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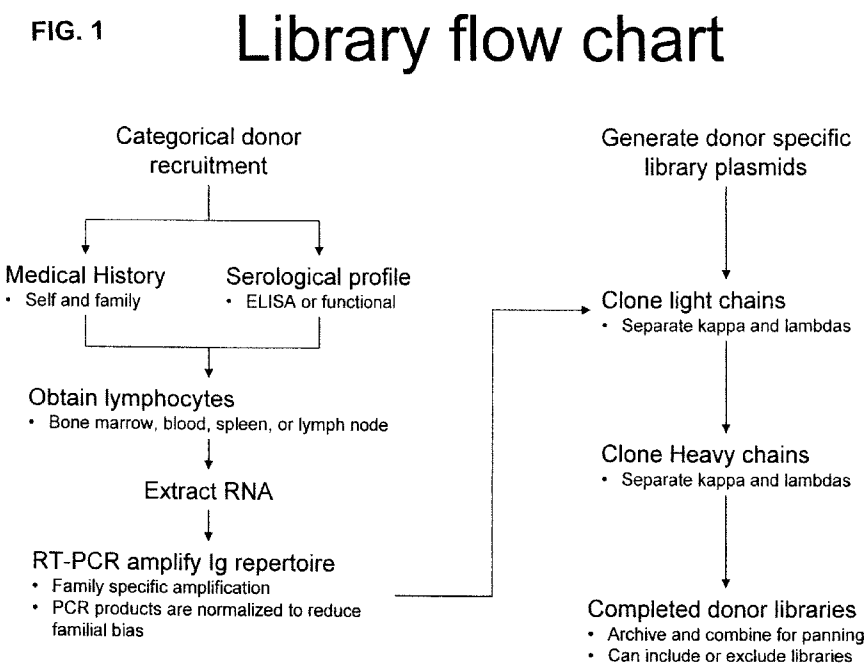
(54) **Title:** DONOR SPECIFIC ANTIBODY LIBRARIES

FIG. 1



(57) **Abstract:** The present application concerns donor-specific antibody libraries derived from a patient donor who has suffered from, or is suffering from one or more diseases discussed herein. The present application also concerns the method of making and using the donor-specific antibodies. The present application further concerns the neutralizing antibodies obtained from the donor-specific antibody libraries and the methods of using these antibodies for the prevention/treatment of human disease.

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DONOR SPECIFIC ANTIBODY LIBRARIES

Field of the Invention

The present invention concerns donor-specific antibody libraries and methods of making and using thereof. The present invention also concerns neutralizing antibodies obtained from such donor-specific antibody libraries and methods of using the antibodies obtained for the prevention and/or treatment of various human diseases and conditions.

Background of the Invention

The generation and identification of specific agents for the diagnosis, prevention, and treatment of human diseases requires access to vast collections of useful chemistries. With the advent and rapid development of a variety of techniques for the creation and screening of antibody libraries, monoclonal antibodies against disease targets have become one of the major categories of new drug candidates. Since for human use, in addition to specificity and efficacy, safety is of primary concern, libraries of human monoclonal antibodies have become of particular importance.

At present, the development of human antibody-based drug candidates are typically identified by screening of human antibody libraries comprising a random collection of antibody sequences from human repertoires that are typically unrelated to their intended application or applications. Each antibody library created from a specific human donor potentially contains antibodies to every component, physiology, and metabolic alteration stemming from, or creating, every unique challenge that the donor has encountered, challenged, and surmounted over the course of that individual's lifetime. As typical human antibody libraries made with the current approaches are constructed without the knowledge of the health history of donors, little is known of what would be expected in the resulting immunoglobulin repertoires.

Thus, it is of great interest to create antibody libraries from individuals who have successfully survived or are surviving an encounter with specific diseases because their resulting repertoires include antibodies that were used by the donor to defend specifically against a relevant disease. It is also important to provide methods for the efficient screening and handling of such libraries, including the ability to remove or isolate negative or positive elements, eliminate undesirable content, and produce human antibodies with improved properties.

The present invention addresses these needs by providing methods and means for the creation, screening and handling of donor-specific antibody libraries from individuals who have been exposed to and survived or are surviving an encounter with a specific target disease.

Summary of the Invention

In one aspect, the invention concerns a vector collection comprising a repertoire of nucleic acid molecules encoding antibody light or heavy chains or fragments thereof, derived from a human patient donor who has suffered from, or is suffering from, a disease evoking antibody production to a target antigen, wherein the collection is identified with a unique barcode.

In one embodiment, the vector collection comprises a repertoire of nucleic acid molecules encoding antibody light chains or fragments thereof, such as antibody λ light chains, or antibody κ light chains, or fragments thereof.

In another embodiment, the vector collection comprises a repertoire of nucleic acid molecules encoding antibody heavy chains or fragments thereof.

In yet another embodiment, the barcode is a nucleotide sequence linked to or incorporated in the vectors present in the collection, and/or linked to or incorporated in the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof such that it does not interfere with the expression of the nucleic acid molecules.

Thus, the barcode may be contiguous non-coding nucleotide sequence of one to about 24 nucleotides, which may, for example, be linked to the 3' or 5' non-coding region of the nucleic acid molecules.

In a further embodiment, the barcode is a nucleotide sequence that is a coding sequence of one or more silent mutations incorporated into the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof.

In a still further embodiment, the barcode is a non-contiguous nucleotide sequence. At least part of the non-contiguous nucleotide sequence may be linked to or incorporated in the vectors present in the collection. Alternatively, at least part of the non-contiguous nucleotide sequence may be incorporated into the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof such that it does not interfere with the expression of said nucleic acid molecules.

In another embodiment, the barcode is a peptide or polypeptide sequence.

In a different embodiment, the vectors present in the vector collection are phagemid vectors, which may, for example, contain a bacteriophage gene III and a stop codon between the nucleic acid molecules encoding antibody light or heavy chains or fragments thereof and the bacteriophage III gene, and may have a barcode, such as a non-coding contiguous nucleotide sequence inserted in the untranslated region following the stop codon.

In another aspect, the invention concerns host cells comprising the vector collection of the present invention. The host cells may by eukaryotic or prokaryotic host cells, such as, for example, *E. coli* cells.

In a further aspect, the invention concerns a donor-specific antibody library comprising library members expressing a collection of antibodies or antibody fragments to a target antigen wherein the antibodies or antibody fragments are derived from a human donor who has suffered from, or is suffering from, a disease evoking antibody production to said target antigen, wherein said antibody library is identified with at least one unique barcode.

In one embodiment, the antibody heavy and light chains are separately identified each with a barcode unique to the human donor from whom it derived.

In another embodiment, the donor-specific antibody library is identified with one unique barcode.

In yet another embodiment, the antibodies or antibody fragments are composed of antibody heavy and light chains or fragments thereof encoded by nucleic acid molecules present in a vector.

In a further embodiment, the barcode is a nucleotide sequence linked to or incorporated in the vectors present in the library, and/or linked to or incorporated in the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof such that it does not interfere with the expression of the nucleic acid molecules.

In a still further embodiment, the barcode is a contiguous non-coding nucleotide sequence of one to about 24 nucleotides, which may, for example, be linked to the 3' or 5' non-coding region of the nucleic acid molecules.

In a different embodiment, the barcode is encoded by a coding sequence of one or more silent mutations incorporated into the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof.

In another embodiment, the barcode is encoded by a non-contiguous nucleotide sequence.

In a further embodiment, a least part of the non-contiguous sequence encoding the barcode is linked to or incorporated in the vectors present in the library.

In a still further embodiment, at least part of the non-contiguous sequence encoding the barcode is incorporated into the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof such that it does not interfere with the expression of such nucleic acid molecules.

In different embodiment, the barcode is a peptide or polypeptide sequence.

In another embodiment, vectors are phagemid vectors, may, for example, contain a bacteriophage gene III and a stop codon between the nucleic acid molecules encoding antibody light or heavy chains or fragments thereof and the bacteriophage III gene.

In yet another embodiment, the medical history of the human patient donor shows that the donor has suffered from, or is suffering from said disease. In some embodiments, it is independently confirmed that the human donor suffered from, or is suffering from the disease.

In an additional embodiment, the donor-specific antibody library is substantially devoid of antibodies and antibody fragments specifically binding antigens different from said target antigen.

In one embodiment, the target antigen is an influenza A virus, such as an isolate of influenza A virus H1, H2, H3, H5, H7, or H9 subtype.

In another embodiment, the library expresses at least one antibody or antibody fragment specifically binding to more than one influenza A virus subtype.

In yet another embodiment, the library expresses at least one antibody or antibody fragment binding to and neutralizing the H5N1 subtype of influenza virus A.

In a further embodiment, the human donor has suffered from, or is suffering from a disease selected from the group consisting of the diseases listed in Table 1 below.

In a still further embodiment, the antibody library expresses at least one antibody or antibody fragment binding to an antigen associated with the target disease.

In an additional embodiment, the antibody library expresses at least one antibody or antibody fragment binding to and neutralizing an antigen associated with the target disease.

The donor-specific antibody library may, for example, be a phage library, in an embodiment, it may contain sequences encoding more than 10^6 different members of antibodies or antibody fragments, or more than 10^9 different members of antibodies or antibody fragments.

In other embodiments, the donor-specific antibody library is, without limitation, a spore-display library, a ribosome display library, a mRNA display library, a microbial cell display library, a yeast display library, or a mammalian display library.

In an embodiment, the nucleic acid encoding the antibodies or antibody fragments present in the library is reverse-transcribed from mRNA extracted from lymphocytes of the human patient donor, where the lymphocytes may, for example, originate from from bone marrow, blood, spleen, or lymph node.

If desired, a serological profile of said human donor may be generated prior to extraction of said mRNA.

Alternatively, or in addition, the medical history of the human donor, and optionally the donor's family, is examined prior to or following extraction of the mRNA.

In another aspect, the invention concerns a method of making a donor-specific library expressing a collection of antibodies or antibody fragments to a target antigen, comprising the steps of:

a) obtaining mRNA from lymphocytes of a human patient donor who has suffered from, or who is suffering from a disease evoking antibody production to said target antigen;

b) generating a collection of nucleic acids comprising sequences encoding an immunoglobulin repertoire of the patient by reverse transcription of said obtained mRNA; and

c) identifying the donor-specific library with an unique barcode labeling said nucleic acids.

The method may further comprise steps of generating a serological profile of said patient and/or examining medical history of the patient prior or subsequent to step a).

The method may further comprise the steps of d) inserting said nucleic acids into expression vector; e) expressing the immunoglobulin repertoire; and f) displaying the immunoglobulin repertoire in a display system.

In another embodiment, the method further comprises the step of selecting members of the library based their ability to neutralize or activate the target antigen.

In a still further embodiment, the method yields at least one neutralizing antibody.

In another embodiment, the method further comprises the step of creating one or more sub-libraries comprising library members that were found to neutralize or activate the target antigen.

In yet another embodiment, the method comprises the step of sequencing at least one library member identified.

In a further aspect, the invention concerns a method of treating or preventing a disease associated with a target antigen neutralized or activated by an antibody selected by

the method described above, comprising administering to a human patient in need an effective amount of the antibody selected.

The antibody may, for example, be a neutralizing antibody, such as a neutralizing antibody to at least one influenza A virus subtype.

In another embodiment, the disease is selected from the group consisting of the diseases listed in Table 1 below.

Brief Description of the Drawings

Figure 1 is a flow chart schematically illustrating a typical method for the creation of the human antibody libraries of the present invention.

Figure 2 illustrates a typical panning enrichment scheme for increasing the reactive strength towards two different targets, A and B. Each round of enrichment increases the reactive strength of the pool towards the individual target(s).

Figure 3 illustrates a strategy for the selection of clones cross-reactive with targets A and B, in which each successive round reinforces the reactive strength of the resulting pool towards both targets.

Figure 4 illustrates a strategy for increasing the reactive strengths towards two different targets (targets A and B), by recombining parallel discovery pools to generate/increase cross-reactivity. Each round of selection of the recombined antibody library increases the reactive strength of the resulting pool towards both targets.

Figure 5 illustrates a strategy for increasing cross-reactivity to a target B while maintaining reactivity to a target A. First, a clone reactive with target A is selected, then a mutagenic library of the clones reactive with target A is prepared, and selection is performed as shown, yielding one or more antibody clones that show strong reactivity with both target A and target B.

Figure 6 illustrates a representative mutagenesis method for generating a diverse multifunctional antibody collection by the "destinational mutagenesis" method.

Figure 7 shows the amino acid sequences of 15 known hemagglutinin (H) protein subtypes.

Figure 8 shows the H5 hemagglutinin (HA) serology results for blood samples obtained from six human survivors of a Turkish H5N1 bird flu outbreak. The data demonstrate the presence of antibodies to the HA antigen.

Figure 9 shows serology results obtained with serum samples of twelve local donors, tested on H5 antigen (A/Vietnam/1203/2004) and H1N1 (A/New Caledonia/ 20/99) and H3N2 (A/Panama/2007/99) viruses.

Figure 10 illustrates the unique barcoding approach used in the construction of antibody phage libraries.

Figure 11 shows the analysis of antibody binding to hemagglutinins from different influenza A subtypes.

Figure 12 shows the positions of H5 hemagglutinin binding Group 1 required and dominant mutations on the crystal structure at Fab 47e.

Figures 13 and 14 illustrate the use of destinational mutagenesis to create diverse antibody heavy and light chain libraries using the antibody heavy and light chain sequences identified by analysis of sera and bone marrow of Turkish bird flu survivors.

Detailed Description

A. Definitions

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton *et al.*, *Dictionary of Microbiology and Molecular Biology* 2nd ed., J. Wiley & Sons (New York, NY 1994), provides one skilled in the art with a general guide to many of the terms used in the present application.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

The phrase "conserved amino acid residues" is used to refer to amino acid residues that are identical between two or more amino acid sequences aligned with each other.

The terms "disease," "disorder" and "condition" are used interchangeably herein, and refer to any disruption of normal body function, or the appearance of any type of pathology. The etiological agent causing the disruption of normal physiology may or may not be known. Furthermore, although two patients may be diagnosed with the same disorder, the particular symptoms displayed by those individuals may or may not be identical.

An "effective amount" is an amount sufficient to effect beneficial or desired therapeutic (including preventative) results. An effective amount can be administered in one or more administrations.

A "composition," as used herein, is defined as comprising an active ingredient, such as a neutralizing antibody generated from the present invention, and at least one additive, such as a pharmaceutically acceptable carrier, including, without limitation, water, minerals, proteins, and/or other excipients known to one skilled in the art.

As used herein, the term "treating" or "treatment" is intended to mean an amelioration of a clinical symptom indicative of a disease.

As used herein, the term "preventing" or "prevention" is intended to mean a forestalling of a clinical symptom indicative of a disease

The terms "subject" and "patient," as used herein, are used interchangeably, and can refer to any to animal, and preferably a mammal, that is the subject of an examination, treatment, analysis, test or diagnosis. Thus, subjects or patients include humans, non-human primates and other mammals, who may or may not have a disease or other pathological condition.

The term "amino acid" or "amino acid residue" typically refers to an amino acid having its art recognized definition such as an amino acid selected from the group consisting of: alanine (Ala); arginine (Arg); asparagine (Asn); aspartic acid (Asp); cysteine (Cys); glutamine (Gln); glutamic acid (Glu); glycine (Gly); histidine (His); isoleucine (Ile): leucine (Leu): lysine (Lys); methionine (Met); phenylalanine (Phe); proline (Pro); serine (Ser); threonine (Thr); tryptophan (Trp); tyrosine (Tyr); and valine (Val) although modified, synthetic, or rare amino acids may be used as desired. Thus, modified and unusual amino acids listed in 37 CFR 1.822(b)(4) are included within this definition and expressly incorporated herein by reference. Amino acids can be subdivided into various sub-groups. Thus, amino acids can be grouped as having a nonpolar side chain (*e.g.*, Ala, Cys, Ile, Leu, Met, Phe, Pro, Val); a negatively charged side chain (*e.g.*, Asp, Glu); a positively charged side chain (*e.g.*, Arg, His, Lys); or an uncharged polar side chain (*e.g.*, Asn, Cys, Gln, Gly, His, Met, Phe, Ser, Thr, Trp, and Tyr). Amino acids can also be grouped as small amino acids (Gly, Ala), nucleophilic amino acids (Ser, His, Thr, Cys), hydrophobic amino acids (Val, Leu, Ile, Met, Pro), aromatic amino acids (Phe, Tyr, Trp, Asp, Glu), amides (Asp, Glu), and basic amino acids (Lys, Arg).

The term "variant" with respect to a reference polypeptide refers to a polypeptide that possesses at least one amino acid mutation or modification (i.e., alteration) as compared to a native polypeptide. Variants generated by "amino acid modifications" can be produced, for example, by substituting, deleting, inserting and/or chemically modifying at least one amino acid in the native amino acid sequence.

An "amino acid modification" refers to a change in the amino acid sequence of a predetermined amino acid sequence. Exemplary modifications include an amino acid substitution, insertion and/or deletion.

An "amino acid modification at" a specified position, refers to the substitution or deletion of the specified residue, or the insertion of at least one amino acid residue adjacent to the specified residue. By insertion "adjacent" to a specified residue is meant insertion within one to two residues thereof. The insertion may be N-terminal or C-terminal to the specified residue.

An "amino acid substitution" refers to the replacement of at least one existing amino acid residue in a predetermined amino acid sequence with another different "replacement" amino acid residue. The replacement residue or residues may be "naturally occurring amino acid residues" (i.e. encoded by the genetic code) and selected from the group consisting of: alanine (Ala); arginine (Arg); asparagine (Asn); aspartic acid (Asp); cysteine (Cys); glutamine (Gln); glutamic acid (Glu); glycine (Gly); histidine (His); isoleucine (Ile): leucine (Leu); lysine (Lys); methionine (Met); phenylalanine (Phe); proline (Pro); serine (Ser); threonine (Thr); tryptophan (Trp); tyrosine (Tyr); and valine (Val). Substitution with one or more non-naturally occurring amino acid residues is also encompassed by the definition of an amino acid substitution herein.

A "non-naturally occurring amino acid residue" refers to a residue, other than those naturally occurring amino acid residues listed above, which is able to covalently bind adjacent amino acid residues(s) in a polypeptide chain. Examples of non-naturally occurring amino acid residues include norleucine, ornithine, norvaline, homoserine and other amino acid residue analogues such as those described in Ellman et al. Meth. Enzym. 202:301 336 (1991). To generate such non-naturally occurring amino acid residues, the procedures of Noren et al. Science 244:182 (1989) and Ellman et al., supra, can be used. Briefly, these procedures involve chemically activating a suppressor tRNA with a non-naturally occurring amino acid residue followed by in vitro transcription and translation of the RNA.

An "amino acid insertion" refers to the incorporation of at least one amino acid into a predetermined amino acid sequence. While the insertion will usually consist of the insertion of one or two amino acid residues, the present application contemplates larger "peptide insertions", e.g. insertion of about three to about five or even up to about ten amino acid residues. The inserted residue(s) may be naturally occurring or non-naturally occurring as disclosed above.

An "amino acid deletion" refers to the removal of at least one amino acid residue from a predetermined amino acid sequence.

The term "polynucleotide(s)" refers to nucleic acids such as DNA molecules and RNA molecules and analogs thereof (*e.g.*, DNA or RNA generated using nucleotide analogs or using nucleic acid chemistry). As desired, the polynucleotides may be made synthetically, *e.g.*, using art-recognized nucleic acid chemistry or enzymatically using, *e.g.*, a polymerase, and, if desired, be modified. Typical modifications include methylation, biotinylation, and other art-known modifications. In addition, the nucleic acid molecule can be single-stranded or double-stranded and, where desired, linked to a detectable moiety.

The term "mutagenesis" refers to, unless otherwise specified, any art recognized technique for altering a polynucleotide or polypeptide sequence. Preferred types of mutagenesis include error prone PCR mutagenesis, saturation mutagenesis, or other site directed mutagenesis.

"Site-directed mutagenesis" is a technique standard in the art, and is conducted using a synthetic oligonucleotide primer complementary to a single-stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the single-stranded phage DNA, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells that harbor the phage. Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. Plaques of interest are selected by hybridizing with kinased synthetic primer at a temperature that permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques that hybridize with the probe are then selected, sequenced and cultured, and the DNA is recovered.

The term "vector" is used to refer to a rDNA molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of genes encoding for one or more polypeptides are referred to herein as "expression vectors. The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a

promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

Percent amino acid sequence identity may be determined using the sequence comparison program NCBI-BLAST2 (Altschul et al., Nucleic Acids Res. 25:3389-3402 (1997)). The NCBI-BLAST2 sequence comparison program may be downloaded from http://www.ncbi.nlm.nih.gov or otherwise obtained from the National Institute of Health, Bethesda, MD. NCBI-BLAST2 uses several search parameters, wherein all of those search parameters are set to default values including, for example, unmask = yes, strand = all, expected occurrences = 10, minimum low complexity length = 15/5, multi-pass e-value = 0.01, constant for multi-pass = 25, dropoff for final gapped alignment = 25 and scoring matrix = BLOSUM62.

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" are used herein to refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express FcRs (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. Various immune cells express different Fc receptors (FcRs). Thus, the primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII.

The terms "influenza A subtype" or "influenza A virus subtype" are used interchangeably, and refer to influenza A virus variants that are characterized by various combinations of the hemagglutinin (H) and neuraminidase (N) viral surface proteins, and thus are labeled by a combination of an H number and an N number, such as, for example, H1N1 and H3N2. The terms specifically include all strains (including extinct strains) within

each subtype, which usually result from mutations and show different pathogenic profiles. Such strains will also be referred to as various "isolates" of a viral subtype, including all past, present and future isolates. Accordingly, in this context, the terms "strain" and "isolate" are used interchangeably.

The term "influenza" is used to refer to a contagious disease caused by an influenza virus.

B. General Techniques

Techniques for performing the methods of the present invention are well known in the art and described in standard laboratory textbooks, including, for example, Ausubel *et al.*, Current Protocols of Molecular Biology, John Wiley and Sons (1997); Molecular Cloning: A Laboratory Manual, Third Edition, J. Sambrook and D. W. Russell, eds., Cold Spring Harbor, New York, USA, Cold Spring Harbor Laboratory Press, 2001; O'Brian et al., Analytical Chemistry of Bacillus Thuringiensis, Hickle and Fitch, eds., Am. Chem. Soc., 1990; Bacillus thuringiensis: biology, ecology and safety, T.R. Glare and M. O'Callaghan, eds., John Wiley, 2000; Antibody Phage Display, Methods and Protocols, Humana Press, 2001; and Antibodies, G. Subramanian, ed., Kluwer Academic, 2004. Mutagenesis can, for example, be performed using site-directed mutagenesis (Kunkel et al., Proc. Natl. Acad. Sci USA 82:488-492 (1985)). PCR amplification methods are described in U.S. Pat. Nos. 4,683,192, 4,683,202, 4,800,159, and 4,965,188, and in several textbooks including "PCR Technology: Principles and Applications for DNA Amplification", H. Erlich, ed., Stockton Press, New York (1989); and PCR Protocols: A Guide to Methods and Applications, Innis et al., eds., Academic Press, San Diego, Calif. (1990).

