

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
28 September 2006 (28.09.2006)

PCT

(10) International Publication Number
WO 2006/099747 A1

(51) International Patent Classification:

<i>C12N 15/13</i> (2006.01)	<i>C12N 1/11</i> (2006.01)
<i>C07K 1/00</i> (2006.01)	<i>C12N 15/70</i> (2006.01)
<i>A61K 39/395</i> (2006.01)	<i>C12Q 1/70</i> (2006.01)
<i>C07K 14/52</i> (2006.01)	<i>C40B 30/00</i> (2006.01)
<i>C07K 14/705</i> (2006.01)	<i>C40B 40/02</i> (2006.01)
<i>C07K 14/725</i> (2006.01)	<i>C40B 40/10</i> (2006.01)
<i>C07K 16/00</i> (2006.01)	<i>G01N 33/50</i> (2006.01)

(21) International Application Number:

PCT/CA2006/000451

(22) International Filing Date: 24 March 2006 (24.03.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/664,954 25 March 2005 (25.03.2005) US

(71) Applicant (for all designated States except US): NATIONAL RESEARCH COUNCIL OF CANADA [CA/CA]; 1200 Montreal Road, M-58, Eg-06b, Ottawa, Ontario K1A 0R6 (CA).

(72) Inventor; and

(75) Inventor/Applicant (for US only): TANHA, Jamshid [CA/CA]; 6292 Sablewood Place, Orleans, Ontario K1C 7M6 (CA).

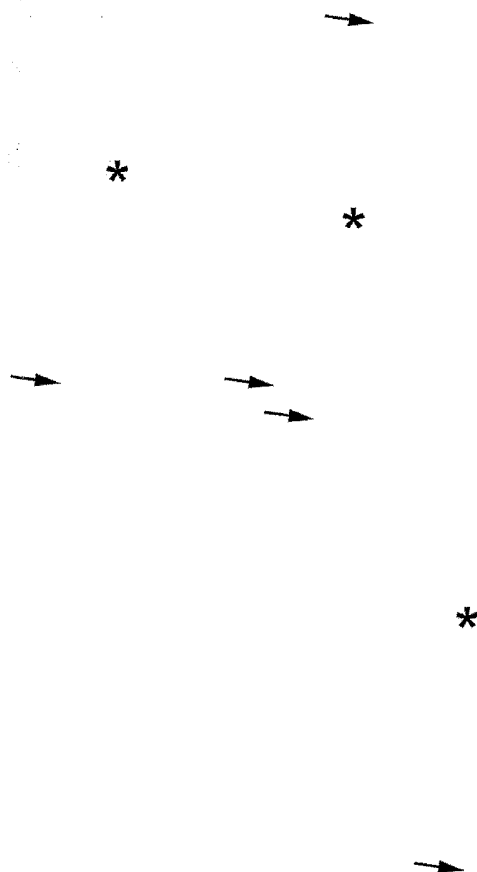
(74) Agent: CASSAN MACLEAN; Suite 401, 80 Aberdeen Street, Ottawa, Ontario K1S 5R5 (CA).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

[Continued on next page]

(54) Title: METHOD FOR ISOLATION OF SOLUBLE POLYPEPTIDES



(57) Abstract: Polypeptides with desirable biophysical properties such as solubility, stability, high expression, monomericity, binding specificity or non-aggregation, including monomeric human VHs and VLs, are identified using a high throughput method for screening polypeptides, comprising the steps of obtaining a phage display library, allowing infection of a bacterial lawn by the library phage, and identifying phage which form larger than average plaques on the bacterial lawn. Sequences of monomeric human VHs and VLs are identified, which may be useful for immunotherapy or as diagnostic agents. Multimer complexes of human VHs and VLs are also identified. The VHs and VLs identified may be used to create further libraries for identifying additional polypeptides. Further, the VHs and VLs may be subjected to DNA shuffling to select for improved biophysical properties.

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European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:

— *with international search report*

Method for Isolation of Soluble Polypeptides

Field of the Invention

- 5 This invention relates to the isolation, identification and manipulation of polypeptides, especially monomeric human antibody fragments.

Background of the Invention

10 Antibodies in vertebrates are typically composed of paired heavy (H) and light (L) chains. The first domain of the combined H and L chains, the V_H and V_L , are more variable in sequence, and this is the portion of the antibody that recognizes and binds to the antigen. The V_H and V_L domains recognize the antigen as a pair.

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The immune repertoire of camelidae (camels, dromedaries and llamas) is unique in that it possesses unusual types of antibodies referred to as heavy-chain antibodies (**Hamers, Casterman C. *et al.*, 1993**). These antibodies lack light chains and thus their combining sites consist of one domain, termed V_HH .

20

Recombinant V_HH single-domain antibodies (sdAbs) provide several advantages over single-chain Fv (scFv) fragments derived from conventional four-chain antibodies. While sdAbs are comparable to their scFv counterparts in terms of affinity, they outperform scFvs in terms of solubility, stability, resistance to aggregation, refoldability, expression yield, and ease of DNA manipulation, library construction and 3-D structural determinations. Many of the aforementioned properties of V_HH sdAbs are desired in applications involving antibodies.

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However, the non-human nature of V_HH s limits their use in human immunotherapy due to immunogenicity. In this respect, human V_H and V_L sdAbs are ideal candidates for immunotherapy applications because they are expected to be least immunogenic.

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Human V_{HS} and V_{LS}, however, are by and large prone to aggregation, a characteristic common to V_{HS} and V_{LS} derived from conventional antibodies (Davies, J. *et al.*, 1994; Tanha, J. *et al.*, 2001; Ward, E. S. *et al.*, 1989).

5 Thus, attempts have been made to obtain monomer human V_{HS} and V_{LS} suitable for antibody applications. Such V_{HS} and V_{LS} have also displayed other useful properties typical of V_{HS} such as high expression yield, high refoldability and resistance to aggregation. Synthetic libraries built on these V_{HS} and V_{LS} as library scaffolds might serve as a promising source of
10 therapeutic proteins.

Camelization as well as llamination which involves incorporating key solubility residues from camel and llama V_{HS}, respectively, into human V_{HS} or V_{LS} have been employed to generate monomeric human V_{HS} and V_{LS}.
15 Synthetic sdAb libraries constructed based on these V_{HS} and V_{LS} and generated by CDR randomization were shown to be functional in terms of yielding binders to various antigens (Davies, J. *et al.*, 1995; Tanha, J. *et al.*, 2001).

20 In another approach, fully human monomeric V_{HS} and V_{LS} were isolated from human synthetic V_H and V_L libraries without resorting to engineering of the sort mentioned above. In one experiment a monomeric human V_H, was discovered when a human V_H library was panned against hen egg lysozyme (Jespers, L. *et al.*, 2004b). More recently, a selection method
25 based on reversible unfolding and affinity criteria yielded many monomeric V_{HS} from synthetic human V_H libraries (Jespers, L. *et al.*, 2004a). This finding underlined the fact that an appropriate selection method is key to efficient capturing of rare monomer human V_{HS} with desirable biophysical properties.

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Objects of the Invention

A first object of the invention is to provide a high throughput screening method for identifying polypeptides, especially antibody fragments, with

improved biophysical properties, including solubility, high expression, and/or stability (such as high refolding after thermal denaturation, high resistance to chemical denaturant, and high resistance to proteases, in particular gastrointestinal proteases such as trypsin).

5

A second object of the invention is to provide a high throughput screening method for identifying monomeric human V_{HS} and V_{LS} .

10 A third object of the invention is to identify, isolate and characterize monomeric human V_{HS} and V_{LS} .

A fourth object of the invention is to construct and characterize multimers of antibody fragments, especially monomeric human V_{HS} and V_{LS} .

15 A fifth object of the invention is to construct display libraries from polypeptides, especially antibody fragments, and most especially monomeric human V_{HS} and V_{LS} .

20 A sixth object of the invention is to provide a DNA shuffling method for producing polypeptides, especially antibody fragments, and most especially monomeric human V_{HS} and V_{LS} with improved biophysical properties.

Summary of the Invention

25 A method is provided for isolating polypeptides, preferably antibody fragments, and most preferably human V_{HS} and V_{LS} with desirable biophysical properties (solubility, stability, high expression, monomericity, non-aggregation, binding specificity). The method includes the steps of obtaining a phage display library capable of expressing a variety of polypeptide
30 sequences, allowing infection of a bacterial lawn by the library phage, and identifying phage which form larger than average plaques on the bacterial lawn. The phage are then isolated, and steps are taken to sequence or otherwise characterize the polypeptide sequences.

The invention also provides for polypeptides, especially monomeric human V_{HS} and V_{LS}, identified by the above method, which may be useful for immunotherapy, and/or as diagnostic or detection agents. The monomeric human V_{HS} and V_{LS} may also be combined to form dimers, trimers, pentamers
5 or other multimers, which may be useful for immunotherapy and/or as diagnostic or detection agents.

The polypeptides identified by the above method, including human V_{HS} and V_{LS}, can be manipulated by methods such as DNA shuffling to select for
10 improved biophysical properties such as solubility, stability, monomericity, high expressibility, binding specificity and human origin.

The polypeptides identified by the above method, including human V_{HS} and V_{LS}, may also be used to generate further display libraries, which can then in
15 turn be used to isolate further polypeptides by the above method.

In a first aspect, the present invention provides a method of identifying target polypeptides, comprising a) obtaining a phage display library capable of expressing a variety of polypeptide sequences, b) allowing infection of a
20 bacterial lawn by the library phage and c) identifying phage which form larger than average plaques on the bacterial lawn.

In a second aspect, the present invention provides polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NO:8-54
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In a third aspect, the present invention provides a V_H antibody fragment comprising at least one amino acid sequence selected from the group consisting of: SEQ ID NO:8-22.

30 In a fourth aspect, the present invention provides a V_L antibody fragment comprising at least one amino acid sequence selected from the group consisting of: SEQ ID NO:23-54.

In a fifth aspect, the present invention provides A method for producing polypeptides with desirable biophysical properties, comprising the steps of a) providing at least one first nucleic acid sequence that encodes an antibody fragment as claimed in claim 41,42,44,45,47,48, 59 or 70 or that encodes a polypeptide sequence as claimed in claim 24,27,37 or 39, and having a first desirable property; b) providing at least one second nucleic acid sequence that encodes an antibody fragment having a second desirable property; c) cutting the at least one first and at least one second nucleic acid sequences into random fragments; d) reassembling the random fragments; e) expressing the random fragments; and f) screening the expressed random fragments for the first and second desirable properties.

Detailed Description of the Drawings

Figure legends

Figure 1. A pictorial representation of selected example results: The contrast in plaque size between phages displaying a soluble V_H (HVHP428) and those displaying an insoluble one (BT32/A6). The photo depicts a part of the bacterial lawn agar plate which was magnified to enhance plaque visualization. Although the plate contained an equal number of each of the two plaque types, the photo essentially contains the large, HVHP428 plaques. The majority of the BT32/A6 plaques were too small to produce clear, well-defined images in the photo. The plaques marked by arrows, thus, represent a minor proportion of BT32/A6 phages which were large enough to be visible in this image. Asterisks marks representative plaque sizes for HVHP428 phages. The identities of plaques were determined by DNA sequencing.

Figure 2. Amino acid sequence of the human V_H s selected based on affinity for protein A and plaque size. The dots in the sequence entries indicate amino acid identity with HVHP2M10 or HVHP44. Dashes are included for sequence alignment. Residues at the key solubility positions and residue 57T which associates with V_H s/ V_H h with protein A binding property are in bold. The Kabat numbering system is used. The total "frequency" value is 114.

CDR = complementarity determining region; FR = framework region; gln seq = germline sequence

Figure 3. Aggregation tendencies of the human V_H s. Gel filtration chromatograms comparing the oligomerization state of a human V_H isolated in this study (HVHP428) to that of a llama V_H (H11C7) and a typical human V_H (BT32/A6). The peak eluting last in each chromatogram corresponds to monomeric V_H . The dimeric H11C7 peak is marked by an arrow. *B*, One-dimensional ^1H NMR spectra of HVHP414 at 800 MHz (i), HVHP423 at 500 MHz (ii) and HVHP428 at 800 MHz (iii). The spectra in the left panel are scaled up by a factor of two to enable better viewing of low-intensity signals.

