy chain) may be defined according to any known method in the art including, for example, Kabat, Chothia or IMGT. According to Kabat, LCDR1 comprises amino acid residues 24 to 34, LCDR2 comprises amino acid residues 50 to 56, LCDR3 comprises amino acid residues 89 to 97, HCDR1 comprises amino acid residues 31 to 35b, HCDR2 comprises amino acid residues 50 to 65 and HCDR3 comprises amino acid residues 95 to 102. According to Chothia, LCDR1 comprises amino acid residues 24 to 34, LCDR2 comprises amino acid residues 50 to 56, LCDR3 comprises amino acid residues 89 to 97, HCDR1 comprises amino acid residues 26 to 32, HCDR2 comprises amino acid residues 52 to 56 and HCDR3 comprises amino acid residues 95 to 102. According to IMGT, LCDR1 comprises amino acid residues 27 to 32, LCDR2 comprises amino acid residues 50 to 52, LCDR3 comprises amino acid residues 89 to 97, HCDR1 comprises amino acid residues 26 to 33, HCDR2 comprises amino acid residues 51 to 57 and HCDR3 comprises amino cid residues 93 to 102. Residues numbers for the Kabat, Chothia and IMGT CDRs are given as Kabat position numbers.

[00100] The present disclosure provides methods of mutagenesis of a parent nucleic acid sequence encoding a protein by obtaining one or more primers that each comprise at least one 2 to 12 fold degenerate codon, wherein the primers are complementary to a sequence in the parent nucleic acid sequence and wherein the primers code for amino acid mutations at one amino acid position encoded by the parent nucleic acid sequence; and subjecting the parent nucleic acid sequence to replication or polymerase based amplification using the obtained primers, wherein

replication or amplification of the parent nucleic acid sequence with the primers generates variant nucleic acid sequences and wherein the variant nucleic acid sequences comprise amino acid mutations at the one position in the parent nucleic acid sequence with the exception of cysteine and methionine.

[00101] Several different regions of a protein may be mutagenized simultaneously. This approach may enable the evaluation of amino acid substitutions in conformationally related regions such as the regions which, upon folding of the protein, are associated to make up a functional site such as the catalytic site of an enzyme or the binding site of an antibody. For example, the six hypervariable regions of an immunoglobulin, which make up the unique aspects of the antigen binding site (*e.g.*, Fv region), can be mutagenized simultaneously, or separately within the V_H or V_L chains, to study the three dimensional interrelationship of selected amino acids in this site.

[00102] Mutations may be introduced into a parent nucleic acid sequence by PCR mutagenesis using primers that comprise one or more degenerate codons. For example, basic amino acid changes may be introduced using the degenerate codon ARG (R=A/G), which codes for arginine/lysine. Polar amino acid changes may be introduced using the degenerate codons WMC (W=A/T; M=A/C), which codes for serine/threonine/asparagine/tyrosine and/or CAS (S=C/G), which codes for histidine/glutamine. Acidic amino acid changes may be introduced using the degenerate codon GAS (S=C/G), which codes for glutamic acid/aspartic acid. Non-polar changes may be introduced using the degenerate codons NTC (N=A/G/C/T), which codes for leucine/phenylalanine/isoleucine/valine, KGG (K=G/T), which codes for tryptophan/glycine and/or SCG (S=C/G), which codes for proline/alanine.

[00103] An oligonucleotide comprising one or more of the degenerate codons of the present disclosure may be synthesized by known methods for DNA synthesis. Such methods may involve the use of solid phase beta-cyanoethyl phosphoramidite chemistry (see, *e.g.*, U.S. Pat. No. 4,725,677).

[00104] Methods are provided for making modified proteins that comprise changes at one or more positions in a parent protein in order to modify one or more biological properties of the parent protein. For example, one or more positions in a parent antibody may be modified in order to enhance the binding affinity of an antibody by means of producing targeted amino acid changes in the antibody's variable domain. Engineered amino acid changes may be introduced at positions likely to produce enhanced affinity based upon an amino acid residue's proximity group.

[00105] In an exemplary method, amino acid changes are engineered at one or more amino acid residues categorized as preferably contacting (C), peripheral (P), supporting (S) and/or interfacial on the "prox" lines of Figures 2A, 2B, 3A, and/or 3B. In other embodiments, amino acid residues categorized in more than one group may be selected for change. Less preferably one or more distant (D) amino acid residues may additionally or alternatively be changed.

[00106] Modified proteins are synthesized by mutating the nucleic acid encoding a parent protein, inserting the modified nucleic acid into an appropriate cloning vector and expressing the modified nucleic acid to produce modified proteins. Exemplary protocols are described below.

1. Making Modified Nucleic Acid

[00107] Modified proteins that comprise one or more amino acid sequence changes (*e.g.,* substitutions) relative to a parent protein sequence may be produced by methods known in the art using the degenerate primers of the present disclosure. For example, amino acids may be preferably incorporated into a position of interest by utilizing seven different degenerate codons. Basic amino acid changes can be produced with a single oligonucleotide that contains the codon mixture of ARG (R=A/G), encoding Arg/Lys. Polar amino acid changes can be produced with two oligonucleotides. For example, the first oligonucleotide contains the codon mixture WMC (W=A/T; M=A/C), encoding Ser/Thr/Asn/Tyr, while the second polar oligonucleotide utilizes the codon mixture CAS (S=C/G), encoding His/Gln. Acidic amino acid changes can be produced with a single codon mixture of GAS, encoding Glu/Asp. Non-polar functional amino acid changes can be produced with four oligonucleotide mixtures: NTC (N=A/G/C/T), encoding Leu/Phe/Ile/Val, KGG (K=G/T), encoding Trp/Gly, and SCG, encoding Pro/Ala.

[00108] In some embodiments, all seven of the degenerate primers are used to perform one PCR reaction. In other embodiments, each degenerate primer is used in a separate PCR reaction. Any combination of PCR primers may be used in a PCR reaction.

[00109] DNA encoding modified proteins may be prepared by a variety of methods known in the art. These methods include, but are not limited to, preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared modified protein or a parent protein. These techniques may utilize antibody nucleic acid (DNA or RNA), or nucleic acid complementary to the protein nucleic acid.

[00110] In an exemplary method, Kunkel mutagenesis may be performed by placing a plasmid that contains a protein to be mutated into an ung$^-$ dut$^-$ strain of *E. coli* bacteria. Dut $^-$ (lacking dUTPase) bacteria accumulate dUTP and ung$^-$ (lacking uracil deglycosidase) bcteria cannot remove dUTP that gets incorporated into new DNA strands. The end result is that the plasmid is converted to DNA that lacks T's and contains U's instead. The U-containing target DNA may then be incubated with a mutagenic primer that base pairs with the target except at the location of the desired mutation. This mixture may then be incubated with Klenow, dNTP's and later Ligase and ATP to produce double-stranded plasmid with one strand containing U's and the new one containing only T's. Finally, the hybrid old/new double-stranded DNA may be transformed into bacteria that destroy the old U-containing DNA and produce a T-containing strand using the new and mutagenized DNA strand as a template.

[00111] DNA encoding a modified protein with more than one amino acid to be changed may be generated in one of several ways. If the amino acids are located close together in the protein chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid changes. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

[00112] In the first method, a separate oligonucleotide is generated for each amino acid to be changed. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid changes.

[00113] The alternative method involves two or more rounds of mutagenesis to produce the desired mutant antibody. The first round is as described for the modified variable domain which comprise one amino acid change: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid change(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid change(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

2. Insertion of DNA into a Cloning Vehicle

[00114] The cDNA or genomic DNA encoding the modified protein may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

[00115] For example, the cDNA or genomic DNA encoding the modified protein may be inserted into a modified phage vector (*i.e*. phagemid). Construction of phage display libraries exploits the bacteriophage's ability to display peptides and proteins on their surfaces, *i.e*., on their capsids. Often, filamentous phage such as M13, f1 or fd are used. Filamentous phage contain single-stranded DNA surrounded by multiple copies of genes encoding major and minor coat proteins, *e.g.*, pIII. Coat proteins are displayed on the capsid's outer surface. DNA sequences inserted in-frame with capsid protein genes are co-transcribed to generate fusion proteins or protein fragments displayed on the phage surface. Peptide phage libraries thus can display peptides representative of the diversity of the inserted genomic sequences. Significantly, these epitopes can be displayed in “natural” folded conformations. The peptides expressed on phage display libraries can then bind target molecules, *i.e*., they can specifically interact with binding partner molecules such as antibodies (Petersen (1995) *Mol. Gen. Genet.* 249:425-31), cell surface receptors (Kay (1993) *Gene* 128:59-65), and extracellular and intracellular proteins (Gram (1993) *J. Immunol. Methods* 161:169-76).

[00116] The concept of using filamentous phages, such as M13, fd or fl, for displaying peptides on phage capsid surfaces was first introduced by Smith (1985) Science 228:1315-1317. Peptides have been displayed on phage surfaces to identify many potential ligands (see, *e.g.*, Cwirla (1990) *Proc. Natl. Acad. Sci. USA* 87:6378-6382). There are numerous systems and methods for generating phage display libraries described in the scientific and patent literature (see, *e.g.*, Sambrook and Russell, Molecule Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, Chapter 18, 2001; “Phage Display of Peptides and Proteins: A Laboratory Manual, Academic Press, San Diego, 1996; Crameri (1994) *Eur. J. Biochem.* 226:53-58; de Kruif (1995) *Proc. Natl. Acad. Sci. USA* 92:3938-42; McGregor

(1996) *Mol. Biotechnol.* 6:155-162; Jacobsson (1996) *Biotechniques* 20:1070-1076; Jespers (1996) *Gene* 173:179-181; Jacobsson (1997) *Microbiol Res.* 152:121-128; Fack (1997) *J. Immunol. Methods* 206:43-52; Rossenu (1997) *J. Protein Chem.* 16:499-503; Katz (1997) *Annu. Rev. Biophys. Biomol. Struct.* 26:27-45; Rader (1997) *Curr. Opin. Biotechnol.* 8:503-508; Griffiths (1998) *Curr. Opin. Biotechnol.* 9:102-108).

[00117] Typically, exogenous nucleic acid to be displayed are inserted into a coat protein gene, *e.g.* gene III or gene VIII of the phage. The resultant fusion proteins are displayed on the surface of the capsid. Protein VIII is present in approximately 2700 copies per phage, compared to 3 to 5 copies for protein III (Jacobsson (1996), supra). Multivalent expression vectors, such as phagemids, can be used for manipulation of exogenous genomic or antibody encoding inserts and production of phage particles in bacteria (see, *e.g.*, Felici (1991) *J. Mol. Biol.* 222:301-310).

[00118] Phagemid vectors are often employed for constructing the phage library. These vectors include the origin of DNA replication from the genome of a single-stranded filamentous bacteriophage, *e.g.*, M13, f1 or fd. A phagemid can be used in the same way as an orthodox plasmid vector, but can also be used to produce filamentous bacteriophage particle that contain single-stranded copies of cloned segments of DNA.

[00119] Other phage can also be used. For example, T7 vectors can be employed in which the displayed product on the mature phage particle is released by cell lysis.

[00120] In addition to phage epitope display libraries, analogous epitope display libraries can also be used. For example, the methods of the disclosure can also use yeast surface displayed epitope libraries (see, *e.g.*, Boder (1997) Nat. Biotechnol. 15:553-557), which can be constructed using such vectors as the pYD1 yeast expression vector. Other potential display systems include mammalian display vectors and *E. coli* libraries.

[00121] An modified protein including, for example, an antibody or antibody fragment, *e.g.*, a scFv, Fab or Fv may be displayed on the surface of a phage using phage display techniques. Exemplary antibody phage display methods are known to those skilled in the art and are described, *e.g.*, in Hoogenboom, *Overview of Antibody Phage-Display Technology and Its Applications*, from *Methods in Molecular Biology: Antibody Phase Display: Methods and Protocols* (2002) 178:1-37 (O'Brien and Aitken, eds., Human Press, Totowa, N.J.). For example, a library of antibodies or antibody fragments (*e.g.*, scFvs, Fabs, Fvs with an engineered intermolecular disulfide bond to

stabilize the V_H-V_L pair, and diabodies) can be displayed on the surface of a filamentous phage, such as the nonlytic filamentous phage fd or M13. Antibodies or antibody fragments with the desired binding specificity can then be selected.

[00122] An antibody phage-display library can be prepared using methods known to those skilled in the art (see, *e.g.*, Hoogenboom, *Overview of Antibody Phage-Display Technology and Its Applications*, from *Methods in Molecular Biology: Antibody Phage Display: Methods and Protocols* (2002) 178:1-37 (O'Brien and Aitken, eds., Human Press, Totowa, N.J.).

[00123] In some embodiments, cDNA is cloned into a phage display vector, such as a phagemid vector (*e.g.*, pCES1, p XOMA Fab or pXOMA Fab-gIII). In certain embodiments, cDNA encoding both heavy and light chains may be present on the same vector. In some embodiments, cDNA encoding scFvs are cloned in frame with all or a portion of gene III, which encodes the minor phage coat protein pIII. The phagemid directs the expression of the scFv-pIII fusion on the phage surface. In other embodiments, cDNA encoding heavy chain (or light chain) may be cloned in frame with all or a portion of gene III, and cDNA encoding light chain (or heavy chain) is cloned downstream of a signal sequence in the same vector. The signal sequence directs expression of the light chain (or heavy chain) into the periplasm of the host cell, where the heavy and light chains assemble into Fab fragments. Alternatively, in certain embodiments, cDNA encoding heavy chain and cDNA encoding light chain may be present on separate vectors. In certain embodiments, heavy chain and light chain cDNA may be cloned separately, one into a phagemid and the other into a phage vector, which both contain signals for in vivo recombination in the host cell.

[00124] The techniques for constructing and analyzing phage display libraries uses recombinant technology well known to those of skill in the art. General techniques, *e.g.*, manipulation of nucleic encoding libraries, epitopes, antibodies, and vectors of interest, generating libraries, subcloning into expression vectors, labeling probes, sequencing DNA, DNA hybridization are described in the scientific and patent literature, see *e.g.*, Sambrook and Russell, eds., Molecular Cloning: a Laboratory Manual (3rd), Vols. 1-3, Cold Spring Harbor Laboratory Press, (2001); Current Protocols in Molecular Biology, Ausubel, ed. John Wiley & Sons, Inc., New York (1997-2001) ("Ausubel"); and, Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization with Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993). Sequencing methods typically use dideoxy

sequencing, however, other methodologies are available and well known to those of skill in the art.

3. Transformation of Host Cells

[00125] Suitable host cells for cloning or expressing the vectors herein may include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, Bacilli such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa, Salmonella typhimurium*, or *Serratia marcescens*.

[00126] For example, recombinant phagemid or phage vectors may be introduced into a suitable bacterial host, such as *E. coli*. In some embodiments using phagemid, the host may be infected with helper phage to supply phage structural proteins, thereby allowing expression of phage particles carrying the antibody-pIII fusion protein on the phage surface.

Methods for Identifying a Modified Protein with Altered Activity as Compared to a Parent Protein

[00127] Methods are provided for identifying a modified protein with altered activity as compared to a parent protein. For example, a modified antibody variable domain having enhanced binding affinity for a binding partner may be identified by contacting a parent antibody variable domain with the binding partner under conditions that permit binding; contacting modified antibody variable domains made by the methods of the present disclosure with the binding partner under conditions that permit binding; and determining binding affinity of the modified antibody variable domains and the parent antibody variable domain for the binding partner, wherein modified antibody variable domains that have a binding affinity for the binding partner greater than the binding affinity of the parent antibody variable domain for the binding partner are identified as having enhanced binding affinity.

[00128] Isolated antibody variable domains may exhibit binding affinity as single chains, in the absence of assembly into a heteromeric structure with their respective V_H or V_L subunits. As such, populations of V_H and V_L altered antibody variable domains can be expressed alone and screened for binding affinity having substantially the same or greater binding affinity compared to the parent antibody V_H or V_L variable domain.

[00129] Alternatively, populations of antibody V_H and V_L altered variable domains proteins can be co-expressed so that they self-assemble into heteromeric

altered antibody variable domain binding fragments. The heteromeric binding fragment population can then be screened for species exhibiting enhanced binding affinity to a binding partner compared to the binding affinity of the parent antibody variable domain.

[00130] The expressed population of modified antibody variable domains can be screened for the identification of one or more altered antibody variable domain species which exhibit enhanced binding affinity to a binding partner as compared with the parent antibody variable domain. Screening can be accomplished using various methods well known in the art for determining the binding affinity of a protein or compound. Additionally, methods based on determining the relative affinity of binding molecules to their partner by comparing the amount of binding between the modified antibody variable domain and the binding partner can similarly be used for the identification of species exhibiting binding affinity substantially the same or greater than the parent antibody variable domain to the binding partner. The above methods can be performed, for example, in solution or in solid phase. Moreover, various formats of binding assays are well known in the art and include, for example, immobilization to filters such as nylon or nitrocellulose; two-dimensional arrays, enzyme linked immunosorbant assay (ELISA), radioimmuno-assay (RIA), panning and plasmon resonance (*see*, *e.g.*, Sambrook *et al*., supra, and Ansubel *et al*., supra).

[00131] For the screening of populations of proteins such as the modified antibody variable domains produced by the methods of the disclosure, immobilization of the modified antibody variable domains to filters or other solid substrates is particularly advantageous because large numbers of different species can be efficiently screened for binding to a binding partner. Such filter lifts allow for the identification of modified antibody variable domains that exhibit enhanced binding affinity compared to the parent antibody variable domain to the binding partner. Alternatively, the modified antibody variable domains may be expressed on the surface of a cell or bacteriophage. For example, panning on an immobilized binding partner can be used to efficiently screen for the relative binding affinity of species within the population of modified antibody variable domains and for those which exhibit enhanced binding affinity to the binding partner than the parent antibody variable domain.

[00132] Another affinity method for screening populations of modified antibody variable domains is a capture lift assay that is useful for identifying a binding molecule having selective affinity for a ligand. This method employs the selective immobilization of modified antibody variable domains to a solid support and then screening of the selectively immobilized modified antibody variable domains for selective binding

interactions against the binding partner. Selective immobilization functions to increase the sensitivity of the binding interaction being measured since initial immobilization of a population of modified antibody variable domains onto a solid support reduces non-specific binding interactions with irrelevant molecules or contaminants which can be present in the reaction.

[00133] Another method for screening populations or for measuring the affinity of individual modified antibody variable domains is through surface plasmon resonance (SPR). This method is based on the phenomenon which occurs when surface plasmon waves are excited at a metal/liquid interface. Light is directed at, and reflected from, the side of the surface not in contact with sample, and SPR causes a reduction in the reflected light intensity at a specific combination of angle and wavelength. Biomolecular binding events cause changes in the refractive index at the surface layer, which are detected as changes in the SPR signal. The binding event can be either binding association or disassociation between a receptor-ligand pair. The changes in refractive index can be measured essentially instantaneously and therefore allows for determination of the individual components of an affinity constant. More specifically, the method enables accurate measurements of association rates (k_{on}) and disassociation rates (koff).

[00134] Measurements of k_{on} and k_{off} values can be advantageous because they can identify modified antibody variable domains with enhanced binding affinity for a binding partner. For example, a modified antibody variable domain can be more efficacious because it has, for example, a higher k_{on} valued compared to the parent antibody variable domain. Increased efficacy is conferred because molecules with higher k_{on} values can specifically bind and inhibit their binding partner at a faster rate. Similarly, a modified antibody variable domain can be more efficacious because it exhibits a lower k_{off} value compared to molecules having similar binding affinity. Increased efficacy observed with molecules having lower k_{off} rates can be observed because, once bound, the molecules are slower to dissociate from their binding partner.

[00135] Methods for measuring the affinity, including association and disassociation rates using surface plasmon resonance are well known in the arts and can be found described in, for example, Jonsson and Malmquist, Advances in Biosensors, 2:291–336 (1992) and Wu *et al*. Proc. Natl. Acad. Sci. USA, 95:6037–6042 (1998).

[00136] Using any of the above described screening methods, a modified antibody variable domain having binding affinity substantially the same or greater than the parent variable domain is identified by detecting the binding of at least one altered variable domain within the population to its binding partner.

[00137] Detection methods for identification of species within the population of modified variable domains can be direct or indirect and can include, for example, the measurement of light emission, radioisotopes, calorimetric dyes and fluorochromes. Direct detection includes methods that operate without intermediates or secondary measuring procedures to assess the amount of the binding partner bound by the modified antibody variable domain. Such methods generally employ ligands that are themselves labeled by, for example, radioactive, light emitting or fluorescent moieties. In contrast, indirect detection includes methods that operate through an intermediate or secondary measuring procedure. These methods generally employ molecules that specifically react with the binding partner and can themselves be directly labeled or detected by a secondary reagent. For example, a modified antibody variable domain specific for a binding partner can be detected using an antibody capable of interacting with the modified antibody variable domain, again using the detection methods described above for direct detection. Indirect methods can additionally employ detection by enzymatic labels. Moreover, for the specific example of screening for catalytic antibodies, the disappearance of a substrate or the appearance of a product can be used as an indirect measure of binding affinity or catalytic activity.

[00138] In some embodiments, the modified antibody variable domain has a binding affinity for the binding partner greater than the binding affinity of the parent variable domain for the binding partner and thus is identified as having enhanced binding affinity.

[00139] In some embodiments, a modified antibody variable domain exhibits enhanced binding affinity to a binding partner compared to the binding affinity between the parent variable domain and the binding partner. In some embodiments, a modified variable domain exhibits an at least 10%, at least 15%, at least 25%, at least 50%, at least 75%, at least 100% (or two-fold), at least 5-fold, at least 10-fold, at least 50-fold, at least 100-fold, or more, higher affinity to a binding partner than the corresponding parent antibody variable domain.

[00140] In other embodiments, the modified antibody variable domain has a binding affinity for the binding partner less than the binding affinity of the parent

antibody variable domain for the binding partner and thus is identified as having reduced binding affinity for the binding partner.

[00141] This disclosure is further illustrated by the following examples which are provided to facilitate the practice of the disclosed methods. These examples are not intended to limit the scope of the disclosure in any way.

EXAMPLES

Example 1: Design of Primers for Synthesis of Nucleic Acid Encoding Modified Protein

[00142] Each amino acid residue in a parent protein may be changed with other amino acid residues (*e.g.*, alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine) by performing PCR with an oligonucleotide containing one of seven different degenerate codons (*e.g.*, ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G)). For example, contacting residues identified from the "prox" lines in Figure 1, 3A and/or 3B may be changed with other amino acid residues by performing PCR with an oligonucleotide containing one of seven different degenerate codons (*e.g.*, ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G)).

[00143] In an exemplary substitution method, use of seven primers, each comprising one of the seven degenerate codons, may be employed to change one or more contacting (C) amino acid positions in a parent nucleic acid molecule to 18 other amino acid residues. An alternate substitution method may employ the use of three primers each comprising a different degenerate codon to produce eighteen amino acid changes at one or more contacting resides in a parent nucleic acid molecule. For example, the codons may include: NHT (where N=A/G/C/T, H=A/C/T), which codes for Phe/Ser/Tyr/Leu/Pro/His/Ile/Thr/Asn/Val/Ala/Asp; VAA (where V=A/C/G), which codes for Gln/Lys/Glu; and BGG (where B=C,G,T), which codes for Trp/Arg/Gly. This allows production of all eighteen amino acids at equal ratios if oligonucleotides comprising NHT is used at a 4:1:1 ratio with oligonucleotides comprising VAA and oligonucleotides comprising BGG, since NHT encodes twelve amino acids and VAA and BGG both encode three amino acids.

[00144] Primers containing one or more degenerate codons may be used to introduce a desired class of amino acid residue at a contacting (C) position by hybridizing to a parent nucleic acid (*e.g.*, the nucleotide sequence encoding the degenerate codon pairs with a contacting (C) position to be changed). Basic amino acid changes can be produced at a contacting (C) position with a single oligonucleotide that contains the codon mixture of ARG (R=A/G), encoding Arg/Lys. Further, polar amino acid changes can be introduced at a contacting (C) position with two oligonucleotides. The first oligonucleotide contains the codon mixture WMC (W=A/T; M=A/C), encoding Ser/Thr/Asn/Tyr, while the second oligonucleotide utilizes the codon mixture CAS (S=C/G), encoding His/Gln. Additionally, acidic amino acid changes may be introduced at a contacting (C) position with a single codon mixture of GAS, encoding Glu/Asp. Last, non-polar amino acid changes may be introduced at a contacting (C) position with a mixture of three primers with degenerate codons: NTC (N=A/G/C/T), encoding Leu/Phe/Ile/Val, KGG (K=G/T), encoding Trp/Gly, and SCG, encoding Pro/Ala.

Example 2: Construction of a Library Containing Modified Proteins

[00145] Modified proteins containing amino acid changes at one or more positions in a parent protein may be synthesized by PCR amplification from a parent nucleic acid molecule using synthetic oligonucleotides containing a degenerate codon. For example, modified antibody variable domains containing amino acid changes at one or more contacting (C) residues present within an exemplary antibody, for example, ING-1 (a mouse-human chimeric antibody containing the Br-1 mouse variable region domains and human constant regions domains which selectively binds to Ep-CAM (US Patent 5,576,184), heavy chain sequence represented by SEQ ID NO: 579, light chain sequence represented by SEQ ID NO: 580) may be synthesized by PCR amplification from a parent nucleic acid molecule using synthetic oligonucleotides containing a degenerate codon (SEQ ID NO: 1- 285 or SEQ ID NO: 583-699). Similarly, modified antibody variable domains containing amino acid changes at one or more contacting (C) residues present within an exemplary antibody, for example, IL-1 antibody (heavy chain sequence represented by SEQ ID NO: 581, kappa chain sequence represented by SEQ ID NO: 582) may be synthesized by PCR amplification from a parent nucleic acid molecule using synthetic oligonucleotides containing a degenerate codon (SEQ ID NO: 286- 578 or SEQ ID NO: 700-806).

[00146] For example, each library oligonucleotide containing the degenerate codon described above for ING-1 may be used in a PCR reaction to synthesize a DNA fragment which incorporates an amino acid change and a 3′ restriction site. In an exemplary method, PCR may be conducted at a contacting (C) position (*e.g.*, H3-3) by utilizing the CDRH3 oligonucleotide H3-3NP2 (SEQ ID NO: 267): 5'-GCTACATATTTCTGTGCAAGATTTGGCTCTKGGGTGGACTACTGGGGTCAAGG-3', and the reverse primer *Not*I-R (SEQ ID NO: 285): 5'-AGCGGCCGCACAAGATTTGGGCTCAACTCTC-3') (*see*, Figure 5) under standard conditions (see, *e.g.*, Sambrook and Russell, Molecule Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, 2001). After PCR amplification, fragments are obtained which comprise either a tryptophan or glycine residue at the internal codon (underlined above). Further, six other PCR reactions may be performed at the H3-3 position, utilizing SEQ ID NO: 285 with one of SEQ ID NOs: 262-266 and 268 under the conditions described above to obtain other amino acid changes at the site. Next, the products from these reactions may be combined at equal mass, except for reactions which used SEQ ID NO: 263 and 266 as a primer (this mixture is termed the pooled H3-3 library). Due to the degeneracy of these primers, twice the mass of the sample obtained with SEQ ID NO: 263 and 266 is added to produce an equimolar ratio of encoded amino acids.

[00147] An additional PCR reaction may be performed to create a fragment (called the H3-R fragment) which contains a 5′ restriction site and an overlapping complementary region to the library fragments described above. As an example, for the H3-3 position, a PCR reaction may be performed utilizing the Asc-F2 (SEQ ID NO: 284) and one of the H3R (SEQ ID NO: 247) primer. The 3’ portion of this molecule contains a region that is identical to the 5’ portion of the molecules created above which permits the use of a PCR reaction to create a contiguous molecule containing a 5′ and 3′ restriction site.