The methods of the present invention are not limited by any particular technology used for the display of antibodies. Although the invention is illustrated with reference to phage display, antibodies of the present invention can also be identified by other display and enrichment technologies, such as, for example, ribosome or mRNA display (Matthcakis *et al., Proc. Natl. Acad. Sci. USA* 91:9022-9026 (1994); Hanes and Pluckthun, *Proc. Natl. Acad. Sci. USA* 94:4937-4942 (1997)), microbial cell display, such as bacterial display (Georgiou *et al., Nature Biotech.* 15:29-34 (1997)), or yeast cell display (Kieke *et al., Protein Eng.* 10:1303-1310 (1997)), display on mammalian cells, spore display (Isticato *et al., J. Bacteriol.* 183:6294-6301 (2001); Cheng *et al., Appl. Environ. Microbiol.* 71:3337-3341 (2005) and co-pending provisional application Serial No. 60/955,592, filed August 13, 2007), viral display, such as retroviral display (Urban *et al., Nucleic Acids Res.* 33:e35

(2005), display based on protein-DNA linkage (Odegrip et al., Proc. Acad. Natl. Sci. *USA* 101:2806-2810 (2004); Reiersen *et al., Nucleic Acids Res.* 33:e10 (2005)), and microbead display (Sepp *et al., FEBS Lett.* 532:455-458 (2002)).

In ribosome display, the antibody and the encoding mRNA are linked by the ribosome, which at the end of translating the mRNA is made to stop without releasing the polypeptide. Selection is based on the ternary complex as a whole.

In a mRNA display library, a covalent bond between an antibody and the encoding mRNA is established via puromycin, used as an adaptor molecule (Wilson *et al., Proc. Natl. Acad. Sci. USA* 98:3750-3755 (2001)). For use of this technique to display antibodies, see, *e.g.*, Lipovsek and Pluckthun, *J. Immunol. Methods.* 290:51-67 (2004).

Microbial cell display techniques include surface display on a yeast, such as *Saccharomyces cerevisiae* (Boder and Wittrup, *Nat. Biotechnol.* 15:553-557 (1997)). Thus, for example, antibodies can be displayed on the surface of *S. cerevisiae* via fusion to the α-agglutinin yeast adhesion receptor, which is located on the yeast cell wall. This method provides the possibility of selecting repertoires by flow cytometry. By staining the cells by fluorescently labeled antigen and an anti-epitope tag reagent, the yeast cells can be sorted according to the level of antigen binding and antibody expression on the cell surface. Yeast display platforms can also be combined with phage (see, *e.g.*, Van den Beucken *et al., FEBS Lett.* 546:288-294 (2003)).

For a review of techniques for selecting and screening antibody libraries see, *e.g.*, Hoogenboom, *Nature Biotechnol.* 23(9):1105-1116 (2005).

C. Detailed Description of Preferred Embodiments

I. Preparation of Donor-Specific Antibody Libraries

The present invention concerns donor-specific antibody libraries from individuals who have successfully survived or are surviving an encounter with a specific disease. The resulting antibody repertoires will include antibodies that were used by the donor to defend specifically against a relevant disease, and thus are important tools, for example, for developing neutralizing antibodies for the prevention and/or treatment of a target disease.

While the present invention is applicable to any target disease that evokes antibody production in a human subject, representative, non-limiting, examples of such diseases are listed in Table 1.

TABLE 1

Type of disorder	Representative examples
infectious disorder	Influenza viral infection, hepatitis C virus (HCV) infection, herpes simplex virus (HSV) infection, human immunodeficiency virus (HIV) infection, Methicillin-resistant Staphylococcus aureus (MRSA) infection, Epstein-Barr virus (EBV) infection, respiratory syncytial virus (RSV) infection, Pseudomonas. Candida infections
Respiratory disorder	Asthma, Allergies, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), adult respiratory distress syndrome (ARDS)
metabolic disorder	Frailty, cachexia, sarcopenia, Obesity, type II diabedyslipidemia, metabolic syndrome-associated myocardial infarction (MI), chronic renal failure (CRF), osteoporosis
digestive disorder	irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), Chron's disease, fatty liver disease, fibrosis, drug-induced liver disease
Neurological disorder	Alzheimer's disease, multiple sclerosis (MS), Parkinson's disease, bovine spongiform encephalopathy (BSE, mad cow disease)
Cancer	e.g., breast, renal, stomach, melanoma, lung. colon, glioma, lymphoma

A method of creating the donor-specific libraries of the present invention is schematically illustrated in Figure 1. As a first step, potential donors are identified. The patient donor may currently suffer from or may have recovered from and survived a target disease. Thus, for example, as illustrated in the Examples, the donor-specific libraries herein may be created from the bone marrow of convalescent patients of prior influenza infections, including seasonal influenza outbreaks, epidemics, and pandemics.

When selecting or identifying a patient donor, it is important to confirm that the patient indeed had or is having the target disease. Part of the confirmation is the examination of the medical history of the patient donor. In addition to the medical history, various other factors, such as the medical history of the patient's family, the patient's sex, weight, health

state, etc., should be taken into consideration. If the patient history is not available or unreliable, or for any other reason, such as a further confirmation measure, the serological profile of the patient may be determined. Serological assays are well known in the art and can be performed in various formats, such as in the form of various ELISA assay formats. Thus, for example, the presence of antibodies to an influenza virus can be detected by the well-known hemagglutinin inhibition (HAI) assay (Kendal, A. P., M. S. Pereira, and J. J. Skehel. 1982. Concepts and procedures for laboratory-based influenza surveillance. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, Georgia), or the microneutralization assay (Harmon *et al., J. Clin. Microbiol.* 26:333-337 (1988)). This step might not be necessary if the serum sample has already been confirmed to contain influenza neutralizing antibodies.

In order to prepare donor-specific human antibody libraries, samples containing lymphocytes are collected from individuals (patient donors) known to have developed a target disease, such as at least one disease from those listed in Table 1. The sample may, for example, derive from bone marrow, blood, spleen, lymph nodes, tonsils, thymus, and the like. Bone marrow is a preferred source of the antibody libraries herein, since it represents the complete "fossil archive" of individual donor's mature antibody repertoire.

Samples containing lymphocytes can be collected from the patient donor at various time points. In one embodiment, lymphocytes are collected from a patient who has recovered from the targeted disease(s) at least for 1, 5, 10, 15, 20, 25 days, at least for 1, 2, 3, 4, 5, 6, 7, 8, 9,10, 11 months, or at least for 1, 2, 3, 4, 5, 6, 7, 8, 9,10 years. In another embodiment, lymphocytes are collected from a patient who is having the targeted disease(s) at the time of collection, and has been diagnosed as having the disease(s) at least 1, 5, 10, 15, 20, 25 days, or at least 1, 2, 3, 4, 5, 6, 8, 9, 10 months, or 1, 2, 3, 4, or 5 years prior to collection.

Peripheral blood samples, especially from geographically distant sources, may need to be stabilized prior to transportation and use. Kits for this purpose are well known and commercially available, such as, for example, BD Vacutainer® CPT™ cell preparation tubes can be used for centrifugal purification of lymphocytes, and guanidium, Trizol, or RNAlater used to stabilize the samples. Methods and kits for isolating lymphocytes from other sources, such as lymphoid organs are also well known and commercially available.

Upon receipt of the stabilized lymphocytes or whole bone marrow, RNA is extracted and RT-PCR is performed to rescue antibody heavy and light chain repertoires, using immunoglobulin oligo primers known in the art.

Methods for preparation of RNA from bone marrow lymphocytes, or lymphocytes from any other source, are well known in the art. General methods for mRNA extraction are disclosed in standard textbooks of molecular biology, including Ausubel *et al.*, Current Protocols of Molecular Biology, John Wiley and Sons (1997). RNA purification kits are available from commercial manufacturers, such as Qiagen, and can be used according to the manufacturer's instructions.

Since RNA cannot serve as a template for PCR, it is first reverse transcribed into cDNA, which is subjected to PCR amplification. The two most commonly used reverse transcriptases are avilo myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukemia virus reverse transcriptase (MMLV-RT). The reverse transcription step is typically primed using specific primers, random hexamers, or oligo-dT primers, depending on the circumstances and the goal of expression profiling. For example, extracted RNA can be reverse-transcribed using a GeneAmp RNA PCR kit (Perkin Elmer, CA, USA), following the manufacturer's instructions. The derived cDNA can then be used as a template in the subsequent PCR reaction.

In order to create phage-display libraries, the PCR repertoire products may be combined with linker oligos to generate scFv libraries to clone directly in frame with m13 pIII protein, following procedures known in the art. Libraries using other display techniques, such as those discussed above, can be prepared by methods well known in the art.

In a typical protocol, whole RNA is extracted by Tri BD reagent (Sigma) from fresh or RNAlater stabilized tissue. Subsequently, the isolated donor total RNA is further purified to mRNA using Oligotex purification (Qiagen). Next first strand cDNA synthesis, is generated by using random nonamer oligonucleotides and or oligo $(dT)_{18}$ primers according to the protocol of AccuScript reverse transcriptase (Stratagene). Briefly, 100 ng mRNA, 0.5 mM dNTPs and 300 ng random nonamers and or 500 ng oligo $(dT)_{18}$ primers in Accuscript RT buffer (Stratagene) are incubated at 65 °C for 5 min, followed by rapid cooling to 4 °C. Then, 100 mM DTT, Accuscript RT, and RNAse Block are added to each reaction and incubated at 42 °C for 1h, and the reverse transcriptase is inactivated by heating at 70 °C for 15 minutes. The cDNA obtained can be used as a template for RT-PCR amplification of the antibody heavy and light chain V genes, which can then be cloned into a vector, or, if phage

display library is intended, into a phagemid vector. This procedure generates a repertoire of antibody heavy and light chain variable region clones (V_H and V_L libraries), which can be kept separate or combined for screening purposes. The vector, such as a phagemid vector, can then be introduced into a host cell, such as an *E coli* host, to generate a vector collection comprising a repertoire of nucleic acid molecules encoding antibody light chains or heavy chains or fragments thereof. In each case, the vector collection may comprise a single or more than one antibody light chain or heavy chain subtype. Thus, the vector collection may comprise sequences encoding antibody κ and/or λ light chains.

In the methods of the present invention, typically antibody light chains and antibody heavy chains are at first cloned separately, as discussed above, also separating the κ and λ light chain libraries. The libraries can be archived, and, when needed, the heavy chain library can be combined with the segregated κ and λ light chain libraries and heavy and light chain pairings can be identified, e.g. by panning, in the case of phage display. It is possible to repeat these steps multiple times with various libraries or sub-libraries, depending on the goal to be attained. The methods of the present invention provide great flexibility in including or excluding libraries, sub-libraries or clones, as needed during panning in order to maximize success.

In particular, because the sequences present in the vector collection harbor the coding sequences of the antibody heavy and light chains (or fragments) separately, the sequences may be excised and inserted into one or more expression vectors for expression of the antibody heavy and light chains, or fragments thereof. Preferably, the coding sequences of the antibody heavy and light chains, or fragments thereof, are inserted into the same expression vector for coexpression of the heavy and light chains to produce the library of the antibodies or antibody fragments.

The expression vectors of the present invention contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

Expression vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement

auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibodies-encoding nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)].

Expression and cloning vectors usually contain a promoter operably linked to the antibody-encoding nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding antibodies

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657.

Transcription of the heavy chain or light chain genes in the expression vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding the antibody genes by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody coding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding antibody heavy and light chains.

Still other methods, vectors, and host cells suitable for adaptation to the synthesis of polypeptide, in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

In a particular embodiment, the antibody library is produced in the form of a phage library, where the coding sequences of the antibody heavy and light chains, or fragments thereof, are cloned into a phagemid vector, such as a vector comprising the bacteriophage gene III. Phagemid vectors are well known and commercially available, including, for example, the pBluescript vector SKII+ (Stratagene, Genbank Accession X52328), and other pBluscript vectors. Phage display technology enables the generation of large repertoires of

human antibodies, and the biopanning procedure allows the selection of individual antibodies with any desired specificity or other properties.

For example, immunoglobulin repertoires from peripheral lymphocytes of survivors of earlier epidemics and pandemics, such as the 1918 Spanish Flu, can be retrieved, stabilized, rescued and expressed in a manner similar to that described above. For additional H1 and H3 libraries, repertoires can be recovered from properly timed vaccinated locally-sourced donors. As an additional option, commercially available bone marrow total RNA or mRNA can be purchased from commercial sources to produce libraries suitable for H1 and H3, and, depending upon the background of donor, also suitable for H2 antibody screening. In general, for target diseases where vaccination is an available treatment option, antibodies can be isolated from biological samples obtained from immunized human donors as well. From immunized patients that have developed titers of antibody recognizing the particular antigen, bone marrow, blood, or another source of lymphocytes, is collected, and antibodies produced are isolated, amplified and expressed as described above.

As discussed above, for For each donor, antibody light and heavy chain libraries can be cloned separately. Thus, for each donor, various κ and λ light chain families can be separately pooled and cloned in equimolar amounts. Similarly, for each donor, various heavy chain families can be pooled and cloned in equimolar amounts. By enabling gene family specific rescue of antibodies, the methods of the present invention yield libraries more completely representing the antibody repertoire of the donor, including antibodies that are less abundant and, in the case of pooled antibody libraries, guaranteeing immunoglobulin contributions from any and all individuals. For example, as illustrated in Example 1by the Examples, in preparing the influenza heavy and light chain libraries herein, 6 κ light chain families, 11 λ light chain families and 4 heavy chain families were rescued.

A typical screen can yield zero, one, or more than one target specific positive clone(s). If a particular combinatorial antibody library or libraries have been exhaustively screened and no further solutions seem attainable this may not be a failure of the heavy and light chain repertoire(s) ability to bind target, but rather the collection may have failed to bring together the necessary heavy and light chain pair required to bind target. A typical rescued repertoire of light chains from any individual may contain between about 10^5 – 10^6 unique light chains and between about 10^6-10^8 unique heavy chains. The possible combinatorial products of such pairings range from 10^{11} to 10^{14}. Such a collection exceeds the practical limits of most display systems, such as phage display, by several orders of

magnitude. Consequently, with current display technologies (such as phage display), only a fraction of the combinatorial possibilities are captured and assessed in any single phage antibody library. Therefore, recloning the original set of heavy chains with the original collection of light chains will generate an entirely new set of shuffled heavy and light chain combinations with likely novel antibodies to a particular target. Such newly reshuffled collections were found to transform previously existing poorly performing donor specific libraries into highly productive collections. Specifically, for a collection from a single donor previously only 0.3x-fold enrichment could be achieved compared to background after three rounds of selection. However, when this collection was recloned and reshuffled it became capable of 15-fold enrichment following 3 rounds of panning resulting in 55 novel sequences from 92 selected clones.

As mentioned previously, a typical screen can yield any number of target specific positive clones. The present invention enables the identification of the origins of any clone by their embedded barcode. As a typical antibody screen may combine phage antibodies from numerous donor specific libraries it is possible that some of the libraries and their combinatorial clones are not completely represented as antibody bearing phage particles. In which case a positive clone may have resulted from only a limited physical set of all the possible cloned solutions present in the sub-library phage population being screened. In such an instance it is of considerable interest to more fully interrogate the collection of donor specific phage. In this case the barcode from a positive clone guides one to the specific library responsible for the clone and allows to exclusively and more deeply screen the collection of interest.

In instances where desired antibodies must have functional capabilities beyond those initially used as the basis for the initial library construction, such as neutralization, or activation, we can prospectively profile individual donor sera for evidence of such activities. If the desired activities are present at reasonable titers in any particular donor sera, one can select those corresponding libraries are selected to screen against the target of interest. In other instances the relevant selection criteria may be unrelated to serology, but related to donor characteristics such as age, gender, or medical histories. In any event, donor profiles are logical guides for library selection and possible only in donor specific and segregated antibody libraries.

It is not unusual to complete a phage panning screen and discover the presence of immunodominant clones. Furthermore, it is also not unusual to rediscover such clone upon repeated panning screening regimens. In the case of a dominant clone or clones, where

either more or different clones are desirable, it is important to avoid the library material responsible for the presence of this clone. In typical phage antibody libraries the specific library or materials responsible for the clones origin are not separable from the collection, however in donor specific libraries it is possible to rescreen the libraries and simply omit the undesirable donor sublibrary or sublibraries, thereby forcing positive selection away from previously identified dominant clones.

Although, for simplicity, the libraries are described as heavy or light chain libraries, it will be apparent to those of ordinary skill in the art that the same description applies to the libraries of antibody fragments, fragments of antibody heavy and/or light chains, and libraries of antibody-like molecules.

In a particular embodiment, antibodies with dual specificities, such as, for example, showing reactivity with two different influenza A subtypes and/or with two strains (isolates) of the same subtype, and/or with human and non-human isolates, can be discovered and optimized through controlled cross-reactive selection and/or directed combinatorial and/or mutagenic engineering.

In a typical enrichment scheme, illustrated in Figure 2, a library including antibodies showing cross-reactivity to two targets, designated as targets A and B, are subjected to multiple rounds of enrichment. If enrichment is based on reactivity with target A, each round of enrichment will increase the reactive strength of the pool towards target A. Similarly, if enrichment is based on reactivity with target B, each round of enrichment will increase the reactive strength of the pool towards target B. Although Figure 2 refers to panning, which is the selection method used when screening phage display libraries (see below), the approach is equally applicable to any type of library discussed above, or otherwise known in the art, and to any type of display technique. Targets A and B include any targets to which antibodies bind, including but not limited to various isolates, types and sub-types of influenza viruses.

If the goal is to identify neutralizing antibodies with multiple specificities, a cross-reactive discovery selection scheme can be used. In the interest of simplicity, this scheme is illustrated in Figure 3 showing the selection of antibodies with dual specificities. In this case, an antibody library including antibodies showing reactivity with two targets, targets A and B, is first selected for reactivity with one of the targets, *e.g.*, target A, followed by selection for reactivity with the other target, *e.g.*, target B. Each successive selection round reinforces the reactive strength of the resulting pool towards both targets. Accordingly, this method is particularly useful for identifying antibodies with dual specificity. Of course, the

method can be extended to identifying antibodies showing reactivity towards further targets, by including additional rounds of enrichment towards the additional target(s). Again, if the library screened is a phage display library, selection is performed by cross-reactive panning, but other libraries and other selection methods can also be used.

A combination of the two methods discussed above includes two separate enrichment rounds for reactivity towards target A and target B, respectively, recombining the two pools obtained, and subsequent cross-reactive selection rounds, as described above. This approach is illustrated in Figure 4. Just as in the pure cross-reactive selection, each round of selection of the recombined library increases the reactive strength of the resulting pool towards both targets.

In a further embodiment, illustrated in Figure 5, first a clone showing strong reactivity with a target A, and having detectable cross-reactivity with target B is identified. Based on this clone, a mutagenic library is prepared, which is then selected, in alternating rounds, for reactivity with target B and target A respectively. This scheme will result in antibodies that maintain strong reactivity with target A, and have increased reactivity with target B. Just as before, selection is performed by panning, if the libraries screened are phage display libraries, but other libraries, other display techniques, and other selection methods can also be used, following the same strategy.

As discussed above, targets A and B can, for example, be two different subtypes of the influenza A virus, two different strains (isolates) of the same influenza A virus, subtypes or isolates from two different species, where one species is preferably human. Thus, for example, target A may be an isolate of the 2004 Vietnam isolate of the H5N1 virus, and target B may be a 1997 Hong Kong isolate of the H5N1 virus. It is emphasized that these examples are merely illustrative, and antibodies with dual and multiple specificities to any two or multiple targets can be identified, selected and optimized in an analogous manner.

Once neutralizing antibodies with the desired properties have been identified, it might be desirable to identify the dominant epitope or epitopes recognized by the majority of such antibodies. Methods for epitope mapping are well known in the art and are disclosed, for example, in Morris, Glenn E., Epitope Mapping Protocols, Totowa, N.J. ed., Humana Press, 1996; and Epitope Mapping: A Practical Approach, Westwood and Hay, eds., Oxford University Press, 2001.

II. Identifying Donor-specific Antibody Library with Unique Barcoding

According to the present invention, following amplification of the antibody heavy and light chain repertoires from cDNA, such as bone marrow cDNA, prepared as described above, preferably antibody heavy and light chain libraries are cloned separately for each patient donor, where the individual libraries can be distinguished using unique barcodes.

The barcodes preferably are selected such that they are capable of propagating along with the clone(s) labeled, without interfering with the expression of the desired antibody chain or fragment thereof. In an exemplary embodiment of the present invention, the barcode is inserted into the sequence of the expression vector, preferably, the 3' untranslated region following the terminal pIII stop codon when a phagemaid vector is used. Upon clonal isolation, a vector's unique sequence is determined and subsequently dedicated to a single defined library. This defined library can be derived not only from a single donor, but also from discrete pools of donors, or a synthetic repertoire or a semi-synthetic collection. In another embodiment, the barcode is inserted into the coding sequence of the antibody heavy and/or light chain or fragment thereof, at a position or in a form that does not interfere with the expression of the respective chains.

Thus the barcodes can be non-coding DNA sequences of about 1-24 contiguous non-coding nucleotides in length that can be deconvoluted by sequencing or specific PCR primers. This way, a collection of nucleic acids, such as an antibody repertoire, can be linked at the cloning step. In a exemplary embodiment of the present invention, the barcode is 3 or 5 bases of randomly generated sequence.

In another example, the barcodes are coding sequences of silent mutations. If the libraries utilize restriction enzymes that recognize interrupted palidromes (e.g. Sfi GGCCNNNNNGGCC), distinct nucleotides can be incorporated in place of the "N's" to distinguish various collections of clones, such as antibody libraries. This barcoding approach has the advantage that the repertoire is linked at the amplification step.

