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(54) **Title**: IMPROVED ANTIBODY LIBRARIES

(57) **Abstract**: The present invention features improved in vitro RNA display libraries to allow reliable expression and selection of scFv antibody molecules from expression libraries. The scFv antibody libraries of the invention contain an optimized, shortened inter-domain linker that improves expression scFv antibody expression. The scFv antibody libraries also include short nucleic acid barcodes that allow for identification of individual library clones, libraries or subsets thereof. Primers for generating, amplifying and spectratyping the scFv antibody libraries of the invention are also provided.

IMPROVED ANTIBODY LIBRARIES

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 61/101,483, filed September 30, 2008, the contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The invention relates to improved antibody libraries and methods and materials for making same.

BACKGROUND OF THE INVENTION

Antibodies that bind with high specificity and affinity to almost any structural epitope are routinely used as research tools and as FDA approved therapeutics. As a result, therapeutic and diagnostic monoclonal antibodies constitute a multi-billion dollar market worldwide.

Classical methods of immunizing animals to obtain antibodies are slow and cumbersome. As a consequence, several methods have been developed for *ex vivo* selection of an antibody to a desired target molecule using synthetic antibody libraries. In some methods, libraries of antibodies, or fragments thereof, are displayed on the surface of an organism (for example, a yeast cell, bacterial cell or mammalian cell) or a sub-microscopic agent (for example, a bacteriophage or virus), and the organism or sub-microscopic agent is selected for expression of the desired antibody. In other methods, antibody libraries are expressed and selected in a cell free *in vitro* system. Current *in vitro* expression systems, although good at expressing single antibody variable domains, are inefficient at expressing multi-domain antibodies such as single chain antibody (scFv) molecules. This is due to both the structure of current scFv antibody libraries and to the reaction conditions of the current *in vitro* expression systems.

There is, therefore, a need in the art for improved antibody libraries for selection of scFv antibodies against a desired target.

SUMMARY OF THE INVENTION

The invention solves the foregoing problems by providing improved *in vitro* display RNA libraries to allow reliable expression and selection of scFv antibody molecules.

The invention has several advantages, which include but are not limited to, the following:

- providing an improved *in vitro* display scFv antibody library containing an optimized inter-domain linker for improved expression;
- providing an improved *in vitro* display scFv antibody library containing short nucleic acid barcodes;
- providing primers to generate the improved *in vitro* display scFv antibody libraries;
- providing primers to spectratype the CDR3 regions of the heavy chain variable regions of the scFv antibody molecules in the libraries of the invention; and
- methods of making the improved in vitro display libraries.

In one aspect, the invention provides an oligonucleotide consisting of a nucleic acid sequence as set forth in any one of SEQ ID NOs: 1-14, 19-42, and 58-210. In another aspect, the invention provides an oligonucleotide comprising a nucleic acid sequence as set forth in any one of SEQ ID NOs: 1-14, 19-42, and 58-210.

In another aspect, the invention provides an oligonucleotide consisting of a nucleic acid sequence as set forth in any one of SEQ ID NOs: 14-16, and 43-57. In another aspect, the invention provides an oligonucleotide comprising a nucleic acid sequence as set forth in any one of SEQ ID NOs: 14-16, and 43-57.

In yet another aspect, the invention provides an oligonucleotide consisting of a nucleic acid sequence as set forth in SEQ ID NOs: 17 or 18. In another aspect, the invention provides an oligonucleotide comprising a nucleic acid sequence as set forth in SEQ ID NOs: 17 or 18.

In an embodiment, the invention provides for the use of any of the sequences set forth in SEQ ID NOs: 1-210 for library amplification, library reverse transcription, and/or library spectratyping.

In another aspect, the invention provides for a nucleic acid library for expression of single chain antibodies (scFv), the library comprising a repertoire of sequences

encoding heavy chain variable domains and light chain variable domains, wherein each member of said library contains an open reading frame comprising a heavy chain variable domain, a light chain variable domain, and a linker region, and wherein said library is generated using one or more of the oligonucleotides set forth in SEQ ID NOs: 1-210.

In an embodiment, the library further comprises a linker region that encodes less than 20 amino acids. In another embodiment, the library further comprises a linker region that encodes 15 amino acids.

In an embodiment, each member of the library further comprises a promoter operably linked to the open reading frame. In another embodiment, the promoter is selected from the group consisting of T7, SP6, and T3. In yet another embodiment, the promoter is a T7 promoter.

In an embodiment, each member of the library further comprises a 5'untranlated region (5'UTR) capable of enhancing transcription of a gene to which it is operably linked. In another embodiment, the 5'UTR is a Tobacco Mosaic Virus 5'UTR or an active fragment thereof. In another embodiment, each member of the library further comprises a polyadenine sequence.

In yet another embodiment, the library further comprises a nucleic acid barcode. In another embodiment, the nucleic acid barcode comprises 8 nucleotides.

In another embodiment, each member of the library further comprises a nucleic acid sequence encoding an epitope tag. In yet another embodiment, the epitope tag is a FLAG tag. In yet another embodiment, the nucleic acid sequence is part of the linker region of the scFv. In another embodiment, the library further comprises a nucleic acid sequence encoding an antibody constant region, or fragment thereof.

In an embodiment, the library further comprises a ribosome pause sequence.

In an embodiment, the library further comprises a peptide acceptor. In another embodiment, the peptide acceptor is covalently attached via a linker comprising a Psoralen C6 molecule. In yet another embodiment, the linker is 5'(Psoralen C6) 2'Ome (U AGC GGA UGC) XXX XXX CC (Puromycin), wherein X is a triethylene glycol linker or PEG-150 and CC is a DNA backbone.

In another aspect, the invention provides for a method of producing a nucleic acid library for expression of single chain antibodies (scFv) comprising (a) providing a nucleic acid composition, wherein at least a portion of the nucleic acids in the

composition comprises at least one open reading frame encoding an antibody variable domain and (b) amplifying a plurality of antibody variable domains using one or more oligonucleotides set forth in SEQ ID NOs 1-210.

In another aspect, the invention provides for a method of spectratyping a nucleic acid comprising at least one open reading frame encoding an antibody variable domain comprising (a) providing a nucleic acid composition, wherein at least a portion of the nucleic acids in said composition comprise at least one open reading frame encoding an antibody variable domain and (b), amplifying the CDR3 regions of said variable domains using one or more oligonucleotides set forth in SEQ ID NOs 1-210.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a general scheme for the mRNA-scFv display technology in certain embodiments of the invention.

Figure 2 depicts a general scheme for the mRNA-scFv display technology in certain embodiments of the invention.

Figure 3 depicts a general depiction of a library DNA construct.

Figure 4 depicts the functional scFv generated as mRNA-scFv molecules.

Figure 5 depicts the results showing that an scFv attached in an mRNA-scFv molecule format is functionally equivalent to a free scFv molecule.

Figure 6 depicts four 8-bp tags that were inserted between the TMV-UTR and the Kozak consensus sequence of the 17/9 mRNA-scFv construct.

Figure 7 depicts the exemplary constructs and control sequences.

Figure 8 depicts the random tag sequences identified in three rounds of selection.

Figure 9 depicts the results quantifying 17/9 scFv before and after one round of mRNA-scFv selection.

Figure 10 depicts the chimeras between D2E7 and 2SD4.

Figure 11 depicts the K_D curves for different TNFα binders.

Figure 12 depicts the thermostability of mRNA-scFv molecules.

Figure 13 depicts the results showing that RNA is recovered after high temperature treatment of mRNA-scFv molecules.

Figure 14 depicts the age, ethnicity and gender distribution of the PBMCs donors in a naive human PBMC kappa scFv PROfusion library.

Figure 15 depicts the VH family-specific PCR fragments in the constructed naive human PBMC kappa scFv PROfusion library.

Figure 16 depicts the Vκ family-specific PCR fragments in the constructed naive human PBMC kappa scFv PROfusion library.

Figure 17 depicts the VH-Vκ scFv PCR products in the constructed naive human PBMC kappa scFv PROfusion library.

Figure 18 depicts the VH and Vκ family distribution in the constructed naive human PBMC kappa scFv PROfusion library.

Figure 19 depicts the spectratyping analysis of naive human PBMC antibody CDR3 sizes.

Figure 20 depicts the quality control of VH/Vκ library by spectratyping analysis.

Figure 21 depicts the Vλ family-specific PCR fragments in a naive human PBMC lambda scFv PROfusion library.

. *Figure 22* depicts the VH-Vλ scFv PCR products in the constructed naive human PBMC lambda scFv PROfusion library.

Figure 23 depicts the VH and Vλ family distribution in the constructed naive human PBMC lambda scFv PROfusion library.

Figure 24 depicts the schema of PROfusion library constructions in a naive human lymph node kappa and lambda scFv PROfusion libraries.

Figure 25 depicts the VH family-specific PCR fragments in the constructed naive human lymph node kappa and lambda scFv PROfusion libraries.

Figure 26 depicts the Vκ family-specific PCR fragments in the constructed naive human lymph node kappa and lambda scFv PROfusion libraries.

Figure 27 depicts the Vλ family-specific PCR fragments in the constructed naive human lymph node kappa and lambda scFv PROfusion libraries.

Figure 28 depicts the VH-Vκ and VH-Vλ scFv PCR products in the constructed naive human lymph node kappa and lambda scFv PROfusion libraries.

Figure 29 depicts the VH and Vκ family distribution in a constructed VH-Vκ scFv library.

Figure 30 depicts the VH and Vλ family distribution in the constructed VH-Vλ scFv library.

Figure 31 depicts the quality control of VH-Vκ and VH-Vλ libraries by spectratyping analysis in the constructed VH-Vλ scFv library.

DETAILED DESCRIPTION OF THE INVENTION

In order that the present invention may be more readily understood, certain terms are first defined.

I. Definitions

The term "antibody" includes monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), chimeric antibodies, CDR-grafted antibodies, humanized antibodies, human antibodies, murine antibodies and fragments thereof, for example, an antibody light chain (VL), an antibody heavy chain (VH), a single chain antibody (scFv), an F(ab')2 fragment, an Fab fragment, an Fd fragment, an Fv fragment, and a single domain antibody fragment (DAb).

The term "antibody library" refers to a plurality of DNA or RNA molecules containing an open reading frame (ORF) that encodes an antibody or fragment thereof. It also includes a plurality of antibody proteins and nucleic acid/antibody fusion molecules expressed from said DNA or RNA molecules.

The term "heavy chain variable domain" refers to the nucleic acid encoding an antibody heavy chain variable region and to the protein product of said nucleic acid.

The term "light chain variable domain" refers to the nucleic acid encoding an antibody light chain variable region and to the protein product of said nucleic acid.

The term "spectratyping" refers to a PCR based method that separates genetic sequences encoding antibodies on the basis of CDR3 length. Changes in CDR3 length distribution is correlated with changes in the antibody repertoire (Janeway *et al.* "Immunobiology", 5th ed. Garland Publishing, New York and London, (2001)).

The term "epitope tag" refers a short amino acid sequence specifically recognized by an antibody that is attached chemically or genetically to a molecule to allow for its detection by said antibody, for example, a FLAG tag, an HA tag, a MYC tag or a T7 tag,

The term "nucleic acid barcode" refers to a short nucleic acid included in the untranslated region of the libraries of the invention. The barcode is a random or predetermined sequence that serves to provide a unique identifier to an individual clone or a plurality of library members.

The term “non-antibody sequences” refers to any nucleic acid or amino acid sequences that appear in the antibody libraries of the invention that are not part of the original antibody sequence. Such sequences include, for example, epitope tags, or nucleic acid barcodes.

The term "control sequences" refers to the nucleic acid sequences or genetic elements necessary for the expression of an operably linked coding sequence in a particular host organism, sub-microscopic agent or *in vitro* expression system. Such sequences are well known in the art. 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. Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, 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 nucleic acid sequences being linked are contiguous. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites, for example. If such sites do not exist, synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term “specific binding”' or “specifically binds to” refers to the ability of a binding molecule to bind to a target with an affinity of at least about 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M, 1×10^{-10} M, 1×10^{-11} M, 1×10^{-12} M, or more, and/or bind to a target with an affinity that is at least two-fold greater than its affinity for a nonspecific antigen.

The term "target" refers to an antigen or epitope recognized by an antibody. Targets include, for example, any peptide, proteins, saccharides, nucleic acids, lipids, and small molecules for which a specific antibody is generated. In one embodiment, antibodies are against a human protein, for example, TNFalpha, IL-12 or IL-1alpha.

A "conservative amino acid substitution" is one in which an amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art.

The term "RNA display" or "mRNA display” refers to an *in vitro* technique wherein, expressed proteins or peptides are linked covalently or by tight non-covalent

interaction to their encoding mRNA to form "RNA/protein fusion" molecules. The protein or peptide component of an RNA/protein fusion is selected for binding to a desired target and the identity of the protein or peptide determined by sequencing of the attached encoding mRNA component. Such methods are well known in the art and are described, for example, in U.S. Patent Nos. 7,195,880; 6,951,725; 7,078,197; 7,022,479, 6,518,018; 7,125,669; 6,846,655; 6,281,344; 6,207,446; 6,214,553; 6,258,558; 6,261,804; 6,429,300; 6,489,116; 6,436,665; 6,537,749; 6,602,685; 6,623,926; 6,416,950; 6,660,473; 6,312,927; 5,922,545; and 6,348,315.

The term "single chain Fv antibody" or "scFv" refers to an antigen binding portion of a light chain variable region and an antigen binding portion of a heavy chain variable region, joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. U.S.A 85:5879-5883).

The term "functional moiety" refers to any biological or chemical entity that imparts additional functionality to a molecule to which it is attached.

The term "selecting" refers to substantially partitioning a molecule from other molecules in a population. As used herein, a "selecting" step provides at least a 2-fold, preferably, a 30-fold, more preferably, a 100-fold, and, most preferably, a 1000-fold enrichment of a desired molecule relative to undesired molecules in a population following the selection step. As indicated herein, a selection step may be repeated any number of times, and different types of selection steps may be combined in a given approach.

The term "pause sequence" refers to a nucleic acid sequence that causes a ribosome to slow or stop its rate of translation.

The term "solid support" refers to, without limitation, any column (or column material), bead, test tube, microtiter dish, solid particle (for example, agarose or sepharose), microchip (for example, silicon, silicon-glass, or gold chip), or membrane (for example, the membrane of a liposome or vesicle) to which an affinity complex may be bound, either directly or indirectly (for example, through other binding partner intermediates such as other antibodies or Protein A), or in which an affinity complex may be embedded (for example, through a receptor or channel).

The term "linker region" refers to a region of nucleic acid connecting the nucleic acid sequences encoding the antibody VH and VL domains in a scFv antibody gene. A linker region is in-frame with the nucleic acid sequences encoding the antibody VH and VL such that a continuous open reading frame containing the VH, VL and linker regions is formed. The term also refers to the region connecting the VH and VL in an scFv protein.

The term "peptide acceptor" refers to any molecule capable of being added to the C-terminus of a growing protein chain by the catalytic activity of a ribosomal peptidyl transferase. Typically, such molecules contain (i) a nucleotide or nucleotide-like moiety (for example, puromycin and analogues thereof)), (ii) an amino acid or amino acid-like moiety (for example, any of the 20 D- or L-amino acids or any amino acid analog thereof (for example, O-methyl tyrosine or any of the analogs described by Ellman et al. (1991) Meth. Enzymol. 202:301), and (iii) a linkage between the two (for example, an ester, amide, or ketone linkage at the 3' position or, less preferably, the 2' position); preferably, this linkage does not significantly perturb the structure of the ring from the natural ribonucleotide conformation. In addition, this term encompasses, without limitation, a peptide acceptor molecule that is covalently bonded (either directly or indirectly through intervening nucleic acid sequence) to the protein coding sequence, as well as one that is joined to the protein coding sequence by some non-covalent means, for example, through hybridization using a second nucleic acid sequence that binds at or near the 3' end of the protein coding sequence and that itself is bound to a peptide acceptor molecule.

II. Overview

The present invention features improved *in vitro* RNA display libraries to allow reliable expression and selection of scFv antibody molecules from expression libraries. RNA display methods generally involve expression of a library of proteins or peptides, wherein the expressed proteins or peptides are linked covalently or by tight non-covalent interaction to their encoding mRNA to form RNA/protein fusion molecules. The protein or peptide component of an RNA/protein fusion is selected for binding to a desired target and the identity of the protein or peptide determined by sequencing of the attached encoding mRNA component.

The scFv antibody libraries of the invention contain an optimized, shortened inter-domain linker that improves expression scFv antibody expression. The scFv antibody libraries also include short nucleic acid barcodes that allow for identification of individual library clones, libraries or subsets thereof.

The present invention also provides novel primers for generating, amplifying and spectratyping the scFv antibody libraries of the invention.

III. Library Construction

As an antibody technology development to generate monoclonal antibody drug candidates, this invention discloses the development of two recombinant antibody generation approaches, PROfusion (mRNA display) and Yeast Surface Display. The PROfusion mRNA display technology is an *ab initio* method for screening human antibody libraries. The yeast surface display technology is a cellular method for screening monoclonal antibodies specifically displayed on the yeast surface.

In one aspect, the invention features novel antibody libraries capable of expressing antibody molecules. Libraries of the invention are generated from any antibody fragment capable of binding to a target. In one embodiment, libraries of antibody variable domains are generated. In an embodiment, these are VH and/or VL domains. In another embodiment, scFv libraries are generated.

The libraries of the invention may also include antibody nucleic acid sequences encoding regions outside of the variable regions, for example, a constant region or fragment thereof, or a hinge region.

Nucleic acid libraries of the invention can comprise RNA, DNA, or both RNA and DNA elements.

1) Generation of Nucleic Acid Input Diversity

The nucleic acid sequences used to generate the antibody libraries of the invention may be obtained from any source. In one embodiment, the libraries of the invention may be obtained from the antibody repertoire of any animal including, but not limited to, rodents, primates, camelids, sharks, or any transgenic animal containing a repertoire of human immunoglobulin genes. Techniques for the isolation and cloning of nucleic acids encoding the variable regions of the antibody complement of an organism are well known in the art. Indeed, many cDNA libraries containing nucleic acids

encoding the variable regions of antibodies are commercially available, for example, libraries of human antibody variable regions generated from various immune cells, for example, peripheral blood mononuclear cells (PBMC), spleen or lymph node. In another embodiment, the libraries of the invention may be obtained by *ab initio* synthesis of nucleic acids encoding one more antibodies.

The libraries of the invention may require the introduction of additional diversity by introducing nucleic substitutions and/or deletions that result in one or more amino acid substitutions and/or deletions in the expressed antibodies molecules. Any art recognized methods of mutagenesis are contemplated, for example, random mutagenesis, "walk through mutagenesis, and "look through mutagenesis. Such mutagenesis of an antibody may be achieved by using, for example, error-prone PCR, "mutator" strains of yeast or bacteria, or incorporation of random or defined nucleic acid changes during *ab inito* synthesis of all or part of an antibody. In one embodiment, a library of antibody molecules may be created in which one or more amino acids are randomly mutated. In another embodiment, a library of antibody molecules may be created in which one or more amino acids are mutated to one or more predetermined amino acid.

2) Control Sequences

The nucleic acid libraries of the invention may contain additional control sequences to facilitate the expression and screening of the encoded antibodies in vitro.

One such control sequence may be a promoter to be used in conjunction with a desired RNA polymerase for mRNA synthesis. As described herein, any promoter capable of directing synthesis from a linear double-stranded DNA may be used, for example, the T7, SP6 or T3 phage promoters.

A second control sequence may be termed the 5' untranslated region (or 5'UTR) and corresponds to the RNA upstream of the translation start site. Any other appropriate 5' UTR may be utilized (see, for example, Kozak (1983) Microbiol. Rev. 47:1). In one embodiment, the 5'UTR (termed "TE") may be a deletion mutant of the Tobacco Mosaic Virus 5' untranslated region and, in particular, corresponds to the bases directly 5' of the TMV translation start; the sequence of this UTR is as follows: rGrGrG rArCrA rArUrU rArCrU rArUrU rUrArC rArArU rUrArC rA (with the first 3 G nucleotides being inserted to augment transcription).

A third element may be a translation start site. In general, this is an AUG codon. However, there are examples where codons other than AUG are utilized in naturally-occurring coding sequences, and these codons may also be used in the selection scheme of the invention. This translation start site is preferably in a suitable sequence context termed a "Kozak" sequence (see, for example, Kozak (1983) Microbiol. Rev. 47:1).

A fourth element may be a polyadenylation (poly A) sequence containing a 5' stop codon. The poly A sequence may be placed after the antibody coding sequence within the nucleic acid library construct. Such sequences are well known in the art and any such sequence is contemplated.

3) Additional Nucleic Acid Sequence Elements

The nucleic acid libraries of the invention may also include additional sequence elements that are incorporated into mRNA transcripts, encoding the antibodies. These may include non-antibody sequences.

In one embodiment, a short nucleic acid sequence, or "nucleic acid barcode", may be incorporated into the untranslated portion of an antibody mRNA transcript. These barcode sequences may serve as unique identifiers to distinguish individual members of a nucleic acid library or to distinguish between different libraries. The short nucleic acid sequences preferably contain less than 50 bases, less than 20 bases or less than 10 bases. In one embodiment, the short nucleic acid sequences comprise 8 bases.

In other embodiments, nucleic acid sequence elements encoding a specific non-antibody amino sequence may be incorporated into the open reading frame (ORF) of the nucleic acid libraries of the invention such that the encoded amino acid sequence is incorporated into the expressed antibody. In one embodiment, a non-antibody nucleic acid sequence element may be incorporated into the ORF of a scFv between the VH and VL regions to serve as a linker region. Any nucleic acid sequence that encodes a continuous amino acid sequence that lacks a stop codon may be contemplated for the linker region. The length of the linker region is less than 50 amino acids, or less than 20 amino acids, or less than 16 amino acids.

In another embodiment, nucleic acid sequence elements encoding one or more epitope tags (for example, a FLAG tag) may be incorporated into the antibody coding sequence. These sequences may result in production of an antibody with an epitope

tag present at any position, for example, at the N-terminus, at the C-terminus, or in the linker region between the VH and VL domains of an scFv antibody molecule. In one embodiment, sequences encoding an antibody constant region or fragment thereof may be included in the 3' portion of the ORF of the nucleic acid libraries of the invention. This antibody constant region or fragment thereof is identical in all members of a particular library.

In other embodiments, nucleic acid sequence elements encoding a specific non-antibody amino sequence may be incorporated into the vectors, which are utilized to specifically express the nucleic acid library in this invention on the surface of yeast cells. These elements may include, but are not limited to, transmembrane domains known in the art. In one embodiment, these elements may be incorporated into the ORF of the nucleic acid libraries of the invention such that the encoded amino acid sequence is incorporated into the expressed antibody. In another embodiment, these elements may be incorporated into the vector sequence but not the ORF of the nucleic acid libraries of the invention. These elements may help the expression, stability, folding and epitope presentation, or other characteristics of the nucleic acid library in this invention and mentioned above.

4) Oligonucleotide Primers

In one aspect, the invention features nucleic oligonucleotide primers suitable for the synthesis and/or amplification of the antibody libraries of the invention. Exemplary primers include SEQ ID NOs: 1-13 (Table 6).

In another aspect the invention features nucleic oligonucleotide primers suitable for reverse transcription of mRNA produced from the libraries of the invention (Table 3). Exemplary primers include SEQ ID NOs: 14-16 (Table 3).

In another aspect the invention features oligonucleotide primers suitable for spectratype PCR analyzes of VH CDR3 size distributions in the library or its selection outputs (Table 4). Spectratyping may be a useful tool for assessing antibody library diversity and the progression of selections. Exemplary spectratyping PCR primers include SEQ ID NOs: 17-18.

5) Linkage of Nucleic Acid to Peptide Acceptors

In an embodiment, the antibody nucleic acid libraries of the invention may be modified to contain a peptide acceptor moiety. This facilitates the covalent attachment of individual member of nucleic acid expression libraries to their cognate protein products. Any art recognized means of attachment of a peptide acceptor to a nucleic acid are contemplated.

In one aspect, the invention features novel methods and compositions for the attachment of a peptide acceptor to nucleic acid libraries. In one embodiment, a linking molecule may be synthesized that comprises a Psoralen C6 molecule and a peptide acceptor molecule, wherein the Psoralen C6 molecule and a peptide acceptor molecule may be fused to a nucleic acid sequence, wherein the nucleic acid sequence may be complementary to sequences at the 3' end of the nucleic acid library. Such linking molecules may bind, via complementary base pairing, to the 3' end of nucleic acid library clones. Psoralen C6 is sensitive to ultraviolet (UV) light and will cross link the linker to the nucleic acid library clones, thus covalently linking the peptide acceptor to the nucleic acid library clones. In another embodiment, the nucleic acid portion of the linker molecule may contain modified nucleotides, for example, 2 prime methoxy (2'OMe) ribonucleotides. In another embodiment, the linker molecule may further comprise a Triethylene glycol or PEG-150 linker separating the nucleic acid region containing the Psoralen C6 molecule and a peptide acceptor molecule. In one embodiment the linker may be: 5' (Psoralen C6) 2'OMe(U AGC GGA UGC) XXX XXX CC (Puromycin) 3', (where X is a Triethylene glycol or PEG-150 and CC is standard DNA backbone). In an embodiment, such linkers are custom-synthesized by, for example, TriLink BioTechnologies, Inc (San Diego, CA).

IV. Methods of Spectratyping

Spectratype analysis is a method used in clinical and basic immunological settings in which antigen receptor length diversity is assessed as a surrogate for functional diversity (see, for example, Cochet, M., *et al.* (1992) *Eur. J. Immunol.*, 22:2639–2647; Pannetier, C., *et al.* (1993) *Proc. Natl Acad. Sci. USA*, 90:4319–4323; Pannetier, C., *et al.* (1997) In Austin, O.J.R. (Ed.). *The Antigen T Cell Receptor: Selected Protocols and Applications*, TX Landes, pp. 287–325). Spectratype assays may

use, for example, CD4 or CD8 T cells isolated from a peripheral blood sample from the subject, while in other cases total CD3 or PBMC cells are used.

In this invention, PCR may be used to specifically replicate the variable-length region (CDR3) for analysis of genetic sequences encoding antibodies on the basis of CDR3 length. Changes in CDR3 length distribution are correlated with changes in the antibody repertoire. In some embodiments, primers specific to individual libraries constructed in the practice of the invention may be used to provide independent spectratypes for each library. In one preferred embodiment, a fluorescent dye-labeled 5' forward primer (6-FAM-PanVHFR3-Fwd, 5'-GACACGGCCGTGTATTACTGT-3', SEQ ID NO: 17) and a reverse primer (PanJH-Rev, 5'-GCTGAGGAGACGGTGACC-3', SEQ ID NO: 18) that respectively anneal to the VH's framework 3 region and to the J region may be used to amplify across the CDR3 regions of VH domains by PCR. In other embodiments, other primers known in the art may be used, with specifically annealing to the same region or other regions on the polynucleotide sequences encoding the library of antibodies. In one preferred embodiment, the resulting mixture of CDR3 replicons may be size-separated by electrophoresis, and quantified by densitometry. In other embodiments, other methods known in the art may be used to characterize the resulting CDR3 replicons.

In one preferred embodiment, spectratyping analysis of CDR3 size distribution among different VH families may be carried out on VH cDNA fragments. In an embodiment, the exemplary VH families may be obtained from a single germline or from different VH families such as VH1-46, VH2, VH5, and VH6. In one preferred embodiment, the templates for spectratyping may be selected from human lymph node libraries, yeast spleen libraries, naïve human lamda libraries, human PBMC kappa libraries, VH-Vλ scFv library, VH-Vκ scFv library, for example. In other embodiments, the templates for spectratyping may be selected from other libraries.

V. General Screening Methods

In one aspect, the invention features methods of screening the expression libraries of the invention to identify antibodies capable of binding to a desired target. Any *in vitro* or *in vivo* screening method that allows for selection of an antibody from an expression library, based upon the antibody binding to a target molecule, is contemplated.

In one embodiment, the expression libraries of the invention may be screened using an art recognized *in vitro* cell-free phenotype-genotype linked display. Such methods are well known in the art and are described, for example, in U.S. Patent Nos. 7,195,880; 6,951,725; 7,078,197; 7,022,479; 6,518,018; 7,125,669; 6,846,655; 6,281,344; 6,207,446; 6,214,553; 6,258,558; 6,261,804; 6,429,300; 6,489,116; 6,436,665; 6,537,749; 6,602,685; 6,623,926; 6,416,950; 6,660,473; 6,312,927; 5,922,545; and 6,348,315. These methods involve transcription of protein *in vitro* from a nucleic acid in such a way that the protein is physically associated or bound to the nucleic acid from which it originated. By selecting for an expressed protein with a target molecule, the nucleic acid that codes for the protein may also be selected.

To improve the expression of scFv proteins, the above referenced *in vitro* screening assays may require the addition or removal of certain reagents. In one embodiment, protein disulphide isomerase enzymes may be added to the *in vitro* expression system to improve the production of functional scFv molecules. In another embodiment, a mild oxidizing agent (for example, GSSG (oxidized glutathione) /GSH (reduced glutathione), for example 100mM GSSG /10mM GSH) may be added to *in vitro* translation reaction mixture of the scFv proteins to allow intra-chain disulphide bond formation in the VH and VL regions of the scFv molecule. In another embodiment, reducing agents (for example, dithiothreitol (DTT)) may be removed from the *in vitro* translation reaction mixture of the scFv.

In another embodiment, one or more labeled amino acids, or derivatives thereof, may be added to the *in vitro* translation system such that the labeled amino acid(s) becomes incorporated into the resultant antibody. Any art recognized labeled amino acid is contemplated, for example, a radiolabelled amino acid, for example, ^{35}S-labelled methionine or cysteine.

In one embodiment, the *in vitro* screening assays of the invention require that after *in vitro* selection of an antibody or plurality of antibodies the mRNA that is physically associated with the antibody or plurality of antibodies may be reverse transcribed to generate cDNA encoding said antibody or plurality of antibodies. Any suitable method for reverse transcription is contemplated, for example, enzyme mediated, for example, Moloney murine leukemia virus reverse transcriptase.

The screening methods employed in the invention may require amplification of the nucleic acid that encodes antibodies that bind specifically to a desired target. In one

embodiment, mRNA that is physically associated with an antibody or plurality of antibodies may be amplified to produce more mRNA. Any art recognized method of RNA replication is contemplated, for example, using an RNA replicase enzyme. In another embodiment, mRNA that is physically associated with an antibody or plurality of antibodies is first reverse transcribed into cDNA before being amplified by PCR. In one embodiment, PCR amplification is accomplished using a high fidelity, proof – reading polymerase, for example, the KOD1 thermostable DNA polymerase from *Thermococcus kodakaraensis* or Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA). In another embodiment, PCR amplification may be performed under conditions that result in the introduction of mutations into amplified DNA, *i.e.,* error-prone PCR.

Screening methods employed in the invention may also require that the stringency of the target-binding screening assay be increased to select for antibodies with improved affinity for target. Any art recognized methods of increasing the stringency of an antibody-target interaction assay are contemplated. In one embodiment, one or more of the assay conditions may be varied (for example, the salt concentration of the assay buffer) to reduce the affinity of the antibody molecules for the desired target. In another embodiment, the length of time permitted for the antibodies to bind to the desired target may be reduced. In another embodiment, a competitive binding step may be added to the antibody-target interaction assay. For example, the antibodies may first be allowed to bind to a desired immobilized target. A specific concentration of non-immobilized target may then be added, which serves to compete for binding with the immobilized target such that antibodies with the lowest affinity for antigen are eluted from the immobilized target, resulting in an enrichment for antibodies with improved antigen binding affinity. In an embodiment, the stringency of the assay conditions may further be increased by increasing the concentration of non-immobilized target that is added to the assay.