[00148] A PCR reaction may be performed to fuse the above PCR products together into a single molecule. Products from the two PCR reactions described above may be melted and re-annealed to allow for the region of overlap from the two molecules to hybridize. For example, an equal mass of the pooled H3-3 library (approximately two uL of each pooled PCR reaction) and the H3-R fragment may be annealed at their regions of overlap. Next, amplification of annealed molecules with both the Asc-F2 primer (SEQ ID NO: 284) and the NotI-R primer (SEQ ID NO: 247) allows for the synthesis of a single contiguous molecule (see, *e.g.*, Sambrook and

Russell, Molecule Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, 2001).

[00149] The DNA fragment synthesized by the methods above may be cloned into a pXOMA Fab or pXOMA Fab-gIII vector. Briefly, the DNA fragment is purified by using the QIAGEN® PCR purification kit and sequentially digesting the fragment with *Not*I (NEW ENGLAND BIOLABS®, Ipswich, MA) and *Asc*I (NEW ENGLAND BIOLABS®, Ipswich, MA) (*See*, Methods in Molecular Biology, vol. 178: *Antibody Phage Display: Methods and Protocols* Edited by: P.M. O'Brien and R. Aitken, Humana Press, "Standard Protocols for the Construction of Fab Libraries, Clark, M. A., 39-58) (see, *e.g.*, Figure 6). Next, the vectors may be ligated with the mutagenized insert using T4 Ligase (NEW ENGLAND BIOLABS®, Ipswich, MA) and transformed into TG1 cells by electroporation.

Example 3: Selection of High Affinity Binders

[00150] Phage containing a modified proteins including, for example, modified antibody variable domains that bind to an antigen (*e.g.*, Ep-Cam or IL-1β) with high affinity may be selected by standard panning protocols (*see*, *e.g.*, Methods in Molecular Biology, vol. 178: *Antibody Phage Display: Methods and Protocols* Edited by: P.M. O'Brien and R. Aitken, Humana Press, "Panning of Antibody Phage-Display Libraries", Coomber, D. W. J. pp133-145, and "Selection of Antibodies Against Biotinylated Antigens", Chames, P. *et al*. p.147-157).

[00151] In an exemplary method, library phage for the panning procedure are amplified by inoculating fifty milliliters of 2YT with library TG1 cells and grown to an OD_{600} of 0.6-0.8. Helper phage VCSM13 are added to the inoculated 2YT culture at a multiplicity of infection (M.O.I.) of 10 (*e.g.,* in 50 mL of cells with OD_{600}=0.6 there are $0.6x3^8$ x 50= $9x10^9$ cells, M.O.I. of 10 is therefore 9^{10} helper phage, which corresponds to about 10µl of 1^{13} stock phage). The helper phage are used to infect the TG1 cells by gently mixing the phage with the cells with no shaking for thirty minutes. The culture is then shaken for an additional thirty minutes at 180 rpm. Following infection, the culture is spun down at 2500 rpm for ten minutes. The resulting cell pellet is resuspended in fifty milliliters of 2TYAmpKan and grown overnight at 30°C and the supernatant is removed and discarded.

[00152] Exemplary methods of panning include coating one well of a NUNC® MAXISORP plate with fifty µl of Ep-Cam or IL-1β at 0.1 µg/ml in DULBECCO'S® PBS with Calcium and Magnesium chloride (Invitrogen, Carlsbad, CA) and incubating the

plates overnight at 4°C. The wells are then blocked with 5% milk in PBS for one hour at room temperature. Separately 0.5 ml of phage supernatant from the overnight culture described above are blocked with 300 µL of 10% milk in PBS for one hour at room temperature. Blocked phage (*e.g.*, approximately 200 µl) are added to the blocked wells in 3% BSA-PBS and incubated at room temperature with shaking for one to two hours. After incubation, the wells are emptied and washed five times with PBST quick wash (*e.g.*, PBS + 0.05% Tween 20), then washed five times with PBST five minute wash, followed by five washes with PBS quick wash and lastly washed five times with PBS five minute wash. Phage bound to the wells are eluted by incubating with 200 µL/well of freshly prepared 100 mM TEA (prepared by adding 140 µL of 7.18 M Triethylamine stock to ten ml H_2O for 20 minutes at room temperature (see, *e.g.*, Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, 2001). The eluate is transferred to a Falcon tube containing 0.5 ml 2M TRIS-HCl pH 7.4. The pH of the eluate is checked with pH paper to ensure that it is about pH 7 and adjusted if necessary.

[00153] Eluted phage from the exemplary panning method are amplified by infecting TG1 cells. In an exemplary method, TG1 cells are grown to an OD_{600}=0.6 (*e.g.*, mid log phase) and ten ml of the culture is added to the phage eluate from the panning method described above. The eluted phage are used to infect the TG1 cells at 37°C for thirty minutes without shaking and then continued for an additional thirty minutes at 37°C with shaking at 240 rpm. After the infection, the culture is centrifuged at 2500 rpm for five minutes. Next, the supernatant is removed and the cell pellet is resuspended in 700 µL of 2YTAG. The re-suspension is plated on two 15 cm 2YTAG agar plates and incubated at 30°C overnight. After the overnight incubation, the cells are scraped from the two plates using five to ten milliliters of 2YTAG per plate, and transferred to a fifty milliliter falcon tube where they are used to make a glycerol stock.

[00154] In an alternative exemplary method, panning may be performed with biotinylated Ep-Cam or IL-1β. Briefly, two hundred microliters of streptavidin beads (Dynal) are blocked in 5% BSA-PBS (100 µl of the blocked beads are used for the de-selection and 100 µL for the selection). Using a magnet, the beads are removed from the 5% BSA-PBS and rinsed twice in PBS. To the rinsed beads is added one milliliter of 5% BSA-PBS and the beads are incubated at room temperature for one hour with very gentle rotation. After the incubation, the beads are split into two tubes, with the supernatant removed from one tube for the de-selection. Phage solution is added to the tube with beads designated for the de-selection and resuspended. The phage-

bead solution is incubated at room temperature for forty-five minutes with gentle rotation. After the incubation, the phage supernatant (de-selected phage solution) is transferred to a new tube using a magnet. Next, the de-selected phage solution is incubated at room temperature for sixty minutes with one hundred pmols of biotinylated Ep-Cam or IL-1β. The phage-biotinylated Ep-Cam or IL-1β solution is then added to a new aliquot of streptavidin beads (with the supernatant removed) and incubated at room temperature for sixty minutes. After the incubation, the beads are separated from the supernatant using a magnet. Next the beads are washed five times with one ml of 0.5% BSA-PBST by adding the wash to the tube, closing the tube and resuspending the pellet, putting back in the magnet waiting a few seconds until the beads are attached to the magnet side of the tube and removing the wash with a pipetman. Further, the beads are washed five times in 0.5% BSA-PBST for five minutes for each wash, washed five times with one milliliter of 0.5% BSA-PBS, washed five times for five minutes each wash in five milliliters of 0.5% BSA, and washed one time with PBS. Bound phage are eluted by incubating the beads with 500 µL of freshly prepared 100 mM TEA (add 140 µL of 7.18 M Triethylamine stock to 10 ml H_2O) for thirty minutes at room temperature with gentle rotation. The eluate is separated from the beads by using a magnet and transferred to a fifty milliliter falcon tube containing 250 µl of 1M TRIS pH 7.4 to neutralize the TEA and can be used for infection and/or amplification (see, *e.g.*, Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, 2001). For example, log phase TG1 cells may be infected with phage eluate at 37°C for one hour at ninety rpm. After infection of the cells, the culture is centrifuged at 2500 rpm for five minutes and the supernatant removed. Next, the cell pellet is resuspended in 700 µl of 2YTAG, plated onto two 15 cm 2YT-ampicillin-2% glucose agar plates and incubated at 30°C overnight.

Example 4: Screening of an Affinity Matured Protein Using the DELFIA® Competition Assay

[00155] Individual proteins including, for example, Fabs obtained from the affinity-based selection of libraries of the ING-1 antibody clone are tested for their ability to inhibit the binding of Ep-Cam to the parent chimeric ING-1 IgG antibody using a competitive screening assay (*e.g.*, the microplate based competitive screening DELFIA® assay (PERKIN ELMER®, Waltham, Massachusetts)). Ninety-six well plates containing two hundred and fifty milliliters of 2YT media are inoculated with glycerol stock of Fab-expressing E. coli transformed with the pXOMA-Fab vector. The culture is

grown at 37°C until cloudy (approximate OD600=0.5) and inoculated with IPTG to a final concentration of 1 mM. The cultures are grown overnight at 30°C. In addition, a Costar plate 3922 (White) is coated with 1.25 ug/mL of parental ING-1 chimeric IgG O/N at 4°C.

[00156] Periplasmic extracts (PPE) of the overnight expression constructs are prepared by spinning the overnight expression plates at 3000 rpm for fifteen minutes, discarding supernatant and adding 60 microliters of PPB buffer to each well. The pellets are resuspended, and 90 microliters of cold PPB diluted 1:5 with cold water are added to each well. This mixture is incubated on ice for one hour and subsequently spun down at 3000 rpm for fifteen minutes. This PPE supernatant is transferred to a new plate. The PPE is diluted into 10% PPE in PBS, 5% PPE in PBS, and 1% PPE in PBS. For the coated Costar plate, it is washed three times with PBS-tween and blocked with 350 microliters of 3% BSA in PBS for one hour.

[00157] The blocked Costar plate is washed three times with PBS and then biotinylated Ep-Cam is added to the diluted PPE to a final concentration of 3 nM. The diluted PPE and biotinylated Ep-Cam solution is then added to the coated Costar plate and incubated for one and a half hours at room temperature. The plates are washed three times with PBST and fifty microliters of 1:250 dilution of Europrium-Streptavidin in Delfia Assay Buffer (PERKIN ELMER®, Waltham, Massachusetts) is added. The mixture is incubated at room temperature for one hour, and the Time-Resolved Fluorescence Plate reader is setup (Gemini microplate reader, Molecular Devices), interval 200-1600 microseconds, 20 reads/well, excitation 345 nm, emission 618 nm and cutoff 590 nm. The plates are washed seven times with Delfia Wash Buffer (PERKIN ELMER®, Waltham, Massachusetts), followed by the addition of fifty µl of Delfia Enhancement buffer (PERKIN ELMER®, Waltham, Massachusetts) and incubated for five minutes. The plates are read on the Gemini plate reader. Plates with decreased signal compared with control parental antibody show greater binding by the affinity matured Fab and can be further characterized by Biacore (e.g., Biacore 2000 or A100) and other affinity measuring techniques (see, *e.g.*, Tables 4 and 5).

[00158] Similarly, XPA23 antibody clones may be tested for their ability to inhibit the binding of IL-1β to the parent chimeric XPA23 IgG using a competitive screening assay as described above.

Table 4: *Delfia Screening of 10% Periplasmic Extract*

	1	2	3	4	5	6	7	8	9	10	11	12

A	46.9	37.1	71.2	75.7	51.3	22.3	65.8	72.9	58.8	81.7	56.2	96.7
B	**2.6**	55.2	39.2	54.8	31.7	41.3	57.1	56.7	21.6	77.8	**1.8**	102.0
C	53.2	42.3	72.5	61.2	16.2	78.0	41.2	57.2	63.8	28.6	13.6	100.7
D	49.0	45.5	**8.9**	**1.0**	21.5	82.8	105.8	67.3	68.5	61.8	63.5	100.6
E	49.1	72.1	68.6	**0.3**	91.8	57.6	53.1	**8.3**	58.3	60.4	82.2	-0.4
F	61.7	72.1	71.8	45.6	44.6	53.1	15.3	73.2	84.7	15.1	59.0	0.1
G	58.4	26.4	**1.0**	59.4	62.3	19.9	**-0.1**	49.0	52.4	76.2	46.8	0.3
H	36.1	67.7	65.2	27.4	34.3	50.3	60.0	60.1	56.8	83.0	49.3	-0.4

Percentage of inhibition is shown in each well using the average signal from wells A12-D12 as positive control, 100% inhibition and the average signal in well E12-H12 as 0% inhibition negative control wells. Wells bolded show strong competition in the Delphia assay.

Table 5: *Delfia Screening of 5% Periplasmic Extract*

	1	2	3	4	5	6	7	8	9	10	11	12
A	58.4	59.3	86.6	87.8	74.8	52.4	71.7	84.4	68.6	84.8	74.2	99.4
B	**14.5**	73.0	71.5	77.5	63.1	75.4	79.2	76.4	53.2	82.6	**44.9**	101.8
C	56.2	56.3	92.3	76.5	55.1	94.0	81.1	79.9	77.6	60.2	53.7	100.5
D	46.8	57.8	43.0	**33.0**	52.4	92.6	115.3	91.6	74.7	77.1	74.7	98.2
E	58.8	60.1	82.5	**28.5**	101.3	88.5	71.6	**51.9**	76.2	74.9	94.8	0.2
F	58.3	65.8	69.2	64.6	67.3	78.0	65.3	87.7	88.2	38.9	70.8	0.1
G	52.8	47.4	**22.2**	72.2	65.8	55.7	**10.0**	68.1	69.2	73.5	64.6	-0.2
H	42.2	68.8	68.5	46.3	62.7	63.6	73.7	70.9	76.2	85.1	68.7	-1.1

Percentage of inhibition is shown in each well using the average signal from wells A12-D12 as positive control, 100% inhibition and the average signal in well E12-H12 as 0% inhibition negative control wells. Wells bolded show strong competition in the Delphia assay. Boxed wells retain strong inhibition and are prioritized for affinity testing.

Example 5: Screening of an Affinity Matured Protein Using Kinetic Titration Analysis

[00159] Kinetic properties of affinity matured proteins including, antibodies, for example, as represented by XPA23 clones such as Y208L may be determined by kinetic titration analysis. In an exemplary method, an antigen such as IL-1β is amine coupled to a CM5 sensor chip. Each sample (*e.g.*, from lowest to highest concentration) may be injected for 240 seconds at a flow rate of 30 □l/min at a selected temperature (*e.g.*, 25ºC). Sample are allowed to dissociate for 30 seconds except the highest concentration which may be permitted 300 seconds to dissociate. The assay is run at 25°C.

[00160] Biaevaluation software (*e.g.*, Biacore 2000 evaluation software) is used to calculate dissociation rates of individual samples and the relative amount of sample bound to each test surface. The data is fit to an appropriate kinetic model

(*e.g.*, the kinetic titration model). For example, XPA23 had a ka= 2.5e5 and a kd= 1.2e-2 KD = 4.6e-8, while the modified XPA23 Y208L mutant had a ka=3.57E+05kd= 5.80E-03 KD=1.62E-08.

Example 6: ELISA Measurement for Fab Expression, EpCam Binding or IL-1 Binding

[00161] Additionally or alternatively to the Biacore assay described below in Example 10, an ELISA assay may be used for the identification of modified antibody variable domains that bind its binding partner or for verifying expression of Fab domains.

[00162] In an exemplary method, ELISA plates (*e.g.*, Nunc MAXISORP™) are coated with 1µg/ml EpCam, 1 µg/mL EpCam for EpCam ELISA, 1 µg/mL IL-1 (Peprotech), or anti-human IgG, $F(ab')_2$ fragment specific antibody (Jackson Immunoresearch) in PBS at 50 µg/ml. The ELISA plates are then covered and incubated at 4°C overnight. After the incubation, the coated ELISA plates are washed three times with PBS. The plates are then filled with 370 µl of 3% milk (*e.g.*, Carnation, nonfat) and incubated for one hour at room temperature. Separately, 150 µl of periplasmic extract is blocked by adding 50 µl of 15% milk and incubating the extract for one hour at room temperature. The blocked plates are washed three times with PBS and 50 µL of the blocked periplasmic extract is added to each well of the antigen coated ELISA plates. The plates are incubated for two hours at room temperature and then washed four times with TBST.

[00163] Secondary antibodies are added to each ELISA plate. For the Ep-Cam or IL-1 ELISA, 50 µl of mouse anti-human c-myc antibody (9E10 Ab, Roche) at 2.5 µg/ml in 3% milk is added to each well. For the anti-Fab ELISA, 50 µl of biotin-SP-conjugated anti-human IgG F(ab')2 fragment specific antibody (Jackson Immunoresearch) at 1:2000 dilution in 3% milk is added to each well. The plates from both ELISAs are incubated at room temperature for one hour. After the incubation, the plates are washed four times with TBST. After the washes, a tertiary antibody may be added to the plates in both ELISAs. For the Ep-Cam or IL-1 ELISA, 50 µl of goat anti-mouse IgG-HRP (Pierce) diluted 1:10,000 in 3% milk is added to each well. For the anti-Fab ELISA, 50 µl of extravidin-HRP conjugate (Sigma) at a 1:500 dilution in 3% milk is added to each well. Again the plates from both ELISAs are incubated for one hour at room temperature. After the incubation, the plates are washed four times with TBST. Next, 50 µl of the TMB substrate (Calbiochem) is added to each well and

incubated until the color develops (do not incubate long enough to see the negative control turn blue). The reaction is stopped by adding 50 µl of 2N H_2SO_4 to each well and the plates are read at 450 nm.

Example 7: Methods for Off-Rate Ranking of Antibody Fragments

[00164] A high-throughput off-rate ranking method is used for rapid prioritization of modified proteins including, for example, modified antibody variable domains that bind to their binding partner by analyzing their relative off-rates (using, *e.g.*, Biacore 2000 or A100).

[00165] In an exemplary method, modified antibody variable domains (*e.g.*, Epcam-binding) are produced in ninety-six well plates by inoculating two hundred and fifty microliters of 2YT media with a glycerol stock of Fab-expressing *E. coli* transformed with a pXOMA-Fab vector comprising a modified Epcam-binding variable domain. The culture is grown at 37°C until cloudy (*e.g.*, approximate OD_{600}=0.5), inoculated with IPTG to a final concentration of 1 mM and grown overnight at 30°C.

[00166] Next, periplasmic extracts (PPE) of the overnight expression constructs are prepared by spinning the overnight expression plates at 3000 rpm for fifteen minutes, discarding the supernatant and adding 60 µl of PPB buffer to each well. The pellets are resuspended, and 90 µl of cold PPB diluted 1:5 with cold water is added to each well. This mixture is incubated on ice for one hour and subsequently spun down at 3000 rpm for fifteen minutes. The supernatant is transferred to a new plate and the periplasmic extracts are used for the Biacore (*e.g.*, Biacore 2000 or A100) determination.

[00167] Epcam from the periplasmic extracts is amine coupled (*e.g.*, 10µg/mL Epcam in pH 4.5 acetate, seven minute injection at 5 µl/minute) to a CM5 sensor chip and periplasmic extracts containing the antibody fragments are injected over the sensor, resulting in binding of the Fab to the immobilized Epcam. Non specific binding of the antibody fragment to the sensor surface is corrected by subtracting the interaction of the antibody fragment with a blank flow cell (*e.g.*, having no immobilized Epcam) from the interaction of the antibody fragment with the Epcam immobilized flow cell. The instrument settings are: a flow rate of 20 microliters/minute, an injection time of three minutes, a dissociation time of five minutes and an instrument temperature set to 25ºC. Biaevaluation software is used to calculate dissociation rates of individual samples and the relative amount of sample bound to each test surface. Samples are then ranked according to their dissociation rates. Sensograms depicting the off-rates

for heavy chains (Figure 15) and light chains (Figure 16) are shown. The off rates for the improved clones are tabulated for the heavy chain (Figure 11) and the light chain (Figure 12).

[00168] Likewise, modified XPA23 variable domains (*e.g.*, IL-1β -binding) may be ranked according to their dissociation rates using the high-throughput off-rate ranking method described above. The instrument settings are: a flow rate of 30 microliters/minute, an injection time of three minutes, a dissociation time of ten minutes and an instrument temperature set to 25ºC. The off rates for the improved clones are tabulated for the heavy chain (Figure 13) and the light chain (Figure 14).

[00169] The modified antibody variable domains of the present disclosure may have a k_{off} that is greater than (see, *e.g.*, Figure 20), less than (see, *e.g.*, Figure 18) or equal to (see, *e.g.*, Figure 19) than an unmodified antibody variable domain.

Example 8: Reformatting of Candidate Clones to IgG

[00170] Two of the improved off-rate clones from the k_{off} analysis were reformatted into IgG_1 format by PCR amplification of the heavy and light chain variable domains and cloning the PCR amplified regions into a mammalian expression vector containing the Fc and the light chain constant domain respectively. The heavy chain is cloned into a mammalian expression vector containing a CMV promoter using BsmI and NheI sites for the 5' and 3' ends respectively and is cloned in frame with the heavy chain secretion signal on the 5' end and the constant CH1,CH2, and CH3 portions of the IgG molecule on the 3' end. The amplification sequences are as follows: (ING-HC-IgGF 5'-ATATATTGCATTCCCAGATCCAGTTGGTGCAGTC-3'), ING-HC-IgGR (5'-ATATATGCTAGCTGAGCTGACGGTGACCGAGGTTCC-3'). The light chain is cloned into a similarly constructed expression vector utilizing a blunt 5' cloning site and the BsiWI site on the 3' end and is cloned in frame with the light chain secretion signal on the 5' end and the light chain constant region on the 3' end. The PCR amplification primer sequences are as follows: (ING-LC-IgGF 5'-CAAATTGTGATGACGCAGGC-3') and (ING-LC-IgGR 5'-ATATATCGTACGTTTCATCTCTAGTTTGGTGCC-3'). The PCRs are performed under standard conditions: see, *e.g.*, Sambrook and Russell, Molecule Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, 2001. Improved off-rate clones reformatted into IgG_1 vectors are transiently co-transfected in a 2:1 light chain to heavy chain DNA ratio into HEK 292 cells using Lipofectamine 2000 (Invitrogen) using the manufacturer's guidelines. Secreted IgGs secreted from HEK 292 cells are purified using protein A SEPHAROSE® (GE-AMERSHAM®, Piscataway,

NJ) using the manufacturer's gudielines and tested by BIACORE® (*e.g.*, Biacore 2000 or A100) for affinity (see, *e.g.*, Figures 11 and 15) and Example 8.

Example 9: Expression and Testing of Modified Proteins with a Combination of Amino Acid Changes

[00171] Modified proteins including, for example, antibody variable domains with improved off-rates and affinities as compared to a parent protein may be identified by employing the DELFIA® competition assay and/or BIACORE® (*e.g.*, Biacore 2000 or A100) off-rate ranking. Clones with improved k_{off} are sequenced and aligned by both their light and heavy chain. Identified amino acid changes in the light and heavy chain that increase affinity can be combined in one modified antibody variable domain for potential additive and synergistic combinations. Modifications for combination may utilize the residues that have improved off-rates greater than or equal to 4.9 fold compared with the parental antibodies (*see, e.g.*, Figure 11, 12). For any given amino acid position, the change that leads to the greatest improvement is chosen for study. This compilation is described in Table 6, and will lead to 21 combinations of heavy and light chains (*e.g.*, 7 heavy chains combined in all variations with three light chains).

Table 6: *Heavy and Light Chain CDR1, CDR2 and/or CDR3 Combinations*

	Heavy Chain Combinations	
CDR1	**CDR2**	**CDR3**
G33F	wt	wt
wt	T53I	wt
wt	wt	G100R
G33F	T53I	wt
wt	T53I	G100R
G33F	wt	G100R
G33F	T53I	G100R
	Light Chain Combinations	
wt	Q55R	wt
wt	wt	E98T
wt	Q55R	E98T

[00172] Alternatively, the initial modifications for combination may utilize the residues that have improved off-rates greater than or equal to approximately 2.5-fold compared with the parental antibodies (see, *e.g.*, Figure 13 ,14). For any given amino acid change, the change that leads to the greatest improvement is chosen for study. The amino acids with greater than or equal to approximately 2.5 fold improved k_{off} are

compiled in Table 7. There are two amino acids in CDR1 (position 28), two amino acids in position 100, three amino acids in position 101, and five amino acids in position 102. In all, there are 60 (2x2x3x5=60) combinations.

Table 7: *Heavy Chain CDR1 and CDR3 Combinations*

CDR1	CDR3
28T (wt)	100G(wt)
28I	100R
	101S(wt)
	101I
	101G
	102A (wt)
	102Y
	102F
	102W
	102G

[00173] A PCR based strategy may be used to create a modified antibody light chain containing more than one amino acid change (*see, e.g.*, Figure 7). In an exemplary method, PCR may be used to amplify three segments of the V_k gene, two of which may be engineered to contain an amino acid change. For example, to create a light chain containing the mutations Q55R and E98T, PCR product 1 may be synthesized using the HindIII-F (SEQ ID NO: 814) and L2R primer (SEQ ID NO: 74), PCR product 2 may be synthesized using L2-Q55R primer (SEQ ID NO: 808) and the L3R primer (SEQ ID NO: 110) and PCR product 3 may be synthesized using L3-E98T primer (SEQ ID NO: 807)and the AscI-R primer (SEQ ID NO: 812). The PCR products are then melted and re-annealed such that their regions of overlap hybridize. Subsequently, all three PCR products may be joined into one molecule by PCR amplification using the forward primer from PCR product 1 (HindIII-For) (SEQ ID NO: 814) and the reverse primer from PCR product 3 (AscI-R) (SEQ ID NO: 812). In an exemplary method to create a heavy chain containing the mutations outlined above and described in Figure 7, product 1 may be synthesized using the AscI-F (SEQ ID NO: 813) and H1R primer (SEQ ID NO: 146), PCR product 2 may be synthesized using H1-28TI primer and the H3R primer (SEQ ID NO: 247) and PCR product 3 may be synthesized using each H3 combination primer (6 primers, 6 rxns) and the NotI-R primer (SEQ ID NO: 285). The PCR products are then melted and re-annealed such

that their regions of overlap hybridize. Subsequently, all three PCR products may be joined into one molecule by PCR amplification using the forward primer from PCR product 1 (AscI-F) (SEQ ID NO: 813) and the reverse primer from PCR product 3 (NotI-R) (SEQ ID NO: 285).

[00174] In an exemplary method, a 50 µL PCR reaction for the production of PCR product 1, 2 and 3 may be performed with 25 pmol of each of the forward and reverse primers, 10 ng of template DNA, 5 µL PFU buffer, 2.5 µL of 10 µM dNTPs, 1 µL PFU and water to 50 µL. The PCR reaction is heated to 94°C for two minutes, followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 54°C, and one minute at 72°C. After the 25 cycles, a final 72°C incubation may be performed for five minutes.

[00175] An equal mass of the three PCR products may be combined in a PCR reaction to produce a modified variable domain with several amino acid changes which enhance affinity. Briefly, the PCR may be conducted by adding approximately 2 µL of each pooled PCR reaction to 5 µL PFU buffer, 25 pmol of both HindIII-f primer (SEQ ID NO: 814)and AscI-R primers (SEQ ID NO: 812), 2.5 µL of 10 µM dNTPs, 1 µL PFU polymerase and water to 100 µL. Next, the PCR reaction is heated to 94°C for two minutes, followed by twenty-five cycles of thirty seconds at 94°C, 30 seconds at 54°C, and finally one minute at 72°C. After the twenty cycles, a final 72°C incubation is performed for five minutes.

[00176] The resulting DNA fragment may be purified (*e.g.*, using the QIAGEN® PCR purification kit (Valencia, CA)) and sequentially digested with *Hind*III (NEB) and then *Asc*I (NEW ENGLAND BIOLABS®, Ipswich, MA) such that it may be cloned into the pXOMA Fab or pXOMA Fab-gIII vector.

