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| (56) | Related Art<br><b>US 5,679,512 (Laney et al.) 21 October 1997</b><br><b>Hemmings-Mieszczak &amp; Hohn, RNA (1999), vol. 5, no. 9, pp. 1149-1157</b><br><b>Little et al., J. Immunol. Meth. (1999), vol. 231, no. 1-2, pp. 3-9</b><br><b>Orum et al., Nuc. Acids Res. (1993), vol. 21, no. 19, pp. 4491-4498</b><br><b>Li et al., Nuc. Acids Res. (2000), vol. 28, no. 13, pp. 2605-2612</b> |                                |  |

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A3

(54) Title: ENGINEERED TEMPLATES AND THEIR USE IN SINGLE PRIMER AMPLIFICATION

(57) **Abstract:** Methods of amplifying nucleic acid have now been discovered which include the steps of: a) annealing a primer to a template nucleic acid sequence, the primer having a first portion which anneals to the template and a second portion of predetermined sequence; b) synthesizing a polynucleotide that anneals to and is complementary to the portion of the template between the location at which the first portion of the primer anneals to the template and the end of the template, the polynucleotide having a first end and a second end, wherein the first end incorporates the primer; c) separating the polynucleotide synthesized in step (b) from the template; d) annealing a nested oligonucleotide to the second end of the polynucleotide synthesized in step (b), the nested oligonucleotide having a first portion that anneals to the second end of the polynucleotide and a second portion having the same predetermined sequence as the second portion of the primer; e) extending the polynucleotide synthesized in step (b) to provide a terminal portion thereof that is complementary to the predetermined sequence; and f) amplifying the extended polynucleotide using a single primer having the predetermined sequence.

**WO 2003/025202 A3**

**ENGINEERED TEMPLATES AND THEIR USE  
IN SINGLE PRIMER AMPLIFICATION**

**Related Applications**

This application claims priority to U.S. Provisional Application No. 60/323,455 filed September 19, 2001.

**Technical Field**

This disclosure relates to engineered templates useful for amplification of a target nucleic acid sequence. More specifically, templates which are engineered to contain complementary sequences at opposite ends thereof are provided by a nested oligonucleotide extension reaction (NOER). The engineered template allows Single Primer Amplification (SPA) to amplify a target sequence within the engineered template. In particularly useful embodiments, the target sequences from the engineered templates are cloned into expression vehicles to provide a library of polypeptides or proteins, such as, for example, an antibody library.

**Background of Related Art**

Methods for nucleic acid amplification and detection of amplification products assist in the detection, identification, quantification and sequence analysis of nucleic acid sequences. Nucleic acid amplification is an important step in the construction of libraries from related genes such as, for example antibodies. These libraries can be screened for antibodies having specific, desirable activities. Nucleic acid analysis is important for detection and identification of pathogens, detection of gene alteration leading to defined phenotypes, diagnosis of genetic diseases or the susceptibility to a disease, assessment of gene expression in development, disease and in response to defined stimuli, as well as the

various genome projects. Other applications of nucleic acid amplification method include the detection of rare cells, detection of pathogens, and the detection of altered gene expression in malignancy, and the like. Nucleic acid amplification is also useful for qualitative analysis (such as, for example, the detection of the presence of defined nucleic acid sequences) and quantification of defined gene sequences (useful, for example, in assessment of the amount of pathogenic sequences as well as the determination of gene multiplication or deletion, and cell transformation from normal to malignant cell type, etc.). The detection of sequence alterations in a nucleic acid sequence is important for the detection of mutant genotypes, as relevant for genetic analysis, the detection of mutations leading to drug resistance, pharmacogenomics, etc.

10 There are many variations of nucleic acid amplification, for example, exponential amplification, linked linear amplification, ligation-based amplification, and transcription-based amplification. One example of exponential nucleic acid amplification method is polymerase chain reaction (PCR) which has been disclosed in numerous publications.

15 See, for example, Mullis et al. Cold Spring Harbor Symp. Quant. Biol. 51:263-273 (1986); Mullis K. EP 201,184; Mullis et al. U.S. Pat. No. 4,582,788; Erlich et al. EP 50,424, EP 84,796, EP 258,017, EP 237,362; and Saiki R. et al. U.S. Pat. No. 4,683,194. In fact, the polymerase chain reaction (PCR) is the most commonly used target amplification method. PCR is based on multiple cycles of denaturation, hybridization of 20 two different oligonucleotide primers, each to opposite strand of the target strands, and primer extension by a nucleotide polymerase to produce multiple double stranded copies of the target sequence.

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Amplification methods that employ a single primer, have also been disclosed.

See, for example, U.S. Pat. Nos. 5,508,178; 5,595,891; 5,683,879; 5,130,238; and 5,679,512. The primer can be a DNA/RNA chimeric primer, as disclosed in U.S. Pat. No. 5,744,308.

5 Some amplification methods use template switching oligonucleotides (TSOs) and blocking oligonucleotides. For example, a template switch amplification in which chimeric DNA primer are utilized is disclosed in U.S. Pat. Nos. 5,679,512; 5,962,272; 6,251,639 and by Patel et al. Proc. Natl. Acad. Sci. U.S.A. 93:2969-2974 (1996).

10 However the previously described target amplification methods have several drawbacks. For example, the transcription base amplification methods, such as Nucleic Acid Sequence Based Amplification (NASBA) and transcription mediated amplification (TMA), are limited by the need for incorporation of the polymerase promoter sequence into the amplification product by a primer, a process prone to result in non-specific amplification. Another example of a drawback of the current amplification methods is the 15 requirement of two binding events which may have optimal binding at different temperatures as well as the use of primers containing naturally occurring sequences. This combination of factors results in increased likelihood of mis-priming and resultant amplification of sequences other than the target sequence.

20 Therefore, there is a need for improved nucleic acid amplification methods that overcome these drawbacks. The invention provided herein fulfills this need and provides additional benefits.

Reference to any prior art in the specification is not, and should not be taken as, an acknowledgment, or any form of suggestion, that this prior art forms part of the common general knowledge in Australia or any other jurisdiction or that this prior art could reasonably be expected to be ascertained, understood and regarded as relevant by a person skilled in the art.

**Summary**

Novel methods of amplifying nucleic acid have now been discovered which include the steps of: a) annealing a primer to a template nucleic acid sequence, the primer having a first portion which anneals to the template and a second portion of predetermined sequence; b) synthesizing a polynucleotide that anneals to and is 5 complementary to the portion of the template between the location at which the first portion of the primer anneals to the template and the end of the template, the polynucleotide having a first end and a second end, wherein the first end incorporates the primer; c) separating the polynucleotide synthesized in step (b) from the template; d) annealing a nested oligonucleotide to the second end of the polynucleotide synthesized in 10 step (b), the nested oligonucleotide having a first portion that anneals to the second end of the polynucleotide and a second portion having the same predetermined sequence as the second portion of the primer; e) extending the polynucleotide synthesized in step (b) to provide a terminal portion thereof that is complementary to the predetermined sequence; and f) amplifying the extended polynucleotide using a single primer having the 15 predetermined sequence.

In an alternative embodiment, the method includes the steps of a) annealing a primer and a boundary oligonucleotide to a template nucleic acid sequence, the primer having a first portion which anneals to the template and a second portion of predetermined sequence; b) synthesizing a polynucleotide that anneals to and is 20 complementary to the portion of the template between the location at which the first portion of the primer anneals to the template and the portion of the template to which the boundary oligonucleotide anneals, the polynucleotide having a first end and a second end,

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wherein the first end incorporates the primer; c) separating the polynucleotide synthesized in step (b) from the template; d) annealing a nested oligonucleotide to the second end of the polynucleotide synthesized in step (b), the nested oligonucleotide having a first portion that anneals to the second end of the polynucleotide and a second 5 portion having the same predetermined sequence as the second portion of the primer; e) extending the polynucleotide synthesized in step (b) to provide a terminal portion thereof that is complementary to the predetermined sequence; and f) amplifying the extended polynucleotide using a single primer having the predetermined sequence.

It is also contemplated that a engineered nucleic acid strand having a 10 predetermined sequence at a first end thereof and a sequence complementary to the predetermined sequence at the other end thereof is itself a novel aspect of this disclosure.

In another aspect, this disclosure provides a new method of amplifying a nucleic acid strand that includes the steps of providing an engineered nucleic acid strand having a predetermined sequence at a first end thereof and a sequence complementary to the 15 predetermined sequence at the other end thereof; and contacting the engineered nucleic acid strand with a primer having the predetermined sequence in the presence of a polymerase and nucleotides under conditions suitable for polymerization of the nucleotides.

The amplification processes and engineered templates described herein can be 20 used to prepare amplified products that can be ligated into a suitable expression vector. The vector may then be used to transform an appropriate host organism using standard methods to produce the polypeptide or protein encoded by the target sequence. In particularly useful embodiments, the techniques described herein are used to amplify a family of related sequences to build a complex library, such as, for example an antibody 25 library.

As used herein, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to exclude other additives, components, integers or steps.

**Brief Description of Drawings**

Fig. 1 is a schematic illustration of a primer and boundary oligo annealed to a template;

Fig. 2A is a schematic illustration of a restriction oligo annealed to a nucleic acid strand;

Fig. 2B is a schematic illustration of a primer annealed to a template that has a shortened

5' 5' end;

Fig. 3 is a schematic illustration of an alternate embodiment wherein multiple rounds of polymerization are performed and a restriction oligonucleotide is annealed to the newly synthesized strands, rather than to the original template;

Fig. 4 is a schematic illustration of a nested oligo annealed to a newly synthesized nucleic acid strand;

Fig. 5 is a schematic illustration of an engineered template in accordance with this disclosure;

Fig. 6 is a schematic illustration of the single primer amplification of an engineered template;

Fig. 7 shows the sequence of the nested oligo designated TMX24CMnpt;

Figs. 8a-c show the sequences of isolated Fabs produced in Example 3; and

Figs. 9a-d show the sequences of isolated Fabs produced in Example 5.

**Detailed Description of Preferred Embodiments**

The present disclosure provides a method of amplifying a target nucleic acid sequence. In particularly useful embodiments, the target nucleic acid sequence is a gene encoding a polypeptide or protein. The disclosure also describes how the products of the amplification may be cloned and expressed in suitable expression systems. In particularly useful embodiments, the techniques described herein are used to amplify a

family of related sequences to build a complex library, such as, for example an antibody library.

The target nucleic acid sequence is exponentially amplified through a process that involves only a single primer. The ability to employ a single primer (i.e., without the 5 need for both forward and reverse primers each having different sequences) is achieved by engineering a strand of nucleic acid that contains the target sequence to be amplified. The engineered strand of nucleic acid (sometimes referred to herein as the "engineered template") is prepared from two templates; namely, 1) a starting material that is a natural or synthetic nucleic acid (e.g., DNA or cDNA) containing the sequence to be amplified 10 and 2) a nested oligonucleotide. The starting material can be considered the original template. The nested oligonucleotide is used as a template to extend the nucleotide sequence of the original template during creation of the engineered strand of nucleic acid. The engineered strand of nucleic acid is created from the original template by a series of manipulations that result in the presence of complementary sequences at opposite ends 15 thereof. It is these complementary sequences that allow amplification using only a single primer.

Any nucleic acid, in purified or nonpurified form, can be utilized as the starting material for the processes described herein provided it contains or is suspected of containing the target nucleic acid sequence to be amplified. Thus, the starting material 20 employed in the process may be, for example, DNA or RNA, including messenger RNA, which DNA or RNA may be single stranded or double stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of any of these nucleic acids may also be employed, or the nucleic acids produced from a previous

amplification reaction herein using the same or different primers may be utilized. The target nucleic acid sequence to be amplified may be a fraction of a larger molecule or can be present initially as a discrete molecule. The starting nucleic acid may contain more than one desired target nucleic acid sequence which may be the same or different.

5 Therefore, the present process may be useful not only for producing large amounts of one target nucleic acid sequence, but also for amplifying simultaneously more than one different target nucleic acid sequence located on the same or different nucleic acid molecules.

The nucleic acids may be obtained from any source, for example: genomic or  
10 cDNA libraries, plasmids, cloned DNA or RNA, or from natural DNA or RNA from any source, including bacteria, yeast, viruses, and higher organisms such as plants or animals. The nucleic acids can be naturally occurring or may be synthetic, either totally or in part. Techniques for obtaining and producing the nucleic acids used in the present invention are well known to those skilled in the art. If the nucleic acid contains two strands, it is  
15 necessary to separate the strands of the nucleic acid before it can be used as the original template, either as a separate step or simultaneously with the synthesis of the primer extension products. Additionally, if the starting material is first strand DNA, second strand DNA may advantageously be created by processes within the purview of those skilled in the art and used as the original template from which the engineered template is  
20 created.

First strand cDNA is a particularly useful original template for the present methods. Suitable methods for generating DNA templates are known to and readily selected by those skilled in the art. In a preferred embodiment, 1<sup>st</sup> strand cDNA is

synthesized in a reaction where reverse transcriptase catalyzes the synthesis of DNA complementary to any RNA starting material in the presence of an oligodeoxynucleotide primer and the four deoxynucleoside triphosphates, dATP, dGTP, dCTP, and TTP. The reaction is initiated by annealing of the oligo-deoxynucleotide primer to the 3' end of 5 mRNA followed by stepwise addition of the appropriate deoxynucleotides as determined by base pairing relationships with the mRNA nucleotide sequence, to the 3' end of the growing chain. As those skilled in the art will appreciate, all mRNA in a sample can be used to generate first strand cDNA through the annealing of oligo dT to the polyA tail of the mRNA.

10 Once the original template is obtained, a primer 20 and a boundary oligonucleotide 30 are annealed to the original template 10. (See Fig. 1.) A strand of nucleic acid complementary to the portion of the original template beginning at the 3' end of the primer up to about the 5' end of the boundary oligonucleotide is polymerized.

The primer 20 that is annealed to the original template includes a first portion 22 15 of predetermined sequence that preferably does not anneal to the original template and a second portion 25 that anneals to the original template, and optionally includes a restriction site 23 between the first and second portions. The primer anneals to the original template adjacent to the target sequence 12 to be amplified. It is contemplated that the primer can anneal to the original template upstream of the target sequence to be 20 amplified, or that the primer may overlap the beginning of the target sequence 12 to be amplified as shown in Fig. 1. The predetermined sequence of the non-annealing portion 22 of the primer is not native in the original template and is selected so as to provide a sequence to which the single primer used during the amplification process can hybridize

as described in detail below. Optionally, the predetermined sequence may include a restriction site useful for insertion of a portion of the engineered template into an expression vector as described more fully hereinbelow.

The boundary oligonucleotide 30 that is annealed to the original template serves 5 to terminate polymerization of the nucleic acid. Any oligonucleotide capable of terminating nucleic acid polymerization may be utilized as the boundary oligonucleotide 30. In a preferred embodiment the boundary oligonucleotide includes a first portion 35 that anneals to the original template 10 and a second portion 32 that is not susceptible to an extension reaction. Techniques to prevent the boundary oligo from acting as a site for 10 extension are within the purview of one skilled in the art. By way of example, portion 32 of the boundary oligo 30 may be designed so that it does not anneal to the original template 10 as shown in Fig. 1. In such embodiments, the boundary oligonucleotide 30 prevents further polymerization but does not serve as a primer for nucleic acid synthesis because the 3' end thereof does not hybridize with the original template 10. Alternatively, 15 the 3' end of the boundary oligo 30 might be designed to include locked nucleic acid to achieve the same effect. Locked nucleic acid is disclosed for example in WO 99/14226, the contents of which are incorporated herein by reference. Those skilled in the art will envision other ways of ensuring that no extension of the 3' end of the boundary oligo occurs.

20 Primers and oligonucleotides described herein may be synthesized using established methods for oligonucleotide synthesis which are well known in the art. Oligonucleotides, including primers of the present invention include linear oligomers of natural or modified monomers or linkages, such as deoxyribonucleotides,

ribonucleotides, and the like, which are capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to monomer interactions such as Watson-Crick base pairing. Usually monomers are linked by phosphodiester bonds or their analogs to form oligonucleotides ranging in size from a few monomeric units e.g., 5 3-4, to several tens of monomeric units. A primer is typically single-stranded, but may be double-stranded. Primers are typically deoxyribonucleic acids, but a wide variety of synthetic and naturally occurring primers known in the art may be useful for the methods of the present disclosure. A primer is complementary to the template to which it is designed to hybridize to serve as a site for the initiation of synthesis, but need not reflect 10 the exact sequence of the template. In such a case, specific hybridization of the primer to the template depends on the stringency of the hybridization conditions. Primers may be labeled with, e.g., chromogenic, radioactive, or fluorescent moieties and used as detectable moieties.

Polymerization of nucleic acid can be achieved using methods known to those 15 skilled in the art. Polymerization is generally achieved enzymatically, using a DNA polymerase which sequentially adds free nucleotides according to the instructions of the template. Several different DNA polymerases are suitable for use in the present process. In a certain embodiments, the criteria for selection includes lack of exonuclease activity or DNA polymerases which do not possess a strong exonuclease . DNA polymerases 20 with low exonuclease activity for use in the present process may be isolated from natural sources or produced through recombinant DNA techniques. Illustrative examples of polymerases that may be used, are, without limitation, T7 Sequenase v. 2.0, the Klenow Fragment of DNA polymerase I lacking exonuclease activity, the Klenow Fragment of

Taq Polymerase, exo.- Pfu DNA polymerase, Vent. (exo.-) DNA polymerase, and Deep Vent. (exo-) DNA polymerase.

In a particularly useful embodiment, the use of a boundary oligonucleotide is avoided by removing unneeded portions of the starting material by digestion. In this 5 embodiment, which is shown schematically in Fig. 2A, a restriction oligonucleotide 70 is annealed to the starting material 100 at a preselected location. The restriction oligonucleotide provides a double stranded portion on the starting material containing a restriction site 72. Suitable restriction sites, include, but are not limited to Xho I, Spe I, Nhe1, Hind III, Nco I, Xma I, Bgl II, Bst I, and Pvu I. Upon exposure to a suitable 10 restriction enzyme, the starting material is digested and thereby shortened to remove unnecessary sequence while preserving the desired target sequence 12 (or portion thereof) to be amplified on what will be used as the original template 110. Once the original template 110 is obtained, a primer 20 is annealed to the original template 110 (see Fig. 2B) adjacent to or overlapping with the target sequence 12 as described above in 15 connection with previous embodiments. A strand of nucleic acid 40 complementary to the portion of the original template between the 3' end of the primer 20 and the 5' end of the original template 110 is polymerized. As those skilled in the art will appreciate, in this embodiment where a restriction oligonucleotide is employed to generate the original template, there is no need to use a boundary oligonucleotide, because primer extension 20 can be allowed to proceed all the way to the 5' end of the shortened original template 110.

Once polymerization is complete (i.e., growing strand 40 reaches the boundary oligonucleotide 30 or the 5' end of the shortened original template 110), the newly

synthesized complementary strand is separated from the original template by any suitable denaturing method including physical, chemical or enzymatic means. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or the enzyme RecA, which has helicase activity and in the presence of riboATP is known to 5 denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are described by Cold Spring Harbor Symposia on Quantitative Biology, Vol. XLIII "DNA: Replication and Recombination" (New York: Cold Spring Harbor Laboratory, 1978), B. Kuhn et al., "DNA Helicases", pp. 63-67, and techniques for using RecA are reviewed in C. Radding, Ann. Rev. Genetics, 16:405-37 (1982).