Figure 4. Stability of the human V_H s in terms of their resistance to trypsin at 37°C and integrity following long incubation at 37°C. *A*, SDS-PAGE comparing the mobilities of the untreated and trypsin-treated HVHP414 V_H at 15, 30 and 60 min relative to a 21 kDa marker. HVHP414-cMyc denotes HVHP414 V_H lacking the c-Myc. *B*, Molecular mass profiles obtained by mass spectrometry of untreated and trypsin-treated (60 min) HVHP414 V_H . The mass spectrometry profile of the treated V_H is superimposed onto that for the untreated one to provide a better visual comparison. The experimental molecular mass of the untreated V_H is 14,967.6 Da, which is essentially identical to the expected molecular mass, 14,967.7 Da. The observed molecular mass of the trypsin-treated V_H (13,368.5 Da) indicates loss of 13 amino acids at the C-terminus by cleavage at K (Lys) in the c-Myc tag to give an expected molecular mass of 13,368.0 Da. The trypsin cleavage site is shown by a vertical arrow above the amino acids sequence of HVHP414. *C*, Gel filtration chromatograms comparing the oligomerization state of the 37°C-treated HVHP420 V_H (upper profile) to that of untreated V_H (lower profile). The chromatograms were shifted vertically because they were indistinguishable when superimposed. The major and minor peaks in each chromatogram correspond to monomeric and dimeric V_H s, respectively. The dimeric V_H constitutes 3% of the total protein. The inset shows the sensorgram overlays for the binding of 37°C-treated HVHP420 to protein A at

various concentrations. The V_{HS} used for temperature stability studies were from stocks which had already been at 4°C for several months.

Figure 5. Sensogram overlays showing the binding of native (thick lines) and refolded (thin lines) HVHP423 to immobilized protein A at 75, 100, 150 and 200 nM concentrations. K_{Dn} and K_{Dref} were calculated from respective sensograms and used to determine RE as described below.

Figure 6. Amino acid sequences of the human V_L s selected based on affinity for protein L and plaque size. The dots in the sequence entries indicate amino acid identity with HVLP333. Dashes are included for sequence alignment. See the *V BASE* (http://vbase.mrc-cpe.cam.ac.uk/index.php?module=pagemaster&PAGE_user_op=view_page&PAGE_id=7&MMN_position=5:5) for sequence numbering and CDR designation. L6, A27, L2, L16, O2/O12, A30 and 1b are V germline designation. J germline designations are in the brackets. NF, not found.

Figure 7. Size exclusion chromatograms of human V_L domains. In A, the V_L s were applied at a concentration of 0.6 mg/ml. In B, the V_L s were applied at their highest concentration available: HVLP342, 1.0 mg/ml; HVLP3103, 5.9 mg/ml; HVLP335, 4.9 mg/ml; HVLP351, 0.89 mg/ml. “#” and “*” represent aggregate and monomer peaks, respectively. The aggregates elute in the exclusion volume. The peak marked by an arrow in the HVLP342 panel (B) is the carry over from a previous run.

Figure 8. Sensorgram overlays showing the binding of V_L s to immobilized protein L at concentrations of 0.2, 0.5, 0.75, 1, 2, 3, 5 and 10 μ M (HVLP389, HVLP351 and HVLP364); 1, 2, 3, 5, 7.5 and 10 nM (HVLP342); 0.2, 0.5, 1, 2, 3, 5 and 10 μ M (HVLP335); 0.2, 0.5, 1, 1.5, 2 and 5 μ M (HVLP325), 0.2, 0.5, 0.75, 1, 1.5, 2, 3 and 5 μ M (HVLP3103) and 1, 2, 3, 4, 5 and 6 nM (HVLP324). The sensorgrams for HVLP324 and HVLP342 bindings to the

low affinity site of protein L are not included but the calculated K_D s are recorded in **Table 3**.

Figure 9. Bindings of HVHP328PTV2 to protein A and HVLP335PTV2 to protein L in surface plasmon resonance experiments. **(A)** Sensorgram overlays showing the binding of HVH28PTV2 to immobilized protein A at 1, 2, 3, 4, 6, 8 and 10 nM concentrations. **(B)** Sensorgram overlays showing the binding of HVLP335PTV2 to immobilized protein L at 1, 2, 2.5, 3, 3.5, 4 and 4.5 nM concentrations. The binding data are recorded in Table 4.

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Figure 10. Figure showing the results of the microagglutination experiments with *S. aureus* cells. The concentration of the pentamers decreases two-fold from well 1 to well 11 with well 12 having the pentamers replaced with PBS buffer. The top row wells contain HVHP328PTV2 pentamer and the bottom ones HVLP335PTV2 pentamer. The concentrations of the pentamers in wells 1 to 6 are 215, 108, 54, 27, 13 and 7 μ g/ml, respectively.

15

Detailed Description of the Invention

It is desirable to identify polypeptides, especially antibody fragments, that are of human origin, soluble, stable, resistant to aggregation, refoldable, highly expressed, easily manipulated at the DNA level, ideal for library construction and for 3-D structural determinations. Such antibody fragments are useful for a wide variety of immunotherapeutical applications, and also as diagnostic and detection agents. Human monomeric V_H and V_L antibodies are of particular interest, as they are likely to have many of the above-mentioned properties.

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Polypeptides with the above-mentioned properties may be identified by high throughput screening of libraries capable of expressing a variety of polypeptide sequences. For example, phage display libraries (preferably filamentous phage such as M13 or fd) may be screened by infecting a field of bacteria susceptible to the phage (a bacterial lawn) with the phage, then

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determining which phages have successfully lysed the bacteria by looking for clear, bacteria-free areas known as plaques. Phages displaying monomeric llama V_Hs and V_Ls form larger plaques on bacterial lawns than phages displaying fully human V_Hs with aggregation tendencies. Thus, plaque size
5 may be used as a means of identifying rare, naturally-occurring monomer V_Hs and V_Ls from the human V_H repertoire.

The method disclosed herein is also useful in identifying soluble, stable (stability covers a number of characteristics, including but not limited to high
10 thermal refolding efficiency, high melting temperature, maintaining functionality after long (several days) incubation at 37°C, resistant to chemical denaturants, resistant to proteases, having a long shelf life at below 0°C, and 4°C, and at room temperature, maintaining functionality in intracellular environments, and maintaining functionality inside the human body, such as in
15 the bloodstream) and high expressing proteins of differing origins, including:

1. V_Hs, V_Ls, Fabs, scFvs and whole antibodies such as IgGs, more specifically human ones
2. Protein variants based on non-antibody scaffolds single-chain T-cell receptors, T-cell receptor domains, transferrin, lipocalins, kunitz domains,
20 ankyrin repeats, and cytotoxic T-lymphocyte-associated antigen (CTLA-4), including human ones
3. Vaccines such as viral and bacterial protein vaccines
4. Therapeutic proteins, e.g., insulin, growth hormone, erythropoietin
5. Proteinaceous diagnostic and biochemical reagents, e.g., protein A, protein
25 G.

Once polypeptides have been identified by this method, they can be used to construct additional libraries. This is done by selecting a nucleic acid sequence of, for example, a V_H. Oligonucleotides with randomized codons
30 are created and incorporated into the V_H sequence. Thus, each unique oligonucleotide is incorporated into a V_H gene, and the modified V_H genes constitute a library of sequences with slight variations. Typically, the oligonucleotides are designed such that the CDRs or loops of the V_H are randomized. For example, one, two or all three of V_H CDRs may be

randomized. The VH library is then cloned into an appropriate vector, depending on the type of library to be used, and the nucleic acid sequences are expressed as polypeptides. The library is screened for molecules that bind to the library polypeptides, typically by panning. The libraries may be
5 phage display libraries, or other display libraries such as ribosome display and yeast display.

Polypeptides identified by the method discussed herein may be used for immunotherapy by, for example, the cross-linking of monomers to form
10 dimers, trimers, pentamers and other multimers. This may result in better affinity for antigen molecules and slower dissociation rates for some antigens. Another possible approach is to link or fuse polypeptides to a variety of molecules with various functions. For example, antibody fragments may be linked to radionuclides, cytotoxic drugs, toxins, peptides, proteins, enzymes,
15 liposomes, lipids, T-cell superantigens or viruses in order to target and destroy or modify specific cells or molecules.

Once the V_{HS} or V_{LS} identified by the selection method described herein have been isolated, they can be further manipulated to select for improved
20 biophysical properties such as solubility, stability, monomericity, binding specificity, human origin or high expressability. This can be achieved by *in vitro* recombination techniques such as DNA shuffling or a staggered extension process. DNA shuffling involves cutting the nucleic acid sequence of first (donor) and second (acceptor) polypeptides, such as antibody
25 fragments, into random fragments, then reassembling the random fragments by a PCR-like reaction. The reassembled fragments are then screened to select for the desired properties.

For example, one or more VHs with high stability (donors) can be mixed with
30 one or more VHs lacking sufficient stability (acceptors) and subjected to DNA shuffling. This generates mutants of the acceptor VHs which have incorporated stability residues from the donor VHs. The newly stable mutants can be identified by the methods described herein, or through other evolutionary protein screening systems such as ribosome display, yeast

display, bacterial cell display and phage display. Similarly, this technique can be used to transfer desirable traits such as solubility, monomericity, and high expression.

- 5 This technique may be used where both donor and acceptor V_H s have desirable properties, to produce a V_H with both properties. For example, an unstable donor V_H which binds to an important therapeutic or diagnostic ligand can be shuffled with a stable acceptor V_H . In order to ensure that new generated stable V_H s also have the ability to bind to the ligand, the screening
10 system may involve a ligand binding step.

- DNA shuffling may also be useful for humanizing non-human V_H s such as camelid heavy chain antibody variable domains and nurse shark and wobbegong shark variable domains, or non-human V_L s which bind to
15 therapeutic targets. Human V_H s and V_L s with desirable properties such as solubility, stability, monomericity and high expressability may be used as donors. For example, one or more human V_H s with good stability (donors) can be mixed with one or more non-human therapeutic V_H s (acceptors) and subjected to DNA shuffling. This generates mutants of the acceptor V_H s
20 which are both stable and humanized. The newly generated humanized and stable mutants can be identified by the methods described herein, or through other evolutionary protein screening systems such as ribosome display, yeast display, bacterial cell display and phage display. In a further example, the acceptor V_H could be a therapeutic V_H (camelid heavy chain antibody
25 variable domain).

- Further, this technique is also useful for selecting desirable properties of polypeptides other than V_H s and V_L s. As discussed above, the donor polypeptide and the acceptor polypeptide may be both human, or the donor
30 may be human and the acceptor non-human.

A possible approach for imparting solubility, monomericity, high expressability or stability to V_H s and V_L s may be through grafting complementarity determining regions (CDRs) onto acceptor V_H s and V_L s. Since CDRs are

known to be involved in the solubility and stability of single-domain antibodies, and accordingly the grafting of these regions, such as the CDRs from V_{HS} and V_{LS} isolated by the methods described herein, may impart solubility and/or stability to acceptor V_{HS} and V_{LS}.

5

Human Monomeric V_{HS} and V_{LS}

Several monomeric human V_{HS} with different germline and overall sequences were identified (see Figure 1 and SEQ ID NO. 8 through 22) from a naïve human V_H phage display library by this selection method based on phage plaque size. The V_{HS} remain functional and monomeric following trypsin treatment at 37°C, weeks of incubations at 37°C or months of storage at 4°C, have high thermal refolding efficiencies, are produced in good yields in *E. coli* and possess protein A binding activity.

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In addition, several monomeric human V_{LS} were identified (see Figure 6 and SEQ ID NO. 23 through 54). The V_{LS} are also produced in good yields in *E. coli* and possess protein L binding activity.

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Such properties will also be manifested by V_{HS} from synthetic libraries that utilize the above V_{HS} as scaffolds. Thus, such libraries may yield therapeutic or diagnostic V_{HS} which would have good efficacy at physiological temperature, extended shelf life and a cost-effective production. High thermal refolding efficiency characteristic would further extend the biotechnological applications of these libraries to situations where V_H binders are required to maintain their activity after exposure to transient high temperatures. The V_{HS} should also be very suitable for intrabody applications because of their desirable biophysical properties. The protein A binding property will simplify V_H purification and detection in diagnostic tests, immunoblotting and immunocytochemistry and can be exploited to enhance library performance by removing nonfunctional V_{HS} from the libraries. Similarly, libraries that utilize V_{LS} as scaffolds will yield therapeutic or diagnostic V_{LS} which have similarly desirable properties. Since V_{LS} bind with protein L, V_L purification

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and detection is simplified by taking advantage of this protein L binding property.

Display libraries built on the present V_{HS} and V_{LS} may also be a useful
5 source of diagnostics and detection agents.