[00177] For the heavy chain modifications, a similar PCR based strategy may be used to create a modified antibody heavy chain containing more than one amino acid change (see, *e.g.*, Figure 8). In an exemplary method, PCR may be used to amplify four segments of the V_H gene, three of which may be engineered to contain the G33F, T53I and G100R amino acid changes. For example, PCR product 1 may be synthesized using the AscI-F (SEQ ID NO: 813) and H1R primers (SEQ ID NO: 146), PCR product 2 may be synthesized using the H1-G33F primer (SEQ ID NO: 809) and H2R primer (SEQ ID NO: 182), PCR product 3 may be synthesized using H2-T3I primer (SEQ ID NO: 810) and H3R primer (SEQ ID NO: 247) and PCR product 4 may be synthesized using H3-G100R primer (SEQ ID NO: 811) and the NotI-R primer (SEQ ID NO: 285). The PCR products are then melted and re-annealed such that their regions of overlap hybridize. All four PCR products may then be joined into one

molecule by PCR amplification using the forward primer from PCR product 1 (AscI-F) (SEQ ID NO: 813) and the reverse primer from PCR product 3 (NotI-R) (SEQ ID NO: 285).

[00178] In an exemplary method, a 50 µL PCR reaction for the production of PCR products 1, 2, 3 and 4 may be performed with 25 pmol each of the forward and reverse primers, 10 ng of template DNA, 5 µL PFU buffer, 2.5 µL of 10 µM dNTPs, 1 µL PFU and water to 50 µL. The PCR reaction is heated to 94°C for 2 minutes, followed by 25 cycles of 30 sec at 94°C, 30 seconds at 54°C, and one minute at 72°C. After the 25 cycles, a final 72°C incubation may be performed for five minutes.

[00179] An equal mass of the four PCR products may be combined in a PCR reaction to produce a modified variable domain with several amino acid changes which enhance affinity. Briefly, the PCR may be conducted by adding approximately 2 µL of each pooled PCR reaction to 5 µL PFU buffer, 25 pmol of both AscI-F primer (SEQ ID NO: 813) and NotI-R primer (SEQ ID NO: 285), 2.5 µL of 10 µM dNTPs, 1 µL PFU polymerase and water to 100 µL. Next, the PCR reaction is heated to 94°C for two minutes, followed by twenty-five cycles of thirty seconds at 94°C, 30 seconds at 54°C, and finally one minute at 72°C. After the twenty cycles, a final 72°C incubation is performed for five minutes.

[00180] The heavy chain PCR fragments and the vector will be digested with *Asc*I (NEW ENGLAND BIOLABS®, Ipswich, MA) and *NotI* (NEW ENGLAND BIOLABS®, Ipswich, MA) such that it may be cloned into the pXOMA Fab or pXOMA Fab-gIII vector.

Example 10: Biacore Measurement of Protien Affinity

[00181] Proteins including, for example, IgGs that bind a binding partner (*e.g.*, Epcam) are tested by BIACORE® for affinity (see, *e.g.*, Figure 15). For example, kinetic analysis of anti-Epcam mAb's are conducted on a Biacore 2000®.

[00182] In an exemplary method, the ING1 antibody is diluted to 0.5 µg/mL in HBS-EP running buffer and injected for two minutes at 5 µl/ minute over a high density protein A/G surface. Next, six serial 3 fold dilutions of Epcam are prepared in running buffer and injected in triplicate in random order over the high density protein A/G surface with buffer injections evenly distributed throughout the run. The sample injections are then double referenced against the blank flow cells and buffer injections to correct for any bulk shift or non-specific binding. Data are then analyzed with the

Biaevaluation software from Biacore and sensorgrams are fit utilizing the 1:1 langmuir model (*see, e.g.*, Figure 15).

Example 11: Construction of Arrays of Modified Proteins

[00183] Arrays of modified proteins including, for example, antibody variable domains (*e.g.*, modified ING-1 variable domains) with amino acids changes at desired positions (*e.g.*, contacting (C) residues) may be generated and tested for enhanced binding affinity compared to the parent protein (*e.g.*, ING-1). Modified variable domains used in the array may be obtained directly from a library of modified variable domains as described in Example 2 or may first be screened for those modified variable domains that exhibit enhanced binding as compared to the parent variable domain as described in Examples 3, 4 and 5.

[00184] In an exemplary method, each contacting (C) residue in the heavy and light chain variable region of ING-1 is separately changed (*e.g.*, by PCR mutagenesis) with alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine to generate modified ING-1 variable domains. CDNAs encoding the modified ING-1 variable domains are then inserted into a pXOMA vector and used to transform electrocompetent TG1 cells. The clones are plated on 2YT- Amp_{100}/2%Glucose plates (Teknova) and the plates filled with 250 µl of 2YT-Amp_{100}/well (Teknova). Each well is inoculated with a single colony comprising a single amino acid change at a contacting (C) residue. The colonies are grown by incubating the plates at 37ºC for two to four hours with shaking at 450 rpm. After the incubation, the plates are duplicated to sequencing plates by filling new deep-well culture plates (Thomson) with one milliliter of 2YT-$Amp_{100}Gluc_{2\%}$/well from the grown cultures. The Genetix 96-pin replicator is used to transfer cells from the master plate to the new sequencing plates. The sequencing plates are grown overnight at 37ºC with shaking at 450 rpm. After the incubation, the sequencing plate is spun down at 5000 rpm for ten minutes and the supernatant is discarded. Samples from the plate are sequenced (*e.g.*, samples may be submitted for automated miniprep and automated sequencing (Elim biopharmaceuticals). After the incubation, Master Plates are made by adding glycerol to a final concentration of 15% to the wells on the glycerol plate and storing the plates at -80ºC. The unique clones and their well position in the master plate are identified after sequencing results are returned.

[00185] Eighteen different clones, each containing an amino acid change at a contacting (C) residue in ING-1, are identified (typically 96 sequenced clones yield all eighteen clones). Unique clones from the master plates are rearrayed to a new 96-well master plate containing 2YT- Amp_{100} by transferring ten microliters of glycerol stock from the master plate to the rearrayed master plate. Alternatively, automation, such as the QPIX II is used to transfer the glycerol stock containing the unique clones to the new master plate. The new rearrayed glycerol master plates are replicated into new expression plates to perform Biacore (*e.g.*, Biacore A100) analysis (*see*, *e.g.*, Table 8 and Table 9). Arrays may also be constructed for XPA 23 modified antibodies (see, *e.g.*, Table 10 and 11).

Table 8: *Biacore Analysis of Modified Light Chain Variable Regions*[1, 2 3]

						NP						NP							NP	
					Aromatic															
		Neg		Pos		Polar						Aliphatic				Small				
		D	E	R	K	H	Y	W	F	Q	N	S	T	V	I	L	A	C	G	P	M
CDR1	K 27	1.26	-1.00	1.26	?	1.06	nd	-1.00	1.62	1.52	-1.00	-1.00	-1.00	nd	1.15	1.39	1.10	nd	1.11	**1.29**	nd
	S 28	1.63	1.02	**2.78**	2.32	1.90	2.02	nd	2.38	1.99	-1.00	1.08	1.19	2.15	2.35	2.60	1.53	nd	nd	2.42	nd
	L 29	-1.00	-1.00	-1.00	nd	nd	-1.00	-1.00	-1.00	-1.00	1.85	**2.03**	nd	nd	-1.00	?	1.97	nd	1.53	-1.00	nd
	L 30	1.47	-1.00	-1.00	-1.00	1.45	1.53	-1.00	1.56	1.60	1.45	1.43	**1.89**	1.69	0.96	0.97	1.27	nd	1.64	0.82	nd
	H 31	0.71	0.68	0.06	0.05	0.95	**2.16**	1.66	nd	0.57	0.50	0.82	1.94	1.17	1.14	0.59	1.26	nd	0.73	1.19	nd
	S 32	0.94	nd	**1.79**	1.32	1.13	1.37	1.27	1.64	1.10	-1.00	?	nd	0.93	1.17	1.12	1.35	nd	1.39	0.83	nd
	N 33	0.49	0.65	0.71	0.70	0.73	0.80	1.04	0.93	0.73	**1.38**	0.48	0.60	-1.00	nd	nd	0.76	nd	0.72	0.67	nd
	I 35	0.19	0.16	0.92	0.61	0.59	0.51	0.34	0.66	0.41	0.50	0.55	0.87	1.08	nd	0.60	0.46	nd	0.39	0.70	nd
	T 36	0.05	1.60	-1.00	1.15	0.79	nd	1.30	nd	1.04	0.74	1.10	0.94	0.98	0.76	**1.64**	1.02	nd	1.09	0.67	nd
	Y 37	nd	0.01	nd	0.02	**4.07**	0.95	0.85	0.63	0.02	0.06	0.09	-1.00	-1.00	0.90	nd	0.04	nd	1.30	nd	nd
CDR2	Y 54	0.03	0.05	-1.00	**3.62**	-1.00	0.92	0.96	0.94	1.23	-1.00	0.90	0.61	1.32	-1.00	3.44	0.08	nd	1.85	0.86	nd
	Q 55	0.05	0.05	**5.31**	0.46	3.82	nd	4.11	0.86	0.95	0.36	0.56	0.66	1.53	1.42	0.71	0.64	nd	0.70	0.95	nd
	M 56	1.36	0.71	0.92	0.98	1.32	1.21	1.29	**1.40**	1.12	0.99	1.05	0.80	-1.00	1.37	0.74	0.86	nd	0.80	1.38	nd
	S 57	0.95	0.93	1.17	1.54	1.01	-1.00	**2.34**	0.96	-1.00	1.17	1.00	0.86	0.89	0.98	1.38	1.15	nd	-1.00	nd	nd
	N 58	nd	0.97	1.77	1.40	1.16	1.43	1.99	1.03	1.55	0.95	1.65	1.42	2.84	2.51	1.47	1.79	nd	1.87	**3.47**	nd
CDR3	L 97	-1.00	0.75	-1.00	0.61	0.42	0.98	1.59	0.93	0.91	0.48	0.79	0.54	1.44	**2.62**	0.93	0.50	nd	-1.00	0.95	nd
	E 98	1.62	0.98	3.08	2.22	1.23	1.23	1.10	1.43	1.41	-1.00	-1.00	**4.90**	nd	nd	2.82	1.35	nd	1.63	-1.00	nd
	L 99	0.02	0.01	0.04	0.02	0.05	1.00	0.89	0.43	0.02	2.00	0.04	0.09	1.04	**2.07**	0.93	1.43	nd	0.02	0.01	nd
	P 100	0.02	0.06	0.05	0.03	0.05	**1.94**	1.51	1.65	0.04	0.05	0.06	0.08	0.14	0.14	0.03	0.05	nd	1.62	1.01	nd
	R 101	-1.00	-1.00	0.93	0.04	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	**1.33**	-1.00	nd	-1.00	-1.00	nd

1. A value of -1 indicates no binding
2. Bolded values indicate the highest affinity o affinity (as measured by how many "fold" differences in affinity. The mutant is in comparison to original, *e.g.*, 2.0 as twice as strong and 0.5 as half as strong) obtained for an amino acid change at the position
3. nd indicates that binding affinity was not determined

Table 9: *Biacore Analysis of Modified Heavy Chain Variable Regions*[1, 2, 3]

		NP											NP							NP
						Aromatic														
		Neg		Pos		Polar							Aliphatic			Small				
		D	E	R	K	H	Y	W	F	Q	N	S	T	V	I	L	A	C	G	P	M
CDR1	**T 28**	0.98	1.23	1.23	**1.77**	1.15	1.86	1.08	1.28	-1.00	0.69	1.07	nd	2.08	**2.45**	nd	1.56	nd	0.92	**2.16**	nd
	T 30	0.63	0.73	**1.39**	0.94	nd	**2.00**	1.26	nd	nd	0.73	0.91	0.93	1.30	nd	1.26	0.89	nd	-1.00	0.93	nd
	K 31	0.66	0.54	0.76	0.96	0.78	1.00	1.02	nd	nd	-1.00	**0.49**	nd	**1.41**	1.17	0.60	0.39	nd	1.02	nd	nd
	Y 32	nd	0.08	0.43	0.08	0.60	0.84	nd	**1.05**	0.10	nd	**0.01**	nd	0.11	0.03	0.05	0.01	nd	0.02	0.01	nd
	G 33	-1.00	-1.00	0.03	-1.00	0.02	6.16	-1.00	**7.19**	0.06	-1.00	nd	**0.01**	0.04	0.55	2.27	0.06	nd	nd	6.31	nd
CDR2	**W 50**	**3.27**	-1	0.10	0.04	0.02	0.04	0.97	0.09	0.01	0.03	0.02	0.03	0.02	0.07	0.04	0.03	nd	0.03	-1	nd
	N 52	0.02	-1	-1	-1	0.02	-1	-1	-1	-1	**0.98**	-1	-1	-1	-1	-1	-1	nd	-1	-1	nd
	T 53	-1	-1	-1	1.79	-1	-1	-1	-1	-1	-1	0.17	1.19	2.44	**11.40**	nd	9.03	nd	-1	-1	nd
	Y 54	0.05	0.07	**3.72**	3.62	1.00	0.92	0.96	0.65	0.66	2.11	0.49	0.36	1.32	0.28	1.80	0.47	nd	**3.72**	0.86	nd
	T 55	0.03	-1	0.14	0.45	0.05	0.03	0.10	0.03	0.03	0.17	0.42	nd	0.28	nd	**0.95**	nd	nd	0.02	nd	nd
	E 56	0.81	0.95	1.34	1.27	**1.74**	1.04	1.17	0.78	1.23	1.01	1.46	1.21	0.86	0.85	0.64	1.67	nd	1.37	0.01	nd
	E 57	1.17	1.07	**1.71**	nd	1.16	1.37	1.39	1.06	-1.00	1.57	1.41	1.44	nd	1.34	-1	1.65	nd	1.45	-1.00	nd
	P 58	0.54	0.44	nd	1.14	nd	0.99	1.11	0.98	1.11	0.90	1.07	1.00	1.01	nd	0.64	1.03	nd	**1.31**	1.06	nd
	T 59	0.87	0.51	1.22	1.43	0.40	nd	**2.24**	0.43	-1.00	0.96	nd	0.99	1.03	0.76	0.95	nd	nd	0.35	-1.00	nd
CDR3	**G 100**	-1	-1	**7.51**	1.59	-1	-1	-1	-1	-1.00	1.55	1.68	0.61	2.17	nd	0.65	1.99	nd	nd	-1	nd
	S 101	0.21	0.76	nd	2.20	1.35	1.79	1.22	1.16	2.18	0.97	nd	0.84	1.92	**3.53**	-1	1.15	nd	3.31	nd	nd
	A 102	0.28	0.51	2.18	1.48	2.40	3.01	3.13	2.97	1.01	0.94	0.94	0.68	1.20	0.79	-1	nd	nd	**3.58**	0.87	nd
	D 104	nd	0.14	-1	-1	-1	-1	-1	-1	-1	0.73	**1.87**	-1	-1	nd	-1	-1	nd	0.41	-1	nd
	Y 105	-1	-1	0.66	-1	**0.94**	nd	0.84	0.91	0.87	nd	0.09	0.12	0.18	0.23	0.22	0.08	nd	-1.00	-1	nd

1. A value of -1 indicates no binding
2. Bolded values indicate the highest affinity o affinity (as measured by how many "fold" differences in affinity. The mutant is in comparison to original, *e.g.*, 2.0 as twice as strong and 0.5 as half as strong) obtained for an amino acid change at the position
3. nd indicates that binding affinity was not determined

Table 10: *Biacore Analysis of Modified C5A (XPA23) Light Chain Variable Regions*[1, 2, 3]

		NP							NP											NP
					Aromatic															
		Neg		Pos		Polar						Aliphatic				Small				
		D	E	R	K	H	Y	W	F	Q	N	S	T	V	I	L	A	C	G	P	M
CDR1	**Q27**	1.07	1.08	0.89	-1.00	1.09	nd	-1.00	2.96	1.06	-1.00	**3.30**	0.64	-1.00	0.82	0.83	1.47	nd	-1.00	-1.00	nd
	D28	1.00	0.74	0.94	0.82	1.23	1.45	3.81	1.43	nd	1.00	5.25	1.19	0.85	-1.00	**9.64**	0.88	nd	1.21	1.17	nd
	N30	0.81	0.64	0.74	0.61	1.00	1.40	1.06	**1.59**	0.60	1.08	0.72	0.97	0.89	0.65	0.68	0.61	nd	0.71	0.69	nd
	R31	**11.11**	-1.00	1.06	1.18	-1.00	-1.00	0.43	0.92	0.27	0.38	0.44	0.24	0.44	0.68	0.51	8.48	nd	0.92	-1.00	nd
	W32	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	**0.99**	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	nd	-1.00	-1.00	nd
CDR2	**H49**	0.11	-1.00	0.21	0.10	**1.10**	0.52	0.21	0.50	-1.00	0.31	0.14	-1.00	-1.00	-1.00	0.07	0.23	nd	-1.00	0.86	nd
	S50	-1.00	0.02	-1.00	0.10	0.05	0.05	0.02	-1.00	0.21	0.25	**1.04**	0.87	0.21	0.25	0.49	0.13	nd	0.14	-1.00	nd
	A51	0.13	0.29	0.18	nd	0.61	0.45	0.24	-1.00	0.24	0.30	0.95	0.76	0.31	-1.00	0.16	1.09	nd	**3.84**	0.13	nd
	T52	0.72	0.61	**3.37**	3.23	0.91	1.01	0.87	1.05	0.83	0.83	0.78	1.02	0.82	0.80	0.78	0.66	nd	-1.00	1.08	nd
	S53	-1.00	1.13	3.29	4.07	nd	1.23	1.11	1.24	1.09	1.23	1.08	0.95	1.42	1.42	0.99	1.07	nd	1.03	**4.92**	nd
CDR3	**A91**	1.08	0.10	-1.00	nd	**1.12**	-1.00	-1.00	-1.00	-1.00	0.10	0.90	0.67	0.51	-1.00	0.39	-1.00	nd	-1.00	-1.00	nd
	D92	0.83	0.99	-1.00	0.23	0.63	-1.00	0.12	0.26	0.56	0.67	**6.59**	0.34	-1.00	0.33	0.14	0.40	nd	0.26	-1.00	nd
	S93	4.59	3.71	0.91	0.86	1.08	1.45	**5.49**	1.32	1.54	3.76	1.20	1.47	3.81	1.35	1.08	1.16	nd	0.75	-1.00	nd
	F94	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	**1.14**	-1.00	-1.00	-1.00	-1.00	0.20	0.88	0.55	-1.00	nd	-1.00	0.17	nd
	P95	1.32	0.88	-1.00	-1.00	-1.00	-1.00	-1.00	0.98	-1.00	-1.00	**4.05**	-1.00	-1.00	-1.00	-1.00	3.83	nd	-1.00	-1.00	nd
	L96	-1.00	-1.00	-1.00	-1.00	0.07	-1.00	**4.49**	0.28	0.21	0.06	0.23	0.06	nd	0.67	1.16	0.26	nd	-1.00	-1.00	nd

1. A value of -1 indicates no binding
2. Bolded values indicate the highest affinity (as measured by how many "fold" differences in affinity. The mutant is in comparison to original, *e.g.*, 2.0 as twice as strong and 0.5 as half as strong) obtained for an amino acid change at the position
3. nd indicates that binding affinity was not determined

Table 11: *Biacore Analysis of Modified C5A (XPA23) Heavy Chain Variable Regions*[1, 2, 3]

		NP							NP										NP
					Aromatic														
		Neg		Pos		Polar							Aliphatic				Small		
		D	E	R	K	H	Y	W	F	Q	N	S	T	V	I	L	A	C	G	P	M
CDR1	**T28**	nd	-1.00	-1.00	-1.00	-1.00	nd	-1.00	nd	-1.00	nd	nd	-1.00	nd	nd	nd	nd	nd	-1.00	nd	nd
	S30	-1.00	-1.00	0.10	-1.00	-1.00	-1.00	0.77	-1.00	-1.00	0.15	0.85	0.06	-1.00	0.08	**0.91**	0.77	nd	0.13	-1.00	nd
	K31	0.04	-1.00	0.90	**1.30**	1.11	0.04	0.84	0.05	-1.00	-1.00	-1.00	-1.00	0.81	0.74	1.08	nd	nd	nd	-1.00	nd
	Y32	0.68	0.12	nd	nd	0.62	nd	nd	0.75	-1.00	0.04	**1.33**	0.80	1.07	nd	0.75	-1.00	nd	1.00	0.68	nd
	F33	0.92	0.86	-1.00	0.85	-1.00	0.77	0.79	-1.00	0.78	**0.97**	0.73	0.78	0.76	0.75	0.88	0.75	nd	0.76	0.91	nd
	F35	0.06	-1.00	-1.00	0.68	-1.00	0.85	-1.00	-1.00	-1.00	0.86	0.09	0.74	**0.89**	0.85	0.78	0.07	nd	0.04	0.06	nd
CDR2	**V50**	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	0.03	-1.00	0.03	**0.19**	0.10	0.09	0.09	nd	0.03	-1.00	nd
	I51	0.07	0.73	0.10	0.11	0.08	**1.75**	-1.00	0.90	0.83	0.68	0.69	0.86	0.99	0.95	0.79	1.04	nd	0.94	-1.00	nd
	S52	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	nd	0.04	0.04	-1.00	-1.00	**0.05**	nd	0.03	0.05	nd
	P53	-1.00	0.03	nd	0.04	-1.00	0.03	0.05	0.05	0.03	0.06	0.03	nd	**1.10**	0.08	0.04	0.05	nd	0.02	0.94	nd
	S54	0.12	0.03	1.43	**1.53**	nd	0.99	1.07	0.87	1.01	0.08	nd	0.14	1.00	0.86	0.91	1.00	nd	1.43	0.06	nd
	G55	-1.00	0.11	0.95	0.11	nd	0.08	nd	nd	0.90	0.10	**1.02**	nd	nd	0.86	0.08	nd	nd	0.99	nd	nd
	G56	0.05	1.21	**2.11**	2.05	0.82	1.27	1.51	1.41	1.31	1.06	nd	1.11	nd	1.71	1.41	1.21	nd	nd	0.85	nd
	M57	-1.00	-1.00	0.06	0.04	0.08	0.86	nd	**1.03**	0.02	0.03	0.04	0.06	0.03	0.03	0.06	0.03	nd	nd	-1.00	nd
	T58	0.12	-1.00	1.14	-1.00	0.98	1.01	0.93	1.04	-1.00	0.90	0.82	1.04	0.99	0.95	0.95	0.14	nd	0.95	**1.81**	nd
	R59	-1.00	-1.00	-1.00	0.94	-1.00	-1.00	0.09	0.92	0.86	0.86	0.13	0.76	-1.00	-1.00	0.08	-1.00	nd	**1.04**	0.07	nd
CDR3	**V99**	nd	-1.00	nd	-1.00	nd	nd	nd	0.03	-1.00	-1.00	-1.00	**0.10**	nd	nd	-1.00	0.03	nd	nd	-1.00	nd
	G100	-1.00	nd	-1.00	-1.00	0.06	0.09	0.05	-1.00	-1.00	nd	**2.80**	0.12	nd	nd	-1.00	nd	nd	1.02	-1.00	nd
	Y101	-1.00	0.03	0.85	nd	0.04	1.00	0.81	0.85	0.06	0.06	nd	0.06	0.09	0.83	**1.91**	0.04	nd	-1.00	0.04	nd
	G102	nd	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	nd	-1.00	**1.17**	nd	nd	-1.00	nd	-1.00	-1.00	nd	nd	-1.00	nd
	G103	-1.00	-1.00	-1.00	-1.00	nd	nd	-1.00	-1.00	-1.00	nd	nd	nd	**2.56**	nd	nd	0.03	nd	0.93	nd	nd
	N104	-1.00	-1.00	-1.00	-1.00	0.07	-1.00	-1.00	-1.00	0.03	1.02	1.02	0.95	1.27	nd	1.19	**1.61**	nd	1.21	nd	nd
	S105	nd	1.17	-1.00	nd	0.05	-1.00	-1.00	1.25	0.98	0.85	1.09	0.11	0.08	0.07	-1.00	0.84	nd	0.12	**1.41**	nd
	D106	**0.98**	0.04	0.06	0.04	0.02	-1.00	-1.00	-1.00	0.02	0.07	0.03	-1.00	-1.00	-1.00	-1.00	-1.00	nd	0.03	-1.00	nd
	Y107	0.85	0.90	0.85	0.82	-1.00	-1.00	0.89	0.90	0.87	-1.00	0.89	0.83	0.93	**0.96**	0.86	0.83	nd	0.10	0.04	nd

1. A value of -1 indicates no binding
2. Bolded values indicate the highest affinity o affinity (as measured by how many "fold" differences in affinity. The mutant is in comparison to original, *e.g.*, 2.0 as twice as strong and 0.5 as half as strong) obtained for an amino acid change at the position
3. nd indicates that binding affinity was not determined

Example 12: Construction of Arrays of Modified Proteins

[00186] Arrays of modified proteins including, for example, antibody variable domains (*e.g.*, modified ING-1 variable domains) with amino acids changes at desired positions (*e.g.*, contacting (C) residues) may be generated and tested for enhanced binding affinity compared to the parent protein (*e.g.*, ING-1). Modified variable domains used in the array may be obtained directly from a library of modified variable domains as described in Example 2 or may first be screened for those modified variable domains that exhibit enhanced binding as compared to the parent variable domain as described in Examples 3, 4 and 5.

[00187] In an exemplary method, each contacting (C) residue in the heavy and light chain variable region of ING-1 is separately changed (*e.g.*, by PCR mutagenesis) with alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine to generate modified ING-1 variable domains. cDNAs encoding the modified ING-1 variable domains are then inserted into a pXOMA vector and used to transform electrocompetent TG1 cells. The clones are plated on 2YT- Amp_{100}/2%Glucose plates (Teknova) and the plates filled with 250 µl of 2YT-Amp_{100}/well (Teknova). Each well is inoculated with a single colony comprising a single amino acid change at a contacting (C) residue. The colonies are grown by incubating the plates at 37ºC for two to four hours with shaking at 450 rpm. After the incubation, the plates are duplicated to expression plates by filling new plates (Costar) with two hundred and fifty microliters of 2YT-Amp100 media (Teknova). The Genetix 96-pin replicator is used to transfer cells from the Master plate to the new expression plates The culture is grown at 37°C until cloudy (*e.g.*, approximate OD_{600}=0.5), inoculated with IPTG to a final concentration of 1 mM and grown overnight at 30°C.

[00188] Next, periplasmic extracts (PPE) of the overnight expression constructs are prepared by spinning the overnight expression plates at 3000 rpm for fifteen minutes, discarding the supernatant and adding 60 µl of PPB buffer to each well. The pellets are resuspended, and 90 µl of cold PPB diluted 1:5 with cold water is added to each well. This mixture is incubated on ice for one hour and subsequently spun down at 3000 rpm for fifteen minutes. The supernatant is transferred to a new plate and the periplasmic extracts are used for the Biacore (*e.g.*, Biacore A100) determination.

[00189] After Biacore determination, wells that contain clones with improved off rates are sequenced and further characterized (*e.g.* IgG reformatting and affinity determination).

Example 13: Affinity Optimization of Protein by Targeted Mutagenesis of Selected Amino Acid Residues.

[00190] Affinity optimized proteins including, for example, antibodies or fragments thereof may be obtained by mutation of one or more selected amino acid residues in a parent protein with other amino acid residues (*e.g.*, alanine, arginine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine or valine). Methods for optimization of an exemplary antibody variable domain may comprise the stages as set forth below.

A. Selection of Amino Acid Residues for Mutation

[00191] Amino acid residues at one or more positions in a parent antibody or binding fragment thereof are selected for mutagenesis. Such methods may include, for example, identifying the proximity assigned to amino acid positions in the variable domain of the antibody using the "prox" line as shown in Figure 3A, 3B, 3C and/or 3D. One or more amino acid resdies identified as C, P, S and/or I residues may be selected for mutation.

B. Design of Primers for Mutagenesis

[00192] Primers are designed to mutagenize a parent nucleic acid sequence that codes for an antibody or binding fragment thereof.