10 The newly synthesized complementary strand thus includes sequences provided by the primer 20 (e. g., the predetermined sequence 22, the optional restriction site 23 and the annealing portion 25 of the primer) as well as the newly synthesized portion 45 that is complementary to the portion of the original template 10 between the location at which the primer 20 was annealed to the original template 10 and either the portion of the 15 original template 10 to which the boundary oligonucleotide 30 was annealed or the shortened 5' end of the original template. See Fig. 4.

20 Optionally, multiple rounds of polymerization (preferably, 15-25 rounds) using the original template and a primer are performed to produce multiple copies of the newly synthesized complementary strand for use in subsequent steps. Making multiple copies of the newly synthesized complementary strand at this point in the process (instead of waiting until the entire engineered template is produced before amplifying) helps ensure that accurate copies of the target sequence are incorporated into the engineered templates ultimately produced. It is believed that multiple rounds of polymerization based on the

original template provides a greater likelihood that a better representation of all members of the library will be achieved, therefore providing greater diversity compared to a single round of polymerization.

In an alternative embodiment, newly synthesized strands are produced by annealing primer 20 as described above to original template 10 and performing multiple rounds of polymerization, without either the presence of a blocking oligonucleotide or removing a portion of the original template. In this embodiment, which is shown schematically in Fig. 3, the primer is extended along the full length of the original template to provide a full length newly synthesized strand 140. Next, a restriction oligonucleotide 170 is hybridized to the full length newly synthesized strand. The restriction oligonucleotide provides a double stranded portion on the newly synthesized strand containing a restriction site. Suitable restriction sites, include, but are not limited to Xho I, Spe I, Nhe I, Hind III, Nco I, Xma I, Bgl II, Bst I, Pvu I, Xcm I, Bsa J I, Hpa I, Apa L I, Sac I, Dra III and Sma I. Upon exposure to a suitable restriction enzyme, the newly synthesized strand is digested and thereby shortened. A nested oligonucleotide 50 can then be hybridized to the shortened newly synthesized strand 142 to complete preparation of the engineered template, as described in more detail below.

The next step in preparing the engineered template involves annealing a nested oligonucleotide 50 to the 3' end of the newly synthesized complementary strand, for example as shown in Fig. 4. As seen in Fig. 4, the nested oligonucleotide 50 provides a template for further polymerization necessary to complete the engineered template. Nested oligonucleotide 50 includes a portion 52 that does not hybridize and/or includes modified bases to the newly synthesized complementary strand, thereby preventing the

nested oligonucleotide from serving as a primer. Nested oligonucleotide 50 also includes a portion 55 that hybridizes to the 3' end of the newly synthesized complementary strand. Portion 55 may be coterminous with newly synthesized portion 45 or may extend beyond newly synthesized portion 45 as shown in Fig. 4. Nested oligonucleotide 50 may 5 optionally also include a portion 56 defining a restriction site. The final portion 58 of nested oligonucleotide 50 contains the same predetermined sequence as portion 22 of primer 20. From the point at which portion 55 extends beyond the 3' end of the beginning the newly synthesized complementary strand, the nested oligonucleotide serves as a template for further polymerization to form the engineered template. It should be 10 understood that the nested oligo may contain part of the target sequence (if part thereof was truncated in forming the original template) or may include genes that encode a polypeptide or protein (or portion thereof) such as, for example, one or more CDR's or Framework regions or constant regions of an antibody. It is also contemplated that a collection of nested oligonucleotides having different sequences can be employed, 15 thereby providing a variety of templates which results in a library of diverse products. Thus, polymerization will extend the newly synthesized complementary strand by adding additional nucleic acid 60 that is complementary to the nested oligonucleotide as shown in Fig. 4. Techniques for achieving polymerization are within the purview of one skilled in the art. As previously noted, selecting a suitable polymerase, an enzyme lacking 20 exonuclease activity may be preferred in certain embodiments.

Once polymerization is complete, the engineered template 120 is separated from the nested oligonucleotide 50 by techniques well known to those skilled in the art such as, for example, heat denaturation. The resulting engineered template 120 contains a portion

derived from the original primer 20, portion 45 that is complementary to a portion of the original template, and portion 65 that is complementary to a portion of the nested oligonucleotide (see Fig. 5). Significantly, the 3' end of engineered template 120 includes portion 68 containing a sequence that is complementary to the predetermined sequence of portion 22 of primer 20. This allows for amplification of the desired sequence contained within engineered template 120 using a single primer having the same sequence as the predetermined sequence of primer portion 22 using techniques known to those of ordinary skill in the art. During single primer amplification, the presence of a polymerase having exonuclease activity is preferred because such enzymes are known to provide a "proofreading" function and have relatively higher processivity compared to polymerases lacking exonuclease activity.

Fig. 6 illustrates the steps involved in the single primer amplification of the newly synthesized cDNA template. When the primer is present in the reaction mixture it hybridizes to the sequences flanking the template and amplifies the template. When there is no primer present, it is believed that there is internal self annealing between the 5' end predetermined sequence and the 3' end sequence which is complementary to the predetermined sequence. In a preferred embodiment, the predetermined sequence and complementary predetermined sequence may be designed to anneal at higher temperatures in order to avoid miss-priming during the single primer amplification reaction.

After amplification is performed, the products may be detected using any of the techniques known to those skilled in the art. Examples of methods used to detect nucleic acids include, without limitation, hybridization with allele specific oligonucleotides,

restriction endonuclease cleavage, single-stranded conformational polymorphism (SSCP), analysis.gel electrophoresis, ethidium bromide staining, fluorescence resonance energy transfer, hairpin FRET assay, and TaqMan assay.

Once the engineered nucleic acid is amplified a desired number of times,  
5 restriction sites 23 and 66 or any internal restriction sites can be used to digest the strand so that the target nucleic acid sequence can be ligated into a suitable expression vector. The vector may then be used to transform an appropriate host organism using standard methods to produce the polypeptide or protein encoded by the target sequence.

In particularly useful embodiments, the methods described herein are used to  
10 amplify target sequences encoding antibodies or portions thereof, such as, for example the variable regions (either light or heavy chain) using cDNA of an antibody. In this manner, a library of antibodies can be amplified and screened. Thus, for example, starting with antibody mRNA, first strand cDNA can be produced and digested to provide an original template. A primer can be designed to anneal upstream to a selected  
15 complementary determining region (CDR) so that the newly synthesized nucleic acid strand includes the CDR. By way of example, if the target sequence is heavy chain CDR3, the primer may be designed to anneal to the heavy chain framework one (FR1) region. Those skilled in the art will readily envision how to design appropriate primers to anneal to other upstream sites or to reproduce other selected targets within the antibody  
20 cDNA based on this disclosure.

The following Examples are provided to illustrate, but not limit, the present invention(s):

**EXAMPLE 1**

Amplification of a repertoire of IgM heavy chain variable genes

**1<sup>st</sup> strand cDNA synthesis and modification**

Human peripheral blood lymphocyte (PBL) mRNA was used to generate 5 traditional 1<sup>st</sup> strand cDNA with an oligo dT primer using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) essentially according to kit instructions. The first stand cDNA product was cleaned up over a QIAGEN spin column (PCR Purification Kit). A restriction oligonucleotide was added to the first strand cDNA in order to generate a double stranded DNA region that could be digested by the 10 restriction endonuclease EcoR I. The sequence of the restriction oligonucleotide (CMEcoR I) was 5' TCC TGT GAG AAT TCC CCG TCG 3' (Seq. ID No. 1). The reaction was set up with 1<sup>st</sup> strand cDNA and 0.1uM oligonucleotide. The sample was heated to 95°C for 2 minutes and then held at 64°C for two minutes to allow specific annealing to occur. An appropriate amount of 10X restriction buffer H (Roche 15 Diagnostics) was added to the sample and further cooled to 37°C. The restriction endonuclease EcoR I (New England Biolabs) was added and incubated at 37° C for 30 minutes. The restriction enzyme was heat inactivated at 65° C for 20 minutes and then the sample was cooled to 4°C.

**2<sup>nd</sup> Strand Linear Amplification and Nested Oligo Extension**

20 EcoR I digested 1<sup>st</sup> strand cDNA was used as the original template in a 2<sup>nd</sup> strand cDNA reaction along with primer "TMX24VH3a" (0.4 uM final), dNTPs, AmpliTaq enzyme and its 10X reaction buffer (Applied Biosystems). The primer was designed to contain the predetermined TMX24 sequence, an Xho I restriction site and a region that

anneals to the 1<sup>st</sup> strand cDNA in the framework 1 region of the human antibody heavy chain genes. The sequence of "TMX24VH3a" was 5' GTG CTG GCC GTT GGA AGA GGA GTG CTC GAG GAR GTG CAG CTG GTG GAG 3' (Seq. ID No. 2) where R stands for an equal molar mixture of bases A and G. The sample was heat denatured at 5 95° C for 1 minute then cycled 20 times through 95° C for 5 seconds, 56° C for 10 seconds and 68° C for 1 minute. This allows linear amplification of the 2nd strand cDNA. A nested oligo designated "TMX24CM0" was then added on ice to a final concentration of 0.08 uM. The sequence of "TMX24CM0" was 5' GTG CTG GCC GTT GGA AGA GGA GTG ACT AGT AAT TCT CAC AGG AGA CGA GGG GGA 3' (Seq. ID No. 3), which contains a Spe I restriction endonuclease site to be used in 10 subsequent cloning steps. The 3' end of the nested oligo is designed to prevent elongation by incorporation of a reverse linked (3'-3' rather than 3'-5') adenine. The 2<sup>nd</sup> strand cDNAs were further elongated off the nested oligo by heat denaturing at 94°C for 5 seconds, then cycled 4 times for annealing and elongating at 68° C for 10 seconds 15 and 95° for 5 seconds, followed by 68° for 30 seconds and 4°C. The resulting 2<sup>nd</sup> strand cDNA or engineered template was then cleaned up using a QIAGEN spin column (PCR Purification Kit). This step removes the oligonucleotides and allows simple buffer exchange for downstream protocols.

#### Single Primer Amplification (SPA)

20 The engineered template was amplified using Advantage 2 polymerase mix (Clontech) and its 10X reaction buffer, dNTPs, and a single primer (TMX24) having the sequence of 5' GTG CTG GCC GTT GGA AGA GGA GTG 3' (Seq. ID No. 4). The samples were heat denatured at 95°C for 1 minute then cycled 35 times through 95° for 5

seconds and 68° C for 1 minute. This was followed by an additional 3 minutes at 68° C and a 4° C hold.

Cloning and Sequencing

Amplification products of approximately 450 bp were gel purified and then 5 digested by Xho I and Spe I and cloned into pBluescript KS+ (Stratagene). Individual clones were picked and their DNA sequence determined. All of the 16 clones analyzed were IgM heavy chain, each possessing a different CDR3 sequences of varying length thereby indicating that a diverse population of antibody chains were amplified by this method (see Table 1).

Table 1

|    |                          | <u>FR3</u> | <u>HCDR3</u>          | <u>FR4</u> |
|----|--------------------------|------------|-----------------------|------------|
| 10 | CLONE 1 (Seq. ID No.5)   | YYCAR      | EGSSSGAFDI            | WGQ        |
|    | CLONE 2 (Seq. ID No.6)   | YYCAR      | AAFYCSGGSCYFDYYYYGMDV | WGQ        |
|    | CLONE 3 (Seq. ID No.7)   | YYCAK      | DIGGLGVLNFDY          | WGQ        |
| 15 | CLONE 5 (Seq. ID No.8)   | YYCAK      | GVLAIRICDY            | WGQ        |
|    | CLONE 6 (Seq. ID No.9)   | YYCAR      | DPGVYDYVWGSYRYPPDAFDI | WGQ        |
|    | CLONE 7 (Seq. ID No.10)  | YYCAR      | GMIVGATSYPDY          | WGQ        |
|    | CLONE 8 (Seq. ID No.11)  | YYCLL      | GYCSSTSCPDAFDI        | WGQ        |
|    | CLONE 9 (Seq. ID No.12)  | YYCVI      | GGAVFSGGSYRQQIDY      | WGQ        |
| 20 | CLONE 10 (Seq. ID No.13) | YYCTR      | DRGGSYTSHLGAFDI       | WGQ        |
|    | CLONE 11 (Seq. ID No.14) | YYCAK      | DNDLGGDYYYYGMDV       | WGQ        |
|    | CLONE 12 (Seq. ID No.15) | YYCAR      | DRRFPTDLFDI           | WGQ        |
|    | CLONE 13 (Seq. ID No.16) | YYCAR      | EDGYNNSGWSYNWFDP      | WGQ        |
|    | CLONE 14 (Seq. ID No.17) | YYCAK      | DCVSGSYHYFDY          | WGQ        |
| 25 | CLONE 16 (Seq. ID No.18) | YYCAK      | DSYCSGGSCYYYYGVDV     | WGQ        |
|    | CLONE 17 (Seq. ID No.19) | YYCAR      | EVVPAAIIDYYYYGMDV     | WGQ        |
|    | CLONE 18 (Seq. ID No.20) | YYCAK      | DLGIAVVVPAH           | WGQ        |

**EXAMPLE 2**

30 In order to clone VH products into a vector so that the native IgM CH1 constant region could be reconstituted, a site other than the EcoR I in CH1 was utilized for the 1<sup>st</sup>

strand cDNA endonuclease digestion. As those skilled in the art will appreciate, when Taq polymerase is used for this protocol, a terminal A is added to many of the newly synthesized DNA strands. In order to maximize diversity, the presence of that terminal A was taken into account in the design of the nested oligonucleotide. However, the 5 presence of that extra A results in the loss of the EcoR I recognition site. Analysis of the IgM constant region revealed other native restriction sites that could potentially be used for this method, such as Dra III. The result of using the Dra III native restriction site in the CH1 domain is that the upstream EcoR I site remains unmodified and can be used for cloning the heavy chain repertoire. The heavy chain inserts are cloned by Xho I and 10 EcoR I into an appropriate vector which has the remaining IgM CH1 domain from EcoR I to the CH2 domain.

1<sup>st</sup> strand cDNA synthesis and modification

Human peripheral blood lymphocyte (PBL) mRNA was used to generate traditional 1<sup>st</sup> strand cDNA with an oligo dT primer. This was done using SuperScript 15 First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) according essentially to kit instructions. A restriction oligonucleotide was added to the first strand cDNA in order to generate a double stranded DNA region that could be digested by the restriction endonuclease Dra III. The sequence of the restriction oligonucleotide (CMDra III) was 5' GAC GAA CAC GTG GTG TGC AAA G 3' (Seq. ID No. 21). The 20 reaction was set up with 1<sup>st</sup> strand cDNA and 1uM oligonucleotide. The sample was heated to 95°C for 2 minutes and then held at 64°C for two minutes to allow specific annealing to occur. An appropriate amount of 10X restriction buffer H (Roche Diagnostics) was added to the sample and further cooled to 37°C. The restriction

endonuclease Dra III (New England Biolabs) was added and incubated at 37° C for 30 minutes. The restriction enzyme was heat inactivated at 65° C for 20 minutes and then the sample was cooled to 4°C.

2<sup>nd</sup> Strand Linear Amplification and Nested Oligo Extension

5 Dra III digested 1<sup>st</sup> strand cDNA was used as the original template in a 2<sup>nd</sup> strand cDNA reaction along with primer “TMX24VH1a” (0.4 uM final), dNTPs, AmpliTaq enzyme and its 10X reaction buffer (Applied Biosystems). The primer was designed to contain the predetermined TMX24 sequence, an Xho I restriction site and a region that anneals to the 1<sup>st</sup> strand cDNA in the framework 1 region of the human antibody heavy 10 chain genes. The sequence of “TMX24VH1a” was

5'GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTCKCAGCTGGTGCAG 3'  
(Seq. ID No. 22) where K stands for an equal molar mixture of bases G and T. The sample was heat denatured at 94° C for 1 minute then cycled 20 times through 94° C for 5 seconds, 56° C for 10 seconds and 68° C for 2 minutes. This allowed linear 15 amplification of the 2nd strand cDNA. A nested oligo designated “TMX24CMnpt” was added on ice to a final concentration of 0.2 uM. As shown in Fig. 7, the sequence of “TMX24CMnpt” (Seq. ID No. 23) includes three 3' terminal nucleotides having modified structures which were designed to prevent elongation of the oligonucleotide. Specifically, the nested oligo has three terminal nucleotides modified with 20 phosphorthioate and 2' OMe which is designed to prevent extension and protect against exo- and endonuclease activity. The 3' end nucleotide of this oligo is non-hybridizing (g instead of c). The 2<sup>nd</sup> strand cDNAs were further elongated off the nested oligo by heat denaturing at 94°C for 1 minute, annealing and elongating at 68° C for 2 minutes,

followed by 4°C. The resulting 2<sup>nd</sup> strand cDNA or engineered template was then cleaned up using a QIAGEN spin column (PCR Purification Kit). This step removes the oligonucleotides and allows simple buffer exchange for downstream protocols. This procedure was repeated and extended to the rest of the VH primer panel (see primer list) 5 to generate a library of immunoglobulin products that can be cloned into an appropriate vector.

**VH Framework 1 Specific Primers:**

10 PRIMER TMX24VH1a (Seq. ID No. 25)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTCAGCTGGTGCAG

15 PRIMER TMX24VH1b (Seq. ID No. 26)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTCAGCTGGTGCAG

20 PRIMER TMX24VH1c (Seq. ID No. 27)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGSAGGTCCAGCTGGTACAG

25 PRIMER TMX24VH1d (Seq. ID No. 28)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCARATGCAGCTGGTGCAG

30 PRIMER TMX24VH2a (Seq. ID No. 29)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGATCACCTGAAGGAG

35 PRIMER TMX24VH2b (Seq. ID No. 30)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTACCTTGARGGAG

40 PRIMER TMX24VH3a (Seq. ID No. 31)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGGARGTGCAGCTGGTGGAG

PRIMER TMX24VH3b (Seq. ID No. 32)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTGCAGCTGGTGGAG

PRIMER TMX24VH3c (Seq. ID No. 33)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGGAGGTGCAGCTGTTGGAG

PRIMER TMX24VH4a (Seq. ID No. 34)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGSTGCAGCTGCAGGAG

PRIMER TMX24VH4b (Seq. ID No. 35)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTGCAGCTACAGCAG

PRIMER TMX24VH5a (Seq. ID No. 36)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGGARGTGCAGCTGGTGCAG

PRIMER TMX24VH6a (Seq. ID No. 37)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTACAGCTGCAGCAG

5 PRIMER TMX24VH7a (Seq. ID No. 38)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTSCAGCTGGTGCAA

In the foregoing sequences, R is an equal mixture of A and G, K is an equal mixture of G and T, and S is an equal mixture of C and G.

#### Single Primer Amplification (SPA)

10 The engineered template was amplified using Advantage 2 polymerase mix (Clontech) and its 10X reaction buffer, dNTPs and a single primer (TMX24) having the sequence 5' GTG CTG GCC GTT GGA AGA GGA GTG 3' (Seq. ID No. 4). The samples were heat denatured at 95°C for 1 minute then cycled 30 times through 95° C for 5 seconds and 68° C for 1 minute. This was followed by an additional 3 minutes at 68°C  
15 and a 4° hold.