Screening methods of the invention may also require multiple rounds of selection to enrich for one or more antibodies with improved target binding. In one embodiment, at each round of selection further amino acid mutations may be introduced into the antibodies using art recognized methods. In another embodiment, at each round of

selection the stringency of binding to the desired target may be increased to select for antibodies with increased affinity for a desired target.

Screening methods of the invention may require purification of RNA-antibody fusion proteins from the components of an in vitro translation system. This may be accomplished using any art recognized method of separation. In one embodiment, the RNA-antibody fusion proteins may be separated by chromatography using a polydeoxythimidine (polydT) resin. In another embodiment, the RNA-antibody fusion proteins may be separated by chromatography using an antibody specific for an epitope present in the antibody component of the RNA-antibody fusion protein. In an embodiment, the epitope may be an amino acid sequence tag, for example, FLAG or HA tags, incorporated into the amino acid sequence of the antibody component of the RNA-antibody fusion protein, for example, at the N-terminal, C-terminal or in the inter variable region linker.

Selection of antibodies from the libraries of the invention may require the use of immobilized target molecules. In one embodiment, the target molecule may be directly linked to a solid substrate for example, agarose beads. In another embodiment, the target molecule may first be modified, for example, biotinylated and the modified target molecule may be bound via the modification to a solid support, for example, streptavidin-M280, neutravidin-M280, SA-M270, NA-M270, SA-MyOne, NA-MyOne, SA-agarose, and NA-agarose.

This invention is further illustrated by the following examples that should not be construed as limiting.

EXEMPLIFICATION OF THE INVENTION

Throughout the examples, the following materials and methods were used unless otherwise stated.

Materials and Methods

In general, the practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, recombinant DNA technology, immunology (especially, e.g., immunoglobulin technology), and animal husbandry. See, *e.g.*, Sambrook, Fritsch and Maniatis, Molecular Cloning: Cold Spring Harbor Laboratory Press (1989); Antibody Engineering Protocols (Methods in

Molecular Biology), 510, Paul, S., Humana Pr (1996); Antibody Engineering: A Practical Approach (Practical Approach Series, 169), McCafferty, Ed., Irl Pr (1996); Antibodies: A Laboratory Manual, Harlow *et al.*, C.S.H.L. Press, Pub. (1999); Current Protocols in Molecular Biology, eds. Ausubel *et al.*, John Wiley & Sons (1992).

EXAMPLE 1

MRNA DISPLAY PROTOCOL FOR SCFV MOLECULES

mRNA display may be conducted according to the method shown in Figure 2. The particular embodiments of this method are described in greater detail below. These embodiments are intended to illustrate the methods of the invention, and should not be construed as limiting.

1. Design of Antibody Library Templates

Library DNA constructs are designed according to the diagram depicted in Figure 3. The double-stranded DNA constructs generally contain the following functional elements, from the 5' to the 3'end. A T7 promoter may be useful for the RNA transcription *in vitro.* A TMV-UTR (tobacco mosaic virus untranslated region) may be useful for protein translation *in vitro*. An optional tag contains an 8 base-pair sequence unique to each library, which may be useful for identifying constructs belonging to a given library. A Kozak consensus sequence facilitates the initiation of protein translation. The antibody library of interest may contain sequences encoding an scFv, VH, or VL. In a preferred embodiment, the antibody library encodes scFv. In one embodiment, a partial antibody constant region sequence that is invariable at the 3' end of all antibody libraries is also included. In some embodiments, constructs additionally include a FLAG tag useful for affinity purification. In other embodiments, constructs contain a linker annealing sequence comprising of an annealing site , where a psoralen and puromycin modified DNA oligonucleotide linker may be crosslinked to the construct in subsequent steps of the protocol. In an embodiment, a polyadenylation sequence with a 5' stop codon may be useful for mRNA stability and purification through oligo-dT cellulose batch purification.

2. *Preparation of the Target Antigen*

Generally the mRNA display antibody library may be selected against biotinylated antigens. While the best antigen for each target should be determined on a case-by-case basis, the following considerations may be used as a general guideline. A target antigen is typically well characterized, and is the relevant or dominant genetic isotype, as determined by polymorphism (SNP and haplotype) and/or pharmacogenetic analysis. A target antigen additionally may have reasonable bioactivity (comparable to the native antigen), good solubility and good chemical and physical properties, and may be prepared in sufficient quantities for library selections or screenings and downstream bioassays. Exemplary quantities of target antigen useful for library selection are noted in Table 1 below.

Table 1: Target antigen quantity required for library selection

Technology	Choice of human antibody scFv libraries	Antigen requirements (to be multiplied by # of libraries)		Contingent antigen requirement	Deliverables
		for each library selection	for each library output screening		
PROfusion mRNA Display	2 PBMC (κ/λ) 2 Spleen (κ/λ) 2 Lymph nodes (κ/λ)	1 nmole biotinylated	2.5 nmole biotinylated	2 nmole biotinylated	Bioactive IgG candidates that will likely need LO
Yeast Display	2 PBMC (κ/λ) 1 Spleen (κ) 1 Lymph nodes (κ)	6.5 nmole (preferably biotinylated)	3 nmole biotinylated	3 nmole biotinylated	

3. *Preparation of the Library DNA*

The library DNA and its selection outputs may be amplified by PCR. Exemplary primers for library amplification are shown in Table 2.

Table 2: Primers for library amplification

Primer	Amplifies	Sequence
5' forward primer		
T7TMVUTR (SEQ ID NO:1)	All scFv and VH libraries	TAATACGACTCACTATAGGGACAATT ACTATTTACAATTACA
VL-T7TMVTag3GS-Fwd[a] (SEQ ID NO:2)	Vκand Vλ PBMC libraries	TAATACGACTCACTATAGGGACAATT ACTATTTACAATTACAGGCTTTGGACC ATGGGGTCTGGCGGCGGAGGTAGCG
3' reverse primer		
CK5FLAGA20 Rev (SEQ ID NO:7)	All κ scFv and Vκ libraries	TTTTTTTTTTTTTTTTTTTTAAATAGCG GATGCCTTGTCGTCGTCGTCCTTGTAG TCGAAGACAGATGGTGCAGCCACA
CL5FLAGA20 Rev (SEQ ID NO:12)	All λ scFv and Vλ libraries	TTTTTTTTTTTTTTTTTTTTAAATAGCG GATGCCTTGTCGTCGTCGTCCTTGTAG TCAGTGACAGTGGGGTTGGCCTTG
VH-GSFLAGA20-Rev[a] (SEQ ID NO:13)	VH library from human PBMC	TTTTTTTTTTTTTTTTTTTTAAATAGCG GATGCTTTGTCATCATCATCTTTATAA TCGCTACCTCCGCCGCCAGAC

PCR amplification may be performed using methods known in the art. PCR reactions typically contain the DNA template, a reaction buffer, dNTP, the primers used for amplification, DNA polymerase, and water. Multiple reaction tubes are set up simultaneously from a master mix to increased amplified DNA yield. 25 cycles of PCR typically give sufficient amplification, but as many as 35 cycles may be used to gain more products.

4. Library DNA Purification

If products from the above PCR are the correct size (~850 bp for scFv, ~500 bp for VH or VL library) and contain minimal non-specific products, they may be used directly in the transcription reaction. Alternatively, the products may be gel purified. If gel purification is performed for PCR products, the products may be separated on a

preparative agarose gel and the specific band containing the PCR products may be cut out. DNA may then be purified from the band by gel extraction, using standard methods known in the art, and its concentration measured on a spectrophotometer.

5. RNA Transcription

The RNA transcription from library DNA may be performed using standard methods known in the art. A large reaction volume may be used to transcribe sufficient DNA templates to sample the entire library diversity. In an exemplary embodiment, 1 x 10^{13} copies of library templates may be used in the RNA transcription reaction. An RNA transcription reaction typically contains 5-10 μg of PCR product, a reaction buffer, plus ATP, CTP, GTP, UTP, and T7 RNA polymerase. The RNA transcription reaction may be run at 37°C for between 2 hours to overnight. Shorter times may be used following initial rounds of selection. After the RNA transcription, DNA templates may be removed from the reaction mixture using DNase I.

6. RNA Purification by NAP Column Chromatography

Following the RNA transcription, RNA may be fractionated using a NAP-10 column (GE Healthcare, Piscataway, NJ). Up to 1 mL of transcription reaction may be loaded onto a NAP-10 column for RNA purification. The column may be equilibrated using diethylpyrocarbonate (DEPC)-treated dH_2O prior to fractionation. The total elution volume should be less than 150% of the transcription reaction volume. RNA may be additionally or alternatively fractionated using a NAP-25 column (GE Healthcare, Piscataway, NJ).

7. RNA Quality Control and Quantitation

The size and yield of RNA samples may be monitored using gel electrophoresis. The RNA yield typically reaches a maximum at ~20 nmol/mL transcription reaction.

8. RNA Ligation to Linker

A DNA linker that contains a peptide acceptor molecule at its 3' end may be covalently ligated to the 3' ends of each RNA molecule. The peptide acceptor, which can enter the ribosomal A site and covalently couple to the carboxyl terminus of the nascent polypeptide chain, will ultimately enable the covalent association of the mRNA

(genotype) to the protein encoded by this mRNA (phenotype). An exemplary PEG6/10 linker may have the following formula:

5' (Psoralen C6) 2'OMe(U AGC GGA UGC) XXX XXX CC (Puromycin) 3'.

The Psoralen C6 5' modification is light sensitive and functions to create a covalent bond between the linker and the mRNA by UV crosslinking. A 2'OMe (U AGC GGA UGC) backbone region anneals to the linker annealing site 3' to the FLAG sequence on mRNA (see Figure 1). In the sequence above, X denotes "Spacer 9", alternatively known as Triethylene glycol or PEG-150. This spacer has been optimized to provide flexibility for puromycin insertion into the eukaryotic ribosome A site. CC comprises a standard DNA backbone. A puromycin 3' modification inserts into the ribosome A site to create a stable link between the linker and the nascent peptide. The extinction coefficient for the linker described herein is 147.7 OD_{260}/μmole. Since this linker is light sensitive, solutions containing this linker should be protected from light.

For initial rounds of library selections, a large-scale ligation reaction (3.1×10^{15} transcribed RNA molecules) is recommended to sample a library's entire diversity. This RNA quantity may be set to ensure enough templates are put into translation reactions and produce ~10 pmol functional mRNA display molecules. In later rounds, RNA input may be reduced to 0.5 nmol per selection. In an exemplary embodiment, an RNA ligation reaction may contain the following components: the RNA, water, a chemical ligation buffer, and the PEG6/puromycin linker (1mM). In an exemplary embodiment, the total reaction volume is 100 μL. In a preferred embodiment, the linker/RNA molar ratio may be greater than 1.5. In one embodiment, the final linker concentration in the reaction may be about 15 μM, and the RNA concentration in the reaction may range from about 3 – 10 μM (= 0.3 – 1 nmol RNA input). As a reference, an 850 nt scFv RNA at 1 mg/mL = 3.56 μM, and the attainable maximal ligation concentration is 3.16 μM (= 0.32 nmol).

The annealing reaction (which anneals the linker to the transcribed RNA) may be performed in a thermal cycler. In a preferred embodiment, the annealing reaction may be conducted by incubating samples at about 85°C for 30 seconds, then at about 4°C, using a ramp rate of about 0.3°C per second. Reactions may then be held at 4°C.

Ligation of the annealed linker/RNA may be accomplished by UV crosslinking. This may be conducted using any method known to one of skill in the art. In one embodiment, reaction tubes may be placed over the center of a handheld UV lamp (long

wavelength, about 365 nm) and crosslinked for about 15 minutes. A freezer pack may be placed on top of the lamp to help dissipate heat generated during UV irradiation. Typical ligation efficiency is about 50 – 90%, and purification is usually not required. The ligation products may be stored at –80 °C.

9. Translation Reaction

In an exemplary embodiment, about ~0.1% of input RNA may be made into mRNA display molecules after all reactions and purifications. *In vitro* translation may be conducted using methods and reagents known to one of skill in the art. In one embodiment, the translation reaction using the scFv library may use about 5 nmol of RNA template with about 10 mL of reticulocyte lysate in a reaction volume of about 15 mL.

In preparation for the translation reaction, solutions of GSSG/GSH (oxidized glutathione/reduced glutathione) may be prepared at a final concentration of about 100 mM GSSG/10 mM GSH. PDI (Protein Disulfide Isomerase) may be prepared by dissolving PDI powder into dH_2O to reach a concentration of about 1 Unit/μL. The PDI solution may be stored at –20°C.

An exemplary translation reaction may be set up as follows:

RNA (100 pmol)	X	X μL
dH_2O	to 73.7	to 370 μL
Amino acid master mix (Met)	15	75 μL
100 mM GSSG/10 mM GSH	3.3	16.5 μL
PDI (1 U/μL)	6	30 μL
[^{35}S]Methionine	2	10 μL
Reticulocyte lysate	200	1000 μL
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Total volume	300	1500 μL

Translation reactions are incubated in 30 °C water bath for 1 - 2 hours. A significant decrease in RNA/protein fusion yield was observed when the translation volume exceeds 1.5 mL. Therefore a master mix of the translation reaction may be prepared if the reaction volume will be larger than 1.5 mL, before dividing it to smaller aliquots.

10. RNA/Protein Fusion Formation

After the translation reaction, about 100 µL 2M KCl and about 20 µL 1M $MgCl_2$ may be added for every 300 µL of translation reaction mixture, and incubated for 1 hour at room temperature. This stabilizes the paused ribosomes at the end of mRNA templates and allows puromycin at the end of the DNA linker to enter the A sites of paused ribosomes, which permanently links the translated scFv proteins to their mRNA templates. The room temperature incubation may be shortened if the reaction will be stored at –20 °C overnight. The reaction may be terminated by adding 50 µL 0.5 M EDTA to disrupt the ribosomes. Reactions may be stored at –20 °C. A 5 µL aliquot may be removed for scintillation counting later.

11. RNA/Protein Fusion Purification by Oligo-dT Cellulose

This step purifies mRNA display molecules and remaining RNA templates from the translation/fusion reaction. For oligo-dT binding, the amount of pre-washed oligo-dT cellulose needed to capture all RNA templates may be estimated. A sufficient volume of oligo-dT binding buffer may be added to the fusion reaction to reach about a 1X final concentration. Pre-washed oligo-dT cellulose may then be added, and the reactions carried out for 1 hour at 4 °C. Reactions may optionally be spun down at about 1500 rpm for 5 minutes at 4°C, and the supernatant discarded. Oligo-dT cellulose beads may be transferred and washed about 6 times with 1X Oligo-dT binding buffer using spin columns, and the buffer may typically be removed by spinning columns at about 1000 rpm for 10 seconds. The flow-through may be discarded, but the last wash may be saved for scintillation counting. mRNA display molecules (and free RNA templates) may be eluted by adding dH_2O to beads and incubating for 5 minutes at room temperature. The eluate may be collected by spinning at about 4000 rpm for 20 seconds. The elution may typically be repeated once, and the eluates combined. 5 µL of eluate may be removed for scintillation counting. The efficiency of oligo-dT purification may also be assessed by OD at 260 nm (OD_{260}) on a NanoDrop spectrophotometer machine (NanoDrop Technologies, Wilmington, DE). All remaining RNA templates and mRNA display molecules are theoretically recovered by the oligo-dT beads. 5X FLAG binding buffer may be added to the eluates to reach about a 1X final concentration. Samples may be stored at –80 °C if not proceeding to the next FLAG purification step.

Oligo-dT recovery may be calculated as follows. About 5 μL of input (from fusion reaction), 100 μL from the last wash, and 5 μL of output (eluate from oligo-dT purification) are counted. The last wash is used to assess extent of washing, and the other two counts are used to calculate RNA/protein fusion recovery from original RNA template input. RNA/protein fusion yield (pmol) = ($CPM_{output} \times Volume_{output} \times 5$ μM × $Volume_{lysate}$) / [$CPM_{input} \times Volume_{input} \times$ (# of methionine in product)]. This formula assumes a 5 μM methionine concentration in the reticulocyte lysate, and all volumes used in calculation expressed as μL. For earlier rounds of selection the yield of mRNA display molecules is typically 0.5 – 2%, but may increase to 10% in later rounds.

12. RNA/Protein Fusion Purification by anti-FLAG M2 Agarose

This step purifies mRNA display molecules from remaining RNA templates. The amount of pre-washed anti-FLAG M2 agarose beads needed to capture all mRNA display molecules may be estimated. In one embodiment, the binding capacity of the beads is about 6 nmol fusion protein per mL of 50% slurry. To have a sufficient bead volume for manipulation during binding and washing, it is not recommended to use less than 200 μL of pre-washed beads. The example given below is for an initial 300 μL translation reaction.

For FLAG purification, a wide-bore pipette tip may be used to transfer 300 μL pre-washed anti-FLAG M2 agarose to the oligo-dT purified output. The mixture may be mixed and incubated by rotation for 1 hour at 4 °C. Incubation with anti-FLAG M2 agarose may continue overnight. Anti-FLAG M2 agarose may optionally be spun at about 1500 rpm in a centrifuge for 1 minute at 4 °C, and the supernatant may be discarded. Anti-FLAG beads may be washed about 5 times with 1X FLAG binding buffer, using spin columns and centrifugation at about 1000 rpm for 10 seconds for each wash. The flow-through may be discarded. The beads may additionally be washed 2 times with 700 μL selection buffer (see below) by centrifugation at about 1000 rpm for 10 seconds. The last wash may be saved for scintillation counting. mRNA display molecules may be eluted by adding about 400 μL 100 μg/mL FLAG peptide (in selection buffer) and incubating for 5 minutes at room temperature. The eluate may be collected by spinning at about 3000 rpm for 20 seconds and eluted one more time by adding about 400 μL 100 μg/mL FLAG peptide. Both elutes may be combined, and 5 μL of the combined elutes may be removed for scintillation counting. This volume of

FLAG peptide is typically sufficient for elution from up to about 1 mL of 50% slurry, and may be cut in half (200 µL) if less slurry was used and/or higher RNA/protein fusion concentration is desired. To prevent RNA degradation during storage and antigen selection, an appropriate amount of RNase inhibitor known in the art (*i.e.*, 1 – 2 U/µL RNaseOUT and 0.02 µg/mL yeast tRNA) may be added to the purified mRNA display library. Samples are stored at –80 °C if not proceeding to the next antigen selection step.

To quantitate the FLAG recovery, about 5 µL elution output and about 100 µL from the last wash may be counted on a beta counter. A recovery of 10-30% or higher may be expected, and may be calculated according to the following formula: PROfusion molecule recovery % = $(CPM_{output} \times Volume_{output}) / (CPM_{input} \times Volume_{input})$.

13. Library Selection by Biotinylated antigens

Selection is designed to enrich molecules that specifically bind to a target of interest. A negative selection (pre-clear) may be necessary to remove non-specific and matrix binders. Depending on the target format, the selection protocol varies. The following is an exemplary selection protocol for use with biotinylated targets. This protocol may be modified to accommodate target antigens in other formats, and may be scaled up or down depending on the desired output.

A. Preparations Before Selection

Streptavidin (SA) magnetic beads may be used for capture, and are typically pre-blocked prior to use. SA beads may be transferred from the original bottle to 1.5 or 2 mL tubes, and washed twice with 2 mL of 1X FLAG binding buffer. The beads may then be blocked with 2 mL of the selection buffer for 2 hours to overnight at 4 °C with rotation. Enough beads should be prepared for both pre-clear and selection capture. Pre-blocked beads are stored at 4 °C. About 100 µL of beads are typically used for every 10 pmol of biotinylated antigen.

1.5 mL or 2 mL microfuge tubes are pre-blocked with 1X FLAG binding buffer for about 1 hour to overnight. The pre-blocked tubes may be used for all pre-clear and selection steps. Typically four tubes are needed for each sample: 2 for pre-clearing, 1 for the beads, and 1 for selection.

Optimal results may be obtained by pre-clearing the library. FLAG-purified mRNA display library may be added to the SA beads (separated from buffer). The volume of SA bead may be equal to half of the capture volume. The total mixture may be incubated with rotation at 30 °C for 30 minutes before the separation of pre-cleared mRNA display library from SA beads using a magnet. This pre-clearing step is repeated one more time and the second pre-clear SA beads may be washed and counted as described in above to determine if background is high. This may also serve as a "no antigen" negative control.

B. Library Selection: Binding

For first rounds of selection, biotinylated target may be added (100 nM) to the whole pre-cleared library and incubated with rotation at 30 °C for 1 hour. For later selection rounds when recovery of antigen-binding molecules is expected to exceed 1%, the pre-cleared library may be divided into 2 equal aliquots. Biotinylated antigen may be added to one aliquot, and the other serves as the "No antigen" negative control. Alternatively, the washed second pre-clear beads may also be considered as a "No antigen" control, as noted above, although these beads will have one less 'pre-clearing' procedure. The antigen concentration in later rounds may be dropped when recovery of antigen-binding molecules exceeds 5%.

C. Library Selection: Capture

Pre-blocked SA beads (separated from buffer) may be added to the binding reaction and incubated with rotation at 30 °C for 5 to 10 minutes. The amount of SA beads for capture should be calculated based on the capacity and the target concentration used in selection (see above). The amount of SA beads should be reduced when lowering the target concentration to avoid the SA bead binders, but typically not less than 50 μL of beads is used.

D. Library Selection: Washing

The SA beads may be collected using a magnet and may be washed with 1 mL of the selection buffer for 1 minute. The beads are collected again using a magnet and washed for about 5 more times (about 6 times total). The wash time may be increased in later rounds to incorporate off rate selection strategy to some targets. The beads may be

washed one last time with 1 mL of 1X buffer suitable for reverse transcription. The beads are collected with a magnet and re-suspended in water (one fourth of the capture bead volume calculated above).

E. Library Selection: Counting and Recovery Calculation

Starting from Round 3, about 10 – 20% of the last wash and the beads are counted. Typically only less than 100 μL of beads is counted, because more beads can quench the counts. Library selection recovery is calculated according to the following formula:

Selection recovery % = $100 \times CPM_{\text{Total Beads}} / CPM_{\text{Total Input.}}$

14. Reamplification of Library DNA by RT-PCR

Reverse transcription may be performed using the material captured from the library. Reagents and protocols known in the art are suitable for performing the reverse transcription reaction. The volume of the reaction may be scaled up or down according to the bead volume after selection.

Exemplary primers useful for reverse transcription are shown in Table 3, although additional primers may be designed using methods known in the art. The Cκ reverse primer is used for kappa libraries, CJL reverse primer is used for lambda libraries, and Lib-GS-Rev is used for human PBMC VH library.

Table 3: Exemplary primers suitable for reverse transcription

Ck Reverse (SEQ ID NO:14)	GTCGTCGTCGTCCTTGTAGTCGAAGACAGATGGTGCAGCCACAGTTCG
CJL Reverse (SEQ ID NO:15)	GTCGTCGTCGTCCTTGTAGTCAGTGACAGTGGGGTTGGCCTTGGGCTGACCKAGGACGGT
Lib-GS-Rev (VH, PBMC) (SEQ ID NO:16)	CGCTACCTCCGCCGCCAGAC

An exemplary reverse transcription reaction may contain the beads from the library selection (in water), about 10 μM reverse primer, and about 10 mM dNTP. Reactions are incubated at 65 °C for 5 minutes and chilled on ice. First strand synthesis buffer, 0.1M DTT, and RNase inhibitor are then typically added to the reaction. The reverse transcription reactions are incubated at 42°C for 2 minutes before adding the

reverse transcriptase enzyme. Reactions are then incubated at 42 °C for 50 minutes with occasional agitation and further incubated at 95 °C for 5 minutes. The beads are then collected by magnet, and the supernatant transferred to new tubes, which may be pooled if it is from same selection output. The beads are resuspended in water (half of RT volume), and incubated in tubes at 95 °C for 5 minutes. The beads are again collected using a magnet, and the supernatant is pooled with the previously transferred supernatant. This contains the cDNA template for PCR amplification of selection output.

Spectratyping PCR may be used to analyze VH CDR3 size distributions in the library or its selection outputs. It is a useful tool to assess the library diversity and the progression of selections. The initial few rounds of library selection outputs and the library before selection should be very diverse and the CDR3 size distribution approximates a Gaussian distribution. Exemplary spectratyping PCR primers are shown in Table 4.

Table 4: Exemplary spectratyping PCR primers

6-FAM-PanVHFR3-Fwd (SEQ ID NO:17)	GACACGGCCGTGTATTACTGT
PanJH-Rev (SEQ ID NO:18)	GCTGAGGAGACGGTGACC

An exemplary spectratyping PCR reaction is shown below in Table 5, although the reaction components may be substituted with comparable reagents known in the art, and the reaction volume may be adjusted to accommodate the scale of the selection reaction.

Table 5: Exemplary spectratyping PCR reaction

cDNA template	2.0	µL
dH_2O	18.1	µL
5X thermal stable DNA polymerase reaction buffer	6.0	µL
25 mM $MgCl_2$	1.8	µL
10 mM dNTP	0.6	µL
5' forward primer (10 µM)	0.6	µL
3' reverse primer (10 µM)	0.6	µL
thermal stable DNA polymerase	0.3	µL

Total volume	30.0 µL

Thermal stable DNA polymerases known in the art are suitable for this reaction. In an exemplary embodiment, the final Mg^{2+} concentration is 1.5 mM. As an exemplary thermal cycling program, the reaction is incubated at 94 °C for 2 minutes and then subjected to 30 thermal cycles to elongate the DNA. For each cycle, the reaction is incubated at 94 °C for 20 seconds, at 55°C for 20 seconds, and then at 72°C for 30 seconds. After 30 cycles, the reaction is further incubated at 72°C for 5 minutes and then stored at 4°C. After PCR, 10 µL of PCR product is loaded onto a 2% agarose gel to confirm that the reaction was successful. The reaction and the remaining product are analyzed by spectratyping electrophoresis.

The amplified DNA product has the following organization:

5'-FR3 (27 bp)–VH CDR3–FR4 (35 bp)-3'.

The VH CDR3 size may be deduced from the apparent DNA product size. This may be determined by the Rox dye size marker using the following calculation:

$Size_{VH\ CDR3} = (Size_{Apparent\ DNA\ product\ size} - 60) / 3$

Where $60 = (62_{Frameworks\ on\ both\ ends} - 1_{3'\ A\ overhang} + 3_{DNA\ marker\ underestimation})$.

15. PCR for Library DNA Template Amplification

For selecting outputs from first and second rounds, cDNA (supernatants from RT reactions) may be dialyzed against water using an 8 kDa cut-off and the entire amount of cDNA may be used as the PCR template. For selecting outputs from later rounds, 10% of cDNA is used as template for PCR, and the dialysis is typically not necessary. The exemplary amplification primers are shown in Table 6.

Table 6: Amplification primers

Primer	Sequence (5' – 3')
Forward primer for all scFv and VH libraries	
T7TMVUTR[a] (SEQ ID NO:1)	TAATACGACTCACTATAGGGACAATTACTATTTACAATTACA
Forward primer for all Vκand Vλ libraries	
VL-T7TMVTag3GS-Fwd (SEQ ID NO:2)	TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAGGCTTTGGACCATGGGGTCTGGCGGCGGAGGTAGCG
Reverse primers for all κ scFv and Vκ libraries	
Ck1-FlagA20 Rev (SEQ ID NO:3)	TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTAGTCGAA GACAGAT
Ck2-FlagA20 Rev (SEQ ID NO:4)	TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTAGTCGAAGACAGATGGT
Ck3-FlagA20 Rev (SEQ ID NO:5)	TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTAGTCGAAGACAGATGGTGCA
Ck4-FlagA20 Rev (SEQ ID NO:6)	TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTAGTCGAAGACAGATGGTGCAGCC
Ck5-FlagA20 Rev [b](SEQ ID NO:7)	TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTAGTCGAAGACAGATGGTGCAGCCACA
Reverse primers for all λ scFv and Vλ libraries	
CL1FLAGA20 Rev [b](SEQ ID NO:8)	TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTAGTCAGTGACAGTG
CL2FLAGA20 Rev (SEQ ID NO:9)	TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTAGTCAGTGACAGTGGGG
CL3FLAGA20 Rev (SEQ ID NO:10)	TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTAGTCAGTGACAGTGGGGTTG
CL4FLAGA20 Rev (SEQ ID NO:11)	TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTAGTCAGTGACAGTGGGGTTGGCC
CL5FLAGA20 Rev (SEQ ID NO:12)	TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTAGTCAGTGACAGTGGGGTTGGCCTTG
Reverse primer for all VH libraries	
VH-GSFLAGA20-	TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCTTTGTCATC

Rev (SEQ ID NO:13)	ATCATCTTTATAATCGCTACCTCCGCCGCCAGAC
[a] T7TMVTag-primer (sequence of the Tag depends on the library) can also be used. [b] Preferred primer	

An exemplary PCR reaction for library DNA template amplification is shown in Table 7 below.

Table 7: The exemplary PCR reaction for library DNA template amplification

cDNA template	X µL
dH_2O	add to 790 µL
10X High Fidelity Taq DNA Polymerase buffer	100µL
$MgSO_4$ (50 mM)	40 µL
10 mM dNTP	20 µL
5' forward primer (10 µM)	20 µL
3' reverse primer (10 µM)	20 µL
High Fidelity Taq DNA Polymerase	10 µL

Total volume	1000 µL

In an exemplary embodiment, 1 mL PCR reactions are used for round 1 and 2 outputs, and 0.5 mL reactions are used for outputs from later rounds. Aliquots of 100 µL reactions should be made from a master mix. The exemplary thermal cycling condition for the amplification of library DNA templates is shown in Table 8 below.

Table 8: Thermal cycling conditions for library DNA template amplification

94 °C	2 minutes	
	↓	
94 °C	20 seconds	⎫
55 °C	20 seconds	⎬ 25 cycles*
68 °C	1 minute	⎭

↓

68 °C 5 minute

↓

4 °C Hold forever

*Note: 25 cycles typically gives sufficient amplification but it may be increased to as many as 35 cycles to gain more products. Non-specific products of various sizes may become more apparent with additional amplification cycles, and the product may need to be gel purified. If possible, it may be helpful to increase the DNA template input rather than the number of amplification cycles.

After PCR, 5 to 10 µL products are loaded on a 1.2% agarose gel with an appropriate DNA size marker to check the result. If products are the correct size (~ 850 bp for scFv, ~ 500 bp for VH or VL library) and have minimal non-specific products, they may be used directly in transcription reaction of the next round. The products may need to be gel purified. If gel purification will be done for PCR products, all remaining products on a preparative agarose gel are separated and the specific band containing the products may be cut out for gel extraction. Quantitation of gel purified DNA may be misleading, as residual EtBr in the DNA tends to interfere with the UV absorbance. A more extensive wash step during gel extraction may help alleviate this interference. If possible, DNA concentration should be measured on a spectrophotometer, as the UV scanning traces are quite different between a clean DNA sample and a DNA with residual EtBr. This protocol is subsequently repeated to conduct multiple rounds of selection.