[00193] For a PCR-based mutagenesis method, a primer may be designed such that the forward primer sequence flanks both sides (*e.g.*, 20 base pairs) of the position to be mutated. Additionally, it is preferred that the primer be 70 bases or les in length. A representative CDR comprising amino acid residues 1-8 is shown below.

```
        aa#              1   2  3  4   5  6  7   8
                         G   F  T  F   S  K  Y   F
 5′-G TCTTTCTTGC GCTGCTTCCG GATTCACTTT CTCTAAGTAC TTTATGTTTT GGGTTCGCCAAGC-
3′ (SEQ ID NO: 964)
 3′-C AGAAAGAACG CGACGAAGGC CTAAGTGAAA GAGATTCATG AAATACAAAA CCCAAGCGGTTCG-
5′ (SEQ ID NO: 965)
```

[00194] If the CDR is too long to incorporate all the desired mutations and remain under 70 nucleotides, the mutagenesis region may be broken up into two regions. An example of this process is shown below, where the 8 amino acid CDR as shown above is broken into two 4 amino acid regions (region 1 and region 2, respectively).

Region 1:

```
        aa#              1   2  3  4
                         G   F  T  F
 5'-G TCTTTCTTGC GCTGCTTCCG GATTCACTTT CTCTAAGTAC TTTATGTTTT GGGTTC-3' (SEQ
ID NO: 966)
 3'-C AGAAAGAACG CGACGAAGGC CTAAGTGAAA GAGATTCATG AAATACAAAA CCCAAG-5'
(SEQ ID NO: 967)
```

Region 2:

```
        aa#               5  6  7   8
                          S  K  Y   F
 5'- GCTGCTTCCG GATTCACTTT CTCTAAGTAC TTTATGTTTT GGGTTCGCCAAGC-3'
(SEQ ID NO: 968)
 3'- CGACGAAGGC CTAAGTGAAA GAGATTCATG AAATACAAAA CCCAAGCGGTTCG-5'
(SEQ ID NO: 969)
```

[00195] Sets of primers may be constructed to incorporate all 18 amino acid mutations at each positon in region 2. Each codon selected for mutation may be replaced with NHT, VAA or BGG in the sense direction. Exemplary primer sets for mutation of each of positions 5-8 are shown below.

[00196] Mutation of the S position (aa5) in region 2 above may be accomplished by the following primers:R2-5-NHT 5'- GCTGCTTCCGGATTCACTTT-CNHTAAGTACTTTATGTTTTGGGTTCGCCAAGC-3'(SEQ ID NO: 970); R2-5-VAA 5'-GCTGCTTCCGGATTCACTTTCVAAAAGTACTTTATGTTTTGGGTTCGCCAAGC-3' (SEQ ID NO: 971); and R2-5-BGG 5'- GCTGCTTCCGGATTCACTTTCBGGAAGTAC-TTTATGT-TTTGGGTTCGCCAAGC-3' (SEQ ID NO: 972).

[00197] Mutation of the K position (aa6) in region 2 above may be accomplished by the following primers: R2-6-NHT 5'- GCTGCTTCCGGATTCACTTT CTCTNHTTACTTTATGTTTTGGGTTCGCCAAGC-3' (SEQ ID NO: 973); R2-6-VAA 5'-GCTGCTTCCGGATTCACTTTCTCTVAATACTTTATGTTTTGGGTTCGCCAAGC-3' (SEQ ID NO: 974); and R2-6-BGG 5'- GCTGCTTCCGGATTCACTTTCTCTBGGTAC TTTATGTTTTGGGTTCGCCAAGC-3' (SEQ ID NO: 975).

[00198] Mutation of the Y position (aa7) in region 2 above may be accomplished by the following primers: R2-7-NHT 5'- GCTGCTTCCGGATTCACTTT CTCTAAGNHTTTTATGTTTTGGGTTCGCCAAGC-3' (SEQ ID NO: 976); R2-7-VAA 5'-GCTGCTTCCGGATTCACTTTCTCTAAGVAATTTATGTTTTGGGTTCGCCAAGC-3' (SEQ ID NO: 977); and R2-7-BGG 5'- GCTGCTTCCGGATTCACTTTCTCTAAGBGG TTTATGTTTTGGGTTCGCCAAGC-3' (SEQ ID NO: 978).

[00199] Mutation of the F position (aa8) in region 2 above may be accomplished by the following primers: R2-8-NHT 5'- GCTGCTTCCGGATTCACTTT CTCTAAGTACNHTATGTTTTGGGTTCGCCAAGC-3' (SEQ ID NO: 979); R2-8-VAA 5'-GCTGCTTCCGGATTCACTTTCTCTAAGTACVAAATGTTTTGGGTTCGCCAAGC-3' (SEQ

ID NO: 980); and R2-8-BGG 5'- GCTGCTTCCGGATTCACTTTCTCTAAGTAC BGGATGTTTTGGGTTCGCCAAGC-3' (SEQ ID NO: 981).

[00200] Alternatively, modified antibody variable domains containing amino acid changes at one or more contacting (C) residues present within an exemplary antibody may be synthesized by QUIKCHANGE™ site-directed mutagenesis (STRATAGENE, Texas).

[00201] In an exemplary method, QUIKCHANGE™ site-directed mutagenesis may be performed to replace one or more codons in an antibody variable region (*e.g.*, a CDR) such as XPA-23. Mutagenic primers are designed to contain the desired mutation and anneal to the same sequence on opposite strands of a plasmid comprising a nucleotide coding for XPA-23. Preferably, the desired mutation in the middle of the primer contains 20 bases of correct sequence on both sides of the nucleic acid flanking the mutation. The XPA-23 CDR1 coding region is shown below.

```
        aa#              1   2  3  4   5  6  7   8
                         G   F  T  F   S  K  Y   F
 5'-G TCTTTCTTGC GCTGCTTCCG GATTCACTTT CTCTAAGTAC TTTATGTTTT GGGTTCGCCAAGC-
3' (SEQ ID NO: 851)
 3'-C AGAAAGAACG CGACGAAGGC CTAAGTGAAA GAGATTCATG AAATACAAAA CCCAAGCGGTTCG-
5' (SEQ ID NO: 852)
```

[00202] Primers for QUIKCHANGE™ site-directed mutagenesis are synthesized such that they are complementary to a parent nucleic acid sequence with the exception that they comprise a NHT, a VAA, or a BGG codon in the sense direction, and a ADN, a TTB, or a CCV codon in the antisense direction at the position to be mutagenized in the parent nucleic acid. Exemplary primers for mutagenesis of each of the eight amino acid residues in the XPA-23 heavy chain CDR1 are shown below and comprise a degenerate codon (underlined nucleotide triplet):

[00203] Mutation of the G position (aa1) may be accomplished by the following primers: 5'-GTCTTTCTTGCGCTGCTTCCNHTTTCACTTTCTCTAAGTACTTTATG-3' (SEQ ID NO: 853) and 3'-CAGAAAGAACGCGACGAAGGNDAAAGTGAAAG-AGATTCATGAAATAC-5' (SEQ ID NO: 854); 5'-GTCTTTCTTGCGCTGCTTCCVAAT-TCACTTTCTCTAAGTACTTTATG-3' (SEQ ID NO: 855) and 3'-CAGAAAGAACGC-GACGAAGGBTTAAGTGAAAGAGATTCATGAAATAC-5' (SEQ ID NO: 856); and 5'-GTC-TTTCTTGCGCTGCTTCCBGGTTCACTTTCTCTAAGTACTTTATG-3' (SEQ ID NO: 857) and 3'-CAGAAAGAACGCGACGAAGGVCCAAGTGAAAGAGATTCATGAA-ATAC-5' (SEQ ID NO: 858).

[00204] Mutation of the F position (aa2) may be accomplished by the following primers: 5'-CTTTCTTGCGCTGCTTCCGGANHTACTTTCTCTAAGTACTTTATG-3' (SEQ ID

NO: 859) and 3'-GAAAGAACGCGACGAAGGCCTNDATGAAAGAGATTCAT-GAAATAC-5' (SEQ ID NO: 860); 5'-CTTTCTTGCGCTGCTTCCGGAVAAACTTTC-TCTAAGTACTTTATG-3' (SEQ ID NO: 861) and 3'-GAAAGAACGCGACGAAGGC-CTBTTTGAAAGAGATTCATGAAATAC-5' (SEQ ID NO: 862); and 5'-CTTTCTTGC-GCTGCTTCCGGABGGACTTTCTCTAAGTACTTTATG-3' (SEQ ID NO: 863) and 3'-GAAAGAACGCGACGAAGGCCTVCCTGAAAGAGATTCATGAAATAC-5' (SEQ ID NO: 864).

[00205] Mutation of the T (aa3) position may be accomplished by the following primers: 5'-CTTGCGCTGCTTCCGGATTCNHTTTCTCTAAGTACTTTATGTTTTG-3' (SEQ ID NO: 865) and 3'-GAACGCGACGAAGGCCTAAGNDAAAGAGATTCATGA-AATACAAAAC-5' (SEQ ID NO: 866); 5'-CTTGCGCTGCTTCCGGATTCVAATT-CTCTAAGTACTTTATGTTTTG-3' (SEQ ID NO: 867) and 3'-GAACGCGACGAAGGC-CTAAGBTTAAGAGATTCATGAAATACAAAAC-5' (SEQ ID NO: 868); and 5'-CTTGCGCTGCTTCCGGATTCBGGTTCTCTAAGTACTTTATGTTTTG-3' (SEQ ID NO: 869) and 3'-GAACGCGACGAAGGCCTAAGVCCAAGAGATTCATGAAATACAAAAC-5' (SEQ ID NO: 870).

[00206] Mutation of the F (aa4) position may be accomplished by the following primers: 5'-CGCTGCTTCCGGATTCACTNHTTCTAAGTACTTTATGTTTTGGG-3' (SEQ ID NO: 871) and 3'-G CGACGAAGGCCTAAGTGANDAAGATTCATGAAA-TACAAAACCC-5' (SEQ ID NO: 872); 5'-CGCTGCTTCCGGATTCACTVAATCTAA-GTACTTTATGTTTTGGG-3' (SEQ ID NO: 873) and 3'-GCGACGAAGGCCTAAGTGA-BTTAGATTCATGAAATACAAAACCC-5' (SEQ ID NO: 874); and 5'-CGCTGCTTCC-GGATTCACTBGGTCTAAGTACTTTATGTTTTGGG-3' (SEQ ID NO: 875) and 3'-G CGACGAAGGCCTAAGTGAVCCAGATTCATGAAATACAAAACCC-5' (SEQ ID NO: 876).

[00207] Mutation of the S (aa5) position may be accomplished by the following primers: 5'-CTGCTTCCGGATTCACTTTCNHTAAGTACTTTATGTTTTGGGTTCG-3' (SEQ ID NO: 877) and 3'-GACGAAGGCCTAAGTGAAAGNDATTCATGAAAT-ACAAAACCCAAGC-5' (SEQ ID NO: 878); 5'-CTGCTTCCGGATTCACTTTCVAAAA-GTACTTTATGTTTTGGGTTCG-3'(SEQ ID NO: 879) and 3'-GACGAAGGCCT-AAGTGAAAGBTTTTCATGAAATACAAAACCCAAGC-5'(SEQ ID NO: 880); and 5'-CTGCTTCCGGATTCACTTTCBGGAAGTACTTTATGTTTTGGGTTCG-3'(SEQ ID NO: 881) and 3'-GACGAAGGCCTAAGTGAAAGVCCTTCATGAAATACAAAACCCAAGC-5'(SEQ ID NO: 882).

[00208] Mutation of the K (aa6) position may be accomplished by the following primers: 5'-CTTCCGGATTCACTTTCTCTNHTTACTTTATGTTTTGGGTTCGCC-3'(SEQ ID NO: 883) and 3'-GAAGGCCTAAGTGAAAGAGANDAATGAAATACAAAAC-CCAAGCGG-

5'(SEQ ID NO: 884); 5'-CTTCCGGATTCACTTTCTCTVAATACTTT-ATGTTTTGGGTTCGCC-3'(SEQ ID NO: 885) and 3'-GAAGGCCTAAGTGAAAGAG-ABTTATGAAATACAAAACCCAAGCGG-5'(SEQ ID NO: 886); and 5'-CTTCCGGA-TTCACTTTCTCTBGGTACTTTATGTTTTGGGTTCGCC-3'(SEQ ID NO: 887) and 3'-GAAGGCCTAAGTGAAAGAGAVCCATGAAATACAAAACCCAAGCGG-5'(SEQ ID NO: 888).

[00209] Mutation of the Y (aa7) position may be accomplished by the following primers: 5'-CCGGATTCACTTTCTCTAAGNHTTTTATGTTTTGGGTTCGCCAAG-3'(SEQ ID NO: 889) and 3'-GGCCTAAGTGAAAGAGATTCNDAAAATACAAAA-CCCAAGCGGTTC-5'(SEQ ID NO: 890); 5'-CCGGATTCACTTTCTCTAAGVAATT-TATGTTTTGGGTTCGCCAAG-3'(SEQ ID NO: 891) and 3'-GGCCTAAGTGAAAGA-GATTCBTTAAATACAAAACCCAAGCGGTTC-5'(SEQ ID NO: 892); and 5'-CCGGATTCACTTTCTCTAAGBGGTTTATGTTTTGGGTTCGCCAAG-3'(SEQ ID NO: 893) and 3'-GGCCTAAGTGAAAGAGATTCVCCAAATACAAAACCCAAGCGGTTC-5'(SEQ ID NO: 894).

[00210] Mutation of the F (aa8) position may be accomplished by the following primers: 5'-GGATTCACTTTCTCTAAGTACNHTATGTTTTGGGTTCGCCAAGC-3' (SEQ ID NO: 895) and 3'-CCTAAGTGAAAGAGATTCATGNDATACAAAACCCAA-GCGGTTCG-5' (SEQ ID NO: 896); 5'-GGATTCACTTTCTCTAAGTACVAAATGTTTT-GGGTTCGCCAAGC-3' (SEQ ID NO: 897) and 3'-CCTAAGTGAAAGAGATTCAT-GBTTTACAAAACCCAAGCGGTTCG-5' (SEQ ID NO: 898); and 5'-GGATTCACTTT-CTCTAAGTACBGGATGTTTTGGGTTCGCCAAGC-3' (SEQ ID NO: 899) and 3'-CCTAAGTGAAAGAGATTCATGVCCTACAAAACCCAAGCGGTTCG-5' (SEQ ID NO: 900).

C. Synthesis of Full-Length Mutagenized Antibody

[00211] Full-length mutagenized antibodies may be produced by recombinant DNA technologies.

[00212] For the PCR-based method, a first PCR reaction (PCR1) is performed with a R2-rev primer and a 5'-SfiI primer, which incorporates a 5' SfiI restriction site into the amplified fragment. For each library oligonucleotide containing the mutations described above, the PCR2 reaction is performed to create the DNA fragment incorporating the primer mutation and the 3′ SfiI restriction site. For the mutations in region 2, twelve PCR2 reactions will be performed with forward primers denoted R2-5 through R2-8 above (denoted primer-F in PCR2 below). The reverse primer for the mutagenic reaction will be 3'-SfiI. An appropriate amount of the following reagents may be used for PCR1: PfuUltra buffer; dNTPs [10□M], template (10 ng total), 5'-SfiI [25 pmol], R2-rev [25 pmol], PfuUltra (2.5 U/□L), dH2O to 50 □L total. An appropriate amount of the following reagents may be used for PCR2:

PfuUltra buffer, dNTPs [10□M], template (10 ng total), Primer-F [10 pmol], 3'-SfiI [25 pmol], PfuUltra (2.5 U/□L), dH2O to 50 □L total. PCR1 and PCR2 may be conduced according to standard protocols includinig an initial denatural step, a number of cycles including a denaturation, annealing and extension step and a final extension step for appropriate times and temperatures.

[00213] A full-length antibody fragment may be produced by performing a separate reaction for each PCR2 product. For this step, an approximately equimolar amount of PCR product 1 and 2 is combined (*e.g.*, 0.5 microliters of each PCR is combined). An appropriate amount of the following reagents may be used generation of a full-length antibody fragment: PfuUltra buffer, dNTPs [10□M], PCR1 product, PCR2 product, PfuUltra (2.5 U/□L), dH2O to 50 □L total. PCR may be conduced according to standard protocols includinig an initial denatural step, a number of cycles including a denaturation, annealing and extension step for appropriate times and temperatures.

[00214] The full-length fragment may then be amplified by directly adding to the above reaction an appropriate amount of the following reagents: PfuUltra buffer, dNTPs [10μM], 5'-SfiI [25 pmol], 3'-SfiI [25 pmol], PfuUltra (2.5 U/μL), dH2O to 50 μL total. PCR may be conduced according to standard protocols including an initial denaturation step, a number of cycles that comprise a denaturation, annealing and extension step for approapte times and temperatures and a final extension step. The PCR product may be examined on an agarose gel to ensure that the amplified DNA segment is the correct length.

[00215] Next, a vector and the DNA inserts obtained from the above PCR are digested with SfiI (NEB) according to the manufacturer's instructions and gel purified. The DNA synthesized fragment may be cloned into a pXOMA Fab or pXOMA Fab-gIII vector. Briefly, the DNA fragment is purified by using the QIAGEN® PCR purification kit and sequentially digesting the fragment with *Not*I (NEW ENGLAND BIOLABS®, Ipswich, MA) and *Asc*I (NEW ENGLAND BIOLABS®, Ipswich, MA) (*See*, Methods in Molecular Biology, vol. 178: *Antibody Phage Display: Methods and Protocols* Edited by: P.M. O'Brien and R. Aitken, Humana Press, "Standard Protocols for the Construction of Fab Libraries, Clark, M. A., 39-58) (see*, e.g.*, Figure 6). Next, the vectors may be ligated with the mutagenized insert using T4 Ligase (NEW ENGLAND BIOLABS®, Ipswich, MA) and transformed into TG1 cells by electroporation.

[00216] Alternatively, for the DPN-based method, a double-stranded DNA (*e.g.*, dsDNA) vector with an antibody insert isolated from a dam+ host is used as template for mutagenesis. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to DpnI digestion. Two synthetic oligonucleotide primers containing the desired mutation each complementary to opposite strands of the vector, are extended during

temperature cycling by DNA polymerase (*e.g.*, PfuTurbo). PCR reactions may comprise an appropriate amout of PfuUltra buffer, dNTPs [10 mM] each dNTP, template (50 ng total), Primer-F [5μM], Primer-R [5μM], PfuUltra (2.5 U/μL), DMSO, and dH2O up to 50 μL total and be conducted with the following cycling parameters: an initial denaturation, subsequent cycles of denaturation, annealing and extension and a final extension step. Incorporation of the mutagenesis primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the PCR product is treated with DpnI and incubated at an appropriate temperature (e.g, at 37ºC for 4-5 hours). The DpnI endonuclease (target sequence: 5´-Gm6ATC-3´) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. The nicked vector DNA containing the desired mutations is then transformed into supercompetent cells (*e.g.*, XL1-Blue).

D. Sequencing of Mutagenized Antibodies

[00217] A library of mutagenized antibodies may comprise each of 18 unique amino acid mutations at each postion mutated. To identify all possible unique mutations an appropriate number of clones obtained from each degenerate codon are analyzed. For example, the NHT codon encodes 12 amino acids such that 72 clones from this reaction are sequenced for each mutated position. The VAA codon encodes 3 amino acids such that 12 clones are sequenced from this reaction for each mutated position. The BGG codon encodes 3 amino acids such that 12 clones from this reaction are sequenced for each mutated position. Unique clones are rearrayed into 96-well plates.

E. Expression of Mutagenized Antibodies

[00218] Mutagenized antibodies may be expressed. In an exemplary method, starting cultures may be produced by filling a plate (*e.g.*, a 96 well plate) with an appropriate growth media (*e.g.*, 2YTAG (2YT+ 2% glucose +100 □gs/ml Ampicillin) and inoculating the plate with glycerol stocks of the mutagenized antibodies. The cultures are then grown overnight (*e.g.*, in an ATR plate shaker incubator at 37°C with shaking at 450 rpm). Next, plates are filled with an appropriate growth medium (*e.g.*, 1.2 mL per well of Superbroth + 100 □gs.ml Ampicillin +0.2% glucose). The plates are then Innoculated with an appropriate amout of the overnight culture (*e.g*, 25□L of overnight culture). The cultures are then grown with incubation (*e.g.*, ATR plate shaker incubator at 37°C) and shaking (*e.g.*, at 700 rpm until Abs600nm = 1.5). Expression in the cultures is then induced (*e.g.*, by adding 12 uL of 100mM IPTG per well to get a final concentration of 1mM IPTG final) and incubated overnight (*e.g.*, in an ATR plate shaker incubator at 30°C with shaking at 700 rpm). Next,

the plates are spun (*e.g.*, at 4000 rpm using Beckman Coulter table top centrifuge for 10 minutes) and the supernatant decanted. The cells are then vortexed to disturb and loosen the pellet. The pellets are resuspended (*e.g.*, with 75□L per well of cold PPB) and incubated one ice (*e.g.*, for 10 mintues). Next, water (*e.g.*, 225□L per well) is added and the cells resuspended. The suspension is incubated on ice (*e.g.*, for 1 hour) and the plates are then spun (*e.g.*, at 4000 rpm using Beckman Coulter table top centrifuge for 20 minutes). Last, the supernatants are collected for use in assays as described in detail below.

F. ELISA Screening of Mutagenized Antibodies

[00219] An assay including, for example, an ELISA may be performed to ensure that the mutagenized antibodies are capable of binding to their respective antigen.

[00220] In an exemplary ELISA, plates (*e.g.*, 96-well Nunc Maxisorp plates) are coated with an antibody to the mutagenized antibody (*e.g.*, 50□L per well of 1□g/ml Goat anti Human IgG $(Fab)_2$ Jackson immunoresearch, Cat. 109-005-006) and the plates are then incubated overnight at 4°C. After incubation, the plates may be washed (*e.g.*, 3X with PBS-Tween at 350□L/well) and then blocked (*e.g.*, by adding 350□L/ well with 5% Milk+ PBS).

[00221] Next, periplasmic extracts (PPE) containing the mutagenized antibody are blocked (*e.g.*, by milk(diluted in PBS) to 200□L of PPE to get a final milk percent of 5%). The PPEs are then mixed and incubated (*e.g.*, at room temperature still for 1 hour) before using as samples to screen on ELISA and then washed (*e.g.*, 3X with PBS-Tween at 350□L/well). The blocked PPE samples (*e.g.*, 50□L) are then added to the blocked ELISA plates and incubated (*e.g.*, at room temperature for 1-2 hours). Again the PPEs are washed (*e.g.*, 3X with PBS-Tween at 350□L/well). Next, an antibody specific for the mutagenized antibody is added to the PPEs (*e.g.*, 50□L/well of 1□g/ml monoclonal anti-V5 antibody, Sigma Cat.# V8012-50UG) and the PPEs incubated (*e.g.*, at room temperature for 1 hour). Again the PPEs are washed (*e.g.*, 3X with PBS-Tween at 350□L/well). Next, a secondary antibody conjugated to a enzymatic label is added to the PPEs (*e.g.*, 1:10000 diluted Goat anti mouse HRP conjugated, Biorad, Cat. 170-5047) and incubated wth the PPEs (*e.g.*, for 1 hour at room temperature). Again the PPEs are washed (*e.g.*, 3X with PBS-Tween at 350□L/well). Next, an appropriate amount of substrate for the enzymatic label is added to the PPEs *(e.g.,* 50□L/well of TMB, soluble, Calbiochem, Cat. 613544) and the enzyme is allowed time to act on the substrate (*e.g.*, until sufficiently blue color develops). The reaction may be stopped by the addition of an agent that sequesters the substrate and/or and agent that inhibits the enzymatic activity of the secondary antibody (*e.g*, 50□L per well of 2N H_2SO_4). Last, absorbance of the samples are read at 450nm.

G. Ranking of Mutagenized Antibodies

[00222] Mutagenized antibodies may be ranked based on their dissociation rate from their respective antigen.

[00223] In an exemplary method, a Biacore A100 screening protocol may be used to rank mutagenized antibody clones. For example, a CM5 chip may be docked and normalized using normalization solution (*e.g.*, using A100 normalization solution and use and an appropriate running buffer (*e.g.*, HBS-N (0.01 M HEPES pH 7.4, 0.15 M NaCl). After normalization, software is set to immobilize antigen on desired spots of each flow cell. For antigen surface preparation the surface may be activated (*e.g.*, with NHS/EDC mixture from the amine coupling kit for 5 minutes at 10□l/min). Antigen is then diluted (*e.g.*, in 10 mM sodium acetate buffer) and the surface of the CM5 chip is blocked (*e.g.*, with 1 M ethanolamine HCl pH 8.5 for 5 min at 10□l/min). Next, each sample comprising a mutagenized antibody is injected over the CM5 chip (*e.g.*, for 3 min at 30 □l/min flow rate with 600s dissociation) at an appropriate temperature (*e.g.*, 25°C). Biaevaluation software (*e.g.*, Biacore A100 evaluation software) is then used to calculate dissociation rates of individual samples and the relative amount of sample bound to each test surface. The data is fit to an appropriate kinetic model (*e.g.*, the kinetic titration model).

[00224] While the present disclosure has been described and illustrated herein by references to various specific materials, procedures and examples, it is understood that the disclosure is not restricted to the particular combinations of materials and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art. It is intended that the specification and examples be considered as exemplary, only, with the true scope and spirit of the disclosure being indicated by the following claims. All references, patents, and patent applications referred to in this application are herein incorporated by reference in their entirety.

CLAIMS:

1. A method of mutagenesis of a parent nucleic acid encoding a protein to generate modified proteins, said method comprising:

(a.) obtaining one or more primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in the parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid; and

(b.) mutating the parent nucleic acid by replication or polymerase based amplification using the one or more primers obtained in (a),

wherein replication or amplification of the parent nucleic acid with the one or more primers generates mutated nucleic acids that encode modified proteins.

2. The method of claim 1, wherein the amino acid mutations are selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at each position.

3. The method of claim 1, wherein the primers code for eighteen amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

4. The method of claim 3, wherein three primers that each comprise at least one 2 to 12 fold degenerate codon are obtained.

5. The method of claim 3, wherein seven primers that each comprise at least one 2 to 12 fold degenerate codon are obtained.

6. The method of claim 4, wherein the degenerate codons are selected from the group consisting of: NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T).

7. The method of claim 5, wherein the degenerate codons are selected from the group consisting of: ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G).

8. The method of claim 1, wherein the primers code for basic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

9. The method of claim 8, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is obtained

10. The method of claim 9, wherein the one primer comprises a degenerate codon which codes for arginine and lysine.

11. The method of claim 10, wherein the degenerate codon is represented by ARG (where, R=A/G).

12. The method of claim 1, wherein the primers code for polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

13. The method of claim 12, wherein two primers that comprise at least one 2 to 12 fold degenerate codon is obtained.

14. The method of claim 13, wherein the two primers comprise degenerate codons that collectively code for serine, threonine, asparagine and tyrosine.

15. The method of claim 14, wherein the degenerate codons are represented by WMC (where, W=A/T; M=A/C) and CAS (where S=C/G).

16. The method of claim 1, wherein the primers code for acidic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

17. The method of claim 16, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is obtained.

18. The method of claim 17, wherein the one primer comprises a degenerate codon that codes for glutamic acid and aspartic acid.

19. The method of claim 18, wherein the degenerate codon is represented by GAS (where S=C/G).

20. The method of claim 1, wherein the primers code for non-polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

21. The method of claim 20, wherein three primers that comprise at least one 2 to 12 fold degenerate codon are obtained.

22. The method of claim 21, wherein the three primers comprise degenerate codons that collectively code for glutamic acid and aspartic acid.