#### Cloning and Sequencing

Amplification products of approximately 450 bp are gel purified and digested by Xho I and EcoR I. The inserts are cloned into the any suitable expression vector containing the remaining portion of the IgM CH1 domain from the native EcoR I site up 20 to, or including a portion of, the CH2 domain and a compatible restriction site for cloning the amplified fragments.

#### **EXAMPLE 3**

Construction of a phagmid display library from mRNA of a Hepatitis B positive donor.

1<sup>st</sup> strand cDNA synthesis and modification for IgG Heavy and Kappa Light Chains

Human peripheral blood lymphocyte (PBL) mRNA from a Hepatitis B vaccinated donor was used to generate traditional 1<sup>st</sup> stand cDNA with an oligo dT primer. This was done using SuperScript First-Strand Synthesis System for RT-PCR (INVITROGEN LIFE 5 TECHNOLOGIES) according essentially to kit instructions. Restriction oligonucleotide "CGApaL I" for IgG or "CKSac I" for kappa light chain was added to the first strand cDNA in order to generate a double stranded DNA region that could be digested by the restriction endonuclease ApaL I for IgG or Sac I for kappa light chain. "CGApaL I" sequences is 5'CCA GCG GCG TGC ACA CCT TCC3' (Seq ID No. 39). "CKSac I" 10 sequence is 5'AGG GCC TGA GCT CGC CCG TC 3' (Seq ID No. 40). The reaction was set up with 1<sup>st</sup> strand cDNA, 1uM oligonucleotide, and appropriate amount of 10X restriction buffer A(Roche Diagnostics). The sample was heated to 95°C for 2 minutes and then held at 64°C for two minutes to allow specific annealing to occur and cooled to 37°C. The restriction endonuclease ApaL I or Sac I (New England Biolabs) was added 15 and incubated at 37° C for 30 minutes. The restriction enzyme was heat inactivated for Sac I at 65° C for 20 minutes and then the sample was cooled to 4°C.

Digestion of the 1<sup>st</sup> strand cDNAs by each restriction endonuclease was verified by PCR amplification using techniques known to those skilled in the art. These products were not used for cloning the antibody genes. Positive amplification of the digested 1<sup>st</sup> 20 strand cDNA was observed in reactions using the 5' VBVH1a and the 3' CG0 internal control primer for IgG and 5' VBVK1a and the 3' CK0 internal control primer for kappa. Good amplification with primers 5'VBVH1a / 3' CG0 or 5' VBVK1a/3' CK0 and minimal amplification with primers 5' VBVH1a / 3' CG1Z or 5' VK1a/3' CK1dx2

indicate successful digestion of the 1<sup>st</sup> strand cDNA template with each restriction endonuclease. Sequences of the primers used for check PCR were VBVH1a: 5' GAG CCG CAC GAG CCC CTC GAG CAG GTK CAG CTG GTG CAG 3' (Seq. ID No. 41), CG0: 5' GRG CGC CTG AGT TCC ACG ACA CCG 3' (Seq. ID No. 42), VBVK1a: 5' 5 GAC GCG CAC AAC ACG GAG CTC RAC ATC CAG ATG ACC CAG 3' (Seq. ID No. 43), CK0: 5' GTG ACT TCG CAG GCG TAG ACT T 3' (Seq. ID No. 44), CG1z: 5' GCA TGT ACT AGT TTT GTC ACA AGA TTT GGG 3' (Seq. ID No. 45), CK1dx2: 5' AGA CAG TGA GCG CCG TCT AGA ATT AAC ACT CTC CCC TGT TGA AGC TCT TTG TGA CGG GCG AAC TCA G 3' (Seq. ID No. 46).

10 Light Chain 2<sup>nd</sup> Strand Linear Amplification and Nested Oligo Extension

Sac I digested 1<sup>st</sup> strand cDNA was used as the original template to set up multiple 2<sup>nd</sup> strand cDNA reactions using a framework 1 specific primer (0.4 uM final), dNTPs, AmpliTaq enzyme and its 10X reaction buffer (Applied Biosystems). The primers were designed to contain predetermined TMX24K sequence 5'GAC GAC CG G 15 CTA CCA AGA GGA GTG3' (Seq. ID No. 47) for kappa, an Xba I restriction site, and a region that annealed to 1<sup>st</sup> strand cDNA in the framework 1 region of human antibody kappa light chain genes. Those annealing sequences were derived from the VBase database primers ([www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html](http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html)) that were designed based on the known sequences of human antibodies and are reported to cover 20 the entire human antibody repertoire of kappa light chain genes.

**Kappa light Chain Framework 1 Specific Primers:**

TMX24vk1a (Seq. ID No. 48) Xba I  
GACGACCGGCTACCAAGAGGAGTGTCTAGARACATCCAGATGACCCAG  
5

TMX24vk1b (Seq. ID No. 49)  
GACGACCGGCTACCAAGAGGAGTGTCTAGAGMCATCCAGTTGACCCAG

TMX24vk1c (Seq. ID No. 50)  
10 GACGACCGGCTACCAAGAGGAGTGTCTAGAGCCATCCRGATGACCCAG

TMX24vk1d (Seq. ID No. 51)  
GACGACCGGCTACCAAGAGGAGTGTCTAGAGTCATCTGGATGACCCAG

15 TMX24vk2a (Seq. ID No. 52)  
GACGACCGGCTACCAAGAGGAGTGTCTAGAGATATTGTGATGACCCAG

TMX24vk2b (Seq. ID No. 53)  
20 GACGACCGGCTACCAAGAGGAGTGTCTAGAGATRTTGATGACTCAG

TMX24vk3a (Seq. ID No. 54)  
GACGACCGGCTACCAAGAGGAGTGTCTAGAGAAATTGTGTTGACRCAG

25 TMX24vk3b (Seq. ID No. 55)  
GACGACCGGCTACCAAGAGGAGTGTCTAGAGAAATAGTGATGACGCAG

TMX24vk3c (Seq. ID No. 56)  
GACGACCGGCTACCAAGAGGAGTGTCTAGAGAAATTGTAATGACACAG

30 TMX24vk4a (Seq. ID No. 57)  
GACGACCGGCTACCAAGAGGAGTGTCTAGAGACATCGTGATGACCCAG

TMX24vk5a (Seq. ID No. 58)  
35 GACGACCGGCTACCAAGAGGAGTGTCTAGAGAAACGACACTCACGCAG

TMX24vk6a (Seq. ID No. 59)  
GACGACCGGCTACCAAGAGGAGTGTCTAGAGAAATTGTGCTGACTCAG

TMX24vk6b (Seq. ID No. 60)  
40 GACGACCGGCTACCAAGAGGAGTGTCTAGAGATGTTGTGATGACACAG

In the foregoing sequences, R is an equal mixture of A and G, M is an equal mixture of A and C, Y is an equal mixture of C and T, W is an equal mixture of A and T, and S is an equal mixture of C and G.

The samples were heat denatured at 94° C for 1 minute then cycled 20 times  
5 through 94° C for 5 seconds, 56° C for 10 seconds, and 68° C for 2 minutes. This allowed linear amplification of the 2nd strand cDNA. A nested oligo designated “TMX24CKnpt” for kappa chains was added on ice to a final concentration of 0.2 uM. “TMX24CKnpt” contains predetermined sequence TMX24K and the sequence was 5’  
GAC GAC CGG CTA CCA AGA GGA GTG CTC GAG CTC AGG CCC TGA TGG  
10 GTG ACT TCG CT 3’ (Seq. ID No. 61). The 2<sup>nd</sup> strand cDNAs were further elongated off the nested oligo by heat denaturing at 94°C for 1 minute, annealing and elongating at 68° C for 2 minutes, followed by 4°C. The resulting 2<sup>nd</sup> strand cDNA (engineered template) were cleaned up using a QIAGEN spin column (PCR Purification Kit). This step removes the free oligonucleotides and allows simple buffer exchange for  
15 downstream protocols.

#### Light Chain Single Primer Amplification (SPA)

The engineered template was amplified using Advantage 2 polymerase mix (Clontech) and its 10X reaction buffer, dNTPs, and primer “TMX24K” for kappa chains. The sequence for “TMX24K” is 5’GAC GAC CGG CTA CCA AGA GGA GTG 3’ (Seq. ID No. 62). The samples were heat denatured at 95°C for 1 minute then cycled 30 times through 95° C for 5 seconds and 68° C for 1 minute. This was followed by an additional 3 minutes at 68° C and a 4° C hold.

Light Chain Cloning

Kappa amplification products were gel purified and then digested by Xba I and Sac I. The inserts were cloned into a suitable expression vector that contains the remaining portion of the kappa light chain constant region. The ligated product was 5 introduced into an *E. coli* by electroporation and grown overnight at 37° C. The following morning a DNA maxi prep (QIAGEN) was performed to recover the light chain library DNA. The light chain library DNA was then used in subsequent steps to clone in the heavy chain Fd fragments by Xho I / Age I to complete the construction of the library.

10 Heavy Chain 2<sup>nd</sup> Strand Linear Amplification and Nested Oligo Extension

ApaL I digested 1<sup>st</sup> strand cDNA was used as the original template to set up multiple 2<sup>nd</sup> strand cDNA reactions using a framework 1 specific primer (0.4 uM final), dNTPs, AmpliTaq enzyme and its 10X reaction buffer (Applied Biosystems). The primers were designed to contain the predetermined TMX24 sequence, an Xho I 15 restriction site, and a region that annealed to 1<sup>st</sup> strand cDNA in the framework 1 region of human antibody heavy chain genes. Those annealing sequences were derived from the VBase database primers that were designed based on the known sequences of human antibodies and are reported to cover the entire human antibody repertoire of heavy chain genes.

20 **Heavy chain Framework 1 Specific Primers:**TMX24VH1a (Seq. ID No. 63)

GTGCTGGCCGTTGGAAGAGGGAGTGCTCGAGCAGGTCKCAGCTGGTGCAG

TMX24VH1b (Seq. ID No. 64)

25 GTGCTGGCCGTTGGAAGAGGGAGTGCTCGAGCAGGTCCAGCTTGTGCAG

5 TMX24VH1c (Seq. ID No. 65)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGSAGGTCCAGCTGGTACAG

10 TMX24VH1d (Seq. ID No. 66)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCARATGCAGCTGGTGCAG  
TMX24VH2a (Seq. ID No. 67)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGATCACCTGAAGGAG

15 TMX24VH2b (Seq. ID No. 68)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTACACCTGARGGAG

20 TMX24VH3a (Seq. ID No. 69)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGGARGTGCAGCTGGTGGAG

25 TMX24VH3b (Seq. ID No. 70)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTGCAGCTGGTGGAG

30 TMX24VH3c (Seq. ID No. 71)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGGAGGTGCAGCTGGTGGAG

35 TMX24VH4a (Seq. ID No. 72)  
GTGCTGGCCGTTGGAACACGAGTGCTCGAGCAGSTGCAGCTGCAGGAG

TMX24VH4b (Seq. ID No. 73)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTGCAGCTACAGCAG

TMX24VH5a (Seq. ID No. 74)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGGAGGTGCAGCTGGTGCAG

40 TMX24VH6a (Seq. ID No. 75)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTACAGCTGCAGCAG

TMX24VH7a (Seq. ID No. 76)  
GTGCTGGCCGTTGGAAGAGGAGTGCTCGAGCAGGTSCAGCTGGTCAA

In the foregoing sequences, R is an equal mixture of A and G, K is an equal mixture of G and T, and S is an equal mixture of C and G.

The samples were heat denatured at 94° C for 1 minute then cycled 20 times through 94° C for 5 seconds, 56° C for 10 seconds, and 68° C for 2 minutes. This 40 allowed linear amplification of the 2nd strand cDNA. A nested oligonucleotide designated “TMX24CGnpt” (sequence 5’ GTG CTG GCC GTT GGA AGA GGA GTG TGT TTG CAC GCC GCT GGT CAG RGC GCC TGA GTT G 3’ (Seq. ID No. 77)) was added on ice to a final concentration of 0.2 uM. As shown in Fig. 1 for the IgM nested oligo, the three 3’ terminal nucleotides were modified to prevent oligo extension. The 2<sup>nd</sup>

strand cDNAs were further elongated off the nested oligo by heat denaturing at 94°C for 1 minute, annealing and elongating at 68° C for 2 minutes, followed by 4°C. The resulting 2<sup>nd</sup> strand cDNA (the engineered template) was then cleaned up using a QIAGEN spin column (PCR Purification Kit). This step removed the free 5 oligonucleotides and allowed simple buffer exchange for downstream protocols.

#### Single Primer Amplification (SPA)

The engineered template was amplified using Advantage 2 polymerase mix (Clontech) and its 10X reaction buffer, dNTPs, and primer “TMX24”. The samples was heat denatured at 95°C for 1 minute then cycled 30 times through 95° C for 5 seconds 10 and 68° C for 1 minute. This was followed by 3 additional minutes at 68° C and a 4° C hold.

#### Heavy Chain Cloning & Production of the Library

The amplified products were pooled and then gel purified. DNA was recovered with QIAquick PCR purification kit (QIAGEN). The DNA was sequentially digested 15 with Xho I and Age I restriction enzymes and then gel purified. Age I site is naturally present in the CH1 of IgG constant region upstream of ApaL I site. DNA was recovered with QIAquick Gel extraction Kit (QIAGEN).

The light chain library DNA was digested sequentially with Xho I and Age I and then gel purified. The light chain library DNA was ligated with the heavy chain 20 fragments. Ligated DNA was placed over a spin column (PCR purification Kit , QIAGEN) to remove the reaction buffer and to concentrate the DNA. Final transformation was done in electrocompetent XL-1 Blue cells (Stratagene).

Panning and Screening of Library on HBs Ag

The library was panned on immobilized HBs Ag for 4 rounds essentially as described in Barbas III, CF, Burton, DR, Scott, JK, and Silverman, GJ (2001) Phage Display: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. Individual clones from the 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> rounds of panning were 5 screened by ELISA on HBs Ag.

1<sup>st</sup> Strand cDNA Synthesis and Modification for Lambda Light Chain.

Human PBL mRNA from a Hepatitis B vaccinated donor was used to generate traditional 1<sup>st</sup> strand cDNA with an oligo dT primer. This was done using SuperScript 10 First-Strand Synthesis for RT-PCR (INVITROGEN LIFE TECHNOLOGIES) according essentially to kit instructions. Restriction oligonucleotide "CLSma I" was added to the first strand cDNA in order to generate a double stranded DNA region that could be digested by the restriction endonuclease Sma I. "CLSma I" sequence is 5'GAC TTC TAC 15 CCG GGA GCY GTG3' (Seq. ID No. 78) where Y is a mixture of C and T. The reaction was set up with 1<sup>st</sup> strand cDNA, 1 uM oligonucleotide, and appropriate amount of 10X restriction buffer A (Roche Diagnostics). The sample was heated to 95°C for 2 minutes and then held at 64°C for two minutes to allow specific annealing to occur and cooled to 20 37°C. The restriction endonuclease Sma I (New England Biolabs) was added and incubated at 37°C for 30 minutes. The restriction enzyme was heat inactivated at 65°C and then the sample was cooled to 4°C.

Digestion of the 1<sup>st</sup> strand cDNA by restriction endonuclease was verified by PCR amplification using techniques known to those skilled in the art. These products were not used for cloning the antibody genes. Positive amplification of the digested 1<sup>st</sup> strand

cDNA was observed in reaction using the 5'VBVL1a and 3'CL0 internal control primer.

Good amplification with primers 5'VBVL1a/3'CL0 and minimal amplification with

primers 5'VBVL1a/3'CL2dx2 indicated successful digestion of the 1<sup>st</sup> strand cDNA

template with Sma I. Sequences of the primers for check PCR were VBVL1a 5' GAC

5 GCG CAC A AC ACG GAG CTC CAG TCT GTG CTG ACT CAG 3' (Seq. ID No. 79),

CL0 5'CCT CAG AGG AGG GYG GG A ACAG3' (Seq. ID No. 80) and CL2dx2 5'

AGA CAG TGA CGC CGT CTA GAA TTA TGA ACA TTC TGT AGG 3' (Seq. ID No.

81).

Lambda Light Chain 2<sup>nd</sup> Strand Linear Amplification and Nested Oligo Extension

10 Sma I digested 1<sup>st</sup> strand cDNA was used as the original template to set up multiple 2<sup>nd</sup> strand cDNA reactions using a framework 1 specific primer (0.4 uM final), dNTPs, AmpliTaq enzyme and its 10X reaction buffer (Applied Biosystems). The primers are designed to contain predetermined TMX24L sequence, an Xba I site, and a region that anneals to 1<sup>st</sup> strand cDNA in the framework region of human antibody lambda light

15 chain genes. Those annealing sequences are derived from the VBase database primers ([www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html](http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html)) that are designed based on the known sequences of human antibodies and are reported to cover the entire human antibody repertoire of lambda light chain genes. Lambda light chain framework 1 specific primers are those used in Example 4.

20 The samples were heat denatured at 94°C for 1 minute and then cycled 20 times through 94°C for 5 seconds, 56°C for 10 seconds, and 68°C for 2 minutes. This allowed linear amplification of the 2<sup>nd</sup> strand cDNA. A nested oligo nucleotide designated "TMX24CLnpt" as shown in Example 4 was added on ice to a final concentration of 0.2

uM. As shown in Fig. 7 for the IgM nested oligonucleotide "TMX24CMnpt", the 3' terminal nucleotides of "TMX24CLnpt" are modified to prevent oligo extension. The 2<sup>nd</sup> strand cDNAs were further elongated off the nested oligonucleotide by heat denaturing at 94°C for 1 minutes, annealing and elongating at 68°C for 2 minutes, followed by 4°C.

5 The resulting 2<sup>nd</sup> strand cDNAs (the engineered template) were cleaned up using PCR Purification Kit (QIAGEN). This step removes the free oligonucleotides and allows simple buffer exchange for downstream protocols.

Lambda Light Chain Single Primer Amplification (SPA)

10 The engineered template was amplified using Advantage 2 polymerase mix (Clontech) and its 10x reaction buffer, dNTPs, and primer "TMX24L". The predetermined sequence of TMX24L is 5'GAC GAC CGG CTA CCA AGA GGA CAG3' (Seq. ID No. 82). The samples were heat denatured at 95°C for 1 minutes then cycled 30 times through 95°C for 5 seconds and 68°C for 1 minute. This was followed by an additional 3 minutes at 68°C and a 4°C hold.

15 Lambda Light Chain Cloning

Lambda light chain amplification products were cleaned up by PCR purification kit (QIAGEN) and digested by Xba I and Sac I. The insert was gel purified using a gel extraction kit (QIAGEN) and cloned into an appropriate vector that contains the remaining portion of the lambda light chain constant region. The ligated product was 20 introduced into an E. coli by electroporation and grown overnight at 37°C. The following morning a DNA maxi prep (QIAGEN) was performed to recover the lambda light chain library DNA.