In a further embodiment, the barcodes are non-contiguous nucleotide sequences, which may be present in the vector sequence and/or the coding sequence of the desired antibody chain. Thus, a barcode with a non-contiguous sequence provides a great degree of flexibility in identifying the origins of the various individual sequences, and monitoring their subsequent handling.

In a different example, the barcodes are coding sequences that encode immunologically distinct peptide or protein sequences fused to phage particles. Examples include, for example, epitope (e.g. Myc, HA, FLAG) fusions to pIII, pVIII, pVII, or pIX

phages. The epitopes can be used singly or in various combinations, and can be provided in cis (on the library-encoding plasmid) or in trans (specifically modified helper phage) configuration.

Other examples of possible barcodes include, without limitation, chemical and enzymatic phage modifications (for phage libraries) with haptens or fluorescent chromophores. Such tags are preferred for a single round of selection.

The individual heavy and light chain libraries obtained from individual donors, or other barcoded clone or collections, can be pooled, without losing the ability to distinguish the source of individual sequences.

III. Optimizing Neutralizing Antibodies From the Donor-specific Antibody Libraries

If desired, cross-reactivity of the neutralizing antibodies with dual or multiple specificity can be further improved by methods known in the art, such as, for example, by Look Through Mutagenesis (LTM), as described in US. Patent Application Publication No. 20050136428, published June 23, 2005, the entire disclosure of which is hereby expressly incorporated by reference.

Look-through mutagenesis (LTM) is a multidimensional mutagenesis method that simultaneously assesses and optimizes combinatorial mutations of selected amino acids. The process focuses on a precise distribution within one or more complementarity determining region (CDR) domains and explores the synergistic contribution of amino acid side-chain chemistry. LTM generates a positional series of single mutations within a CDR where each wild type residue is systematically substituted by one of a number of selected amino acids. Mutated CDRs are combined to generate combinatorial single-chain variable fragment (scFv) libraries of increasing complexity and size without becoming prohibitive to the quantitative display of all variants. After positive selection, clones with improved properties are sequenced, and those beneficial mutations are mapped. To identify synergistic mutations for improved binding properties, combinatorial libraries (combinatorial beneficial mutations, CBMs) expressing all beneficial permutations can be produced by mixed DNA probes, positively selected, and analyzed to identify a panel of optimized scFv candidates. The procedure can be performed in a similar manner with Fv and other antibody libraries.

Mutagenesis can also be performed by walk-through mutagenesis (WTM), as described above.

Another useful mutagenic method to intentionally design cross-reactivity of the antibodies herein with more than one influenza A subtype and/or more than one isolate of the same subtype, is referred herein as "destinational" mutagenesis. Destinational mutagenesis can be used to rationally engineer a collection of antibodies based upon one or more antibody clones, preferably of differing reactivities. In the context of the present invention, destinational mutagenesis is used to encode single or multiple residues defined by analogous positions on like sequences such as those in the individual CDRs of antibodies. In this case, these collections are generated using oligo degeneracy to capture the range of residues found in the comparable positions. It is expected that within this collection a continuum of specificities will exist between or even beyond those of the parental clones. The objective of destinational mutagenesis is to generate diverse multifunctional antibody collections, or libraries, between two or more discrete entities or collections. To create a destinational mutagenesis library, the CDR sequences for both antibodies are first attained and aligned. Next all positions of conserved identity are fixed with a single codon to the matched residue. At non-conserved positions a degenerate codon is incorporated to encode both residues. In some instances the degenerate codon will only encode the two parental residues at this position. However, in some instances additional co-products are produced. The level of co-product production can be dialed in to force co-product production or eliminate this production dependent upon size limits or goals.

Thus, for example, if the first position of the two antibodies respectively are threonine and alanine, the degenerate codon with A/G-C- in the first two positions would only encode threonine or alanine, irrespective of the base in the third position. If, for example, the next position residues are lysine and arginine the degenerate codon A-A/G-A/G will only encode lysine or arginine. However, if the degenerate codon A/C-A/G-A/G/C/T were used then asparagine, histidine, glutamine, and serine coproducts will be generated as well.

As a convenience it is simpler to use only antibodies with matched CDR lengths. One way to force this is to screen a size restricted library for the second antigen, based on the CDR length and potentially even framework restrictions imparted by the initially discovered antibody. It is noted, however, that using CDRs of equal length is only a convenience and not a requirement. It is easy to see that, while this method will be useful to create large functionally diverse libraries of influenza A virus neutralizing antibodies, its applicability is much broader. This mutagenesis technique can be used to produce functionally diverse libraries or collections of any antibody. Thus, Figure 6 is included herein to illustrate the use

of the destinational mutagenesis method using CDRs of a TNF- α antibody and a CD11a antibody as the parental sequences mutagenized.

Other exemplary mutagenesis methods include saturation mutagenesis and error prone PCR.

Saturation mutagenesis (Hayashi *et al., Biotechniques* 17:310-315 (1994)) is a technique in which all 20 amino acids are substituted in a particular position in a protein and clones corresponding to each variant are assayed for a particular phenotype. (See, also U.S. Patent Nos. 6,171,820; 6,358,709 and 6,361,974.)

Error prone PCR (Leung *et al., Technique* 1:11-15 (1989); Cadwell and Joyce, *PCR Method Applic.* 2:28-33 (1992)) is a modified polymerase chain reaction (PCR) technique introducing random point mutations into cloned genes. The resulting PCR products can be cloned to produce random mutant libraries or transcribed directly if a T7 promoter is incorporated within the appropriate PCR primer.

Other mutagenesis techniques are also well known and described, for example, in In Vitro Mutagenesis Protocols, J. Braman, Ed., Humana Press, 2001.

Optimization can be based on any of the libraries discussed above, or any other types of libraries known in the art, alone or in any combination.

IV. Production of neutralizing antibodies

Once antibodies with the desired neutralizing properties are identified, such antibodies, including antibody fragments can be produced by methods well known in the art, including, for example, hybridoma techniques or recombinant DNA technology.

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.* 133:3001 (1984); and Brodeur *et al.*, Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

Recombinant monoclonal antibodies can, for example, be produced by isolating the DNA encoding the required antibody chains and co-transfecting a recombinant host cell with the coding sequences for co-expression, using well known recombinant expression vectors. Recombinant host cells can be prokaryotic and eukaryotic cells, such as those described above.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims *et al., J. Immunol.* 151:2296 (1993); Chothia *et al., J. Mol. Biol.* 196:901 (1987)). It is important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences.

In addition, human antibodies can be generated following methods known in the art. For example, transgenic animals (*e.g.*, mice) can be made that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of

endogenous immunoglobulin production. See, *e.g.*, Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA* 90:2551 (1993); Jakobovits *et al.*, *Nature* 362:255-258 (1993); Bruggermann *et al.*, *Year in Immuno.* 7:33 (1993); and U.S. Patent Nos. 5,591,669, 5,589,369 and 5,545,807.

V. Use of neutralizing antibodies

The neutralizing antibodies of the present invention can be used for the prevention and/or treatment of the targeted diseases. For therapeutic applications, the antibodies or other molecules, the delivery of which is facilitated by using the antibodies or antibody-based transport sequences, are usually used in the form of pharmaceutical compositions. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Co. (Easton, Pa. 1990). See also, Wang and Hanson "Parenteral Formulations of Proteins and Peptides: Stability and Stabilizers," Journal of Parenteral Science and Technology, Technical Report No. 10, Supp. 42-2S (1988).

Antibodies are typically formulated in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The antibodies also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

The neutralizing antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA* 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA* 77:4030 (1980); U.S. Patent Nos. 4,485,045 and 4,544,545; and WO97/38731 published October 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al. J. Biol. Chem.* 257:286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon *et al. J. National Cancer Inst.* 81(19)1484 (1989).

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of infection to be treated, the severity and course of the disease, and whether the antibody is administered for preventive or therapeutic purposes. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to about 15 mg/kg of antibody is a typical initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion.

Further details of the invention are illustrated by the following non-limiting Examples.

Example 1

Antibody Libraries from Survivors of Prior Bird Flu Outbreaks and Preparation of Neutralizing Antibodies

Materials and Bone Marrow Protocol and Sera Preparation

Blood was obtained by standard venopuncture, allowed to clot, and processed to recover serum. The serum was stored at -20 °C for 3-4 days until they were shipped on dry ice. Donors were anaesthetized with an injection of a local anesthetic and 5ml of bone marrow was removed from the pelvic bone of each H5N1 survivor. Next the 5ml of bone marrow was placed into a sterile 50-ml tube containing 45 ml RNAlater (Ambion). The

mixture was gently inverted approximately 8-20 times, until there were no visible clumps and the marrow and RNAlater were mixed well. Next the specimen was refrigerated the between 2-10 °C overnight. Following the overnight refrigeration, the specimens were stored at -20 °C for 3-4 days until they were shipped on dry ice. Upon receipt the RNAlater/marrow and sera containing tubes were stored at -80 °C until processed.

Serology: HA ELISA

ELISA plates (Thermo, Immulon 4HBX 96W) were coated with 100 µl of 100 ng/mL H5 hemagglutinin (Protein Sciences, A/Vietnam/1203/2004) in 1X ELISA Plate Coating Solution (BioFX) by overnight incubation at room temperature. The next day plates were washed three times with 300 µl PBS/ 0.05% Tween-20 (PBST). Following the wash, 300 µl of a blocking solution (4% Non-Fat dry Milk in PBS/ 0.05% Tween-20) was added and incubated for 1 hour at RT. Following the blocking step, the plates were washed three times with 300 µl PBS/ 0.05% Tween-20. Next, 100 µl serum samples diluted 1:20,000 in PBS/ 0.05% Tween were incubated for 1-2 hours at RT and then washed three times with 300 µl PBS/ 0.05% Tween-20. 100 µl of an anti-human Fc-HRP conjugate diluted 1:5,000 in PBS/ 0.05% Tween was incubated for 1-2 hours at RT and then washed three times with 300 µl PBS/ 0.05% Tween-20. Following this final wash, 100 µl of chromogenic substrate solution was added (TMB1 Substrate, BioFx) and after sufficient amount of time terminated by the addition of 100 µl of STOP Solution (BioFx). Absorbances at 450nm were read on a plate reader (Molecular Devices Thermomax microplate reader with Softmax Pro software), data recorded, and subsequently plotted using Excel (Microsoft).

Bone Marrow: RNA Extraction and mRNA Purification

Bone marrow (~2.5 ml in 20 ml RNA later), previously stored at -80 °C, was recovered by centrifugation to remove RNA later and then resuspended in 11.25 ml TRI BD reagent (Sigma) containing 300 µl Acetic Acid. The pellet was then vortexed vigorously. Next 1.5 ml BCP (1-bromo-3-chloropropane, Sigma) was added, mixed by vortexing, incubated at RT for 5 min, and then centrifuged at 12000 x g for 15 min at 4°C. The aqueous phase was carefully removed to not disturb the interface. Total RNA from the aqueous phase was next precipitated by addition of 25 ml isopropanol, incubation at RT for 10 minutes, and centrifugation at 12000 x g for 10 min at 4°C. Following the addition of isopropanol, two phases were formed due to residual RNAlater, resulting in the precipitated RNA settling at the interface. To eliminate the residual RNAlater and allow maximal recovery of RNA, 5 ml aliquots of 50% isopropanol in H_20 were added and mixed until no

phase separation was noticeable, at which point the RNA was pelleted by centrifugation at 12000 x g for 10 min at 4°C. The RNA pellet was washed with 75% EtOH, transferred to an RNAse-free 1.6 ml microcentrifuge tube, and again recovered by centrifugation. Finally the RNA pellet was resuspended in 100 µl 1mM Na-phosphate, pH 8.2 and the A_{260} and A_{280} were read to assess RNA purity.

Prior to reverse transcription mRNA was purified from total RNA according to Qiagen Oligotex mRNA purification kit. Briefly, 50-200 µg bone marrow RNA was brought to 250 µl with RNase-free water and mixed with 250 µl of OBB buffer and Oligotex suspension followed by incubation for 3 min at 70°C. Hybridization between the oligo dT_{30} of the Oligotex particle and the mRNA poly-A-tail was carried out at room temperature for 10 min. The hybridized suspensions were then transferred to a spin column and centrifuged for 1 min. The spin column was washed twice with 400 µl Buffer OW2. Purified mRNA was then eluted twice by centrifugation with 20 µl hot (70°C) Buffer OEB. Typical yields were 500 ng to 1.5 µg total RNA.

Reverse transcription using N9 and Oligo dT on bone marrow mRNA

Reverse transcription (RT) reactions were accomplished by mixing together 75-100 ng mRNA with 2 µl 10X Accuscript RT Buffer (Stratagene), 0.8 µl 100 mM dNTPs, and either N9 (300 ng) or oligo dT primer (100 ng) and then brought to a final volume of 17 µl with water. The mixtures were heated at 65°C for 5 min, and then allowed to cool to room temperature. Next 2 µl DTT, 0.5 µl RNase Block (Stratagene), 0.5 µl AccuScript RT (Stratagene) were added to each reaction. Next, the N9 primed reactions were incubated for 10 minutes at room temperature and the oligo-dT primed reactions were incubated on ice for 10 minutes. Finally, both reactions were incubated at 42°C for 60 minutes followed by 70°C for 15 minutes to kill the enzyme.

PCR from bone marrow-derived cDNA

Antibody heavy and light chain repertoires were amplified from bone marrow cDNA essentially using previously described methods and degenerate primers (O'Brien, P.M., Aitken R. Standard protocols for the construction of scFv Libraries. Antibody Phage Display - Methods and Protocols, vol. 178, 59-71, 2001, Humana Press) based upon human germline V and J regions.

Briefly, PCR reactions using Oligo dT primed cDNA (from 75 ng mRNA) for lambda light chains and N9 primed cDNA (from 75 ng mRNA for kappa light chains, from 100 ng mRNA for heavy chains) were mixed together with 5 µl 10X amplification buffer

(Invitrogen), 1.5 µl dNTPs (10 mM), 1 µl MgSO4 (50 mM), 2.5 µl V_{region} primers (10 uM) and 2.5 µl J_{region} primers (10 uM) -10 uM for V_H, 0.5 µl Platinum Pfx Polymerase (Invitrogen), and sterile dH_2O to final volume of 50 µl. PCR parameters were as follows: step 1-95°C 5 minutes, step 2- 95°C 30 seconds, step 3-58°C 30 seconds, step 4- 68°C 1 minute, step 5- cycle step 2-4 40 times, step 6- 68°C 5 minutes. Light chain PCR products were cleaned up using Qiagen PCR Cleanup kit. Heavy chains PCR products were gel purified from 1.5% agarose gel using Qiagen Gel Extraction Kit and then reamplified. Heavy chain reamplification was carried out as follows: Mixed 10 µl 10X amplification buffer (Invitrogen), 3 µl dNTPs (10mM), 2 µl MgSO4 (50 mM), 5 µl each V_H primers (10 uM) and J_H primers (10 uM), 5 µl Heavy chain Primary PCR product, 1 µl Platinum Pfx, volume adjusted to 100 µl with water. Cycling parameters were as follows: step 1-95°C 5 minutes, step 2- 95°C 30 seconds, step 3-58°C 30 seconds, step 4- 68°C 1 minute, step 5- cycle step 2-4 20 times, step 6- 68°C 5 minutes. Re-amplified heavy chain PCR products were cleaned up from a 1.5% agarose-TAE gel using Qiagen Extraction Kit.

Antibody phage library construction

Separate antibody libraries for each individual bird flu survivor were constructed using unique identifying 3-nucleotide barcodes inserted in the untranslated region following the stop codon of the pIII gene of filamentous phage.

Light Chain cloning:

1 µg each of pooled kappa light chain and pooled lambda light chain per donor were digested with NotI and BamHI and gel purified from a 1.5% agarose-TAE gel using Qiagen Gel Extraction Kit. 5 µg of each vector (pAMPFab) was digested with NotI and BamHI and gel purified from a 1% agarose-TAE gel using Qiagen Gel Extraction Kit. Library ligations were performed with 200 ng of gel purified Kappa or Lambda inserts and 1 µg of gel purified vector in 60 µl for 1 hour at RT or overnight at 14°C. Ligations were desalted using Edge BioSystem Perfroma spin columns. The library was transformed in five electroporations in 80 µl TG-1 or XL-1 Blue aliquots, each recovered in 1 ml SOC, pooled and outgrown for one hour at 37°C. Total number of transformants was determined following this outgrowth by plating an aliquot from each of the transformations. The remaining electroporation was amplified by growing overnight at 37°C in 200 ml 2YT + 50 µg/ml Ampicillin + 2% glucose. The subsequent light chain library was recovered by plasmid purification from these overnight cultures using a Qiagen High Speed Maxiprep Kit.

Heavy Chain Cloning:

1.5-2 μg each of the donor-specific heavy chains (V_H1, V_H2, 5, 6 pool, V_H3, and V_H4) were digested with a 40 Unit excess/μg DNA with SfiI and XhoI and gel purified from a 1.5% agarose-TAE gel using Qiagen Gel Extraction Kit. 15 μg of each light chain library vector was digested with 40 Unit/μg DNA with SfiI and XhoI and gel purified from a 1% agarose-TAE gel using Qiagen Gel Extraction Kit. Library ligations were set up by combining 1.2 μg SfiI/XhoI digested, gel purified heavy chain donor collections and 5 μg of each light chain library (kappa and lambda) overnight at 14°C. The library ligations were then desalted with Edge BioSystem Pefroma spin columns and then transformed through 20 electroporations per library in 80 μl TG-1 aliquots, each recovered in 1 ml SOC, pooled and outgrown for one hour at 37°C. Again following this outgrowth an aliquot of each was used to determine the total number of transformants with the remainder transferred to 1L 2YT + 50 μg/ml Ampicillin + 2% glucose and grown at 37C with vigorous aeration to an OD_{600} of ~0.3. Next M13K07 helper phage was then added at a multiplicity of infection (MOI) of 5:1 and incubated for 1 hour at 37°C, with no agitation. Next the cells were harvested by centrifugation and resuspended in 1L 2YT + 50 μg/ml Ampicillin, 70 μg/ml Kanamycin and grown overnight at 37°C with vigorous aeration to allow for scFv phagemid production. The next morning the cells were collected by centrifugation and supernatant containing phagemid was collected. The phagemids were precipitated from the supernatant by the addition of 0.2 volumes 20% PEG/5 M NaCl solution and incubation for 1 hour on ice. The phagemid library stocks were then harvested by centrifugation and resuspended in 20 ml sterile PBS. Residual bacteria were removed by an additional centrifugation and the final phagemid libraries were stored at -20°C in PBS+50% glycerol.

Phagemid panning and amplification

ELISA plates (Immulon 4HBX flat bottom, Nunc) were coated with 100 μl of 100 ng/ml, H5 hemagglutinin protein(Protein Sciences, A/Vietnam/1203/2004) in ELISA Coating Solution (BioFX) by overnight incubation at room temperature. The next day plates were washed three times with 300 μl PBST. Following the wash, 300 μl of a blocking solution (4% Non-Fat dry Milk in PBS/ 0.05% Tween-20) was added and incubated for 30 mins on ice. Following the blocking step, the plates were washed three times with 300 μl PBST. Just prior to phage panning, the glycerol was removed from the frozen phagemid stocks using Millipore Amicon Ultra columns and then blocked in 4% nonfat dry milk for 15 minutes. Next, 100 μl aliquots of phagemid were distributed into 8 wells (total phage

~1x10^{12} CFU) and incubated for 2 hours at 4°C followed by washing 6-8 times with 300 µl PBST. Phagemid were collected following a 10 min at room temperature in 100 µl/well Elution buffer (0.2M glycine-HCl, pH 2.2, 1 mg/ml BSA). The eluate was then neutralized by the addition of 56.25 µl 2M Tris base per ml eluate. Following neutralization, 5 ml TG1 cells (OD_{600} ~0.3) were infected with 0.5 ml neutralized phage at 37°C for 30 minutes in 2-YT with no shaking. Following this step some cells were plated onto LB AMP Glucose plates to determine total phagemid recovery. The remaining inoculum was placed into 10 ml 2-YTAG (final concentration 2% glucose and 50ug/ml ampicillin) and grown at 37°C with vigorous aeration to OD_{600} ~0.3. Next the cultures were infected with M13K07 helper phage at an MOI of 5:1 and incubated at 37°C for 30-60 minutes with no shaking. The cells were collected by centrifugation and resuspended in 25ml 2-YTAK (Ampicillin 50 µg/ml, Kanamycin 70 µg/ml), transferred to a fresh culture flask, and grown ON at 37°C with shaking. Subsequent rounds were similarly recovered and amplified.

scFv ELISA

Individual colonies of *E. coli* HB2151 transformed cells from biopanned phage were grown overnight at 37°C in 1 ml of 2YT+ 100 µg/ml AMP. The following morning the cells were harvested by centrifugation and resuspended in 1.5 ml periplasmic lysis buffer (1ml BBS (Teknova) + 0.5 ml 10mg/ml lysozyme + EDTA to 10 mM final concentration). The cells were again pelleted by centrifugation and the scFv containing periplasmic lysates were collected. The scFv lysates were combined 1:1 with dilution buffer (PBS/0.05% BSA) and 100 µl was added to wells that had been previously antigen coated with and blocked with dilution buffer. The samples were incubated for 2 hours at room temperature and then washed three times with PBS/ 0.05% Tween. Next 100 µl of 1:5000 diluted Biotin Anti-Histidine mouse (Serotec) in dilution buffer was added to each well and incubated for 1 hr at room temperature. Following this incubation the wells were washed three times with PBS/ 0.05% Tween and then to each well 100 µl of 1:2500 Streptavidin:HRP (Serotec) was added and incubated for 1 hr at room temperature and then washed three times with PBS/ 0.05% Tween. Following this final wash, 100 µl of chromogenic substrate solution was added (TMB1 Substrate, BioFx) and after sufficient amount of time terminated by the addition of 100 µl of STOP Solution (BioFx). Absorbances at 450nm were read on a plate reader (Molecular Devices Thermomax microplate reader with Softmax Pro software), data recorded, and subsequently plotted using Excel (Microsoft).

Sequencing

To deduce the heavy and light chain sequences, individual clones were grown and plasmid DNA extracted (Qiagen). The plasmid DNA was subjected to standard DNA sequencing.

Hemagglutinin Inhibition (HAI) Assays

Hemagglutination Inhibition was performed essentially following the method of Rogers *et al.*, *Virology* 131:394-408 (1983), in round bottom microtiter plates (Corning) using 4 HAU (hemagglutinating units) of virus or protein/well. For HAI determinations 25 µl samples of purified single chain variable fragments (scFv) were mixed with 25 µl of PBS containing 4 HAU of the test virus in each microtiter well. Following a preincubation of 15 minutes at room temperature, 25 µl of 0.75% human erythrocytes were added, and mixed. HAI antibody activity was determined by visual inspection following a 60 min incubation at room temperature.