16. Exemplary Reagents and Buffer Compositions

10X Chemical Ligation Buffer

Tris, pH 7	250 mM
NaCl	1 M

Oligo-dT Binding Buffer	1X	2X	3X
Tris, pH 8	100	200	300 mM
NaCl	1	2	3 M
Triton X-100	0.05	0.1	0.15%

FLAG Binding Buffer	1X	5X
Phosphate-based buffer		
PBS	1X	5X
Triton X-100	0.025	0.125%
Alternative HEPES-based buffer		
HEPES	50	250 mM
NaCl	150	750 mM
Triton X-100	0.025	0.125%

Selection Buffer	
Phosphate-based buffer	
PBS	1X
BSA	1 mg/mL
Salmon sperm DNA	0.1 mg/mL
Triton X-100	0.025%
Yeast tRNA (optional, add before use)	20 ng/mL

Alternative HEPES-based buffer	
HEPES	50 mM
NaCl	150 mM
BSA	1 mg/mL
Salmon sperm DNA	0.1 mg/mL
Triton X-100	0.025%
Yeast tRNA (optional, add before use)	20 ng/mL

First strand buffer	
Tris-HCl, pH 8.3	250 mM
KCl	375 mM
$MgCl_2$	15 mM

50X FLAG stock solution	
FLAG peptide	25 mg
Selection buffer	5 mL

Make 1 mL aliquots and store at –20 °C.

FLAG elution solution

50X FLAG stock solution	1 mL
Selection buffer	49 mL

Make 1 mL aliquots and store at –20 °C

Oligo-dT cellulose preparation

2.5 g of oligo-dT cellulose may be transferred into a 50 mL tube and mixed with 25 mL of 0.1 N NaOH. The mixture may be spun down at 1500 rpm for 3 minutes and the supernatant discarded. The oligo-dT cellulose may then be washed with 25 mL of 1X Oligo-dT binding buffer and spun down at 1500 rpm for 3 minutes. The supernatant may be discarded. The wash step may be repeated for 3 more times and the pH of the supernatant measured. The pH should be the same as wash buffer (~ pH 8.5). The oligo-dT cellulose may be re-suspended to a final volume of 25 mL by adding 1X Oligo-dT binding buffer to make an approximately 50% slurry and stored at 4 °C. The Final concentration = 100 mg/mL = 1 nmol RNA capacity.

Anti-FLAG M2 agarose preparation

25 mL of M2 agarose beads may be transferred into a 50 mL of tube and spun down for 5 minutes at 1000 rpm in a Beckman centrifuge (Beckman Coulter, Fullerton, CA). The supernatant may be removed by aspiration. The resulting beads may be re-suspended, washed in equal volume of 10 mM glycine (pH 3.5) and spun down for 5 minutes at 1000 rpm. The supernatant is again removed by aspiration. The beads are re-suspended with one column volume of 1X FLAG binding buffer and spun down for 5 minutes at 1000 rpm. The supernatant is removed by aspiration. This wash step may be repeated 3 times and the beads re-suspended with one column volume of 1X binding buffer (containing 1 mg/mL BSA and 100 mg/mL salmon sperm DNA). The mixture may be rotated for 1 hour or overnight at 4 °C and split into aliquots in 2 mL fractions, if desired, and keep at 4 °C.

EXAMPLE 2
DEMONSTRATION OF FUNCTIONAL MRNA-SCFV MOLECULES

Four antibodies are used to demonstrate that functional mRNA-scFv molecules can be displayed and bind to their respective antigen: D2E7 (human anti-hTNF), Y61 (human anti-hIL-12), 17/9 (mouse anti-HA), and MAK195 (mouse anti-hTNF). The MAK195 scFv is generated by PCR using the following primers in Table 9.

Table 9: Oligonucleotide primers used for the construction of MAK195 mRNA-scFv constructs

Primers	Sequences
T7-MAK195VH-Fwd (SEQ ID NO: 19)	TAATACGACTCACTATAGGGACAATTACTATTT ACAATTACACCATGGAGGTGCAGCTGAAGGAG TCAGG
MAK195VHGS-Rev (SEQ ID NO: 20)	CGATCCGCCACCGCCAGAGCCACCTCCGCCTGA ACCGCCTCCACCTGCAGAGACAGTGACCAGAGT CC
MAK195VLGS-Fwd (SEQ ID NO: 21)	GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGG CGGTGGCGGATCGGACATTGTGATGACCCAGTC TC
MAK195VL-Rev (SEQ ID NO: 22)	GATGGTGCAGCCACCGTACGTTTTATTTCCAAC TTTGTCCCCGAG

An anti-HA 17/9 scFv (see Schulze-Gahmen *et al.* (1993) J. Mol. Biol. 234(4): 1098-118) is generated by PCR using the following primers based on protein sequences A31790 and B31790 downloaded from NCBI's database (see Table 10 below).

Table 10: Oligonucleotide primers used for the construction of 17/9 mRNA-scFv constructs

Primers	Sequences
T7TMVUTR-17/9 VH-1 Fwd (SEQ ID NO: 23)	GGACAATTACTATTTACAATTACACCATGGAAG TGCAGCTGGTGGAAAGCGGCGGCGATCTGGTG AAACC

Primers	Sequences
17/9 VH-2 Rev (SEQ ID NO: 24)	GCTGCTAAAGCTAAAGCCGCTCGCCGCGCAGCT CAGTTTCAGGCTGCCGCCCGGTTTCACCAGATC GCCG
17/9 VH-3 Fwd (SEQ ID NO: 25)	GGCTTTAGCTTTAGCAGCTATGGCATGAGCTGG GTGCGCCAGACCCCGGATAAACGCCTGGAATG GGTGG
17/9 VH-4 Rev (SEQ ID NO: 26)	GCCTTTCACGCTATCCGGATAATAGGTATAGCC GCCGCCGTTGCTAATGGTCGCCACCCATTCCAG GCGT
17/9 VH-5 Fwd (SEQ ID NO: 27)	CCGGATAGCGTGAAAGGCCGCTTTACCATTAGC CGCGATAACGCGAAAAACACCCTGTATCTGCAG ATG
17/9 VH-6 Rev (SEQ ID NO: 28)	GTTCGCGGCGCGCGCAATAATACATCGCGCTAT CTTCGCTTTTCAGGCTGCTCATCTGCAGATACA GGGT
17/9 VH-7 Fwd (SEQ ID NO: 29)	ATTGCGCGCGCCGCGAACGCTATGATGAAAAC GGCTTTGCGTATTGGGGCCAGGGCACCCTGGTG ACCGT
17/9 VH-8 GS Rev (SEQ ID NO: 30)	CGATCCGCCACCGCCGCTGCCACCTCCGCCTGA ACCGCCTCCACCCGCGCTCACGGTCACCAGGGT GCCC
GS-17/9 VL-1 Fwd (SEQ ID NO: 31)	AGCGGCGGTGGCGGATCGGATATTGTGATGACC CAGAGCCCGAGCAGCCTGACCGTGACCGCGGG CGAAA
17/9 VL-2 Rev (SEQ ID NO: 32)	TGTTTGCCGCTGTTAAACAGGCTCTGGCTGCTG GTGCAGCTCATGGTCACTTTTTCGCCCGCGGTC ACGG
17/9 VL-3 Fwd (SEQ ID NO: 33)	GTTTAACAGCGGCAAACAGAAAAACTATCTGA CCTGGTATCAGCAGAAACCGGGCCAGCCGCCG AAAGTG
17/9 VL-4 Rev (SEQ ID NO: 34)	CGGTAAAGCGATCCGGCACGCCGCTTTCGCGGG TGCTCGCCCAATAAATCAGCACTTTCGGCGGCT

Primers	Sequences
	GGCC
17/9 VL-5 Fwd (SEQ ID NO: 35)	TGCCGGATCGCTTTACCGGCAGCGGCAGCGGCA CCGATTTTACCCTGACCATTAGCAGCGTGCAGG CGGA
17/9 VL-6 Rev (SEQ ID NO: 36)	AAAGGTCAGCGGGTTGCTATAATCGTTCTGGCA ATAATACACCGCCAGATCTTCCGCCTGCACGCT GCTA
17/9 VL-7 Fwd (SEQ ID NO: 37)	AGCAACCCGCTGACCTTTGGCGGCGGCACCAAA CTGGAACTGAAACGTACGGTGGCTGCACCATCT GTCT
17/9 VL-8 Flag Rev (SEQ ID NO: 38)	TTAAATAGCGGATGCCTTGTCGTCGTCGTCCTT GTAGTCGATGAAGACAGATGGTGCAGCCACC

The 17/9 antibody sequence is retrieved from NCBI database using the accession numbers A31790 and B31790.

The DNA constructs for these scFv are transcribed *in vitro* and then translated in the rabbit reticulocyte lysate either as a mRNA-scFv (the protein is attached to the mRNA via a linker with puromycin modification) or as a free scFv (protein is not attached to mRNA). Both types of molecules are purified and subjected to pull-down assays by corresponding biotinylated antigens (see Figure 4).

The data in Figure 4 show that the functional mRNA-scFv (bound to biotinylated antigen) molecules are pulled down by streptavidin-magnetic beads, albeit at lower percent recovery than free scFv molecules. Further experiments show that this difference is simply due to the large RNA molecule tethered to the scFv. RNase degradation of the RNA portion from mRNA-scFv molecules restores the scFv recovery by antigens to the same level as that of free scFv molecules (see Figure 5).

EXAMPLE 3
MRNA-SCFV LIBRARY CONSTRUCTION

Human peripheral blood mononuclear cells (PBMC) from 18 donors are obtained from SeraCare. Table 11 below shows the PBMC analysis by the Fluorescence-

activated cell sorting (FACS) method. The poly A RNA is then extracted for the library construction.

Table 11: PBMC analysis by FACS

Lot	Total cell number per vial, x10^6	CD20+ cells			CD27+/CD20+ total
		CD27-	CD27+	Total	
#012505	22	2.8%	5.6%	8.4%	67%
#020805	21	12.1%	2.4%	14.5%	17%
#022205A	23	4.9%	3.7%	8.5%	43%
#030305A	14	7.6%	3.5%	11.1%	32%
#032905A	11	3.8%	2.8%	6.7%	42%
#041205A	23	4.9%	4.8%	9.7%	49%
#041405A	23	5.9%	3.2%	9.1%	35%
#041905A	18	5.1%	2.2%	7.3%	30%
#042604B	26	9.3%	2.5%	11.9%	21%
#042805B	24	11.4%	1.9%	13.3%	14%
#050305B	17	7.4%	3.1%	10.5%	30%
#050505B	20	6.2%	2.4%	8.6%	28%
#051005B	18	6.7%	3.1%	9.7%	32%
#051205B	17	7.6%	2.4%	10.1%	24%
#051705B	16	7.3%	2.1%	9.4%	23%
#051905A	27	4.8%	0.7%	5.5%	13%
#0524051	28	7.4%	2.2%	9.6%	23%
#122105	14	11.4%	1.4%	12.8%	11%

EXAMPLE 4
LIBRARY TAG SELECTION

Four 8-base pair tags (SEQ ID NOs: 39-42) are selected and inserted in between TMV-UTR and Kozak consensus sequences of the 17/9 mRNA-scFv construct (see Figure 6). The tag sequences are designed to not include adenosines and are identified

after three rounds of selection. As seen in Figure 6, the first position prefers G, and the second position prefers T. The random sequence tags are generated by designing 5' primers with eight random (B=G, C, T) nucleotide insertions between TMV and Kozak consensus sequences of 5'UTR (see Figure 7). The 17/9 scFv is then amplified and selected through 2-3 rounds of selection, where subsequent rounds are reamplified with 5' primers. Sequence outputs are then processed to identify tags that passed through the selection process. The different output tags from each round are shown in Figure 8. Some repeated sequences are seen inside each round, however no tag sequence is seen in multiple rounds. It should be noted that there is one possible mutation in the tag in round 2, as an ATG sequence should not be possible in a tag sequence.

EXAMPLE 5
LIBRARY SELECTION FOR 17/9 SCFV

To demonstrate that an mRNA-scFv molecule can be enriched by several rounds of selection using the mRNA display methods described here, an scFv library with a diversity of 25 is constructed by the overlapping PCR method. To create the scFv library, the VH and VL fragments of 17/9, D2E7, 2SD4, Y61 and MAK195 are used as described above. The 17/9 scFv is then selected from this library by biotinylated HA tag. After selection, 17/9 enrichment is examined by cloning and colony PCRs. The results quantifying 17/9 scFv before and after one round of mRNA-scFv selection are shown in Figure 9.

EXAMPLE 6
MRNA DISPLAY TECHNOLOGY IS USED TO DISCRIMINATE SCFV BINDERS WITH DIFFERENT AFFINITY

To determine whether mRNA display technology, *i.e.,* as described above, is used to discriminate scFv binders with different affinity, chimeras between D2E7 and 2SD4 are made. 2SD4 is the D2E7 scFv precursor that exhibits low affinity (KD ~ 200 nM as free protein) for TNFα. Figure 10 depicts the chimeras.

Titration is performed for free proteins. Figure 11 shows the percent of recovery after antigen binding between the different chimeras, as well as the normalized percent

of recovery after antigen selection. The above results show that mRNA display technology as described herein can be used to discriminate binders with different affinity.

EXAMPLE 7
THERMOSTABILITY OF MRNA-SCFV MOLECULES

To determine the thermostability of mRNA-scFv molecules, D2E7-scCk and Y61-scCk are translated and purified in the mRNA-scFv format, as described herein. The mRNA-scFv molecules are then incubated at different temperatures for 30 minutes prior to antigen selection. The normalized percent of recovery after antigen selection is shown in Figure 12.

Figure 13 shows that RNA can be recovered after the high temperature treatment of mRNA-scFv molecules. Here, RT-PCR is performed on the beads with recovered Y61-scCl mRNA-scFv molecules.

EXAMPLE 8
CONSTRUCTION OF A NAÏVE KAPPA PROFUSION SCFV LIBRARY FROM HUMAN PBMC RNA

The following example describes the generation of a human naïve kappa scFv library suitable for the selection using the PROfusion technology.

Human peripheral blood mononuclear cells (PBMCs) are purchased from SeraCare (Milford, MA, cat. # 72000). Cells from different donors are characterized by staining with anti-human CD20-FITC (BD Pharmingen, San Diego, CA, cat. # 556632) and anti-human CD27-PE (BD Pharmingen, cat. # 555441) antibodies. Total RNA isolated from PBMCs using RNeasy Midi Kit (QIAGEN, Valencia, CA, cat. # 75144), according to manufacturer's protocol. Briefly, frozen cells are quickly thawed at 37 °C, resuspended in a buffer containing guanidine isothiocyanate and homogenized by passing through a 21G needle for multiple times. Ethanol is added and lysate is applied to RNeasy midi columns (18 columns total). Columns are washed, and total RNA is eluted with RNase-free water. RNA concentration and yield are determined by measuring OD 260 nm absorbance. Then mRNA is isolated, according to the kit

manual, from the total RNA using Invitrogen Fastrack MAG Maxi mRNA Isolation kit (cat #K1580-02). RNase inhibitor (Invitrogen , Carlsbad, CA, cat #10777-019) is added during the procedure to minimize RNA degradation. Total RNA is first treated with DNase (Invitrogen, cat #18-68-015) to minimize genomic DNA contamination. Briefly, the oligo-dT-magnetic beads are first washed and then added to the total RNA, incubated at 65 °C for 10 minutes, and then allowed to bind at room temperature for 30 minutes. The beads are washed several times, and the bound mRNA is eluted by RNAse-free water. mRNA is quantified by measuring OD_{260} nm absorbance.

Reverse Transcription

First strand cDNA is then synthesized from 37 μg of mRNA by the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, cat. # 18064-014) and a mixture of 15 primers (100 nM total concentration) (Table 12), according to manufacturer's protocol. Reaction is done in two aliquots of 0.9 ml each. RT reaction (1.8 ml total volume) is purified by passing through 36 MicroSpin S-200 HR columns (Amersham Biosciences, Piscataway, NJ, cat. # 27-5120-01) (50 μl per column). Columns eluate is incubated with RNaseH for 20 minutes at 37 °C.

Table 12 Reverse Transcription primers

Name	Oligo sequence
FcγRev1 (SEQ ID NO: 43)	AGTTCCACGACACC
FcγRev2 (SEQ ID NO: 44)	GAAGGTGTGCACG
FcγRev3 (SEQ ID NO: 45)	CCACGCTGCTGAG
FcμRev1 (SEQ ID NO: 46)	ACTTTGCACACCAC
FcμRev2 (SEQ ID NO: 47)	TTTGTTGCCGTTGG
FcμRev3 (SEQ ID NO: 48)	GGGAATTCTCACAGG
FcδRev1 (SEQ ID NO: 49)	GCTGCTTGTCATGT
FcδRev2 (SEQ ID NO: 50)	TGCCTTTGGAGACT
FcδRev3 (SEQ ID NO: 51)	GACCACGCATTTGT
CκRev1 (SEQ ID NO: 52)	TCCACCTTCCACTG
CκRev2 (SEQ ID NO: 53)	CAGGCACACAACAG
CκRev3 (SEQ ID NO: 54)	GAGTGTCACAGAGC
CλRev1 (SEQ ID NO: 55)	GGGAACAGAGTGAC
CλRev2 (SEQ ID NO: 56)	GTGTGGCCTTGTTG
CλRev3 (SEQ ID NO: 57)	CCATCTGCCTTCCA

VH cDNA Amplification

One third of above RT reaction is subjected to limited amplification by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, cat. # 11304-102) in reactions containing a mixture of VH leader sequence (LS)-specific forward primers (200 nM total concentration) and a mixture of JH-specific reverse primers (200 nM total concentration) (Table 13). PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 10 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (60 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step. PCR products are then purified by QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, cat. # 28106), according to manufacturer's protocol. A small-scale pilot experiment confirms at least 100-fold amplification of cDNA.

Table 13 Primers used for VH fragments amplification

Name	Oligo sequence
VH1/7LS (SEQ ID NO: 58)	ATCCTCTTYTTGGTGGSAGC
VH1-46LS (SEQ ID NO: 59)	GGTCTTCTGCTTGCTGGCTG
VH2LS (SEQ ID NO: 60)	CCTGCTGCTGACCAYCCCTTC
VH3LS (SEQ ID NO: 61)	GCTATTTTWVRAGGTGTCCARTGT
VH4LS (SEQ ID NO: 62)	GCRGCTCCCAGATGGGTCCTG
VH5LS (SEQ ID NO: 63)	ATGGGGTCAACCGCCATCCT
VH6LS (SEQ ID NO: 64)	TGGGCCTCCCATGGGGTGTC
JH1/2sRev (SEQ ID NO: 65)	CTGAGGAGACRGTGACCAGGGTGC
JH4/5sRev (SEQ ID NO: 66)	CTGAGGAGACGGTGACCAGGGTTC
JH6sRev (SEQ ID NO: 67)	CTGAGGAGACGGTGACCGTGGTCC
JH3sRev (SEQ ID NO: 68)	CTGAAGAGACGGTGACCATTGTCC

1/100th of purified PCR product is used as a template for amplification of each VH family-specific cDNA. Amplification is carried out by PCR with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a reaction containing the individual VH LS-specific forward primer (200 nM) and a mixture of JH-specific reverse primers (200 nM total concentration) (Table 13). PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step.

The above PCR products are purified by QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) according to manufacturer's protocol. Each PCR product is subsequently amplified by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a PCR reaction containing corresponding VH-specific nested forward primer (200 nM) and a mixture of JH-specific reverse primers (200 nM total concentration) (Table 14). The forward primers in each reaction carry an 8-nucleotide "tag" (underlined), which is introduced to increase the specificity during subsequent library amplifications. PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step.

Table 14 Primers used for nested VH-specific PCR

Name	Oligo sequence
VH1Tag2Forward	TTTACAATTACAGTGTTGCGACCATGGAGGTGCAGCTGGT
(SEQ ID NO: 69)	GCAGTCTGG**RS**CT
VH2Tag2Forward	TTTACAATTACAGTGTTGCGACCATGGAGRTCACCTTGAR
(SEQ ID NO: 70)	GGAGTCTGGT
VH3Tag2Forward	TTTACAATTACAGTGTTGCGACCATGGAGGTGCAGCTG**K**T
(SEQ ID NO: 71)	GGAGTCT**S**G**R**GGA
VH4Tag2Forward	TTTACAATTACAGTGTTGCGACCATGGAGGTGCAGCTGCA
(SEQ ID NO: 72)	G**S**AGT**SS**GGC
VH5Tag2Forward	TTTACAATTACAGTGTTGCGACCATGGAGGTGCAGCTGGT
(SEQ ID NO: 73)	GCAGTCTGGAGCA
VH6Tag2Forward	TTTACAATTACAGTGTTGCGACCATGGAGGTACAGCTGCA
(SEQ ID NO: 74)	GCAGTCAG
VH7Tag2Forward	TTTACAATTACAGTGTTGCGACCATGGAGGTGCAGCTGGT
(SEQ ID NO: 75)	GCAATCTGGGT
JHReverse1/2	CGCTACCTCCGCCGCCAGACCCGCCTCCACCTGAGGAGAC
(SEQ ID NO: 76)	RGTGACCAGGGTGC
JHReverse4/5	CGCTACCTCCGCCGCCAGACCCGCCTCCACCTGAGGAGAC
(SEQ ID NO: 77)	GGTGACCAGGGTTC
JHReverse6	CGCTACCTCCGCCGCCAGACCCGCCTCCACCTGAGGAGAC
(SEQ ID NO: 78)	GGTGACCGTGGTCC
JHReverse3	CGCTACCTCCGCCGCCAGACCCGCCTCCACCTGAAGAGAC
(SEQ ID NO: 79)	GGTGACCATTGTCC

PCR products are subjected to 1% agarose gel electrophoresis, and purified by QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, cat. # 28704). Aliquots of these PCR products are further amplified on large scale in a PCR reaction by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and 200 nM of the following universal primers:

T7TMVTag2 (SEQ ID NO: 80):

TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAGTGTTGCGAC

Library-GS-Reverse (SEQ ID NO: 16): CGCTACCTCCGCCGCCAGAC.

These primers add a T7 promoter and a TMV-UTR sequence to the 5' end of PCR products, and a partial glycine-serine (G4S)-linker to the 3' end of PCR products. PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 26 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step.

PCR products are purified with QIAquick PCR Purification Kit (QIAGEN, Valencia, CA), quantified by UV absorbance at 260 nm, and aliquots of these products are visualized by 1% agarose gel-electrophoresis to confirm purity. Aliquots of VH family-specific cDNA fragments are cloned using the TOPO TA Cloning Kit (Invitrogen, cat. # 45-0641), and individual clones are analyzed by sequencing.

Vκ cDNA Amplification

One third of cDNA generated from the aforementioned RT reaction is subjected to limited amplification by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in reaction containing a mix of Vκ leader sequence (LS)-specific forward primers (total concentration 200 nM) and Cκ-specific reverse primer (200 nM) (Table 15). PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 10 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (60 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step. A separate small-scale experiment confirms at 10 to 100-fold amplification of cDNA by such PCR.

PCR products are purified with QIAquick PCR Purification Kit (QIAGEN, Valencia, CA,) according to manufacturer's protocol. $1/500^{th}$ of purified PCR product is used as a template for amplification of each Vκ family-specific cDNA. Amplification is done by PCR with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a reaction containing individual Vκ LS-specific forward primers (200 nM) and Cκ-specific reverse primers (200 nM) (Table 16). PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step.

Table 15 Primers used for Vκ fragments amplification

Name	Oligo sequence
Vκ1LS (SEQ ID NO: 81)	GCTCCTGGGRCTYCTGC
Vκ2LS (SEQ ID NO: 82)	CTYCTGGGGCTGCTAATG
Vκ3LS (SEQ ID NO: 83)	CTCTGGCTCMCAGATACCAC
Vκ4LS (SEQ ID NO: 84)	GGATCTCTGGTGCCTACGG
Vκ5LS (SEQ ID NO: 85)	GGATCTCTGATACCAGGGCA
Vκ6LS (SEQ ID NO: 86)	CTGGGTTCCAGCCTCCAG
Cκ-sReverse (SEQ ID NO: 87)	GAAGACAGATGGTGCAGCCACAGTTCG

Table 16 Primers used for nested Vκ-specific PCR

Name	Oligo sequence
Vκ1 Forward	GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGACA
(SEQ ID NO:88)	TCC**R**G**W**TGACCCAGTCTCC**W**T
Vκ2 Forward	GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGATA
(SEQ ID NO:89)	TTGTGATGAC**Y**CAG**W**CTCCAC
Vκ3 Forward	CTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGAAATT
(SEQ ID NO:90)	GTG**W**TGAC**R**CAGTCTCCAG**S**CA
Vκ4/6 Forward	GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGACA
(SEQ ID NO:91)	TCGTG**M**TGAC**Y**CAGTCTCCAGA
Vκ5 For-Redo	CTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGAAACG
(SEQ ID NO:92)	ACACTCACGCAGTCTCCAGCAT
Vκ6 For-NEW	CTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGATGTC
(SEQ ID NO: 93)	GTGATGACACAGTCTCCAGCTT
Cκ Reverse	GTCGTCGTCGTCCTTGTAGTCGAAGACAGATGGTGCAGC
(SEQ ID NO:14)	CACAGTTCG

PCR products are purified with QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's protocol. Each PCR product is subsequently amplified by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a PCR reaction containing corresponding Vκ-specific nested forward primers (200 nM) and Cκ-specific reverse primers (200 nM) (Table 13). PCR conditions are as follows: an

initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step.

PCR products are subjected to 1% agarose gel-electrophoresis, and purified by QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). Aliquots of these PCR products are further amplified on large scale in a PCR reaction containing Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and the following universal primers (200 nM):

Library-GS-Forward (SEQ ID NO: 94): GTCTGGCGGCGGAGGTAGCG

FlagA20Rev (SEQ ID NO: 95):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA GTC.

These primers add a partial G4S-Linker to the 5' end of PCR product and a FLAG tag, linker annealing site and poly A tail to the 3' end of the resulting PCR products.

PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 26 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (30 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step. PCR products are purified with QIAquick PCR Purification Kit (QIAGEN, Valencia, CA), quantified by UV absorbance at 260 nm, and aliquots of these products are visualized by 1% agarose gel-electrophoresis to confirm purity. Part of the obtained Vκ family-specific cDNA fragments are cloned using TOPO TA Cloning Kit (Invitrogen, cat. # 45-0641), and individual clones are analyzed by sequencing.

VH-Vκ scFv Construction

VH and Vκ cDNA fragments are mixed according to number of germlines in each family (Table 17 and 18).

Table 17 Mixing ratio of VH fragments

VH fragment	VH1/7	VH1-46	VH2	VH3	VH4	VH5	VH6	Total
# of germlines	10	1	3	22	7	1	1	45
% of Total	22.2%	2.7%	6.7%	48.9%	15.5%	2.7%	2.7%	100%

Table 18 Mixing ration for Vκ fragments

Vκ fragment	Vκ1	Vκ2	Vκ3	Vκ4	Vκ5	Vκ6	Total
# of germlines	21	11	8	1	1	3	45
% of Total	46.7%	2.4%	17.8%	2.7%	2.7%	8.3%	100%

Total of 10 μg of VH cDNA fragments ($2x10^{13}$ molecules) and total of 10 μg of Vκ cDNA fragments ($2x10^{13}$ molecules) is used as template for overlapping PCR. PCR is done with Platinum Taq DNA Polymerase High Fidelity and primers T7TMVTag2 (200 nM) and FlagA20Rev (200 nM), in a volume of 30 ml. PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 17 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (60 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step. Aliquot of PCR product is cloned using TOPO TA Cloning Kit (Invitrogen, cat. # 45-0641), and individual clones are analyzed by sequencing.

Spectratyping

A fluorescent dye-labeled 5' forward primer (6-FAM-PanVHFR3-Fwd, 5'-GACACGGCCGTGTATTACTGT-3', SEQ ID NO: 17) and a reverse primer (PanJH-Rev, 5'-GCTGAGGAGACGGTGACC-3', SEQ ID NO: 18) that respectively anneal to the VH's framework 3 region and to the J region are used to amplify across the CDR3 regions of VH domains by PCR. Fifty ng of scFv library DNA template is used in a reaction volume of 30 μl containing 200 nM 6-FAM-PanVHFR3-Fwd primer, 200 nM PanJH-Rev primer, 200 μM dNTP, 1 x GoTaq buffer, and 1.5 U of GoTaq (Promega, Madison, WI). PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 30 cycles of 94° C (20 seconds), 55 °C (20 seconds), and 72 °C (30 seconds); followed by 5 minutes of 72 °C extension and 4 °C storage step. After PCR, 10 μl of products is loaded onto a 2% agarose gel to confirm successful reactions and the remaining products are subjected to spectratyping electrophoresis using an ABI sequencer (Applied Biosystems, Foster City, CA). The CDR3 lengths are calculated by subtracting 60 bp flanking framework sequences from the product lengths that are determined by ROX-dye labeled DNA markers.

Results

Human PBMC Characterization

Prescreening PBMC samples from 20 donors for B ($CD20^+$) and memory B ($CD27^+$) cells by flow cytometry results in the selection of 10 donors (> 9 % B in PMBC and < 35% memory B cells in total B cells) and allows for the optimization of donor age, gender, and ethnicity distribution to the best extent of donor availability (Figure 14).

RNA Purification

To obtain antibody cDNA repertoire for library construction, 1.9 mg of total RNA is obtained from 2.3×10^9 human PBMCs with an estimated 2.6×10^8 B cells. A subsequent poly A mRNA purification from the total RNA yields 42.2 μg of mRNA.

Amplification of VH cDNA Fragments

Aliquots of VH family-specific cDNA fragments of ~ 500 bp amplified by the VH universal primers (T7TMVTag2, SEQ ID NO: 80 and library-GS-Rev, SEQ ID NO: 16) are visualized by agarose gel-electrophoresis (Figure 15).

Sequencing analysis of individual VH clones is presented in Table 19. Comparison of cloned VH sequences to known VH germline sequences confirms highly specific amplifications by the VH family-specific primers.

Amplification of Vκ Fragments

Aliquots of Vκ family-specific cDNA fragments, amplified with universal primers (Library-GS-Fwd, SEQ ID NO: 94 and FlagA20Rev, SEQ ID NO: 95), are visualized by agarose gel-electrophoresis (Figure 16). Sequencing analysis of individual Vκ clones is presented in Table 20. The data confirms the primer specificity for each of the Vκ germline family.