Heavy Chain Cloning & Production of the IgG Lambda Library

The lambda light chain library DNA prepared above was sequentially digested by Xho I

and Age I for the insertion of the heavy chain Fd fragments prepared also for the IgG

5 kappa library as described previously. The ligated product was then introduced into an E.

coli by electroporation and grown overnight at 37°C. The following morning a DNA

maxi prep (QIAGEN) was performed to recover the complete IgG lambda library DNA.

Panning and Screening of Library on HBs Ag

The panning and screening was performed as described previously for IgG kappa library.

10 DNA Sequencing Analysis and Characterization of Isolated Fabs

Clones that showed specific binding to HBsAg and minimal binding to a non-specific

protein, ovalbumin by ELISA screening were analyzed by DNA sequencing. See

Figs. 8a-e. Total of 38 distinct IgG kappa Fabs (25 heavy chains and 37 light chains) and

17 distinct IgG lambda Fabs (13 heavy chains and 16 light chains) to HBsAg were

15 isolated from the libraries made from the PBL mRNA from a Hepatitis B vaccinated

donor.

**EXAMPLE 4**

Construction of a phage display antibody library from human PBL mRNA.

20 1<sup>st</sup> strand cDNA Synthesis and Modification for Light Chains

Human PBL mRNA from donor was to generate traditional 1<sup>st</sup> stand cDNA

with an oligo dT primer using SuperScript First-Strand Synthesis System for RT-PCR

(Invitrogen Life Technologies) essentially according to kit instructions. Kappa and

lambda light chain reactions are set up separately. Restriction oligonucleotide "CKSac I" or "CLSma I" is added to the first strand cDNA in order to generate a double stranded DNA region that could be digested by the restriction endonuclease Sac I for kappa or Sma I for lambda light chains. As there are multiple lambda constant regions (C1, C2, 5 C3, and C6) it is important to note that the Sma I site is conserved among all functional lambda constant domains (C1, C2, C3, and C6). "CKSac I" sequence is 5' AGG GCC TGA GCT CGC CCG TC 3' (Seq ID No. 179), "CLSma I" sequence is 5' GAC TTC TAC CCG GGA GCY GTG 3' (Seq ID No. 180) where Y is a mixture of C and T. The reactions are set up with 1<sup>st</sup> strand cDNA and 1uM oligonucleotide. The sample is heated 10 to 95°C for 2 minutes and then held at 64°C for two minutes to allow specific annealing to occur. An appropriate amount of 10X restriction buffer A (Roche Diagnostics) is added to the samples and further cooled to 37°C. The restriction endonuclease Sac I or Sma I (New England Biolabs) is added and incubated at 37° C for 30 minutes. The restriction enzyme is heat inactivated at 65° C for 20 minutes and then the sample is 15 cooled to 4°C.

Light Chain 2<sup>nd</sup> Strand Linear Amplification and Nested Oligo Extension

Sac I digested kappa 1<sup>st</sup> strand cDNA or Sma I digested lambda 1<sup>st</sup> strand cDNA are used as the original templates to set up multiple 2<sup>nd</sup> strand cDNA reactions using a framework 1 specific primer (0.4 uM final), dNTPs, AmpliTaq enzyme and its 10X 20 reaction buffer (Applied Biosystems). The primers are designed to contain TMX24K (for kappa) or TMX24L (for lambda) sequence, an Xba I restriction site, and a region that annealed to 1<sup>st</sup> strand cDNA in the framework 1 region of human antibody kappa or lambda chain genes. Those annealing sequences are derived from the VBase database

primers ([www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html](http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html)) that are designed based on the known sequences of human antibodies and are reported to cover the entire human antibody repertoire of light chain genes. Kappa light chain framework 1 specific primers are those used in Example 3. See the following list of primers for use in lambda

5 amplification.

**Lambda light chain Framework 1 Specific Primers:**

TMX24VL1a (Seq. ID No. 181)  
GACGACCGGCTACCAAGAGGACAGTCTAGACAGTCTGTGCTGACTCAG

10 TMX24VL1b (Seq. ID No. 182)  
GACGACCGGCTACCAAGAGGACAGTCTAGACAGTCTGTGTYGACGCAG

15 TMX24VL1C (Seq. ID No. 183)  
GACGACCGGCTACCAAGAGGACAGTCTAGACAGTCTGTGCTGACGCAG

15 TMX24VL2 (Seq. ID No. 184)  
GACGACCGGCTACCAAGAGGACAGTCTAGACAGTCTGCCCTGACTCAG

20 TMX24VL3a (Seq. ID No. 185)  
GACGACCGGCTACCAAGAGGACAGTCTAGATCCTATGWGCTGACTCAG

20 TMX24VL3b (Seq. ID No. 186)  
GACGACCGGCTACCAAGAGGACAGTCTAGATCCTATGAGCTGACACAG

25 TMX24VL3c (Seq. ID No. 187)  
GACGACCGGCTACCAAGAGGACAGTCTAGATCTTGAGCTGACTCAG

25 TMX24VL3d (Seq. ID No. 188)  
GACGACCGGCTACCAAGAGGACAGTCTAGATCCTATGAGCTGATGCAG

30 TMX24VL4 (Seq. ID No. 189)  
GACGACCGGCTACCAAGAGGACAGTCTAGACAGCYTGTGCTGACTCAA

30 TMX24VL5 (Seq. ID No. 190)  
GACGACCGGCTACCAAGAGGACAGTCTAGACAGSCTGTGCTGACTCAG

35 TMX24VL6 (Seq. ID No. 191)  
GACGACCGGCTACCAAGAGGACAGTCTAGAAAATTATGCTGACTCAG

40 TMX24VL7 (Seq. ID No. 192)  
GACGACCGGCTACCAAGAGGACAGTCTAGACAGRCTGTGGTGACTCAG

45 TMX24VL8 (Seq. ID No. 193)  
GACGACCGGCTACCAAGAGGACAGTCTAGACAGACTGTGGTGACCCAG

45 TMX24VL4/9 (Seq. ID No. 194)  
GACGACCGGCTACCAAGAGGACAGTCTAGACACWGCTGTGCTGACTCAG

TMX24VL10 (Seq. ID No. 195)  
GACGACCGGCTACCAAGAGGACAGTCTAGACAGGCAGGGCTGACTCAG

5 In the foregoing sequences, R is an equal mixture of A and G, M is an equal mixture of A and C, Y is an equal mixture of C and T, W is an equal mixture of A and T, and S is an equal mixture of C and G.

The samples are heat denatured at 94° C for 1 minute then cycled 20 times through 94° C for 5 seconds, 56° C for 10 seconds, and 68° C for 2 minutes. This 10 allowed linear amplification of the 2nd strand cDNA. A nested oligonucleotide designated “TMX24CKnpt” for kappa chains or “TMX24CLnpt” for lambda chains are added on ice to a final concentration of 0.2 uM. The nested oligonucleotide sequences are; “TMX24CKnpt” 5’ GAC GAC CGG CTA CCA AGA GGA GTG CTC GAG CTC AGG CCC TGA TGG GTG ACT TCG CT 3’ (Seq. ID No. 196) and “TMX24CLnpt” 5’ 15 GAC GAC CGG CTA CCA AGA GGA CAG AAG AGC TCC TGG GTA GAA GTC ACT KAT SAG RCA CAG 3’ (Seq. ID No. 197). As shown in Fig. 7 for the IgM nested oligo, the three 3’ terminal nucleotides are modified to prevent oligo extension. The 2<sup>nd</sup> strand cDNAs are further elongated off the nested oligos by heat denaturing at 94°C 1 minute, annealing and elongating at 68° C for 2 minutes, followed by 4°C. The resulting 20 2<sup>nd</sup> strand cDNA (the engineered templates) are cleaned up using a QIAGEN spin column (PCR Purification Kit). This step removes the free oligonucleotides and allows simple buffer exchange for downstream protocols.

#### Light Chain Single Primer Amplification (SPA)

The engineered template is amplified using Advantage 2 polymerase mix 25 (Clontech) and its 10X reaction buffer, dNTPs, and primer “TMX24K” for kappa chains,

or "TMX24L" for lambda chains. The sequence for "TMX24K" is 5'GAC GAC CGG CTA CCA AGA GGA GTG 3' (Seq. ID No. 198), and for "TMX24L" it is 5' GAC GAC CGG CTA CCA AGA GGA CAG 3' (Seq. ID No. 199). The samples are heat denatured at 95°C for 1 minute then cycled 30 times through 95° C for 5 seconds and 68° C for 1  
5 minute. This is followed by an additional 3 minutes at 68° C and a 4° C hold.

#### Light Chain Cloning

Kappa and Lambda amplification products were cleaned using a PCR purification kit (QUIAGEN) and were separately gel purified. Those products are digested by Xba I and Sac I. The inserts are cloned into an appropriate vector that contains the remaining  
10 portion of the respective light chain constant region. The ligated product is introduced into *E. coli* by electroporation and grown overnight at 37° C. The following morning a DNA maxiprep is performed to recover the light chain library DNA. The light chain library DNA preps are used as the cloning vector for insertion of the heavy chain Fd fragments by Xho I / EcoR I to complete the construction of the library.

#### 1<sup>st</sup> strand cDNA Synthesis and Modification for Heavy Chains

Human PBL mRNA from a donor is used to generate traditional 1<sup>st</sup> stand cDNA with an oligo dT primer. This is done using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) according essentially to their instructions. Restriction oligonucleotide CMDra III is added to the first strand cDNA in order to  
20 generate a double stranded DNA region that could be digested by the restriction endonuclease Dra III. The reaction is set up with 1<sup>st</sup> strand cDNA and 1uM oligonucleotide. The sample is heated to 95°C for 2 minutes and then held at 64°C for

two minutes to allow specific annealing to occur. An appropriate amount of 10X restriction buffer H (Roche Diagnostics) is added to the sample and further cooled to 37°C. The restriction endonuclease Dra III (New England Biolabs) is added and incubated at 37° C for 30 minutes followed by cooling at 4° C.

5 Digestion of the 1<sup>st</sup> strand cDNAs by Dra III is verified by PCR amplification. Amplification products will not be used for cloning antibody fragments. Positive amplification of the digested 1<sup>st</sup> strand cDNA is observed in reactions using the 5' VBVH1a and the 3' CM0 internal control primer under two different buffer conditions. Good amplification with primers 5'VBVH1a / 3' CM0 and minimal amplification with 10 primers 5' VBVH1a / 3' CM1 indicate successful Dra III digestion of the 1<sup>st</sup> strand cDNA template.

#### Heavy Chain 2<sup>nd</sup> Strand Linear Amplification and Nested Oligo Extension

Dra III digested 1<sup>st</sup> strand cDNA is used as the original template to set up multiple 2<sup>nd</sup> strand cDNA reactions using a framework 1 specific primer (0.4 uM final), dNTPs, 15 AmpliTaq enzyme and its 10X reaction buffer (Applied Biosystems). The primers are designed to contain the TMX24 sequence, an Xho I restriction site, and a region that annealed to 1<sup>st</sup> strand cDNA in the framework 1 region of human antibody heavy chain genes. Those annealing sequences are derived from the VBase database primers that are designed based on the known sequences of human antibodies and are reported to cover 20 the entire human antibody repertoire of heavy chain genes as described above in example 3. Heavy chain framework 1 specific primers used are those as listed in example 3.

The samples are heat denatured at 94° C for 1 minute then cycled 20 times through 94° C for 5 seconds, 56° C for 10 seconds, and 68° C for 2 minutes. This

allowed linear amplification of the 2nd strand cDNA. A nested oligo nucleotide designated “TMX24CMnpt” (as used in Example 3) is added on ice to a final concentration of 0.2 uM. The 2<sup>nd</sup> strand cDNAs are further elongated off the nested oligo by heat denaturing at 94°C for 1 minute, annealing and elongating at 68° C for 2 minutes, 5 followed by 4°C. The resulting 2<sup>nd</sup> strand cDNA (engineered template) is then cleaned up using a QIAGEN spin column (PCR Purification Kit). This step removed the free oligonucleotides and allowed simple buffer exchange for downstream protocols.

#### Heavy Chain Single Primer Amplification (SPA)

The engineered template is amplified using Advantage 2 polymerase mix 10 (Clontech) and its 10X reaction buffer, dNTPs, and primer “TMX24”. The samples are heat denatured at 95°C for 1 minute then cycled 30 times through 95° C for 5 seconds and 68° C for 1 minute. This is followed by 3 additional minutes at 68° C and a 4° C hold.

#### Heavy Chain Cloning & Production of the Library

15 Amplification products of approximately 500 bp are gel purified and then digested by Xho I and EcoR I. The inserts are cloned into an appropriate vector contains the remaining portion of the IgM CH1 domain. The ligated product containing a Fab library is introduced into *E. coli* by electroporation.

In order to produce the Fab library on the surface of bacteriophage, a suppressor 20 strain of cells such as XL1BLUE (Stratagene) is used. Following electroporation, the cells are shaken for 1 hour at 37° then carbenicillin is added to 20 ug/ml. After one hour shaking at 37° C the carbenicillin is increased to 50 ug/ml for an additional hour at 37° C.

VCS-M13 helper phage (Stratagene) are then added to provide all the necessary components for generation of phagemid particles and the volume of the culture is increased to 100mls of SB media. After an hour at 37°C kanamycin is added to 70 ug/ml to select for those bacteria containing helper phage DNA. The culture is shaken at 37°

5 overnight. During that time the bacteria produce new phagemid particles that have Fab displayed on its surface. The following morning the phagemid particles can be isolated by spinning out the bacterial cells and then precipitating the phagemid particles from the supernate with 4% PEG 8000 and 0.5 M NaCl on ice for 30 minutes. Precipitated phage pellet on centrifugation at 14,300 Xg. The pellet can be resuspended in PBS/1%BSA.

10 The preparation can be filtered to remove bacterial debris. The resulting library is stored at 4°.

**EXAMPLE 5**

Construction of a phagemid display library from mRNA of mice immunized with IgE or a recombinant IgE Fc CH2~4.

15 1<sup>st</sup> strand cDNA synthesis and modification for IgG Heavy and Kappa Light Chains

Mouse spleen mRNA from mice immunized with human IgE or recombinant human IgE was used to generate traditional 1<sup>st</sup> strand cDNA with an oligo dT primer. This was done using SuperScript First-Strand Synthesis for RT-PCR (Invitrogen Life

20 Technologies) according essentially to kit instructions. Restriction oligonucleotide "mCG1Xcm I" for IgG1, "mCG2aBsaJ I" for IgG2a, or "mCKHpa I" for kappa light chain was added to the first strand cDNA in order to generate a double stranded DNA region that could be digested by the restriction endonuclease Xcm I for IgG1, BsaJ I for IgG2a, or Hpa I for kappa light chain. "mCG1Xcm I" sequences is 5'CTAACTCCAT

25 GGTGACCCTGGGATG3' (Seq. ID No. 200). "mCG2aBsaJ I" sequence is

5'CAACTGGCTCCTCGGT GACTCTAG3' (Seq. ID No. 201), "mCKHpa I" sequence is 5'CAGTGAGCAGTTAACATCTGGAGG3' (Seq. No. 202). The reaction was set up with 1<sup>st</sup> strand cDNA 1uM oligonucleotide, and appropriate amount of 10x NEBuffer (New England Biolab) or 10X restriction buffer A(Roche Diagnostics). The sample was 5 heated to 95°C for 2 minutes and then held at 64°C for two minutes to allow specific annealing to occur and cooled to 37°C for Xcm I and Hpa I and 60 °C for BsaJ I. The restriction endonuclease Xcm I, BsaJ I, or Hpa I (New England Biolabs) was added and incubated at 37°C for 30 minutes, 60 °C for 30 min, and 37°C for 10 minutes, respectively. The restriction enzyme was heat inactivated for Xcm I at 65°C for 20 10 minutes and for BsaJ I at 80°C for 20 min and then the sample was cooled to 4°C.

Digestion of the 1<sup>st</sup> strand cDNAs by each restriction endonuclease was verified by PCR amplification using techniques known to those skilled in the art. These products were not used for cloning the antibody genes. Positive amplification of the digested 1<sup>st</sup> strand cDNA was observed in reactions using the 5' TMX24mVHIIIBshort and the 3' 15 mCG1 internal control primer for IgG1, 5' TMX24mVHIIIBshort and the 3' mCG2a internal control primer for IgG2a, and 5' TMX24mVKIVshort and the 3' mCK0 internal control primer for kappa. Good amplification with primers 5' TMX24mVHIIIBshort / 3' mCG1 or primers 5' TMX24mVHIIIBshort / 3' mCG2a or 5' TMX24mVKIVshort /3' mCK0 and minimal amplification with primers 5' TMX24mVHIIIBshort / 3' mCG1B or 20 5' TMX24mVHIIIBshort / 3' mCG2aB or 5' TMX24mVKIVshort /3' mCKB indicate successful digestion of the 1<sup>st</sup> strand cDNA template with each restriction endonuclease. Sequences of the primers used for check PCR were

TMX24mVHIIIBshort (Seq. ID No. 203)

5'GACGTGGCCGTTGGAAGAGGAGTGCTCGAGGTCCAATGCAGCAGYC3'

mCG1 (Seq. ID No. 204) 5'CATGGAGTTAGTTGGGCAGCAG3'

mCG1B (Seq. ID No. 205) 5'CAACGTTGCAGGTGACGGTCTC3'

mCG2a (Seq. ID No. 206) 5'CGAGGAGGCCAGTTGTATCTCCAC3'

5 mCG2aB (Seq. ID No. 207) 5'CCACATTGCAGGTGATGGACTG3'

TMX24mVKIVshort (Seq. ID No. 208)

5'GACGACCGGCTACCAAGAGGAGTGTCTAGAGAAAWTGTGCTACCCAGTC  
TC3'

mCK0 (Seq. ID No. 209) 5'CTGCTCACTGGATGGTGGGAAG3'

10 mCKB (Seq. ID No. 210) 5'GAGTGGCCTCACAGGTATACTG3'

#### Light Chain 2<sup>nd</sup> Strand Linear Amplification and Nested Oligo Extension

Hpa I digested 1<sup>st</sup> strand cDNA was used as the original template to set up multiple 2<sup>nd</sup> strand cDNA reactions using a framework 1 specific primer (0.4 uM final),  
15 dNTPs, AmpliTaq enzyme and its 10X reaction buffer (Applied Biosystems). The primers were designed to contain TMX24mK sequence for kappa, and Xba I restriction site, and a region that annealed to 1<sup>st</sup> strand cDNA in the framework 1 region of mouse antibody kappa light chain genes. Those annealing sequences were designed based on the known sequences of mouse antibodies derived from Kabat database  
20 (<http://immuno.bme.nwu.edu/>) to cover the entire mouse antibody repertoire of kappa light chain genes.