In particular, the following protocols were used:

Antibodies, Proteins and viruses.

IgG_1 proteins were generated from either scFv or Fabs by subcloning their coding regions into a pCI-based (Promega) full length mammalian protein expression and then transfecting them into 293 Freestyle cells (Invitrogen) according to manufacturers guidelines. Briefly 20 µg of light chain and 10 µg heavy chain encoding plasmid were combined with 1.0 ml 293fectin and incubated for 60 minutes. Following this preincubation the DNA mixture was combined with 3 X 10^7 cells in 30 ml media and the resulting cell suspension was grown according to manufacturers suggestion for 7 days. After seven days the secreted immunoglobulins were purified from the culture supernatants using protein A chromatography (Calbiochem). The resulting purified antibodies were buffer exchanged into sterile PBS using centrifugal size filtration (Centricon Plus-20) and their protein concentrations determined by colorimetric BCA assay (Pierce).

Cross-reaction IgG ELISA.

Microtiter plates were coated with 0.1 ml of the following antigens diluted in coating buffer and incubated overnight at room temperature: 100 ng/ml H5N1 Vietnam 1203/04, 250 ng/ml H5N1 Turkey/65596/06, 1 µg/ml H5N1 Indonesia/5/05, 700 ng/ml H1N1 New Caledonia/20/99, 1µg/ml H1N1 North Carolina/1/18, 100 ng/ml and H3N2 Wisconsin/67/05. Blocking was done with 0.3ml of blocking buffer (4% Non-fat dry milk in PBS/ 0.05% Tween-20). Following blocking antibodies diluted to 0.5 µg/ml in 2% non-fat dry milk blocking buffer were incubated for two hours at 4C, washed, and later detected using a

1:3000 dilution of peroxidase conjugated anti-human F_c antibody (Jackson ImmunoResearch) in 2% non-fat dry milk blocking buffer and standard TMB substrate detection (BioFX). Absorbance at 450nm was read, data recorded, and reported herein

Viral Microneutralization.

Indonesia and Turkey hemagglutinin genes were synthetically assembled using human codon optimized sequences (DNA 2.0) and then used to generate recombinant engineered viruses. Recombinant influenza viruses were generated using reverse genetics as previously described (Fodor, E. *et al.* J Virol. 1999;73(11): 9679-82). Briefly, 1ug each of 10 plasmids was transfected into 293 T cells in monolayer. Each transfection contained ambisense plasmids (for the expression of both vRNAs and mRNAs) for the A/Puerto Rico/8/34/PA, PB1, PB2, NP, M, and NS segments, in addition to vRNA (pPOL1 type) and protein expression plasmids (pCAGGS type) for A/Vietnam/1203/04 HA and NA (pCAGGS expression plasmid was kindly provided by J. Miyazaki, Osaka University, Osaka, Japan) (Miyazaki, J. *et al.* Gene 1989;79(2):269-77). Twenty hours following transfection, 293T cells were resuspended in cell culture supernatant, and used to inoculate 10-day-old embryonated eggs.

Antibodies were screened for neutralizing activity against viruses as follows. Two fold serial dilutions of each Mab were incubated with 100 $TCID_{50}$ of virus in PBS at 37° for 1 h. Madin-Darby Canine Kidney cell monolayers in 24 well plates were washed once with PBS and inoculated with virus-antibody mixtures. Following incubation for 1 h at 37°C in 5% CO_2, the inoculum was removed and monolayers were again washed once with PBS. Opti-MEM supplemented with 0.3% BSA, 0.01% FBS and 1ug/ml TPCK-treated trypsin was added and cells were incubated for 72 h at 37°C. The presence of virus in cell culture supernatants was assessed by HA assays using 0.5% chicken red blood cells.

RESULTS

Bone marrow and blood samples were collected from six survivors of the H5N1 bird flu outbreak that had taken place in Turkey in January 2006, approximately four months after the outbreak. For all six survivors the initial diagnosis of bird flu was made following by physical examination, clinical laboratory testing, and molecular diagnostic determination, sanctioned by the Turkish Ministry of Health. Four of these survivors were additionally confirmed by the World Health Organization (WHO). Serum samples were analyzed to confirm the presence of antibodies to H5 hemagglutinin (A/Vietnam/1203/2004) using the serology protocol described above. As shown in Figure 8, the blood samples of all six

patients (designated SLB H1-H6, respectively) demonstrated the presence of antibodies to the H5 antigen. Following this confirmation, RNA was extracted from the bone marrow samples of these individuals, and bone marrow mRNA was purified and reverse transcribed using the protocols described above. The antibody heavy and light chain repertoires were then amplified from the bone marrow cDNA as described above, and individual antibody heavy and light chain phage libraries were cloned separately for each survivor, using the above-described three-nucleotide bar coding to distinguish the individual libraries.

Using this vector with its coding system, we successfully cloned repertoires from the bone marrow of five of the six survivors in both single chain (scFv) and Fab phagemid formats. Each collection from an individual survivor has a diversity of greater than 1.0×10^8 members. Furthermore, we created additional bar coded libraries comprised of mixed survivor light and heavy chains with a final diversity of 1.1×10^9. Collectively the 5 donor-specific collections and the pooled libraries from all donors have a total diversity of 1.0×10^9 as a scFv collection and 4.2×10^9 as a Fab-displayed collection (Table 4).

Table 2 shows the light chain and full library total transformants in both scFv and Fab formats. Total diversity represented by all libraries is 5.6×10^9.

Table 2

scFv	Light Chains		Completed Libraries	
	Kappa	Lambda	Kappa	Lambda
H5-1	3.00E+06	4.00E+06	1.50E+08	1.20E+08
H5-2	3.00E+06	3.00E+06	4.00E+07	1.60E+07
H5-3	8.20E+05	1.70E+06	5.30E+07	1.50E+08
H5-5	7.00E+06	5.60E+06	6.50E+08	5.60E+07
H5-6	1.50E+06	5.00E+06	ND	1.00E+07
H5Pool	Not Determined		1.80E+08	5.70E+08
		Totals	1.10E+09	9.20E+08
		scFv Total	**2.00E+09**	

Fab	Light Chains		Completed Libraries	
	Kappa	Lambda	Kappa	Lambda
H5-1	1.50E+06	2.90E+06	2.90E+08	4.60E+08
H5-2	3.10E+06	9.40E+05	4.40E+08	4.30E+08
H5-3	2.80E+06	2.30E+06	3.90E+08	3.90E+08
H5-5	7.00E+06	5.60E+06	7.20E+08	1.70E+08
H5-6	1.50E+06	5.00E+06	ND	ND
H5Pool	1.90E+07		2.60E+08	
		Totals	2.10E+09	1.45E+09
		Fab Total	**3.60E+09**	

Bone marrow and blood samples were also collected from twelve local donors who were treated for flu symptoms in the year of 2006. Serology was performed as described above to confirm the presence of antibodies to H1, H3 and H5 hemagglutinin, respectively. As shown in Figure 8, all serum samples tested positive for antibodies to H1 and/or H3 hemagglutinins, where the dominance of a certain subtype depended on the influenza A virus subtype to which the particular donor was exposed most throughout his or her lifetime. Interestingly, there were donors whose serum contained a significant level of antibodies of H5 hemagglutinin as well (donors SLB1 and SLB5 in Figure 9). Following this confirmation, RNA was extracted from the bone marrow samples of the donors, and bone marrow mRNA was purified and reverse transcribed using the protocols described above. The antibody heavy and light chain repertoires were then amplified from the bone marrow cDNA as described above, and individual antibody heavy and light chain phage libraries were cloned separately for each donor, using the above-described three-nucleotide bar coding to distinguish the individual libraries.

Selecting binding antibodies

The H5N1 survivor libraries summarized in Table 2, were panned against inactivated virus containing the Vietnam/1203/04 virus HA and NA proteins or recombinant purified hemagglutinin (Barbas, C. *et al.* (2001) *Phage Display, A Laboratory Manual* (Cold Spring Harbor Laboratory Press)). Following three to four rounds of phage panning, individual clones from enriched phage pools were analyzed by ELISA against H5N1 virus or purified hemagglutinin and the positive clones were sequenced to determine their heavy and light chain sequences and to read their survivor bar code (D. W. Coomber, *Methods Mol Biol* **178**, 133 (2002)). From this, we isolated specific H5 hemagglutinin binding clones from all five of the individual survivor libraries. In total, more than 300 hundred different antiviral antibodies were recovered, of which 146 specifically bind the H5 hemagglutinin protein.

General Features of the Selected Clones

Overall, the individual donors use different germ lines for both heavy and light chains, demonstrating that individual patients have found different solutions to the same potentially lethal immunological challenge. The major features of combinatorial antibody libraries that can be used both to give confidence as to the quality of the obtained repertoire and to provide information as to the chemistry of antibody binding and/or neutralization are seen in these clones. These clones contain all the hallmarks of the previously described repeated clones ("jackpot solution") to antigen binding that is found in the natural progression of affinity maturation, as well as in selected synthetic antibody libraries (Lerner,

R.A. *Angew Chem Int Ed Engl* **45**, 8106 (Dec 11, 2006); A. Rajpal *et al.*, *Proc Natl Acad Sci USA* **102**, 8466 (Jun 14, 2005)). The presence of "jackpots" in these large collections validates the screening procedure because, unless the phage were selected on the basis of activity, the chance of obtaining the same clone multiple times is highly improbable. Moreover, when one analyzes the heavy chain differences within groups, it was observed that many of the amino acid substitutions were chemically and structurally conservative (Table 4). As with repeated clones, the appearance of multiple amino acid substitutions that are chemically reasonable is unlikely to be a random event.

Binding Specificity of recovered antibodies

Six clones were selected from three survivors that recognized two different epitopes for conversion into full IgG_1 proteins. The binding of four of these antibodies was mapped to the HA1 subunit of the hemagglutinin protein, while the remaining two did not. As the HA1 subunit has tremendous relevance in infection we further analyzed the activities of those four antibodies as described below.

One goal of these studies was to recover those rare antibodies that broadly neutralize divergent viral strains. There was a suggestion that some of our antibodies might be broadly reactive because the serum from the donors had high titer antibodies against a divergent subfamily of H5N1 viruses that extended beyond the virus with which they were infected. To determine the degree of cross reactivity at the level of individual antibodies, we first analyzed binding of our clones to different influenza hemagglutinin antigens (Figure 11). Not surprisingly, these antibodies recognize hemagglutinin from the corresponding infecting Turkey/65596/06 strain, and in addition recognize the heterologous hemagglutinin from the Vietnam/1203/04 strain used for selection. Moreover, they recognize the antigenically divergent Indonesian/5/05 H5 hemagglutinin. Furthermore, we found that four prototype antibodies bound hemagglutinin from the closely related subtype H1N1 contemporary reference strain New Caledonia/20/99. Notably, the three neutralizing antibodies belonging to survivor 5 also bound hemagglutinin from the H1N1 South Carolina/1/18 isolate that emerged during the 1918 Spanish Flu pandemic. Conversely, none of these four antibodies bound hemagglutinin from the contemporary H3N2 Wisconsin/67/05 reference strain, indicating that even though the antibodies display broad spectrum binding amongst and between Influenza subtypes, the reactivity did not extend to all influenza subtypes. Figure 11 shows the cross-reactivity of H5N1 antibodies from two survivors with hemagglutinins from H1N1 viruses. (A) Bars are H5N1 Vietnam 1203/04(dark grey), H5N1 Turkey/65596/06 (white), H5N1 Indonesia/5/05 (diagonal stripes), H1N1 New

Caledonia/20/99 (vertical stripes), H1N1 South Carolina/1/18 (crosshatch stripes), and H3N2 Wisconsin/67/05 (light gray).

Neutralization Studies

Initially the antibodies were assayed for their ability to neutralize an H5 HA (Vietnam/1203/04) containing influenza virus. One antibody derived from survivor 2 and 3 from survivor 5 that recognized a common epitope (epitope "A") were all neutralizing whereas the two antibodies derived from survivor 1 that recognized a second epitope (epitope "B") were not.

Based on the striking sequence similarity of clones separately isolated from survivor 5 against either H5N1 or H1N1 hemagglutinin, we predicted that their cross reactivity would extend beyond simple binding and they would also have the highly unusual property of neutralizing both H5N1 and H1N1 virus. To test the cross neutralizing activity of the IgGs, we tested representative antibodies from the H5N1 screen in a neutralization assay to see if they would also neutralize H1N1 or H3N2 virus (Table 3). We studied the H1 bearing virus A/New Cal/20/99 and the H3 bearing virus A/Hong Kong/68. A collection of viruses bearing H5 subtype hemagglutinin was also tested (A/Vn/1203/04; A/Indo/5/05; A/Turkey/65596/06; A/Egypt/06). The antibodies showed no activity against H3 subtype influenza. However, three of the monoclonal antibodies (1-3) that neutralized H5 containing viruses also strongly neutralized all viruses bearing HA from subtypes H1 (Table 5).

Table 3

	Virus*						
	H5					H1	H3
	A/Vietnam 1203/04 ‡	A/Vietnam 1203/04 ‡	A/Indonesia 5/05	A/Turkey 65596/06	A/Egypt 14725/06	A/New Cal 20/99	A/Hong Kong/68
Ab 1†	32-64	7-28	28	28	3.5-7	28	> 1000
Ab 2†	188	162-650	80	325	20-40	162-325	> 1000
Ab 3†	175	53	47	94	12-23	23-47	> 1000
Ab 4†	5-19	1.6- 6.6	> 1000	Not done	Not done	> 1000	> 1000

Immunochemical basis of neutralization

One advantage of antibody libraries is that when one obtains large numbers of antibodies, they can be grouped as to their relatedness. Thus, when a function for a given antibody in the collection is observed one can predict that other members of the group to which it belongs will have similar activity.

Table 4 shows example sequences displaying the immunochemical basis of neutralization discovered from Survivor 5 libraries following H5N1 Vietnam panning. The 61 unique heavy chain sequences aligned with their germline variable regions from the 114 unique heavy and light chain combinations. Requisite mutations are shown in bolded, underlined text (column 5 -- PI to GM and A to T; column 6 – KS to EL or EM or XL) and predominant mutations are shown in italicized, underlined text (column 2 -- A to T; column 3 -- IS to VT; column 5 -- G to A; column 8 -- K to Q or R). Heavy chains sequences also discovered in H1N1 New Caledonia panning are highlighted in gray. Antibody regions and Kabat numbering ranges are listed at the top of each sequence column. The heavy chain/light chain pairing is indicated in the first column as follows: * - paired with 2 unique light chains, † - paired with 3 unique light chains, ‡ - paired with 4 unique light chains, § - paired with 5 unique light chains, and ¶ - paired with 12 unique light chains.

Table 4

Group 1 heavy chains	FR1 1-29	CDR1 30-35	FR2 36-46	CDR2 47-56	FR3 59-62	CDR3 93-101	FR4 102-113
Vh1e	QVQLVQSGAEVKKPGSSVKVSCKASGGTF	SSYAIS	WVRQAPGQGLE	WMGGIIPIFGTAN	YAQKFQGRVTITADKSTSTAYMELSSLRSEDTAVYYC	ARGSYYYESSLD	YWGQGTLVTVSS
1	----------	------	----------	------GM---T-	----------EL----------	----------	---K--T-----
2	----------	------	----------	---A--GM---T-	----------L---EL----------	----------	---K--M-----
3	----------	------	----------	---A--GM---T-	----------EL----------D-------	----------	---K--M-----
4*	----------	----VT	----------	---A--GM---T-	----------L---EL----------	----------	----------
5	----------	----VT	----------	---A--GM---T-	----------EL----------D-------	----------	----------
6	----------	----VT	----------	---A--GM---T-	----------L---EL----------	----------	---K--M-----
7†	----------T-----	----VT	----------	------GM---T-	----------ELR----------	----------	----------
8*	----------T-----	----VT	----------	------GM---T-	----------EL----------	----------	---R-------
9†	----------T-----	----VT	----------	---A--GM---T-	----------EL----------D-------	----------	----------
10	----------T-----	----VT	----------	---A--GM---T-	----------EL----------D-------	----------	---R--M-----
11*	----------T-----	----VT	----------	---A--GM---T-	----------EL----------D-------	----------	------M-----
12¶	----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	----------
13*	----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	------M-----
14	----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	---K--M-----
15	----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------X-------	----------	----------
16*	----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	----------
17	----------R----T-----	----VT	----------	------GM---T-	--------L-----EM----------	--------TT--	---R-------
18	----------T---S-	----VT	----------	------GM---T-	----------EL----------	----------	------M-----
19	----------TT-----	----VT	----------	------GM---T-	----------EM----------	----------	---K--M-----
20§	----------TT-----	----VT	----------	------GM---T-	----------EM----------	----------	----------
21	----------TT-----	----VT	----------	------GM---T-	----------EM----------	----------	---K-------
22	----------TT-----	----VT	----------	------GM---T-	----------EM----------	----------	------M-----
23	----------X-----	----VT	----------	---A--GM---T-	----------EL----------D-------	----------	----------
24	E----------	------	----------	---A--GM---T-	----------EL----------D-------	----------	----------
25	E-------T----------L----------	----VT	----------	------GM---T-	---------L-----EM----------	--------TT--	---R--M-----
26	E-------T----------V-----	----VT	----------	---A--GM---T-	----------EL----------D-------	----------	---G--T-----
27	E----------Q----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	----------
28	E----------A----------A-	----VT	----------	------GM---T-	----------EL----------	----------	------M-----
29†	E----------R----T-----	----VT	----------	------GM---T-	--------L-----EM----------	--------TT--	----------
30†	E----------R----T-----	----VT	----------	------GM---T-	---------L-----EM----------	--------TT--	---K-------
31	E----------R----T-----	----VT	----------	------GM---T-	---------L-----EM----------	--------TT--	---R--M-----
32	E----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	---R-------
33*	E----------T-----	----VT	----------	---A--GM---T-	----------EL----------D-------	----------	---K-------
34†	E----------T-----	----VT	----------	---A--GM---T-	----------EL----------D-------	----------	----------
35	E----------T-----	----VT	----------	---A--GM---T-	----------EL----------D-------	----------	------M-----
36	E----------T-----	----VT	----------	---A--GM---T-	----------EL----------D-------	----------	---K-------
37	E----------T-----	----VT	----------	---A--GM---T-	----------EL----------D-------	----------	---K--T-----

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Table 4 (continued)

Group 1 heavy chains	FR1 1-29	CDR1 30-35	FR2 36-46	CDR2 47-56	FR3 59-62	CDR3 93-101	FR4 102-113
Vh1e	QVQLVQSGAEVKKPGSSVKVSCKASGGTF	SSYAIS	WVRQAPGQGLE	WMGGIIPIFGTAN	YAQKFQGRVTITADKSTSTAYMELSSLRSEDTAVYYC	ARGSYYYESSLD	YWGQGTLVTVSS
38	E----------------------T-----	----VT	-----------	---A--GM---T-	-------------EL--------------D-------	------------	---K--M-----
39§	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L--EL----------------------	------------	------------
40§	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L--EL----------------------	------------	---K--------
41*	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L--EL----------------------	------------	---K--M-----
42†	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L--EL----------------------	------------	---R--------
43	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L--XL----------------------	------------	---R--------
44	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L--EL----------------------	------------	---R--M-----
45	E----------------------T-----	----VT	-----------	------GM---T-	----------M--EM----------------------	------------	---K--------
46	E----------------------T-----	----VT	-----------	------GM---T-	-------------EL----------------------	------------	------------
47	E----------------------T-----	----VT	-----------	------GM---T-	-------------EL----------------------	------------	---K--------
48†	E----------------------T-----	----VT	-----------	------GM---T-	-------------EM----------------------	------------	---R--------
49	E----------------------T-----	----VT	-----------	------GM---T-	-------------EM----------------------	------------	---R--M-----
50†	E----------------------TT----	----VT	-----------	------GM---T-	-------------EM----------------------	------------	------------
51	E----------------------TT----	----VT	-----------	------GM---T-	-------------EM----------------------	------------	------M-----
52	G----------------------T-----	----VT	-----------	---A--GM---T-	----------L--EL----------------------	------------	---K--M-----
53	G----------------------T-----	----VT	-----------	---A--GM---T-	-------------EL--------------D-------	------------	---R--------
54	-M---------------------T-----	----VT	-----------	---A--GM---T-	-------------EL--------------D-------	------------	---K--M-----
55†	-M---------------------T-----	----VT	-----------	---A--GM---T-	----------L--EL----------------------	------------	------------
56	----L------------------TT----	----VT	-----------	------GM---T-	-------------EM----------------------	------------	---K--------
57*	----Q------------------T-----	----VT	-----------	---A--GM---T-	----------L--EL----------------------	------------	------------
58	----Q------------------T-----	----VT	-----------	---A--GM---T-	-------------EL--------------D-------	------------	---K--M-----
59	----Q------------------T-----	----VT	-----------	------GM---T-	-------------EL----------------------	--------N---	---K--------
60*	----Q-------------R----T-----	----VT	-----------	------GM---T-	--------L----EM----------------------	--------TT--	------M-----
61*	----Q-------------R----T-----	----VT	-----------	------GM---T-	--------L----EM----------------------	--------TT-	---K--------

Members of the group that contained the neutralizing antibody collection against epitope "A" from survivor 5, are shown in Table 4. The group is comprised of 61 unique members that most closely resemble the V_H1e germ line heavy chain. Some heavy chains are paired with more than one light chain. In total these heavy chains have 114 unique pairings to both kappa and lambda light chains. Comparing these heavy chains to the highly related VH1e germline, we observe three types of point substitutions. Some changes appear to be required, others are dominant, and some residues have only been changed sporadically. The changes that are required occur in every clone in the group within CDR2 at position 52A (Pro>Gly), 53 (Ile>Met), and 57 (Ala>Thr), as well as in the framework 3 region at position 73 (Lys> Glu) and 74 (Ser>Leu or Met), all of which vary from the germline side chain chemistries, suggesting that these mutations are critical to antigen binding and neutralization. The second set of mutations is dominant and found in most clones. The first, in framework 1 at position 24 (Ala>Thr), represents a significant chemical change. The next three are conservative changes in CDR1 at positions 34(Ile>Val) and 35 (Ser>Thr) and also in CDR2 at position 50 (Gly>Ala). All four of these dominant substitutions, however, are dispensable, suggesting that, while beneficial, they are not essential. The sporadic changes found throughout framework regions 1, 3, and 4, as well as CDR3, are all conservative and likely represent minor optimization events.