23. The method of claim 22, wherein the degenerate codons are represented by NTC (where, N=A/G/C/T), KGG (where, K=G/T), and SCG (where S=C/G).

24. The method of claim 1, wherein the parent nucleic acid encodes a binding molecule.

25. The method of claim 24, wherein the binding molecule is an antibody or fragment thereof.

26. The method of claim 1 further comprising selecting the one or more positions in the parent nucleic acid sequence for mutation.

27. The method of claim 1 further comprising transforming the mutated nucleic acid sequences into competent cells.

28. A library/array of mutated nucleic acid sequences generated by the method of claim 1.

29. A method for mutagenesis of a protein to obtain modified proteins with mutated amino acid sequences, the method comprising:
    a. identifying one or more amino acid positions in the protein for mutagenesis;
    b. substituting one or more of the identified amino acid residues in the protein with other amino acid residues excluding cysteine and methionine to generate a library or an array of modified proteins with mutated amino acid sequences;
    c. screening the library or array of modified proteins in an assay for a biological activity of the protein; and

d. obtaining modified proteins having the biological activity of the protein.

30. The method of claim 29, wherein the amino acid substitutions are selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at each position.

31. The method of claim 29, wherein step (b) of substituting is performed with one or more primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid and code for an amino acid substitution with the exception of cysteine and methionine at one amino acid position encoded by the parent nucleic acid.

32. The method of claim 31, wherein the primers code for eighteen amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

33. The method of claim 32, wherein three primers that each comprise at least one 2 to 12 fold degenerate codon are used.

34. The method of claim 32, wherein seven primers that each comprise at least one 2 to 12 fold degenerate codon are used.

35. The method of claim 33, wherein the degenerate codons are selected from the group consisting of: NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T).

36. The method of claim 34, wherein the degenerate codons are selected from the group consisting of: ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G).

37. The method of claim 31, wherein the primers code for basic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

38. The method of claim 37, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is used

39. The method of claim 38, wherein the one primer comprises a degenerate codon which codes for arginine and lysine.

40. The method of claim 39, wherein the degenerate codon is represented by ARG (where, R=A/G).

41. The method of claim 31, wherein the primers code for polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

42. The method of claim 41, wherein two primers that comprise at least one 2 to 12 fold degenerate codon are used.

43. The method of claim 42, wherein the two primers comprise degenerate codons that collectively code for serine, threonine, asparagine and tyrosine.

44. The method of claim 43, wherein the degenerate codons are represented by WMC (where, W=A/T; M=A/C) and CAS (where S=C/G).

45. The method of claim 31, wherein the primers code for acidic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

46. The method of claim 45, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is used.

47. The method of claim 46, wherein the one primer comprises a degenerate codon that codes for glutamic acid and aspartic acid.

48. The method of claim 47, wherein the degenerate codon is represented by GAS (where S=C/G).

49. The method of claim 31, wherein the primers code for non-polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

50. The method of claim 49, wherein three primers that comprise at least one 2 to 12 fold degenerate codon are used.

51. The method of claim 50, wherein the three primers comprise degenerate codons that collectively code for glutamic acid and aspartic acid.

52. The method of claim 51, wherein the degenerate codons are represented by NTC (where, N=A/G/C/T), KGG (where, K=G/T), and SCG (where S=C/G).

53. The method of claim 29, wherein the protein is a binding molecule.

54. The method of claim 53, wherein the binding molecule is an antibody or fragment thereof.

55. A method for generating an array of nucleic acids encoding modified proteins, said method comprising:
    a. obtaining a collection of nucleic acids encoding modified proteins containing amino acid mutations other than cysteine and methionine at amino acid residues of a parent protein sequence by mutagenesis of a nucleic acid encoding the protein sequence using primers that each comprise at least one 2 to 12 fold degenerate codon;
    b. sequencing the collection of nucleic acids encoding the modified proteins; and
    c. arranging each sequenced nucleic acid encoding a modified protein to generate an array of nucleic acid sequences each encoding a modified protein.

56. A method for generating an array of nucleic acid sequences encoding modified proteins, said method comprising:
    a. preparing a plurality of nucleic acid sequences by mutagenesis that encode a plurality of modified proteins that vary from a parent protein sequence at one or more amino acid positions and contain one of eighteen different amino acids excluding cysteine and methionine at each position mutated from the parent protein sequence; and
    b. arranging each nucleic acid sequence prepared in step (a) to generate an array of nucleic acid sequences each encoding a modified protein.

57. The method of claim 55 or 56, wherein the amino acid mutations are selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at each position.

58. The method of claim 55, wherein the primers code for eighteen amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

59. The method of claim 55, wherein three primers that each comprise at least one 2 to 12 fold degenerate codon are used.

60. The method of claim 55, wherein seven primers that each comprise at least one 2 to 12 fold degenerate codon are used.

61. The method of claim 59, wherein the degenerate codons are selected from the group consisting of: NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T).

62. The method of claim 60, wherein the degenerate codons are selected from the group consisting of: ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G).

63. The method of claim 55, wherein the primers code for basic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

64. The method of claim 63, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is used

65. The method of claim 64, wherein the one primer comprises a degenerate codon which codes for arginine and lysine.

66. The method of claim 65, wherein the degenerate codon is represented by ARG (where, R=A/G).

67. The method of claim 55, wherein the primers code for polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

68. The method of claim 67, wherein two primers that comprise at least one 2 to 12 fold degenerate codon are used.

69. The method of claim 68, wherein the two primers comprise degenerate codons that collectively code for serine, threonine, asparagine and tyrosine.

70. The method of claim 69, wherein the degenerate codons are represented by WMC (where, W=A/T; M=A/C) and CAS (where S=C/G).

71. The method of claim 70, wherein the primers code for acidic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

72. The method of claim 71, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is used.

73. The method of claim 72, wherein the one primer comprises a degenerate codon that codes for glutamic acid and aspartic acid.

74. The method of claim 73, wherein the degenerate codon is represented by GAS (where S=C/G).

75. The method of claim 55, wherein the primers code for non-polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

76. The method of claim 75, wherein three primers that comprise at least one 2 to 12 fold degenerate codon are used.

77. The method of claim 76, wherein the three primers comprise degenerate codons that collectively code for glutamic acid and aspartic acid.

78. The method of claim 77, wherein the degenerate codons are represented by NTC (where, N=A/G/C/T), KGG (where, K=G/T), and SCG (where S=C/G).

79. The method of claim 55, wherein the protein is a binding molecule.

80. The method of claim 79, wherein the binding molecule is an antibody or fragment thereof.

81. The method of claim 55 further comprising transforming the mutated nucleic acid sequences into competent cells.

82. A library/array of mutated nucleic acid sequences generated by the method of claim 55.

83. A method for generating an array of clones comprising nucleic acids encoding modified proteins, said method comprising:
    a. preparing a plurality of nucleic acids by mutagenesis that encode a plurality of modified proteins that vary from a parent protein sequence at one or more amino acid positions and contain one of eighteen different amino acids excluding cysteine and methionine at each position varied from the parent protein sequence;
    b. transfecting the nucleic acids prepared in step (a) into host cells and selecting clones comprising the transfected nucleic acids; and
    c. arranging each clone selected from step (b) to generate an array of clones with each arrayed clone capable of expressing a modified protein.

84. The method of claim 83, wherein the different amino acids are selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at each position.

85. The method of claim 83, wherein the mutagenesis is performed with one or more primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid and code for an amino acid substitution with the exception of cysteine and methionine at one amino acid position encoded by the parent nucleic acid.

86. The method of claim 85, wherein the primers code for eighteen amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

87. The method of claim 86, wherein three primers that each comprise at least one 2 to 12 fold degenerate codon are used.

88. The method of claim 86, wherein seven primers that each comprise at least one 2 to 12 fold degenerate codon are used.

89. The method of claim 87, wherein the degenerate codons are selected from the group consisting of: NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T).

90. The method of claim 88, wherein the degenerate codons are selected from the group consisting of: ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G).

91. The method of claim 85, wherein the primers code for basic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

92. The method of claim 91, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is used

93. The method of claim 92, wherein the one primer comprises a degenerate codon which codes for arginine and lysine.

94. The method of claim 93, wherein the degenerate codon is represented by ARG (where, R=A/G).

95. The method of claim 85, wherein the primers code for polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

96. The method of claim 95, wherein two primers that comprise at least one 2 to 12 fold degenerate codon are used.

97. The method of claim 96, wherein the two primers comprise degenerate codons that collectively code for serine, threonine, asparagine and tyrosine.

98. The method of claim 97, wherein the degenerate codons are represented by WMC (where, W=A/T; M=A/C) and CAS (where S=C/G).

99. The method of claim 85, wherein the primers code for acidic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

100. The method of claim 99, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is used.

101. The method of claim 100, wherein the one primer comprises a degenerate codon that codes for glutamic acid and aspartic acid.

102. The method of claim 101, wherein the degenerate codon is represented by GAS (where S=C/G).

103. The method of claim 85, wherein the primers code for non-polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

104. The method of claim 103, wherein three primers that comprise at least one 2 to 12 fold degenerate codon are used.

105. The method of claim 104, wherein the three primers comprise degenerate codons that collectively code for glutamic acid and aspartic acid.

106. The method of claim 105, wherein the degenerate codons are represented by NTC (where, N=A/G/C/T), KGG (where, K=G/T), and SCG (where S=C/G).

107. The method of claim 83, wherein the protein is a binding molecule.

108. The method of claim 107, wherein the binding molecule is an antibody or fragment thereof.

109. A method of producing a nucleic acid library with an equal representation of non-redundant amino acid changes at an amino acid position encoded by a parent nucleic acid, the method comprising:

(a.) providing a set of primers that each comprise at least one degenerate codon, wherein each primer comprises at least two oligonucleotide sequence that are complementary to a sequence in the parent nucleic acid and code for an amino acid mutation with the exception of cysteine and methionine at one amino acid position encoded by the parent nucleic acid, wherein the primers code for an equal representation of non-redundant amino acid changes at the one position;

(b.) hybridizing a primer from the set to the parent nucleic acid;

(c.) replicating or amplifying the parent nucleic acid molecule with the primer to generate nucleic acids that code for amino acid changes at the one position;

(d.) repeating steps (b) and (c) with each remaining primer from the set;

(e.) pooling the nucleic acids produced with each primer; and

(f.) obtaining a library of nucleic acids from steps (a)-(e) coding for an equal representation of amino acid changes at the one position.

110. The method of claim 109, wherein the amino acid mutations are selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at each position.

111. The method of claim 109, wherein the primers code for eighteen amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

112. The method of claim 111, wherein three primers that each comprise at least one 2 to 12 fold degenerate codon are provided.

113. The method of claim 111, wherein seven primers that each comprise at least one 2 to 12 fold degenerate codon are provided.

114. The method of claim 112, wherein the degenerate codons are selected from the group consisting of: NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T).

115. The method of claim 113, wherein the degenerate codons are selected from the group consisting of: ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G).

116. The method of claim 109, wherein the primers code for basic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

117. The method of claim 116, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is provided

118. The method of claim 117, wherein the one primer comprises a degenerate codon which codes for arginine and lysine.

119. The method of claim 118, wherein the degenerate codon is represented by ARG (where, R=A/G).

120. The method of claim 109, wherein the primers code for polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

121. The method of claim 120, wherein two primers that comprise at least one 2 to 12 fold degenerate codon are provided.

122. The method of claim 121, wherein the two primers comprise degenerate codons that collectively code for serine, threonine, asparagine and tyrosine.

123. The method of claim 122, wherein the degenerate codons are represented by WMC (where, W=A/T; M=A/C) and CAS (where S=C/G).

124. The method of claim 109, wherein the primers code for acidic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

125. The method of claim 124, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is provided.

126. The method of claim 125, wherein the one primer comprises a degenerate codon that codes for glutamic acid and aspartic acid.

127. The method of claim 126, wherein the degenerate codon is represented by GAS (where S=C/G).

128. The method of claim 109, wherein the primers code for non-polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

129. The method of claim 128, wherein three primers that comprise at least one 2 to 12 fold degenerate codon are provided.

130. The method of claim 129, wherein the three primers comprise degenerate codons that collectively code for glutamic acid and aspartic acid.

131. The method of claim 130, wherein the degenerate codons are represented by NTC (where, N=A/G/C/T), KGG (where, K=G/T), and SCG (where S=C/G).

132. The method of claim 109, wherein the parent nucleic acid encodes a binding molecule.

133. The method of claim 132, wherein the binding molecule is an antibody or fragment thereof.

134. The method of claim 109 further comprising transforming the mutated nucleic acid sequences into competent cells.

135. A method for obtaining a nucleic acid sequence with an improvement in comparison to a parent nucleic acid sequence with respect to at least one molecular or biological property of interest, said method comprising;

(a.) obtaining a set of primers that each comprise at least one 2 to 12 fold degenerate codon that does not code for cysteine and methionine, wherein the primers are complementary to a sequence in the parent nucleic acid sequence and wherein the primers code for

non-redundant amino acid mutations at one amino acid position encoded by the parent nucleic acid sequence;

(b.) mutating the parent nucleic acid sequence by replication or polymerase based amplification using the set of primers obtained in (a) to generate variant nucleic acid sequences;

(c.) producing a library or array of variant nucleic acid sequences from (b) coding for amino acid mutations at the one position in the parent nucleic acid sequence; and

(d) screening the library or array of variant nucleic acid sequences to identify nucleic acid sequences that have a desirable improvement in comparison with the parent nucleic acid sequence with respect to at least one molecular or biological property of interest.

136. The method of claim 135, wherein the amino acid mutations are selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at each position.

137. The method of claim 135, wherein the primers code for eighteen amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

138. The method of claim 137, wherein three primers that each comprise at least one 2 to 12 fold degenerate codon are obtained.

139. The method of claim 137, wherein seven primers that each comprise at least one 2 to 12 fold degenerate codon are obtained.

140. The method of claim 138, wherein the degenerate codons are selected from the group consisting of: NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T).

141. The method of claim 139, wherein the degenerate codons are selected from the group consisting of: ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G).

142. The method of claim 135, wherein the primers code for basic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

143. The method of claim 142, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is obtained

144. The method of claim 143, wherein the one primer comprises a degenerate codon which codes for arginine and lysine.

145. The method of claim 144, wherein the degenerate codon is represented by ARG (where, R=A/G).

146. The method of claim 135, wherein the primers code for polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

147. The method of claim 146, wherein two primers that comprise at least one 2 to 12 fold degenerate codon are obtained.

148. The method of claim 147, wherein the two primers comprise degenerate codons that collectively code for serine, threonine, asparagine and tyrosine.

149. The method of claim 148, wherein the degenerate codons are represented by WMC (where, W=A/T; M=A/C) and CAS (where S=C/G).

150. The method of claim 135, wherein the primers code for acidic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

151. The method of claim 150, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is obtained.

152. The method of claim 151, wherein the one primer comprises a degenerate codon that codes for glutamic acid and aspartic acid.

153. The method of claim 152, wherein the degenerate codon is represented by GAS (where S=C/G).

154. The method of claim 135, wherein the primers code for non-polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

155. The method of claim 154, wherein three primers that comprise at least one 2 to 12 fold degenerate codon are obtained.

156. The method of claim 155, wherein the three primers comprise degenerate codons that collectively code for glutamic acid and aspartic acid.

157. The method of claim 156, wherein the degenerate codons are represented by NTC (where, N=A/G/C/T), KGG (where, K=G/T), and SCG (where S=C/G).

158. The method of claim 135, wherein the parent nucleic acid encodes a binding molecule.

159. The method of claim 158, wherein the binding molecule is an antibody or fragment thereof.

160. The method of claim 135, wherein the biological property of interest is binding.

161. A method of making modified proteins with mutated amino acid sequences, the method comprising:
    a. modifying the amino acid sequence of a protein to produce amino acid mutations at an amino acid residue in the protein to generate a library or an array of modified proteins with mutated amino acid sequences, wherein the amino acid mutations exclude cysteine and methionine; and
    b. selecting modified proteins from the library or the array that have a biological activity of an unmodified protein.

162. The method of claim 161, wherein the amino acid mutations are selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at each position.

163. The method of claim 161, wherein step (a) of modifying is performed with one or more primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid and code for an amino acid substitution with the exception of cysteine and methionine at one amino acid position encoded by the parent nucleic acid.

164. The method of claim 163, wherein the primers code for eighteen amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

165. The method of claim 164, wherein three primers that each comprise at least one 2 to 12 fold degenerate codon are used.

166. The method of claim 164, wherein seven primers that each comprise at least one 2 to 12 fold degenerate codon are used.

167. The method of claim 165, wherein the degenerate codons are selected from the group consisting of: NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T).

168. The method of claim 166, wherein the degenerate codons are selected from the group consisting of: ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G).

169. The method of claim 163, wherein the primers code for basic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

170. The method of claim 169, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is used

171. The method of claim 170, wherein the one primer comprises a degenerate codon which codes for arginine and lysine.

172. The method of claim 171, wherein the degenerate codon is represented by ARG (where, R=A/G).

173. The method of claim 163, wherein the primers code for polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

174. The method of claim 173, wherein two primers that comprise at least one 2 to 12 fold degenerate codon are used.

175. The method of claim 174, wherein the two primers comprise degenerate codons that collectively code for serine, threonine, asparagine and tyrosine.

176. The method of claim 175, wherein the degenerate codons are represented by WMC (where, W=A/T; M=A/C) and CAS (where S=C/G).

177. The method of claim 163, wherein the primers code for acidic amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

178. The method of claim 177, wherein one primer that comprises at least one 2 to 12 fold degenerate codon is used.

179. The method of claim 178, wherein the one primer comprises a degenerate codon that codes for glutamic acid and aspartic acid.

180. The method of claim 179, wherein the degenerate codon is represented by GAS (where S=C/G).

181. The method of claim 163, wherein the primers code for non-polar amino acid mutations at the one amino acid position encoded by the parent nucleic acid.

182. The method of claim 181, wherein three primers that comprise at least one 2 to 12 fold degenerate codon are used.

183. The method of claim 182, wherein the three primers comprise degenerate codons that collectively code for glutamic acid and aspartic acid.

184. The method of claim 183, wherein the degenerate codons are represented by NTC (where, N=A/G/C/T), KGG (where, K=G/T), and SCG (where S=C/G).

185. The method of claim 161, wherein the protein is a binding molecule.

186. The method of claim 185, wherein the binding molecule is an antibody or fragment thereof.

187. A method for selecting modified proteins with mutated amino acid sequences, the method comprising:
    a. obtaining a library or an array of modified proteins comprising amino acid mutations at one amino acid residues in an amino acid sequence of a protein, wherein the amino acid mutations exclude cysteine and methionine;
    b. assaying the modified proteins for a biological activity of an unmodified protein; and
    c. selecting the modified proteins that have a biological activity of the unmodified protein.

188. The method of claim 187, wherein the protein is a binding molecule.

189. The method of claim 188, wherein the binding molecule is an antibody or fragment thereof.

190. The method of claim 187, wherein modified proteins are selected that have increased activity as compared to the unmodified protein.

191. The method of claim 187, wherein modified proteins are selected that have decreased activity as compared to the unmodified protein.

192. The method of claim 187, wherein modified proteins are selected that have equal activity as compared to the unmodified protein.

193. A library/array comprising variants of a protein sequence, wherein the variants each comprise an amino acid mutation at one amino acid position in the protein sequence of a parent protein and wherein the amino acid mutations are not cysteine or methionine.

194. A set of primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid

and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid.

195. The set of primers of claim 194, wherein the amino acid mutation is selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at each position.

196. The set of primers of claim 194, wherein the primers code for eighteen amino acid changes at an amino acid position encoded by the parent nucleic acid.

197. The set of primers of claim 194, wherein the set of primers comprises three primers.

198. The set of primers of claim 194, wherein the set of primers comprises seven primers.

199. The method of claim 197, wherein the primers each comprise one or more degenerate codons as represented by NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T).

200. The set of primers of claim 198, wherein the primers each comprise one or more degenerate codons as represented by ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G).

201. The set of primers of claim 194, wherein the primer set codes for basic amino acid changes at each of one or more positions in the parent nucleic acid.

202. The set of primers of claim 201, wherein the primer set comprises one primer.

203. The set of primers of claim 202, wherein the one primer comprises a degenerate codon which codes for arginine and lysine.

204. The set of primers of claim 203, wherein the one primer comprises one or more degenerate codons as represented by ARG (where, R=A/G).

205. The set of primers of claim 194, wherein the primer set codes for polar amino acid changes at each of one or more positions in the parent nucleic acid.

206. The set of primers of claim 205, wherein the primer set comprises two primers.

207. The set of primers of claim 206, wherein the two primers each comprise a degenerate codon which collectively code for serine, threonine, asparagine and tyrosine.

208. The set of primers of claim 207, wherein the two primers each comprise one or more degenerate codons as represented by WMC (where, W=A/T; M=A/C) and CAS (where S=C/G).

209. The set of primers of claim 194, wherein the primer set codes for acidic amino acid changes at each of one or more positions in the parent nucleic acid.

210. The set of primers of claim 209, wherein the primer set comprises one degenerate codon.

211. The set of primers of claim 210, wherein the one primer comprises a degenerate codon that codes for glutamic acid and aspartic acid.

212. The set of primers of claim 211, wherein the one primer comprises one or more degenerate codons as represented by GAS (where S=C/G).

213. The set of primers of claim 194, wherein the primers code for non-polar amino acid changes at each of one or more positions in the parent nucleic acid.

214. The set of primers of claim 213, wherein the primer set comprises three degenerate codons.

215. The set of primers of claim 214, wherein the three primers each comprise a degenerate codon that collectively code for glutamic acid and aspartic acid.

216. The set of primers of claim 215, wherein the primers each comprise one or more degenerate codons as represented by NTC (where, N=A/G/C/T), KGG (where, K=G/T), and SCG (where S=C/G).

217. A kit for mutagenesis of an amino acid residue encoded by a parent nucleic acid, the kit comprising:
a set of primers that each comprise at least one 2 to 12 fold degenerate codon, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in a parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid.

218. The kit of claim 217, wherein the amino acid mutation is selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, glutamine, glutamine acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at each position.

219. The kit of claim 217, wherein the primers code for eighteen amino acid changes at an amino acid position encoded by the parent nucleic acid.

220. The kit of claim 219, wherein the primers comprises three primers.

221. The kit of claim 219, wherein the primers comprises seven primers.

222. The kit of claim 220, wherein the primers each comprise one or more degenerate codons as represented by NHT or NHC (where N=A/G/C/T, H=A/C/T), VAG or VAA (where V=A/C/G) and BGG or DGG (where B=C/G/T, D=A/G/T).

223. The kit of claim 221, wherein the primers each comprise one or more degenerate codons as represented by ARG (where R=A/G), WMC (where W=A/T and M=A/C), CAS (where S=C/G), GAS (where S=C/G), NTC (where N=A/G/C/T), KGG (where K=G/T) and SCG (where S=C/G).

224. The kit of claim 217, wherein the primer set codes for basic amino acid changes at each of one or more positions in the parent nucleic acid.

225. The kit of claim 224, wherein the primer set comprises one primer.

226. The kit of claim 225, wherein the one primer comprises a degenerate codon which codes for arginine and lysine.

227. The kit of claim 227, wherein the one primer comprises one or more degenerate codons as represented by ARG (where, R=A/G).

228. The kit of claim 217, wherein the primer set codes for polar amino acid changes at each of one or more positions in the parent nucleic acid.

229. The kit of claim 228, wherein the primer set comprises two primers.

230. The kit of claim 229, wherein the two primers each comprise a degenerate codon which collectively code for serine, threonine, asparagine and tyrosine.

231. The kit of claim 230, wherein the two primers each comprise one or more degenerate codons as represented by WMC (where, W=A/T; M=A/C) and CAS (where S=C/G).

232. The kit of claim 217, wherein the primer set codes for acidic amino acid changes at each of one or more positions in the parent nucleic acid.

233. The kit of claim 232, wherein the primer set comprises one degenerate codon.

234. The kit of claim 233, wherein the one primer comprises a degenerate codon that codes for glutamic acid and aspartic acid.

238. The kit of claim 234, wherein the one primer comprises one or more degenerate codons as represented by GAS (where S=C/G).

239. The kit of claim 217, wherein the primers code for non-polar amino acid changes at each of one or more positions in the parent nucleic acid.

240. The kit of claim 239, wherein the primer set comprises three degenerate codons.

241. The kit of claim 240, wherein the three primers each comprise a degenerate codon that collectively code for glutamic acid and aspartic acid.

242. The kit of claim 241, wherein the primers each comprise one or more degenerate codons as represented by NTC (where, N=A/G/C/T), KGG (where, K=G/T), and SCG (where S=C/G).

243. A method for obtaining a nucleic acid sequence with an improvement in comparison to a parent nucleic acid sequence with respect to at least one molecular or biological property of interest, said method comprising:

(a.) mutating the parent nucleic acid by polymerase based amplification using one or more primers that each comprise at least one 2 to 12 fold degenerate codon to generate mutated nucleic acid sequences, wherein each primer comprises at least two oligonucleotide sequences that are complementary to a sequence in the parent nucleic acid and code for an amino acid mutation with the exception of cysteine or methionine at one amino acid position encoded by the parent nucleic acid;

(b.) sequencing the mutated nucleic acid sequences;

(c.) arranging each sequenced mutated nucleic acid sequence comprising one amino acid mutation to generate an array of mutated nucleic acid sequences; and

(d.) screening the array of variant nucleic acid sequences to identify nucleic acid sequences that have a desirable improvement in comparison with the parent nucleic acid sequence with respect to at least one molecular or biological property of interest.

244. The method of claim 243, wherein modified proteins are selected that have increased activity as compared to the unmodified protein.

245. The method of claim 243, wherein modified proteins are selected that have decreased activity as compared to the unmodified protein.

246. The method of claim 243, wherein modified proteins are selected that have equal activity as compared to the unmodified protein.