Table 19 Sequencing analysis of VH family-specific PCR products

Fragment	*# of clones*	*Germline match*	*Family*	*Fragment*	*# of clones*	*Germline match*	*Family*
VH1/7	3	VH7-4.1	VH7	VH4	3	VH4-59	VH4
	1	VH7-4.1/VH7-81	VH7		2	VH4-39	VH4
	7	VH1-8	VH1		8	VH4-34	VH4
	4	VH1-69	VH1		2	VH4-31	VH4
	1	VH1-24	VH1		1	VH4-61	VH4
VH3	3	VH3-23	VH3	VH1-46	15	VH1-46	VH1
	1	VH3-21	VH3				
	1	VH3-48	VH3	VH2	12	VH2-5	VH2
	2	VH3-9	VH3		3	VH2-26	VH2
	1	VH3-43/VH3-9	VH3				
	2	VH3-7	VH3	VH5	16	VH5-51	VH5
	2	VH3-74	VH3				
	1	VH3-30/VH3-33	VH3	VH6	16	VH6-1	VH6

VH-Vκ scFv Construction

Overlapping PCR is carried out to construct VH-Vκ scFv cDNA fragments. Parts of the obtained products are visualized by agarose gel electrophoresis (Figure 17). The generated VH-Vκ scFv fragment has all the necessary elements to be selected by the PROfusion mRNA display technology (Figure 17).Sequencing analysis of individual VH- Vκ scFv clones confirms correct VH-Vκ recombination with a functional intervening G4S linker in a great majority. The distribution of various VH and Vκ families in the constructed scFv library is consistent with previous literature reports (Figure 18, also see Tsuiji *et al.* (2006). Exp. Med.; V.203 (2), pp. 393–400 and Arons *et al.* (2006) British Journal of Haematology V.133, pp. 504-512).

Table 20 Sequencing analysis of Vκ family-specific PCR products

Fragment	*# of clones*	*Germline match*	*Family*	*Fragment*	*# of clones*	*Germline match*	*Family*
Vκ1	3	O18/O8	Vκ1	Vκ3	5	A11/A27	Vκ3
	3	O12/O2	Vκ1		4	L20/L6	Vκ3
	2	A20	Vκ1		2	L16/L2	Vκ3
	2	L19/L5	Vκ1				
	1	L19	Vκ1	Vκ4	14	B3	Vκ4
	1	L1/L15	Vκ1				
	1	L1	Vκ1	Vκ5	13	B2	Vκ5
Vκ2	7	A19/A3	Vκ2	Vκ6	12	A10/A26	Vκ6
	3	A1/A17	Vκ2		1	A14	Vκ6
	1	O1/O11	Vκ2				

Spectratyping

Spectratyping analysis of CDR3 size distribution among different VH families is carried out on VH cDNA fragments just prior to their assembly into scFv library (Figure 19). As would be expected in a cDNA library with a high degree of diversity, the observed CDR3 sizes varies greatly in all families analyzed. The observed peak heights of the different CDR3 sizes assumes a bell shaped curve typical of a normal distribution and is an indication of a very large population size. This is especially evident in the results obtained from a single germline or from very small VH families such as VH1-46, VH2, VH5, and VH6. It is also interesting to note the slight difference of CDR3 sizes in different VH families. For instance, VH1 and VH2 tend to have more CDR3s of 15-16 residues, whereas VH3 have more CDR3s of 13 residues. Taken together, the spectratyping analysis of individual VH families confirms a highly diverse and very large VH sequences in the cDNA library.

Eight scFv library templates are randomly sampled and subjected to spectratyping analysis as previously described. The results obtained from all eight samples are indistinguishable, suggesting the library templates in each aliquot are reproducibly similar (Figure 20). The library VH CDR3 sizes have a normal distribution and the majority fall in between 6 to 24 residues and center in between 13 to 16 residues.

This distribution is as expected and consistent with the individual VH family spectratyping results.

Conclusion

The selection of high quality human antibody leads is a prerequisite for successful therapeutic antibody drug development. In additional to a robust selection technology, the antibody library quality (source, diversity, and construction) greatly determines its usefulness to produce good leads. Many human donors were prescreened for greater diversity and PCR primers designed with extremely high specificity to cover all antibody germline sequences such that all diversity within the donor collection can be captured. The constructed antibody scFv library has a theoretical diversity greater than 2×10^{12} from more than 2×10^{8} B cells, which has been substantiated by sequencing multiple clones and by spectratyping.

EXAMPLE 9
CONSTRUCTION OF A NAÏVE LAMBDA PROFUSION SCFV LIBRARY FROM HUMAN PBMC RNA

Total RNA and mRNA purification, mRNA reverse transcription, and VH cDNA amplification by PCR have been described in Example 1.

Vλ cDNA Amplification

One third of cDNA generated from the aforementioned RT reaction is subjected to limited amplification by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a reaction containing a mix of Vλ leader sequence (LS)-specific forward primers (total concentration 200 nM) and Cλ-specific reverse primer (200 nM) (Table 21). The PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 10 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (60 seconds); followed by 5 minutes of 68 °C extension and 4 °C storage step. A separate small-scale experiment confirms a 10 to 100-fold amplification of cDNA by such PCR.

Table 21 Primers used for Vλ fragments amplification

Name	Oligo sequence
VL1LS1 (SEQ ID NO: 96)	TCACTGTGCAGGGTCCTG
VL1LS3 (SEQ ID NO: 97)	TCACTGCACAGGGTCCTG
VL2LS3 (SEQ ID NO: 98)	TCAGGRCACAGGGTCCTG
VL2LS4 (SEQ ID NO: 99)	TCAGGGCACAGGATCCTG
VL3LS2 (SEQ ID NO: 100)	TGCATAGGTTCTGTGGTTTCTTCTG
VL3LS3 (SEQ ID NO: 101)	ACAGGHTCTGWGGCCTCCTATG
VL3LS4 (SEQ ID NO: 102)	TGCACAGGCTCTGTGACCTCCTATG
VL3LS5 (SEQ ID NO: 103)	TACACAGGCTCTATTGCCTCCTATG
VL4ABLS2 (SEQ ID NO: 104)	TCCACTGSACAGGGTCTCTCT
VL4CLS2 (SEQ ID NO: 105)	CTTCATTTTCTCCACAGGTCTCTGTG
VL5LS (SEQ ID NO: 106)	CACTGCACAGGTTCCCTC
VL6LS (SEQ ID NO: 107)	CTGCACAGGTTCTTGGGC
VL7LS (SEQ ID NO: 108)	CTCACTTGCTGCCCAGGG
VL8LS (SEQ ID NO: 109)	GCTTATGGATCAGGAGTGGATTC
VL9LS (SEQ ID NO: 110)	CACCCTCCTCAGTCTCCTC
VL10LS (SEQ ID NO: 111)	CTCTGCAGTGTCAGTGGTC
CJLS Reverse (SEQ ID NO: 112)	GCCTTGGGCTGACCKAGGACGGT

The PCR product is purified using QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's protocol. 1/250th of the purified PCR product is used as a template for amplification of each Vλ family-specific cDNA. Amplification is done by PCR with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a reaction containing individual Vλ LS-specific forward primer (200nM) and Cλ-specific reverse primer (200 nM) (Table 22). The PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 35 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (60 seconds); followed by 5 minutes of 68 °C extension and 4 °C storage step.

Table 22 Primers used for nested Vλ-specific PCR

Name	Oligo sequence
VL1/10 ForRedo (SEQ ID NO: 113)	GTCTGGCGGCGGAGGTAGCGGCGGTGGCGATCGCAGTC TGKGCTGACKCAGCCRC
VL2 For (SEQ ID NO: 114)	GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGCAGT CTGCCCTGACTCAGCCT
VL3 ForNew (SEQ ID NO: 115)	GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGTCCT ATGAGCTGACDCAG
VL4ab For (SEQ ID NO: 116)	GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGCAGC YTGTGCTGACTCAATC
VL4c For (SEQ ID NO: 117)	GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGCTGC CTGTGCTGACTCAGCCCCCG
VL5/9 ForRedo (SEQ ID NO: 118)	GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGCAGC CTGTGCTGACTCAGCCRBT
VL6 For (SEQ ID NO: 119)	GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGAATT TTATGCTGACTCAGCCC
VL7/8 For (SEQ ID NO: 120)	GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGCAGR CTGTGGTGACYAGGAG
CJL Reverse (SEQ ID NO: 15)	GTCGTCGTCGTCCTTGTAGTCAGTGACAGTGGGGTTGGC CTTGGGCTGACCKAGGACGGT

The PCR products are run on a 2% agarose gel, and purified using QuantumPrep FreezeNSqueeze Columns (Biorad, Hercules, CA) according to the manufacturer's protocol. Each PCR product is subsequently amplified by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a PCR reaction containing corresponding Vλ-specific nested forward primer (200 nM) and Cλ-specific reverse primers (200 nM) (Table 13). The PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 35 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (60 seconds); followed by 5 minutes of 68 °C extension and 4 °C storage step.

PCR products are run on a 2% agarose gel, and purified using QuantumPrep FreezeNSqueeze Columns (Biorad, Hercules, CA). Aliquots of these PCR products are further amplified on a large scale in a PCR reaction containing Platinum Taq DNA

Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and the following universal primers (200 nM):

Library-GS-Forward (SEQ ID NO: 94): GTCTGGCGGCGGAGGTAGCG
FlagA20Rev (SEQ ID NO: 95):
TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCG
TCCTTGTAGTC.

These primers add a partial G4S-Linker to the 5' end of PCR product and a FLAG tag, linker annealing site and poly A tail to the 3' end of the resulting PCR products. The PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 35 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (30 seconds); followed by 5 minutes of 68 °C extension and 4 °C storage step. The PCR products are purified using Purelink PCR Purification Kit (Invitrogen, Carlsbad, CA), quantified by UV absorbance at 260 nm, and aliquots of these products are visualized by 2% agarose gel-electrophoresis to confirm purity.

Part of the obtained Vλ family-specific cDNA fragments are cloned using TOPO TA Cloning Kit (Invitrogen, cat. # 45-0641), and individual clones are analyzed by sequencing.

VH-Vλ scFv Construction

VH and Vλ cDNA fragments are mixed according to the diversity of germlines represented by each PCR product (Tables 23 and 24).

Table 23 Mixing ratio of VH fragments

VH Family	VH1/7	VH1-46	VH2	VH3	VH4	VH5	VH6	Total
% of Total	22.2%	2.7%	6.7%	48.9%	15.5%	2.7%	2.7%	100%

Table 24 Mixing ratio for Vλ fragments

VL Family	VL1	VL2	VL3	VL4	VL5	VL6	VL7	VL8	VL9	VL10	Total
% of Total	18.5%	3.70%	33.30%	11.10%	11.10%	3.70%	7.40%	3.70%	3.70%	3.70%	100%

A total of 10 μg of VH cDNA fragments ($2x10^{13}$ molecules) and 10 μg of Vλ cDNA fragments ($2x10^{13}$ molecules) is used as the template for overlapping PCR. PCR is done with Platinum Taq DNA Polymerase High Fidelity and primers T7TMVTag2 (200 nM) and FlagA20Rev (200 nM), in a volume of 30 ml. The PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 17 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (60 seconds); followed by 5 minutes of 68 °C extension and 4 °C storage step. An aliquot of the PCR product is cloned using TOPO TA Cloning Kit (Invitrogen, cat. # 45-0641), and individual clones are analyzed by sequencing.

Results

Amplification of Vλ Fragments

Aliquots of Vλ family-specific cDNA fragments, amplified with universal primers (Library-GS-Fwd and FlagA20Rev), are visualized by agarose gel-electrophoresis (Figure 21).

Sequencing analysis of individual Vλ clones is presented in Table 25. The data confirmthe primer specificity for each of the Vλ germline family, with one mismatch highlighted in bold (VL1 LS3, Germine V1-3).

Table 25 Sequencing analysis of Vλ family-specific PCR products

VL Fragment	Germline	# of Clones		VL Fragment	Germline	# of Clones
VL1 LS1	V1-16	10		VL3 LS5	V2-1	5
	V1-17	2			V2-6	3
					V2-14	1
VL1 LS3	**V1-3**	**1**			V2-17	3
	V1-9	8				
	V1-13	1		VL4ab LS2	V5-4	3
					V5-6	9
VL2 LS3	V1-3	11				
				VL3c LS2	V5-1	12
VL2 LS4	V1-3	11				
				VL5 LS	V4-1	5
VL3 LS2	V2-13	11			V4-2	7
VL3 LS3	V2-1	9		VL6 LS	V1-22	10
	V2-14	1				
	V2-17	1		VL7 LS	V3-2	10
VL3 LS4	V2-1	7		VL8 LS	V3-4	12
	V2-6	1				
	V2-14	2		VL9 LS	V5-2	12
	V2-17	1				
	V2-19	1		VL10 LS	V1-20	10

VH-Vλ scFv Construction

Overlapping PCR is carried out to construct VH-Vλ scFv cDNA fragments. Parts of the obtained products are visualized by agarose gel electrophoresis (Figure 22). The generated VH-Vλ scFv fragment has all the necessary elements to be selected by the PROfusion mRNA display technology (Figure 23).

Sequencing analysis of individual VH-Vλ scFv clones confirms correct VH-Vλ recombination with a functional intervening G4S linker in a great majority.

EXAMPLE 10
CONSTRUCTION OF A NAÏVE KAPPA AND LAMBDA PROFUSION SCFV LIBRARIES FROM HUMAN LYMPH NODE MRNA

This Example describes the generation of PROfusion human naïve scFv libraries (PBMC kappa and PBMC lambda) from lymph node mRNA.

10.1 Reverse Transcription

First strand cDNA is synthesized from 40 μg of mRNA by SuperScript II Reverse Transcriptase (Invitrogen, cat. # 18064-014) and a mixture of 15 primers (100 nM total concentration) (Table 12) according to manufacturer's protocol. Reaction is done in 16 aliquots of 0.1 ml each. RT reactions are pooled (1.6 ml total volume), incubated with 20 μl of RNaseH for 20 minutes at 37°C and then dialyzed against water.

10.2 PCR

10.2.1 VH cDNA Amplification

One third of above RT reaction is subjected to limited amplification by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, cat. # 11304-102) in reactions containing a mixture of VH leader sequence (LS)-specific forward primers (200 nM total concentration) and a mixture of JH-specific reverse primers (200 nM total concentration) (Table 13). PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 10 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (60 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step. PCR product is then purified by QIAquick PCR Purification Kit (QIAGEN, cat. # 28106), according to manufacturer's protocol. A small-scale pilot experiment confirms at least 10-fold amplification of cDNA.

Half of purified PCR product is divided into 7 equal aliquots, and each aliquot is used as a template for amplification of one of VH family-specific cDNA. Amplification is carried out by PCR with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a reaction containing individual VH LS-specific forward primer (200 nM) and a mixture of JH-specific reverse primers (200 nM total concentration) (Table 13). PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step.

The above PCR products are purified by QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's protocol. Each PCR product is subsequently amplified by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a PCR reaction containing corresponding VH-specific nested forward primer (200 nM) and a mixture of JH-specific reverse primers (200 nM total concentration) (Table 26). The forward primers in each reaction carry an 8-nucleotide "tag" (underlined), which is introduced to increase the specificity during subsequent library amplifications.

PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step.

PCR products are subjected to 1% agarose gel electrophoresis, and purified by QIAquick Gel Extraction Kit (QIAGEN, cat. # 28704). Aliquots of these PCR products are further amplified on large scale in a PCR reaction by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and 200 nM of the universal primers (T7TMVTag4s and Lib-GSv2-Rev). These primers add a T7 promoter and a TMV-UTR sequence to 5' end of PCR product, and partial glycine-serine (G4S)-linker to the 3' end of PCR product. PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step.
PCR products are purified with QIAquick PCR Purification Kit (QIAGEN), quantified by UV absorbance at 260 nm, and aliquots of these products are visualized by 1% agarose gel-electrophoresis to confirm purity. Aliquots of VH family-specific cDNA fragments are cloned using the TOPO TA Cloning Kit (Invitrogen, cat. # 45-0641), and individual clones are analyzed by sequencing.

Table 26 Primers used for nested VH-specific PCR

Name	Oligo sequence
VH1Tag4 Forward (SEQ ID NO: 121)	TTTACAATTACAGCTTCTTCACCATGGAGGTGCAGCTGG TGCAGTCTGGRSCT
VH2Tag4 Forward (SEQ ID NO: 122)	TTTACAATTACAGCTTCTTCACCATGGAGRTCACCTTGAR GGAGTCTGGT
VH3Tag4 Forward (SEQ ID NO: 123)	TTTACAATTACAGCTTCTTCACCATGGAGGTGCAGCTGK TGGAGTCTSGRGGA
VH4Tag4 Forward (SEQ ID NO: 124)	TTTACAATTACAGCTTCTTCACCATGGAGGTGCAGCTGC AGSAGTSSGGC
VH5Tag4 Forward (SEQ ID NO: 125)	TTTACAATTACAGCTTCTTCACCATGGAGGTGCAGCTGG TGCAGTCTGGAGCA
VH6Tag4 Forward (SEQ ID NO: 126)	TTTACAATTACAGCTTCTTCACCATGGAGGTACAGCTGC AGCAGTCAG
VH7Tag4 Forward (SEQ ID NO: 127)	TTTACAATTACAGCTTCTTCACCATGGAGGTGCAGCTGG TGCAATCTGGGT
JH1/2 RevV2 (SEQ ID NO: 128)	CAGACCCTCCACCGCCGCTGCCGCCTCCACCTGAGGAGA CRGTGACCAGGGTGC
JH3 RevV2 (SEQ ID NO: 129)	CAGACCCTCCACCGCCGCTGCCGCCTCCACCTGAAGAGA CGGTGACCATTGTCC
JH4/5 RevV2 (SEQ ID NO: 130)	CAGACCCTCCACCGCCGCTGCCGCCTCCACCTGAGGAGA CGGTGACCAGGGTTC
JH6 RevV2 (SEQ ID NO: 131)	CAGACCCTCCACCGCCGCTGCCGCCTCCACCTGAGGAGA CGGTGACCGTGGTCC
T7TMVTag4s (SEQ ID NO: 132)	TAATACGACTCACTATAGGGACAATTACTATTTACAATT ACAGCTTCTTC
Lib-GSv2-Rev (SEQ ID NO: 133)	CAGACCCTCCACCGCCGCTG

10.2.2 Vκ cDNA Amplification

One third of cDNA generated from the aforementioned RT reaction is subjected to limited amplification by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in reaction containing a mix of Vκ leader sequence (LS)-specific forward

primers (total concentration 200 nM) and Cκ-specific reverse primer (200 nM) (Table 15). PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 10 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (60 seconds); followed by 3 minutes of 68 °C extension and 4°C storage step. A separate small-scale experiment confirms up to 10-fold amplification of cDNA by such PCR.

PCR product is purified with QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's protocol. Half of purified PCR product is divided into 6 equal aliquots, and each aliquot is used as a template for amplification of individual Vκ family-specific cDNA. Amplification is done by PCR with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a reaction containing individual Vκ LS-specific forward primer (200 nM) and Cκ-specific reverse primer (200 nM) (Table 15). PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step.

PCR products are subjected to 1% agarose gel-electrophoresis and purified with QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's protocol. Each PCR product is subsequently amplified by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a PCR reaction containing corresponding Vκ-specific nested forward primer (200 nM) and Cκ-specific reverse primers (200 nM) (Table 27). PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4°C storage step.

Aliquots of these PCR products are further amplified on large scale in a PCR reaction containing Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and the 200 nM universal primers (Lib-GSv2-Fwd and CKReverse).

Primer Lib-GSv2-Fwd adds a partial G4S-linker upstream of Vκ. The sequences of universal primers encoding for G4S linker (Lib-GSv2-Fwd and Lib-GSv2-Rev) are modified from that of primers used for human PBMC libraries construction (Library-GS-Forward and Library-GS-Reverse). This modification is done to avoid the cross-priming problem of primer Library-GS-Reverse annealing to Framework 1 of VH3 family germlines, which creates truncated VH sequences.

Table 27 Primers used for nested Vκ-specific PCR

Name	Oligo sequence
Vκ1FwdV2 (SEQ ID NO:134)	CAGCGGCGGTGGAGGGTCTGGCGGTGGCGGAAGTGACA TCC**R**G**W**TGACCCAGTCTCC**W**T
Vκ2FwdV2 (SEQ ID NO:135)	CAGCGGCGGTGGAGGGTCTGGCGGTGGCGGAAGTGATA TTGTGATGAC**Y**CAG**W**CTCCAC
Vκ3FwdV2 (SEQ ID NO:136)	CAGCGGCGGTGGAGGGTCTGGCGGTGGCGGAAGTGAAA TTGTG**W**TGAC**R**CAGTCTCCAG**S**CA
Vκ4/6FwdV2 (SEQ ID NO:137)	CAGCGGCGGTGGAGGGTCTGGCGGTGGCGGAAGTGACA TCGTG**M**TGAC**Y**CAGTCTCCAGA
Vκ5FwdV2 (SEQ ID NO:138)	CAGCGGCGGTGGAGGGTCTGGCGGTGGCGGAAGTGAAA CGACACTCACGCAGTCTCCAGCAT
Vκ6FwdV2 (SEQ ID NO:139)	CAGCGGCGGTGGAGGGTCTGGCGGTGGCGGAAGTGATG TCGTGATGACACAGTCTCCAGCTT
CκReverse (SEQ ID NO:14)	GTCGTCGTCGTCCTTGTAGTCGAAGACAGATGGTGCAGC CACAGTTCG
Lib-GSv2-Fwd (SEQ ID NO:140)	CAGCGGCGGTGGAGGGTCTG

PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step. PCR products are purified with QIAquick PCR Purification Kit (QIAGEN), quantified by UV absorbance at 260 nm, and aliquots of these products are visualized by 1% agarose gel-electrophoresis to confirm purity. Aliquots of the obtained Vκ family-specific cDNA fragments are cloned using TOPO TA Cloning Kit (Invitrogen, cat. # 45-0641), and individual clones are analyzed by sequencing.

10.2.3 Vλ cDNA Amplification

One third of cDNA generated from the aforementioned RT reaction is subjected to limited amplification by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a reaction containing a mix of Vλ leader sequence (LS)-specific forward primers (total concentration 200 nM) and Cλ-specific reverse primer (200 nM)

(Table 21). The PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 10 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4°C storage step. A separate small-scale experiment confirms up to 10-fold amplification of cDNA by such PCR.

The PCR product is purified using QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's protocol. Half of the purified PCR product is divided into 16 equal aliquots and each aliquot is used as a template for amplification of individual Vλ family-specific cDNA. Amplification is done by PCR with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a reaction containing individual Vλ LS-specific forward primer (200 nM) and Cλ-specific reverse primer (200 nM) (Table 21). The PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 30 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4 °C storage step.

PCR products are purified with QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's protocol. The following fragments are pooled before subsequent amplification: VL-1 LS-1 and VL1 LS-3 fragments are pooled in 3:2 ratio into VL1 LS mix fragments; VL-2 LS-3 and VL2 LS-4 fragment are pooled in 3:2 ration into VL2 LS mix fragments; VL3 LS-2, VL3 LS-3, VL3 LS-4 and VL3 LS-5 fragments are pooled into 1:6:1:1 ratio into VL3 LS mix fragments. PCR product corresponding to individual families are subsequently amplified by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a PCR reaction containing corresponding Vλ-specific nested forward primer (200 nM) and Cλ-specific reverse primers (200 nM) (Table 22). The PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (40 seconds); followed by 3 minutes of 68 °C extension and 4°C storage step.

PCR products were subjected to 1% agarose gel-electrophoresis, and purified by QIAquick Gel Extraction Kit (QIAGEN). Aliquots of these PCR products were further amplified on a large scale in a PCR reaction containing Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and the 200 nM universal primers (Lib-GSv2-Fwd and CJLReverse). The PCR conditions were as follows: an initial 2 minutes of 94 °C denaturation; 25 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 68 °C (30 seconds); followed by 5 minutes of 68 °C extension and 4°C storage step.

PCR products are purified with QIAquick PCR Purification Kit (QIAGEN), quantified by UV absorbance at 260 nm, and aliquots of these products are visualized by 1% agarose gel-electrophoresis to confirm purity. The specificity of VL family-specific primers is confirmed previously.

10.2.4 VH-Vκ scFv Construction

VH and Vκ cDNA fragments are mixed according to number of germlines in each family (based on NCBI) (Tables 17 and 18)

Total of 10 μg of VH cDNA fragments (2×10^{13} molecules) and total of 10 μg of Vκ cDNA fragments (2×10^{13} molecules) is used as template for overlapping PCR. PCR is done with Platinum Taq DNA Polymerase High Fidelity and primers T7TMVUTR (200 nM) and Cκ5-FlagA20 Rev (200 nM), in a volume of 30 ml, with the following steps: an initial 2 minutes of 94 °C denaturation; 12 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (60 seconds); followed by 3 minutes of 68 °C extension and 4°C storage step. Aliquot of PCR product is cloned using TOPO TA Cloning Kit (Invitrogen, cat. # 45-0641), and individual clones are analyzed by sequencing.

T7TMVUTR (SEQ ID NO: 1):
TAATACGACTCACTATAGGGACAATTACTATTTACAATTACA
Ck5-FlagA20 Rev (SEQ ID NO: 7):
TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCGAAGACAGATGGTGCAGCCACA.

Primer Ck5-FlagA20 Rev adds polyA tail at the 3'end of PCR product. Thus the sequence is used for Oligo-dT purification of PROfusion molecules.

10.2.5 VH-Vλ scFv Construction

VH and Vλ cDNA fragments are mixed according to the diversity of germlines represented by each PCR product (Tables 18 and 28). A total of 10 μg of VH cDNA fragments (2×10^{13} molecules) and 10 μg of Vλ cDNA fragments (2×10^{13} molecules) is used as the template for overlapping PCR. PCR is done with Platinum Taq DNA Polymerase High Fidelity and primers T7TMVUTR (200 nM) and CL5 FlagA20 (200 nM), in a volume of 30 ml. The PCR conditions are as follows: an initial 2 minutes of 94

°C denaturation; 10 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 68 °C (60 seconds); followed by 5 minutes of 68 °C extension and 4°C storage step. Aliquot of the PCR product is cloned using TOPO TA Cloning Kit (Invitrogen, cat. # 45-0641), and individual clones are analyzed by sequencing.

Table 28 Mixing ratio for Vλ fragments

VL fragment	VL1	VL2	VL3	VL4	VL5	VL6	VL7	VL8	VL9	VL10	Total
# of germlines	5	5	9	3	3	1	2	1	1	1	31
% of Total	16.1%	16.1%	29.0%	9.7%	9.7%	3.2%	6.4%	3.2%	3.2%	3.2%	100%

CL5 FlagA20 (SEQ ID NO: 12):
TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA GTCAGTGACAGTGGGGTTGGCCTTG

Primer CL5-FlagA20 adds polyA tail at the 3'end of PCR product. Thus the sequence is used for Oligo-dT purification of PROfusion molecules.

10.3 Spectratyping

A fluorescent dye-labeled 5' forward primer (6-FAM-PanVHFR3-Fwd, 5'-GACACGGCCGTGTATTACTGT-3', SEQ ID NO: 17) and a reverse primer (PanJH-Rev, 5'-GCTGAGGAGACGGTGACC-3', SEQ ID NO: 18) that respectively anneal to the VH's framework 3 region and to the J region are used to amplify across the CDR3 regions of VH domains by PCR. Fifty ng of scFv library DNA template is used in a reaction volume of 30 µl containing 200 nM 6-FAM-PanVHFR3-Fwd primer, 200 nM PanJH-Rev primer, 200 µM dNTP, 1 x GoTaq buffer, and 1.5 U of GoTaq (Promega, Madison, WI). PCR conditions are as follows: an initial 2 minutes of 94 °C denaturation; 30 cycles of 94 °C (20 seconds), 55 °C (20 seconds), and 72 °C (30 seconds); followed by 5 minutes of 72 °C extension and 4 °C storage step. After PCR, 10 µl of products is loaded onto a 2% agarose gel to confirm successful reactions and the remaining products are subjected to spectratyping electrophoresis using an ABI sequencer. The CDR3 lengths are calculated by subtracting 60 bp flanking framework sequences from the product lengths that are determined by ROX-dye labeled DNA markers.

Results

Overview of PROfusion ScFv Library Construction

The flow chart representing different steps in PROfusion libraries construction is presented on Figure 24.

Amplification of VH cDNA Fragments

Aliquots of VH family-specific cDNA fragments (~ 500 bp) amplified by the VH universal primers (T7TMVUTR and Lib-GSv2-Rev) are visualized by agarose gel-electrophoresis (Figure 25). Specificity of VH family-specific primers has been previously tested and confirmed again for primers amplifying families with large number of germlines (Table 29).

Amplification of Vκ Fragments

Aliquots of Vκ family-specific cDNA fragments, amplified with universal primers (Lib-GSv2-Fwd: and CK Reverse), are visualized by agarose gel-electrophoresis (Figure 26). Specificity of Vκ family-specific primers is tested before and confirmed again for primers amplifying families with large number of germlines (Table 30).

Table 29 Sequencing analysis of VH family-specific PCR products

Fragment	*# of clones*	*Germline match*	*Family*	*Fragment*	*# of clones*	*Germline match*	*Family*
VH1/7	8	VH-1-69	VH1	VH3	11	VH3-30/VH3-33	VH3
	3	VH1-8	VH7		11	VH3-23	VH3
	2	VH1-18	VH1		7	VH3-7	VH3
	2	VH1-2	VH1		5	VH3-21	VH3
	1	VH1-24	VH1		4	VH3-15	VH3
VH2	11	VH2-5	VH2		2	VH3-9	VH3
	4	VH2-26	VH2		2	VH3-48	VH3
VH4	5	VH4-59	VH4		1	VH3-43	VH3
	4	VH4-55P	VH4		1	VH3-53	VH3
	4	VH4-39	VH4				
	3	VH4-31	VH4				

Table 30 Sequencing analysis of Vκ family-specific PCR products

Fragment	*# of clones*	*Germline match*	*Family*	*Fragment*	*# of clones*	*Germline match*	*Family*
Vκ1	20	O12/O2	Vκ1	Vκ2	13	A19/A3	Vk2
	8	O18/O8	Vκ1		3	A17/A1	Vk2
	4	A20	Vκ1		3	A18/A2	Vk2
	4	A30	Vκ1		3	O1/O11	Vk2
	3	L19/L5	Vκ1	Vκ3	8	A11/A27	Vk3
	2	L1/L15	Vκ1		4	L20/L6	Vk3
	2	L8	Vκ1		3	L16/L2	Vk3
	2	L11	Vκ1	Vκ6	7	A10/A26	Vk6
	1	L12	Vκ1				
	1	L14	Vκ1				
	1	L18/L4	Vκ1				

Amplification of Vλ Fragments

Aliquots of Vλ family-specific cDNA fragments, amplified with universal primers (Lib-GSv2-Fwd, SEQ ID NO: 140, and CJL Reverse, SEQ ID NO: 15), are visualized by agarose gel-electrophoresis (Figure 27). Specificity of Vλ family-specific primers is tested before.

VH-Vκ and VH-Vλ scFv Construction

Overlapping PCRs are carried out to construct VH-Vκ and VH-Vλ scFv cDNA fragments. Part of the obtained products is visualized by agarose gel electrophoresis (Figure 27). The generated VH- Vκ and VH-Vλ scFv fragments have all the necessary elements to be selected by the PROfusion mRNA display technology (Figure 28).

Sequencing analysis of individual VH-Vκ and VH-Vλ scFv clones confirms the correct VH-Vκ and VH-Vλ recombination with a functional intervening G4S linker in a great majority of the clones. The distribution of various VH and Vκ and Vλ families in the constructed scFv library is consistent with previous literature reports (Figure 29, Figure 30, also see Tsuiji *et al.* (2006). Exp. Med.; V.203 (2), pp. 393–400 and Arons *et al.* (2006) British Journal of Haematology V.133, pp. 504-512).