Figure 12 shows the positions of the required mutations in the structure of the antibody superimposed on the crystal structure of a highly related anti-HIV Fab called 47e (1rzi.pdb) (Huang, C.C. *et al.* (2004) *Proc. Nat. Acad. Sci.* **101,** 2706-2711). Figure 12 shows the positions of H5 hemagglutinin binding Group 1 required and dominant mutations on the crystal structure of Fab 47e. The required mutations are shown as G52 (52A (Pro>Gly)), M53 (Ile>Met), T57 (Ala>Thr), E73 (Lys> Glu) and LM74 (Ser>Leu or Met). The dominant mutations are shown as T24 (Ala>Thr), V34 (Ile>Val), T35 (Ser>Thr), and A50 (Gly>Ala). The required and dominant Group1 heavy chain sequences identified in H5 Vietnam/1203/2004 HA biopanning are superimposed on the crystal structure of the highly related anti-HIV Fab 47e. Mutations are shown in both backbone (top) and space-filling (bottom) models. A tight cluster is formed by four of the required mutations in and adjacent to CDR2. The required mutations 52A (Pro>Gly), 53 (Ile>Met), 73 (Lys> Glu) and 74 (Ser>Leu or Met) form a remarkably tight cluster on the exposed surface of the heavy chain variable domain where they form a ridge that prominently protrudes from the protein surface (Figure 12). The remaining required mutation 57 (Ala>Thr) is partially buried at the base of the CDR2 loop. The surface exposed changes in CDR 2 and framework 3 are likely to have

a direct role in antigen binding while the less exposed required mutation and the non-essential dominant mutations may have indirect effects through stabilizing and/or positioning the CDR2 loop.

The antibodies from survivor 2 are comprised of 2 unique heavy chains that most closely resemble the V_H4-4b germ line heavy chain (Table 5). The first heavy chain has been found paired with 5 unique lambda light chains, four of which are from the infrequently used lambda 6 light chain family and the other is paired with a single kappa light chain. Antibody 4 whose neutralization profile was more restricted came from this group.

Table 5

Group 2	FR1 (1-29)	CDR1 (30-35)	FR2 (36-46)	CDR2 (47-58)	FR3 (59-92)	CDR3 (93-101)	FR4 (102-113)
Heavy chain	QVQLQESGPGLVKPSETLSLTCTVSGYSF	DSGYYWG	WLRQPPGKGLE	WIGSIYHSRNTY	YNPSLKSRVTISVDTSKNQFSLQLSSVTAADTAVYYC	ARGTWYSSNLRYWFD	PWGKGTLVRVSS
	FR1 (1-29)	CDR1 (30-35)	FR2 (36-46)	CDR2 (47-58)	FR3 (59-92)	CDR3 (93-101)	FR4 (102-113)
Lambda light chains	FMLTQPHSVSESPGKTVTISCTGSGGN	IARNYVQWY	QQRPGSAPV	TVILEDDKRP	SGIPDRFSGSIDRSSNSASLTISGLRTEDEALYYC	QSYDDSDLV	VFGGGTKLT
	FMLTQPHSVSESPGKTVTISCTGSSGS	IASNYVQWY	QQRPGSAPT	TVIYEDYQRP	SGVPDRFSGSIDSSSNSASLTISGLKTEDEADYYC	QSYDDSDHL	IFGGGTKLTVL
	SVLTQPPSASGTPGQRVTISCSGSSSN	IGSNTVNWY	KQLPGTAPR	LLIYSNDQRP	SGVPDRFSGSKSGTSASLAISGLQSEDEANYYC	AAWDDSLSGW	VFGGGTKLTVL
	SVLTQPPSASGTPGQRVTLSCSGSSSN	IGGNSVNWY	QHVPGTAPK	LLMHSDDQRP	SGVPDRFSGSKSGTSXSLAISGLQSEDEADYYC	AXWDDSLNAW	VFGGXTKVTVL
	PELTQPHSVSESPGKTVTISCTGSGGR	IATNHVQWY	QQRPGSAPT	IVIYENNQRP	SGVPNRFSGSIDDSSNSASLTISALRTEDEADYYC	QSADATNV	FFGGGTKVTVL
	PELTQPPSASGTPGQRVTISCSGSSSN	IGSNTVNWY	QQLPGTAPK	LLIYSNNQRP	SGVPDRFSGSKSGTSASLAISGLQSEDEADYYC	AAWDDSLNGW	VFGGXTKLTVL
	FR1 (1-29)	CDR1 (30-35)	FR2 (36-46)	CDR2 (47-58)	FR3 (59-92)	CDR3 (93-101)	FR4 (102-113)
Kappa light chain	DIQMTQSPSSLSAFVGDRVTITCQASQDI	SNYLNWY	QQKPGKAPK	LLIYDATNLE	TGVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLPL	TFGGGTKVDIKR

The probability that a given mutation is important to the activity of an antibody increases as a function of the number of times it was independently selected. To determine if the required mutations were selected during somatic mutation from independent clones or were from the progeny of a single clone that further mutated during subsequent replications. the codon usage of the dominant mutations were analyzed (Table 6A-6B). The data reveal that although different codons were used they resulted in the same amino acid changes, demonstrating that these mutations arose independently in different clones and were, thus, selected multiple times. This convergent outcome for independently selected events is strong evidence that these dominant mutations play a critical role in the binding to the virus and/or its neutralization.

As illustrated in Tables 6A-6B, codon usage of individual clones shows independent origin of selected H5 HA binding clones. DNA alignment and encoded amino acids for 6 representative Group 1 antibodies against VH1-e germline. The use of different codons for the same amino acids demonstrates that each unique sequence is of a distinct origin. Table 6A corresponds to CDR2 and Table 6B corresponds to Framework 3. Germ line codons are shown as bolded codons. A change from a germ line codon to the same amino acid is shown as a plain text codon. A first change from a germ line amino acid is shown as a bolded, underlined codon. A second change from a germ line amino acid is shown as an italicized, underlined codon. A third change from a germ line amino acid is shown as an underlined, grayed-highlighted codon.

Table 6A

Clone Number		CDR2														
	Kabat Sequence	47	48	49	50	51	52	52A	52B	52C	53	54	55	56	57	58
	VH1-e germline	TGG	ATG	GGA	AGG	ATC	ATC	CCT	ATC	CTT	GGT	ATA	GCA	AAC	TAC	GCA
		W	M	G	R	I	I	P	I	L	G	I	A	N	Y	A
	27	TGG	ATG	GGC	GCG	ATC	ATC	GGT	ATG	TTT	GGT	ACA	ACA	AAC	TAC	GCA
		W	M	G	A	I	I	G	M	F	G	T	T	N	Y	A
	30	TGG	ATG	GGA	*GGG*	ATC	ATC	GGT	ATG	TTT	GGA	ACA	*ACC*	AAC	TAT	GCA
		W	M	G	G	I	I	G	M	F	G	T	T	N	Y	A
	33	TGG	ATG	GGA	GCG	ATC	ATC	GGT	ATG	TTT	GGT	ACA	ACA	AAC	TAC	GCA
		W	M	G	A	I	I	G	M	F	G	T	T	N	Y	A
	41	TGG	ATG	GGC	GCG	ATC	ATC	GGT	ATG	TTT	GGT	ACA	ACA	AAC	TAC	GCA
		W	M	G	A	I	I	G	M	F	G	T	T	N	Y	A
	50	TGG	ATG	GGA	*GGG*	ATC	ATC	GGT	ATG	TTT	GGT	ACA	ACG	AAC	TAT	GCA
		W	M	G	G	I	I	G	M	F	G	T	T	N	Y	A
	17	TGG	ATG	GGA	*GGG*	ATC	ATC	GGT	ATG	TTT	GGT	ACA	ACA	AAC	TAC	GCA
		W	M	G	G	I	I	G	M	F	G	T	T	N	Y	A
	# Codons used	1	1	2	2	1	1	1	1	1	2	1	3	1	2	1

Table 6B

		Framework 3											
	Kabat Sequence	67	68	69	70	71	72	73	74	75	76	77	78
Clone Number	VH1-e germline	**GTC**	**ACG**	ATT	**ACC**	**GCG**	**GAC**	**AAA**	**TCC**	**ACG**	**AGC**	**ACA**	**GCC**
		V	T	I	T	A	D	K	S	T	S	T	A
	27	**GTC**	**ACG**	**CTT**	**ACC**	**GCG**	**GAC**	**GAA**	*TTA*	**ACG**	TCC	**ACA**	**GCC**
		V	T	L	T	A	D	E	L	T	S	T	A
	30	**CTC**	ACA	ATC	**ACC**	**GCG**	**GAC**	*GAG*	**ATG**	**ACG**	TCC	**ACA**	**GCC**
		L	T	I	T	A	D	E	M	T	S	T	A
	33	**GTC**	ACA	ATC	**ACC**	**GCG**	**GAC**	**GAA**	*TTA*	**ACG**	TCC	**ACA**	**GCC**
		V	T	I	T	A	D	E	L	T	S	T	A
	41	**GTC**	**ACG**	**CTT**	**ACC**	**GCG**	**GAC**	**GAA**	*TTA*	**ACG**	TCC	**ACA**	**GCC**
		V	T	L	T	A	D	E	L	T	S	T	A
	50	**GTC**	**ACG**	**ATT**	**ACC**	**GCG**	**GAC**	*GAG*	**ATG**	**ACG**	TCC	**ACA**	**GCC**
		V	T	I	T	A	D	E	M	T	S	T	A
	17	**GTC**	**ACG**	**ATT**	**ACC**	**GCG**	**GAC**	**GAA**	*TTA*	**ACG**	TCC	**ACA**	**GCC**
		V	T	I	T	A	D	E	L	T	S	T	A
	# Codons used	2	2	3	1	1	1	2	2	1	1	1	1

Example 2

Constructing Donor-Specific Antibody Library for Patients Infected with HIV

Bone Marrow Protocol and Sera Preparation

Blood is obtained by standard venopuncture, allowed to clot, and processed to recover serum. The serum is stored at -20 °C for 3-4 days until they are shipped on dry ice. Donors are anaesthetized with an injection of a local anesthetic and 5ml of bone marrow is removed from the pelvic bone of each patient donor. Next the 5ml of bone marrow is placed into a sterile 50-ml tube containing 45 ml RNAlater (Ambion). The mixture is gently inverted approximately 8-20 times, until there are no visible clumps and the marrow and RNAlater are mixed well. Next the specimen is refrigerated the between 2-10 °C overnight. Following the overnight refrigeration, the specimens are stored at -20 °C for 3-4 days until they are shipped on dry ice. Upon receipt the RNAlater/marrow and sera containing tubes are stored at -80 °C until processed. Candidate patient should be tested HIV positive prior to be selected as donors.

Bone marrow extraction and mRNA purification, reverse transcription, PCR, antibody light and heavy chain construction, phagemid panning and amplification, ELISA and sequencing are performed essentially as described in Example 1.

Although in the foregoing description the invention is illustrated with reference to certain embodiments, it is not so limited. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

All references cited throughout the specification are hereby expressly incorporated by reference.

WHAT IS CLAIMED:

1. A vector collection comprising a repertoire of nucleic acid molecules encoding antibody light or heavy chains or fragments thereof, derived from a human patient donor who has suffered from, or is suffering from, a disease evoking antibody production to a target antigen, wherein said collection is identified with a unique barcode.

2. The vector collection of claim 1 comprising a repertoire of nucleic acid molecules encoding antibody light chains or fragments thereof.

3. The vector collection of claim 2 wherein the antibody light chains are λ chains.

4. The vector collection of claim 2 wherein the antibody light chains are κ chains.

5. The vector collection of claim 1 comprising a repertoire of nucleic acid molecules encoding antibody heavy chains or fragments thereof.

6. The vector collection of claim 1 wherein the barcode is a nucleotide sequence linked to or incorporated in the vectors present in the collection, and/or linked to or incorporated in the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof such that it does not interfere with the expression of said nucleic acid molecules.

7. The vector collection of claim 6 wherein said barcode is a contiguous non-coding nucleotide sequence of one to about 24 nucleotides.

8. The vector collection of claim 7 wherein said nucleotide sequence is linked to the 3' or 5' non-coding region of said nucleic acid molecules.

9. The vector collection of claim 6 wherein said nucleotide sequence is a coding sequence of one or more silent mutations incorporated into the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof.

10. The vector collection of claim 6 wherein said nucleotide sequence in non-contiguous.

11. The vector collection of claim 10 wherein at least part of said non-contiguous nucleotide sequence is linked to or incorporated in the vectors present in the collection.

12. The vector collection of claim 10 wherein at least part of said non-contiguous sequence is incorporated into the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof such that it does not interfere with the expression of said nucleic acid molecules.

13. The vector collection of claim 1 wherein the barcode is a peptide or polypeptide sequence.

14. The vector collection of claim 1 wherein the vectors are phagemid vectors.

15. The vector collection of claim 15 wherein the phagemid vectors contain a bacteriophage gene III and a stop codon between the nucleic acid molecules encoding antibody light or heavy chains or fragments thereof and the bacteriophage III gene.

16. The vector collection of claim 15 wherein the barcode is a non-coding contiguous nucleotide sequence inserted in the untranslated region following said stop codon.

17. Host cells comprising the vector collection of claim 1.

18. The host cells of claim 16 which are E. coli host cells.

19. A donor-specific antibody library comprising library members expressing a collection of antibodies or antibody fragments to a target antigen wherein said antibodies or antibody fragments are derived from a human donor who has suffered from, or is suffering from, a disease evoking antibody production to said target antigen, wherein said antibody library is identified with at least one unique barcode.

20. The donor-specific antibody library of claim 19 wherein said antibody heavy and light chains are separately identified each with a barcode unique to the human donor from whom it derived.

21. The donor-specific antibody library of claim 19 which is identified with one unique barcode.

22. The donor-specific antibody library of claim 19 wherein said antibodies or antibody fragments are composed of antibody heavy and light chains or fragments thereof encoded by nucleic acid molecules present in a vector.

23. The donor-specific antibody library of claim 22 wherein the barcode is a nucleotide sequence linked to or incorporated in the vectors present in the library, and/or linked to or incorporated in the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof such that it does not interfere with the expression of said nucleic acid molecules.

24. The donor-specific antibody library of claim 23 wherein said barcode is a contiguous non-coding nucleotide sequence of one to about 24 nucleotides.

25. The donor-specific antibody library of claim 24 wherein said nucleotide sequence is linked to the 3' or 5' non-coding region of said nucleic acid molecules.

26. The donor-specific antibody library of claim 23 wherein said nucleotide sequence is a coding sequence of one or more silent mutations incorporated into the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof.

27. The donor-specific antibody library of claim 23 wherein said nucleotide sequence in non-contiguous.

28. The donor-specific antibody library of claim 27 wherein at least part of said non-contiguous sequence is linked to or incorporated in the vectors present in the library.

29. The donor-specific antibody library of claim 27 wherein at least part of said non-contiguous sequence is incorporated into the nucleic acid molecules encoding the antibody light or heavy chains or fragments thereof such that it does not interfere with the expression of said nucleic acid molecules.

30. The donor-specific antibody library of claim 19 wherein the barcode is a peptide or polypeptide sequence.

31. The donor-specific antibody library of claim 22 wherein the vectors are phagemid vectors.

32. The donor-specific antibody library of claim 31 wherein the phagemid vectors contain a bacteriophage gene III and a stop codon between the nucleic acid molecules encoding antibody light or heavy chains or fragments thereof and the bacteriophage III gene.

33. The donor-specific antibody library of claim 19 expressing a collection of antibody heavy chains or fragments thereof.

34. The donor specific antibody library of claim 33 comprising more than one antibody heavy chain family.

35. The donor-specific antibody library of claim 19 wherein the medical history of said human donor shows that the patient has suffered from, or is suffering from said disease.

36. The donor-specific antibody library of claim 35 wherein it is independently confirmed that said human donor suffered from, or is suffering from said disease.

37. The donor-specific antibody library of claim 19, which is substantially devoid of antibodies and antibody fragments specifically binding antigens different from said target antigen.

38. The donor-specific antibody library of claim 19 wherein said target antigen is an influenza A virus.

39. The donor-specific antibody library of claim 38 wherein said target antigen is an isolate of influenza A virus H1, H2 or H3 subtype.

40. The donor-specific antibody library of claim 38 wherein the target antigen is selected from the group comprising H5, H7 and H9 influenza A virus subtypes.

41. The donor-specific antibody library of claim 38 expressing at least one antibody or antibody fragment specifically binding to more than one influenza A virus subtype.

42. The donor-specific antibody library of claim 39 expressing at least one antibody or antibody fragment binding to and neutralizing the H5N1 subtype of influenza virus A.

43. The donor-specific antibody library of claim 19 wherein the human donor has suffered from, or is suffering from a disease selected from the group consisting of the diseases listed in Table 1.

44. The donor-specific antibody library of claim 43 expressing at least one antibody or antibody fragment binding to an antigen associated with said target disease.

45. The donor-specific antibody library of claim 43 expressing at least one antibody or antibody fragment binding to and neutralizing an antigen associated with said target disease.

46. The donor-specific antibody library of claim 19 expressing a least one neutralizing antibody.

47. The donor-specific antibody library of Claim 19, wherein said donor-specific antibody library is a phage library.

48. The donor-specific antibody library of Claim 47, wherein said collection contains sequences encoding more than 106 different members of antibodies or antibody fragments.

49. The donor-specific antibody library of Claim 47, wherein said collection contains sequences encoding more than 109 different members of antibodies or antibody fragments.

50. The donor-specific antibody library of claim 19, which is selected from the group consisting of a spore-display library, a ribosome display library, a mRNA display library, a microbial cell display library, a yeast display library, and a mammalian display library.

51. The donor-specific antibody library of Claim 50, wherein said nucleic acid is reverse-transcribed from mRNA extracted from lymphocytes of said human donor.

52. The donor-specific antibody library of Claim 51, wherein said lymphocytes are derived from bone marrow, blood, spleen, or lymph node.

53. The donor-specific antibody library of Claim 52, wherein a serological profile of said human donor is generated prior to extraction of said mRNA.

54. The donor-specific antibody library of Claim 53, wherein a medical history of said human donor is examined prior to or following extraction of said mRNA.

55. A method of making a donor-specific library expressing a collection of antibodies or antibody fragments to a target antigen, comprising the steps of:

a) obtaining mRNA from lymphocytes of a human patient donor who has suffered from, or who is suffering from a disease evoking antibody production to said target antigen;

b) generating a collection of nucleic acids comprising sequences encoding an immunoglobulin repertoire of said patient by reverse transcription of said obtained mRNA: and

c) identifying said donor-specific library with an unique barcode labeling said nucleic acids.

56. The method of Claim 55, further comprising steps of generating a serological profile of said patient and/or examining medical history of said patient prior or subsequent to step a).

57. The method of Claim 56, further comprises steps of:

d) inserting said nucleic acids into expression vector;

e) expressing said immunoglobulin repertoire; and

f) displaying said immunoglobulin repertoire in a display system.

58. The method of Claim 57, wherein the display system is a phagemid.

59. The method of claim 58 further comprising the step of selecting members of the library based their ability to neutralize or activate the target antigen.

60. The method of claim 59 that yields at least one neutralizing antibody.

61. The method of claim 59 that yields more than one neutralizing antibody.

62. The method of claim 61 further comprising the step of creating one or more sub-libraries comprising library members that were found to neutralize or activate the target antigen.

63. The method of claim 59 comprising the step of sequencing at least one library member identified.

64. A method of treating or preventing a disease associated with a target antigen neutralized or activated by an antibody selected by the method of claim 59, comprising administering to said human patient in need an effective amount of the antibody selected.