#### **D. Kappa Framework 1 Specific Primers:**

TMX24mVKIshort (Seq. ID No. 211) Xba I

25 5' GACGACCGGCTACCAAGAGGAGTGTCTAGAGACATTGTGATGWCACAGTCTC3 '

5 'GACGACCGGCTACCAAGAGGAGTGTCTAGAGATGTTGTGATGACCCARACTC3'  
TMX24mVKIIbshort (Seq. ID No. 212)  
5 'GACGACCGGCTACCAAGAGGAGTGTCTAGAGACATTGTGATGACKCAGGCTG3'  
5 'GACGACCGGCTACCAAGAGGAGTGTCTAGAGACAWTGTGCTGACCCARTCTC3'  
TMX24mVKIIIsshort (Seq. ID No. 213)  
5 'GACGACCGGCTACCAAGAGGAGTGTCTAGAGACAWTGTGCTGACCCARTCTC3'  
TMX24mVKIVshort (Seq. ID No. 214)  
5 'GACGACCGGCTACCAAGAGGAGTGTCTAGAGACAWTGTGCTCACCCAGTCTC3'  
TMX24mVKVashort (Seq. ID No. 215)  
5 'GACGACCGGCTACCAAGAGGAGTGTCTAGAGACATCCAGATGACMCAGTCTC3'  
TMX24mVKVbshort (Seq. ID No. 216)  
5 'GACGACCGGCTACCAAGAGGAGTGTCTAGAGATATCCAGATGACACAGACTAC3'  
TMX24mVKVcshort (Seq. ID No. 217)  
5 'GACGACCGGCTACCAAGAGGAGTGTCTAGAGACATTGTSATGACCCAGTC3'  
TMX24mVKVIshort (Seq. ID No. 218)  
5 'GACGACCGGCTACCAAGAGGAGTGTCTAGACAAATTGTTCTCACCCAGTCTC3'  
TMX24mVKVIshort (Seq. ID No. 219)  
5 'GACGACCGGCTACCAAGAGGAGTGTCTAGACAAATTGTTCTCACCCAGTCTC3'

Wherein (R is and equal mixture of A and G, M is and equal mixture of A and C, K is and equal mixture of G and T, W is and equal mixture of A and T, and S is and equal mixture of C and G).

The samples were heat denatured at 94° C for 1 minute then cycled 20 times through 94° C for 5 seconds, 56° C for 10 seconds, and 68° C for 2 minutes. This allowed linear amplification of the 2<sup>nd</sup> strand cDNA. A nested oligo designated "TMX24mCKnoer" for kappa chains was added on ice to a final concentration of 0.2 uM.

25 The sequence of; "TM24CKnpt" was 5'GACGACCGGCTACCAAGAGGAGTGTCCG GATGTTAACTGCTCACTGGATGGTGGGAAGATGG2'OMe[A(ps)U(ps)U(ps)](prop yl) 3' (Seq. ID No. 220). The 2<sup>nd</sup> strand cDNAs were further elongated off the nested oligo by heat denaturing at 94°C for 1 minute, annealing and elongating at 68° C for 2 minutes, followed by 4°C. The resulting 2<sup>nd</sup> strand cDNA (engineered template) were 30 cleaned up using a QIAGEN spin column (PCR Purification Kit). This step removes the free oligonucleotides and allows simple buffer exchange for downstream protocols.

Light Chain Single Primer Amplification (SPA)

The engineered template was amplified using Advantage 2 polymerase mix (Clontech) and its 10X reaction buffer, dNTPs, and primer "TMX24mK" for kappa chains. The sequence for "TMX24mK" is 5'GACGACCGGCTACCAAGAGGAGTG3' (Seq. ID No. 221). The samples were heat denatured at 95°C for 1 minute then cycled 25times through 95° C for 5 seconds and 68° C for 1 minute. This was followed by an additional 3 minutes at 68° C and a 4° hold.

Light Chain Cloning

10 Kappa amplification products were gel purified and then digested by Xba I and BspE I. The inserts were cloned into a suitable expression vector that contains the remaining portion of the kappa light chain constant region. The ligated product was introduced into *E. coli* by electroporation and grown overnight at 37° C. The following morning a DNA maxiprep was performed to recover the light chain library DNA. The 15 light chain library DNA was used in subsequent steps to clone in the heavy chain Fd fragments by Xho I / Bln I to complete the construction of the library as described below in Heavy Chain Cloning.

Heavy Chain 2<sup>nd</sup> Strand Linear Amplification and Nested Oligo Extension

20 Xcm I and BsaJ I digested 1<sup>st</sup> strand cDNAs were used to set up multiple 2<sup>nd</sup> strand cDNA reactions using framework 1 specific primer (0.4 uM final), dNTPs, AmpliTaq enzyme and its 10X reaction buffer (Applied Biosystems). The primers were designed to contain the TMX24mH sequence, an Xho I restriction site, and a region that annealed to 1<sup>st</sup> strand cDNA in the framework 1 region of mouse antibody heavy chain genes. Those annealing sequences were designed based on the known sequences of

mouse antibodies derived from Kabat database (<http://immuno.bme.nwu.edu/>) to cover the entire mouse antibody repertoire of heavy chain genes.

**Heavy Chain Framework 1 Specific Primers:**

5

TMX24mVHIAshorter (Seq. ID No. 222) Xho I  
5 'GACGTGGCCGTTGGAAGAGGAGTGCTCGAGGTGCAGCTCAGSAGTC3 '

10

TMX24mVHIBshorter (Seq. ID No. 223)  
5 'GACGTGGCCGTTGGAAGAGGAGTGCTCGAGGTGCAGCTGAAGSAGTC3 '

TMX24mVHIIAshorter (Seq. ID No. 224)

5 'GACGTGGCCGTTGGAAGAGGAGTGCTCGAGGTYCAGCTGCARCARC3 '

15

TMX24mVHIIIBshorter (Seq. ID No. 225)  
5 'GACGTGGCCGTTGGAAGAGGAGTGCTCGAGGTCCAATGCAGCAGYC3 '

TMX24mVHIIICshorter (Seq. ID No. 226)

5 'GACGTGGCCGTTGGAAGAGGAGTGCTCGAGGTTCAGCTGCAGCAGTC3 '

20

TMX24mVHIIIAshorter (Seq. ID No. 227)  
5 'GACGTGGCCGTTGGAAGAGGAGTGCTCGAGGTGAAGCTGGTGGAGWC3 '

TMX24mVHIIIBshorter (Seq. ID No. 228)

5 'GACGTGGCCGTTGGAAGAGGAGTGCTCGAGGTGAAGCTCTGGAGTC3 '

TMX24mVHIIIDshorter (Seq. ID No. 229)

5 'GACGTGGCCGTTGGAAGAGGAGTGCTCGAGGTGMAGCTGGTGGAGTC3 '

30

Wherein (R is an equal mixture of A and G, M is an equal mixture of A and C, Y is an equal mixture of C and T, and S is an equal mixture of C and G).

The samples were heat denatured at 94° C for 1 minute then cycled 20 times through 94° C for 5 seconds, 56° C for 10 seconds, and 68° C for 2 minutes. This allowed linear amplification of the 2<sup>nd</sup> strand cDNA. A nested oligo designated

35

“TMX24mCG1noer” for IgG1 and “TMX24mCG2anoer” for IgG2a was added on ice to

a final concentration of 0.2 uM. The sequence of; “TMX24mCG1noer” was

5'GACGTGGCCGTTGGAAGAGGAGTGCCCTAGGGTTACCATGGAGTTAGTTGG

GCAGCAGA2'OMe[U(ps)C(ps)A(ps)](propyl) 3' (Seq. ID No. 230) and "TMX24mCG2anoer" was 5'GACGTGGCCGTTGGAAGAGGAGTGCCTAGGGTCATCGAGGAGCCAGTTGTA TCTCCACA2'OMe[C(ps)A(ps)U(ps)](propyl) 3' (Seq. ID No. 231).

5 The 2<sup>nd</sup> strand cDNAs were further elongated off the nested oligo by heat denaturing at 94°C for 1 minute, annealing and elongating at 68° C for 2 minutes, followed by 4°C. The resulting 2<sup>nd</sup> strand cDNA (engineered template) were cleaned up using a QIAGEN spin column (PCR Purification Kit). This step removes the free oligonucleotides and allows simple buffer exchange for downstream protocols.

10 Heavy Chain Single Primer Amplification (SPA)

The engineered template was amplified using Advantage 2 polymerase mix (Clontech) and its 10X reaction buffer, dNTPs, and primer "TMX24mH" for heavy chains. The sequence for "TMX24mH" is 5' GACGTGGCCGTTGGAAGAGGAGTG 3' (Seq. ID No. 232). The samples were heat denatured at 95°C for 1 minute then cycled 15 28 times for IgG1 and 30 times for IgG2a through 95° C for 5 seconds and 68° C for 1 minute. This was followed by an additional 3 minutes at 68° C and a 4° hold.

Heavy Chain Cloning

Heavy chain amplification products were gel purified and then digested by Xho I 20 and Bln I. The inserts were cloned into kappa chain library DNAs that contain the remaining portion of the heavy chain constant region for IgG1 and IgG2a. The ligated product was introduced into E. coli by electroporation and grown overnight at 37° C. The following morning a DNA maxiprep was performed to recover the IgG1 kappa or IgG2a kappa library DNA.

Panning and Screening of Libraries on recombinant IgE Fc CH2~4

The libraries panned on recombinant IgE Fc CH2~4 for 4 rounds essentially as described in Barbas III, CF Burton, DR, Scott, JK, and Silverman, GJ (2001) Phage Display: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. Individual clones from the 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> rounds of panning were screened by ELISA on recombinant IgE Fc CH2~4.

Clones that showed specific binding to IgE IgE Fc CH2~4 and minimal binding to a non-specific protein, ovalbumin by ELISA were analyzed by DNA sequencing. A total of 31 distinct Fabs to IgE Fc CH2~4 were isolated from mice libraries. See Figs. 9a-d.

It will be understood that various modifications may be made to the embodiments described herein. Therefore, the above description should not be construed as limiting, but merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the scope and spirit of this disclosure.

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The claims defining the invention are as follows:

1. A method of amplifying nucleic acid comprising:

(a) annealing a primer to a template, the primer having a first portion which anneals to the template and a second portion of predetermined sequence which does not anneal to the template and is not native to the template;

(b) synthesizing a polynucleotide that is complementary to the portion of the template between the location at which the first portion of the primer anneals to the template and the end of the template, the polynucleotide having the primer at a first end thereof and a second end;

(c) separating the polynucleotide synthesized in step (b) from the template;

(d) annealing a template oligonucleotide to the second end of the polynucleotide synthesized in step (b), the template oligonucleotide having a first portion that anneals to the second end of the polynucleotide and a second portion having the same predetermined sequence as the second portion of the primer;

(e) extending the polynucleotide synthesized in step (b) to provide a terminal portion thereof that is complementary to the predetermined sequence; and

(f) amplifying the extended polynucleotide using a single primer having the predetermined sequence.

2. A method as in claim 1 wherein the step of annealing a template oligonucleotide comprises providing a collection of template oligonucleotides having different sequences and contacting the collection of template oligonucleotides with the polynucleotide synthesized in step (b).

3. A method as in claim 1 or 2 wherein the template is digested prior to annealing the primer.

4. A method according to any one of claims 1-3 wherein the polynucleotide synthesized in step (b) is digested prior to annealing the template oligonucleotide.

5. A method of amplifying nucleic acid comprising:

(a) annealing a primer and a boundary oligonucleotide to a template, the primer having a first portion which anneals to the template and a second portion of predetermined sequence which does not anneal to the template and is not native to the template;

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(b) synthesizing a polynucleotide that is complementary to the portion of the template between the location at which the first portion of the primer anneals to the template and the portion of the template to which the boundary oligonucleotide anneals, the polynucleotide having the primer at a first end thereof and a second end;

5 (c) separating the polynucleotide synthesized in step (b) from the template;

(d) annealing a template oligonucleotide to the second end of the polynucleotide synthesized in step (b), the template oligonucleotide having a first portion that anneals to the second end of the polynucleotide and a second portion having the same predetermined sequence as the second portion of the primer;

0 (e) extending the polynucleotide synthesized in step (b) to provide a terminal portion thereof that is complementary to the predetermined sequence;

(f) amplifying the extended polynucleotide using a single primer having the predetermined sequence.

6. A method as in claim 5 wherein the step of annealing a template oligonucleotide  
5 comprises providing a collection of template oligonucleotides having different sequences and contacting the collection of template oligonucleotides with the polynucleotide synthesized in step (b).

7. A method of amplifying a nucleic acid strand comprising:

(a) providing an engineered nucleic acid strand having a predetermined sequence at a  
20 first end thereof and a sequence complementary to the predetermined sequence at the other end thereof;

(b) contacting the engineered nucleic acid strand with a primer having the predetermined sequence in the presence of a polymerase and nucleotides under conditions suitable for polymerization of the nucleotides.

25 8. A method of producing a collection of nucleic acids encoding an antibody library comprising:

(a) providing a diverse population of templates;

(b) contacting the diverse population of templates with at least one primer, the at least  
30 one primer having a first portion which anneals to the templates and a second portion of predetermined sequence which does not anneal to the templates and is not native to the templates;

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(c) synthesizing polynucleotides that are complementary to the portion of the templates between the location at which the first portion of the primer anneals to the template and the end of the templates, the polynucleotides having the primer at a first end thereof and a second end;

5 d) separating the polynucleotides synthesized in step (c) from the templates;

e) annealing at least one template oligonucleotide to the second end of the polynucleotides synthesized in step (c), the at least one template oligonucleotide having a first portion that anneals to the second end of the polynucleotides and a second portion having the same predetermined sequence as the second portion of the primer;

10 f) extending the polynucleotides synthesized in step (c) to provide a terminal portion thereof that is complementary to the predetermined sequence; and

g) amplifying the extended polynucleotides using a single primer having the predetermined sequence.

9. A method of producing an antibody library comprising:

15 a) providing a diverse population of templates;

b) contacting the diverse population of templates with at least one primer, the at least one primer having a first portion which anneals to the templates and a second portion of predetermined sequence which does not anneal to the templates and is not native to the templates;

20 c) synthesizing polynucleotides that are complementary to the portion of the templates between the location at which the first portion of the primer anneals to the template and the end of the templates, the polynucleotides having the primer at a first end thereof and a second end;

d) separating the polynucleotides synthesized in step (c) from the templates;

25 e) annealing at least one template oligonucleotide to the second end of the polynucleotides synthesized in step (c), the at least one template oligonucleotide having a first

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portion that anneals to the second end of the polynucleotides and a second portion having the same predetermined sequence as the second portion of the primer;

f) extending the polynucleotides synthesized in step (c) to provide a terminal portion thereof that is complementary to the predetermined sequence;

5 g) amplifying the extended polynucleotides using a single primer having the predetermined sequence to produce an amplified nucleic acid library; and

h) expressing said antibody library using said amplified nucleic acid library.

10. A method of adding a predetermined sequence to a nucleic acid and amplifying said nucleic acid with said added predetermined sequence, said method comprising:

10 a) annealing an oligonucleotide to a template to form a complex comprising a double-stranded section of nucleic acid comprising a restriction endonuclease recognition site;

b) incubating said complex with a restriction endonuclease that cleaves said complex at said restriction endonuclease recognition site to produce a shortened template with a 5' end and a 3' end;

5 c) annealing a primer to said shortened template, the primer having a first portion which anneals to the shortened template and a second portion of predetermined sequence which does not anneal to the shortened template and is not native to the template;

d) synthesizing a polynucleotide that is complementary to the portion of the shortened template between the location at which the first portion of the primer anneals to the

20 shortened template and the 5' end of the shortened template, the polynucleotide comprising i) the primer at a first end thereof and ii) a second end complementary to the 5' end of the shortened template;

e) separating the polynucleotide synthesized in step (d) from the shortened template;

f) annealing a nested oligonucleotide to the second end of the polynucleotide

25 synthesized in step (d), the nested oligonucleotide having a first portion that anneals to the second end of the polynucleotide and a second portion having the same predetermined sequence as the second portion of the primer;

g) extending the polynucleotide synthesized in step (d) to produce an extended polynucleotide comprising a terminal portion thereof that is complementary to the predetermined

30 sequence; and

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h) amplifying the extended polynucleotide using a single primer having the predetermined sequence.

11. The method of claim 10 wherein said nested oligonucleotide is incapable of being extended in the 3' direction by means of DNA polymerase after being annealed to said second end of the polynucleotide synthesized in step (d).

12. The method of claim 11 wherein said nested oligonucleotide comprises one or more modified bases at its 3' end preventing extension of said 3' end by DNA polymerase.

13. The method of claim 11 wherein said nested oligonucleotide comprises a 3' end that does not anneal to said polynucleotide synthesized in step (d).

14. The method according to any one of claims 10-13 further comprising amplifying said template prior to step (a).

15. The method according to any one of claims 10-14 wherein said nested oligonucleotide encodes a portion of an antibody selected from the group consisting of i) a complementarity determining region, ii) a framework region, and iii) a constant region.

16. A method of adding a predetermined sequence to a nucleic acid and amplifying said nucleic acid with said added predetermined sequence, said method comprising:

- annealing a primer to a template, the primer having a first portion which anneals to the template and a second portion of predetermined sequence which does not anneal to the template and is not native to the template;
- synthesizing a polynucleotide that is complementary to the portion of the template between the location at which the first portion of the primer anneals to the template and the 5' end of the template, the polynucleotide comprising i) the primer at a first end thereof and ii) a second end complementary to the 5' end of the template;
- separating the polynucleotide synthesized in step (b) from the template;
- annealing an oligonucleotide to the separated polynucleotide of step (c) to form a complex comprising a double-stranded section of nucleic acid comprising a restriction endonuclease recognition site;
- incubating said complex with a restriction endonuclease that cleaves said complex at said restriction endonuclease recognition site to produce a shortened polynucleotide with a 5' end and a 3' end;
- annealing a nested oligonucleotide to the 3' end of the shortened polynucleotide,

28 Feb 2008 . the nested oligonucleotide having a first portion that anneals to the 3' end of the polynucleotide and a second portion having the same predetermined sequence as the second portion of the primer;

5 g) extending the shortened polynucleotide to produce an extended polynucleotide comprising a terminal portion thereof that is complementary to the predetermined sequence; and

10 h) amplifying the extended polynucleotide using a single primer having the predetermined sequence.

17. The method of claim 16 wherein said nested oligonucleotide is incapable of being extended in the 3' direction by means of DNA polymerase after being annealed to said 3' end of the shortened polynucleotide synthesized in step (e).

18. The method of claim 17 wherein said nested oligonucleotide comprises one or more modified bases at its 3' end preventing extension of said 3' end by DNA polymerase.

19. The method of claim 16 wherein said nested oligonucleotide comprises a 3' end that does not anneal to said shortened polynucleotide synthesized in step (e).

20. The method according to any one of claims 16-19 further comprising amplifying said template prior to step (a).

21. The method according to any one of claims 16-19 wherein said nested oligonucleotide encodes a portion of an antibody selected from the group consisting of i) a complementarity determining region, ii) a framework region, and iii) a constant region.

22. An engineered nucleic acid strand produced by any one of the methods according to claims 10-21 having a predetermined sequence at a first end thereof and a sequence complementary to the predetermined sequence at the other end thereof.