Figure 1

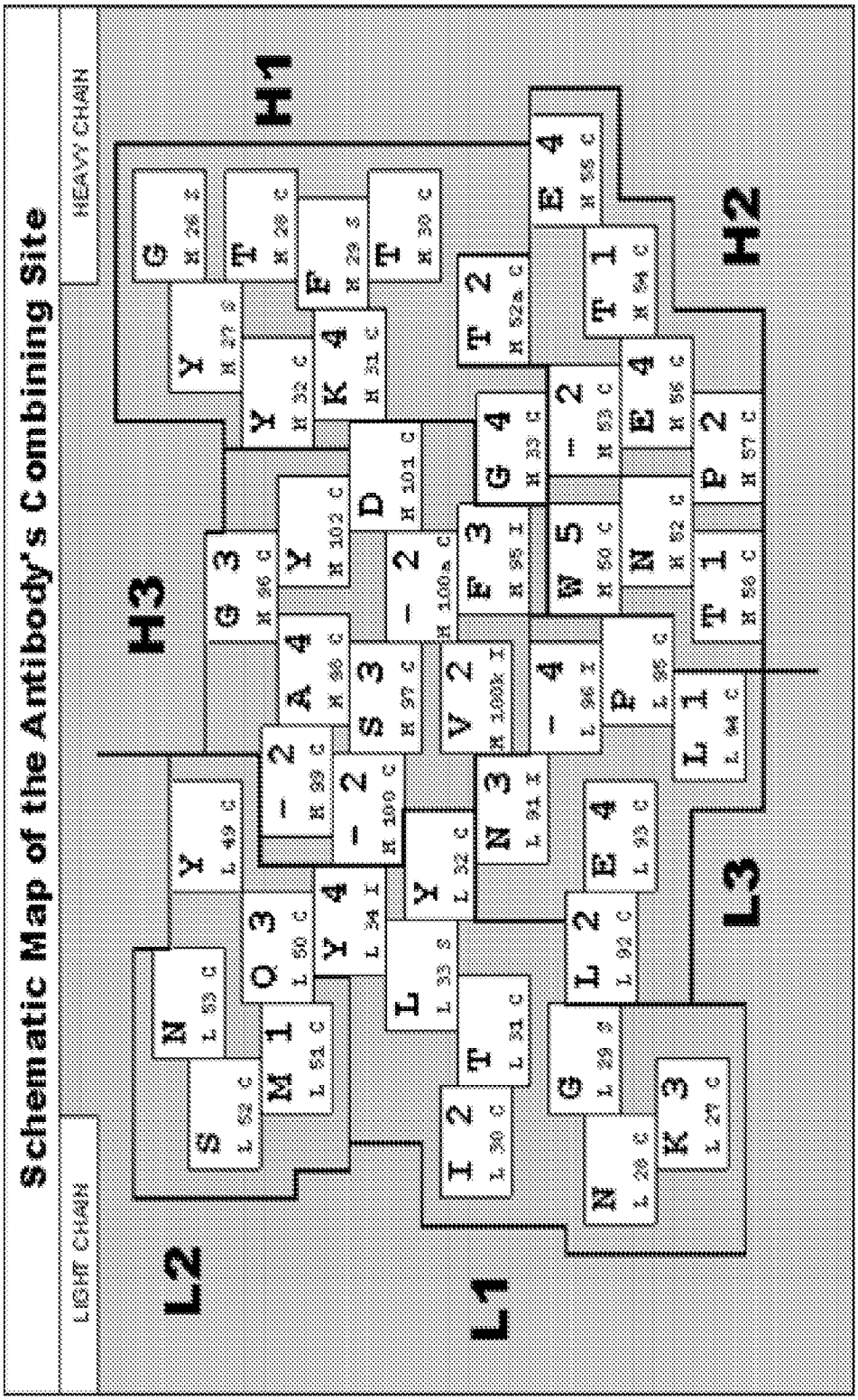

Schematic Map of the Antibody's Combining Site
LIGHT CHAIN
HEAVY CHAIN
L1
L2
L3
H1
H2
H3

Figure 2A

```
pos              10        20       abcdef 30        40        50
prox     .S.S..:::.....:.......:.SPCCCCCCCCSCCCSISI.I.:...:.ISSCCCC
murING1  DIVMTQAAFSNPVTLGTSGSISCRSSKSLLHS-NGITYLYWYLQKPGQSPQLLIYQMS
cspc      1    222 2  1  321       3  1     2   4          3    31
mK2      DVVMTQTPLSLPVSLGDQASISCRSSQSLvHS-NGNTYLeWYLQKPGQSPKLLIYkVS
humING1        S L       E
disc     +-+o+++o++++++o+++++++-+-o-------------o-o+++o+++o+-oo----
expo     +-+-+-+o++o+-++++++-+-+-+-++++++++++-+++-=-=o=++++o=o=--o+++
risk     LHLHLHLMLLMLHLMLLLHLHLHLHMHHHHHHHHHHHHHHHHHHMHLMLLMHMHHHHHHH
pos              10        20       abcdef 30        40        50

pos              60        70        80        90     abcdef  100       a
prox     C.PP..:....S.S.S..S..............I:ISICCCCCCCCCCCISI...........
murING1  NLASGVPDRFSSSGSGTDFTLRISRVEAEDVGVYYCAQNLELPR------TFGGGTKLEI-K  (SEQ ID NO: 815)
cspc      43         2          1         1     3 3241 4     4
mK2      NRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCfQgtHVPP-----yTFGGGTKLEI-KRA (SEQ ID NO: 816)
humING1   R                   K                                  Q
disc     -+oo++o++++-+-+o++-++++++++++++++++-o-------------oo++++++++++
expo     ++o++-o+o-+-+-+-+++-+-+-++-+++-+---=-=-=+++ooooooo=-=-+--+-++++++
risk     HLMMLHMLMHLHLHLHLLLHLHLHLLHLLLHLHHHHHHHHHHHHHHHHHHHHHHHLHHLHLLLLLL
pos              60        70        80        90     abcdef  100       a
```

Figure 2B

```
pos              10         20         30     ab    40         50  abc
prox     PS.S....:....:........:.SPSSCSCCCCSISSSI.I.:...I.ISSCSCCCCCCCCC
murING1  QIQLVQSGPELKKPGETVKISCKASGYTFTKYGMN--WVKQAPGKGLKWMGWINTY--TEEP
cspc     31  2       3    41               4 4   3      2    2 4 1 5   24 21442
mH2a     EVQLQQSGPELVKPGASVKiSCKASGYTFTdYyMnN-WVKQspGKsLEWIGdInP--gnGgT
humING1            V     S                            Q
disc     o-+o++++o++++o+++++++-+oo---------ooo-o+++o+++o+-oo-----------
expo     +-+-+-+-o++o+++++-+-+-+-+--+-++o+-=---=o=++++o=o=--o-o+++++o++
risk     MHLHLHLHMLLMLMLLLHLHLHLHMHHHHHHHHHHHHHHHHMHLMLLMHMHHHHHHHHHHHHHH
pos              10         20         30     ab    40         50  abc

pos       60         70         80  abc       90        100abcdefghijk       110
prox     CPSPP.P....S.S.P..P.S..............I:ISICCCCCCCCCCCCCCCICCI.........
murING1  TYGDDFKGRFAFSLETSASTANLQINNLKSEDTATYFCARFGSA-----------VDYWGQGTSVTVSS (SEQ ID NO: 817)
cspc     1 334    13221113 2    21 111 3    1 2 2    33342222322224422        1
mH2a     sYNQKFKGKATLTvDKSSSTAYMqLsSLTSEDSAVYYCARxxyysssxmxaxxYYaFDYWGQGTtVTVSS (SEQ ID NO: 818)
humING1            T T D  T    Y E SS R                                      L
disc     -oooo+o++++-+-+o++o+-+++++++++++++++-o-------------------o+++++++++
expo     +o-+o-++o-+-+-+o++o+-+-+-++-+++-+-o-=-=-=oo+ooooooooooooo=oo=-+--+-+-++
risk     HMHMMHMLMHLHLHLHLMLLMLHLHLHLLHLLLHLHMHHHHHHHHHHHHHHHHHHHHHHHHHHHLHHLHLHLL
pos       60         70         80  abc       90        100abcdefghijk       110
```

Figure 2C

```
pos                    10        20       abcdef 30        40        50
bind/disc      +-+O+++O++++++O+++++++-+-O-------------O-O+++O+++O+-OO----
bury/exp       +-+-+-+O++O+-+++++-+-+-+-++++++++++-+++-=-=O=++++O=O=--O+++
risk           LHLHLHLMLLMLHLMLLLHLHLHLHMHHHHHHHHHHHHHHHHHMHLMLLMHMHHHHHHH
Prox           .S.S.S..:::.....:.....:.SPCCCCCCCCSCCCSISI.I.:...:.ISSCCCC
cA5            DIQMTQOPSSVSASVGDRLTXICRASQ------DINRWLAWQQTPGNAPKLLIHSAT
Cspc                                          3 142               32 1
A8.2           DIQMTQSPSSVSASAGDRVTITCRASQ------GISTWLAWYQQKPGAPKLLIYAAS
Cspc                                           2 12
hK1 (TAE)      DIQMTQSPSSLSASVGDRVTITCRASQSLV---sIsnyLaWYQQKPGKAPKLLIYaAS
pos                    10        20       abcdef  30        40        50

pos                  60          70        80          90    abcdef   100     a
bind/disc      -+OO++O++++-+-+-+O++-+++++++++++++++-O-------------OO++++++++++
bury/exp       ++O++-O+O-+-+-+-+-+-+-+-++-+++-+---=-=-=+++OOOOOOO=-=-+--+-++++++
risk           HLMMLHMLMHLHLHLHLLHHLHLHLLHLLLHLHHHHHHHHHHHHHHHHHHHHHHLHHLHLLLLLL
Prox           C.PP..:....S.S.S..S..............I:ISICCCCCCCCCISI..........
cA5            SLQSGVPSRFSGSGSOTDFTLTINSLMDFATYYCQQADSFPL------TFGGGTKVEI-K     (SEQ ID NO: 946)
Cspc             3                                  43 2 3
A8.2           SLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQGYRFPF------TFGPGTKVEI-K   (SEQ ID NO: 947)
Cspc             3                                 2   4342 3
hK1 (TAE)      sLeSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQyns1PE-----wTFGQGTKVEI-KRT (SEQ ID NO: 819)
pos                  60          70        80          90     abcdef 100       a
```

Figure 2D

```
pos                 10        20        30     ab   40        50  abc
bind/disc   o-+o++++o++++o+++++++-+oo---------ooo-o+++o+++o+-oo-----------
bury/exp    +-+-+-+-o++o+++++-+-+-+-+--+-++o+-====o=++++o=o=--o-o+++++o++
risk        MHLHLHLHMLLMLMLLLHLHLHLHMHHHHHHHHHHHHHHHHMHLMLLMHMHHHHHHHHHHHHHH
Prox        PS.S....:....:.......SPSSCSCCCCSISSSI.I.:...I.ISSCSCCCCCCCCC E
cAS         EVQLLESGGGLVQPGGSLRLSCAASGFTFSKYFMF--WVRQAPGKGLEWVSVISP--SGGMT
Cspc                                    4 3 3               2   3  3  2
A8.2        EVQLLESGGGLVQPGGSLRLSCAASGFTFSMYDMH--WVRQAPGKGLEWVSYIYP-SGGIT
Cspc                                    2 4 4               2  33  3  2
hH3 (TAE)   EVQLVESGGGLVQPGGSLRLSCAASGFTFSsyaMs--WVRQAPGKGLEWVsvIsgKtdGgst

pos                 10        20        30     ab   40        50  abc

Pos          60       70        80    abc    90      100abcdefghijk        110
bind/disc   -oooo+o++++-+-+o++o+-++++++++++++++-o-------------------o++++++++++
bury/disc   +o-+o-++o-+-+-+o++o+-+-+-++-+++-+-o-=-=-=oo+ooooooooooooo=oo=-+--+-+-++
risk        HMHMMHMLMHLHLHLMLLMLHLHLHLLLHLLLHLHMHHHHHHHHHHHHHHHHHHHHHHHHHHHLHHLHLHLL
Prox        CPSPP.P....S.S.P..P.S..............I:ISICCCCCCCCCCCCCCCCICCI.........
cAS         RYADSVKGRPTISRDNSKNTLYLQMNSLRAEDTAVYYCARVGFGGNS----------DYWGQGTLVTVSS (SEQ ID NO: 948)
Cspc                                            2323322
A8.2        WYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARQGGPNWF----------DPWGQGTLVTVSS (SEQ ID NO: 949)
Cspc                                            2331242           3
hH3 (TAE)   yYADSVKGRFTISRDnSKNTLYLQMNSLRaEDTAVYYCArxxxxxxlsgxyyyyhyFDyWGQGTLVTVSS (SEQ ID NO: 838)
Pos          60       70        80  abc      90       100abcdefghijk       110
```

Figure 3A

```
prox  .S.S..::.....:.....:..SPCCCCCCCCSCCCSISI.I.:.....:.ISSCCCC
pos        10        20        abcdef 30        40        50
hK1   DIQMTQSPSSLSASVGDRVTITCRASQSLV----sIsnyLaWYQQKPGKAPKLLIYaAS
hK2   DIVMTQSPLSLPVTPGEPASISCRSSQSLLHS-DGnnYLnWYLQKPGQsPQLLIYlvS
hK3   EIVLTQSPGTLSLSPGERATLSCRASQS-----vSSSYLAWYQQKPGQAPRLLIYGAS
hK4   DIVMTQSPDSLAVSLGERATINCkSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIYWAS
mK1   DIVMTQSPSSLaVsAGEKVTMSCKSSQSLLnSgnqKNYLAWYQQKPGQsPKLLIYwAS
mK2   DVVMTQTPLSLPVSLGDQASISCRSSQSLvHS-NGNTYLeWYLQKPGQSPKLLIYkVS
mK3   DIVLTQSPASLAVSLGQRATISCRASeSVds—yGnSfMHWYQQKPGQPPKLLIYaAS
mK4   EIVLTQSPAIMsASPGEKVTMTCSASSS-----VSSsYLHWYQQKpGxSPKLWIYrTS
mK5   DIqMTQSpsSLSASlGDRVTItCrASQD-----DIsnyLnWYQQKPggspKLLIYyaS
mK6   QIVLTQSPAIMSASPGEKVTMTCSASS-------SVSYMHWYQQKSGTSPKrWIYDTS
hL1   ZSVLTQPPS-vSgaPGQrVTISCSGSSSNi----IGnNyVsWYQQLPGTAPKLLIYdnN
hL2   ZSALTQPaS-VSGSPGQSiTISCTGTSsDV----ggynaVSWyQQhPGKAPKLliydvt
hL3   SYeLTQPPS-VSVSPGQTArITCSGDn-------LgdkyvhWYQQKPGQaPVLVIYdDx
hL4   -SELTQpPsxVSVAxGQTvrITCxGDS-N-----LGxYdAsWYQQKPxQAPxLVIYGxN
hL5   XSALTQPPS-ASGSPGQSVTISCTGTSSDV----GgynYVSWYQQHaGkAPKviIyEVn
hL6   NFMLTQPHS-VSESPGKTVTISCTgSsGS-----IASnYVQWYQQRPGSAPTTVIYEDN
mL    qAVVTQESA-LTTSPGETVTLTCRSSTGAV----TTSNYANWVQEKPDHLFTGLIGGTN
```

Figure 3B

```
prox  C.PP..:.....S.S.S..S.....................I:ISICCCCCCCCCCISI.............
pos        60        70        80        90        100       abcdef    a
hK1   sLeSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQynslPE-----wTFGQGTKVEI-KRT (SEQ ID NO: 819)
hK2   NRaSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQalQ-PR------TFGQGTKVEI-KRT (SEQ ID NO: 820)
hK3   SRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGsSPP-----lTFGQGTKVEI-KRT (SEQ ID NO: 821)
hK4   TRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYStP-------TFGQGTKVEI-KRT (SEQ ID NO: 822)
mK1   TRESGVPDRFTGSGSGTDFTLTISSVQAEDLAVYYCQndYSYP------LTFGAGTKLEL-KRA (SEQ ID NO: 823)
mK2   NRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCfQgtHVPP-----yTFGGGTKLEI-KRA (SEQ ID NO: 824)
mK3   NLESGVPARFSGSGSGTDFTLnIhPVEedDaATYYCQQSnEdPP-----wTFGGGTKLEI-KRA (SEQ ID NO: 825)
mK4   NLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYCQQwSsyPxG-----lTFGaGTKLEI-KRA (SEQ ID NO: 826)
mK5   rLhsGVPSRFSGSGSGTDYSLTISnLEqEDiAtYfCQQgntlPP-----rTFGGGTKLEI-KRA (SEQ ID NO: 827)
mK6   KLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPPM---PlTFGAGTKLEL-KRA (SEQ ID NO: 828)
hL1   kRPSGvPDRFSGSKSGTSAsLAIxGLQseDEADYYCatWDdSLsaxNSApVFGGGTKLTVLGQP (SEQ ID NO: 829)
hL2   dRPSGvPdRFSGSKSGnTASLTISGLQaEDEADYYCsSYgggsxx----nVFGGGTKxTVLGQP (SEQ ID NO: 830)
hL3   kRPSGIPERFSGSnSGnTATLTISGvqAgDEADYYCQaWDSssdhPG—vVFGGGTKLTVLGQP   (SEQ ID NO: 831)
hL4   NRPSGIPDRFSGSSSGxTASLTITGAQAEDEADYYCNSRDSSGxx----xxFGGGTKLTVLGQP (SEQ ID NO: 832)
hL5   kRPSGVPDRFSGSKSGNTASLTVSGLqAEDEADYYCSSYegsdN-----FVFGtGTKvTVLgQP (SEQ ID NO: 833)
hL6   QRPSGVPDRFSGS-SSNSASLTISGLKTEDEADYYCQSYDsnNh-----wVFGGGTKLTVLGQP (SEQ ID NO: 834)
mL    NRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNHEQFV—WVFGGGTKLTVLGQP   (SEQ ID NO: 835)
```

Figure 3C

```
prox    PS.S.....:.....:..........:..SPSSCSCCCCSISSSI.I.:....I.ISSCSCCCCCCCCC
pos           10        20        30        ab    40        50  abc
hH1     qVQLVQSGAEVKKPGaSVKVSCKASGYTFtSYaIs--WVRQAPGQGLEWMGwInPY-gnGdT
hH2     QVQLQESGPGLVKPSqTLSLTCtvSGgSvSsyxwswnWIRQPPGkGLEWIGrIyYRAysgst
hH3     EVQLVESGGGLVQPGGSLRLSCAASGFTFSsyaMs--WVRQAPGKGLEWVsvIsgKtdGgst
mH1a    eVQLQESGPsLVKPSQtLSLTCSVTGdSITSgYwnNSWIRxFPGNKLEwMGYIsx--YSGST
mH1b    QVQLKESGPGLVAPSQSLSITCTVSGFSLTSYGVHVSWVRQPPGKGLEWLGVIW---aGGST
mH2a    EVQLQQSGPELVKPGASVKiSCKASGYTFTdYyMnN-WVKQspGKsLEWIGdInP--gnGgT
mH2b    QVQLQQPGAELVKPGASVKLSCKASGYTFTSYWMH--WVKQRPGQGLEWIGrIdP--nsGgT
mH2c    EVQLQQSGAELVKPGASVKLSCTASGFNIKDTYMH--WVKQRPEQGLEWIGRIDP--ANGNT
mH3a    EVKLVESGGGLVQPGGSLRLSCATSGFTFSDFYME--WVRQPPGKaLEWiAasRNKANdYTT
mH3b    EVKLLESGGGLVQPGGSLKLSCAASGFDFSRYWMS--WVRQAPGKGLEWIGEINPKADSSTI
mH3c    EVKLEESGGGLVQPGGSMKLSCVASGFTFSNYWMnxxWVRQSPEKGLEWVAeIRLKSdNYAT
mH3d    EVqLVESGGGLVKPGGSLKLSCAASGFTFSsYaMS--WVRQTPEKRLEWVAtISsKSgGgyT
mH5a    EVQLQQSGAELVRAGSSVKMSCKASGYTFTSYGIN--WVKQRPGQGLEWIGYINP--GNGYT
mH5b    EVQLQQSGAELVKAGSSVKMSCSATGYTFSSYGLY--WVRQAPGQGLExxGYISS--SSAYP
mHms    xVQLveSGggLVkPGgSvKlSCkASGfTFssfgMsNFWVRQaPgKgLEWvgwINsKLgggai
```

Figure 3D

```
prox    CPSPP.P....S.S.P..P.S..............I:ISICCCCCCCCCCCCCCCICCI.........
pos      60        70        80  abc      90       100abcdefghijk       110
hH1     nYAQkFQGRVTiTaDtStSTAYMELSSLRSeDTAVYYCARapgygsgggcyrgdyxFDyWGQGTLVTVSS (SEQ ID NO: 836)
hH2     xYnpSLKSRvTIsvDTSKNQFSLkLsSVTaaDTAVYYCARexxxgxxgddyyyxxgfDvWGQGTlVTVSS (SEQ ID NO: 837)
hH3     yYADSVKGRFTISRDnSKNTLYLQMNSLRaEDTAVYYCArxxxxxxlsgxyyyyhyFDyWGQGTLVTVSS (SEQ ID NO: 838)
mH1a    yYNPSLKSRISITRDTSKNQyfLQLNSVTTEDTATYYCARxxygyxxyxydxYYyYFDYWGQGTtvtVSS (SEQ ID NO: 839)
mH1b    NYNSALmSRLSISKDNSKSQVFLKMNSLQTDDTAMYYCARDrGxyyyxsgxxxYYAmDYWGQGTsVTVSS (SEQ ID NO: 840)
mH2a    sYNQKFKGKATLTvDKSSSTAYMqLsSLTSEDSAVYYCARxxyyysssxmxaxxYYaFDYWGQGTtVTVSS (SEQ ID NO: 841)
mH2b    nYNEKFKsKATLTVDKSSSTAYMQLSSLTSEDSAVYYCARyyyggssxxvYx-YwyFDYWGQGTtvTVSS (SEQ ID NO: 842)
mH2c    KYDPKFQGKATITADTSSNTAYLQLSSLTSEDTAVYYCARgyyyydsxVG---YyAMDYWGQGTxVTVSS (SEQ ID NO: 843)
mH3a    EYSASVKGRFtvSRDtSQSILYLQMNALRAEDtAiYYCARdyyygssyyeGPVYWYFDVWGaGTTVTVSS (SEQ ID NO: 844)
mH3b    NYTPSLKDKFIISRDNAKNTLYLQMSKVRSEDTALYYCARlggygyfgSS---YYamdYWGQGTtvTVSS (SEQ ID NO: 845)
mH3c    HYAESVKGRFTISRDDSKSSVYLQMNNLRAEDTGIYYCTtggyggxRRS-----xWFaYWGQGTlVTVSs (SEQ ID NO: 846)
mH3d    YYPDSVKGRFTISRDNAKNTLYLQMSSLrSEDTAmYYCARgxyyyxxgsaPF-DYAmDYWGQGTsVTVSS (SEQ ID NO: 847)
mH5a    kYNEKFKGKTTLTVDKSSSTAYMQLRSLTSEDSAVYFCARSxYYGGSYyyxFAYYyFDYWGQGTTLTVSS (SEQ ID NO: 848)
mH5b    NYAQKFQGRVTITADESTNTAYMELSSLRSEDTAVYFCAVRVISRYF----------DGWGQGTLV---- (SEQ ID NO: 849)
mHms    yYAdtxKGRFTISRDNsKsTLYLQMssLrSEDTAtYyCARxgyyggrrsxxxSYwyFDYWGQGTtVTVSS (SEQ ID NO: 850)
```

Figure 4

Physical Size

(DIVIDE SIZE DIFFERENCES BY THREE AND DROP THE FRACTION AFTER THE DECIMAL)

Nonpolar Size	Polar	Negative	Positive		Special Size	
10	**W**				**10**	
9					**9**	
8		**Y**			**8**	
7	**F**			**R**	**7**	
6				**H**	**6**	
5		**Q**	**E**	**K**	**5**	
4	**L I M**	**N**	**D**		**4**	**x**
3	**V**	**T**			**3**	**p**
2		**S**			**2**	**c**
1	**A**				**1**	
0	**G**				**0**	**-**

Chemical-Function Charge-and-Class

(1 POINT FOR CHANGE OF CLASS, PLUS 1 POINT FOR EACH UNIT OF CHARGE DIFFERENCE)

0 no change in class or charge
1 Nonpolar vs. Polar; Nonconserved vs. (Nonpolar or Polar)
2 (Positive or Negative) vs. (Nonpolar or Polar or Nonconserved)
3 Positive vs. Negative

Nonidentity

1 any change of sidechain, including insertions or deletions

Repeated Identical Mutation

2 each repeat

Figure 5

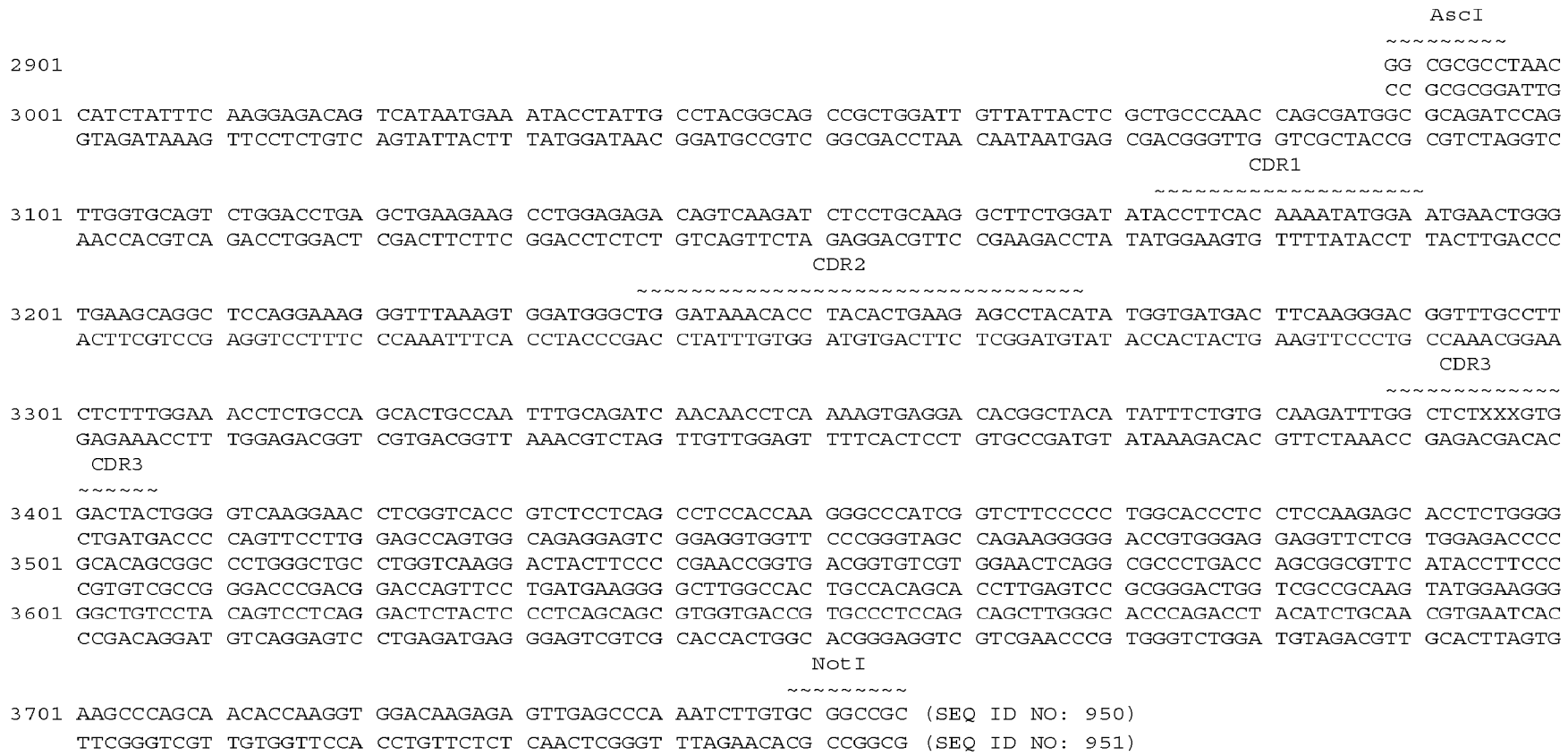

AscI
2901
GG CGCGCCTAAC
CC GCGCGGATTG
3001 CATCTATTTC AAGGAGACAG TCATAATGAA ATACCTATTG CCTACGGCAG CCGCTGGATT GTTATTACTC GCTGCCCAAC CAGCGATGGC GCAGATCCAG
GTAGATAAAG TTCCTCTGTC AGTATTACTT TATGGATAAC GGATGCCGTC GGCGACCTAA CAATAATGAG CGACGGGTTG GTCGCTACCG CGTCTAGGTC
CDR1
3101 TTGGTGCAGT CTGGACCTGA GCTGAAGAAG CCTGGAGAGA CAGTCAAGAT CTCCTGCAAG GCTTCTGGAT ATACCTTCAC AAAATATGGA ATGAACTGGG
AACCACGTCA GACCTGGACT CGACTTCTTC GGACCTCTCT GTCAGTTCTA GAGGACGTTC CGAAGACCTA TATGGAAGTG TTTTATACCT TACTTGACCC
CDR2
3201 TGAAGCAGGC TCCAGGAAAG GGTTTAAAGT GGATGGGCTG GATAAACACC TACACTGAAG AGCCTACATA TGGTGATGAC TTCAAGGGAC GGTTTGCCTT
ACTTCGTCCG AGGTCCTTTC CCAAATTTCA CCTACCCGAC CTATTTGTGG ATGTGACTTC TCGGATGTAT ACCACTACTG AAGTTCCCTG CCAAACGGAA
CDR3
3301 CTCTTTGGAA ACCTCTGCCA GCACTGCCAA TTTGCAGATC AACAACCTCA AAAGTGAGGA CACGGCTACA TATTTCTGTG CAAGATTTGG CTCTXXXGTG
GAGAAACCTT TGGAGACGGT CGTGACGGTT AAACGTCTAG TTGTTGGAGT TTTCACTCCT GTGCCGATGT ATAAAGACAC GTTCTAAACC GAGACGACAC
CDR3
3401 GACTACTGGG GTCAAGGAAC CTCGGTCACC GTCTCCTCAG CCTCCACCAA GGGCCCATCG GTCTTCCCCC TGGCACCCTC CTCCAAGAGC ACCTCTGGGG
CTGATGACCC CAGTTCCTTG GAGCCAGTGG CAGAGGAGTC GGAGGTGGTT CCCGGGTAGC CAGAAGGGGG ACCGTGGGAG GAGGTTCTCG TGGAGACCCC
3501 GCACAGCGGC CCTGGGCTGC CTGGTCAAGG ACTACTTCCC CGAACCGGTG ACGGTGTCGT GGAACTCAGG CGCCCTGACC AGCGGCGTTC ATACCTTCCC
CGTGTCGCCG GGACCCGACG GACCAGTTCC TGATGAAGGG GCTTGGCCAC TGCCACAGCA CCTTGAGTCC GCGGGACTGG TCGCCGCAAG TATGGAAGGG
3601 GGCTGTCCTA CAGTCCTCAG GACTCTACTC CCTCAGCAGC GTGGTGACCG TGCCCTCCAG CAGCTTGGGC ACCCAGACCT ACATCTGCAA CGTGAATCAC
CCGACAGGAT GTCAGGAGTC CTGAGATGAG GGAGTCGTCG CACCACTGGC ACGGGAGGTC GTCGAACCCG TGGGTCTGGA TGTAGACGTT GCACTTAGTG
NotI
3701 AAGCCCAGCA ACACCAAGGT GGACAAGAGA GTTGAGCCCA AATCTTGTGC GGCCGC (SEQ ID NO: 950)
TTCGGGTCGT TGTGGTTCCA CCTGTTCTCT CAACTCGGGT TTAGAACACG CCGGCG (SEQ ID NO: 951)