65. The method of claim 64 wherein the antibody is a neutralizing antibody.

66. The method of claim 65 wherein the disease is an influenza virus A infection.

67. The method of claim 64 wherein the disease is selected from the group consisting of the diseases listed in Table 1.

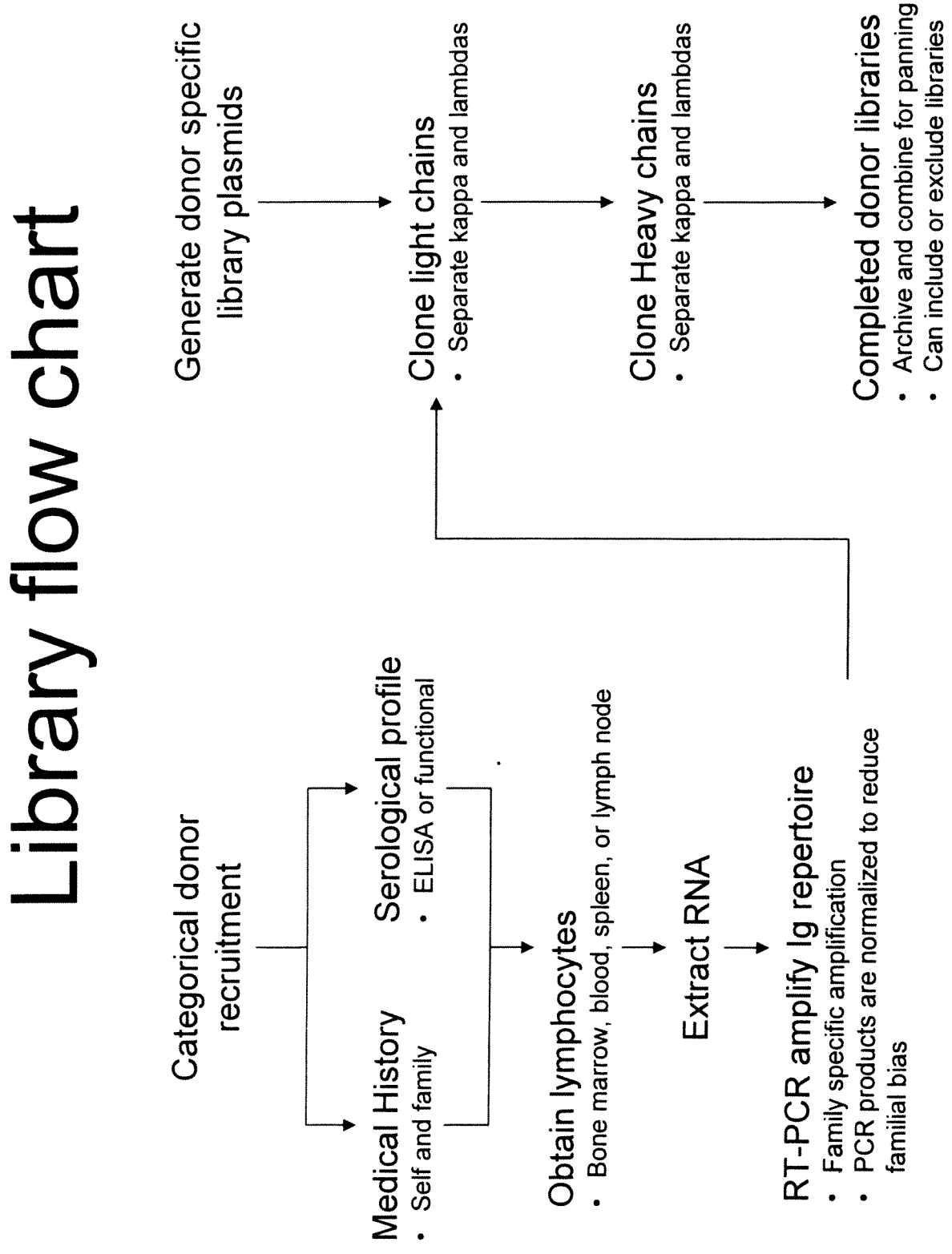
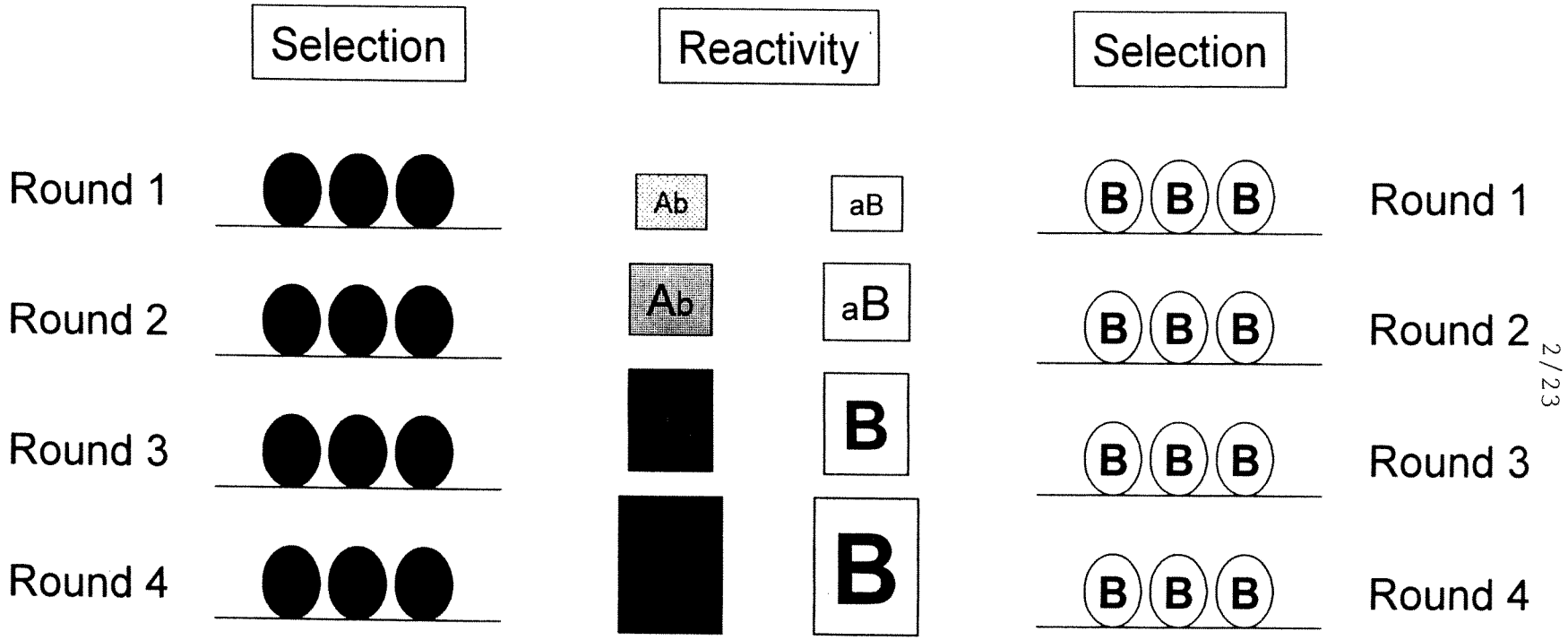

Library flow chart
Categorical donor recruitment
Medical History
• Self and family
Serological profile
• ELISA or functional
Obtain lymphocytes
• Bone marrow, blood, spleen, or lymph node
Extract RNA
RT-PCR amplify Ig repertoire
• Family specific amplification
• PCR products are normalized to reduce familial bias
Generate donor specific library plasmids
Clone light chains
• Separate kappa and lambdas
Clone Heavy chains
• Separate kappa and lambdas
Completed donor libraries
• Archive and combine for panning
• Can include or exclude libraries

FIG. 1

Typical panning enrichment results



Each round of enrichment increases the reactive strength of the pool towards the individual target(s)

FIG. 2

CROSSREACTIVE DISCOVERY SELECTION
SELECTION
REACTIVITY
ROUND 1
A A
A
Ab
ROUND 2
B B
B
AB
ROUND 3
A A
A
Ab
ROUND 4
B B
B
AB
EACH SUCCESSIVE ROUND REINFORCES THE REACTIVE STRENGTH OF THE RESULTING POOL TOWARDS BOTH TARGETS

FIG. 3

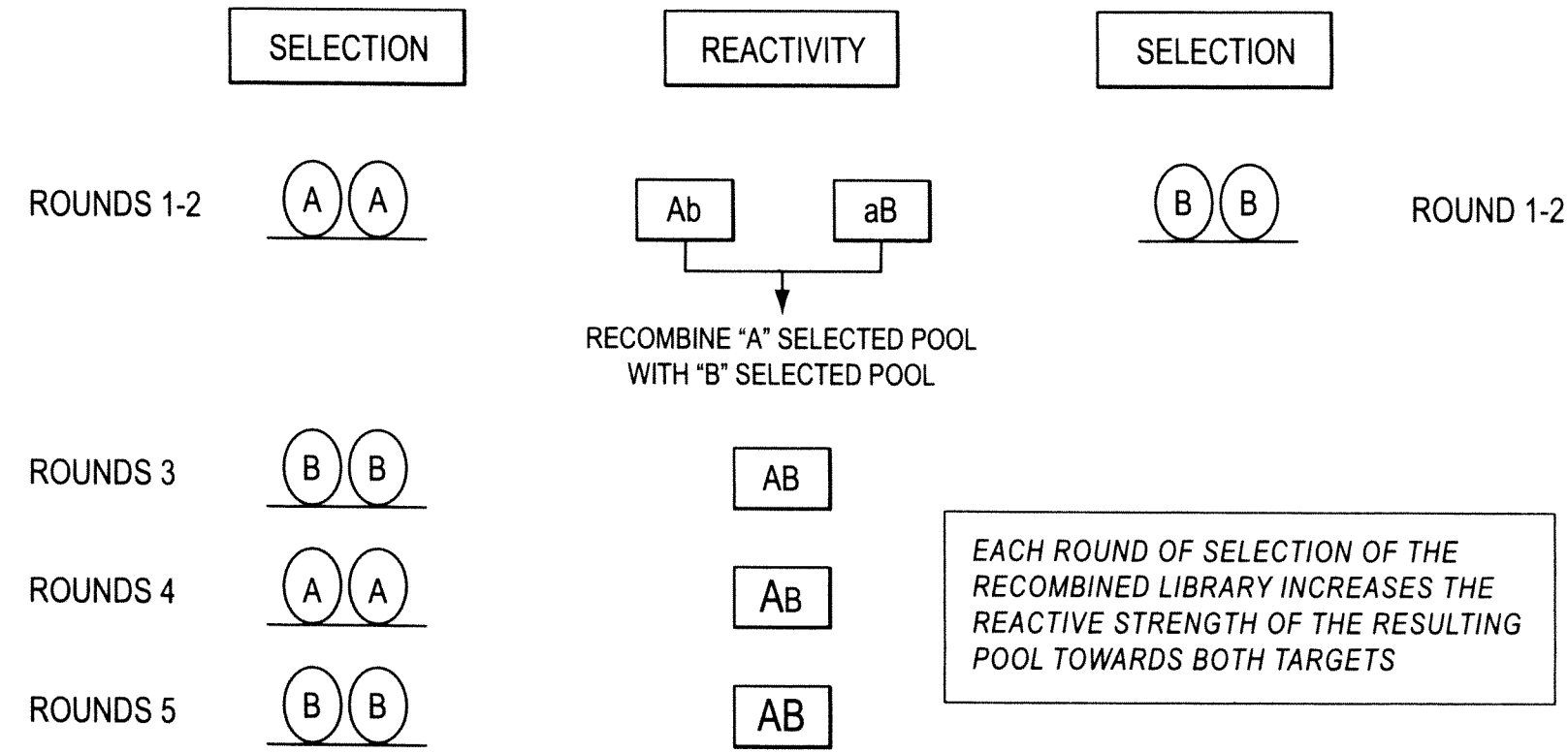

RECOMBINING PARALLEL DISCOVERY POOLS TO GENERATE CROSSREACTIVITY
SELECTION
REACTIVITY
SELECTION
ROUNDS 1-2
A A
Ab
aB
B B
ROUND 1-2
RECOMBINE "A" SELECTED POOL
WITH "B" SELECTED POOL
ROUNDS 3
B B
AB
ROUNDS 4
A A
AB
ROUNDS 5
B B
AB
EACH ROUND OF SELECTION OF THE RECOMBINED LIBRARY INCREASES THE REACTIVE STRENGTH OF THE RESULTING POOL TOWARDS BOTH TARGETS

FIG. 4

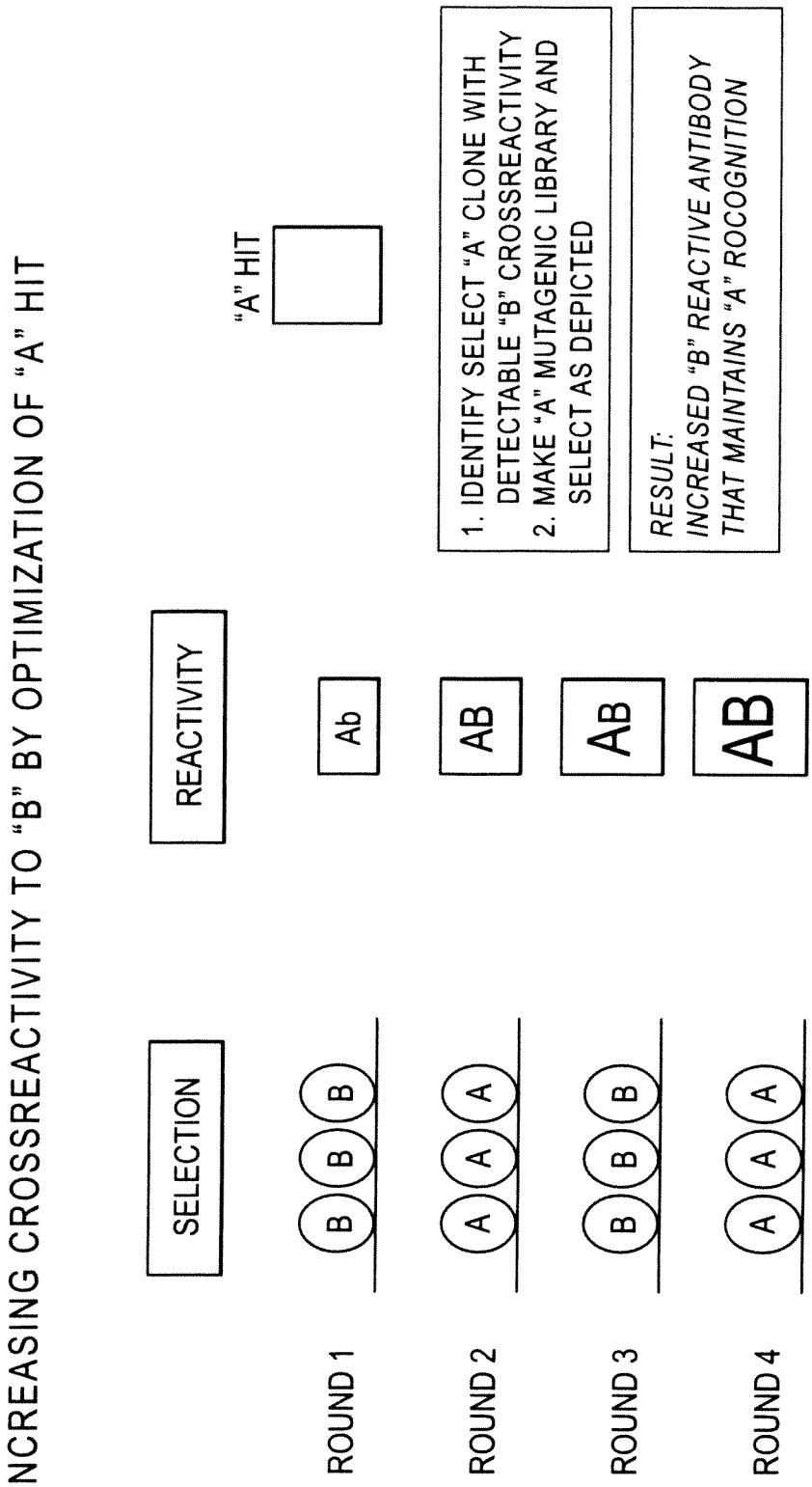

INCREASING CROSSREACTIVITY TO "B" BY OPTIMIZATION OF "A" HIT
SELECTION
REACTIVITY
"A" HIT
ROUND 1
B B B
Ab
ROUND 2
A A A
AB
ROUND 3
B B B
Ab
ROUND 4
A A A
AB
1. IDENTIFY SELECT "A" CLONE WITH DETECTABLE "B" CROSSREACTIVITY
2. MAKE "A" MUTAGENIC LIBRARY AND SELECT AS DEPICTED
RESULT:
INCREASED "B" REACTIVE ANTIBODY THAT MAINTAINS "A" ROCOGNITION

FIG. 5

H3 DESTINATIONAL EXAMPLE
CDR H3

2SD4	T	K	A	S	Y	L	S	T	S	S	S	L	D	
CODONS	ACN	AAR	GCN	TCN	TAY	CTN	AGY	ACN	AGY	AGY	AGY	CTN	GAY	
DEGENERATE OLIGO	RCN	MRN	GSN	WYN	TAY	YTN	WRY	RSN	ASN	ASN	WRY	YTN	GAY	
COCONS	GCN	CGN	GGN	ATH	TAY	TTY	TAY	GGN	ACN	ACN	TAY	YTY	GAY	
Raptiva	A	R	G	I	Y	F	Y	G	T	T	Y	F	D	
DESTINATIONAL COPRODUCTS		AAY		ACN		TTR	AAY	AGY	AGR	AGR	AAY	TTR		
		N		T		L	N	S	R	R	N	L		
		CAY		TTY			TGY	AGR			TGY			
		H		F			C	R			C			
		CAR		TTR				GCN						
		Q		L				A						
		AGY		ATG										
		S		M										PEPTIDE DIVERSITY
POSITIVE RESIDUES	2	6	2	6	1	2	4	5	3	3	4	2	1	414720 HIGH
REDUCED DEGENERACY	2	4	2	4	2	2	2	4	2	2	2	2	2	65538 LOW

FIG. 6

Hemagglutinin alignment

Positions from 1 till 60

```
consensus  MEKIIIILLLLALAACSGALPGNDNSTDKICIGYHANNSTETVDTLTEKNVEVTHATELV
AAA43678   --MA..Y.I..F-T.VR.----------.Q...........K...IL.R..T....KDIL
AAA79775   .Y.VVV.IA..G-.VK-.L---------.R..L.H..VANGTI.K...NEQE...N...T.
AAA96134   .NTQ..VI.V.G-LSMVKS---------....L.H..VANGTK.N....RG...VN...T.
AAC40998   .NTQ.L..A.V.-IIPTN.----------....L.H..VSNGAK.N....RG...VN...T.
AAD17229   ..ARLLV..CAF-..TNA----------.T..........D....VL....T...SVN.L
AAD21159   ----------------------------.Q...........Q...IM....T....QDIL
AAF99711   .LS.V.LF..I.-ENS.QNTY.-----PV..M.H..VANGTM.K..ADDQ...VT.Q...
AAK51718   .KT..ALSYIFC-L.LGQD........ATL.L.H..VPNGTL.K.I.DDQI...N.....
AAX78820   -MIA....AIV.-STSKS---------..............TQ...IL....T...SV..L
ABB87356   .AVKVLH..IIV.GRY.I.---------------LS...SD.......NG.P..SSID..
BAA14334   ...F.A.AT-...-STNAY----------.R.....QS....D..N..I.Q..P..QTM...
BAA14336   ...TLLFAAIFL----.VK.----------.E.....LS....DK...II.N..T..SSV...
BAA14337   ...F..LSTV.--.SFAY----------------QT.......N..S.Q..P..QVE...
BAA14338   .ALNV.AT.T.I-SV.VH.----------.R..V..LST..S.R....L.NG.P..SSID.I
CAB95856   ..T.SL.TI..V-VTA.N.----------.....HQST...........T..P....K..L
```

FIG. 7A

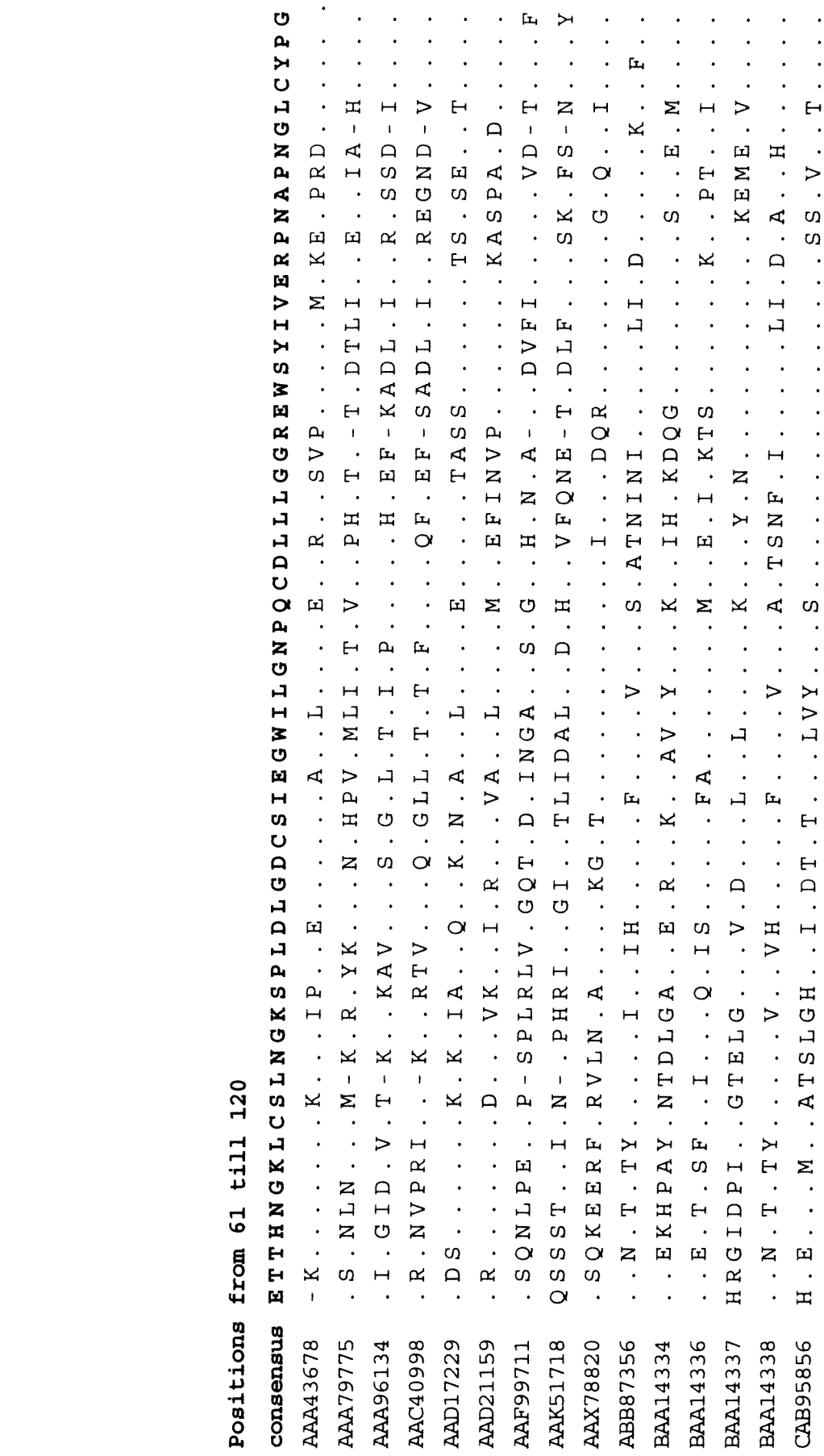

Positions from 61 till 120
consensus ETTHNGKLCSLNGKSPLDLGDCSIEGWILGNPQCDLLLGGREWSYIVERPNAPNGLCYPG
AAA43678 -K.....K.....IP..E.....A..L.....E...R..SVP.....M.KE.PRD.....
AAA79775 .S.NLN.....M-K.R.YK.....N.HPV.MLI.T.V...PH.T.-T.DTLI..E..IA-H.....
AAA96134 .I.GID.V.T-K...KAV.....S.G.L.T.I.P.....H.EF-KADL.I...R.SSD-I.....
AAC40998 .R.NVPRI...-K...RTV.....Q.GLL.T.T.F.....QF.EF-SADL.I...REGND-V.....
AAD17229 .DS.....K.K.IA...Q...K.N.A...L.....E.....TASS.....TS.SE...T.....
AAD21159 .R.....D.....VK...I.R.....VA...L.....M...EFFINVP.....KASPA.D.....
AAF99711 .SQNLPE...P-SPLRLV.GQT.D.INGA...S.G...H.N.A-....DVFFI.....VD-T.....F
AAK51718 QSSST...I.N-..PHRI...GI...TLIIDAL...D.H...VFQNE-T.DLF.....SK.FS-N.....Y
AAX78820 .SQKEERF.RVLN.A.....KG.T.....I.....DQR.....G.Q...I.....
ABB87356 ...N.T.TY.....I...IH.....F.....V.....S.ATNINI.....LI.D.....K...F..
BAA14334 ...EKHPAY.NTDLGA...E.R...K...AV.Y.....K...IH.KDQG.....S...E.M.....
BAA14336 ...E.T.SF...I.....Q.IS.....FA.....M...E.I.KTS.....K...PT...I.....
BAA14337 HRGIDPI...GTELG.....V.D.....L.....K.....Y.N.....KEME.V.....
BAA14338 ...N.T.TY.....V....VH.....F.....V.....A.TSNF.I.....LI.D.A...H.....
CAB95856 H.E.....M...ATSLGH...I.DT.T.....LVY.....S.....SS.V...T.....