Previously reported fully human V_{HS} with favorable biophysical properties were based on a single V germline sequence: DP-47 ((**Jespers, L. et al., 2004b; Jespers, L. et al., 2004a**). The observation that the monomeric
10 human V_{HS} in this study stem from six different germline sequences including DP-47, demonstrates that stable V_{HS} are not restricted in terms of germline gene usage. In fact, it is very likely that we would have isolated monomeric V_{HS} of family and germline origins different from the ones we describe here had we not restricted our selection to a subset of V_{H3} family V_{HS} with protein
15 A binding activity. It is not possible to pinpoint amino acid mutations (**Table 1**) responsible for the observed biophysical behavior of the present V_{HS} due to the occurrence of multiple mutations in V_{HS} and the fact that CDR3 is also known to be involved in shaping the biophysical profiles of sdAbs. It may be, however, that mutations at positions known to be important for sdAbs stability and solubility, eg., V37F in HVHP423 and HVHP44B, or mutations occurring
20 multiple times at the same position, e.g., L5V/Q and V5Q in nine V_{HS} , have a role in determining V_{HS} biophysical properties. In terms of library construction, it would be desirable that the monomericity of the present V_{HS} not be dependent on CDRs, in particular CDR3, so that CDR randomization be
25 performed without the worry of jeopardizing library stability. In this regard, the V_{HS} with smaller CDR3, e.g, HVHB82, may be preferred scaffolds since there would be less dependence on CDR3 for stability.

The diversity of the present V_{HS} and V_{LS} in terms of overall sequence
30 and CDR3 length should allow the construction of better-performing libraries. Synthetic V_H libraries have been constructed on single scaffolds. Such an approach to repertoire generation is in sharp contrast to the natural, *in vivo* "approach" which utilizes a multiplicity of scaffolds. Based on the sequences reported here one can take advantage of the availability of the diverse set of

V_Hs and V_Ls and create libraries which are based on multiple V_H and V_L scaffolds. Such libraries would be a better emulation of *in vivo* repertoires and therefore, would have a more optimal complexity. Of the three CDRs in sdAbs, CDR3 generally contributes most significantly to repertoire diversity and for this reason CDR3 randomization on V_H and V_L scaffolds are typically accompanied by concomitant varying of CDR3 length. While this significantly improves library complexity, it may also compromise library stability by disrupting the length of the parental scaffold CDR3. The heterogeneity of the V_Hs and V_Ls disclosed herein in terms of CDR3 length permit the creation of libraries with both good complexity, good stability and good biophysical characteristics. Such libraries would preferably consist of sub-libraries, where each sub-library is created by CDR3 randomization (and CDR1 and/or CDR2 randomization, if desired) on a single V_H or V_L scaffold without disrupting the parental CDR3 length.

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The versatility of the present V_Hs and V_Ls is also beneficial in terms of choosing an optimal V_H or V_L framework for humanizing V_HHs, V_Hs and V_Ls which are specific to therapeutic targets. High affinity camelid V_HHs against therapeutic targets can be obtained from immune, non-immunized or synthetic V_HH libraries with relative ease and be subsequently subjected to humanization (CDR grafting, resurfacing, deimmunization) to remove possible V_HH immunogenicity, hence providing an alternative to human V_H library approach for production of therapeutic V_Hs. Generating high affinity therapeutic V_Hs by the latter approach may often require additional tedious and time consuming *in vitro* affinity maturation of the lead binder(s) selected from the primary synthetic human V_H libraries.

Nonhuman V_Hs against therapeutic targets can be obtained from immune, non-immunized or synthetic V_H libraries with relative ease and be subsequently subjected to humanization (CDR grafting, resurfacing, deimmunization) to eliminate nonhuman V_H immunogenicity, hence providing an alternative to human V_H library approach for production of therapeutic V_Hs.

Nonhuman V_Ls against therapeutic targets can be obtained from immune, non-immunized or synthetic V_HH libraries with relative ease and be subsequently subjected to humanization (CDR grafting, resurfacing, deimmunization) to eliminate V_HH immunogenicity, hence providing an
5 alternative to human V_L library approach for production of therapeutic V_Ls.

A number of evolutionary approaches for selection of proteins with improved biophysical properties have been described (**Forrer, P. et al., 1999;Waldo, G. S., 2003**); (**Jespers, L. et al., 2004a;Jung, S. et al., 1999;Matsuura, T. et al., 2003**). Typically, stability pressure is required to ensure preferential selection of stable variants over unstable or less stable ones from a library population. For example, in a related work, heat treatment of V_H phage display libraries was required to select aggregation resistant V_{HS} (**Jespers, L. et al., 2004a**). Examples of evolutionary selection approaches
10 involving phage display include conventional phage display, selectively infective phage and the proteolysis approaches. In the first two approaches affinity selection is used to select stable species from a library, based on the assumption that stable proteins possess better binding properties for their ligand than the unstable ones. However, even with the additional inclusion of
15 a stability selection step, these approaches may primarily enrich for higher affinity rather than for higher stability (**Jung, S. et al., 1999**). A binding step requirement also limits the applicability of these approaches to proteins with known ligands. The third, proteolysis approach is based on the fact that stable proteins are generally compact and therefore are resistant to proteases
20 whereas the unstable ones are not. The phage display format is engineered in such a way that the protease stability of the displayed protein translates to phage infectivity. Thus, when a variant phage display library is treated with a protease, only the phages displaying stable proteins retain their infectivity and can subsequently be selected by infecting an *E. coli* host. Since this
25 approach is independent of ligand binding, it has general utility. However, even stable and well folded proteins have protease sensitive sites, e.g., loops and linkers, and this could sometimes hinder the selection of stable species in a proteolysis approach (**Bai, Y. et al., 2004**).

By contrast, in the present evolutionary approach, proteins with superior biophysical properties are simply identified by the naked eye. The approach does not require ligand binding, proteolysis or destabilization steps, and thus, avoids complications which may be encountered in the reported selection approaches. No requirement for a binding step also means that this approach has general utility. As an option, a binding step may be included to ensure that the selected proteins are functional. However, the dependency of the present approach on plating (for plaque visualization) introduces a possible logistical limitation in terms of the number of plates that can be handled and thus limits its application to smaller libraries. Nonetheless, the utility of the current approach can be extended to large libraries, if the library is first reduced to a manageable size. This can be done, for example, by incorporating into the selection system a step which would remove large populations of unstable species, e.g., library adsorption on a protein A surface, or on a hydrophobic interaction column to remove poorly folded proteins with exposed hydrophobic surfaces (**Matsuura, T. et al., 2003**). Here, the approach was used to select V_{HS} and V_{LS} of good biophysical properties in a background of very unstable V_{HS} and V_{LS} . However, it may be more difficult to select the “best” species from a mutant library which is populated with proteins with reasonably good stabilities. In this case, the lead variants may be identified based on the rate of plaque formation by using shorter incubation times, or based on plaque size and frequency criteria.

The present selection approach can be extended to identification of stable and well-folded antibody fragments such as scFvs and Fabs with the optional inclusion, in the selection system, of a binding step involving protein L, A or any ligand, as well as stable non-antibody scaffolds and variants thereof. Moreover, the observed correlation between phage plaque size and V_H expression yield means that one can utilize the present approach for acquiring high-expressing versions of proteins with otherwise poor or unsatisfactory expression from mutant phage display libraries. This application would be particularly appealing in the case of therapeutic proteins or expensive poor-

expressing protein reagents where boosting protein expression would significantly offset protein production cost.

Binding analyses of pentamers

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Both V_L s and V_H s are amenable to pentamerization and the pentamerization can be used to quickly convert a low affinity V_L or V_H monomer to a high affinity V_L or V_H pentamer. Such pentamers are invaluable diagnostics and detection agents. In such applications, the binding of a V_L or V_H pentamer to
10 its target can be detected by a reporter molecule such as an enzyme (for example, horse radish peroxidase or alkaline phosphatase), or a fluorescent molecule conjugated to the pentamer. Alternatively, the binding of the pentamer can be detected by a secondary molecule which is conjugated to a reporter molecule. The secondary molecule can be specific to the pentamer
15 itself or to a tag thereof, such as a 6His tag or c-Myc tag. For example, a typical secondary molecule is an immunoglobulin.

The interactions between the V_H s and protein A and V_L s with protein L are fundamentally different from those between V_H s and V_L s with their target
20 antigens. The antigen binding of a V_H or a V_L involves three antigen binding loops which form the combining site of an antibody domain. The protein A binding of a V_H with protein A binding activity and a V_L with protein L binding activity involve binding sites and residues on the antibody domains that are totally distinct from the antibody combining site. Thus, a V_H with protein A
25 binding activity can simultaneously bind to protein A and its target antigen and a V_L with protein L binding activity can simultaneously bind to protein L and its target antigen. Since the present V_H s and V_L s have affinity for protein A and L, respectively, protein A and L can be used as the secondary molecule for detection and diagnostic applications mentioned above. The human V_H and
30 V_L pentamers can also be used for therapy.

Pathogen detection by the pentamers

The protein A and L binding activity of the V_H s and V_L s can be used to detect bacteria which have protein A and/or L on their surfaces. Protein A is present

on the surface of the pathogenic bacteria, *Staphylococcus aureus*. Thus, the V_{HS} with protein A binding activity such as the ones described here can be used to detect *S. aureus*. Similarly, the V_L monomers and V_L pentamers with protein L binding activity can be used for the detection of bacteria, in particular pathogenic bacteria such as *Peptostreptococcus magnus*, which have protein L on their cell surface.

Protein L is implicated as a virulent factor in the pathogenesis of *P. magnus* (Ricci, S. *et al.*, 2001) in humans. In vaginosis, protein L is thought to exert its effect by cross-linking surface associated IgE. V_L monomers and/or pentamers with protein L binding activity have potential as therapeutics since they could interfere with the IgE cross-linking action of protein L.

Protein A is implicated as a virulent factor in the pathogenesis of *S. aureus* in humans (Fournier, B. *et al.*, 2004). Its virulence has been attributed to its ability to interact with host components including binding to antibodies. V_H monomers and/or pentamers with protein A binding activity have potential as therapeutics since they could interfere with the interaction of of protein A with host components.

EXAMPLES

Identification and sequence analysis of monomeric human V_{HS}

During the course of the construction of fully human and Ilaminated human V_H libraries, it was learned that the phages displaying monomeric Ilaminated V_{HS} formed larger plaques on bacterial lawns than phages displaying fully human V_{HS} with aggregation tendencies. Thus, plaque size was used as a means of identifying rare, naturally-occurring monomer V_{HS} from the human V_H repertoire (**Figure 1**). To this end, a phage library displaying human V_{HS} with a size of 6×10^8 was constructed and propagated as plaques on agar plates. On the titer plates, the library consisted essentially of small plaques interspersed with some large ones. PCR on twenty clones revealed that the small plaques corresponded to the V_H-displaying phages while the large ones represented the wild type phages, i.e., phages lacking V_H sequence inserts.

None of the V_H-displaying phages were found with large plaque morphology. This was not unexpected due to the paucity of the monomeric V_{HS} in the human repertoire and the large size of the library. To facilitate the identification of monomeric V_{HS}, it was decided to reduce the library to a manageable size and remove the interfering wild type phages with large-plaque-size morphology by panning the library against protein A which binds to a subset of human V_{HS} from V_H3 family.