23. A method of amplifying nucleic acid comprising:

24 (a) annealing a primer to a template, the primer having a first portion which anneals to the template and a second portion of predetermined sequence which does not anneal to the template and is not native to the template;

25 (b) synthesizing a polynucleotide that is complementary to the portion of the template between the location at which the first portion of the primer anneals to the template and the end of the template, the polynucleotide having the primer at a first end thereof and a second end;

26 (c) separating the polynucleotide synthesized in step (b) from the template;

27 (d) annealing a template oligonucleotide to the second end of the polynucleotide synthesized in step (b), the template oligonucleotide having a first portion that anneals to the

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second end of the polynucleotide and a second portion having the same predetermined sequence as the second portion of the primer, wherein said template oligonucleotide has a 3'-end which (i) does not hybridize to the polynucleotide synthesized in step (b) or (ii) includes modified bases preventing extension of said 3'-end;

5 (e) extending the polynucleotide synthesized in step (b) to provide a terminal portion thereof that is complementary to the predetermined sequence; and

(f) amplifying the extended polynucleotide using a single primer having the predetermined sequence.

24. A method as in claim 23 wherein the step of annealing a template oligonucleotide 0 comprises providing a collection of template oligonucleotides having different sequences and contacting the collection of template oligonucleotides with the polynucleotide synthesized in step (b).

25. A method as in claim 23 or 24 wherein the template is digested prior to annealing the primer.

5 26. A method according to any one of claims 23-25 wherein the polynucleotide synthesized in step (b) is digested prior to annealing the template oligonucleotide.

27. A method of amplifying nucleic acid comprising:

(a) annealing a primer and a boundary oligonucleotide to a template, the primer having a first portion which anneals to the template and a second portion of predetermined sequence which does not anneal to the template and is not native to the template;

(b) synthesizing a polynucleotide that is complementary to the portion of the template between the location at which the first portion of the primer anneals to the template and the portion of the template to which the boundary oligonucleotide anneals, the polynucleotide having the primer at a first end thereof and a second end;

25 (c) separating the polynucleotide synthesized in step (b) from the template;

(d) annealing a template oligonucleotide to the second end of the polynucleotide synthesized in step (b), the template oligonucleotide having a first portion that anneals to the second end of the polynucleotide and a second portion having the same predetermined sequence as the second portion of the primer, wherein said template oligonucleotide has a 3'-end which i) 30 does not hybridize to the polynucleotide synthesized in step (b) or ii) includes modified bases preventing extension of said 3'-end;

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(e) extending the polynucleotide synthesized in step (b) to provide a terminal portion thereof that is complementary to the predetermined sequence; and

(f) amplifying the extended polynucleotide using a single primer having the predetermined sequence.

5 28. A method as in claim 27 wherein the step of annealing a template oligonucleotide comprises providing a collection of template oligonucleotides having different sequences and contacting the collection of template oligonucleotides with the polynucleotide synthesized in step (b).

10 29. A method of producing a collection of nucleic acids encoding an antibody library comprising:

(a) providing a diverse population of templates;

(b) contacting the diverse population of templates with at least one primer, the at least one primer having a first portion which anneals to the templates and a second portion of predetermined sequence which does not anneal to the templates and is not native to the

15 templates;

(c) synthesizing polynucleotides that are complementary to the portion of the templates between the location at which the first portion of the primer anneals to the templates and the end of the templates, the polynucleotides having the primer at a first end thereof and a second end;

20 (d) separating the polynucleotides synthesized in step (c) from the templates;

(e) annealing at least one template oligonucleotide to the second end of the polynucleotides synthesized in step (c), the at least one template oligonucleotide having a first portion that anneals to the second end of the polynucleotides and a second portion having the same predetermined sequence as the second portion of the primer, wherein said at least one

25 template oligonucleotide has a 3'-end which (i) does not hybridize to the polynucleotides synthesized in step (c) or (ii) includes modified bases preventing extension of said 3'-end;

(f) extending the polynucleotides synthesized in step (c) to provide a terminal portion thereof that is complementary to the predetermined sequence; and

30 (g) amplifying the extended polynucleotides using a single primer having the predetermined sequence.

30. A method of producing an antibody library comprising:

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(a) providing a diverse population of templates;

(b) contacting the diverse population of templates with at least one primer, the at least one primer having a first portion which anneals to the templates and a second portion of predetermined sequence which does not anneal to the templates and is not native to the templates;

(c) synthesizing polynucleotides that are complementary to the portion of the templates between the location at which the first portion of the primer anneals to the templates and the end of the templates, the polynucleotides having the primer at a first end thereof and a second end;

(d) separating the polynucleotides synthesized in step (c) from the templates;

(e) annealing at least one template oligonucleotide to the second end of the polynucleotides synthesized in step (c), the at least one template oligonucleotide having a first portion that anneals to the second end of the polynucleotides and a second portion having the same predetermined sequence as the second portion of the primer, wherein said at least one template oligonucleotide has a 3'-end which i) does not hybridize to the polynucleotides synthesized in step (c) or ii) includes modified bases preventing extension of said 3'-end;

(f) extending the polynucleotides synthesized in step (c) to provide a terminal portion thereof that is complementary to the predetermined sequence;

(g) amplifying the extended polynucleotides using a single primer having the predetermined sequence to produce an amplified nucleic acid library; and

(h) expressing said antibody library using said amplified nucleic acid library

31. A method substantially as hereinbefore described with reference to any one of the examples.

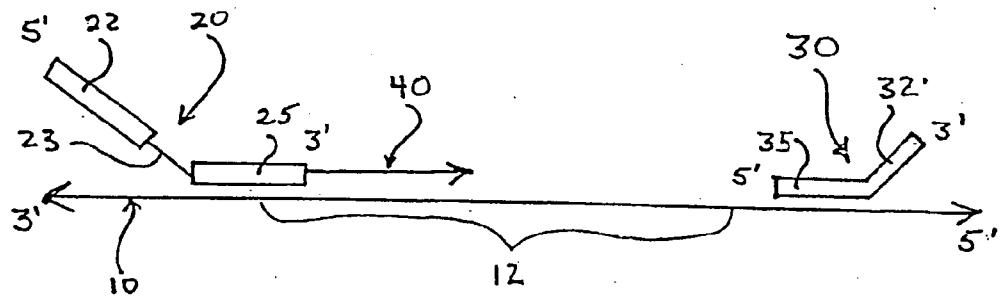


Fig. 1

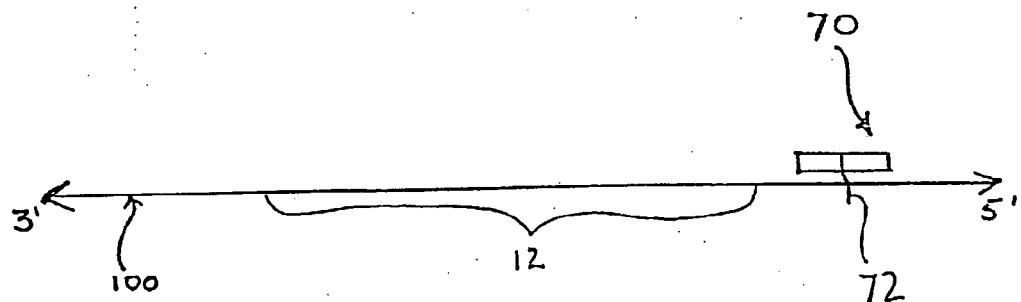


Fig. 2A

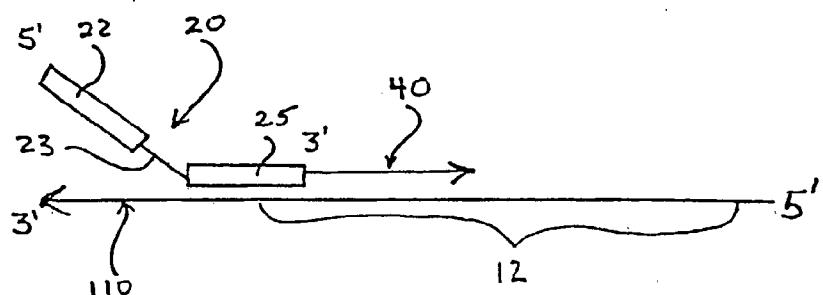
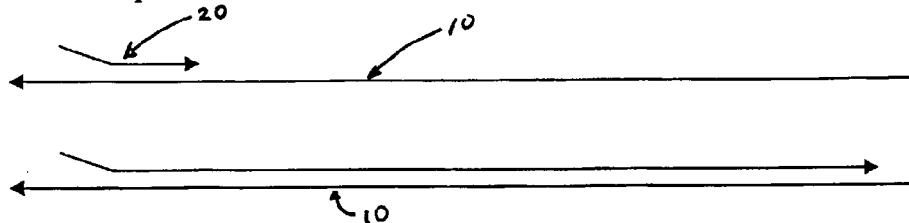


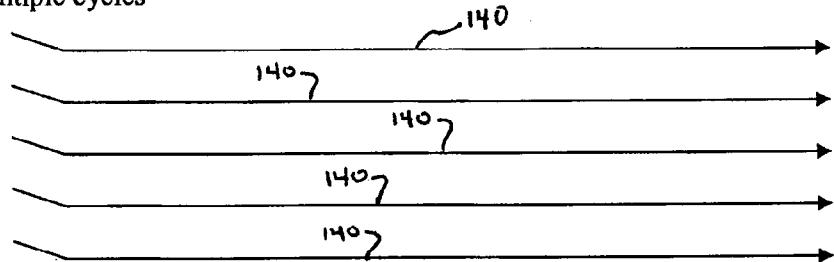
Fig. 2B

### Fig. 3

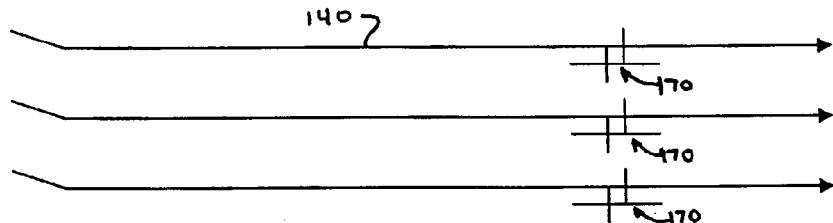
2<sup>nd</sup> strand cDNA synthesis on 1<sup>st</sup> strand cDNA by a primer that has a first portion that hybridizes to the variable region of an antibody gene and a second portion with a predetermined sequence



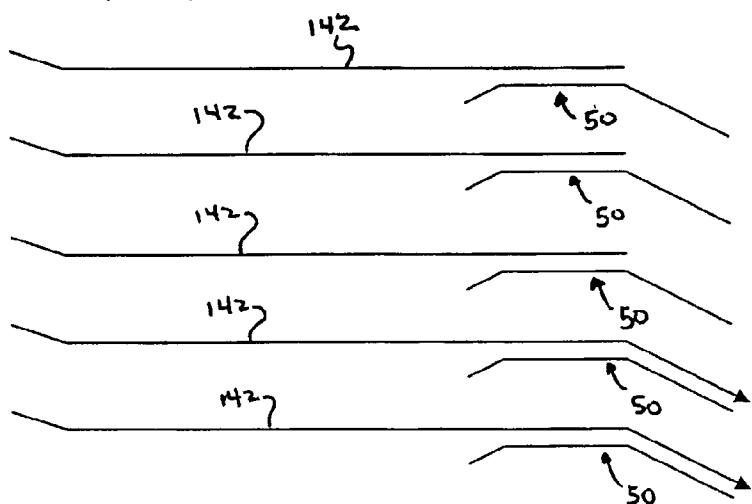
Repeat multiple cycles



Hybridize a restriction oligonucleotide on the desired position in the constant region of an antibody gene and digest with an appropriate restriction enzyme (RED)



Heat denature and add nested oligo nucleotide and perform nested oligonucleotide extension reaction (NOER)



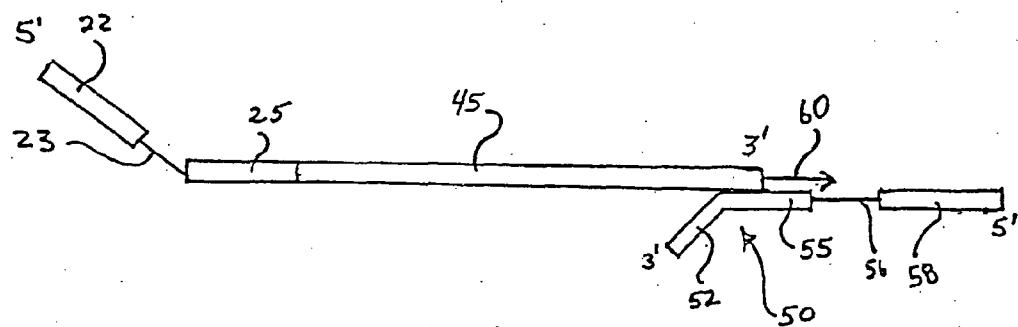


Fig. 4

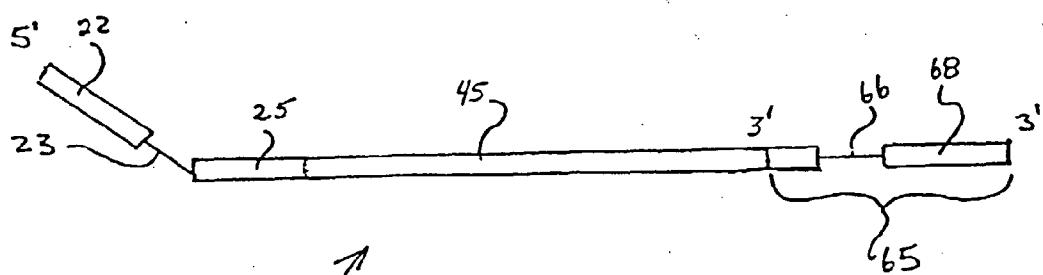
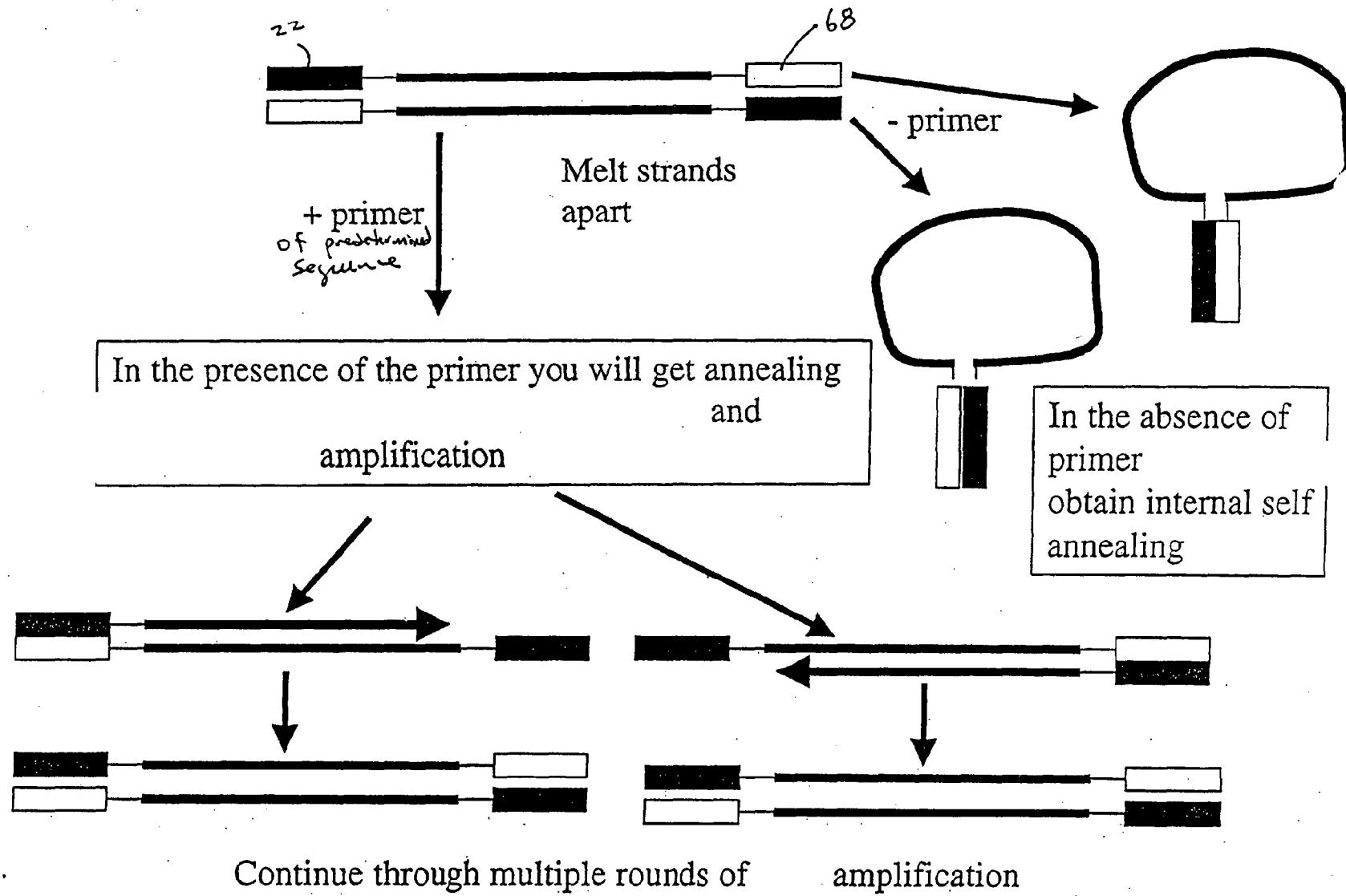