FIG. 7B

```
Positions from 121 till 180
consensus DFENYEELRHLFSSSGSFEKIEIFPKTFTWGNVVTTNGTTKACKDRSGGSSFYRNLVWLT
AAA43678  S.ND.....K..L...VKH.....VK.L...-DR.TQHT..G.-SR..A-V..NP..F..M....
AAA79775  ATI.E.A...QKIME...GIS.MSTG-----.Y.SSI.SA.....M-..N...D....AE.K..V
AAA96134  R.T.E.A...QIIRE...GID.ESMG-----.RY-SGIR.D.A.S.....-.TVS-....SEMK..S
AAC40998  K.V.E.A...QILRK...GID.ETMG-----.Y-SGIR.....A.S...R-....S-....AEMK..L
AAD17229  ..ID.....EQL...VS.....F.....SS.P.HE...K.V.A...S-YA.A.........L....
AAD21159  N.ND.....K...L.RINH.....Q.I...-SS.S.HDASS.VSS...P-YL.R....F...V....I
AAF99711  .VPE.QS...SILANN.K...F.AEE-----.Q.--.T.KQ...KSG.....--.ANVDD.FNR.N..V
AAK51718  .VPD.AS...S.VA....TL.F.TEG-----.....-TG...Q...GSN.....--.GP...G.FSR.N....
AAX78820  ILNEL.....KA.IG.GERVQRF.M.....-S...AG.D.SR.V.....P-YIS.....G...L.II
ABB87356  ELD.NG.........GVN...SRT.LISP-SK...D.LD---.V.AS.L.K-..A.........V
BAA14334  SV....L.....FV.....AA.YKR.RL.DY-SR.---N...RS...S....NAST...Q.....SIN....
BAA14336  TL.SE.....LK...GVLE.N.F.V.TS-NG...A.NSGV.V.A.....FG--.SN...F...M....I
BAA14337  SI...Q.....S.....IKKY.RVKM.DF-TK.---N...YT...S.....NNT.NQG.....SMR....
BAA14338  ELN.NG.....GIR...SRT.LI.P-TS...E.LD---.....S...R.NT.TN.........FI
CAB95856  NV...L.....T.....AS.YQR.Q....D-T.---N...YT...  SR...S-------G.....SMR..I
```

FIG. 7C

```
Positions from 181 till 240
consensus  SKKKGSAYPVIKGTYNNTRGEDILIIWGIHHPPTTTEQTKLYGNADTYVSVGTSTYNRRF
AAA43678   -E-....N....A..S.....S..QM.....V....NDEK..RT..Q.VG.........L.K.S
AAA79775   ..T...QNF.QTTN...R...DTAEH.....................SS.Q.KND....TQSLSI...ES.....QNN.
AAA96134   .SMNNQVF.QLNQ...R....K.PA...V...V....SSSLD...N.....TGNKLIT...S.K.QQS.
AAC40998   .NTDNA.F.QMTKS.K.I.KDPA.....................SGS.A.....SGNKLIT...S.N.QQS.
AAD17229   -.-....S...KLSKS.V.NK.KEV.VL...V.....G.D.QS...Q....A.....S.K.....
AAD21159   -..-.N.S...T...RS.....NQ...L.VL.....NDAA.....Q.PT...I.....L.Q.L
AAF99711   -.SD.N....FQNL.KI.NGDYAR.Y....V....S.S....IN...K.NPGR.T.S.K.SQTSV
AAK51718   -.S-....T....LNV.MP.NDNF.K.Y....V....S.NQ....S...VQ.SGR.T.S.RRSQQTI
AAX78820   -.TESA.....................GSQP..YF...V.....D.N...NT.....SG.R...RM...ESM.FAK
ABB87356   -.-QNDR....VR.D.....T.R.V.VL.....D.E.TA.....V.KNP.TL.S.KEWSK.Y
BAA14334   -..EPDT.DFNE.A.V.NEDG...IFL.....D.K.....T...K...N.LS...T.N.I...S.
BAA14336   -H-QSQT.....R.F....K.R.V...V.....A.L...HQD...KKDSS...A...SE.....
BAA14337   -L.-SGQF...QTDE.K....DS...VFT.A.....SD...V....K.P...LS...T.VEI...S.
BAA14338   -..-.NTR.....SK.....T.R.V.VL.....VSVD.TKT...V.S.P.TL.S.KSWSEKY
CAB95856   -Q.-SGF....QDAQ.T.N...KS...FV.....Y.....N...IRN...TT...T.EDL...T.
```

FIG. 7D

```
Positions from 241 till 300
consensus VPEIGARPKVNGQSGRMDFYWTLLKPGDTITFESNGNLIAPRYAYKLIKGGPSGIEYNGK
AAA43678  T.D.AT.....LGS..E.S.....DMW.....N.....T.....E.GF.IS.R.----------SS
AAA79775  ..VV.....Q.....I...H.....VQ.....N.....SD...G.....SRVS...T----------.R
AAA96134  S.SP.....A...I...H.M...D.....V...TF...AF.....DR.TF.RSNA.........
AAC40998  ..SP.....Q.....I...H.LM.N.N...V...SF...AF.....DR.SF.R----------
AAD17229  T.....A.....RD.A.....NY.....E.....AT.....W...FA.NR.S----------.S
AAD21159  .....AT.....E.F...I.....N.A.N.....F.....E.....IV.K.----------DS
AAF99711  ..D...S...L.R.....VS.....IVE.....L.V.NTI.....GH.....NNQ----------K.
AAK51718  I.N...S...W.R.L.S.ISI.....IV.....VLVIN.....GYF.MRT----------
AAX78820  S.....A.....A.....R...I.Y...SV.....E.LNV.....W.....FTSSN----------N.
ABB87356  EL.....T.IG-D...RSW.KI...H.MH...ER.M.....G.L.....G..-I.EKY----------.T
BAA14334  Q.N...P...L.R...Q.....Y...GI...R.E.LKIRT.....EFG.-..L..E----------SY
BAA14336  T.....NT...R.....A.....T.....KIV.....ES.....AFL.....F-E.VSV----------.N
BAA14337  K.N...P...L.R...Q.....Y...AV.....Q.VKIQT.....E.GH-...T.K----------SH
BAA14338  KL.T.V...GY.....RSW.KI...S.IH...EM.....GFL.....G..-I.EEY----------
CAB95856  K.V...P...L.....LQ.....I.Y...SV.....Q.LRVR.....W.GH-VLS.....----------SH
```

FIG. 7E

```
Positions from 301 till 360
consensus GRIIQSEDLPIGANCNTKCQTPGGAINTSKPFQNISPLTIGECPKYVKSGSLKLATGLRN
AAA43678  .--.MKT-EGTLE--.E......L.....TL..H.VH.............EK.V.......
AAA79775  DLG....-EAL.DNS.ES..FWR..S...KL....L..R.V.Q.....NQR..L....M..
AAA96134  SLG.....-.AQ.DES.EGE.F.YS..T..SPL.....DSRAV.K..R...QS..P..L.MK.
AAC40998  SMG....-EVQVD...EGD.YHS..T.LS.L.....NSR.V....R...QE..L....MK.
AAD17229  .---.T--.A.VH-D........H....S.L.....H.V.........R.TK.RM......
AAD21159  T-.MK.-E.EY.-..........M....S.M..H..H.............NR.V.......
AAF99711  ST.LNT-AI....-S.VS..H.DK.SLS.T.......RIAV.D..R...Q........M..
AAK51718  SS.MR.--.A..D-T.ISE.I..N.S.PND.....VNKI.Y.A......Q.NT......M..
AAX78820  .AVFK.-N...E--.DAV...VA..LR.N.T...V...W..........D..R.......
ABB87356  ...F..-GIRMA-K.......SM.GV..N.T....ERNAL.D....I...Q.........
BAA14334  .....N..I.....-.........YA....S......A.RHYM........KA..R..V....
BAA14336  .KLFR.-E.N.E-S.S.....EI.G...N.S.H.VHRN...D.....NVK.......P..
BAA14337  ...LKN-N..M.-Q.V.E..LNE.VM........T.KHY..K....IP.......I....
BAA14338  ...F...-RIRMS-R.......SV.G...NRT....DKNAL.D....I...Q.........
CAB95856  ...LKT-..KG.-..VVQ...EK.GL.STL..H...KYAF.T.....RVN.....V....
```

FIG. 7F

```
Positions from 361 till 420
consensus VPEIIERRRKSRGLFGAIAGFIEGGWPGLIDGWYGFHHQNAQGTGIAADKKSTQKAIDQI
AAA43678  ..Q.-.-----------------------Q.M.....Y..S.D..S.Y.....E.....F.G.
AAA79775  ...VVQ----G.------------------N..E.MV.....R.....Q....Y.....A.....
AAA96134  ...K.R----T.------------------N..E.....R.....Q.T....Y.....A.....
AAC40998  ....PK----G.------------------N..E..V.....R.....E.T....Y.....S.....
AAD17229  I.S..-Q-----.------------------T.M.....Y.....E..S.Y....Q.....N....G.
AAD21159  T.QR-.....K.------------------Q.MV.....Y..S.E..S.Y....QE.....GV
AAF99711  I...K-A-----.------------------N...Q.....R.....E....T....L.....A...N..
AAK51718  ...K-Q-----T.------------------N..E.M.....R.....SE...Q....L.....A.....
AAX78820  ..QA-.-----T.------------------T.M.....Y..E.S..S.Y.....E.....G.
ABB87356  ..S..-G-----E.------------------.....N.....Q.....E.....A.....NE.
BAA14334  T.SV-.-----P.------------------.....S.M.....S.SE....M....Q.....E....K.
BAA14336  ..A..-A-----.------------------.....N.....Q.RDEE.....E.....
BAA14337  ..QV-Q-----D.------------------.....VA.....Q.....E.....RD.....R....NM
BAA14338  ..A..-S-----N.------------------.....N.....Q.....E.....E.....
CAB95856  ..AR-S-----.------------------.....VA.....Q.S.D..V.M...RD.....K.
```

FIG. 7G

```
Positions from 421 till 480
consensus TNKVNNIIEKMNTQFEAIDHEFSEVEKRINNLNKKVDDGFTDIWSYNAELLVLLENQHTL
AAA43678  ......SV..........VGK....NL.R.LE.......ME....L.V.T..........M..ER..
AAA79775  .G.L.RL....T..E...S.ES.....T.HQ.G.VINWTK.SI.....T..........AM.....I
AAA96134  .G.L.RL....T.K.....L...N...T.....QQ.G.VINWTR.SL.E..........AM.....I
AAC40998  .G.L.RL....T.Q.....L...N...T.....Q.G.VINWTR.SL.EM..........AM.....I
AAD17229  ......SV..........T.VGK...NNL.R...E..........L....T..........ER..
AAD21159  ......S...N..........VGR...NNL.R...E.......ME....L.V.T..........M..ER..
AAF99711  NG.L.RL....T.DKYHQ.EK...EQ...G...QD.ENY.E.TKI.L..........A.....I
AAK51718  NG.L.RV....T.EK.HQ.EK.........G...QD.E.Y.E.T.KI.L..........A.....I
AAX78820  ......S...D..........V.......NL.R.VD.....RME....L.V.T..........ER..
ABB87356  .T.I..........GNTDS.RG...NQ.........M.ADR....AV..........K.....I..DR..
BAA14334  ..........VD....RE...VVN.............MI.D.I...QIE.L.A..........K..
BAA14336  .S.......VDR.....N...SVQ.......I.E.....Q.S.H....SVV..........Q.....EK..
BAA14337  Q...L...V.D.....K.....VVN.............S.....MI.S.I...QI.....A..........K..
BAA14338  .T.I.....D.....GNYDS.RG...NQ.........M.ADRI...AV..........K.....DK..
CAB95856  .S.......VD.....K.Y.I.....................T.L.MI.N.I...QIQ.V.A..........K..
```

FIG. 7H

```
Positions from 481 till 540
consensus DLHDSNVKNLYEKVRRQLRDNAEDDGNGCFEIYHKCDDECMESIRNGTYDHPEYREESKL
AAA43678  .F.........D....M.....VKEL.....F.....N.VK.....Y.K.E.....
AAA79775  .MA..EML.....R..K....Q....E..K.....T....S.....N.....SQ.....AL.
AAA96134  ..A..EMNK....R.....E....E..T.....F.R....Q.....N..N.T.....Q.ALQ
AAC40998  ..A..EMNK....R.....E....E..T.....F.....D..A.....N.....SK.....AIQ
AAD17229  .F.....R.....KS...KN...KEI.....F.....A.....V.....Y.K.S.....
AAD21159  .F.....D....L.....KEL.....F.....N.....VK.....Y.Q.S..AR.
AAF99711  .VT..EMNK.F.R.....E....K.....F.....NN.I.....DI..D.AID
AAK51718  ..T..EMNK.F..T.....E....M.....K.....NA.I.....DV..D.ALN
AAX78820  ....A.........KS.....K.L.....FW.....IN.VK.....Y.K.QD.....
ABB87356  ....A.....H.Q.K.A.KN...I.E.D....NLL.....N.S....T.....N.ED.....Q.
BAA14334  .E.....FDE.K.R.SA...I.A.....D.L.....N.....T.K.....K..E..A..
BAA14336  .....R...H.....M.K.....K.E.....TF.....NK.I.RV.....K.FE.....I
BAA14337  .E..A...R...HDR....V..E...I.T.D.....L.....NN...DT.....N.K..E.....I
BAA14338  .M..A.....H.Q....E.K....I.E.....LL....N.S....T.....T..A.....
CAB95856  .E..A...N....K.A.GS...ME...K.....L.....Q....T.....NRRK.....R.
```

FIG. 7I

```
Positions from 541 till 595
consensus NRQEIDGVKLESGGNVYKILSIYSTVASSLVLAALIAGFIFWACSNGNCRCTICI
AAA43678  ..N...K.....S.M.-...Q...A....A....G...S...IMM...IS...M.....SLQ.R....
AAA79775  ..LN.NP.....S.....-YKD.ILWF.FGE.CF...L.VVM.LV.FCLK.....M.........
AAA96134  ..IM.NP.....S.....-YKDVILWF.FG...CVM.L.IAM.L...MCVK.....L.........
AAC40998  ..IQ...P.....S.....-YKDVILWF.FG...CFI.L.IAM.LV.ICVK.....M.........
AAD17229  ..E.....................M.-...Q...A.....................LVSLGAIS...M.....SLQ.R....
AAD21159  ..E...S.....M.-T.Q.....................A...IMV...LSL.M.....SLQ.R....
AAF99711  ..FQ.Q.....TQ...-YKD.IILWI.FSI.CFL.V...LLA...L.....Q.....I...Q....
AAK51718  ..FQ.K...E.K.....-YKDWILWI.FAI.CFL.CVVLL.....M.....QR.....I...N....
AAX78820  .....S.....NL.-...Q...A.....S.G.....VG...IAMGL.M.....SMP.K....
ABB87356  K.....E.I...KTED.....V.....CI.....I.MVG...LA...M.....S.....FNV...
BAA14334  E.SK.N.....ENT-T.....................A...C...I.....GLILGMQ...S.....MF...
BAA14336  .....E.....D.S.....................CI.....M...M.....S.........
BAA14337  E...KVN.....ENS-T.....S.....LLM.I.GFIFG.Q.....V.....F...
BAA14338  K.....I...K.ED.....A.....CI.....V...VG...LS...M.....S.....FNV...
CAB95856  E...K.E.....E.-T.....T.....MGF.A.L.....N.....S.....N....
```

FIG. 7J

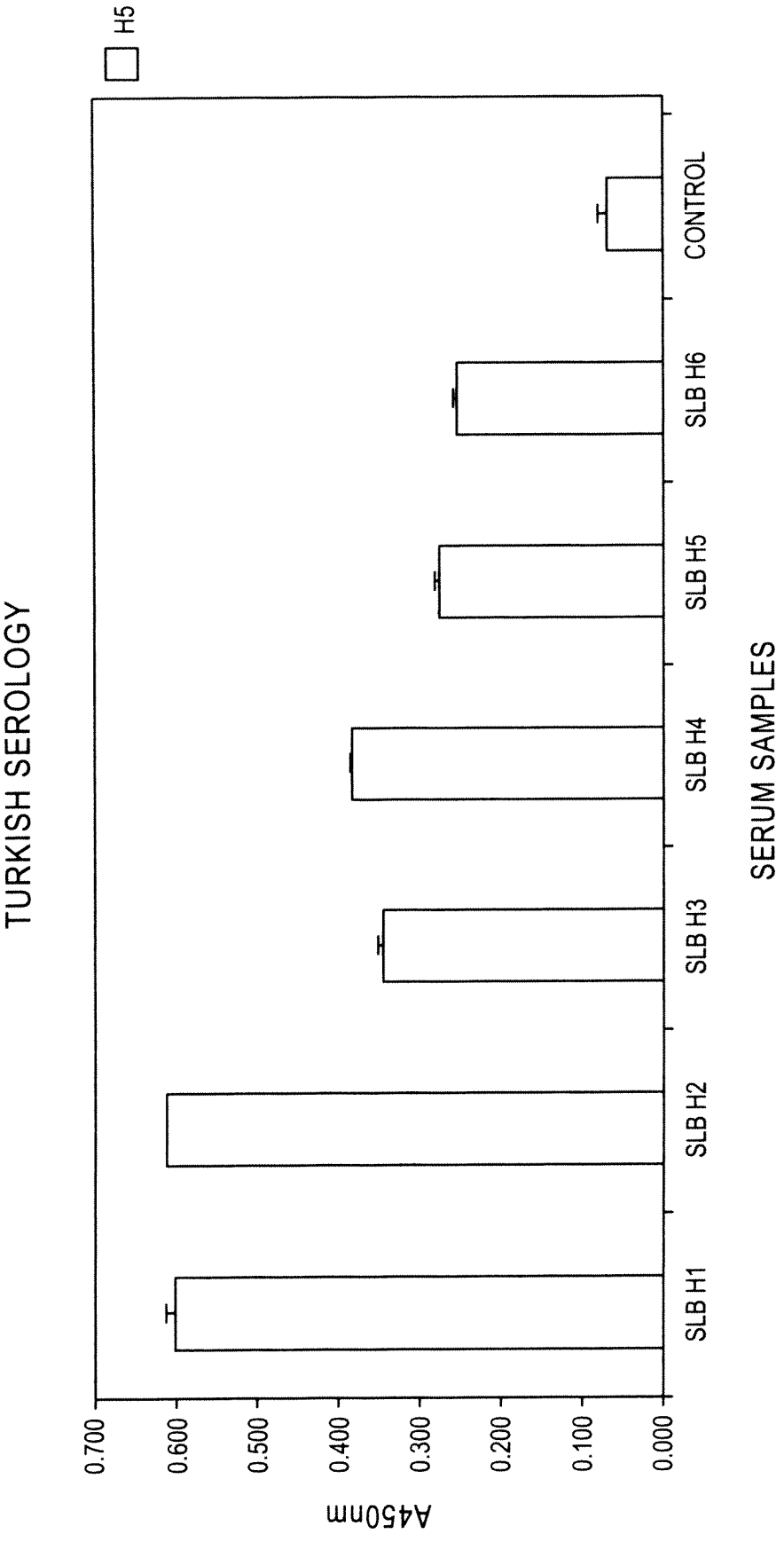

TURKISH SEROLOGY
H5
A450nm
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
SLB H1
SLB H2
SLB H3
SLB H4
SLB H5
SLB H6
CONTROL
SERUM SAMPLES

FIG. 8

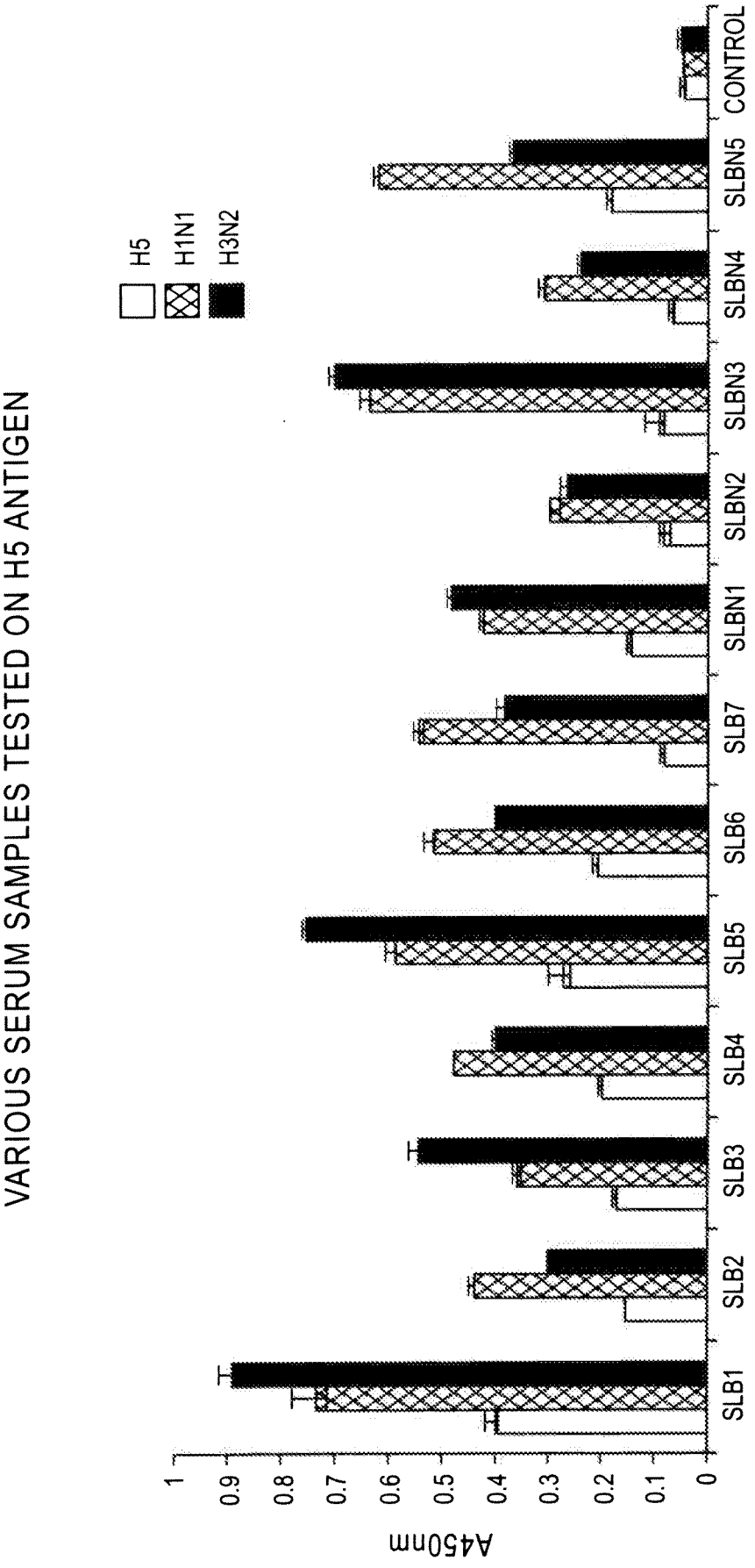

VARIOUS SERUM SAMPLES TESTED ON H5 ANTIGEN
H5
H1N1
H3N2
A450nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
SLB1
SLB2
SLB3
SLB4
SLB5
SLB6
SLB7
SLBN1
SLBN2
SLBN3
SLBN4
SLBN5
CONTROL
SERUM SAMPLES