Following a few rounds of panning, the library became enriched for phage producing large plaques, and PCR and sequencing of more than 110 such plaques showed that all had complete V_H open reading frames. The size of the large plaques which were picked for analysis is represented in Figure 1. Sequencing revealed fifteen different V_{HS} which belonged to the V_H3 family and utilized DP-38, DP-47, V3-49, V3-53, YAC-5 or 8-1B germline V segments (**Table 1; Figure 2**). DP-38 and DP-47 germline sequences have been previously implicated in protein A binding. In addition, all V_{HS} had a Thr residue at position 57 (**Figure 2**), consistent with their protein A binding activity. The most frequently-utilized germline V segment was DP-47 which occurred in over 50% of the V_{HS}, but the most frequent clone (i.e., HVHP428; relative frequency 46%) utilized the V3-49 germline V segment. HVHP429 with a DP-47 germline sequence was the second most abundant V_H with a relative frequency of 21% (**Figure 2**). The V_H CDR3 lengths ranged from 4 amino acids for HVHB82 to 16 amino acids for HVHP430 amino acids, with HVHP430 having a pair of Cys residues in CDR3. Amino acid mutations with respect to the parental germline V segment (residues 1-94) and FR4 (residues 103-113) sequences, were observed in all V_{HS} and ranged from two mutations for HVHP44 (L5V and Q105R) and HVHB82 (E1Q and L5Q) to sixteen mutations for HVHP426 (**Table 1**). Mutations were concentrated in the V segments; only two mutations were detected in all the fifteen FR4s, at positions 105 and 108. HVHP44 and HVHB82 differed from other V_{HS} in that they both had a positively-charged amino acid at position 105 instead of a Gln (**Table 1; Figure 2**). However, while the positively-charged amino acid in HVHP44 was acquired by mutation, the one in HVHB82 was germline-encoded. Except for HVHP423 and HVHP44B, the remaining V_{HS} had the germline residues at the key solubility positions: 37V/44G/45L/47W or

37F/44G/45L/47W (HVHP428); HVHP423 and HVHP44B had a V37F mutation. Mutations at other positions which are shown or hypothesized to be important in V_H solubility included seven E6Q, three S35T/H, one R83G and one K83R, one A84P and one T84A and one M108L. Frequent mutations
5 were also observed at positions 1 and 5 which included eleven E1Q, eight L5V/Q and one V5Q mutations.

Biophysical characterization of the human V_{HS}

All V_{HS} except HVHP44B, which was essentially the same as
10 HVHP423, were expressed in one-litre-culture volumes in *E. coli* strain TG1 in fusion with c-Myc-His₅ tag and purified to homogeneity from periplasmic extracts by immobilized metal affinity chromatography (IMAC). The expression yields ranged from 1.8 to 62.1 mg of purified protein per liter of bacterial culture in shaker flasks with majority of V_{HS} having yields in several
15 milligrams (**Table 2**). In the instance of HVHP423 and HVHP430, another trial under “apparently” the same expression conditions gave yields of 2.4 and 6.4 mg as opposed to 62.1 and 23.7 mg, respectively. This implies that for many of the V_{HS} described here optimal expression conditions should be achieved, without much effort, resulting in expression yields significantly higher than the
20 reported values in **Table 2**. As expected, all the V_{HS} bound to protein A in surface plasmon resonance (SPR) analyses, with K_D s of 0.2-3 μ M, a range and magnitude comparable to the ones reported previously for llama V_{HH} variants with protein A binding activity. None of the V_H s bound to the Fab reference surface.

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The aggregation tendency of the human V_{HS} was assessed in terms of their oligomerization states by gel filtration chromatography and NMR (**Table 2**). All V_{HS} were subjected to Superdex 75 gel filtration chromatography. Similar to a llama V_{HH} , i.e., H11C7, all V_{HS} gave a symmetric single peak at
30 the elution volume expected for a monomer, and were substantially free of any aggregates (see the example for HVHP428 in **Figure 3A**). In contrast, a typical human V_H (i.e., BT32/A6) formed considerable amount of aggregates. For three of the V_{HS} , a minor peak with a mobility expected for a V_H dimer was also observed. SPR analyses of the minor peaks gave off-rate values which

were significantly slower than those for the monomer V_{HS} , consistent with them being dimers. The dimer peak was also observed in the case of the llama V_H H, H11C7. The folding and oligomerization states of the V_{HS} at high concentrations were further studied by NMR spectroscopy. As shown in Table II, all the V_H proteins studied appeared to be relatively soluble and assumed a well-folded three-dimensional structure. One-dimensional NMR spectra of the V_H fragments (Fig. 3B) showed structure folds characteristic of V_H domains. The state of protein aggregation was also assessed by use of an PFG-NMR diffusion experiment for the HVHP414 fragment and two isoforms, VH14 and VH14-cMyc- with and without the c-Myc sequence, of the HVHP414. VH14 is a modified version of HVHP414 with a c-Myc N132E mutation and with an additional methionine residue at the N-terminus. In brief, the PFG-NMR data (not shown) indicated that all the protein samples had expected monomeric molecular weights even at the relatively high protein concentrations used for NMR experiments.

The stability of the V_{HS} was further investigated in terms of their resistance to trypsin at 37°C integrity following long incubations at 37°C. Trypsin cleaves polypeptide amide backbones at the C-terminus of an Arg or a Lys residue. There are 9-13 Arg and Lys residues in the human V_{HS} (**Figure 2**). There is also an additional Lys residue in the C-terminal c-Myc tag which is susceptible to digestion by trypsin. **Figure 4a** is an SDS-PAGE analysis of HVHP414 during trypsin digestion. Within 1 h the original band was completely converted to a single product which had a mobility expected for the V_H with no c-Myc-His₅ tag. The same result was obtained for 12 other V_{HS} following a one-hour incubation with trypsin. Mass spectrometry on a randomly selected sample of the trypsin-treated V_{HS} (i.e., HVHP414, HVHP419, HVHP420, HVHP423, HVHP429, HVHP430 and HVHM81) confirmed that in every case the molecular mass of the digested product corresponded to a V_H with the c-Myc Lys as the C-terminal residue. HVHM41 gave a significantly shorter fragment than the rest upon digestion, and in this case mass spectrometry experiments mapped the cleavage site to the Arg99 in CDR3 (data not shown).

Eleven V_H s ranging in concentration from 0.32 mg/ml (HVHP428) to 3.2 mg/ml (HVHP420) were incubated at 37°C for 17 days. Their stability was subsequently determined in terms of oligomerization state and protein A binding. As shown by gel filtration chromatography, treatment of V_H s at 37°C did not induce any aggregate formation: all V_H s gave chromatogram profiles which were virtually identical to those of untreated V_H s and stayed essentially as monomers (see the example for HVHP420; **Figure 4c**). To ensure that the V_H s maintained their native fold following 37°C treatment, two V_H s, namely, HVHP414 (1.2 mg/ml) and HVHP420 (3.2 mg/ml), were selected at random and their K_D s of binding to protein A were determined by SPR (Data shown for HVHP420; **Figure 4c** inset) and compared to the K_D s obtained for untreated V_H s (Table 2). The calculated K_D s for the heat-treated V_H s were 1.4 μ M and 1.0 μ M for HVHP414 and HVHP420, respectively. These values are essentially identical to the corresponding values for the untreated V_H s (Table 2), demonstrating that 37°C treatment of V_H s did not affect their native fold. The possibility that V_H s may have been in a less compact, non-native fold during the 37°C-incubation periods and resumed their native fold upon returning to room temperature during gel filtration and SPR experiments is unlikely in light of the fact that the V_H s were resistant to trypsin at 37°C (see above), a property typically associated for well folded native proteins.

The refolding efficiency (RE) of the human V_H s was investigated by comparing the K_D s of the binding of the native (K_{Dn}) and heat-treated, refolded (K_{Dref}) V_H s to protein A (**Tanha, J. et al., 2002**). When a fraction of the V_H is inactivated by heat treatment the measured K_D would be higher, since this parameter is based on the concentration of folded, i.e., active, antibody fragment. Thus, the ratio of K_{Dn} to K_{Dref} gives a measure of V_H RE . **Figure 5** compares sensorgrams for HVHP423 binding to immobilized protein A in native (thick lines) and refolded (thin lines) states at several selected V_H concentrations. As can be seen, binding of the refolded V_H to protein A is less in all instances, indicating that the unfolding is not fully reversible. For each of the 14 V_H s, protein A binding in both native and refolded states was measured

at several concentrations, and the K_D s and subsequently RE s were determined (**Table 2**; K_{Dref} values are not shown). The K_D s and RE s of two anti-idiotypic llama V_H Hs, H11F9 and H11 B2, which were used as references, were also determined. Four V_H s had RE s in the range of 92%-95%, similar to
5 the RE s for H11F9 and H11B2, 95% and 100%, respectively. Another five had RE s in the range of 84%-88% and three over 70%. Only two had significantly lower RE : HVHP413 (52%) and HVHP421 (14%). Several published V_H Hs examined previously had RE around 50% (**van der Linden, R. H. et al., 1999**).

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Human V_H phage display library construction and panning. cDNA was synthesized from human spleen mRNA (Ambion Inc., Austin, TX) using random hexanucleotide primers and First Strand cDNA™ kit (GE Healthcare,
15 Baie d'Urfé, QC, Canada). Using the cDNAs as template, V_H genes with flanking C_H sequences were amplified by polymerase chain reaction (PCR) in nine separate reactions using V_H framework region 1 (FR1)-specific primers and an immunoglobulin M-specific primer (**de Haard, H. J. et al., 1999**). The products were gel-purified and used as the template in the second round of
20 PCR to construct V_H genes using the FR1- and FR4-specific primers (**de Haard, H. J. et al., 1999**) that also introduced flanking *Apa*I and *Not* I restriction sites for cloning purposes. The resultant V_H repertoire DNAs were cloned into fd-tetGIIID phage vector and a V_H phage display library was constructed (**Tanha, J. et al., 2001**). Panning against protein A (Amersham
25 Biosciences Inc.) was performed as described (**Tanha, J. et al., 2001**). Germline sequence assignment of the selected V_H s was performed using DNAPLOT software Version 2.0.1 and V BASE version 1.0 (<http://vbase.dnaplot.de/cgi-bin/vbase/vsearch.pl>). Llama V_H Hs H11C7, H11F9 and H11B2 were isolated from a llama V_H H phage display library by
30 panning against H11 scFv as described (**Tanha, J. et al., 2002**).

V_H expression and purification. V_H s were cloned into pSJF2 expression vectors by standard cloning techniques (**Sambrook, J. Fritsch E. F. and Maniatis T, 1989**). Periplasmic expression of sdAbs and subsequent

purification by immobilized metal affinity chromatography (IMAC) were performed as described (**Muruganandam, A. et al., 2002**). Protein concentrations were determined by A_{280} measurements using molar absorption coefficients calculated for each protein (**Pace, C. N. et al., 1995**).

- 5 Gel filtration chromatography of the purified V_{HS} was performed on a Superdex 75 column (GE Healthcare) as described (**Deng, S. J. et al., 1995**).

- Binding and refolding efficiency experiments.** Equilibrium dissociation constants (K_D s) and refolding efficiencies (RE s) of V_{HS}/V_{HH} s were derived
- 10 from surface plasmon resonance (SPR) data collected with BIACORE 3000 biosensor system (Biacore Inc., Piscataway, NJ). To measure the binding of V_{HS} to protein A, 2000 resonance units (RUs) of protein A or a reference antigen-binding fragment (Fab) were immobilized on research grade CM5 sensor chips (Biacore Inc.). Immobilizations were carried out at
 - 15 concentrations of 25 μ g/ml (protein A) or 50 μ g/ml (Fab) in 10 mM sodium acetate buffer pH 4.5, using the amine coupling kit provided by the manufacturer. To measure the binding of the anti-idiotypic llama V_{HH} s to H11 scFv, 4100 RUs of 50 μ g/ml H11 scFv or 3000 RUs of 10 μ g/ml Se155-4 IgG reference were immobilized as described above. In all instances, analyses
 - 20 were carried out at 25°C in 10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.005% P20 at a flow rate of 40 μ l/min, and surfaces were regenerated by washing with the running buffer. To determine the binding activities of the refolded proteins, V_{HS} or V_{HH} s were denatured by incubation at 85°C for 20 min at 10 μ g/ml concentrations. The protein samples were
 - 25 then cooled down to room temperature for 30 min to refold and were subsequently centrifuged in a microfuge at 14,000 rpm for 5 min at room temperature to remove any protein precipitates. The supernatants were recovered and analyzed for binding activity by SPR as described above. For both folded and refolded proteins data were fit to a 1:1 interaction model
 - 30 simultaneously using BIAevaluation 4.1 software (Biacore Inc.) and K_D s were subsequently determined. RE s were determined from

$$RE = \frac{K_{Dn}}{K_{Dref}} \times 100$$

Where K_{Dn} is the K_D of the native protein and K_{Dref} is the K_D of the refolded protein.

Tryptic digest experiments. 3 μ l of a freshly prepared 0.1 μ g/ μ l sequencing grade trypsin (Hoffmann-La Roche Ltd., Mississauga, ON, Canada) in 1 mM HCl was added to 60 μ g V_H in 100 mM Tris-HCl buffer pH 7.8. Digestion reactions were carried out in a total volume of 60 μ l for 1 h at 37°C and stopped by adding 5 μ l of 0.1 μ g/ μ l trypsin inhibitor (Sigma, Oakville, ON, Canada). Following completion of digestion, 5 μ l was removed and analyzed by SDS-PAGE; the remaining was desalted using ZipTip_{C4} (Millipore, Nepean, ON, Canada), eluted with 1% acetic acid in 50:50 methanol:water and subjected to V_H mass determination by MALDI mass spectrometry.