Spectratyping

Spectratyping analysis of VH CDR3 size distribution is carried out on cDNA obtained from lymph node RNA by reverse transcription with mixture of reverse primers (see 10.1), on mixture of VH DNA fragments just prior to their assembly into scFv libraries, and on final VH-Vκ and VH-Vλ scFv fragments (Figure 31). As would be expected in a cDNA library with a high degree of diversity, the observed CDR3 sizes have a normal distribution and the majority fall in between 6 to 22 residues and centerin between 11 to 14 residues.

Conclusion

The selection of high quality human antibody leads is a prerequisite for successful therapeutic antibody drug development. In additional to a robust selection technology, the antibody library quality (source, diversity, and construction) greatly determines its usefulness to produce good leads. In this invention the mRNA from multiple donors are used to increase library diversity and highly specific PCR primers are designed to cover all antibody germline sequences such that all diversity within the donor collection can be captured. VH-Vκ and VH-Vλ ScFv libraries are constructed separately to increase chances of selecting multiple antibodies leads from the same RNA source. Large diversity of both libraries is confirmed by spectratyping and by sequencing of multiple clones. Both libraries are used for PROfusion mRNA display selection against different targets.

EXAMPLE 11
SEQUENCING PRIMERS FOR PROFUSION CONSTRUCTS

This Example provides the sequencing primers used for PROfusion constructs.

Forward primer for all scFv and VH libraries:

T7TMVUTR (SEQ ID NO: 1):

TAATACGACTCACTATAGGGACAATTACTATTTACAATTACA

Forward primer for all Vκ and Vλ libraries:

VL-T7TMVTag3GS-Fwd (SEQ IN NO: 2):

TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAGGCTTTGGAC
CATGGGGTCTGGCGGCGGAGGTAGCG

Reverse primers for all κ scFv and Vκ libraries:

Cκ1FLAGA20 (SEQ ID NO: 3):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCGAAGACAGAT

Cκ2FLAGA20 (SEQ ID NO: 4):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCGAAGACAGATGGT

Cκ3FLAGA20 (SEQ ID NO: 5):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCGAAGACAGATGGTGCA

Cκ4FLAGA20 (SEQ ID NO: 6):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCGAAGACAGATGGTGCAGCC

Cκ5FLAGA20 (SEQ ID NO: 7):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCGAAGACAGATGGTGCAGCCACA

Reverse primers for all λ scFv and Vλ libraries:

CL1FLAGA20 (SEQ ID NO: 8):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCAGTGACAGTG

CL2FLAGA20 (SEQ ID NO: 9):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCAGTGACAGTGGGG

CL3FLAGA20 (SEQ ID NO: 10):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCAGTGACAGTGGGGTTG

CL4FLAGA20 (SEQ ID NO: 11):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCAGTGACAGTGGGGTTGGCC

CL5FLAGA20 (SEQ ID NO: 12):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCAGTGACAGTGGGGTTGGCCTTG

Lib-GS-Rev (SEQ ID NO: 16): CGCTACCTCCGCCGCCAGAC

Reverse primer for all VH libraries:
VH-GSFLAGA20-Rev (SEQ ID NO: 13):
TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCTTTGTCATCATCATCTTTATA
ATCGCTACCTCCGCCGCCAGAC

Primers for synthesis of the first strand cDNA in reverse transcription:
Fcg:
FcγRev1 (SEQ ID NO: 43): AGTTCCACGACACC
FcγRev2 (SEQ ID NO: 44): GAAGGTGTGCACG
FcγRev3 (SEQ ID NO: 45): CCACGCTGCTGAG
Fcm:
FcμRev1 (SEQ ID NO: 46): ACTTTGCACACCAC
FcμRev2 (SEQ ID NO: 47): TTTGTTGCCGTTGG
FcμRev3 (SEQ ID NO: 48): GGGAATTCTCACAGG
Fcd:
FcδRev1 (SEQ ID NO: 49): GCTGCTTGTCATGT
FcδRev2 (SEQ ID NO: 50): TGCCTTTGGAGACT
FcδRev3 (SEQ ID NO: 51): GACCACGCATTTGT

Cκ-RT Primers:
CκRev1 (SEQ ID NO: 52): TCCACCTTCCACTG
CκRev2 (SEQ ID NO: 53): CAGGCACACAACAG
CκRev3 (SEQ ID NO: 54): GAGTGTCACAGAGC
CLRTPrimers
CλRev1 (SEQ ID NO: 55) GGGAACAGAGTGAC
CλRev2 (SEQ ID NO: 56): GTGTGGCCTTGTTG
CλRev3. (SEQ ID NO: 57): CCATCTGCCTTCCA

Primers used for VH fragments amplification:
VH1/7LS (SEQ ID NO: 58): ATCCTCTTYTTGGTGGSAGC

VH1-46LS (SEQ ID NO: 59): GGTCTTCTGCTTGCTGGCTG

VH2LS (SEQ ID NO: 60): CCTGCTGCTGACCAYCCCTTC

VH3LS (SEQ ID NO: 61): GCTATTTTWVRAGGTGTCCARTGT

VH4LS (SEQ ID NO: 62): GCRGCTCCCAGATGGGTCCTG

VH5LS (SEQ ID NO: 63): ATGGGGTCAACCGCCATCCT

VH6LS (SEQ ID NO: 64): TGGGCCTCCCATGGGGTGTC

Primers used for nested VH-specific PCR:

VHPrimersTag2:

VH1Forward (SEQ ID NO: 69):

tttacaattacagtgttgcgaccatggAGGTGCAGCTGGTGCAGTCTGG**RS**CT

VH2Forward (SEQ ID NO: 70):

tttacaattacagtgttgcgaccatggAGRTCACCTTGARGGAGTCTGGT

VH3Forward (SEQ ID NO: 71):

tttacaattacagtgttgcgaccatgGAGGTGCAGCTG**K**TGGAGTCT**S**G**R**GGA

VH4Forward (SEQ ID NO: 72):

tttacaattacagtgttgcgaccatggAGGTGCAGCTGCAG**S**AGT**SS**GGC

VH5Forward (SEQ ID NO: 73):

tttacaattacagtgttgcgaccatgGAGGTGCAGCTGGTGCAGTCTGGAGCA

VH6Forward (SEQ ID NO: 74):

tttacaattacagtgttgcgaccatggAGGTACAGCTGCAGCAGTCAG

VH7Forward (SEQ ID NO: 75):

tttacaattacagtgttgcgaccatggAGGTGCAGCTGGTGCAATCTGGGT

Primers used for Vκ fragments amplification:

T7TMVTag2 (SEQ ID NO: 80):

TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAG TGTTGCGAC

Vκ1LS (SEQ ID NO: 81): GCTCCTGGGRCTYCTGC

Vκ2LS (SEQ ID NO: 82): CTYCTGGGGCTGCTAATG

Vκ3LS (SEQ ID NO: 83): CTCTGGCTCMCAGATACCAC

Vκ4LS (SEQ ID NO: 84): GGATCTCTGGTGCCTACGG

Vκ5LS (SEQ ID NO: 85): GGATCTCTGATACCAGGGCA

Vκ6LS (SEQ ID NO: 86): CTGGGTTCCAGCCTCCAG

Library Gly-Ser overlapping primers:

Lib-GS-Fwd (SEQ ID NO: 94): GTCTGGCGGCGGAGGTAGCG

FLAG-A20.Rev (SEQ ID NO: 95):

TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGT

CGTCCTTGTAGTC

Primers used for Vλ fragments amplification:

VL1LS-1 (SEQ ID NO: 96): TCACTG**TG**CAGGGTCCTG

VL1LS-3 (SEQ ID NO: 97): TCACTGCACAGGGTCCTG

VL2LS-3 (SEQ ID NO: 98): TCA**GG**RCACAGGGTCCTG

VL2LS-4 (SEQ ID NO: 99): TCA**GG**GCACAGGATCCTG

VL3LS-2 (SEQ ID NO: 100): TGCATAGGTTCTGTGGTTTCTTCTG

VL3LS-3 (SEQ ID NO: 101): ACAGGHTCTGWGGCCTCCTATG

VL3LS-4 (SEQ ID NO: 102): TGCACAGGCTCTGTGACCTCCTATG

VL3LS-5 (SEQ ID NO: 103): TACACAGGCTCTATTGCCTCCTATG

VL4Cls-2 (SEQ ID NO: 104): CTTCATTTTCTCCACAGGTCTCTGTG

VL4abLS-2 (SEQ ID NO: 105): TCCAC**TGSACAGGGTCTCTCT**

VL5LS (SEQ ID NO: 106): CACTGCACAGGTTCCCTC

VL6LS (SEQ ID NO: 107): CTGCACAGGTTCTTGGGC

VL7LS (SEQ ID NO: 108): CTCACTTGCTGCCCAGGG

VL8LS (SEQ ID NO: 109): GCTTATGGATCAGGAGTGGATTC

VL9LS (SEQ ID NO: 110): CACCCTCCTCAGTCTCCTC

VL10LS (SEQ ID NO: 111): CTCTGCAGTGTCAGTGGTC

Primers used for nested VH-specific PCR:

VHPrimersTag4:

VH1Forward (SEQ ID NO: 121):

tttacaattacagcttcttcaccatggAGGTGCAGCTGGTGCAGTCTGG**RS**CT

VH2Forward (SEQ ID NO: 122):

tttacaattacagcttcttcaccatggAGR**TCACCTTGARGGAGTCTGGT**

VH3Forward (SEQ ID NO: 123):

ttacaattacagcttcttcaccatgGAGGTGCAGCTG**K**TGGAGTCT**S**G**R**GGA

VH4Forward (SEQ ID NO: 124):

tttacaattacagcttcttcaccatggAGGTGCAGCTGCAG**S**AGT**SS**GGC

VH5Forward (SEQ ID NO: 125):

tttacaattacagcttcttcaccatgGAGGTGCAGCTGGTGCAGTCTGGAGCA

VH6Forward (SEQ ID NO: 126):

tttacaattacagcttcttcaccatggAGGTACAGCTGCAGCAGTCAG

VH7Forward (SEQ ID NO: 127):

tttacaattacagcttcttcaccatggAGGTGCAGCTGGTGCAATCTGGGT

JHReverse1/2 (SEQ ID NO: 128):

CGCTACCTCCGCCGCCAGACCCGCCTCCACCTGAGGAGACRGT GACCAGGGTGC

JHReverse3 (SEQ ID NO: 129):

CGCTACCTCCGCCGCCAGACCCGCCTCCACCTGAAGAGACGGTG ACCATTGTCC

JHReverse4/5 (SEQ ID NO: 130):

CGCTACCTCCGCCGCCAGACCCGCCTCCACCTGAGGAGACGGT GACCAGGGTTC

JHReverse6 (SEQ ID NO: 131):

CGCTACCTCCGCCGCCAGACCCGCCTCCACCTGAGGAGACGGTG ACCGTGGTCC

Primers used for Vλ fragments amplification:

VκPrimersV2:

Vκ1FwdV2 (SEQ ID NO: 134):

C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**GACATCC**R**G**W**TGACCC AGTCTCC**WT**

Vκ2FwdV2 (incl.L10ofVK3) (SEQ ID NO: 135):

C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**GATATTGTGATGAC**Y**C AG**W**CTCCAC

Vκ3FwdV2 (except L10) (SEQ ID NO: 136):

C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**GAAATTGTG**W**TGAC**R**C AGTCTCCAG**S**CA

Vκ4/6FwdV2 (SEQ ID NO: 137):
C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**GACATCGTG**M**TGAC**Y**C
AGTCTCCAGA
Vκ5FwdV2 (SEQ ID NO: 138):
C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**GAAACGACACTCACGC
AGTCTCCAGCAT
Vκ6FwdV2 (SEQ ID NO: 139):
C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**GATGTCGTGATGACAC
AGTCTCCAGCTT

T7-TMV-Seq (9970) (SEQ ID NO: 148): CTC ACT ATA GGG ACA ATT AC
T7TMVTag3 (SEQ ID NO: 149):
TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAGGCTTTGGAC
T7TMVTag4 (SEQ ID NO: 150):
TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAGCTTCTTCAC
T7TMVTag2s (SEQ ID NO: 151):
TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAG TGTTGCG
T7TMVTag3s (SEQ ID NO: 152):
TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAGGCTTTGG
T7TMVTag4s (SEQ ID NO: 132):
TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAGCTTCTTC
T7TMVTag4L (SEQ ID NO: 153):
TAATACGACTCACTATAGGGACAATTACTATTTACAATTACA
GCTTCTTCACCATGG
TMVTag4L (SEQ ID NO: 154):
ACAATTACTATTTACAATTACAGCTTCTTCACCATGG
TMVTag4 (SEQ ID NO: 155): ACAATTACTATTTACAATTACAGCTTCTTCAC
TMVTag4s (SEQ ID NO: 156): ACAATTACTATTTACAATTACAGCTTCTTC
phylflag3's (no poly A) (SEQ ID NO: 157): CCTTGTCGTCGTCGTCCTTGTAGTC
VH-FLAGA20-Rev (FLAG is recoded to minimize X-priming with VL's FLAG sequence) (SEQ ID NO: 158):
TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCTTTGTCATCATCATCTTTATA
ATC

Primers to Make VH Domain Library with Cμ Spacer:

JH1/2Cm-Rev (SEQ ID NO: 159):

GGTTGGGGCGGATGCACTCCCCTGAGGAGACRGTGACCAGGGTGC

JH4/5Cm-Rev (SEQ ID NO: 160):

GGTTGGGGCGGATGCACTCCCCTGAGGAGACGGTGACCAGGGTTC

JH6Cm-Rev (SEQ ID NO: 161):

GGTTGGGGCGGATGCACTCCCCTGAGGAGACGGTGACCGTGG TCC

JH3Cm-Rev (SEQ ID NO: 162):

GGTTGGGGCGGATGCACTCCCCTGAAGAGACGGTGACCATTG TCC

JH1/2sRev (SEQ ID NO: 65): CTGAGGAGACRGTGACCAGGGTGC

JH4/5sRev (SEQ ID NO: 66): CTGAGGAGACGGTGACCAGGGTTC

JH6sRev (SEQ ID NO: 67): CTGAGGAGACGGTGACCGTGGTCC

JH3sRev (SEQ ID NO:68): CTGAAGAGACGGTGACCATTGTCC

Vκ Primers:

Vκ1 Forward (SEQ ID NO: 88):

GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGACATCC**R**G**W**TGACCCA GTCTCC**W**T

Vκ2 Forward (including L10 of Vκ3) (SEQ ID NO: 89):

GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGATATTGTGATGAC**Y**CA G**W**CTCCAC

Vκ3 Forward (except L10) (SEQ ID NO: 90):

CTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGAAATTGTG**W**TGAC**R**CAGT CTCCAG**S**CA

Vκ4/6 Forward (SEQ ID NO: 91):

GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGACATCGTG**M**TGAC**Y**CA GTCTCCAGA

Vκ5 For-NEW (SEQ ID NO:92):

CTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGAAACGACACTCACGCAGT CTCCAGCAT

Vκ6 For-NEW (SEQ ID NO: 93):
CTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGATGTCGTGATGACACAGT
CTCCAGCTT
Vκ5 Forward (SEQ ID NO: 168):
GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGAAACGACACTCACGCA
GTCTC
Vκ6 Forward (SEQ ID NO: 169):
CTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGGATGTCGTGATGACACAGT
CTCCAGCT
Cκ Reverse (SEQ ID NO: 14):
GTCGTCGTCGTCCTTGTAGTCGAAGACAGATGGTGCAGCCACAGTTCG
Cκ5 FLAGA20 (SEQ ID NO: 7):
TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCGAAGACAGATGGTGCAGCCACA

Cκ-s Reverse primer: (SEQ ID NO: 87): GAAGACAGATGGTGCAGCCACAGTTCG
Cκ-Long-Reverse primer(SEQ ID NO: 170): Long Cκ Reverse Primer to add 7 A.A. before FLAG:
gtcgtcgtcgtccttgtagtcCTCATCAGATGGCGGGAAGATGAAGACAGATGGTGCAGC
CACAGTTCG

FLAG hCk primers:
CκL4-FlagA20-Rev (SEQ ID NO: 171):
TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCCTCATCAGATGGCGGGAAGAT
CκL3-FlagA20-Rev (SEQ ID NO: 172):
TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCCTCATCAGATGGCGGGAA
CκL2-FlagA20-Rev (SEQ ID NO: 173):
TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCCTCATCAGATGGCGG

CκL1-FlagA20-Rev (SEQ ID NO: 174):
TTTTTTTTTTTTTTTTTTTTAAATAGCGGATGCCTTGTCGTCGTCGTCCTTGTA
GTCCTCATCAGATGG

VL Primers:
VL1/10 Forward (SEQ ID NO: 175):
GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGCAGTCTG**K**GCTGAC**K**CA
GCC**R**C
VL2 Forward (SEQ ID NO: 114):
GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGCAGTCTGCCCTGACTCA
GCCT
VL3 Forward-New (SEQ ID NO: 115):
GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGTCCTATGAGCTGAC**D**CA
G
VL4ab Forward (SEQ ID NO: 116):
GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGCAGC**Y**TGTGCTGACTCA
ATC
VL4c Forward (SEQ ID NO: 117):
GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGCTGCCTGTGCTGACTCA
GCCCCCG
VL5/9 Forward (SEQ ID NO: 118):
GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGCAGCCTGTGCTGACTCA
GCC**RB**CT
VL6 Forward (SEQ ID NO: 119):
GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGAATTTTATGCTGACTCA
GCCC
VL7/8 Forward (SEQ ID NO: 120):
GTCTGGCGGCGGAGGTAGCGGCGGTGGCGGATCGCAG**R**CTGTGGTGAC**Y**CA
GGAG
CJL Reverse (SEQ ID NO: 15):
GTCGTCGTCGTCCTTGTAGTCAGTGACAGTGGGGTTGGCCTTGGGCTGACC**K**
AGGACGGT
CJL-sReverse (SEQ ID NO: 112): GCCTTGGGCTGACCKAGGACGGT

VH Primers Tag3:

VH1FwdTag3 (SEQ ID NO: 176):

tttacaattacaGGCTTTGGaccatggAGGTGCAGCTGGTGCAGTCTGGRSCT

VH2FwdTag3 (SEQ ID NO: 177):

tttacaattacaGGCTTTGGaccatggAGRTCACCTTGARGGAGTCTGGT

VH3FwdTag3(SEQ ID NO: 178):

tttacaattacaGGCTTTGGaccatgGAGGTGCAGCTGKTGGAGTCTSGR GGA

VH4FwdTag3 (SEQ ID NO: 179):

tttacaattacaGGCTTTGGaccatggAGGTGCAGCTGCAGSAGTSSGGC

VH5FwdTag3 (SEQ ID NO: 180):

tttacaattacaGGCTTTGGaccatgGAGGTGCAGCTGGTGCAGTCTGGA GCA

VH6FwdTag3 (SEQ ID NO: 181):

tttacaattacaGGCTTTGGaccatggAGGTACAGCTGCAGCAGTCAG

VH7FwdTag3 (SEQ ID NO: 182):

TttacaattacaGGCTTTGGaccatggAGGTGCAGCTGGTGCAATCT GGGT

VLPrimers:

VL1/10FwdV2 (SEQ ID NO: 183):

C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**CAGTCTG**K**GCTGAC**K**C AGCC**R**C

VL2FwdV2 (SEQ ID NO: 184):

C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**CAGTCTGCCCTGACTCA GCCT

VL3FwdV2 (SEQ ID NO: 185):
C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**TCCTATGAGCTGAC**D**CA
G
VL4abFwdV2 (SEQ ID NO: 186):
C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**CAGC**Y**TGTGCTGACTCA
ATC
VL4cFwdV2 (SEQ ID NO: 187):
C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**CTGCCTGTGCTGACTCA
GCCCCCG
VL5/9FwdV2 (SEQ ID NO: 188):
C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**CAGCCTGTGCTGACTCA
GCC**RB**CT
VL6FwdV2 (SEQ ID NO: 189):
C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**AATTTTATGCTGACTCA
G CCC
VL7/8FwdV2 (SEQ ID NO: 190):
C**AGC**GGCGGTGGAGGG**TCT**GGCGGTGGCGGA**AGT**CAG**R**CTGTGGTGAC**Y**C
AGGAG
Lib-GSv2-Fwd (SEQ ID NO: 140): CAGCGGCGGTGGAGGGTCTG

Lib-GSv2-Rev (SEQ ID NO: 133): CAGACCCTCCACCGCCGCTG
JH1/2RevV2 (SEQ ID NO: 128):
cagaccctccaccgccgctgccgcctccacCTGAGGAGACRGTGACCAGGGTGC
JH4/5RevV2 (SEQ ID NO: 130):
cagaccctccaccgccgctgccgcctccacCTGAGGAGACGGTGACCAGGGTTC
JH6RevV2 (SEQ ID NO: 131):
cagaccctccaccgccgctgccgcctccacCTGAGGAGACGGTGACCGTGGTCC
JH3RevV2 (SEQ ID NO: 129):
cagaccctccaccgccgctgccgcctccacCTGAAGAGACGGTGACCATTGTCC

Library GlySer linker v2:
Library GS Fwd: C **AGC** GGC GGT GGA GGG **TCT** G ----->

TCC TCA GGT GGA GGC GGC **AGC** GGC GGT GGA GGG **TCT** GGC GGT GGC GGA **AGT**

AGG AGT CCA CCT CCG CCG **TCG** CCG CCA CCT CCC **AGA** CCG CCA CCG CCT **TCA**

Library GS Rev: <----- G **TCG** CCG CCA CCT CCC **AGA** C

S S G G G G S G G G G S G G G G S

Overlapping during PCR

VH3 FR1: CAG CTG GTG GAG TCT **GGG GGA GGC** TTG GTC CAG CCT GGG GGG TTC

TCC TCA GGT GGA GGC GGC **AGC** GGC GGT GGA GGG **TCT** G

G **TCG** CCG CCA CCT CCC **AGA** CCG CCA CCG CCT **TCA**

(for Vk3, Vk6) <-----**CG** CCG CCA CCT CCC **AGA** CCG CCA CCG CCT **TCA**

Primers with New Tag for Human Tonsil Libraries

VH PrimersTag5:

VH1FwdTag5 (SEQ ID NO: 191):

tttacaattacaGTGTCTGTaccatggAGGTGCAGCTGGTGCAGTCTGGR SCT

VH2FwdTag5 (SEQ ID NO: 192):

tttacaattacaGTGTCTGTaccatggAGRTCACCTTGARGGAGTC TGGT

VH3FwdTag5 (SEQ ID NO: 193):

tttacaattacaGTGTCTGTaccatgGAGGTGCAGCTGKTGGAGTCTSGR GGA

VH4FwdTag5 (SEQ ID NO: 194):

tttacaattacaGTGTCTGTaccatggAGGTGCAGCTGCAGSAGTSSGGC

VH5FwdTag5 (SEQ ID NO: 195):

tttacaattacaGTGTCTGTaccatgGAGGTGCAGCTGGTGCAGTCTGGA GCA

VH6FwdTag5 (SEQ ID NO: 196):

tttacaattacaGTGTCTGTaccatggAGGTACAGCTGCAGCAGTCAG

VH7FwdTag5 (SEQ ID NO: 197):
TttacaattacaGTGTCTGTaccatggAGGTGCAGCTGGTGCAATCTGGGT

Primers with New Tag for Human Bone Marrow Libraries
VHPrimersTag6:
VH1FwdTag6 (SEQ ID NO: 198):
tttacaattacaGTTTGGCTaccatggAGGTGCAGCTGGTGCAGTCTGGRS CT
VH2FwdTag6 (SEQ ID NO: 199):
tttacaattacaGTTTGGCTaccatggAGRTCACCTTGARGGAGTCTGGT

VH3FwdTag6 (SEQ ID NO: 200):
tttacaattacaGTTTGGCTCaccatgGAGGTGCAGCTGKTGGAGTCTSG RGGA
VH4FwdTag6 (SEQ ID NO: 201):
tttacaattacaGTTTGGCTaccatggAGGTGCAGCTGCAGSAGTSSGGC
VH5FwdTag6 (SEQ ID NO: 202):
tttacaattacaGTTTGGCTaccatgGAGGTGCAGCTGGTGCAGTCTGGA GCA
VH6FwdTag6 (SEQ ID NO: 203):
tttacaattacaGTTTGGCTaccatggAGGTACAGCTGCAGCAGTCAG
VH7FwdTag6 (SEQ ID NO: 204):
TttacaattacaGTTTGGCTaccatggAGGTGCAGCTGGTGCAATCTGG GT

Reamplifying Tag5 Forward Primers
TMVTag5s (SEQ ID NO: 205): ACAATTACTATTTACAATTACAGTGTCTGT
TMVTag6s (SEQ ID NO: 206): ACAATTACTATTTACAATTACAGTTTGGCT
TMVTag5 (SEQ ID NO: 207): ACAATTACTATTTACAATTACAGTGTCTGTacc
TMVTag6 (SEQ ID NO: 208): ACAATTACTATTTACAATTACAGTTTGGCTacc
T7TMVTag5s (SEQ ID NO: 209):
TAATACGACTCACTATAGGGACAATTACTATTT ACA AT TA CAGTGTCTGT
T7TMVTag6s (SEQ ID NO: 210):
TAATACGACTCACTATAGGGACAATTACTATTTACAATTAC AGTTTGGCT

Incorporation by Reference

The contents of all cited references (including literature references, patents, patent applications, and websites) that maybe cited throughout this application are hereby expressly incorporated by reference in their entirety, as are the references cited therein. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology and cell biology, which are well known in the art.

Equivalents

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting of the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are therefore intended to be embraced herein.

Claims

1. An oligonucleotide consisting of a nucleic acid sequence as set forth in any one of SEQ ID NOs: 1-14, 19-42, and 58-210.

2. An oligonucleotide consisting of a nucleic acid sequence as set forth in any one of SEQ ID NOs: 14-16, and 43-57.

3. An oligonucleotide consisting of a nucleic acid sequence as set forth in SEQ ID NOs: 17 or 18.

4. Use of any of the sequences in the preceding claims for library amplification, library reverse transcription, and/or library spectratyping.

5. A nucleic acid library for expression of single chain antibodies (scFv), said library comprising a repertoire of sequences encoding heavy chain variable domains and light chain variable domains, wherein each member of said library contains an open reading frame comprising a heavy chain variable domain, a light chain variable domain, and a linker region, and wherein said library is generated using one or more oligonucleotides of claim 1.

6. The library of claim 5, wherein the linker region encodes less than 20 amino acids.

7. The library of claim 5, wherein the linker region encodes 15 amino acids.

8. The library of claim 5, wherein each member of said library further comprises a promoter operably linked to the open reading frame.

9. The library of claim 8, wherein said promoter is a promoter selected from the group consisting of T7, SP6, and T3.

10. The library of claim 9, wherein said promoter is a T7 promoter.

11. The library of claim 5, wherein each member of said library further comprises a 5'untranlated region (5'UTR) capable of enhancing transcription of a gene to which it is operably linked.

12. The library of claim 11, wherein said 5'UTR is a Tobacco Mosaic Virus 5'UTR or active fragment thereof.

13. The library of claim 5, wherein each member of said library further comprises a polyadenine sequence.

14. The library of claim 5, wherein each member of said library further comprises a nucleic acid barcode.

15. The library of claim 14, wherein said nucleic acid barcode comprises 8 nucleotides.

16. The library of claim 5, wherein each member of said library further comprises a nucleic acid sequence encoding an epitope tag.

17. The library of claim 16, wherein the epitope tag is a FLAG tag.

18. The library of claim 16, wherein said nucleic acid sequence is part of the linker region of the scFv.

19. The library of claim 5, wherein each member of said library further comprises a nucleic acid sequence encoding an antibody constant region or fragment thereof.

20. The library of claim 5, wherein each member of said library further comprises a ribosome pause sequence.

21. The library of claim 5, wherein each member of said library further comprises a peptide acceptor.

22. The library of claim 21, wherein the peptide acceptor is covalently attached via a linker comprising a Psoralen C6 molecule.

23. The library of claim 22, wherein the linker is 5'(Psoralen C6) 2'Ome (U AGC GGA UGC) XXX XXX CC (Puromycin), wherein X is a triethylene glycol linker or PEG-150 and CC is a DNA backbone.

24. A method of producing a nucleic acid library for expression of single chain antibodies (scFv) comprising:

providing a nucleic acid composition, wherein at least a portion of the nucleic acids in said composition comprise at least one open reading frame encoding an antibody variable domain; and,

amplifying a plurality of antibody variable domains using one or more oligonucleotides of claim 1.

25. A method of spectratyping a nucleic acid comprising at least one open reading frame encoding an antibody variable domain comprising:

providing a nucleic acid composition, wherein at least a portion of the nucleic acids in said composition comprise at least one open reading frame encoding an antibody variable domain; and,

amplifying the CDR3 regions of said variable domains using one or more oligonucleotides of claim 2.