Figure 6

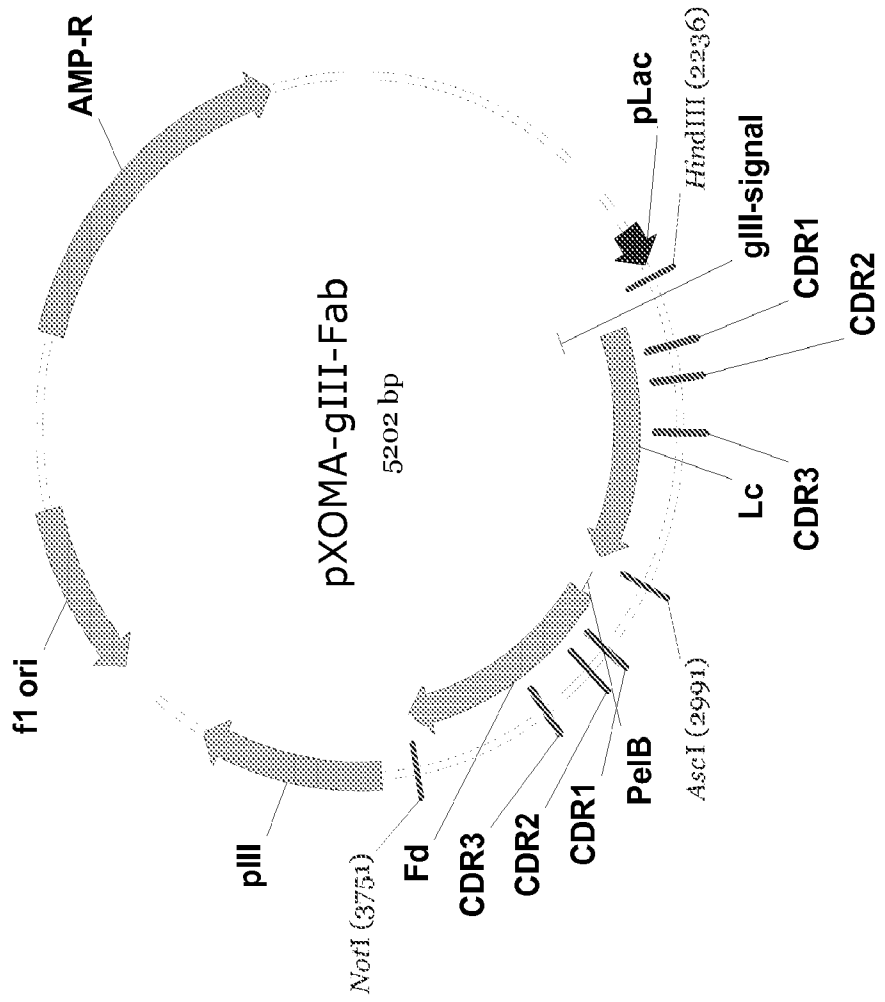

AMP-R
pLac
HindIII (2236)
gIII-signal
CDR1
CDR2
Lc
CDR3
pXOMA-gIII-Fab
5202 bp
f1 ori
pIII
NotI (3751)
Fd
CDR3
CDR2
CDR1
PelB
AscI (2991)

Figure 7

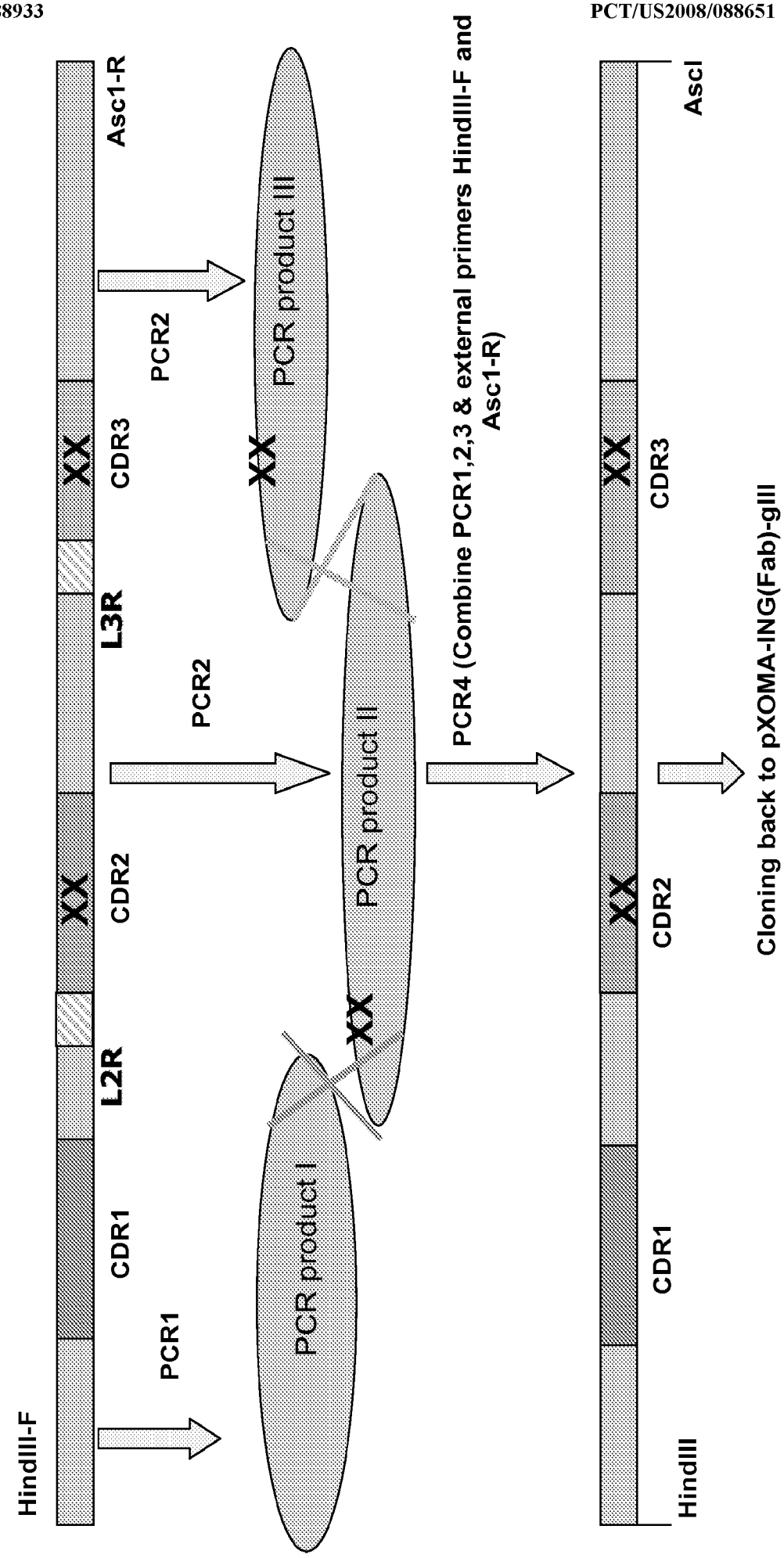

Combination of beneficial ING Vk TAE mutations
HindIII-F
CDR1
L2R
XX
CDR2
L3R
XX
CDR3
Asc1-R
PCR1
PCR2
PCR2
PCR product I
PCR product II
PCR product III
XX
XX
XX
PCR4 (Combine PCR1,2,3 & external primers HindIII-F and Asc1-R)
HindIII
CDR1
XX
CDR2
XX
CDR3
AscI
Cloning back to pXOMA-ING(Fab)-gIII

Figure 8

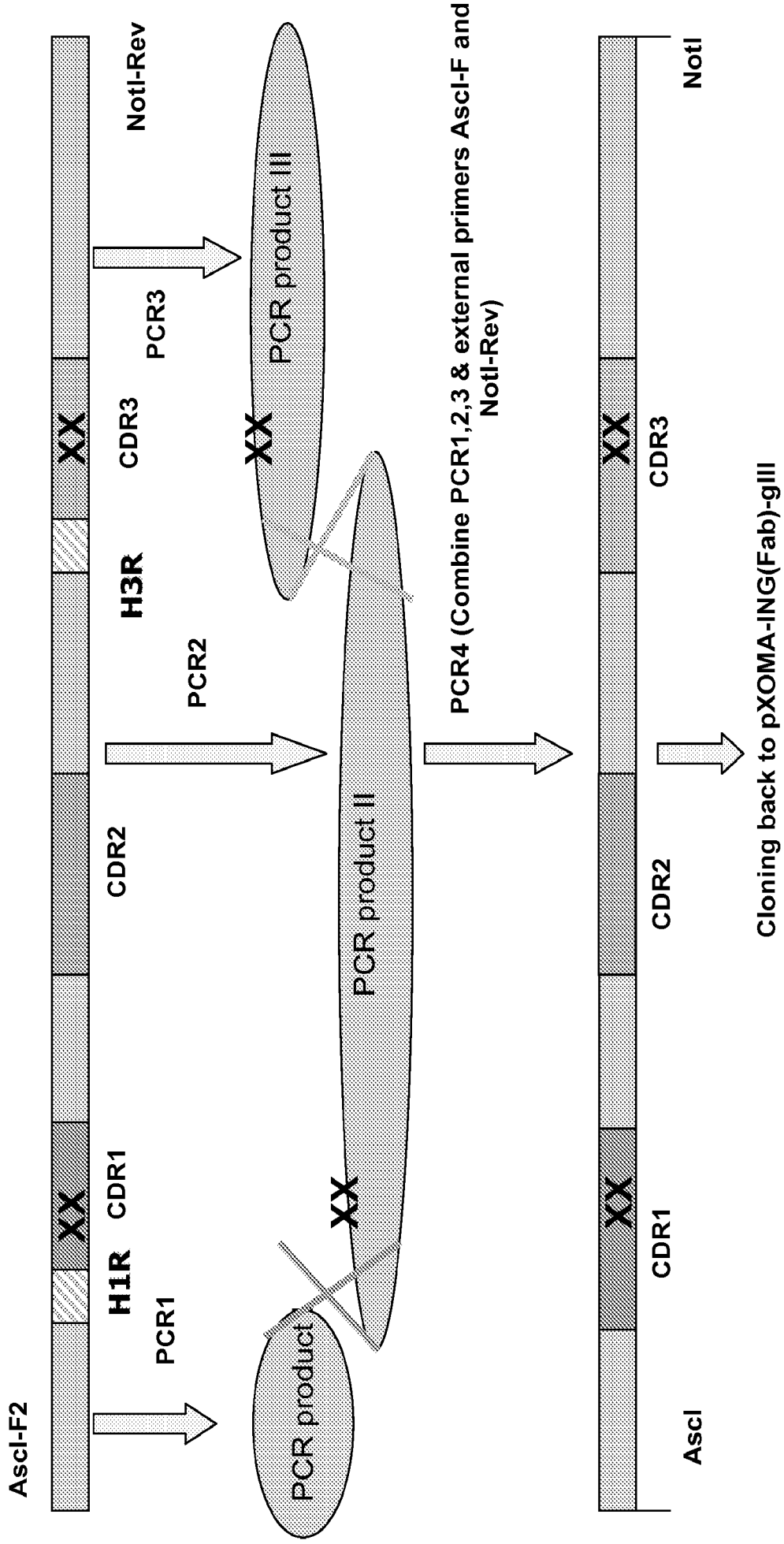

Combination of beneficial ING VH TAE mutations
AscI-F2
XX
CDR1
H1R
PCR1
PCR product
XX
CDR2
H3R
PCR2
PCR product II
XX
CDR3
PCR3
NotI-Rev
PCR product III
PCR4 (Combine PCR1,2,3 & external primers AscI-F and NotI-Rev)
XX
CDR1
XX
CDR2
XX
CDR3
NotI
AscI
Cloning back to pXOMA-ING(Fab)-gIII

Figure 9A

ING-1 KABAT HEAVY CHAIN (KABAT NUMBERING)

```
                                  CDR1                CDR2
1         10         20    26         36             50 52A
QIQLVQSGPELKKPGETVKISCKASGYTFTKYGMNWVKQAPGKGLKWMGWINTYTEEPT
                                              CDR3
       66               82  83           95     103
YGDDFKGRFAFSLETSASTANLQINNLKSEDTATYFCARFGSAVDYWGQGTSVTVSS (SEQ ID NO: 952)
```

ING-1 CHOTHIA HEAVY CHAIN

```
                               CDR1                     CDR2
QIQLVQSGPELKKPGETVKISCKASGYTFTKYGMNWVKQAPGKGLKWMGWINTYTEEPT
                                              CDR3
YGDDFKGRFAFSLETSASTANLQINNLKSEDTATYFCARFGSAVDYWGQGTSVTVSS (SEQ ID NO: 953)
```

ING-1 IMGT HEAVY CHAIN

```
                               CDR1                      CDR2
QIQLVQSGPELKKPGETVKISCKASGYTFTKYGMNWVKQAPGKGLKWMGWINTYTEEPT
                                             CDR3
YGDDFKGRFAFSLETSASTANLQINNLKSEDTATYFCARFGSAVDYWGQGTSVTVSS (SEQ ID NO: 954)
```

ING-1 KABAT LIGHT CHAIN (KABAT NUMBERING)

```
                               CDR1                      CDR2
1                       24 27    28     35               50
QIVMTQAAFSNPVTLGTSGSISCRSSKSLLHSNGITYLYWYLQKPGQSPQLLIYQMS
                                     CDR3
    57                              89                 98
NLASGVPDRFSSSGSGTDFTLRISRVEAEDVGVYYCAQNLELPRTFGGGTKLEMKR (SEQ ID NO: 955)
```

ING-1 CHOTHIA LIGHT CHAIN

```
                               CDR1                      CDR2
QIVMTQAAFSNPVTLGTSGSISCRSSKSLLHSNGITYLYWYLQKPGQSPQLLIYQMS
                                     CDR3
NLASGVPDRFSSSGSGTDFTLRISRVEAEDVGVYYCAQNLELPRTFGGGTKLEMKR (SEQ ID NO: 956)
```

ING-1 IMGT LIGHT CHAIN

```
                                 CDR1                    CDR2
QIVMTQAAFSNPVTLGTSGSISCRSSKSLLHSNGITYLYWYLQKPGQSPQLLIYQMS
                                     CDR3
NLASGVPDRFSSSGSGTDFTLRISRVEAEDVGVYYCAQNLELPRTFGGGTKLEMKR (SEQ ID NO: 957)
```

Figure 9B

XPA23 KABAT HEAVY CHAIN (KABAT NUMBERING)

1 10 26 36 50 52A
EVQLLESGGGLVQPGGSLRLSCAASGFTFS**KYFMF**WVRQAPGKGLEWVS**VISPSGGMTR**
66 82 83 95 103
YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR**VGYGGNSDY**WGQGTLVTVSS (SEQ ID NO: 958)

XPA23 CHOTHIA HEAVY CHAIN

CDR1 CDR2
EVQLLESGGGLVQPGGSLRLSCAAS**GFTFSKY**FMFWVRQAPGKGLEWVSVI**SPSGGMT**R
CDR3
YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR**VGYGGNSDY**WGQGTLVTVSS (SEQ ID NO: 959

XPA23 IMGT HEAVY CHAIN

CDR1 CDR2
EVQLLESGGGLVQPGGSLRLSCAAS**GFTFSKYF**MFWVRQAPGKGLEWVSV**ISPSGGMT**R
CDR3
YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC**ARVGYGGNSDY**WGQGTLVTVSS (SEQ ID NO: 960)

XPA23 KABAT LIGHT CHAIN (KABAT NUMBERING)

CDR1 CDR2
1 24 35 50 57
DIQMTQSPSSVSASVGDRLTIIC**RASQDINRWLA**WYQQTPGNAPKLLIH**SATSLQS**GVP
CDR3
89 98
SRFSGSGSGTDFTLTINSLQPEDFATYYC**QQADSFPLT**FGGGTKVEIKR (SEQ ID NO: 961)

XPA23 CHOTHIA LIGHT CHAIN

CDR1 CDR2
DIQMTQSPSSVSASVGDRLTIIC**RASQDINRWLA**WYQQTPGNAPKLLIH**SATSLQS**GVP
CDR3
SRFSGSGSGTDFTLTINSLQPEDFATYYC**QQADSFPLT**FGGGTKVEIKR (SEQ ID NO: 962)

XPA23 IMGT LIGHT CHAIN

CDR1 CDR2
DIQMTQSPSSVSASVGDRLTIICRAS**QDINRW**LAWYQQTPGNAPKLLIH**SAT**SLQSGVP
CDR3
SRFSGSGSGTDFTLTINSLQPEDFATYYC**QQADSFPLT**FGGGTKVEIKR (SEQ ID NO: 963)

Figure 10A

XPA23 VH

108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
E	**V**	**Q**	**L**	**L**	**E**	**S**	**G**	**G**	**G**	**L**	**V**	**Q**	**P**	**G**	**G**	**S**	**L**	**R**	**L**	**S**	**C**	**A**	**A**

			H1-1		H1-2	H1-3	H12-1	H12-2		H12-3													
25	**26**	**26**	**27**	**27**	**28**	**28**	**29**	**29**	**30**	**30**	**31**	**31**	**32**	**32**	**33**	**33**	**34**	**34**	**35**	**35**	**36**	**36**	**37**
132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155
S	**G**	**F**	**T**	**F**	**S**	**K**	**Y**	**F**	**M**	**F**	**W**	**V**	**R**	**Q**	**A**	**P**	**G**	**K**	**G**	**L**	**E**	**W**	**V**

	H2-1	H2-2	H2-3	H2-4	H2-5	H22-1	H22-2	H22-3	H22-4	H22-5													
156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177		
38	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**		
S	**V**	**I**	**S**	**P**	**S**	**G**	**G**	**M**	**T**	**R**	**Y**	**A**	**D**	**S**	**V**	**K**	**G**	**R**	**F**	**T**	**I**		

178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201
60	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**72**	**73**	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**	**82**	**83**
S	**R**	**D**	**N**	**S**	**K**	**N**	**T**	**L**	**Y**	**L**	**Q**	**M**	**N**	**S**	**L**	**R**	**A**	**E**	**D**	**T**	**A**	**V**	**Y**

				H3-1	H3-2	H3-3	H3-4	H3-5	H32-1	H32-2	H32-3	H32-4											
202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225
84	**85**	**86**	**87**	**88**	**89**	**90**	**91**	**92**	**93**	**94**	**95**	**96**	**97**	**98**	**99**	**100**	**101**	**102**	**103**	**104**	**105**	**106**	**107**
Y	**C**	**A**	**R**	**V**	**G**	**Y**	**G**	**G**	**N**	**S**	**D**	**Y**	**W**	**G**	**Q**	**G**	**T**	**L**	**V**	**T**	**V**	**S**	**S**

Figure 10B

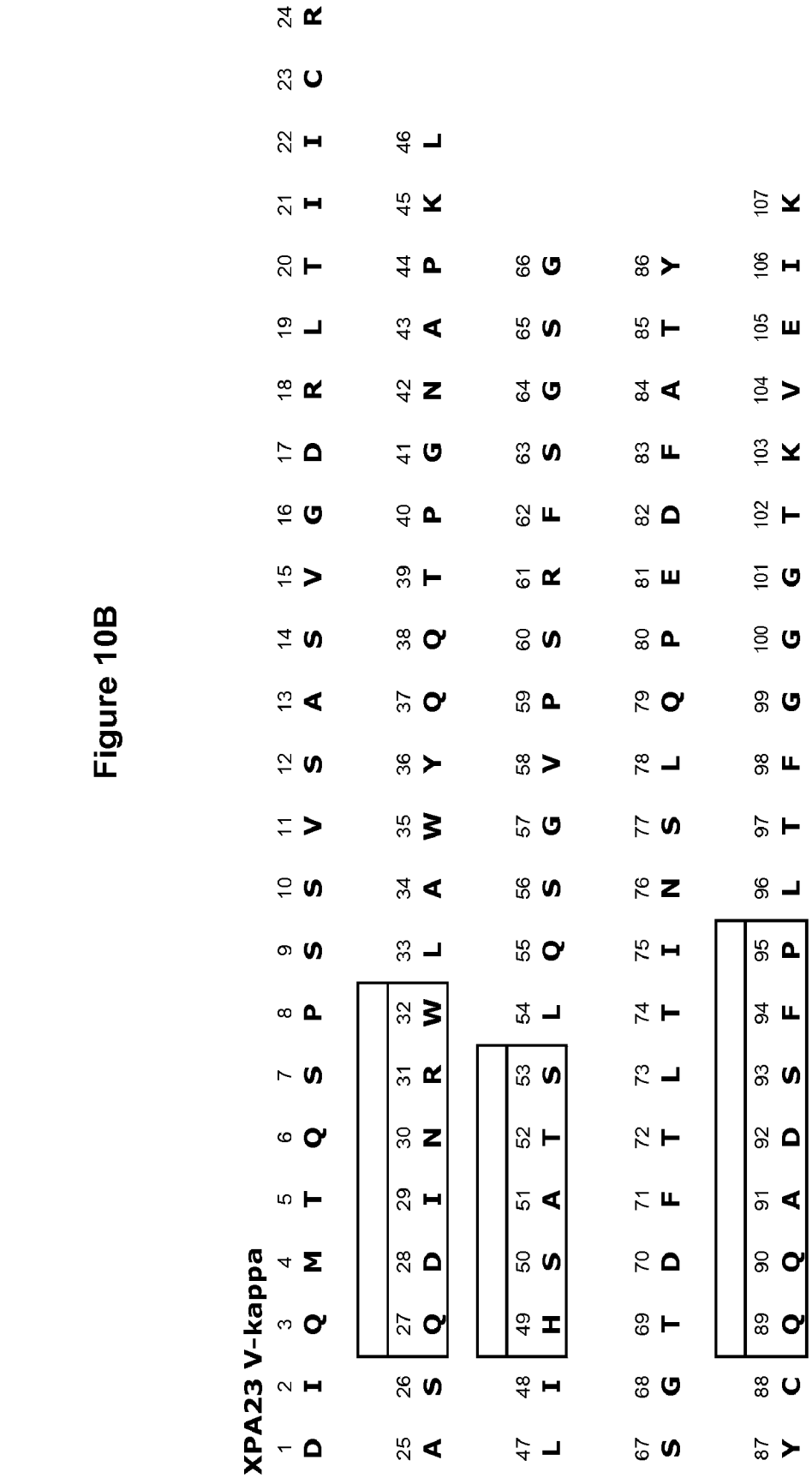

XPA23 V-kappa
1 D 2 I 3 Q 4 M 5 T 6 Q 7 S 8 P 9 S 10 S 11 V 12 S 13 A 14 S 15 V 16 G 17 D 18 R 19 L 20 T 21 I 22 I 23 C 24 R
25 A 26 S 27 Q 28 D 29 I 30 N 31 R 32 W 33 L 34 A 35 W 36 Y 37 Q 38 Q 39 T 40 P 41 G 42 N 43 A 44 P 45 K 46 L
47 L 48 I 49 H 50 S 51 A 52 T 53 S 54 L 55 Q 56 S 57 G 58 V 59 P 60 S 61 R 62 F 63 S 64 G 65 S 66 G
67 S 68 G 69 T 70 D 71 F 72 T 73 L 74 T 75 I 76 N 77 S 78 L 79 Q 80 P 81 E 82 D 83 F 84 A 85 T 86 Y
87 Y 88 C 89 Q 90 Q 91 A 92 D 93 S 94 F 95 P 96 L 97 T 98 F 99 G 100 G 101 G 102 T 103 K 104 V 105 E 106 I 107 K

Figure 10C

Figure 10D

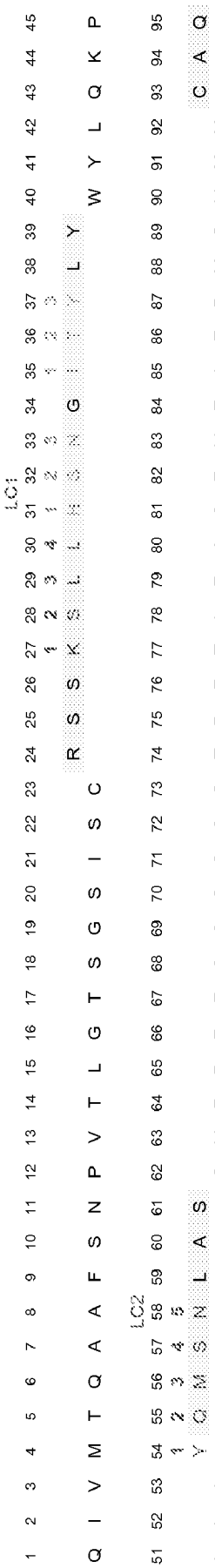

LC1
LC2
Q I V M T Q A A F S N P V T L G T S G G S I S C
R S S K S L L H S N G I T Y L Y W Y L Q K P
L L I Y C M S N L A S G V P D R F S S S G S G T D F T L R I S R V E A E D V G V Y Y C A Q

Figure 11

ING-1 Heavy Chain k_{off} Map

HCDR1	k_{off} fold-improvement
T28V	2.08
T28I	2.45
T28P	2.16
T30Y	2.00
G33F	7.19
G33L	2.27
G33P	6.31

HCDR2	k_{off} fold-improvement
W50D	3.27
T53V	2.44
T53I	11.40
T53A	9.03
Y54R	3.72
Y54K	3.62
Y54N	2.11
Y54G	3.72
T59W	2.24