Protein stability studies at 37°C. Single-domain antibodies (sdAbs) at 0.32-3.2 mg/ml concentrations were incubated at 37°C in PBS buffer for 17 days. Following incubation, the protein samples were spun down in a microfuge at maximum speed for 5 min even in the absence of any visible aggregate formation. The samples were then applied onto a Superdex 75 size exclusion column (GE Healthcare) and the monomeric peaks were collected for SPR analysis against protein A. SPR analyses were performed as described above except that 500 RUs of protein A or reference Fab was immobilized and that immobilizations were carried out at concentration of 50 μ g/ml.

NMR experiments - V_H samples for NMR analysis were dissolved in 10 mM sodium phosphate, 150 mM NaCl, 0.5 mM EDTA, and 0.02% NaN_3 at pH 7.0. The protein concentrations were 40 μ M – 1.0 mM. All NMR experiments were carried out at 298 K on a Bruker Avance-800 or a Bruker Avance-500 NMR spectrometer. One-dimensional (1D) ^1H NMR spectra were recorded with 16,384 data points and the spectral widths were 8,992.81 Hz at 500 MHz and 17,605.63 Hz at 800 MHz, respectively. Two-dimensional ^1H - ^1H NOESY spectra of 2,048 x 400 data points were acquired on a Bruker Avance-800 NMR spectrometer with a spectral width of 11,990.04 Hz and a mixing time of 120 ms. In all NMR experiments, water suppression was achieved using the

WATERGATE method implemented through the 3-9-19 pulse train (Piotto, M. *et al.*, 1992; Sklenar, V. *et al.*, 1993). NMR data were processed and analyzed using the Bruker XWINNMR software package. All PFG-NMR diffusion measurements were carried out with the water-suppressed LED sequence
5 (Altieri, A. S. *et al.*, 1995), on a Bruker Avance-500 NMR spectrometer equipped with a triple-resonance probe with three-axis gradients. One-dimensional proton spectra were processed and analyzed using Bruker Xwinnmr software package. NMR signal intensities were obtained by integrating NMR spectra in the methyl and methylene proton region (2.3ppm
10 to -0.3ppm) where all NMR signals were attenuated uniformly at all given PFG strengths.

Human V_L phage display library construction and panning. cDNAs were synthesized from human spleen mRNA as described above for the human
15 V_{Hs}. The cDNA was used as template in PCR to amplify V_L genes in 50 µl reaction volumes using six V_κ back primers, 11 V_λ back primers (**de Haard, H. J. *et al.*, 1999**), four V_κ For primers and two V_λ For primers (**Sblattero, D. *et al.*, 1998**). The back and forward primers were modified to have flanking *Apa* LI and *Not* I restriction sites, respectively, for subsequent cloning purposes.
20 Forward primers were pooled together in ratios which reflected their degree of degeneracy. V_λ genes were PCR'd in 11 separate reactions using the pooled V_λ For primers and 11 individual V_λ back primers. Similarly, V_κ genes were amplified in 6 separate reactions using the pooled V_κ For primers and 6 individual V_λ back primers. The PCR products were pooled, gel purified and
25 digested with *Apa* LI and *Not* I restriction endonucleases. The library was constructed as described for human V_{Hs}. Plaque PCR was performed on individual library colonies and the amplified V_L genes were sequenced as described (**Tanha, J. *et al.*, 2003**). Panning against protein L (Biolynx Inc., Brockville, ON, Canada) and germline sequence assignment of the selected
30 V_Ls were performed as described above for human V_H library.

V_L expression and purification. V_L expression, purification, concentration determination and gel filtration chromatography were carried out as described for V_Hs in "**V_H expression and purification.**".

- 5 **Expression and purification of V_L and V_H pentamers.** Specific primers were used in a standard PCR to amplify HVHP328 V_H and HVLP335 V_L genes. Standard cloning techniques were used to clone the HVHP328 and HVLP335 genes in fusion with VT1B pentamerization domain gene in an expression vector to yield HVHP328PVT2 and HVLP335PTV2 pentamers,
10 (Zhang, J. *et al.*, 2004). Pentamers were expressed and purified as described (Zhang, J. *et al.*, 2004). Protein concentrations were determined as above.

- Surface plasmon resonance of V_Ls.** The binding kinetics for the interaction
15 of the V_Ls to protein L were determined by SPR using BIACORE 3000 biosensor system (Biacore, Inc., Piscataway, NJ). 680 RUs of protein L or 870 RUs of a Fab reference were immobilized on research grade CM5 sensor chips (Biacore). Immobilizations were carried out at a protein concentration of 50 µg/ml in 10 mM acetate buffer pH 4.5 using the amine coupling kit supplied
20 by the manufacturer. All measurements were carried out at 25°C in 10 mM HEPES buffer pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.005% P20 at a flow rate of 50 µl/min or 100 µl/min. Surfaces were regenerated by washing with the running buffer. Data were evaluated using the BIAevaluation 4.1 software (Biacore, Inc.).

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- Surface plasmon resonance of the pentameric V_L and V_H.** The binding kinetics for the interaction of HVHP328PVT2 with protein A and HVLP335PTV2 with protein L were also determined by SPR. 520 RUs of protein A or a Fab reference were immobilized as above. For the V_L
30 pentamer, the same surfaces prepared above were used. Measurements were carried out as above but at a flow rate of 20 µl/min. Surfaces were regenerated by washing with 50 mM HCl for 3 s. Data were evaluated as described for the monomers.

Cell microagglutination

A single *S. aureus* colony from a BHI plate was used to inoculate 15 mL of BHI media. The bacteria were grown overnight at 37°C at 200 rpm. In the morning, the culture was spun down in a swinging bucket, Sorvall RT6000B refrigerated centrifuge at 4000 rpm for 10 min, the supernatant was removed and the cell pellet was re-suspended in PBS buffer. The cells were re-spun, the supernatant was removed and the cell pellet was re-suspended again in PBS buffer. The cells were diluted to an A_{600} of 1.0, and serial dilutions of the cells were spread on BHI plates at 37°C for overnight growth. The cell titer was determined in the morning. An A_{600} of 1.0 corresponded to 1.5×10^9 cells ml^{-1} . Identical steps were taken to prepare *E. coli* starin TG1 cells for subsequent microagglutination assays, except that the growth media was 2xYT. The viable counts were similar, $A_{600}1.0 = 2.1 \times 10^9$ cells ml^{-1} .

To perform microagglutination assays, two fold dilutions of HVHP328PVT2 in PBS were performed from wells 1 to 11 in a microtiter plate. Well 12 (blank) had only PBS. The total volume in each well was 50 μl . Subsequently, 1×10^8 *S. aureus* cells in 50 μl PBS was added to all wells and the plate was incubated overnight at 4°C. To have a permanent record of the results, a picture was taken from the plate in the morning. For the pentamer control experiment, HVHP328PVT2 was replaced with the V_L pentamer, HVLP335PTV2. In the cell control experiments, the same two sets of experiments were repeated with *E. coli* TG1 cells.

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Identification and sequence analysis of monomeric human V_L s

Essentially the same selection method which was employed to isolate soluble V_H s from a human V_H phage display library was applied to a human V_L library for isolating soluble, monomeric V_L s. A human V_L library with a size of 3×10^6 was constructed. Twenty four plaques from the library titer plates were picked and their V_L genes were PCR'd and sequenced. The sequences were diverse in terms of germ-line origin although 75% of the V_L s were of $V\lambda$ origin (data not shown). Three rounds of panning against protein L resulted in

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5 incubated overnight at 4°C. To have a permanent record of the results, a picture was taken from the plate in the morning. For the pentamer control experiment, HVHP328PVT2 was replaced with the V_L pentamer, HVLP335PTV2. In the cell control experiments, the same two sets of experiments were repeated with *E. coli* TG1 cells.

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15 was constructed. Twenty four plaques from the library titer plates were picked and their V_L genes were PCR'd and sequenced. The sequences were diverse in terms of germ-line origin although 75% of the V_Ls were of V λ origin (data not shown). Three rounds of panning against protein L resulted in enrichment for large plaques. Thirty-nine of large plaques were sequenced
20 and 32 unique sequences were identified (**Figure 6**). HVLP325, HVLP335 and HVLP351 occurred at frequency of 3, 4 and 2, respectively. Except for HVLP389 which is of lambda class (subgroup V λ 1, germline 1b), the remaining 31 V_Ls belonged to the V κ class. Of the 31 kappa V_Ls, 24 fall within the V κ III subgroup and 7 within the V κ 1 subgroup. Sixteen of the 24
25 V κ III sequences utilize L6 germline sequence with the remaining utilizing A27, L2 and L6 germline sequences. The V κ 1 subgroup V_Ls are originated from O2/O12 or A30 germline sequence. Noticeable mutations occurred at position 96. The germline amino acids at this position are aromatic and hydrophobic amino acids Trp, Phe, Tyr, Leu or Ile for kappa V_Ls and Tyr, Val
30 or Ala for lambda V_Ls. But in the selected pool of kappa V_Ls only 5 out of 31 have their germline amino acids at position 96: HVLP325, HVLP349, HVLP388, HVLP3109 and HVLP393. 21 amino acids at position 96 are

charged of which 20 are positively-charged: Arg, Lys or His. Two amino acids are Pro, one Gln, one Ser and one Thr. Of seven kappa V_Ls analyzed by gel filtration chromatography for monomericity, six which had Arg or Lys at position 96 were also monomers, whereas HVLP325 with the germline amino acid Leu at position 96 formed aggregates (see below). Similarly, HVLP389 which was of the lambda class and had a germline mutation to Ser was also monomeric (see below). These data correlates the deviation from the germline amino acids at position 96 (27 out of 32) with improved biophysical properties of V_Ls such as monomericity.

10

Eighteen V_Ls of the kappa class had their last three residues (105-107) replaced with amino acids Thr, Val and Leu which are only found in lambda V_Ls. These substitutions may have had a role in improving the biophysical properties of the kappa V_Ls, resulting in the selection of the aforementioned V_Ls over the parental clones with the original kappa residues at position 105-107.

15

Characterization of the human V_Ls

Eight of the selected V_Ls with different V germline origins were expressed in E. coli in one-liter cultures and purified: HVLP324, HVLP325, HVLP335, HVLP342, HVLP351, HVLP364, HVLP389 and HVLP3103 (Table 6). All were expressed in good yields ranging from 6.2 mg for HVLP324 to around 75 mg for HVLP335 and HVLP364.

20

The aggregation tendency of the human V_Ls was assessed in terms of their oligomerization state by gel filtration chromatography. V_Ls were subjected to Superdex 75 gel filtration chromatography at a concentration of 0.6 mg/ml. All except HVLP325 were essentially free of aggregates and gave symmetric single peaks with the mean apparent molecular mass of 12.7 kDa (range, 6.2-19.2 kDa) (Figure 7A and Table 3). This is in agreement with the expected molecular mass for monomeric V_Ls, 13.4-13.8 kDa. Variation in apparent molecular mass for single-domain antibodies has been reported previously (Jespers, L. *et al.*, 2004a; (Stevens, F. J. *et al.*, 1980). For HVLP325, the

30

aggregates formed 11% of the total protein (aggregate plus monomer). HVLP351, HVLP342, HVLP335 and HVLP3103, were still monomer when tested at their highest concentration available, i.e., 0.89 mg/ml, 1.0 mg/ml, 4.9 mg/ml and 5.9 mg/ml, respectively (**Figure 7B**)

5

V_L s were subjected to Superdex-75 chromatography prior to BIACORE analysis and purified monomer peaks collected even in the absence of any evidence of aggregated material. In SPR analysis, all selected V_L s bound to protein L (Figure 8). This was not unexpected since the V_L s were isolated by panning against protein L. For all, the K_D s of binding to protein L were in 0.6-3 μ M (Table 3). HVLP324 and HVLP342 had additional smaller K_D s, 10 nM and 40 nM, respectively. Low affinity and high affinity bindings of V_L s of V_{KL} subgroup to protein L have been reported previously (Reference). Both, HVLP324 and HVLP342, belong to V_{KL} subgroup (**Table 3**). As expected, the kinetic and equilibrium data were consistent with the monomeric peak being indeed monomeric.