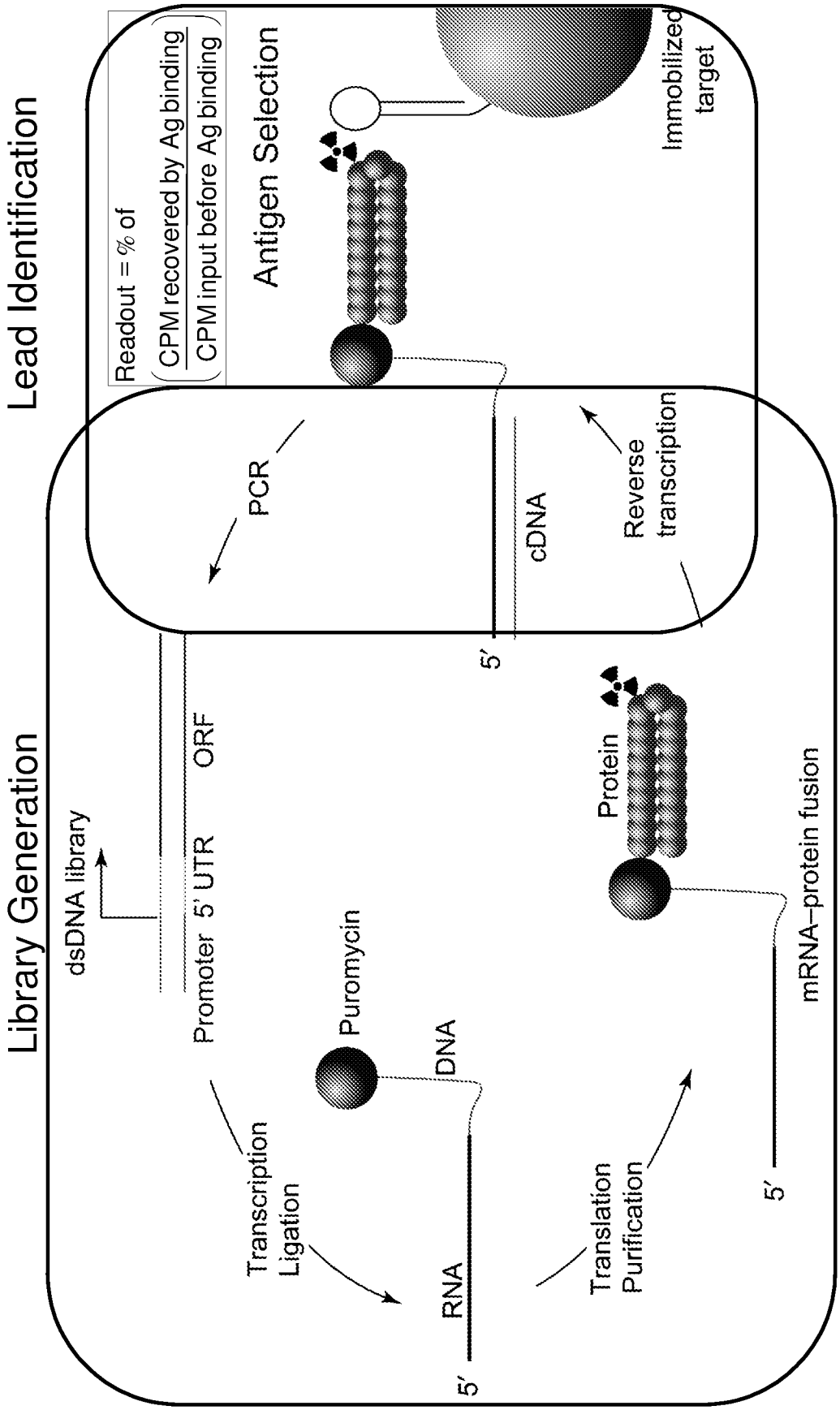
Lead Identification
Library Generation
Antigen Selection
Readout = % of
CPM recovered by Ag binding
CPM input before Ag binding
Immobilized target
dsDNA library
Promoter
5' UTR
ORF
PCR
cDNA
Reverse transcription
Puromycin
DNA
RNA
Transcription Ligation
Translation Purification
Protein
mRNA–protein fusion
5'

Fig. 1

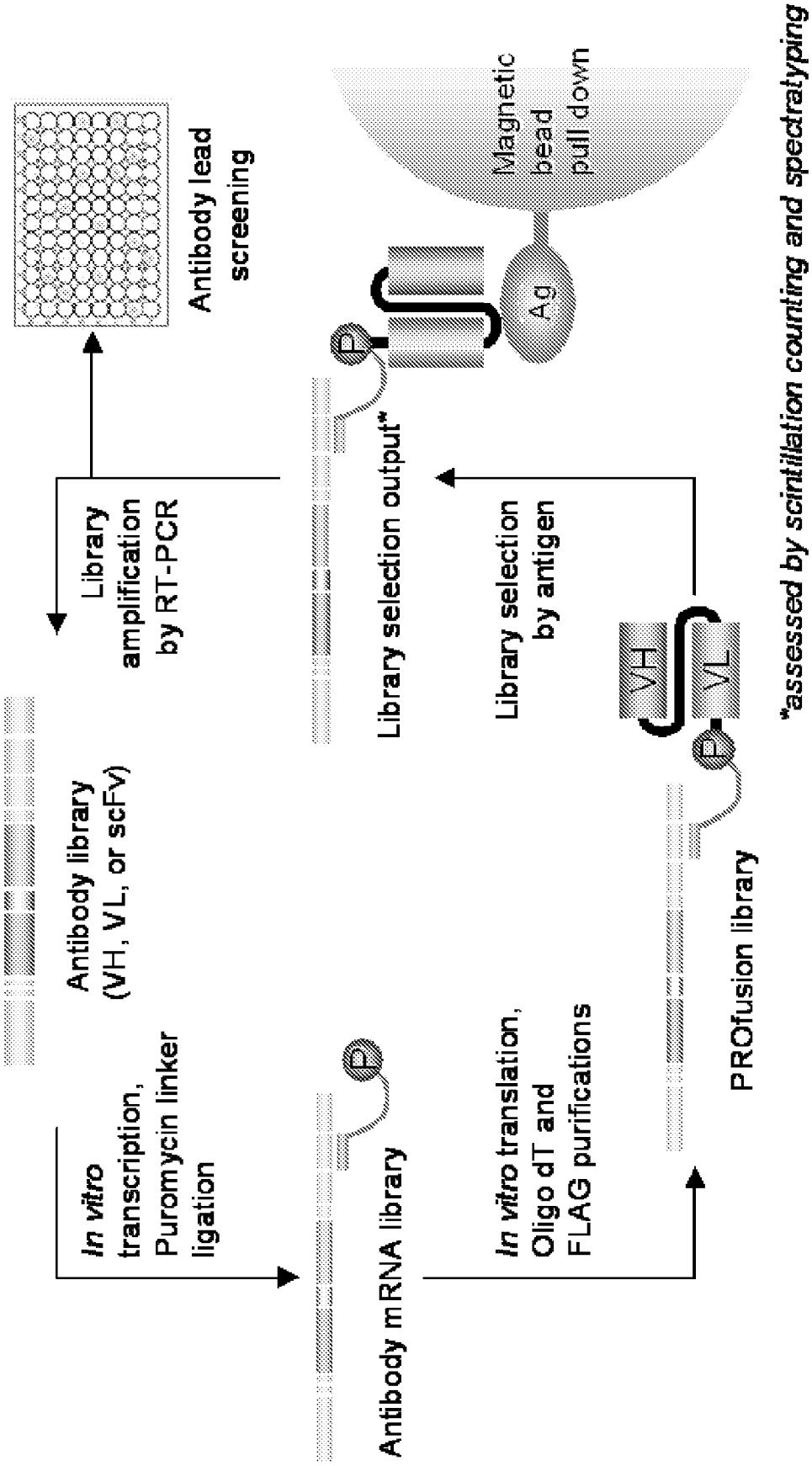
Antibody lead screening
Library amplification by RT-PCR
Library selection output*
Magnetic bead pull down
Ag
Library selection by antigen
VH
VL
*assessed by scintillation counting and spectratyping
Antibody library (VH, VL, or scFv)
In vitro transcription, Puromycin linker ligation
Antibody mRNA library
In vitro translation, Oligo dT and FLAG purifications
PROfusion library

Fig. 2

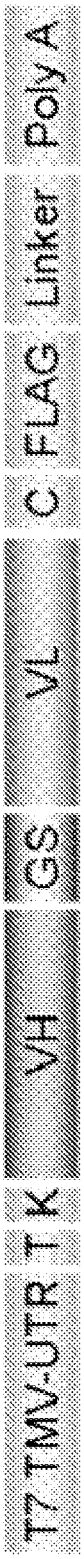

Fig. 3

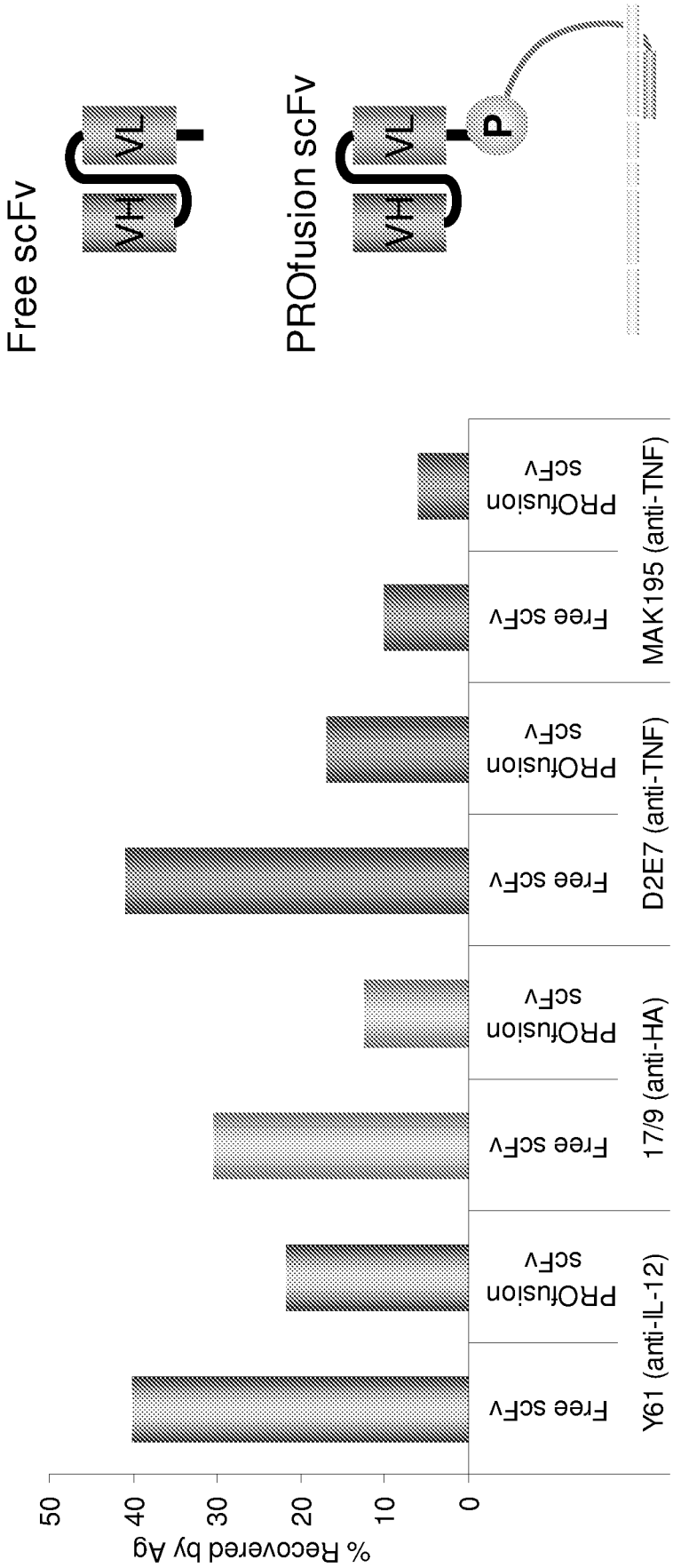
Free scFv
VH
VL
PROfusion scFv
VH
VL
P
% Recovered by Ag
0
10
20
30
40
50
Free scFv
PROfusion scFv
Free scFv
PROfusion scFv
Free scFv
PROfusion scFv
Free scFv
PROfusion scFv
Y61 (anti-IL-12)
17/9 (anti-HA)
D2E7 (anti-TNF)
MAK195 (anti-TNF)

Fig. 4

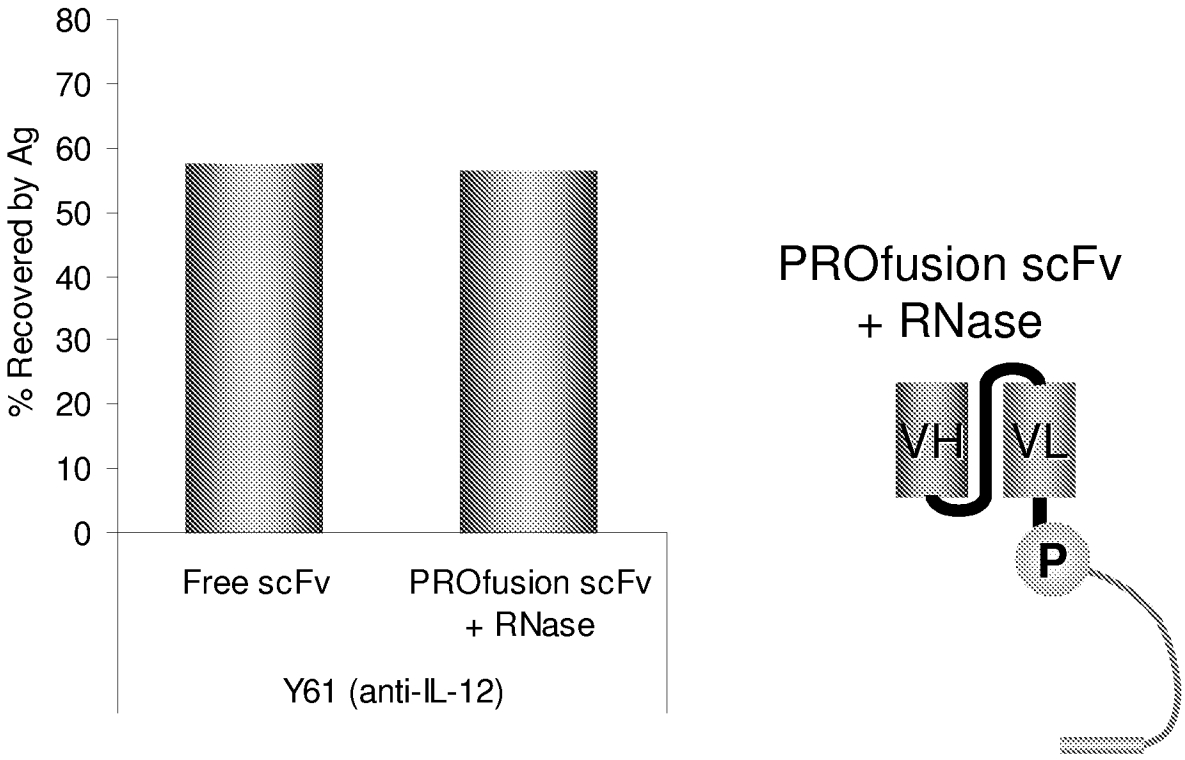
80
70
60
50
40
30
20
10
0
% Recovered by Ag
Free scFv
PROfusion scFv
+ RNase
Y61 (anti-IL-12)
PROfusion scFv
+ RNase
VH
VL
P