HCDR3	k_{off} fold-improvement
G100R	7.51
S101K	2.20
S101Q	2.18
S101V	1.92
S101I	3.53
S101G	3.31
A102R	2.18
A102H	2.40
A102Y	3.01
A102W	3.13
A102F	2.97
A102G	3.58

Figure 12

ING-1 Light chain k_{off} Map

LCDR1	k_{off} fold-improvement
S28R	2.78
S28K	2.32
S28H	1.90
S28Y	2.02
S28F	2.38
S28Q	1.99
S28V	2.15
S28I	2.35
S28L	2.60
S28P	2.42
L29S	2.03
L29A	1.97
H31Y	2.16
H31T	1.94
Y37H	4.07

LCDR2	k_{off} fold-improvement
Y54K	3.62
Y54L	3.44
Q55R	5.31
Q55H	3.82
Q55W	4.11
S57W	2.34
N58W	1.99
N58V	2.84
N58I	2.51
N58P	3.47

LCDR3	k_{off} fold-improvement
L97I	2.62
E98R	3.08
E98K	2.22
E98T	4.90
E98S	2.21
E98L	2.82
L99I	2.07
P100Y	1.94

Figure 13

XPA23 Heavy chain mutations with 2-fold koff improvement

HCDR1	k_{off} fold-improvement
K31Y	2.5
K31W	3.6
K31L	5.4
K31P	4.6
K31H	6.5

HCDR2	k_{off} fold-improvement
S54K	5.0
G56L	2.2
G56Q	3.2
G56I	4.4
G56K	4.8
G56R	4.8

HCDR3	k_{off} fold-improvement
Y101L	4.9
G103V	3.9
N104A	3.4
N104V	2.6
S105E	2.2
S105P	3.7

Figure 14

XPA23 Light chain mutations with >2-fold koff improvement

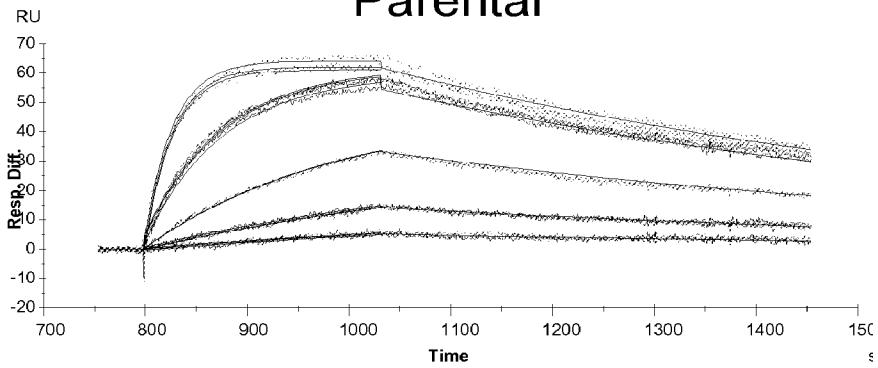
LCDR1	k_{off} fold-improvement
Q27S	3.3
Q27F	3.7
Q27G	4.2
D28L	7.8
D28S	5.2
D28W	3.8

LCDR2	k_{off} fold-improvement
A51G	3.8
S53K	4.1
S53R	3.3

LCDR3	k_{off} fold-improvement
D92S	6.6
S93D	4.6
S93E	3.7
P95S	4.0
P95A	3.9
L96W	5.5

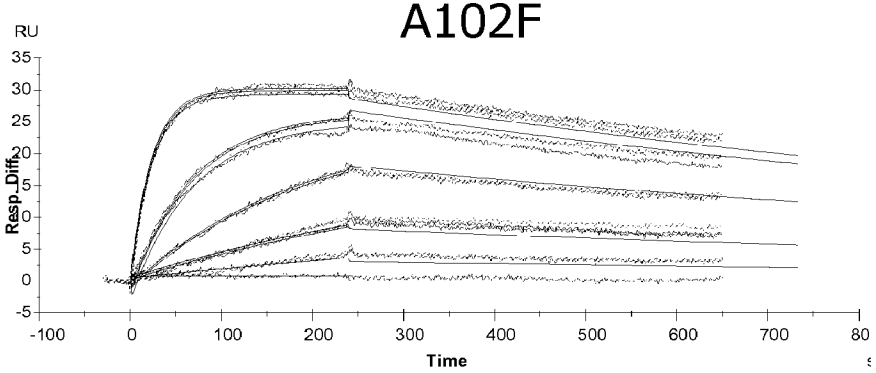
Figure 15

A.



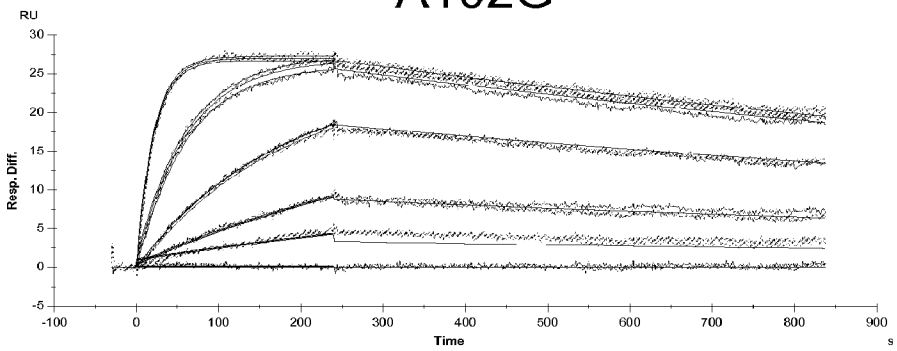
KD= 3 nM

B.



0.9 nM

C.



0.6 nM

D.

	K-on IgG	K-off IgG	KD IgG	Fold Improvement
Ch-ING-1 Fab/IgG (Parental)	4.9e+5	2.0e-3	3.1e-9	
A102F	7.7e+5	7.6e-4	9.9e-10	3.1
A102G	8.8e+5	5.3e-4	6.0e-10	5.1

Figure 16

TAE (ING-1 LC)

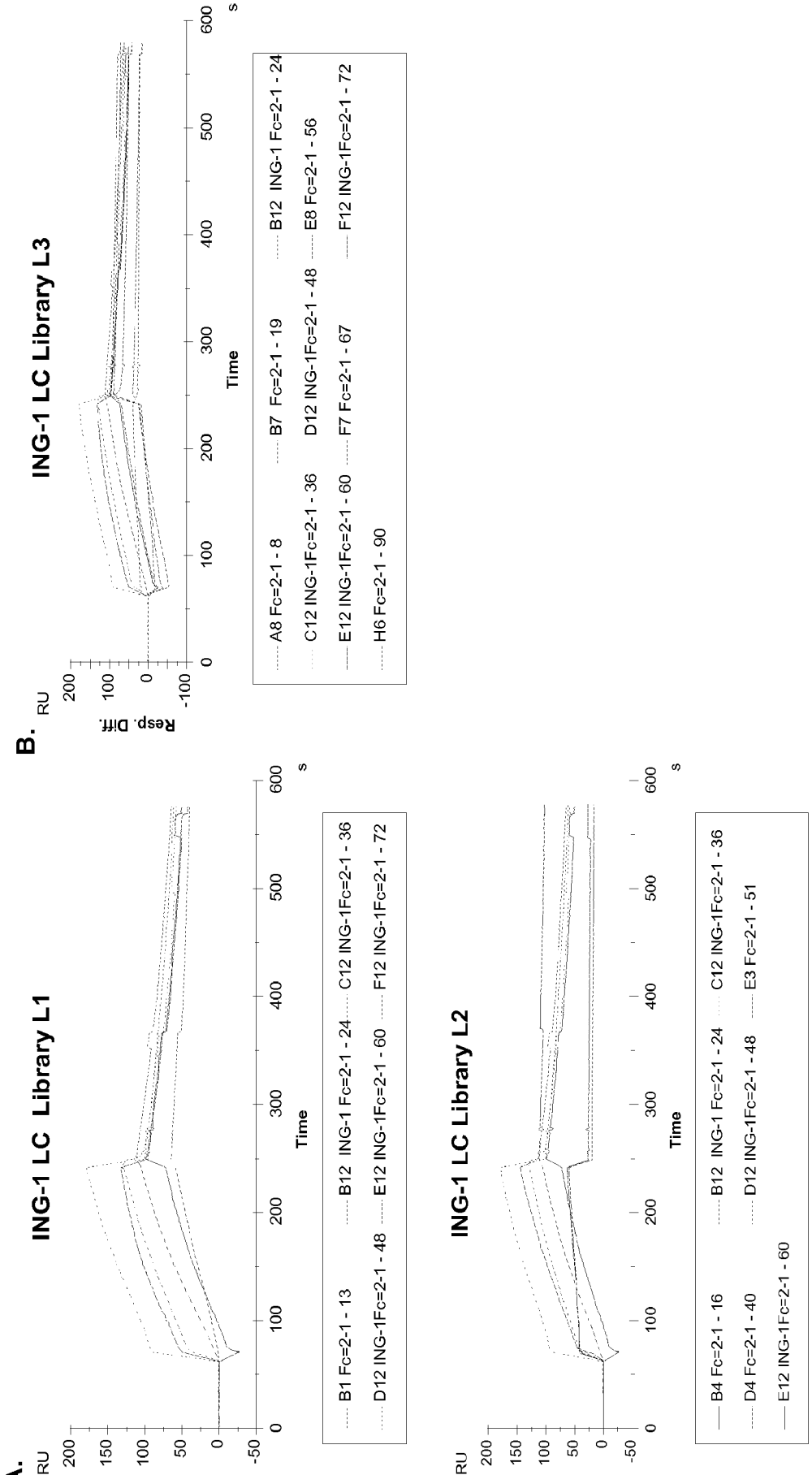


Figure 17

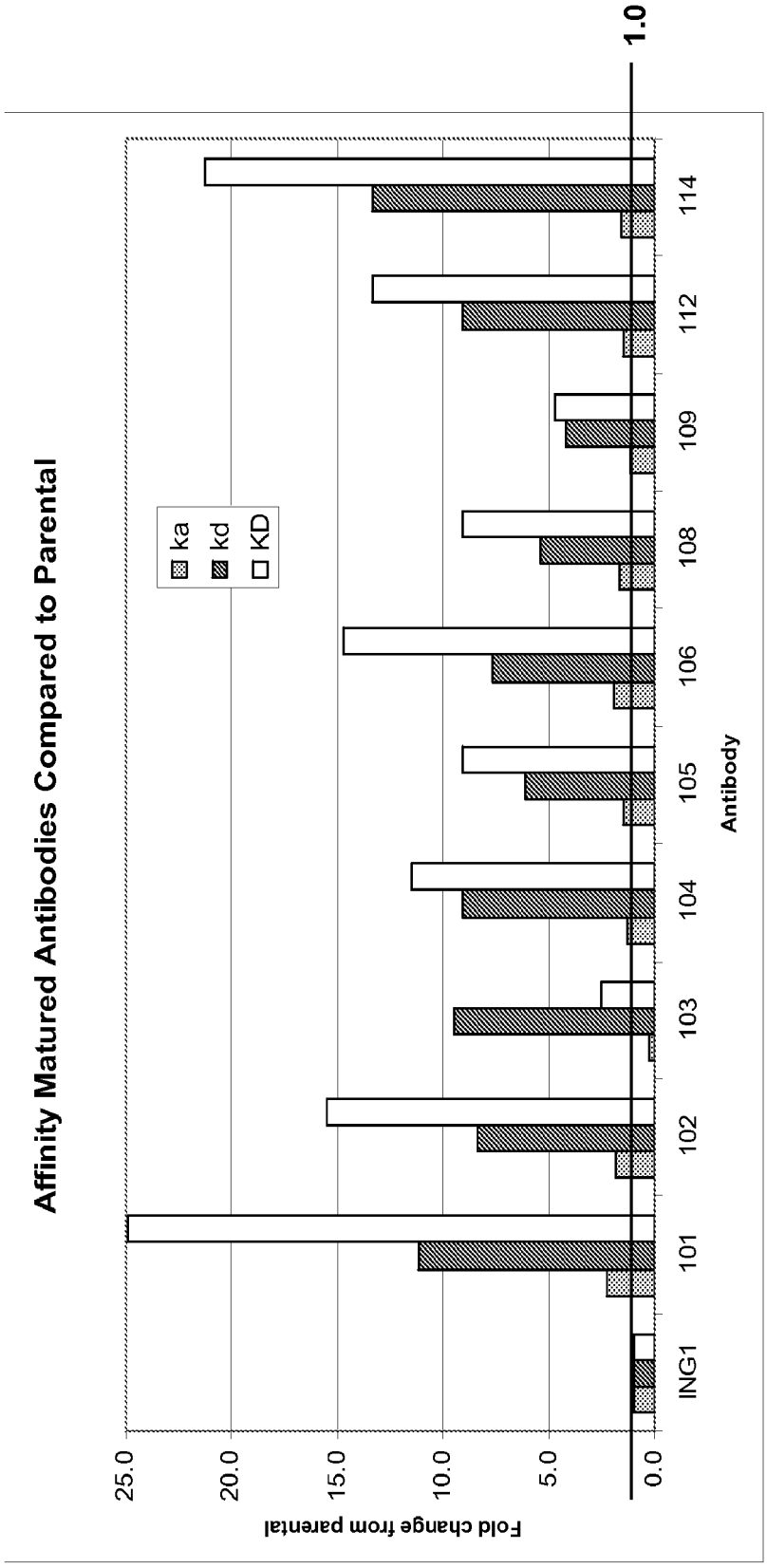

Affinity Matured Antibodies Compared to Parental
ka
kd
KD
1.0
25.0
20.0
15.0
10.0
5.0
0.0
Fold change from parental
ING1
101
102
103
104
105
106
108
109
112
114
Antibody

Figure 18

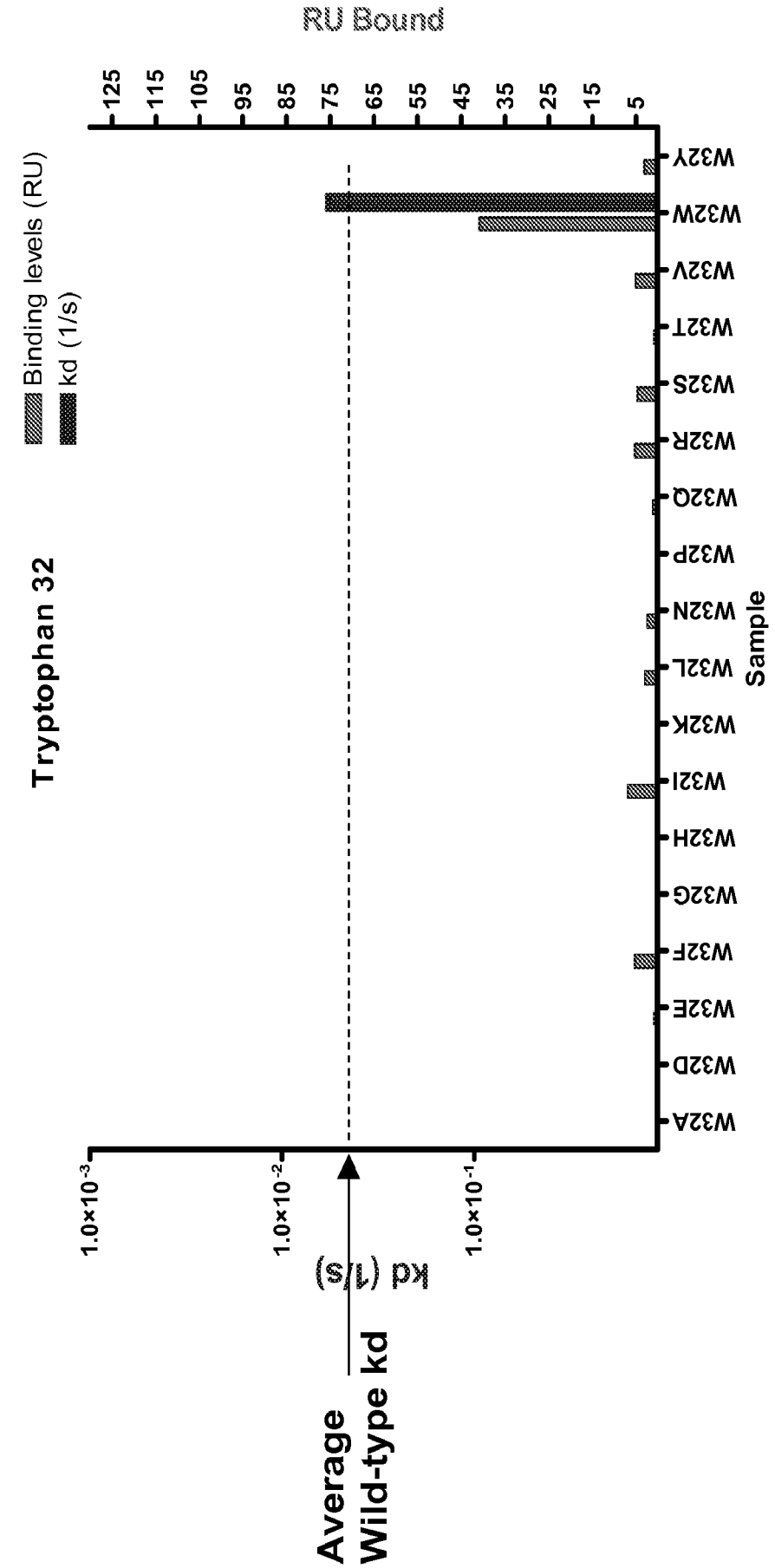

RU Bound
125
115
105
95
85
75
65
55
45
35
25
15
5
Binding levels (RU)
kd (1/s)
Tryptophan 32
W32Y
W32W
W32V
W32T
W32S
W32R
W32Q
W32P
W32N
W32L
W32K
W32I
W32H
W32G
W32F
W32E
W32D
W32A
Sample
1.0×10-3
1.0×10-2
1.0×10-1
kd (1/s)
Average
Wild-type kd

Figure 19

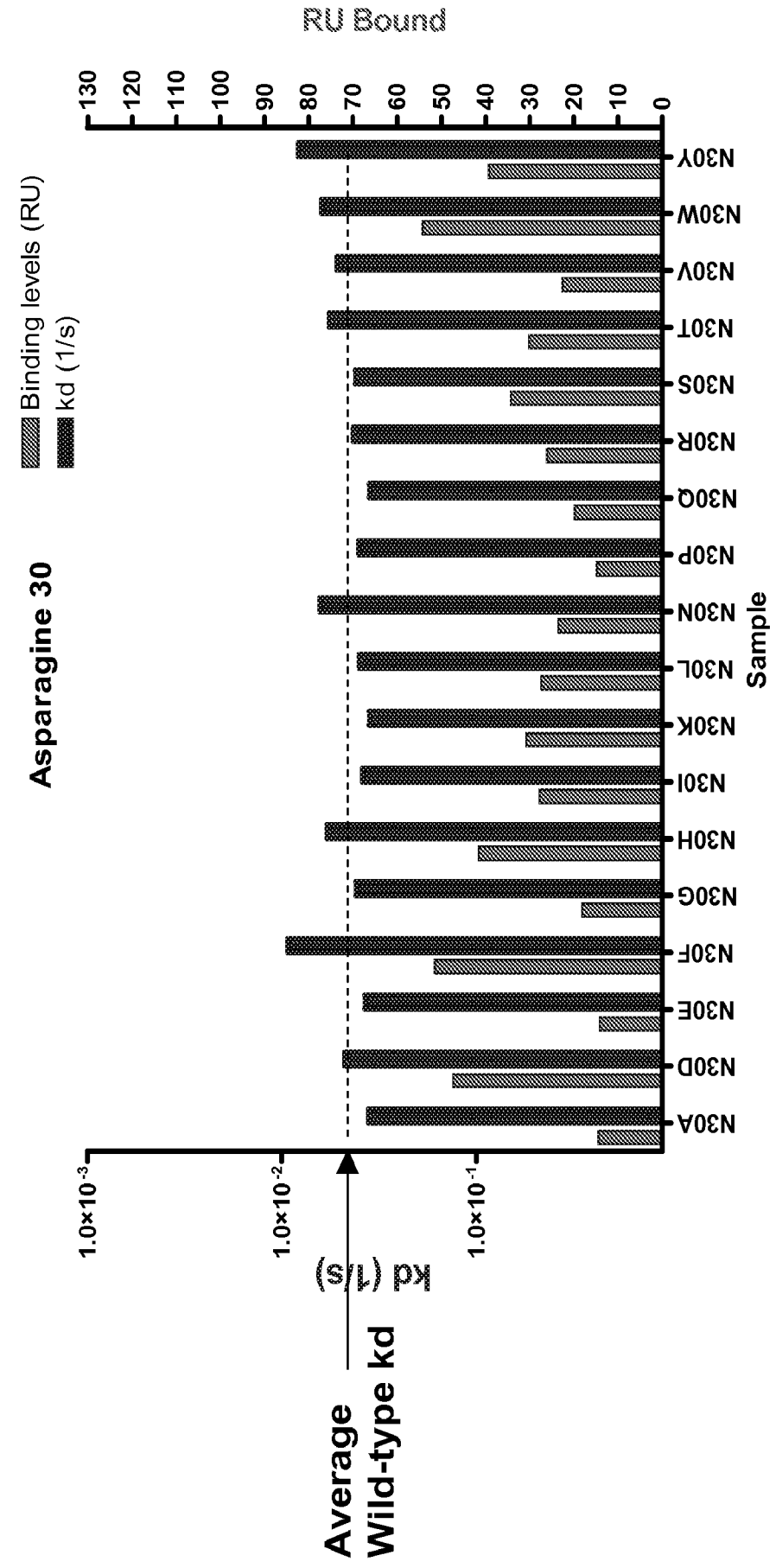
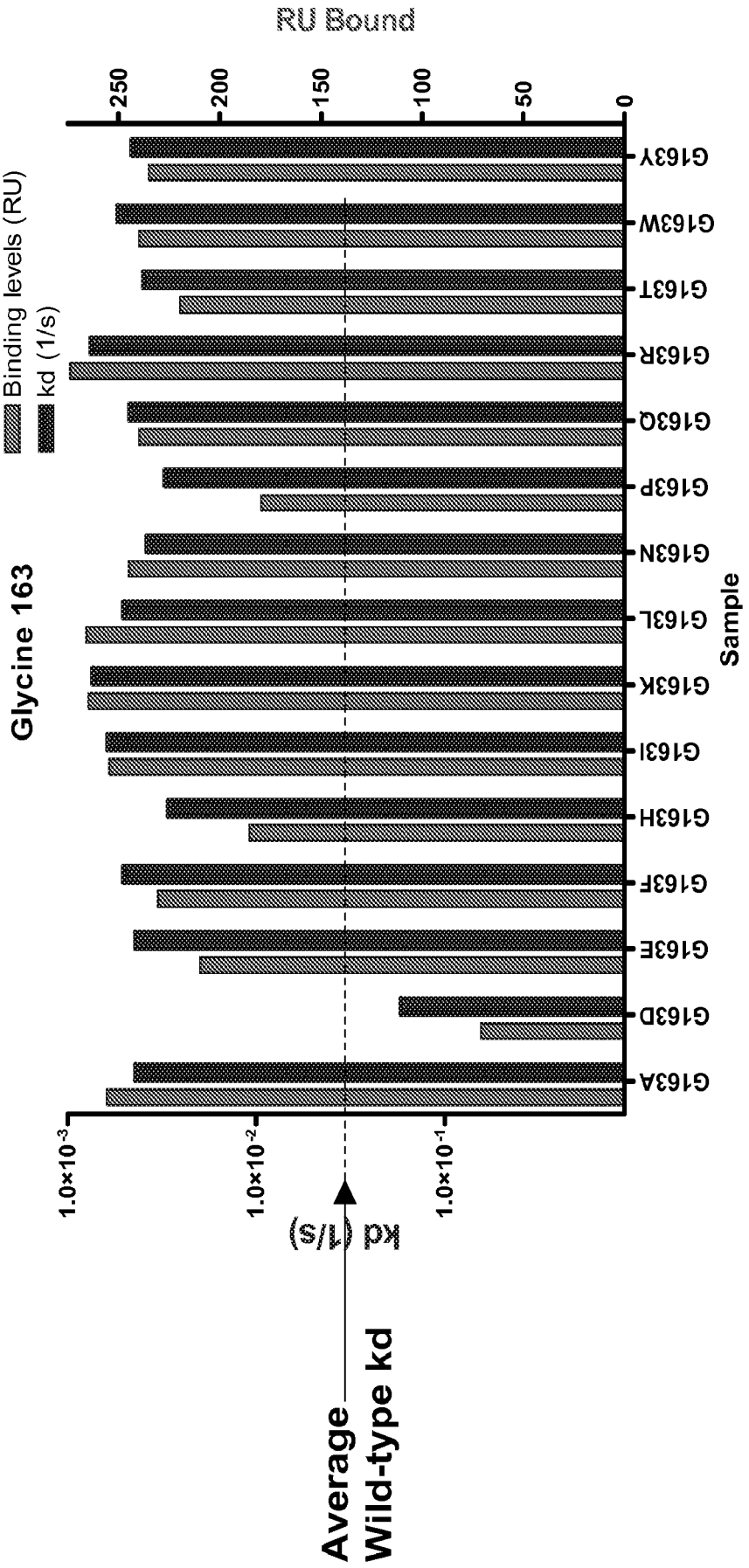

RU Bound
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Binding levels (RU)
kd (1/s)
Asparagine 30
N30Y
N30W
N30V
N30T
N30S
N30R
N30Q
N30P
N30N
N30L
N30K
N30I
N30H
N30G
N30F
N30E
N30D
N30A
Sample
1.0×10-3
1.0×10-2
1.0×10-1
kd (1/s)
Average
Wild-type kd

Figure 20


RU Bound
250
200
150
100
50
0
Binding levels (RU)
kd (1/s)
Glycine 163
G163Y
G163W
G163T
G163R
G163Q
G163P
G163N
G163L
G163K
G163I
G163H
G163F
G163E
G163D
G163A
Sample
1.0×10-3
1.0×10-2
1.0×10-1
kd (1/s)
Average
Wild-type kd

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/088651

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/10

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C07K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
X	AKANUMA SATOSHI ET AL: "Combinatorial mutagenesis to restrict amino acid usage in an enzyme to a reduced set" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC.; US, vol. 99, no. 21, 15 October 2002 (2002-10-15), pages 13549-13553, XP002437014 ISSN: 0027-8424	1,2,4, 28-31, 33,109, 110,112, 135,136, 138, 161-163, 165,187, 191, 193-195, 197,217, 218,220, 243,245
Y	the whole document	1-246
X	US 2006/024308 A1 (CREA ROBERTO [US] ET AL) 2 February 2006 (2006-02-02) figures 2,3	1,2, 28-31

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 March 2009	17/04/2009

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

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/53744 A (DIVERSA CORP [US]) 14 September 2000 (2000-09-14) tables 1,16-24,26,28-41	1,2, 28-31
Y	TOMANDL D AND SCHWIENHORST A: ""Computer-assisted Design of Doped Libraries" in Evolutionary methods in biotechnology" 2004, WILEY-VCH VERLAG , WEINHEIM , XP002520940 ISBN: 3-527-30799-0 the whole document	1-246
A	MAGLIERY, T: "Mixed Codons Worksheet"[Online] 23 July 2003 (2003-07-23), XP002520936 Retrieved from the Internet: URL:http://www.chemistry.ohio-state.edu/{magliery/mixedcodons/mixed_codons_worksheet_v1.zip> [retrieved on 2009-03-23]	
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	LARSSON OLA ET AL: "Quantitative codon optimisation of DNA libraries encoding sub-random peptides: design and characterisation of a novel library encoding transmembrane domain peptides." NUCLEIC ACIDS RESEARCH 1 DEC 2002, vol. 30, no. 23, 1 December 2002 (2002-12-01), page e133, XP002520939 ISSN: 1362-4962	
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2008/088651

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