Binding analyses of pentamers

Bindings of HVHP328PVT2 pentamer to protein A and HVLP335PTV2 pentamer to protein L were determined by surface plasmon resonance (**Figure 9**). The association rates were independently calculated from plots of k_{obs} versus concentration. More than one dissociation rate (k_d) could be calculated due to the heterogeneity in multivalent binding amongst the pentamer population. Therefore, more than one equilibrium dissociation constant, K_D , could be obtained. HVHP328PTV2 and HVLP335PTV2 had minimum K_D s of 2 nM and 200 pM, respectively (**Table 4**). With slower k_d s, HVHP328PTV2 and HVLP335PTV2 had K_D s as low as 900 and 90 pM, respectively.

30 Pathogen detection by V_L s and V_{HS}

The protein A and L binding activity of the V_{HS} and V_L s can be used to detect bacteria which have protein A and/or L on their surfaces. This is possible if the V_{HS} and V_L s are soluble and monomeric (lack of tendency to aggregate)

such as the V_{HS} and V_L s here. Variable domains derived from antibodies which lack light chains such as camelid heavy chain antibodies or nurse shark and wobbegong shark IgNARs are naturally soluble and monomeric. From these, those with protein A and L binding activity can also be used to detect

5 bacteria which have protein A and/or L on their surfaces. Protein A is present on the surface of the pathogenic bacteria, *Staphylococcus aureus*. Thus, the V_{HS} with protein A binding activity such as the ones described here can be used to detect *S. aureus*. We performed a microagglutination assay to detect the ability of HVHP328PVT2 V_H pentamer to bind to *S. aureus*. A constant

10 number of bacterial cells were incubated with two-fold dilutions of HVHP328PVT2 in microtiter wells (wells 1-11) (**Figure 10**). Well 12 had buffer instead of the pentamer. If the V_{HS} bind to the bacterial cells, then the pentamer because of its multimeric nature should be able to cross-link the cells and results in cell agglutination. The agglutinated cells will appear as

15 diffused cells in a microtiter well (**Figure 10**). In the absence of any binding, no agglutination should occur, hence no agglutination, and the cells will appear as a dot at the bottom of the well. As shown in Figure 10, the pentamer binds to the *S. aureus*, since there is agglutination of cells. The agglutination is observed up to well 7. Beyond well 7 the concentration of the

20 pentamer is too low for binding, hence no agglutination. The control V_L pentamer does not show any agglutination, demonstrating the specificity of the V_H pentamer to *S. aureus* (**Figure 10**). The binding is also cell-specific since the V_H pentamer as expected does not agglutinate *E. coli* (TG1 strain) or *Salmonella* cells (data not shown). Similarly, the V_L monomers and V_L

25 pentamers with protein L binding activity can be used for the detection of bacteria, in particular pathogenic bacteria such as *Peptostreptococcus magnus*, which have protein L on their cells surface.

It is understood that the examples described above in no way serve to limit

30 the true scope of this invention, but rather are presented for illustrative purposes.

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Table 1. V_H sequence deviations from parental germline sequences

V _H	V/J germlines	Amino acid deviation from V and FR4 germline sequences
HVHP44	DP47/JH4b	L5V, Q105R
HVHB82	DP47/JH6c	E1Q, L5Q
HVHP421	DP47/JH4b	E1Q, V2L, L5Q, L11V, G16R
HVHP419	DP47/JH4b	E1Q, V2L, L5Q, T77S, R83G, K94R
HVHP430	DP47/JH3b	E1Q, L5V, V12I, Q13K, S31N, G52AS, L78V, A93V, K94R
HVHP429	DP47/JH4	L5V, G10T, S30I, S31N, G42D, E46D, A50T, G52aN, S53N, S56A K75N, A84P, E85D
HVHM41	DP47/JH3a	E1Q, L5V, E6Q, G16R, T28A, S53G, G55D, S56H, M108L
HVHM81	DP47/JH3a	L5V, E6Q, G16R, S30D, S31D, S35H, A50G, G55A, E85G, V89L, K94R
HVHP428	V3-49/JH4b	E1Q, V2L, V5Q, R16G, T23A, G30S, D31S, T60A, G73D, K83R, T84A, V89M, T93A
HVHP420	DP-38/JH4b	E1Q, S35T, S52aT
HVHP414	DP-38/JH3b	E1D, E6Q, A23T, T28P, K52T, A60V
HVHP423	V3-53/JH1	E1Q, V2M, E6Q, L11V, I12V, N32S, Y33R, V37F, K43M, K64R, T68S, V89L
HVHP44B	V3-53/JH1	E1Q, E6Q, N32S, Y33R, V37F, K43M, Y58S, K64R, T68S, V89L
HVHP413	YAC-5/JH3b	E1Q, E6Q, Q13K, V29F, S31D, N32Y, V50F
HVHP426	8-1B/JH3b	E1Q, E6Q, L11V, G16R, T28I, S30D, S31G, N32Y, Y33A, S35H, K43Q, I51T, Y52N, S53N, Y58S, L78V

5

Table 2. Biophysical characteristics of the human V_Hs

V _H /V _H H	Exp.# (mg)	K _D (μM)	Trypsin resistance	RE (%)
HVHP44	8.2	1.3	√	93
HVHB82	5.9	0.2	√	71
HVHP421	5.5	1.0	√	14
HVHP419	3.4	1.6	√	84
HVHP430	6.4,23.7	2.3	√	88
HVHP429	3.4	1.3	√	86
HVHM41	1.8	0.5	X	92
HVHM81	4.3	1.3	√	87
HVHP428	3.1	1.8	√	95
HVHP420	59.0	1.2	√	92
HVHP414	11.8	1.6	√	73
HVHP423	2.4,62.1	3.0	√	86
HVHP413	5.8	0.3	√	52
HVHP426	6.3	0.8	√	70
H11F9*	ND	3.5	ND	95
H11B2*	ND	2.0	ND	100

#expression yield per liter of bacterial culture

* K_D s and RE s were determined against H11 scFv.

5

Table 3. Characteristics of the human V_Ls

V _L	Subgroup	Expression ^a	K _D	Oligomerization state ^b
		mg	μM	
HVLP324	V _K I	6.9	0.2, 0.01 ^c	Monomer
HVLP325	V _K III	6.2	1	Monomer/Aggregate
HVLP335	V _K III	73.5	2	Monomer
HVLP342	V _K I	7.7	0.6, 0.04 ^c	Monomer
HVLP351	V _K III	8.9	2	Monomer
HVLP364	V _K III	77.1	3	Monomer
HVLP389	V _λ I	16.7	1	Monomer
HVLP3103	V _K III	19.0	1	Monomer

^a Expression yield per liter of bacterial culture.^b Oligomerization state was determined by gel filtration chromatography.

10 ^c The smaller K_D values correspond to the binding of the of HVLP324 and HVLP342 to the high affinity sites on protein L.

Table 4. Kinetic and equilibrium constants for the bindings of HVHP328PTV2 and HVLP335PTV2 to protein A and L, respectively

Pentabody	HVHP328PTV2	HVLP335PTV2
k_a ($M^{-1}s^{-1}$)	4.3×10^5	1.7×10^6
k_d (s^{-1})	$< 1 \times 10^{-3}$	$< 4 \times 10^{-4}$
K_D (M)	$< 2 \times 10^{-9}$	$< 2 \times 10^{-10}$

5

What is claimed is:

1. A method of identifying target polypeptides , comprising:
 - (a) obtaining a phage display library capable of expressing a variety of polypeptide sequences;
 - 5 (b) allowing infection of a bacterial lawn by the library phage; and
 - (c) identifying phage which form larger than average plaques on the bacterial lawn.
- 10 2. A method as claimed in claim 1, where the target polypeptides are soluble.
3. A method as claimed in claim 1, where the target polypeptides are monomeric.
- 15 4. A method as claimed in claim 1, where the target polypeptides are non-aggregating.
5. A method as claimed in claim 1, where the target polypeptides are highly expressed.
- 20 6. A method as claimed in claim 1, where the target polypeptides are stable.
7. A method as claimed in claim 1, where the phage is a filamentous phage.
- 25 8. A method as claimed in claim 7, where the phage is M13 or fd.
9. A method as claimed in claim 6, wherein the stable polypeptides have relatively high thermal refolding efficiency.
- 30 10. A method as claimed in claim 6, wherein the stable polypeptides have a relatively high melting temperature.

11. A method as claimed in claim 6, wherein the stable polypeptides have maintain their functionality following long incubation at 37°C.
12. A method as claimed in claim 6, wherein the stable polypeptides are relatively resistant to chemical denaturants.
13. A method as claimed in claim 6, wherein the stable polypeptides are relatively resistant to proteases.
14. A method as claimed in claim 6, wherein the stable polypeptides have a relatively long shelf life at or above room temperature, at 4°C, or below 0°C.
15. A method as claimed in claim 6, wherein the stable polypeptides are functional in intracellular environments.
16. A method as claimed in claim 6, wherein the stable polypeptides are functional when administered internally to humans.
17. A method as claimed in any claim 1, further comprising the steps of:
(d) isolating the larger plaque phage in step (c); and
(e) determining the sequence or other characteristics of the polypeptides expressed by the larger plaque phage.
18. A method as claimed in claim 1, wherein the target polypeptides are antibodies or fragments of antibodies.
19. A method as claimed in claim 18, wherein the target polypeptides are human V_H or V_L antibody fragments.
20. A method as claimed in claim 1, wherein the target polypeptides are vaccines.

21. A method as claimed in claim 1, wherein the target polypeptides are therapeutic proteins.
22. A method as claimed in claim 1, wherein the target polypeptides are
5 selected from the group consisting of: single-chain T-cell receptors, T-cell receptor domains, transferin, lipocalins, kunitz domains, ankyrin repeats, and cytotoxic T-lymphocyte-associated antigen.
23. A method as claimed in claim 1, wherein the target polypeptides are
10 proteinacious diagnostic and biochemical reagents.
24. A polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NO:8-54.
25. A polypeptide having an amino acid sequence that is substantially
15 identical to the amino acid sequence claimed in claim 24.
26. A nucleic acid sequence that encodes a polypeptide as claimed in claim 24 or 25.
27. A polypeptide comprising the CDR3 portion of an amino acid
20 sequence as claimed in claim 24.
28. A nucleic acid sequence that encodes a polypeptide as claimed in
25 claim 27.
29. A polypeptide comprising the FR1 portion of an amino acid sequence as claimed in claim 24.
30. A nucleic acid sequence that encodes a polypeptide as claimed in
30 claim 29.
31. A polypeptide comprising the FR2 portion of an amino acid sequence as claimed in claim 24.

32. A nucleic acid sequence that encodes a polypeptide as claimed in claim 31.
- 5 33. A polypeptide comprising the FR3 portion of an amino acid sequence as claimed in claim 24.
34. A nucleic acid sequence that encodes a polypeptide as claimed in claim 33.
- 10 35. A polypeptide comprising the FR4 portion of an amino acid sequence as claimed in claim 24.
36. A nucleic acid sequence that encodes a polypeptide as claimed in claim 35.
- 15 37. A polypeptide comprising the CDR2 portion of an amino acid sequence as claimed in claim 24.
38. A nucleic acid sequence that encodes a polypeptide as claimed in claim 37.
- 20 39. A polypeptide comprising the CDR1 portion of an amino acid sequence as claimed in claim 24.
- 25 40. A nucleic acid sequence that encodes a polypeptide as claimed in claim 39.
41. A V_H antibody fragment comprising at least one amino acid sequence selected from the group consisting of: SEQ ID NO:8-22.
- 30 42. A V_L antibody fragment comprising at least one amino acid sequence selected from the group consisting of: SEQ ID NO:23-54.

43. A nucleic acid sequence that encodes a V_H or V_L antibody fragment as claimed in claim 41 or 42.
- 5 44. A V_H antibody fragment comprising at least two amino acid sequences as claimed in claim 41.
45. A V_L antibody fragment comprising at least two amino acid sequences as claimed in claim 42.
- 10 46. A nucleic acid sequence that encodes a V_H or V_L antibody fragment as claimed in claim 44 or 45.
47. A V_H antibody fragment comprising at least three amino acid sequences as claimed in claim 41.
- 15 48. A V_L antibody fragment comprising at least three amino acid sequences as claimed in claim 42.
49. A nucleic acid sequence that encodes a V_H or V_L antibody fragment as claimed in claim 47 or 48.
- 20 50. A multimer comprising at least two V_H antibody fragments as claimed in claim 41, 44 or 47.
- 25 51. A multimer comprising at least two V_L antibody fragments as claimed in claim 42, 45 or 48.
52. A multimer comprising at least one V_H antibody fragment as claimed in claim 41, 44 or 47, and at least one V_L antibody fragment as claimed in claim 42, 45 or 48.
- 30 53. A dimer comprising two V_H antibody fragments as claimed in claim 41, 44 or 47.