Fig. 5

```
          1                                                          60
17_9-tag1 TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAGCGTGGGTACCATGGAAG
17_9-tag2 TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAGTGTTGCGACCATGGAAG
17_9-tag3 TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAGGCTTTGGACCATGGAAG
17_9-tag4 TAATACGACTCACTATAGGGACAATTACTATTTACAATTACAGCTTCTTCACCATGGAAG
          T7 Promoter          TMV-UTR                  Tags   Kozak 17/9
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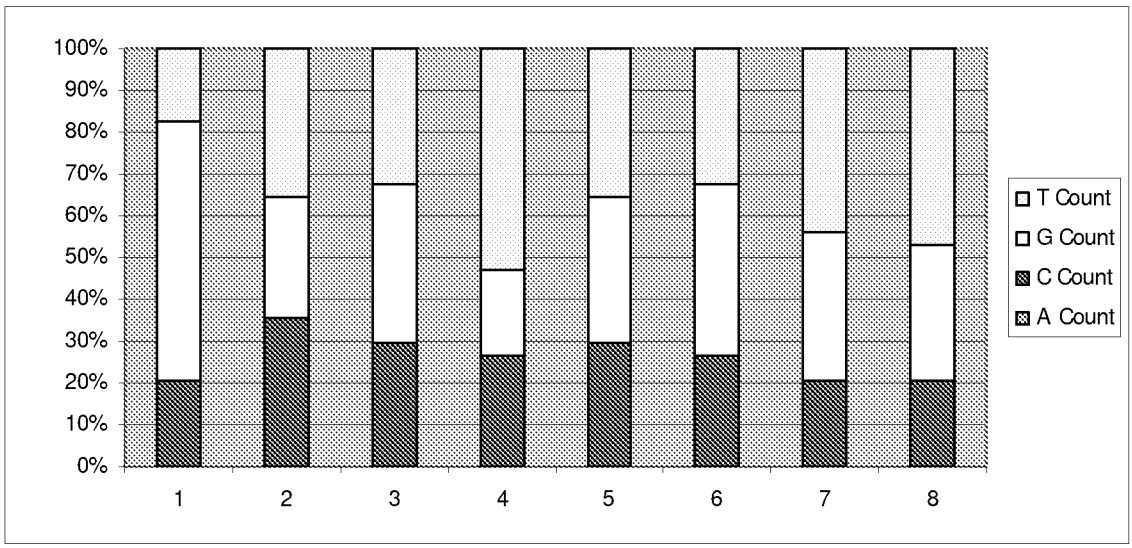


Fig. 6

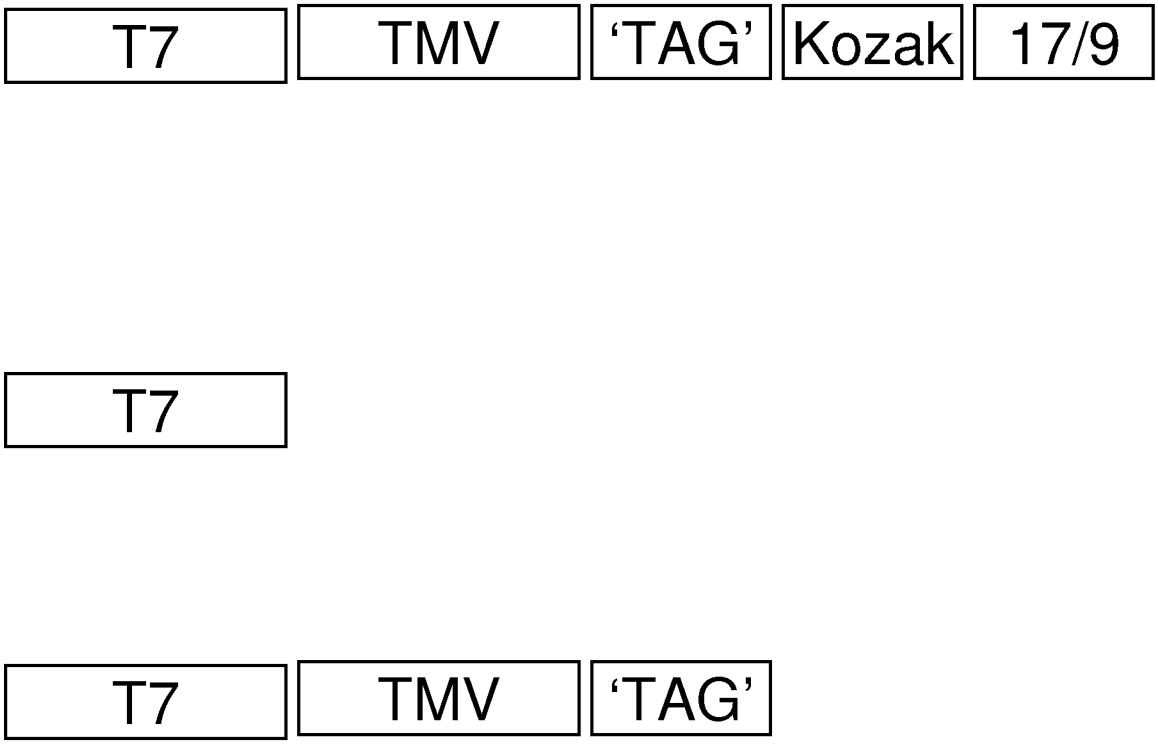


Fig. 7

R1 Output Tags	R2 Output Tags	R3 Output Tags
CGGTCCCT	CCGCGCGG	CCGTCTTT
CTCGTTTG	CCGGGTTG	CCTCTTGT
CTCTGTGT	CCTCCCGT	CCTGTGTT
CTGTGTTG	CGGGCTGG	CGCTGGTT
CTTTTGCT	CGTCGGTC	CGTCTGGT
GCCGCGGT	CGTGGGTT	CTCGCGCC
GGCGCTGT	CGTGGGTT	CTCTTGCG
GGCGCTGT	CTCCTGGC	GCCTTCTC
GGGTGTCT	GCCCGTCT	GCCTTTTT
GGTTCTTT	GCCTGTTC	GCGGCCTT
GGTTGCGG	GCGCTCGT	GCGTGGGT
GTGCTTCT	GCGGCCCT	GCGTGGGT
GTGTGCGT	GCGGCTTG	GCGTGGGT
GTGTTCTC	GCTTCTCG	GCTTCTTC
GTTTGCGC	GGCCTTTG	GCTTTCTT
TCGCGTCC	GGCTGTCC	GGCCGCGG
TCGTGCGT	GGCTT ATG	GGCGCGCG
TGCCCGCG	GGCTT ATG	GGCGGGTG
TGCCGGTG	GGGCGGCT	GGCTTTGG
TGGTCGCT	GGGTGTGT	GGGCCTTG
TGGTCTTG	GGTTGTGC	GGGGGCGT
TTTGCTGC	GTCGGGCG	GGTCCTTC
	GTCGGGTT	GTGCGTCG
	GTGTGCTC	GTGTCTGT
	GTGTTTTT	GTGTTGCG
	TGCTTTGT	GTTCGTCG
	TGCTTTGT	GTTCTTGC
	TGCTTTTG	GTTTGGCT
	TGTTTTCG	TCGTGCGT
	TTCGGCTT	TGTCTGTG
	TTGGGCTT	TTCTCCTT
	TTGTGTTT	TTGGCCTG
	TTTGGCTC	TTGTTGGC
	TTTGGCTC	TTTTGCTC
	TTTTGTTG	

Fig. 8

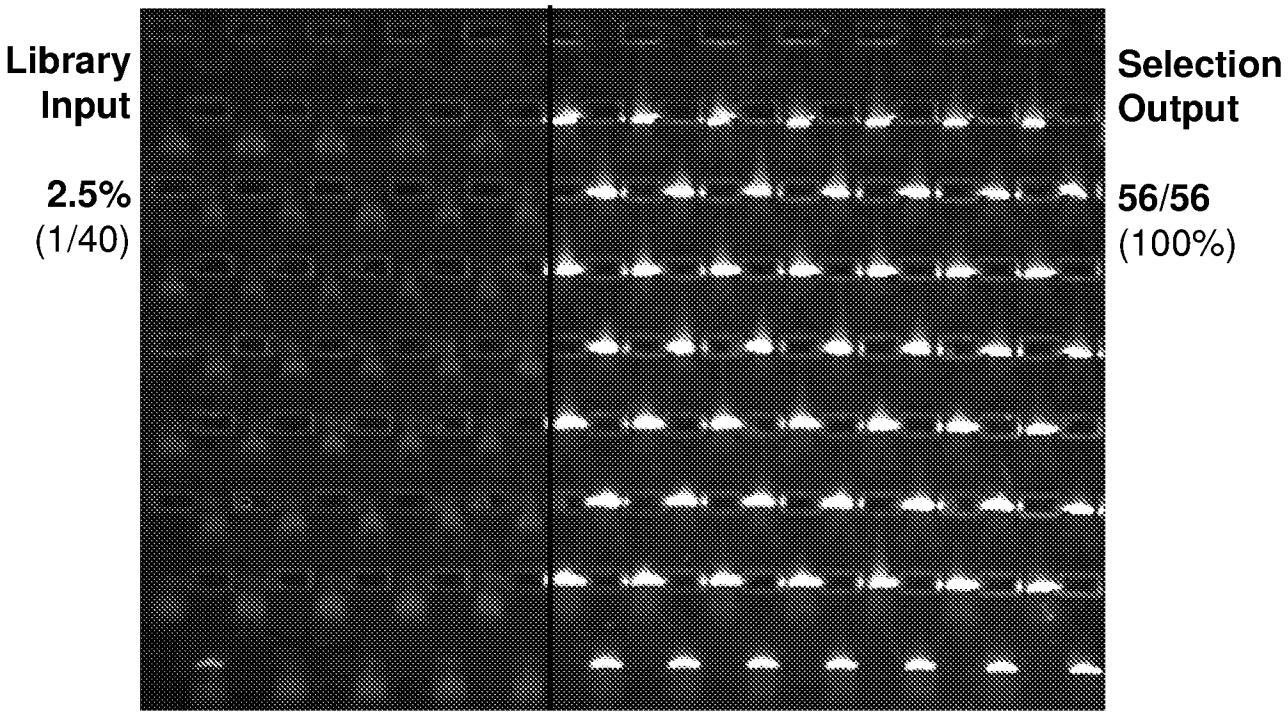


Fig. 9

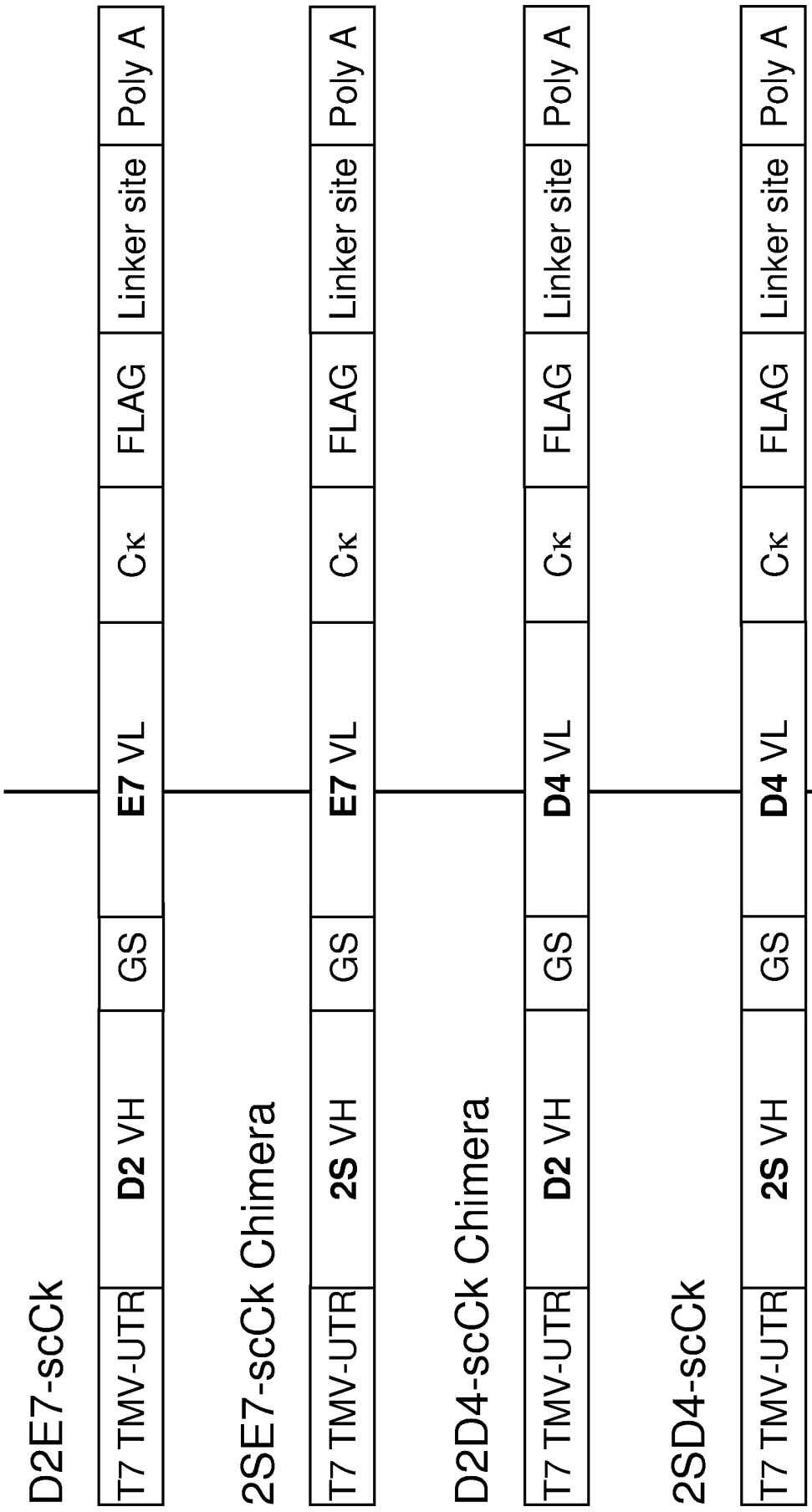
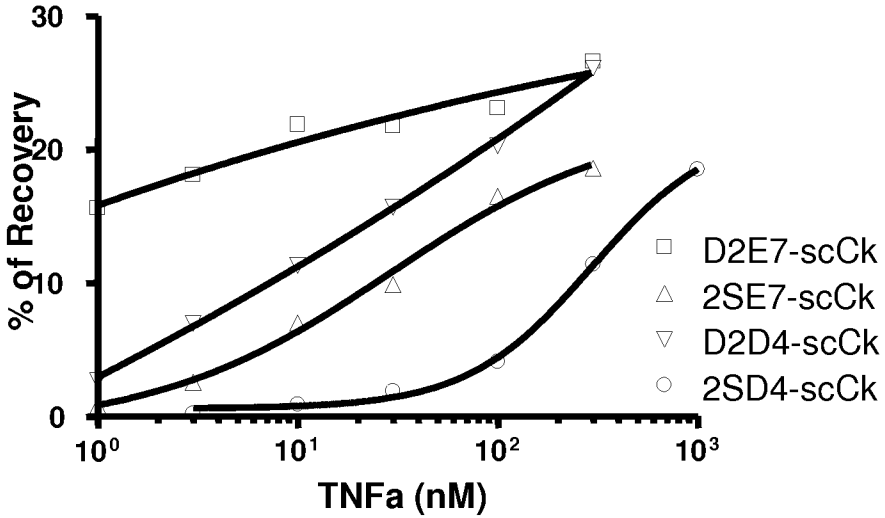
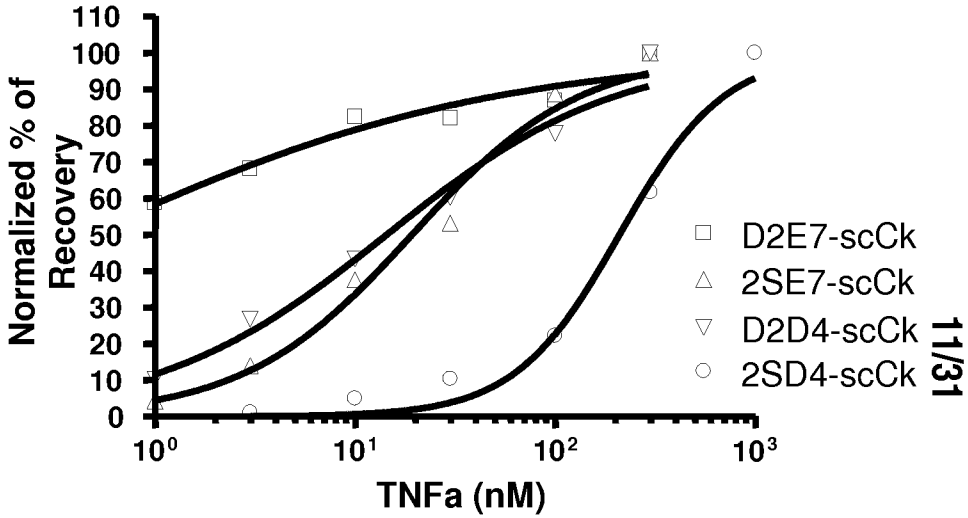

D2E7-scCk
T7 TMV-UTR
D2 VH
GS
E7 VL
Cκ
FLAG
Linker site
Poly A
2SE7-scCk Chimera
T7 TMV-UTR
2S VH
GS
E7 VL
Cκ
FLAG
Linker site
Poly A
D2D4-scCk Chimera
T7 TMV-UTR
D2 VH
GS
D4 VL
Cκ
FLAG
Linker site
Poly A
2SD4-scCk
T7 TMV-UTR
2S VH
GS
D4 VL
Cκ
FLAG
Linker site
Poly A

Fig. 10

Titration was done for free proteins

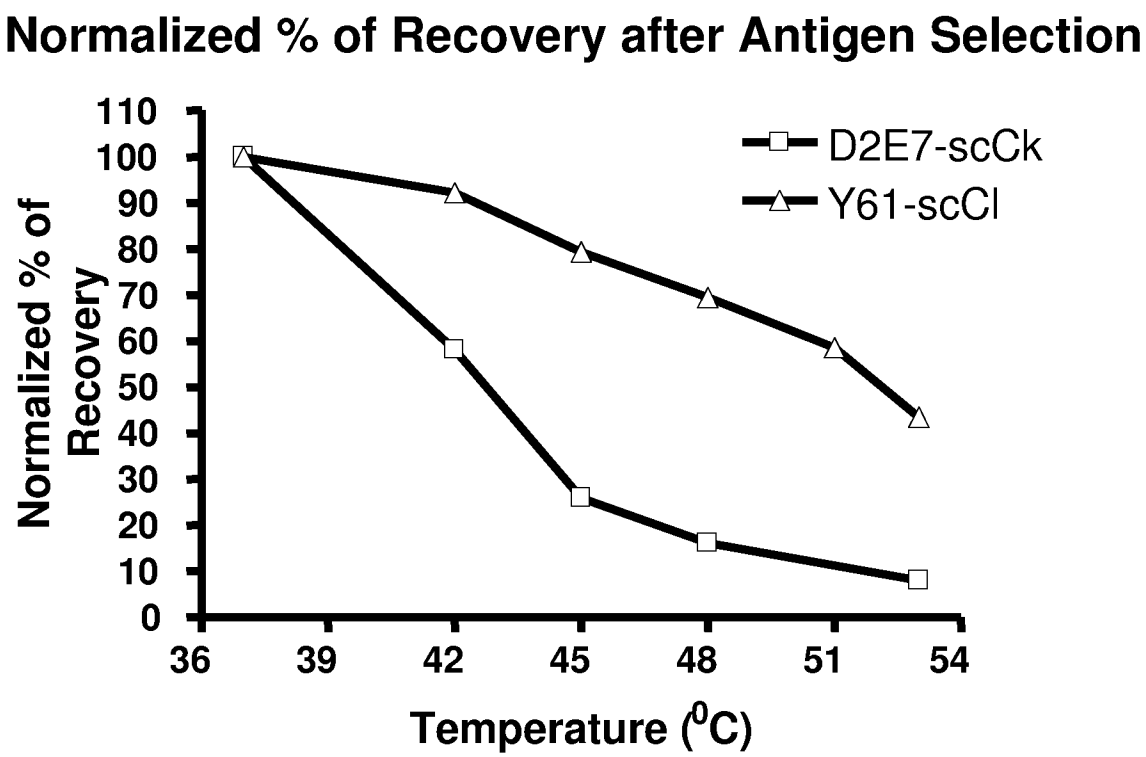




	D2E7-scCk	2SE7-scCk	D2D4-scCk	2SD4-scCk
EC50	0.4506	19.36	14.45	209.8

Conclusion: PROfusion technology can be used to discriminate binders with different affinity

Fig. 11



Notebook 84093-165

Fig. 12

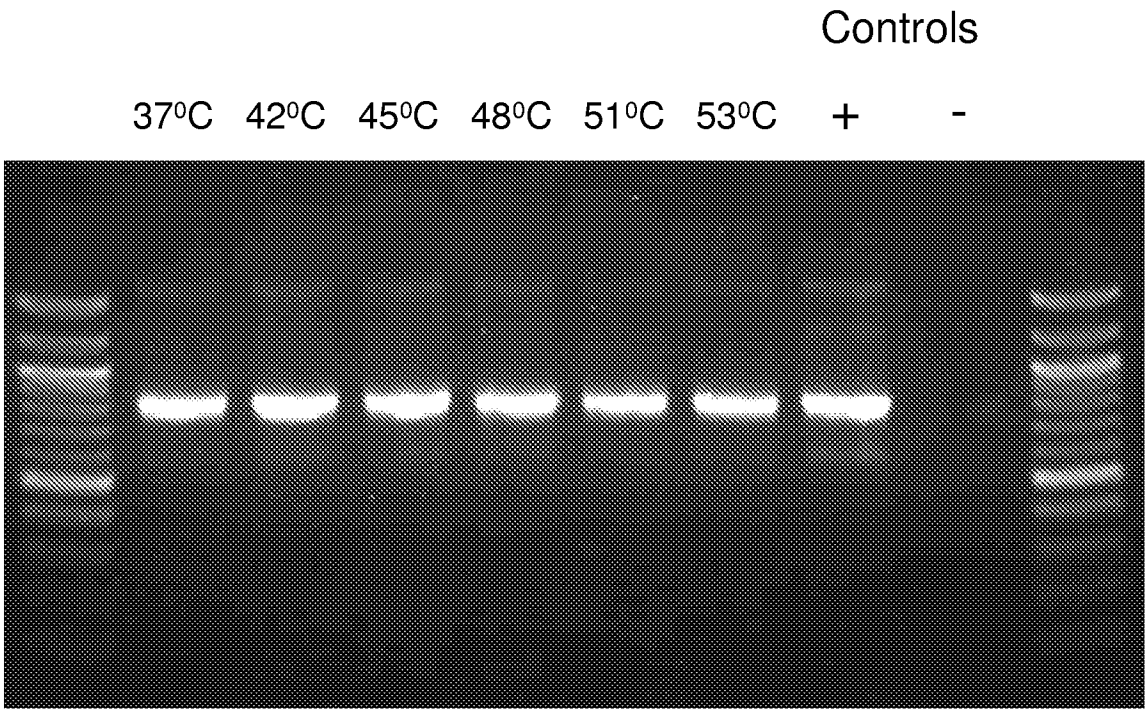


Fig. 13

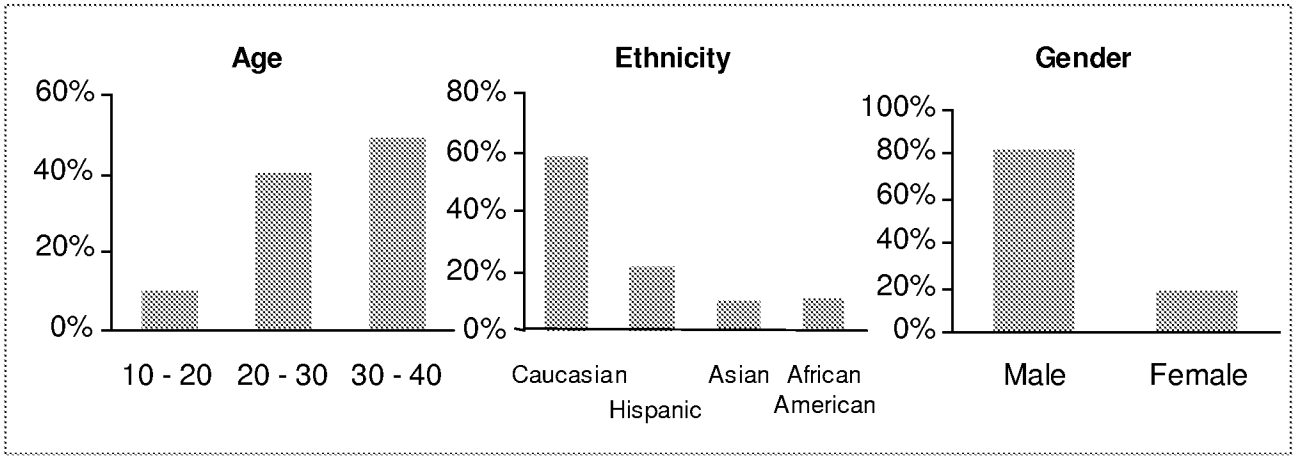

Age
60%
40%
20%
0%
10 - 20
20 - 30
30 - 40
Ethnicity
80%
60%
40%
20%
0%
Caucasian
Hispanic
Asian
African American
Gender
100%
80%
60%
40%
20%
0%
Male
Female

Fig. 14

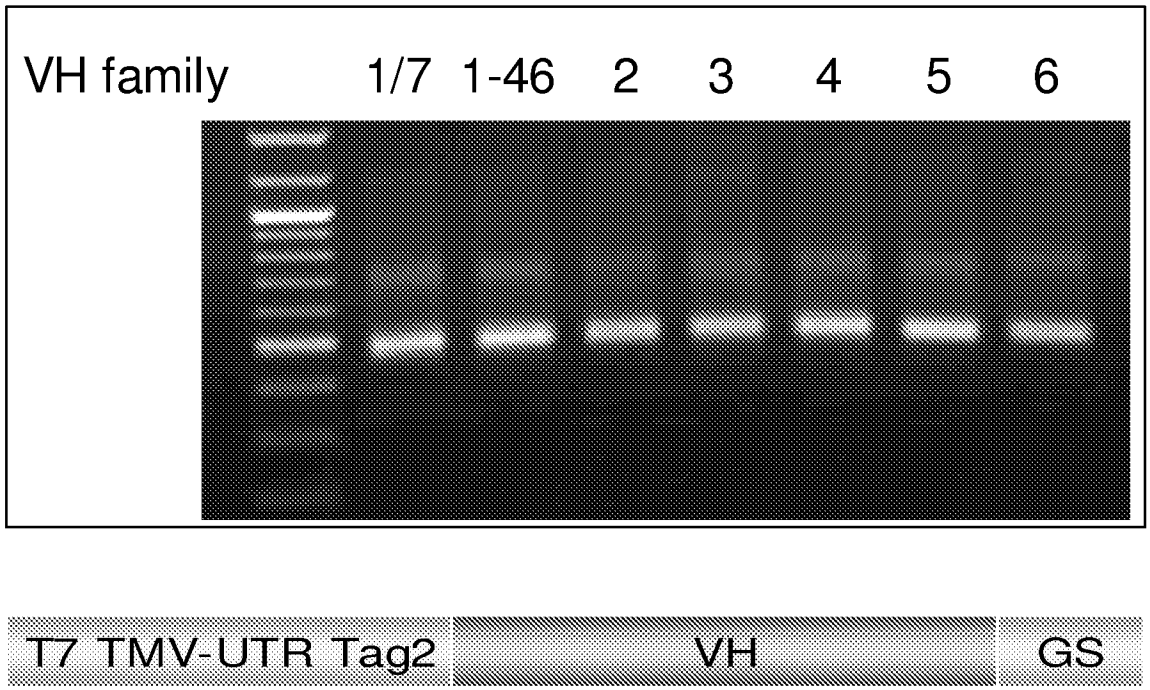


Fig. 15

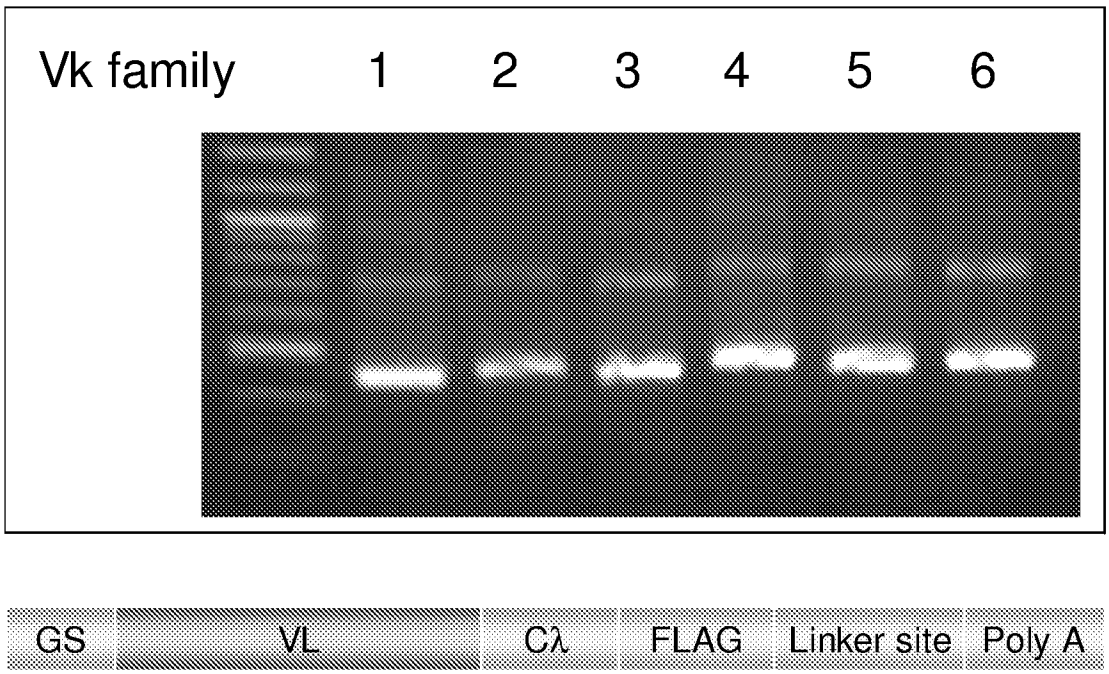
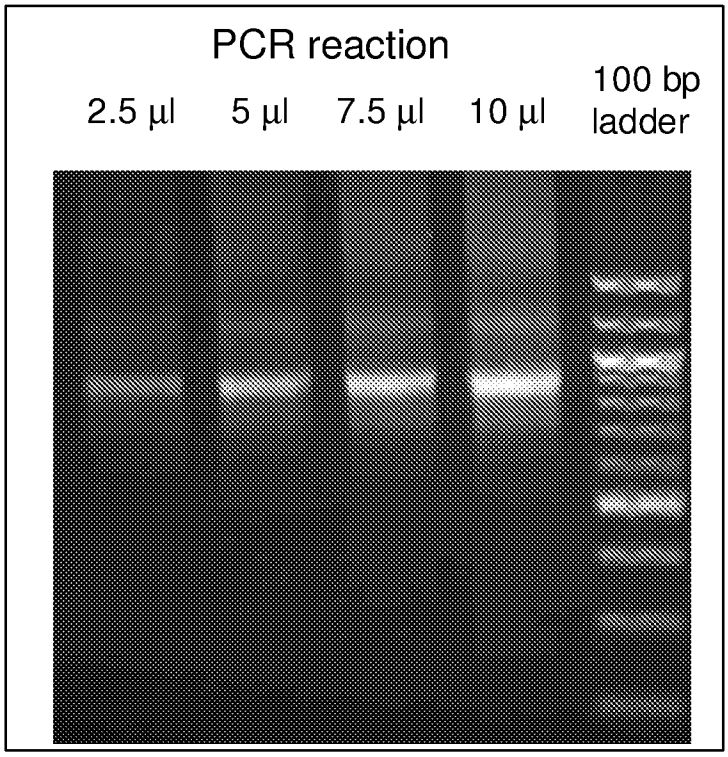


Fig. 16


PCR reaction
2.5 µl
5 µl
7.5 µl