FIG. 9

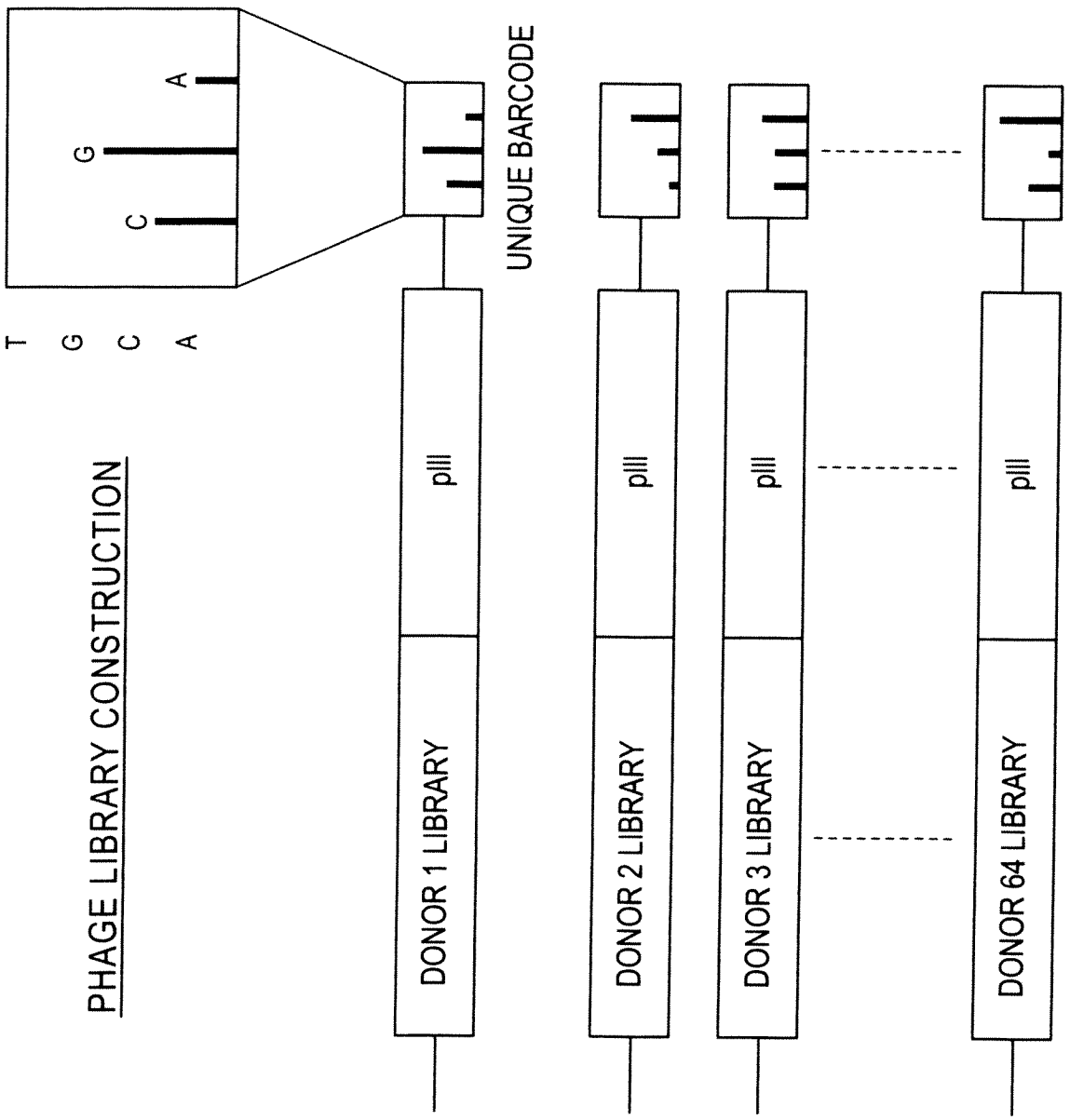

PHAGE LIBRARY CONSTRUCTION
T
G
C
A
UNIQUE BARCODE
pIII
DONOR 1 LIBRARY
DONOR 2 LIBRARY
DONOR 3 LIBRARY
DONOR 64 LIBRARY

FIG. 10

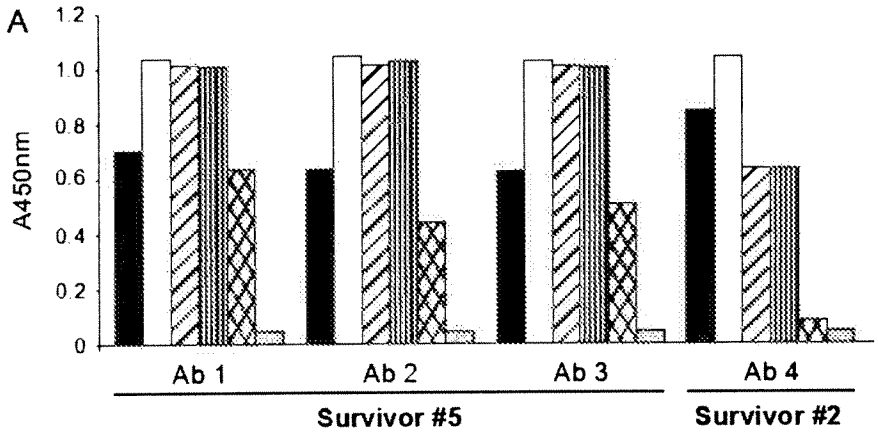

A
1.2
1.0
0.8
0.6
0.4
0.2
0
A450nm
Ab 1
Ab 2
Ab 3
Ab 4
Survivor #5
Survivor #2

FIG. 11

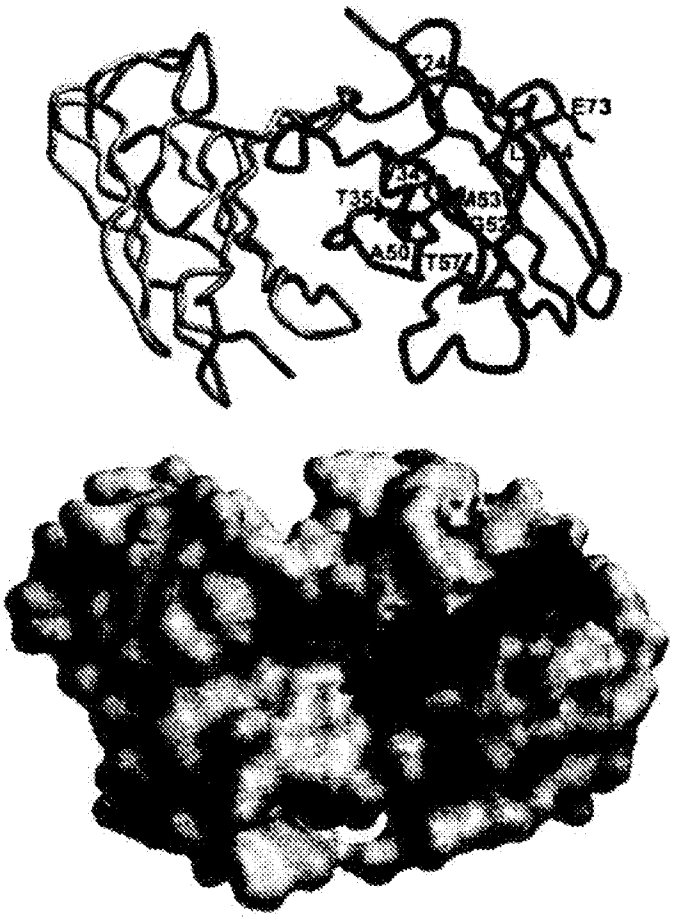


FIG. 12

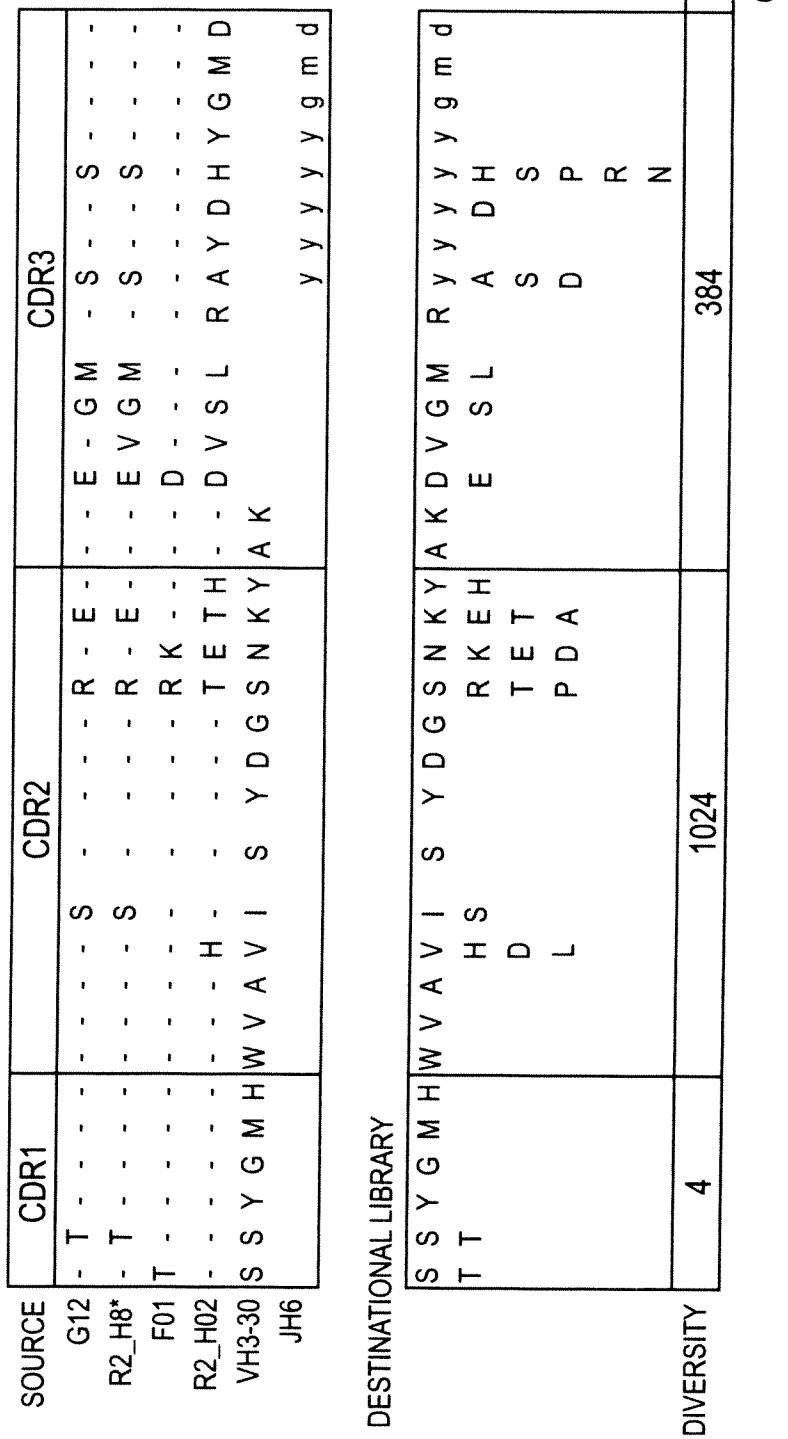

HEAVY CHAIN DESTINATIONAL MUTAGENESIS
SOURCE
CDR1
CDR2
CDR3
G12
R2_H8*
F01
R2_H02
VH3-30
JH6
DESTINATIONAL LIBRARY
DIVERSITY
4
1024
384
1.6E+06
COMBINATORIAL DIVERSITY

FIG. 13

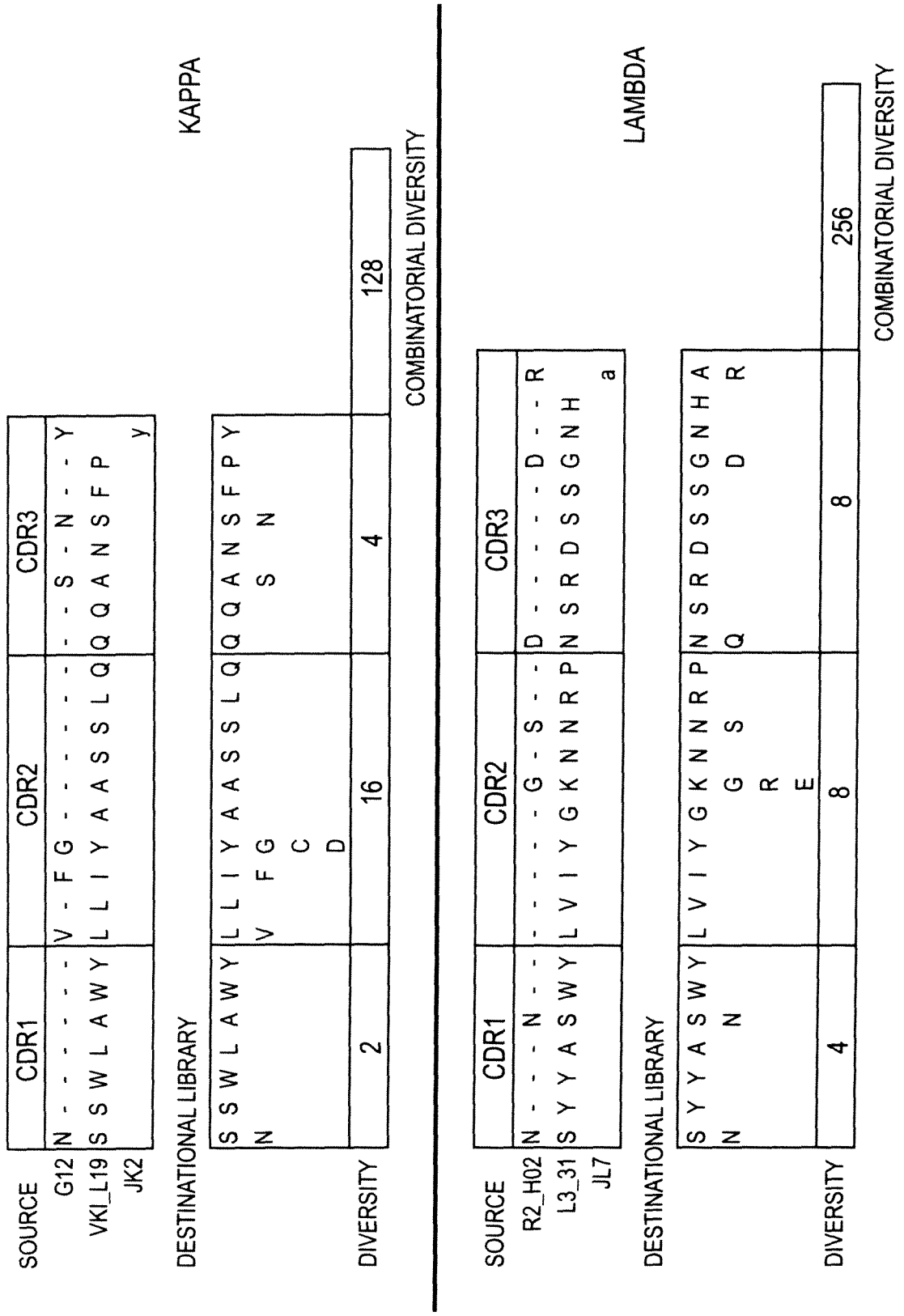

LIGHT CHAIN DESTINATIONAL MUTAGENESIS
KAPPA
SOURCE
CDR1
CDR2
CDR3
G12
N - - - - - - -
V - F G - - - - - - - - -
- - - S - N - - Y
VKI_L19
S S W L A W Y
L L I Y A A S S L Q
Q Q A N S F P
JK2
y
DESTINATIONAL LIBRARY
S S W L A W Y
L L I Y A A S S L Q
Q Q A N S F P Y
N
V F G
S N
C
D
DIVERSITY
2
16
4
128
COMBINATORIAL DIVERSITY
LAMBDA
SOURCE
CDR1
CDR2
CDR3
R2_H02
N - - - N - - -
- - - - - - G - S - - -
D - - - - - - D - - R
L3_31
S Y Y A S W Y
L V I Y G K N N R P
N S R D S S G N H
JL7
a
DESTINATIONAL LIBRARY
S Y Y A S W Y
L V I Y G K N N R P
N S R D S S G N H A
N
N
G S
Q
D
R
R
E
DIVERSITY
4
8
8
256
COMBINATORIAL DIVERSITY

FIG. 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/075998

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/395 A61P31/16 C07K16/00 C07K16/10

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)
A61K A61P 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.
P,X	WO 2007/134327 A (SEA LANE BIOTECHNOLOGIES LLC [US]; BHATT RAMESH R [US]; HOROWITZ LAWRE) 22 November 2007 (2007-11-22) paragraphs [0036], [0128] - [0132], [0156], [0165] - [0167] figure 9 claims 89-93	1-63

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 January 2009	03/02/2009

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	Cilensek, Zoran

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	KASHYAP ARUN K ET AL: "Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC.; US, vol. 105, no. 16, 22 April 2008 (2008-04-22), pages 5986-5991, XP002488075 ISSN: 0027-8424 the whole document	1-63
Y	WO 00/57183 A (BIOVATION LTD [GB]; CARR FRANCIS J [GB]) 28 September 2000 (2000-09-28) page 1, lines 9-15 page 2, lines 19-35 page 6, lines 5-17 page 29, line 15 - page 30, line 34 claims 1-51	1-63
Y	WO 2005/042759 A (ALTHEA TECHNOLOGIES INC [US]; MONFORTE JOSEPH [US]) 12 May 2005 (2005-05-12) paragraphs [0010], [0093], [0097], [0098], [0101], [0102], [0109]; claims 1-13	1-63
Y	WO 03/052101 A (ROSETTA INPHARMATICS INC [US]; MARTON MATTHEW [US]; JONES ALLAN [US]) 26 June 2003 (2003-06-26) page 13, line 23 - page 14, line 33 page 45, lines 11-29	1-63
Y	EP 1 647 600 A (AFFYMETRIX INC A US ENTITY [US]) 19 April 2006 (2006-04-19) paragraphs [0004], [0005], [0027] - [0029], [0078]	1-63
A	HAMMOND P W ET AL: "In vitro selection and characterization of Bcl-X1 proteins from a mix of tissue-specific mRNA display libraries" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOCHEMICAL BIOLOGISTS, BIRMINGHAM,; US, vol. 276, no. 24, 15 June 2001 (2001-06-15), pages 20898-20906, XP002180712 ISSN: 0021-9258 page 2899, left-hand column, paragraph 4 page 2900, left-hand column, paragraph 6 - right-hand column, paragraph 1; figure 1	1-63

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/075998

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	QIU FANG ET AL: "DNA sequence-based "bar codes" for tracking the origins of expressed sequence tags from a maize cDNA library constructed using multiple mRNA sources." PLANT PHYSIOLOGY (ROCKVILLE), vol. 133, no. 2, October 2003 (2003-10), pages 475-481, XP002510727 ISSN: 0032-0889 page 476, paragraph 1; table 1 page 479, right-hand column, paragraphs 2,3	1-63
A	WO 2007/031550 A (CRUCELL HOLLAND BV [NL]; THROSBY MARK [NL]; DE KRUIF CORNELIS ADRIAAN) 22 March 2007 (2007-03-22) page 33, line 5 - page 46, line 22	1-63
A	MAO S ET AL: "Phage-display library selection of high-affinity human single-chain antibodies to tumor-associated carbohydrate antigens sialyl Lewisx and Lewisx" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC.; US, vol. 96, no. 12, 8 June 1999 (1999-06-08), pages 6953-6958, XP002983603 ISSN: 0027-8424 the whole document	1-63
A	THROSBY M ET AL: "ISOLATION AND CHARACTERIZATION OF HUMAN MONOCLONAL ANTIBODIES FROM INDIVIDUALS INFECTED WITH WEST NILE VIRUS" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 80, no. 14, 1 January 2006 (2006-01-01), pages 6982-6992, XP002411390 ISSN: 0022-538X the whole document	1-63
A	KIM S J ET AL: "Neutralizing human monoclonal antibodies to hepatitis A virus recovered by phage display" VIROLOGY, ACADEMIC PRESS,ORLANDO, US, vol. 318, no. 2, 20 January 2004 (2004-01-20), pages 598-607, XP004490196 ISSN: 0042-6822 the whole document	1-63

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2008/075998

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

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

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

2. ☒ Claims Nos.: 64-67
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

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

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 64-67

The present claims 64-67 encompass the methods of treatmet using antibodies produced by the method of claim 59, wherein the method of production does not impart any limitation on the product. The subject-matter is thus not defined in terms of searchable parameters or terms, and the meaningful search over the claimed scope is impossible. Consequently no search has been carried out for claims 64-67.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2)PCT declaration be overcome.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/075998

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007134327 A	22-11-2007	AU 2007249160 A1	22-11-2007
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		EP 1660674 A2	31-05-2006
		JP 2007504831 T	08-03-2007
WO 03052101 A	26-06-2003	NONE	
EP 1647600 A	19-04-2006	NONE	
WO 2007031550 A	22-03-2007	AU 2006290736 A1	22-03-2007
		CA 2622603 A1	22-03-2007