54. A dimer comprising two V_L antibody fragments as claimed in claim 42, 45 or 48.
55. A trimer comprising three V_H antibody fragments as claimed in claim 41, 44 or 47.
56. A trimer comprising three V_L antibody fragments as claimed in claim 42, 45 or 48.
57. A pentamer comprising five V_H antibody fragments as claimed in claim 41, 44 or 47.
58. A pentamer comprising five V_L antibody fragments as claimed in claim 42, 45 or 48.
59. A V_H antibody fragment identified by the method of claim 1.
60. A V_H antibody fragment as claimed in claim 59 that is of human origin.
61. A V_H antibody fragment as claimed in claim 59 having an amino acid sequence that deviates from the corresponding parental germline sequence as shown in Table 1.
62. A V_H antibody fragment as claimed in claim 59 belonging to the V_H3 family.
63. A V_H antibody fragment as claimed in claim 59 belonging to a V germline selected from the group consisting of DP47, V3-49, DP-38, V3-53, YAC-5 and 8-1B.
64. A V_H antibody fragment as claimed in claim 59 belonging to a J germline selected from the group consisting of JH4b, JH6c, JH3b, JH4, JH3a, and JH1.

65. A V_H antibody fragment as claimed in claim 59 and having a residue other than valine at position 37 of its amino acid sequence.
- 5 66. A V_H antibody fragment as claimed in claim 63 having a phenylalanine residue at position 37 of its amino acid sequence.
67. A V_H antibody fragment as claimed in claim 63 having a tyrosine residue at position 37 of its amino acid sequence.
- 10 68. A V_H antibody fragment as claimed in claim 59 having a mutation at a position selected from the group consisting of positions 1,5,6,35,83,84,84a and 108 of its amino acid sequence.
- 15 69. A V_H antibody fragment as claimed in claim 59 which binds to protein A.
70. A V_L antibody fragment identified by the method of claim 1.
- 20 71. A V_L antibody fragment as claimed in claim 70 that is of human origin.
72. A V_L antibody fragment as claimed in claim 70 belonging to subgroup V_κ1, V_κ3 or V_λ1.
- 25 73. A V_L antibody fragment as claimed in claim 70 belonging to the kappa class.
- 30 74. A V_L antibody fragment as claimed in claim 70 belonging to a V germline selected from the group consisting of L6, A27, L2, L16, O2/O12, A30 and 1b.

75. A V_L antibody fragment as claimed in claim 70 belonging to a J germline selected from the group consisting of Jk1, Jk4, Jk2, and Jλ3b.
- 5 76. A V_L antibody fragment as claimed in claim 70 having an arginine, proline, lysine, threonine, leucine, serine, tyrosine, glutamic acid, glutamine or histidine residue at position 96 of its amino acid sequence.
- 10 77. A V_L antibody fragment as claimed in claim 70 having a charged amino acid residue at position 96 of its amino acid sequence.
78. A V_L antibody fragment as claimed in claim 70 which binds to protein L.
- 15 79. A V_L antibody fragment as claimed in claim 70 having a threonine, valine or leucine amino acid residue at position 105, 106 and 107 of its amino acid sequence.
- 20 80. A polypeptide sequence identified by the method of claim 1.
81. A nucleic acid sequence that encodes the polypeptide sequence of claim 80.
- 25 82. A display library constructed using polypeptide sequences as claimed in claim 80.
83. A display library constructed using polypeptide sequences as claimed in claim 24, 59 or 70.
- 30 84. A display library constructed using V_H or V_L antibody fragments as claimed in claim 41, 42, 44, 45, 47 or 48.

85. A display library constructed using polypeptide sequences as claimed in claims 27, 37 or 39.
- 5 86. A display library constructed using the loop (L1, L2, L3, H1, H2, or H3) regions of the polypeptides of claims 24, 59 or 70.
87. A display library as claimed in claim 82, 83, 84, 85 or 86, that is a phage display library.
- 10 88. A display library as claimed in claim 82, 83, 84, 85 or 86 that is a ribosome display, ARM ribosome display, yeast display, bacterial cell display or in vitro compartmentalization library.
- 15 89. A method for producing polypeptides with desirable biophysical properties, comprising the steps of
- a) providing at least one first nucleic acid sequence that encodes an antibody fragment as claimed in claim 41,42,44,45,47,48, 59 or 70 or that encodes a polypeptide sequence as claimed in claim 24,27,37 or 39, and having a first desirable property;
 - 20 b) providing at least one second nucleic acid sequence that encodes an antibody fragment having a second desirable property;
 - c) cutting the at least one first and at least one second nucleic acid sequences into random fragments;
 - 25 d) reassembling the random fragments;
 - e) expressing the random fragments; and
 - f) screening the expressed random fragments for the first and second desirable properties.
- 30 90. A method as claimed in claim 89, wherein the first desirable property is selected from the group consisting of solubility, stability, monomericity, high expression, human origin, and binding specificity.

- 5 91. A method as claimed in claim 89, wherein the second desirable property is selected from the group consisting of solubility, stability, monomericity, high expression, human origin and binding specificity.
92. A method as claimed in claim 89, where the at least one second antibody fragment is of non-human origin.
- 10 93. A method as claimed in claim 89, where the at least one second antibody fragment is of human origin.
94. A method as claimed in claim 89, where the at least one second antibody fragment is a human V_H or V_L .
- 15 95. A method as claimed in claim 89, where screening of the reassembled random fragments is accomplished by ribosome display, yeast display, phage display, bacterial cell display, ARM ribosome display or in vitro compartmentalization.
- 20 96. A pharmaceutical composition comprising the antibody fragment of claim 59 or 70, and a pharmaceutically suitable agent.
- 25 97. A pharmaceutical composition comprising the polypeptide sequence of claim 24 or 80, and a pharmaceutically suitable agent.
98. A recombinant vector comprising the nucleic acid sequence of claim 26 or 81.
- 30 99. A host cell transformed with the recombinant vector of claim 98.

Figure 2

	FR1			CDR1			FR2			CDR2			FR3			CDR3			FR4			Frequency
	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200		
HVHP44	EVQLVESGGGLVQPGGSLRLSCAASGFTFS			SYAMS	WVRQAPGKGLWEWS	AISSG--SGGSYYADSVKVG	RETIISRDNSKNTLIYIQNSIRAE	DTAVIYCAK	DEPRVSGLRGV--VDS	WGRGLIVTVSS											1	
HVHB82	Q...Q...																				1	
HVHP421	QL...Q...V...R...																				3	
HVHP419	QL...Q...																				1	
HVHP430	Q...Q...IK...	N...																			2	
HVHP429	Q...Q...T...	I N...		D...D...	T...N--N..A...																24	
HVHP41	Q...Q...R...	A...																			1	
HVHB1	...Q...R...	D D...H																			1	
HVHP428	QL...Q...			F...	G F RSKAY..T.E..A...																52	
HVHP420	Q...Q...K...	NAW.T			G R.RKTD..T.D..AP...																5	
HVHP414	D...Q...K...	T...P...			G R.TSKTD..T.D..VAP...																4	
HVHP423	Q...Q...V...	V...SR...	F...	M...	V Y...																4	
HVHP44B	Q...Q...I...	V...SR...	F...	M...	V Y...																4	
HVHP413	Q...Q...K...	D.Y.			F Y...																3	
HVHP426	Q...Q...V...R...	IVD G...H		Q...	VTN--N...S...																8	