Fig. 5

Figure 6. Single primer amplification



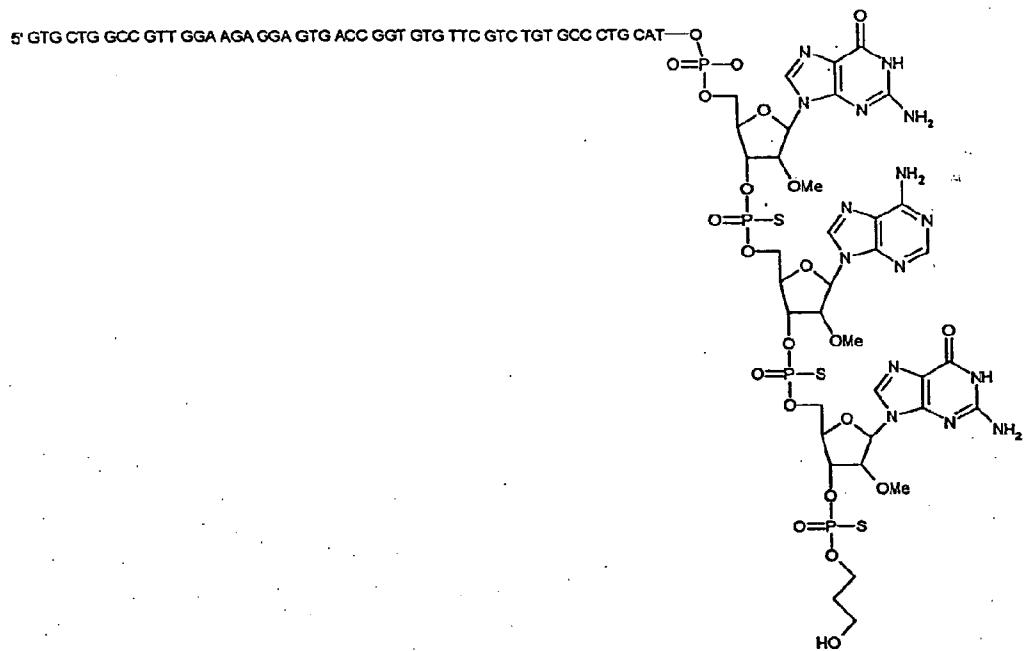


Fig. 7

## IgG kappa clones

## Heavy chain

| Fab      | framework 1                    | CDR1     | framework 2    | CDR2                | IgG kappa clones |  |
|----------|--------------------------------|----------|----------------|---------------------|------------------|--|
|          |                                |          |                |                     | Heavy chain      |  |
| HBPAXK1a | EVQLVESGGGLVQPGGSLRLSCAASGFTFG | S--YTMN  | WVRQAPGKGLEWIS | YIST--TSSSIYYADSVKG | VH3              |  |
| HBPAXK1b | Q***L***V***T***T***           | ***      | ***            | ***                 | VH3              |  |
| HBPAXK1c | Q***                           | ***      | ***            | ***                 | VH3              |  |
| HBPAXK1d | ***L***                        | ***      | ***            | ***                 | VH3              |  |
| HBPAXK2a | EVQLVESGGGLVQPGGSLRLSCAASGFTFG | S--SAMS  | WVRQAPGKGLEWVS | VISG--NGFSTYYADSVKG | VH3              |  |
| HBPAXK2b | ***                            | ***      | ***            | ***                 | VH3              |  |
| HBPAXK2c | ***                            | ***      | ***            | ***                 | VH3              |  |
| HBPAXK2d | ***                            | ***      | ***            | ***                 | VH3              |  |
| HBPAXK2e | ***                            | ***      | ***            | ***                 | VH3              |  |
| HBPAXK3a | EVQLVESGGGLVQPGGSLRLSCAASGFTFS | S--SAMS  | WVRQAPGKGLEWVS | VISG--NGFSTYYADSVKG | VH3              |  |
| HBPAXK3b | ***V***                        | ***V***  | ***            | ***                 | VH3              |  |
| HBPAXK3c | ONQLVQSGEVNGVTCGASVYKCGVTCG    | R--YVTLN | WVRQAPGKGLEWVS | WVNG--WVGVYAKLQG    | VH3              |  |
| HBPAXK3d | ***                            | ***      | ***            | ***                 | VH3              |  |
| HBPAXK5  | QVQVQSGAEVKPGASVKVSCKASGYTFT   | S--YGIC  | WVRQAPGKGLEWMC | WIST--YNGNTMYAQLQG  | VH1              |  |
| HBPAXK6  | EVQLVESGGGLVQPGGSLRLSCAASGFTFD | D--YAMH  | WVRQAPGKGLEWVS | WVNG--WVGVYAKLQG    | VH3              |  |
| HBPAXK7a | QVQLQESGPGLVKPSETLSLTCTVSGGSIS | SLMYFWG  | WIRQPPGKGLEWIG | SIY--YSGTAYYNPSLRS  | VH4              |  |
| HBPAXK7b | ***                            | ***V***  | ***S***        | ***                 | VH4              |  |
| HBPAXK7c | ***                            | ***A***  | ***            | ***T***             | VH4              |  |
| HBPAXK7d | ***L***                        | ***V***  | ***S***        | ***                 | VH4              |  |
| HBPAXK7e | ***                            | ***      | ***            | ***                 | VH4              |  |
| HBPAXK8  | ONQLVQSGEVNGVTCGASVYKCGVTCG    | R--YVTLN | WVRQAPGKGLEWVS | RHKSKEPGKGLDQVADWKG | VH3              |  |
| HBPAXK9a | EVQLVESGGGLVQPGGSLRLSCAASGFTFS | R--YVTLN | WVRQAPGKGLEWVS | YIST--DGSTIYYADSVKG | VH3              |  |
| HBPAXK9b | Q***                           | ***      | ***            | ***                 | VH3              |  |
| HBPAXK9c | EVQLVESGGEVNGVTCGASVYKCGVTCG   | R--YVTLN | WVRQAPGKGLEWMC | WITD--DGRGDNWAKROR  | VH3              |  |
| HBPAXK11 | EVQLVESGGVVVOPGGSLRLSCAASGFTFD | D--YAMH  | WVRQAPGKGLEWVS | LISW--DAISTYYADSVKG | VH3              |  |

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| Fab      | framework 3                        | CDR3                         |                      |                       |
|----------|------------------------------------|------------------------------|----------------------|-----------------------|
|          |                                    | DH                           | JH                   |                       |
| HBPAXK1a | RFSISRDNAKNSLYLQMNSLRDEDTAVYYCAR   | VFFVEGS-----                 | YWSFDLNGRGTLYTVSS    | JH2 (Seq. ID No. 83)  |
| HBPAXK1b | ***                                | ***                          | ***                  | JH2 (Seq. ID No. 84)  |
| HBPAXK1c | ***                                | ***                          | ***                  | JH2 (Seq. ID No. 85)  |
| HBPAXK1d | ***                                | ***                          | ***                  | JH2 (Seq. ID No. 86)  |
| HBPAXK2a | RFSISRDNAKNTLYLQMNSLRDEDTAVYYCAR   | VVWVGRH-----                 | WDRWQSGCPLTVSS       | JH3 (Seq. ID No. 87)  |
| HBPAXK2b | ***                                | ***                          | ***                  | JH3 (Seq. ID No. 88)  |
| HBPAXK2c | ***                                | ***                          | ***                  | JH3 (Seq. ID No. 89)  |
| HBPAXK2d | ***                                | ***                          | ***                  | JH3 (Seq. ID No. 90)  |
| HBPAXK2e | ***                                | ***                          | ***                  | JH3 (Seq. ID No. 91)  |
| HBPAXK3a | RFSISRDNAKNTLYLQMNSLRAEDTAEVYYCAR  | VKYGSGSHF-----               | WEDPPWQGTLTVTVSS     | JH5 (Seq. ID No. 92)  |
| HBPAXK3b | ***                                | ***TK***                     | ***                  | JH5 (Seq. ID No. 93)  |
| HBPAXK5a | RVTVTTRDSTSTAYMELRSILSDDTAVYYCAR   | VRGSGSDT-----                | DAVDINGQGRHLYTVSS    | JH6 (Seq. ID No. 94)  |
| HBPAXK5b | ***                                | ***                          | ***                  | JH6 (Seq. ID No. 95)  |
| HBPAXK5  | RVTMTTDSTSTAYMELRSILSDDTAVYYCAR    | AWPPRGSSQLDR-----            | GQYFQHWGQGTLTVTVSS   | JH1 (Seq. ID No. 96)  |
| HBPAXK6  | RFDISRDNAKNTLYLQMNSLRDEDTAVYYCAR   | QYKQWVQHGLDQYDMDVNGQSPVYTVSS | JH6 (Seq. ID No. 97) |                       |
| HBPAXK7a | RATISVDTSKNQLSLKLMSTVTAADTAEVYYCAR | PSSFYFNQRTSYPPGET-----       | APEIINGQGTTVAVSS     | JH3 (Seq. ID No. 98)  |
| HBPAXK7b | ***V***                            | ***S***                      | ***T***              | JH3 (Seq. ID No. 99)  |
| HBPAXK7c | VS***                              | ***R***                      | ***A***              | JH3 (Seq. ID No. 100) |
| HBPAXK7d | VS***                              | ***S***                      | ***G***              | JH3 (Seq. ID No. 101) |
| HBPAXK7e | VS***                              | ***                          | ***                  | JH3 (Seq. ID No. 102) |
| HBPAXK8  | RVTISRDNAKNTLYLQMNSLRDEDTAVYYCAR   | RPNRYQSGPAPV-----            | DEWYDQGTLTVTVSS      | JH4 (Seq. ID No. 103) |
| HBPAXK9a | RFTISRDNAKNSLQLMISLRDEDTAVYYCAR    | VFFGCGNFR-----               | HWYFDLNGRGTLYTVSS    | JH2 (Seq. ID No. 104) |
| HBPAXK9b | ***                                | ***                          | ***                  | JH2 (Seq. ID No. 105) |
| HBPAXK9c | RITIKESRSISQWVWVQKNSLQSDTAVYYCAR   | SEWSGDP-----                 | WDDYVQGQGTLTVTVSS    | JH4 (Seq. ID No. 106) |
| HBPAX11  | RFTTSRDNAKNFPLYLQMDSLTPEDTALYYCGG  | DQGGRFRL-----                | VDYWGQGTLTVTVSS      | JH4 (Seq. ID No. 107) |

Fig 8a

## Light chain

| Fab     | Clone | framework 1              | CDR1                   | framework 2     | CDR2                         |
|---------|-------|--------------------------|------------------------|-----------------|------------------------------|
| HBPAK1a | 3A1   | EIVMTQSPAALSVSPGERATLSC  | RASQSISS-----SLA       | WYQQKPGQAPRLLIY | AASTRAT VK3                  |
| HBPAK1b | 3A2   | *****T*****              | *****V*****            | -----N**        | *****G***** VK3              |
| HBPAK1c | 3A3   | *****F*****T*****A*****  | *****T*NM-----DV*      | ***R***G*****F  | GT*****P VK3                 |
| HBPAK1c | 3A8   | *****T*****S*****        | ***N*R*****            | ***S*****       | G*****S VK3                  |
| HBPAK1c | 3G6   | *****T*****TT*****       | ***H*VT*****           | ***D*****       | *T*****A VK3                 |
| HBPAK1d | 3C11  | *****T*****              | ***G***V*****          | ***N**          | *****H*****                  |
| HBPAK1d | 3F4   | *****T*****              | ***W*****V*****        | ***H*****       | *****H***** VK3              |
| HBPAK1d | 3A4   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3A5   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3A6   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3B8   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3B11  | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3B12  | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3B16  | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3B19  | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3B20  | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3B21  | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3B4   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3B5   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3B6   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3B7   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK1d | 3C4   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK3a | 3B1   | *****T*****              | *****V*T*****          | -----N**        | *****H***** VK3              |
| HBPAK3b | 3C6   | D*****T*****EA*****      | *****V*N*****          | -----N**        | *****G***** VK3              |
| HBPAK4a | 3D5   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK4a | 3D7   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK3              |
| HBPAK5  | 3B6   | *****T*****              | *****V*****            | -----N**        | *****G***** VK3              |
| HBPAK6  | 3C4   | *****T*****              | *****V*****            | -----N**        | *****G***** VK3              |
| HBPAK7a | 3D1   | ***L*****GT*****L*****   | *****VN*K*****         | -----F**        | ***R***P***** G*****NT** VK3 |
| HBPAK7b | 3D3   | ***L*****GT*****L*****   | *****V*S*****          | -----Y**        | *****G***S*** VK3            |
| HBPAK7b | 3F5   | ***L*****GT*****L*****   | *****I*****            | -----Y**        | *****ST***** VK3             |
| HBPAK7c | 3D7   | ***L*****GT*****L*****   | *****FGNN*****         | -----N**        | ***RL***** G***S*** VK3      |
| HBPAK7d | 3E1   | D*****DS*A*****L*****IN* | KS*****VLYSSNNKNN***** | *****P*****     | W*****ES VK4                 |
| HBPAK7e | 3E8   | *****S*****              | *****S*****            | -----N**        | *****G*N***** VK3            |
| HBPAK8  | 3D2   | *****T*****              | *****W*****            | *****Y*****     | *****W***** VK4              |
| HBPAK9a | 3E3   | *****T*****L*****        | *****GV*****           | -----Y**        | *****Q***** D***N*** VK3     |
| HBPAK9b | 3E7   | *****T*****L*****        | *****V*****            | -----Y**        | *****D***N*** VK3            |
| HBPAK9b | 3G5   | *****T*****L*****        | *****V*****            | -----Y**        | *****D***N*** VK3            |
| HBPAK11 | 3G8   | D*QL*****SS*A*V*DVSIT*   | *****D*N*****          | -----Y*V        | *F*****K**KS*** SLOG VK1     |

Fig 8b

| Fab      | framework | 3                                 | JK                                       | CDR3   |
|----------|-----------|-----------------------------------|--|--|
| HBPAXK1a | 3A1       | GIPARFSGSGSGTEFTLTISSLQSEDVFVVYYC | QQYNNWP--ITFGQGTRLEIR                    | JK5 (Seq. ID No. 108)                        |
| HBPAXK1b | 3A2       | *****                             | A*****                                   | *****K JK5 (Seq. ID No. 109)                 |
| HBPAXK1c | 3A3       | *****DY*****S*****A*****          | *****TY*****G*****P***VDF*               | JK3 (Seq. ID No. 110)                        |
| HBPAXK1c | 3A8       | L*****                            | *****A*****DK*****L*****G*****KV***      | JK4 (Seq. ID No. 111)                        |
| HBPAXK1c | 3G6       | V*****                            | *****A*****H*****K*****V*****G*****KVDL* | JK4 (Seq. ID No. 112)                        |
| HBPAXK1d | 3C11      | A*****                            | *****A*****F*****DK*****P*****N*****K    | JK2 (Seq. ID No. 113)                        |
| HBPAXK1d | 3F4       | A*****D*****                      | *****A*****F*****K*****P*****N*****K     | JK2 (Seq. ID No. 114)                        |
| HBPAXK2a | 3A4       | *****                             | *****                                    | *****K JK1 (Seq. ID No. 115)                 |
| HBPAXK2a | 3A5       | *****                             | *****                                    | *****K JK1 (Seq. ID No. 116)                 |
| HBPAXK2c | 3A5       | *****                             | *****                                    | *****K JK1 (Seq. ID No. 117)                 |
| HBPAXK2c | 3A6       | *****                             | *****                                    | *****K JK1 (Seq. ID No. 118)                 |
| HBPAXK2c | 3A7       | *****                             | *****                                    | *****K JK1 (Seq. ID No. 119)                 |
| HBPAXK2c | 3A7       | *****                             | *****                                    | *****K JK5 (Seq. ID No. 120)                 |
| HBPAXK2c | 3A9       | *****                             | *****                                    | *****K JK5 (Seq. ID No. 121)                 |
| HBPAXK2c | 3A9       | *****                             | *****                                    | *****K JK5 (Seq. ID No. 122)                 |
| HBPAXK2c | 3A9       | *****                             | *****                                    | *****K JK2 (Seq. ID No. 123)                 |
| HBPAXK2c | 3A9       | *****                             | *****                                    | *****K JK5 (Seq. ID No. 124)                 |
| HBPAXK2c | 3A9       | *****                             | *****                                    | *****K JK5 (Seq. ID No. 125)                 |
| HBPAXK2c | 3A9       | *****                             | *****                                    | *****K JK1 (Seq. ID No. 126)                 |
| HBPAXK2c | 3A9       | *****                             | *****                                    | *****K JK1 (Seq. ID No. 127)                 |
| HBPAXK3a | 3B1       | A*****                            | A*****F*****E*****P*****N*****K          | JK2 (Seq. ID No. 128)                        |
| HBPAXK3b | 3C6       | *****                             | T*****A*****D*****                       | *****K JK5 (Seq. ID No. 129)                 |
| HBPAXK4a | 3B3       | *****                             | *****                                    | *****K JK1 (Seq. ID No. 130)                 |
| HBPAXK4b | 3B2       | *****                             | *****                                    | *****K JK2 (Seq. ID No. 131)                 |
| HBPAXK5  | 3B6       | *****                             | A*****                                   | *****TW*****KV**K JK1 (Seq. ID No. 132)      |
| HBPAXK6  | 3C7       | *****                             | *****                                    | *****KV**K JK2 (Seq. ID No. 133)             |
| HBPAXK7a | 3D1       | ***D*****                         | R*EP***AL***                             | ***H*GSS-G-V*****DVK JK5 (Seq. ID No. 134)   |
| HBPAXK7b | 3D3       | ***D*****                         | R*EP***A*****                            | ***GSS-P-I*****K JK5 (Seq. ID No. 135)       |
| HBPAXK7b | 3F5       | ***D*****                         | D*****R*EP***A*****                      | ***GGS--F***P***KV*FK JK3 (Seq. ID No. 136)  |
| HBPAXK7c | 3D7       | A**D*****                         | D*****R*EP***A*****                      | ***GRP--G***G**KV**K JK4 (Seq. ID No. 137)   |
| HBPAXK7d | 3E1       | V*D*****                          | D*A*****A**VA*****                       | ***YST--L***G**KV**K JK4 (Seq. ID No. 138)   |
| HBPAXK7e | 3E8       | DF*****                           | Q*****G*****SA*****HD*****               | Q*****KV*FK JK1 (Seq. ID No. 139)            |
| HBPAXK8  | 3D9       | *****                             | *****                                    | *****K JK4 (Seq. ID No. 140)                 |
| HBPAXK9a | 3E3       | ***V*****                         | D*****EP***A*****                        | ***RS*****N***G**KV**K JK4 (Seq. ID No. 141) |
| HBPAXK9b | 3E7       | *****                             | D*****EP***A*****                        | ***RS*****P***G**KV**K JK4 (Seq. ID No. 142) |
| HBPAXK9b | 3G5       | *****                             | D*****EP***A*****                        | ***RS*****L*****K JK5 (Seq. ID No. 143)      |
| HBPAXK10 | 3F10      | *****                             | *****                                    | *****K JK4 (Seq. ID No. 144)                 |
| HBPAXK11 | 3G8       | V*S*****                          | D*****P***AT***                          | ***H*K*Y--L***G**KV**K JK4 (Seq. ID No. 145) |

Fig 8c

## IgG lambda clones

## Heavy chain

| Fab   | framework 1                            | CDR1  | framework 2    | CDR2              |
|-------|--|-------|----------------|-------------------|
| HBL1  | 3D11 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG   | SYAMS | WVRQAPGKGLEWVS | SISGSGDTIYYADSVRG |
|       | 3C12 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG   | SYAMS | WVRQAPGKGLEWVS | SISGSGDTIYYADSVRG |
|       | 3D9 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG    | SYAMS | WVRQAPGKGLEWVS | SISGSGDTIYYADSVRG |
| HBL2a | 3B5 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG    | SYAMS | WVRQAPGKGLEWVS | SISGSGDTIYYADSVRG |
| HBL2b | 3B6 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG    | SYAMS | WVRQAPGKGLEWVS | SISGSGDTIYYADSVRG |
| HBL2c | 3B7 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG    | SYAMS | WVRQAPGKGLEWVS | SISGSGDTIYYADSVRG |
| HBL2d | 3B8 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG    | SYAMS | WVRQAPGKGLEWVS | SISGSGDTIYYADSVRG |
| HBL2e | 3B9 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG    | SYAMS | WVRQAPGKGLEWVS | SISGSGDTIYYADSVRG |
| HBL2f | 3C4 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG    | SYAMS | WVRQAPGKGLEWVS | SISGSGDTIYYADSVRG |
| HBL3  | 3B10 LEQVQLVESGGGVVQPGGSRLSCAASGFTFS   | NYGMA | WVRQAPGKGLEWVA | YIYDGSKKYYDSVKG   |
| HBL4a | 3C11 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG   | SYAMS | WVRQAPGKGLEWVA | SISGSGDTIYYADSVRG |
| HBL4b | 3D6 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG    | SYAMS | WVRQAPGKGLEWVA | SISGSGDTIYYADSVRG |
| HBL4c | 3D7 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG    | SYAMS | WVRQAPGKGLEWVA | SISGSGDTIYYADSVRG |
| HBL4d | 3D8 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG    | SYAMS | WVRQAPGKGLEWVA | SISGSGDTIYYADSVRG |
| HBL4e | 3D9 LEEVQLLESGGGLVHPGGSRLSCAASGFRFG    | SYAMS | WVRQAPGKGLEWVA | SISGSGDTIYYADSVRG |
| HBL5  | 3D1 LEEVQLLESGGGLVQPGGSRLRISCAASGFRFG  | SYAMS | WVRQAPGKGLEWIS | GIVGTGGDTKYGDSVKG |
| HBL6  | 3D16 LEEVQLLESGGGLVQPGGSRLRISCAASGFRFG | SYAMS | WVRQAPGKGLEWVS | AISGCGGGSYYADSVRG |
| HBL7  | 3G5 LEQVQLVESGGGLVQPGGSRLSCAASGFTFS    | RYDIH | WVRQAPGKGLEWVA | LISYDGMYKSSADSVKG |