10 µl
100 bp ladder


T7 TMV-UTR Tag2
VH
GS
VL
Cκ
FLAG
Linker site
Poly A

Fig. 17

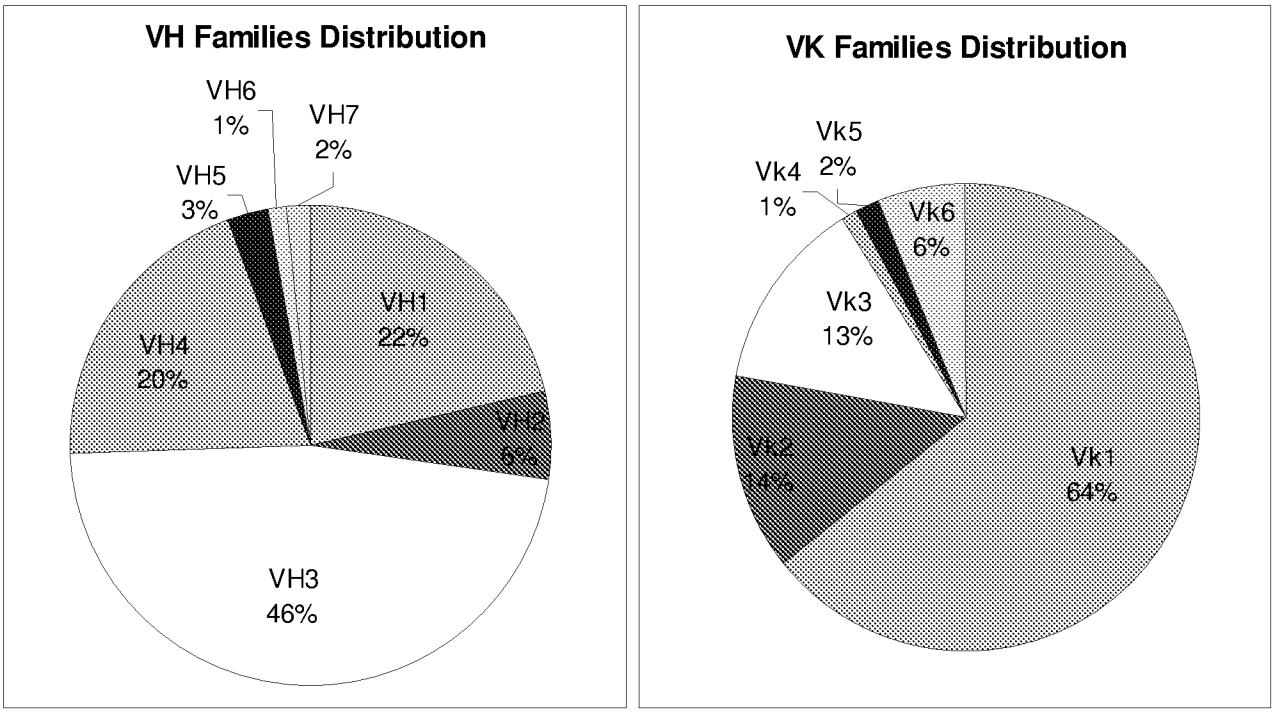

VH Families Distribution
VH6
1%
VH7
2%
VH5
3%
VH1
22%
VH4
20%
VH2
6%
VH3
46%
VK Families Distribution
Vk5
2%
Vk4
1%
Vk6
6%
Vk3
13%
Vk2
14%
Vk1
64%

Fig. 18

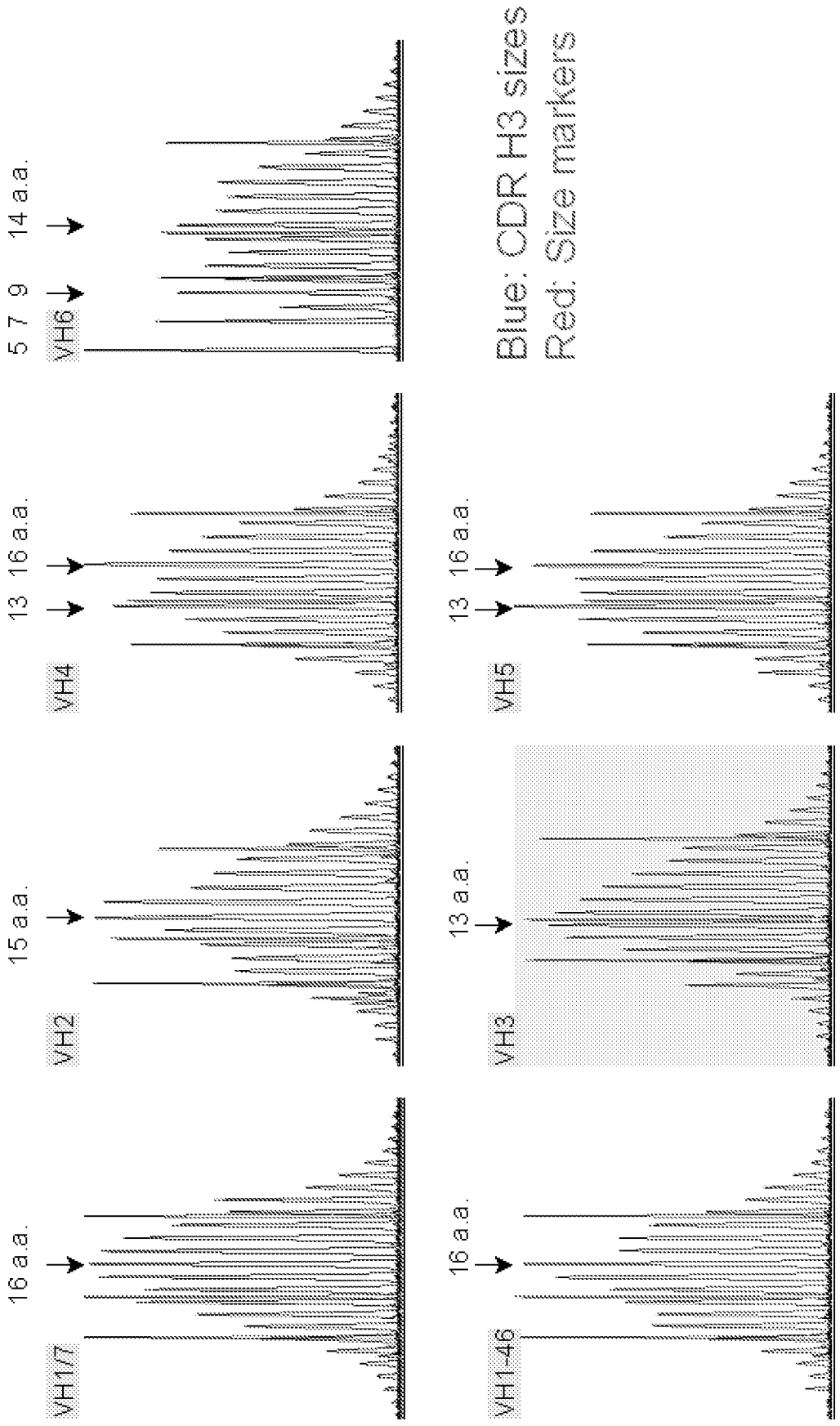

VH6
5 7 9
14 a.a.
Blue: CDR H3 sizes
Red: size markers
VH4
13 16 a.a.
VH5
13 16 a.a.
VH2
15 a.a.
VH3
13 a.a.
VH1/7
16 a.a.
VH1-46
16 a.a.

Fig. 19

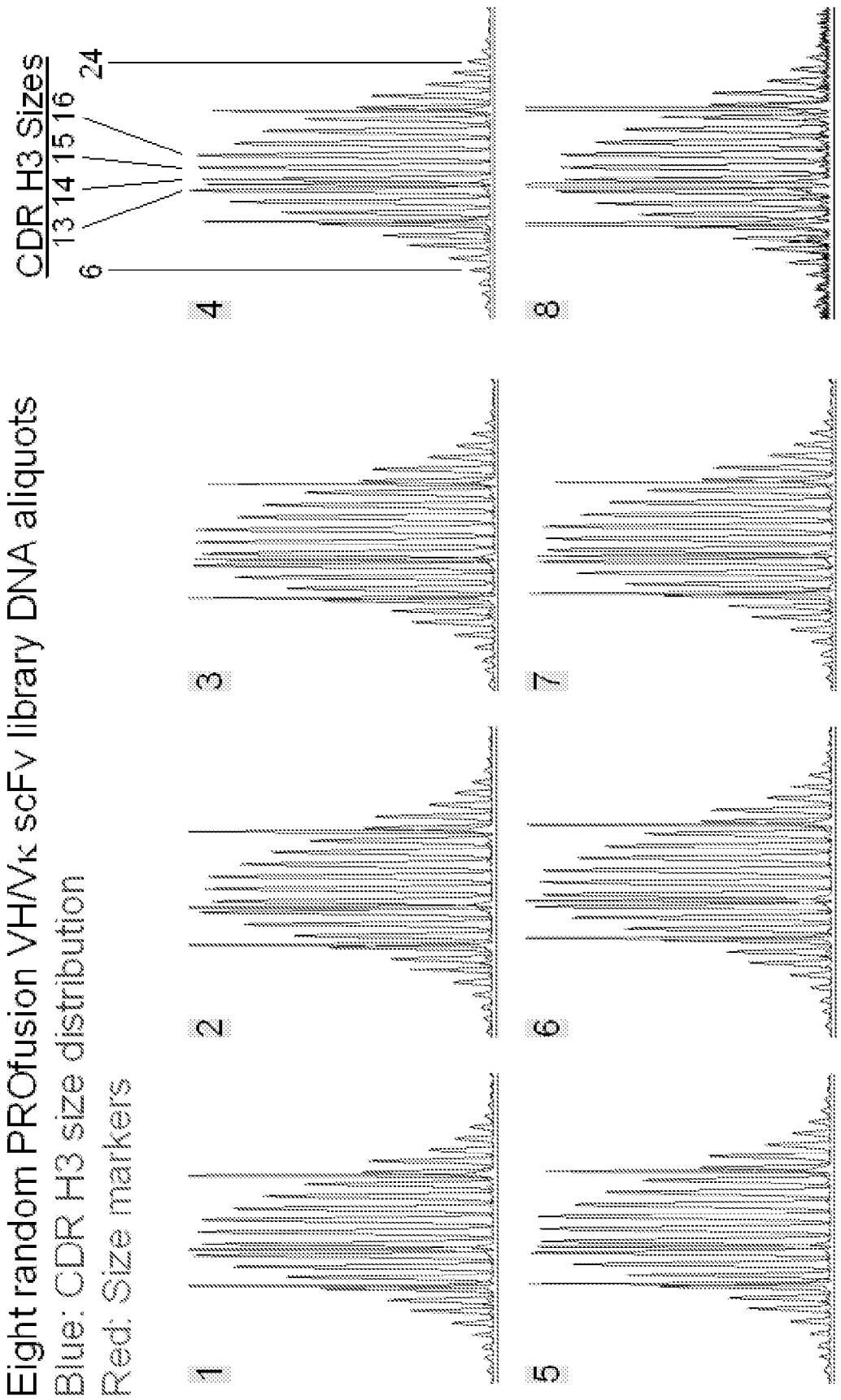
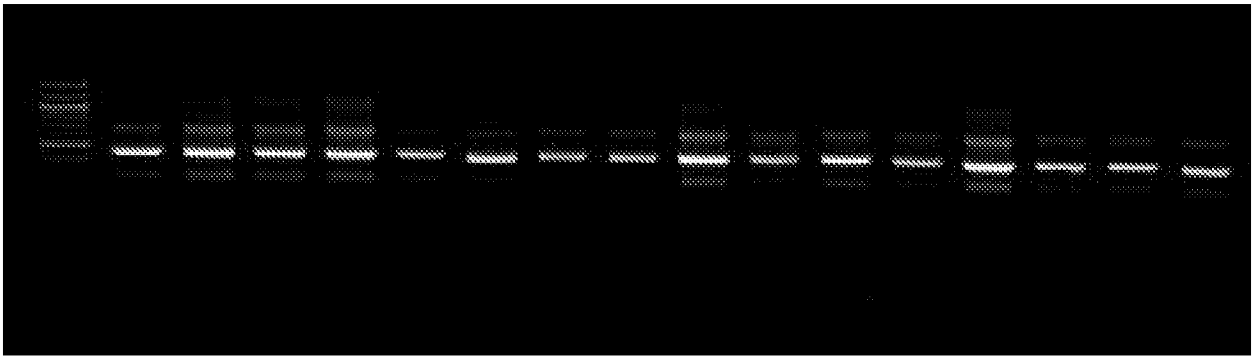

Eight random PROfusion VH/Vκ scFv library DNA aliquots
Blue: CDR H3 size distribution
Red: Size markers
CDR H3 Sizes
6
13 14 15 16
24
1
2
3
4
5
6
7
8

Fig. 20



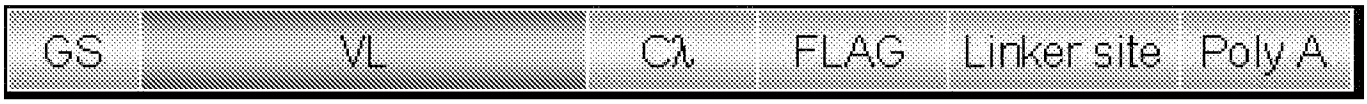


Fig. 21

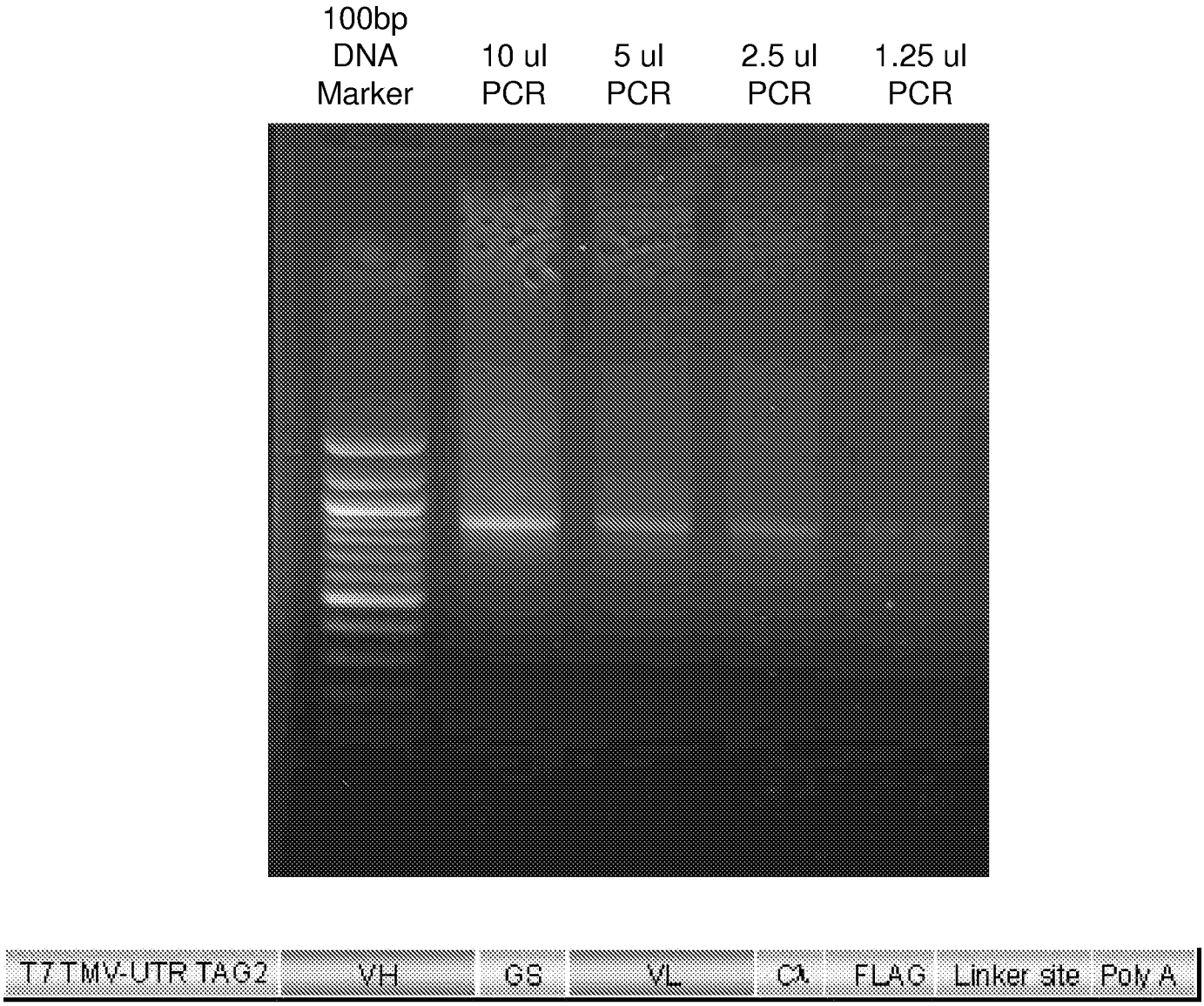

100bp DNA Marker
10 ul PCR
5 ul PCR
2.5 ul PCR
1.25 ul PCR
T7 TMV-UTR TAG2
VH
GS
VL
CA
FLAG
Linker site
Poly A

Fig. 22

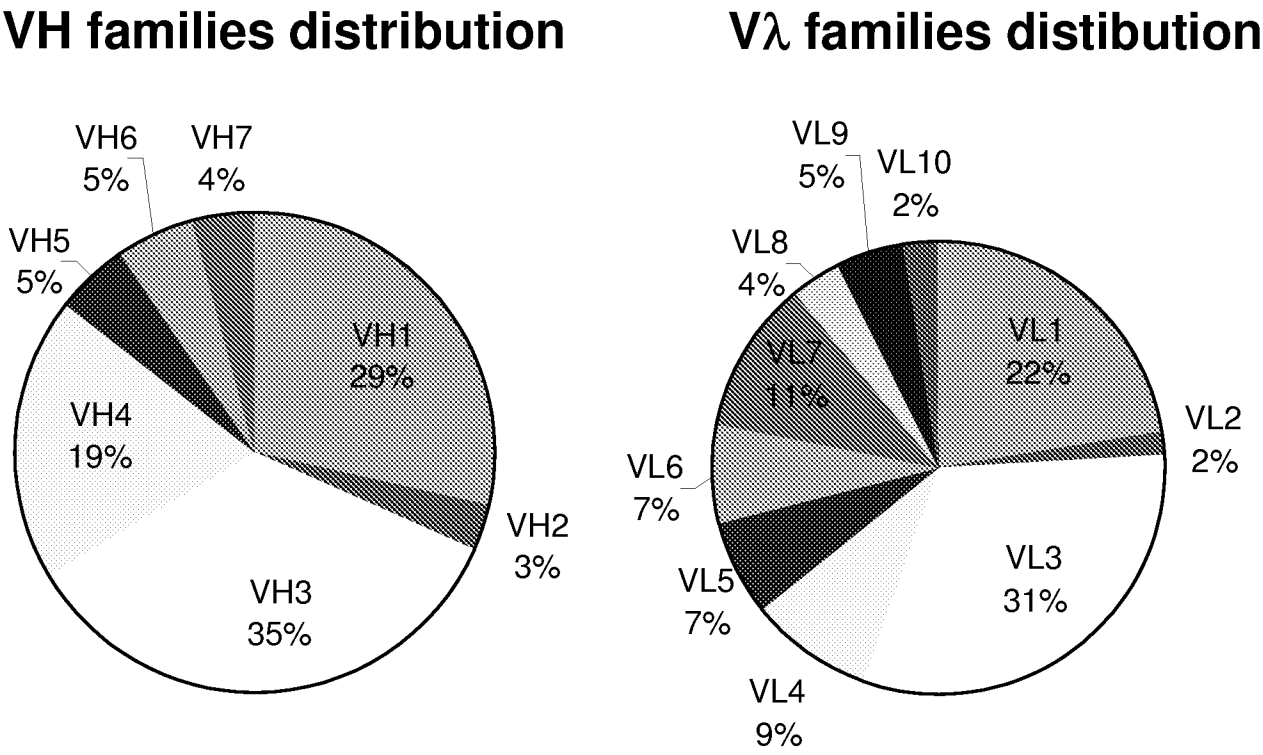

VH families distribution
VH6
5%
VH7
4%
VH5
5%
VH1
29%
VH4
19%
VH2
3%
VH3
35%
Vλ families distibution
VL9
5%
VL10
2%
VL8
4%
VL1
22%
VL7
11%
VL2
2%
VL6
7%
VL3
31%
VL5
7%
VL4
9%

Fig. 23

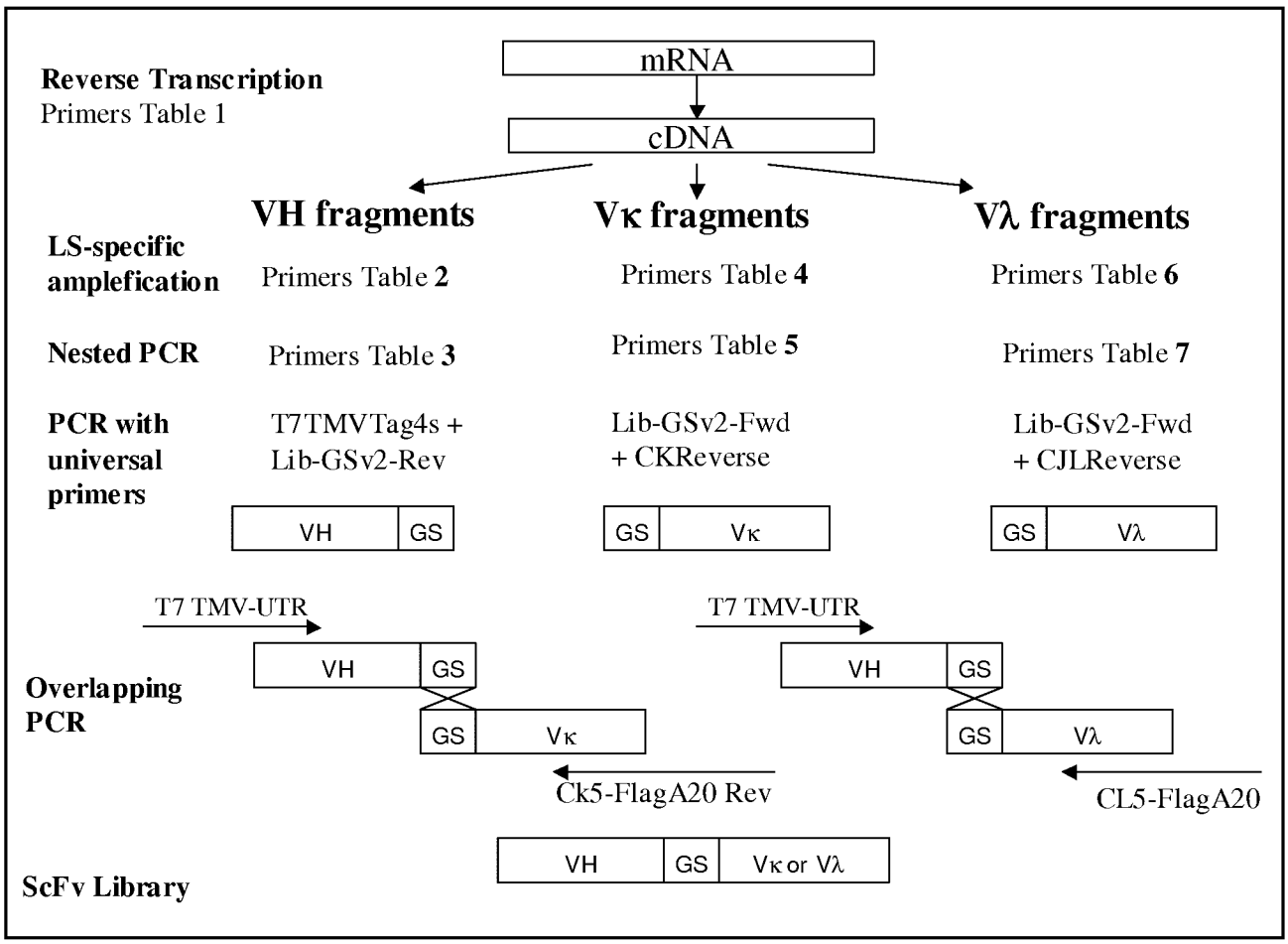
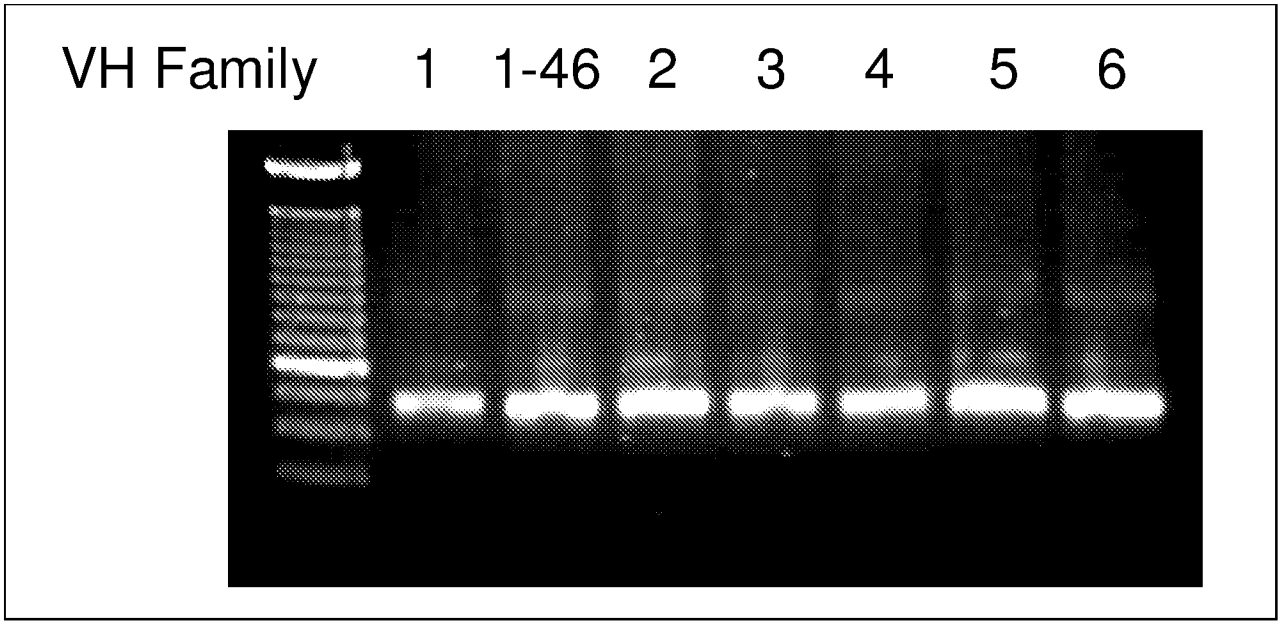

Reverse Transcription
Primers Table 1
mRNA
cDNA
VH fragments
Vκ fragments
Vλ fragments
LS-specific amplefication
Primers Table 2
Primers Table 4
Primers Table 6
Nested PCR
Primers Table 3
Primers Table 5
Primers Table 7
PCR with universal primers
T7TMVTag4s + Lib-GSv2-Rev
Lib-GSv2-Fwd + CKReverse
Lib-GSv2-Fwd + CJLReverse
VH
GS
GS
Vκ
GS
Vλ
T7 TMV-UTR
T7 TMV-UTR
Overlapping PCR
VH
GS
GS
Vκ
VH
GS
GS
Vλ
Ck5-FlagA20 Rev
CL5-FlagA20
VH
GS
Vκ or Vλ
ScFv Library

Fig. 24



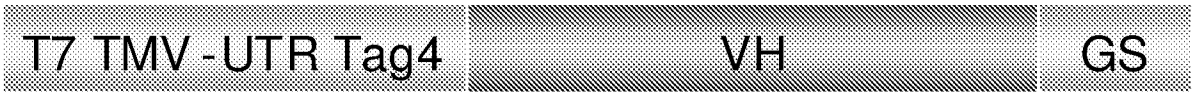
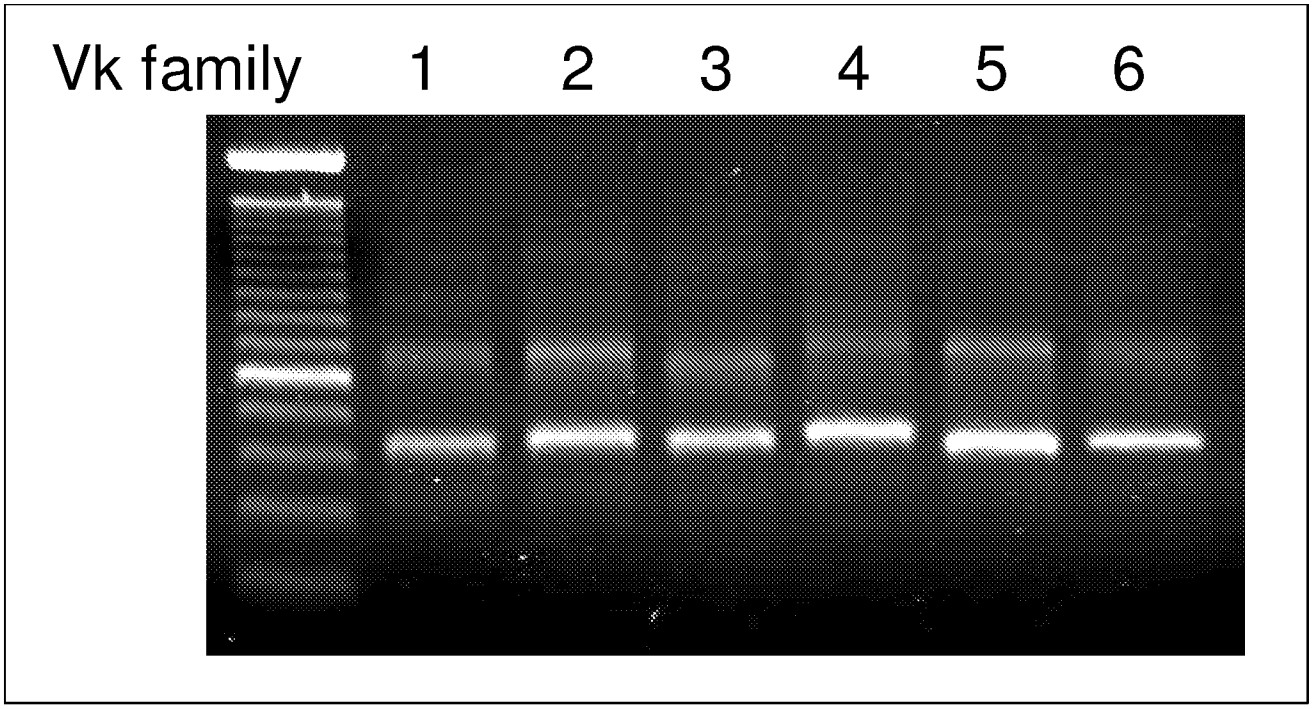


Fig. 25



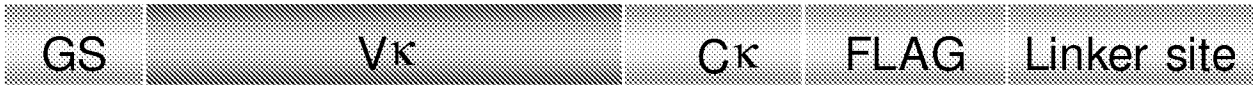
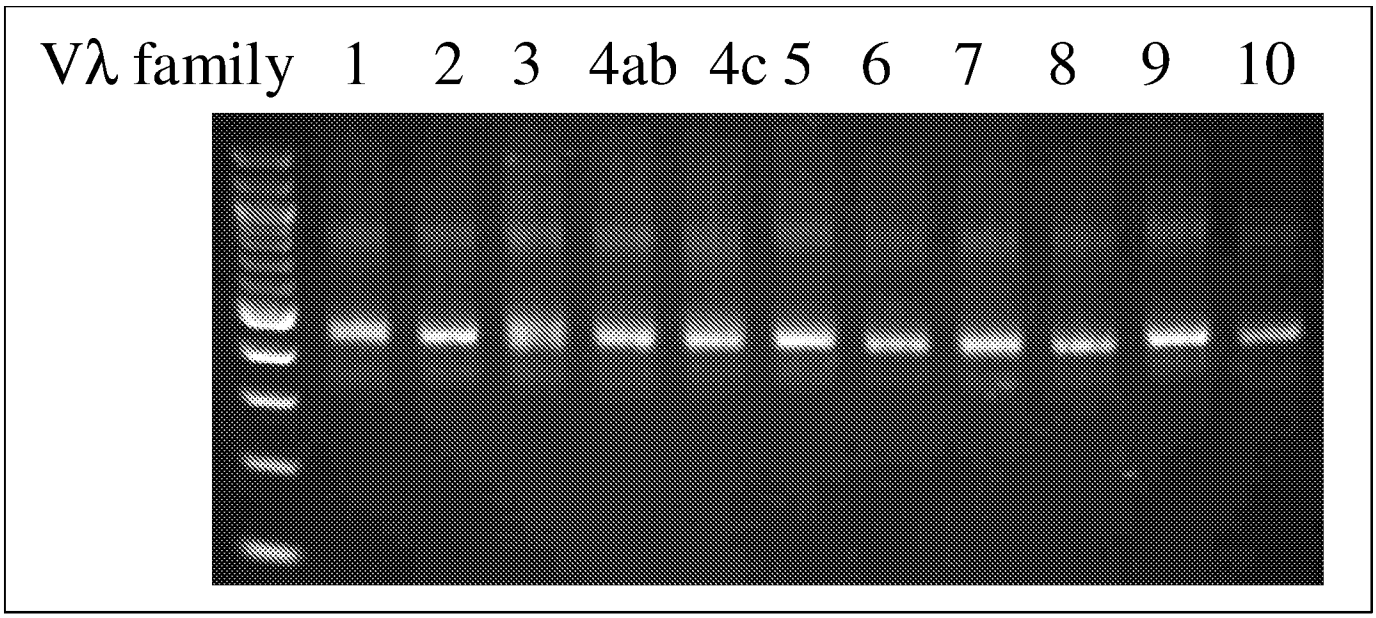


Fig. 26



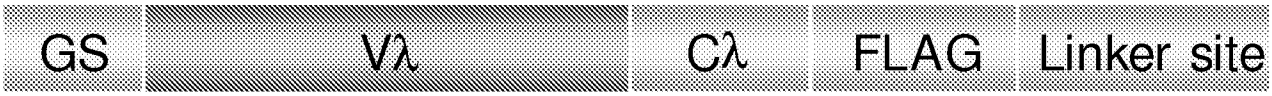


Fig. 27

VH-Vκ scFv VH-Vλ scFv

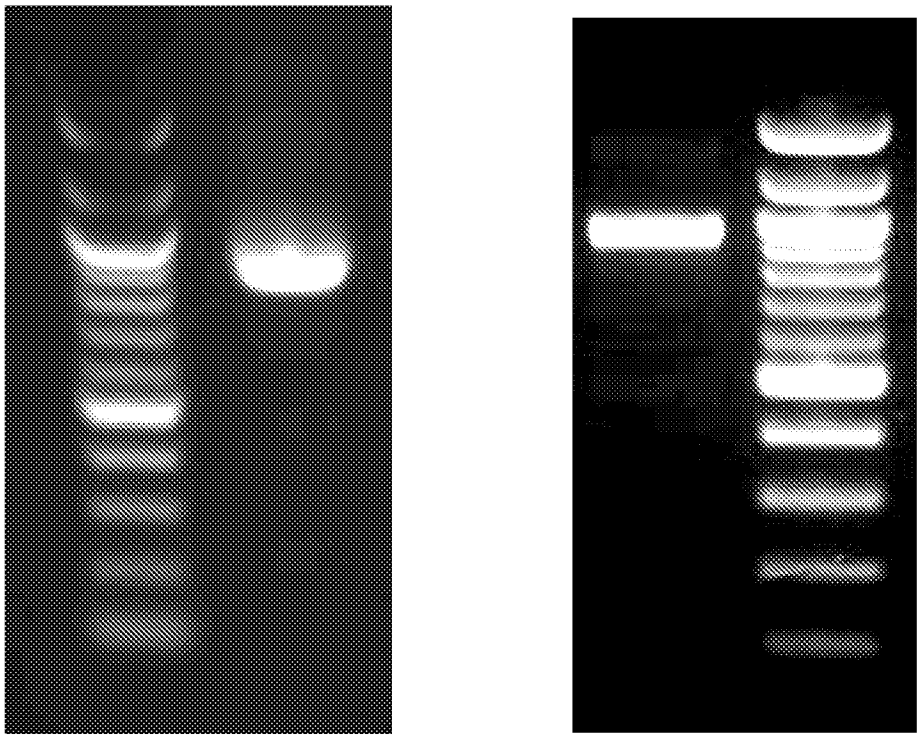


Fig. 28

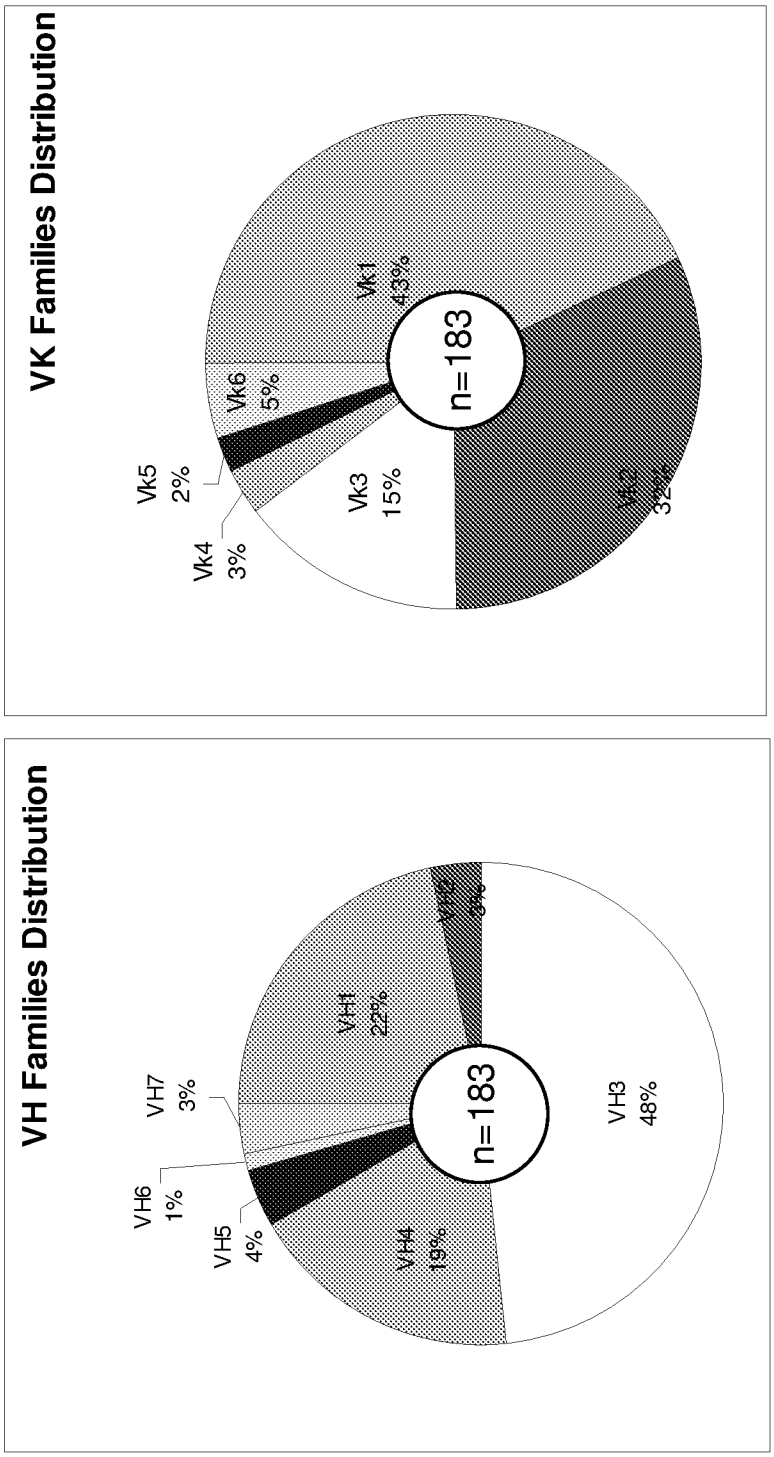

VK Families Distribution
n=183
Vk1 43%
Vk2 32%
Vk3 15%
Vk4 3%
Vk5 2%
Vk6 5%
VH Families Distribution
n=183
VH1 22%
VH2 4%
VH3 48%
VH4 19%
VH5 4%
VH6 1%
VH7 3%

Fig. 29

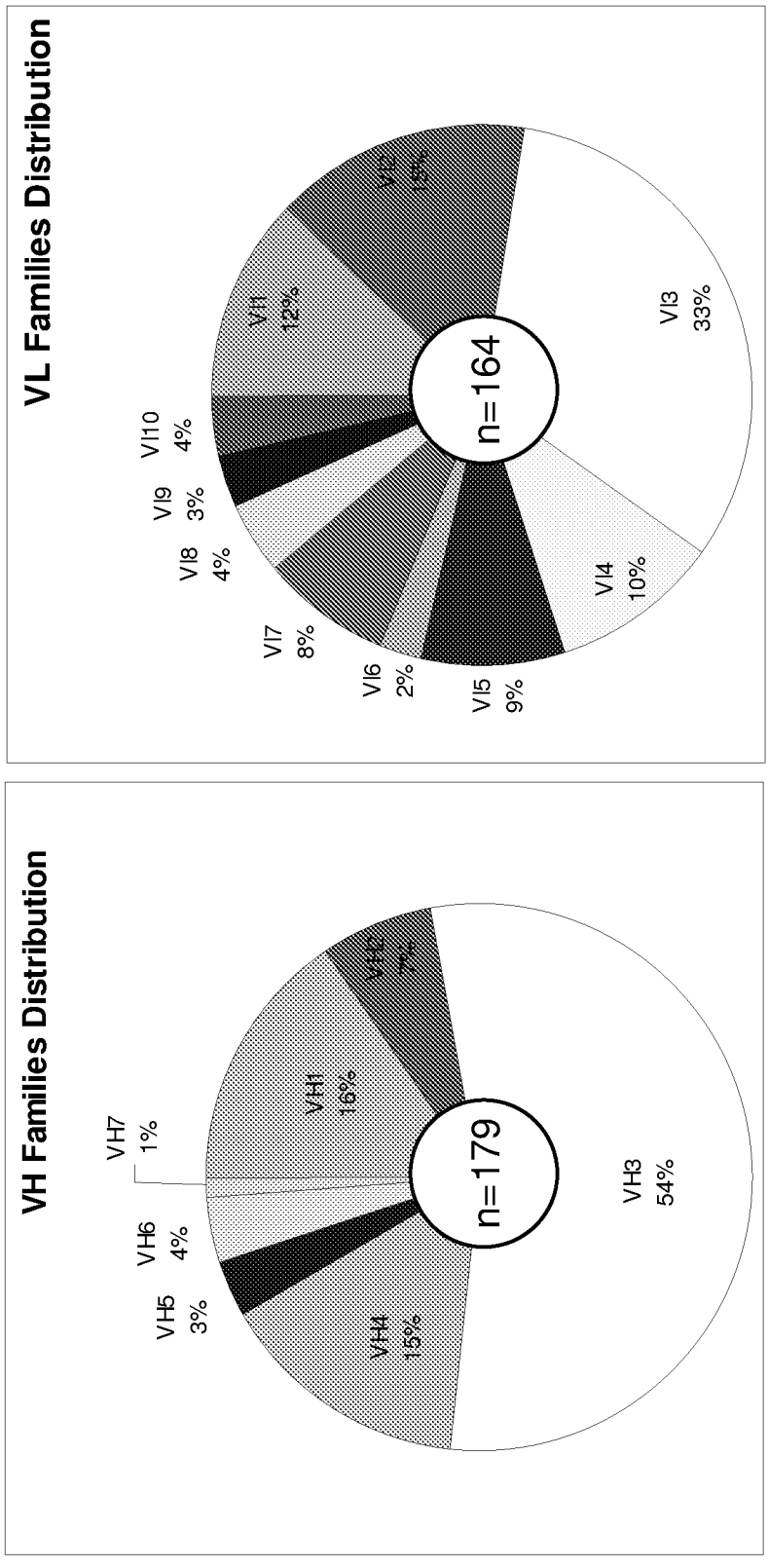

VL Families Distribution
n=164
VL1 12%
VL2 15%
VL3 33%
VL4 10%
VL5 9%
VL6 2%
VL7 8%
VL8 4%
VL9 3%
VL10 4%
VH Families Distribution
n=179
VH1 16%
VH2 7%
VH3 54%
VH4 15%
VH5 3%
VH6 4%
VH7 1%

Fig. 30

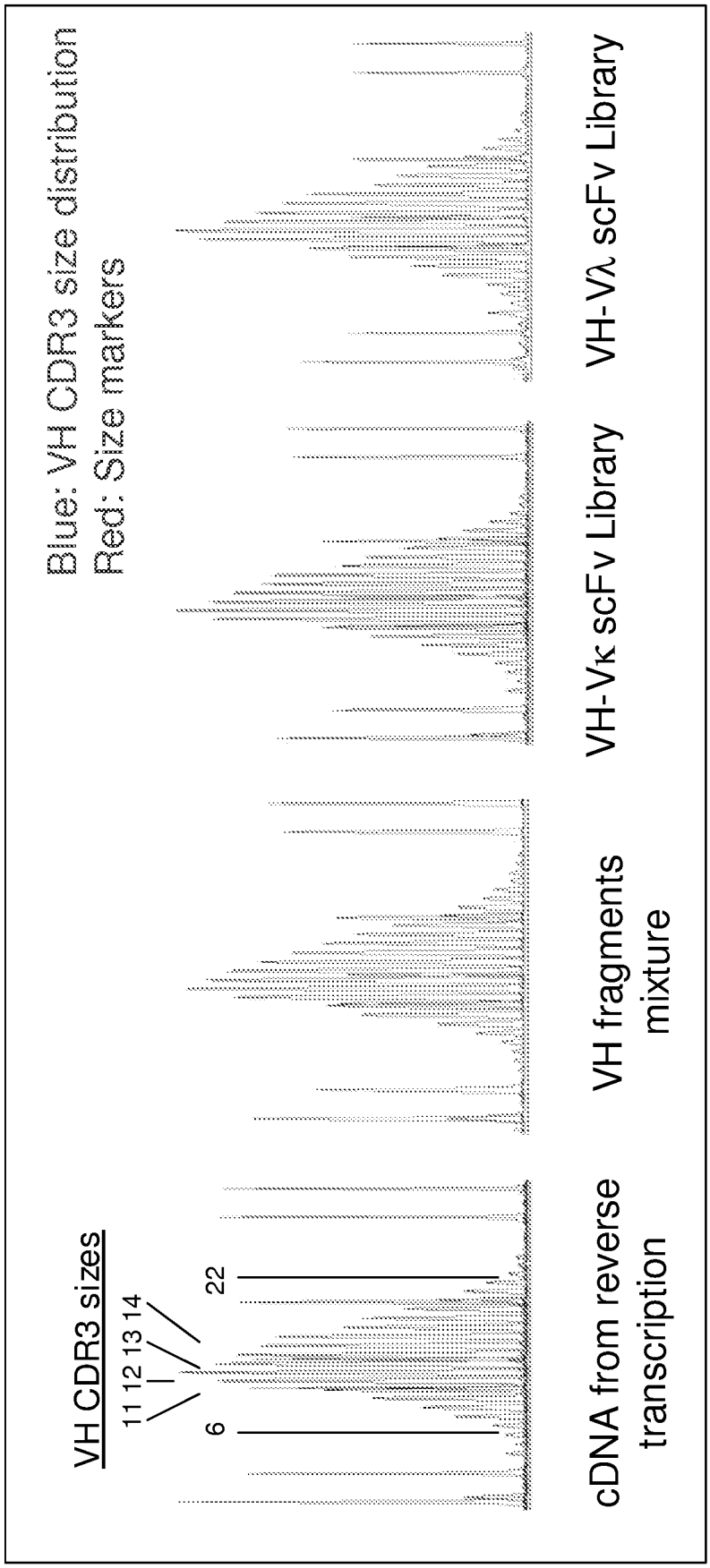

Blue: VH CDR3 size distribution
Red: Size markers
VH CDR3 sizes
11 12 13 14
22
6
cDNA from reverse transcription
VH fragments mixture
VH-Vκ scFv Library
VH-Vλ scFv Library

Fig. 31