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| Fab   | framework 3                           | CDR3            |                                   |
|-------|---------------------------------------|-----------------|-----------------------------------|
|       |                                       | DH              | JH                                |
| HBL1  | 3D11 RFTISKDSSRNTLFLQLNSLRVDDTAVYYCAK | GSIFGTAKVYG     | VDYWGQGALTVSS (Seq. ID No. 146)   |
|       | 3C12 RFTISKDSSRNTLFLQLNSLRVDDTAVYYCAK | GSIFGTAKVYG     | VDYWGQGTLTVSS (Seq. ID No. 147)   |
|       | 3D9 RFTISKDSSRNTLFLQLNSLRVDDTAVYYCAK  | GSIFGTAKVYG     | VDYWGQGTLTVSS (Seq. ID No. 148)   |
| HBL2a | 3B5 RFTISKDSSRNTLFLQLNSLRVDDTAVYYCAK  | DEYVYSGCQFVYCAK | MDYVYSGCQFVYVSS (Seq. ID No. 149) |
| HBL2b | 3B6 RFTISKDSSRNTLFLQLNSLRVDDTAVYYCAK  | DEYVYSGCQFVYCAK | MDYVYSGCQFVYVSS (Seq. ID No. 150) |
| HBL2c | 3B7 RFTISKDSSRNTLFLQLNSLRVDDTAVYYCAK  | DEYVYSGCQFVYCAK | MDYVYSGCQFVYVSS (Seq. ID No. 151) |
| HBL2d | 3B8 RFTISKDSSRNTLFLQLNSLRVDDTAVYYCAK  | DEYVYSGCQFVYCAK | MDYVYSGCQFVYVSS (Seq. ID No. 152) |
| HBL2e | 3B9 RFTISKDSSRNTLFLQLNSLRVDDTAVYYCAK  | DEYVYSGCQFVYCAK | MDYVYSGCQFVYVSS (Seq. ID No. 153) |
| HBL3  | 3B10 RFTVSRDNTSNTLFLQLNSLRPDTAVYYCAK  | DGLLAGGYEGG     | FDYWGQGTLTVSS (Seq. ID No. 154)   |
| HBL4a | 3C11 RFTVSRDNTSNTLFLQLNSLRPDTAVYYCAK  | FDYWGQGTLTVSS   | MDYVYSGCQFVYVSS (Seq. ID No. 155) |
| HBL4b | 3D6 RFTVSRDNTSNTLFLQLNSLRPDTAVYYCAK   | FDYWGQGTLTVSS   | MDYVYSGCQFVYVSS (Seq. ID No. 156) |
| HBL4c | 3D7 RFTVSRDNTSNTLFLQLNSLRPDTAVYYCAK   | FDYWGQGTLTVSS   | MDYVYSGCQFVYVSS (Seq. ID No. 157) |
| HBL4d | 3D8 RFTVSRDNTSNTLFLQLNSLRPDTAVYYCAK   | FDYWGQGTLTVSS   | MDYVYSGCQFVYVSS (Seq. ID No. 158) |
| HBL4e | 3D9 RFTVSRDNTSNTLFLQLNSLRPDTAVYYCAK   | FDYWGQGTLTVSS   | MDYVYSGCQFVYVSS (Seq. ID No. 159) |
| HBL5  | 3D1 RFTISRDNSKNVYVLQMNGLRVEDTAVYYCAK  | SAYYVSGSYYG     | FDYWGQGTRIVSS (Seq. ID No. 160)   |
| HBL6  | 3D16 RFTVSRDNTSNTLFLQLNSLRPDTAVYYCAK  | CHYGRNWNVYV     | FDYWGQGTRIVSS (Seq. ID No. 161)   |
| HBL7  | 3G5 RFTVSRDNTSNTLFLQLNSLRPDTAVYYCAK   | SDVMARARGSG     | FDYWGQGTTVSS (Seq. ID No. 162)    |

Fig 8d

## Light chain

| Fab   |      | framework 1                | CDR1         | framework 2      | CDR2    |
|-------|------|----------------------------|--------------|------------------|---------|
| HBL1  | 3D11 | SRSYELTQPPSVSVA PGQTARITC  | GGNTIGSQSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
|       | 3C12 | SRSYVLTQPPSVSVA PGQTASIAC  | GGNNIGSKSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
|       | 3D9  | SRSYELTQPPSVSVA PRTDGGQITC | GEDKIESKSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
| HBL2a | 3B15 | SRSYELTQPPSVSVA PGQTARITC  | GGNTIGSKSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
| HBL2b | 3B18 | SRSYVLTQPPSVSVA PGQTARITC  | GGDSLNSKSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
|       | 3C6  | SRSYVLTQPPSVSVA PGQTARITC  | GGNTIGSKSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
| HBL2c | 3B9  | SRSYELTQPPSVTVA PGQTARITC  | GGNNIGSKSVH  | WYQQKPGQAPV LLVY | DDSDRPS |
| HBL2d | 3C4  | SRSYVLTQPPSVSVA PGQTARITC  | GGNTIGSKSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
| HBL3  | 3B10 | SRSYVLTQPPSVSVA PGQTAKIIC  | GGNNIGAKSVQ  | WYQQRPGQAPL MVVY | DDTERPS |
| HBL4a | 3C11 | SRSYVLTQPPSVSVA PGQTARITC  | GGNTIGSKSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
|       | 3D6  | SRSYVLTQPPSVSVA PGQTARITC  | GGNTIGSKSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
| HBL4b | 3B5  | SRSYVLTQPPSVSVA PGQTARITC  | GGNNIGRSQSVH | WYQQKPGQAPV LVVY | DDSDRPS |
| HBL4c | 3B8  | SRSYELTQPPSVSVA PGQTARITC  | GGNTIGSKSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
| HBL4d | 3B12 | SRSYVLTQPPSVSVA PGQTARITC  | GGNTIGSKSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
| HBL5  | 3D1  | SRSYVLTQPPSVSVA PGQTARITC  | GGNSIGSKSVH  | WYQQKPGQAPV LVVY | DDSDRPS |
| HBL7  | 3G5  | SRQAVLTQPPSVSVA PGQTARITC  | GGNNIGSKSAH  | WYQQRPGQAPL LVVY | DDSDRPS |

## CDR3

| Fab   |      | framework 3                      | JL  |
|-------|------|----------------------------------|---|
| HBL1  | 3D11 | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSSDH---VVFGGGTRLTVL (Seq. ID No. 163)  |
|       | 3C12 | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDASSDQPY-VVFGGGTRLTVL (Seq. ID No. 164)  |
|       | 3D9  | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSHH---VVFGGGTKLTVL (Seq. ID No. 165)   |
| HBL2a | 3B5  | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSSDH---VVFGGGTKLTVL (Seq. ID No. 166)  |
| HBL2b | 3PA  | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSSDH---VVFGGGTKLTVL (Seq. ID No. 167)  |
|       | 3C6  | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSSDH---VVFGGGTKLTVL (Seq. ID No. 168)  |
| HBL2c | 3B9  | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSSDH---VVFGGGTKLTVL (Seq. ID No. 169)  |
| HBL2d | 3C4  | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSSDH---VVFGGGTKLTVL (Seq. ID No. 170)  |
| HBL3  | 3B10 | AIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWDDSSDH---VVFGGGTKLAVL (Seq. ID No. 171)   |
| HBL4a | 3C11 | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSSDP---VVFGGGTKLTVL (Seq. ID No. 172)  |
|       | 3D6  | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSSDP---VVFGGGTKLTVL (Seq. ID No. 173)  |
| HBL4b | 3B5  | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSSDH---VVFGGGTKLTVL (Seq. ID No. 174)  |
| HBL4c | 3B8  | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSSDH---VVFGGGTKLTVL (Seq. ID No. 175)  |
| HBL4d | 3B12 | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSSSDH---VVFGGGTKLTVL (Seq. ID No. 176)  |
| HBL5  | 3D1  | GIPERFSGNSNGNTATLTISRVEAGDEADYYC | QWWDSTGDR---VVFGGGTKLTVL (Seq. ID No. 177)  |
| HBL7  | 3G5  | GIPERFSGNSNGNAATLTITRVEAGDEADYYC | QWGDGTGDHP---VVFGGGTKLTVL (Seq. ID No. 178) |

HBL2d 3C4 had a "tga" stop codon in CDR1 as indicated by "J".

Fig 8e

## Mouse IgG kappa clones to IgE Fc

## Heavy chain

| Fab              | Framework 1               | CDR1        | Framework 2         | CDR2              |
|------------------|---------------------------|-------------|---------------------|-------------------|
| m2G1R2A8         | QSGAELMKPGASVKISCKATDYTFS | NYWIE       | WVKQRPQHGLEWIG      | EILPGSGSTNFNEKFKG |
| m2G1R2B9         | •••••I•••••RT•••••        | •••R•••••   | ••••T•D•••••R•      |                   |
| m2G1R2B11, C5    | •••••••••••••••••••••     | •I••••••••• | •••••D•••••R••D     |                   |
| m2G1R2C2, F9, c3 | •••••V•••••••••S•••••     | •••••S••••• | •••••D•••••D••V•••• |                   |
| m2G1R2C8         | •••••••••••••••S•••••     | •••••S••••• | •••••D•••••D••V•••• |                   |
| m2G1R2F12        | •••••••••••••••••••••     | •••••       | •••••D•••••         |                   |
| m2G1R2G1, C11    | •••••••••••••••S•••••     | •••••S••••• | •••••T•D•••••R•     |                   |
| m2G1R2H8         | •••••••••••••••••••••     | •••••       | •••••               |                   |
| m2G1R2F7         | •••••••••••••••••••••     | •••••       | D•••••D•••••V••••   |                   |
| m2G1R2D10        | •••••••••••••••••••••     | •••••       | •••••D•••••         |                   |
| m2G1R2F10        | •••••••••••••••••••••     | •••••       | •••••D•••••         |                   |
| m2G1R2H3         | ••••••••••••••S•••••      | •••••S••••• | •••••DA•••••        |                   |
| m2G1R2H7         | ••V•••••••••S•••••        | •••••S••••• | •••••D•••••VS••••   |                   |
| m2G1R2a9         | •••••••••••••••••••••     | •••••       | •••••D•••••         |                   |

| Fab              | Framework 3                      | CDR3            | FR4                              |
|------------------|----------------------------------|-----------------|----------------------------------|
| m2G1R2A8         | KATFTADTSSNTAYMQLSSLTSEDSAVYYCAR | AYFTFS          | LDYWQGQGTTLVSS (Seq. ID No. 233) |
| m2G1R2B9         | •••••••••••••••••••••            | F•••••••••••    | (Seq. ID No. 234)                |
| m2G1R2B11, C5    | •••••••••••••••••••••            | •Y•••           | F••••••••• (Seq. ID No. 235)     |
| m2G1R2C2, F9, c3 | •••••Y•••••••••••••••            | •Y•••           | F••••••••• (Seq. ID No. 236)     |
| m2G1R2C8         | ••••L•Y••••••••••••••            | •Y•••           | F••••••••• (Seq. ID No. 237)     |
| m2G1R2F12        | •••••••••••••••••••••            | •Y•••           | F••••••••• (Seq. ID No. 238)     |
| m2G1R2G1, C11    | •••••••••••••••••••••            | •••••F••••••••• | (Seq. ID No. 239)                |
| m2G1R2H8         | •••••••••••••••••••••            | •••••F••••••••• | (Seq. ID No. 240)                |
| m2G1R2F7         | •••••••••••••••••••••            | •Y•L•           | F••••••••• (Seq. ID No. 241)     |
| m2G1R2D10        | •••••Y•••••••••••••••            | •Y•••           | F••••••••• (Seq. ID No. 242)     |
| m2G1R2F10        | •••••S•••••••••••••••            | •Y•••           | F••••••••• (Seq. ID No. 243)     |
| m2G1R2H3         | •••••Y•••••••••••••••            | •Y•••           | F••••••••• (Seq. ID No. 244)     |
| m2G1R2H3         | •••••Y•••••••••••••••            | •Y•••           | F••••••••• (Seq. ID No. 245)     |
| m2G1R2a9         | •••••••••••••••••••••            | •Y•L•           | F••••••••• (Seq. ID No. 246)     |

Fig. 9a

## Mouse IgG lambda clones to IgE Fc

## Heavy chain

| Fab       | Framework 1                 | CDR1                        | Framework 2             | CDR2                    |
|-----------|-----------------------------|-----------------------------|-------------------------|-------------------------|
| m3G1R3A11 | GAELMKPGASVKISCKATGYTFN     | TYWIE                       | WVKQRPGHGLEWIG          | EILPGTDNTNYNEKFKG       |
| m3G1R3A12 | •••••••••••••••••••••       | •••••                       | •••••••••••••••••••••   | •••••••••••••••••••••   |
| m3G1R3D12 | •••••••••••••••••••••       | •••••                       | •••••••••••••••••••••   | •••••••••••••••••••••   |
| m3G1R3G8  | •••••••••••••••••••••       | •••••                       | •••••••••••••••••••••   | •••••••••••••••••••••   |
| m3G1R3E9  | •••••••••••••••••••••       | •••••                       | •••••••••••••••••••••   | R•••••••••••••••••••••  |
| m3G1R3B10 | GAELMKPGASVKISCKATGYTLS     | SYWIE                       | WVKQRPGHGLEWIG          | EILPGSDNTNYNEKFKG       |
| m3G1R3B11 | oooooooooooooooooooooLS     | ooooo                       | ooooooooooooooo         | ooooo\$ooooooooooooo    |
| m3G1R3F11 | oooooooooooooooooooooLS     | ooooo                       | ooooooooooooooo         | ooooo\$ooooooooooooo    |
| m3G1R3H9  | oooooooooooooooooooooLS     | ooooo                       | ooooooooooooooo         | ooooo\$ooooooooooooo    |
| m3G1R3E7  | LVDPGGGLVQPGGSLRLSCETSGFTFT | DYYLS                       | WVRQPPGKALEWLG          | FIRNKANGYTTEYSASVKG     |
| m3G1R3E10 | •••••••••••••••••••••       | •••MT                       | •••••••••••••••••••••   | •••••••••••••••••••••   |
| m3G1R3F8  | •••••••••••••••••••••       | •••LS                       | •••••••••••••••••••••   | •••••••••••••••••••••   |
| m3G1R3G9  | •••••••••••••••••••••       | •••LS                       | •••••••••••••••••••••   | •••••••••••••••••••••   |
| 1214      | m3G1R3B7                    | CTELMKPGASVKISCKATGYTFN     | DYWIE                   | WVKQRPGHGLEWIG          |
|           | m3G1R3C7                    | ooooooooooooooooooooo\$     | ooooo                   | ooooooooooooooo         |
|           | m3G1R3G12                   | ooooooooooooooooooooo\$     | ooooo                   | ooooooooooooooo         |
|           | m3G2aR3C8                   | ooooooooooooooooooooo\$     | ooooo                   | ooooooooooooooo         |
|           | m3G2aR3E7                   | ooooooooooooooooooooo\$     | ooooo                   | ooooooooooooooo         |
|           | m3G2aR3B10                  | GGGLVQPGNSLRLSCATSGFTFT     | DYYLS                   | WVRQPPGKALEWLG          |
|           | m3G2aR3E10                  | •••••••••••••••••••••       | •••••••••••••••••••••   | •••••••••••••••••••••   |
|           | m3G2aR3D8                   | •••••••••S•••••••M•••••     | •••••A•••••••A•••••A••• | •••••A•••••••A•••••A••• |
|           | m3G2aR3D4                   | HOOSCALEMKPGASVKISCKATGYTFN | SYWIE                   | WVKQRPGHGLEWIG          |
|           | m3G2aR3B4                   | ooooooooooooooooooooo\$     | ooooo                   | ooooooooooooooo         |

Fig. 9b

Fig. 9c

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## Kappa light chain

| Fab        | Framework 1               | CDR1       | Framework 2     | CDR2    |
|------------|---------------------------|------------|-----------------|---------|
| m3G1R3B10  | SRQIVLTQSPAIMSASPGEKVTMTC | SASSSVSYM  | WYQQKSSTSPKLWY  | DTSKLAS |
| m3G1R3H9   | .....                     | .....      | .....           | .....   |
| m3G1R3B7   | SRQIVLTQSPAIMSASPGEKVTMTC | SASSSVN    | WYQQKSCTSPKRWY  | DTSKLAS |
| m3G1R3C7   | .....                     | .....      | .....           | .....   |
| m3G1R3D12  | SRQIVLTQSPAIMSASPGEKVTMTC | SASSSVSYM  | WYQQKSSTSPKLWY  | DTSKLAS |
| m3G1R3E9   | .....                     | .....      | .....           | .....   |
| m3G1R3E7   | SRDTIOMTOSPA              | LSASVGETWY | WYQQKOCKSPOLIVY | DAKTLAR |
| m3G2aR3D8  | .....                     | .....      | .....           | .....   |
| m3G2aR3E10 | .....                     | .....      | .....           | .....   |

| Fab        | Framework 3                        | CDR3    | FR4            |                   |
|------------|------------------------------------|---------|----------------|-------------------|
| m3G1R3B10  | GVPGRFSGSGSGNSLTISMEADVATYYC       | FQGSGYP | LT FGAGTKLEIKR | (Seq. ID No. 270) |
| m3G1R3H9   | .....                              | .....   | .....          | (Seq. ID No. 271) |
| m3G1R3B7   | GVPGRFSGSGSGCTSYSLTISMEADVATYYC    | QWWRNRP | PT FGAGTKLEIKR | (Seq. ID No. 272) |
| m3G1R3C7   | .....                              | .....   | .....          | (Seq. ID No. 273) |
| m3G1R3D12  | GVPGRFSGSGSGNSLTISMEADVATYYC       | FQGSGYP | LT FGAGTKLEIKR | (Seq. ID No. 274) |
| m3G1R3E9   | .....                              | .....   | .....          | (Seq. ID No. 275) |
| m3G1R3E7   | GVPGRFSGSGSGCTDPSLKINSLQPEDEFGSYAC | QHIMCIP | LT FGAGTKLEIKR | (Seq. ID No. 276) |
| m3G2aR3D8  | .....                              | .....   | .....          | (Seq. ID No. 277) |
| m3G2aR3E10 | .....                              | .....   | .....          | (Seq. ID No. 278) |

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Fig. 9d