Figure 3A

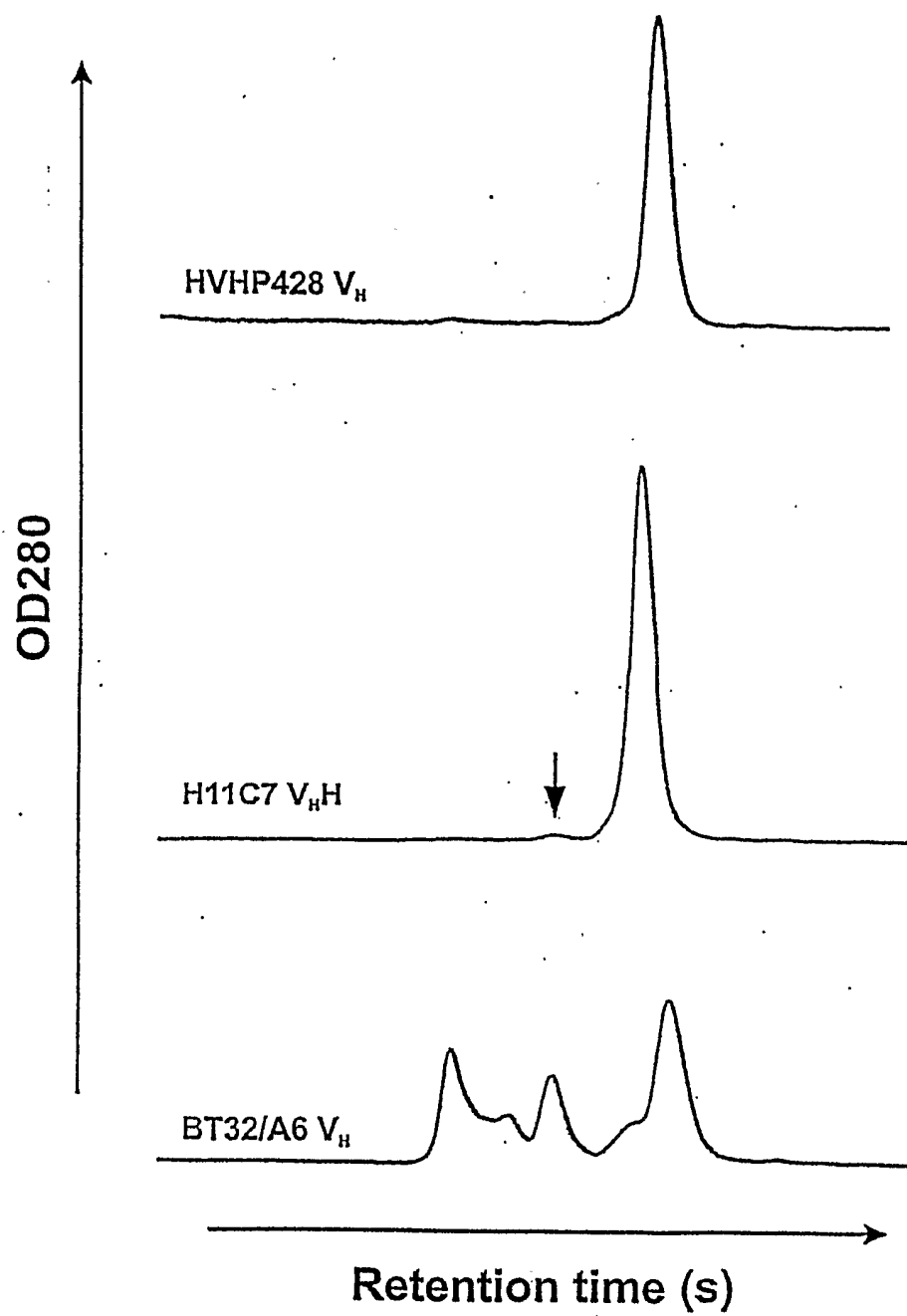


Figure 3B

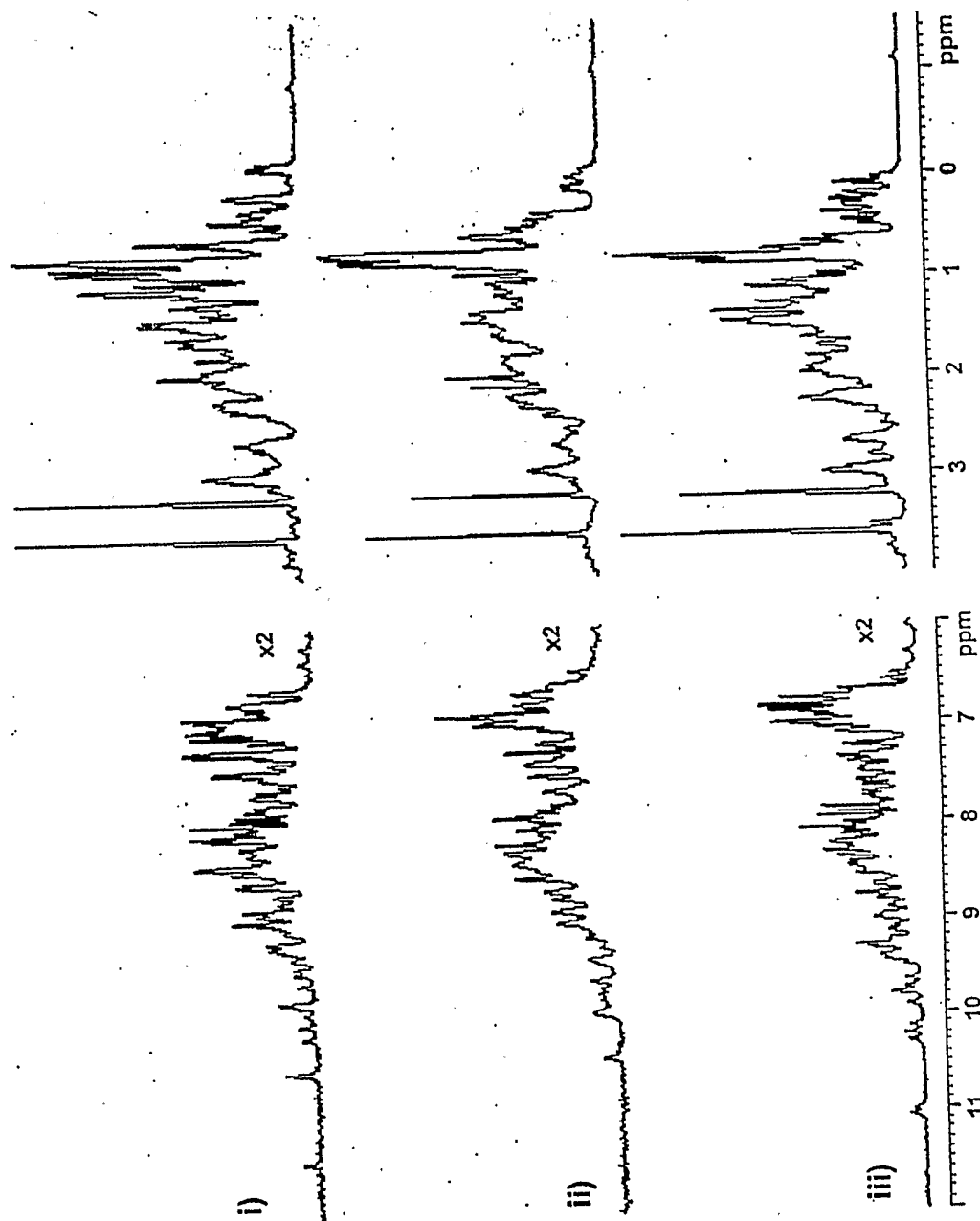


Figure 4A

untreated 15 min 30 min 60 min

21 kDa

HVHP414

HVHP414-cMyc

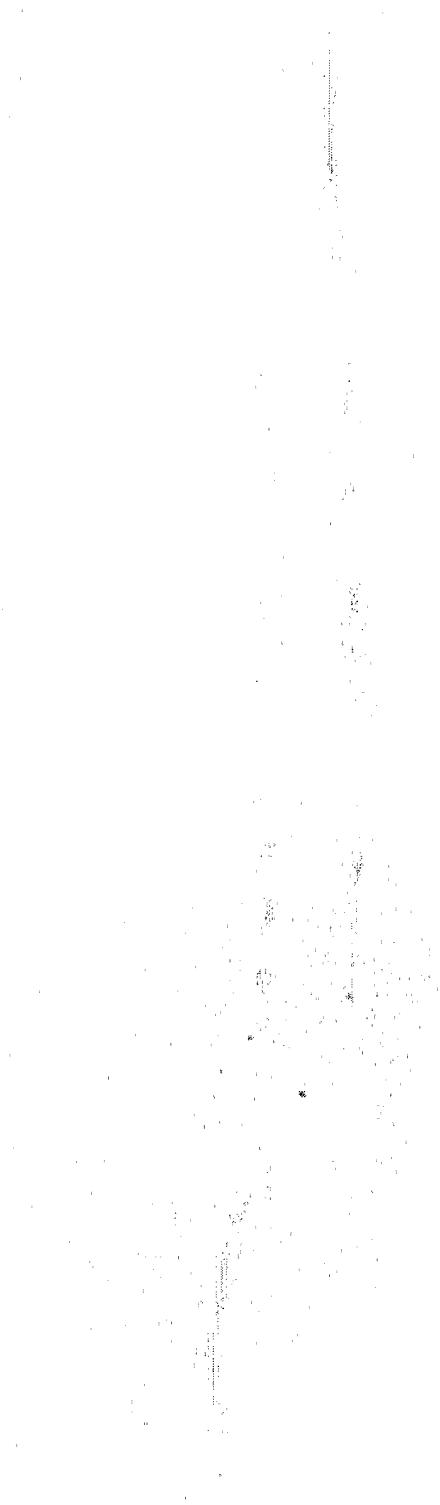


Figure 4B

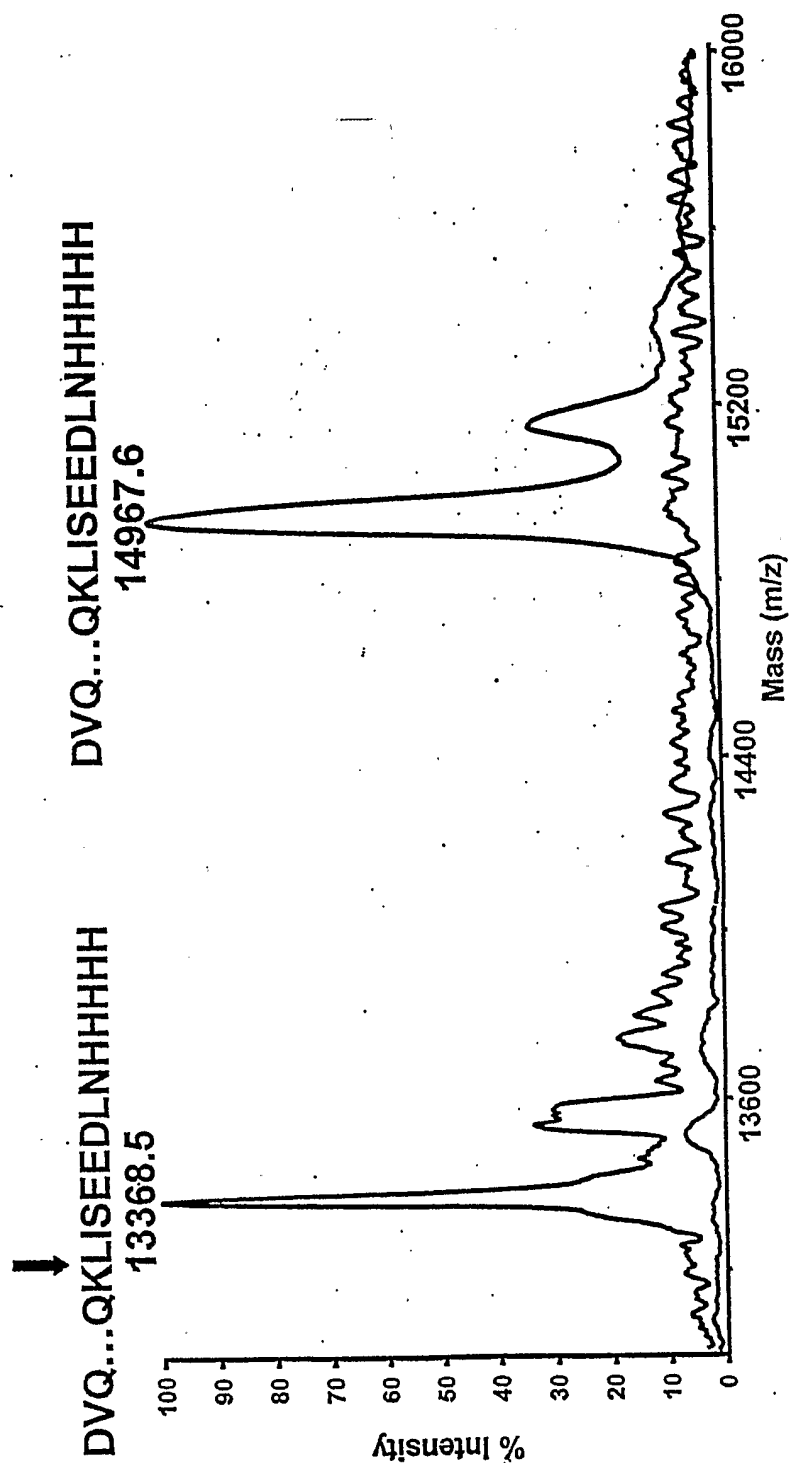


Figure 4C

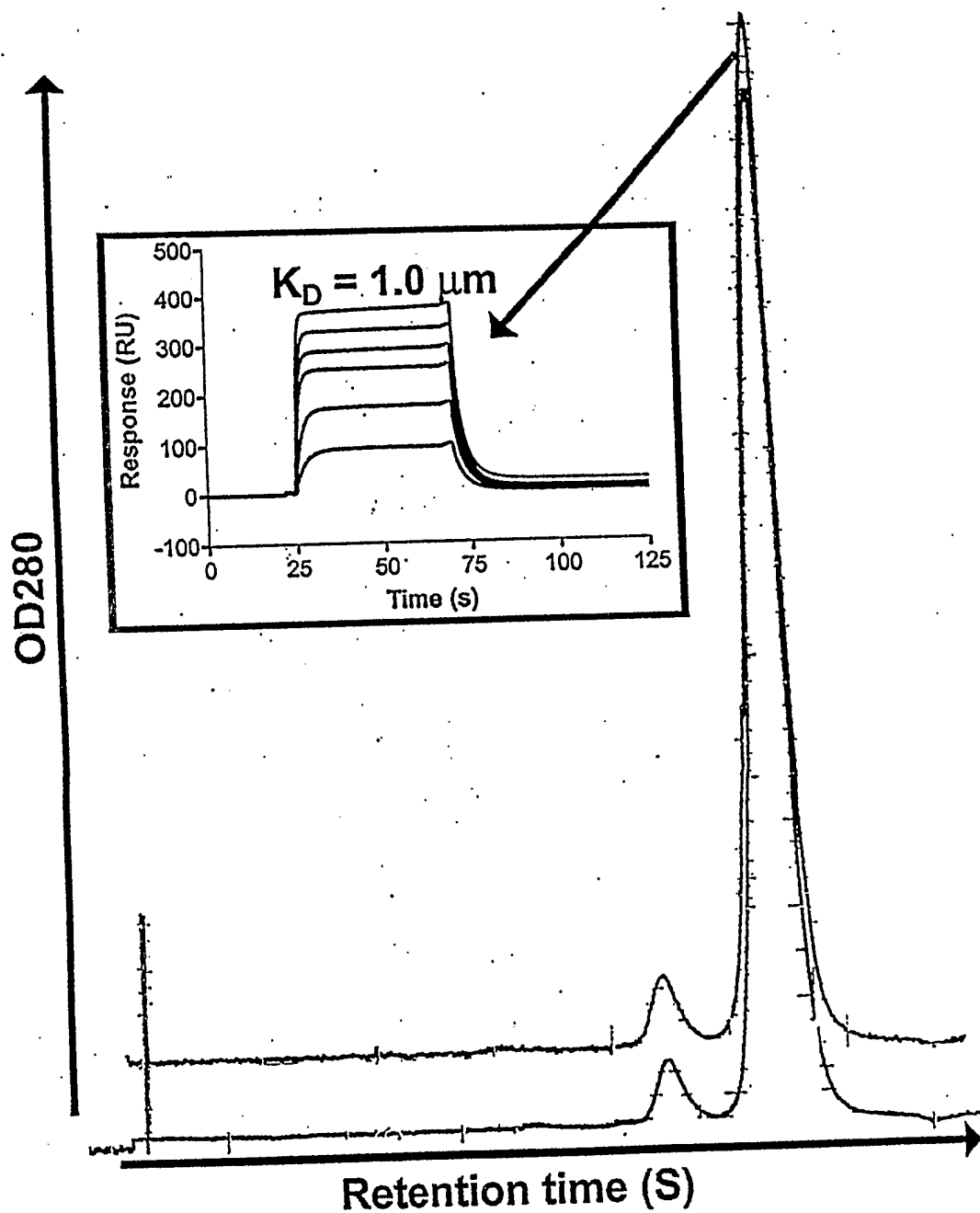
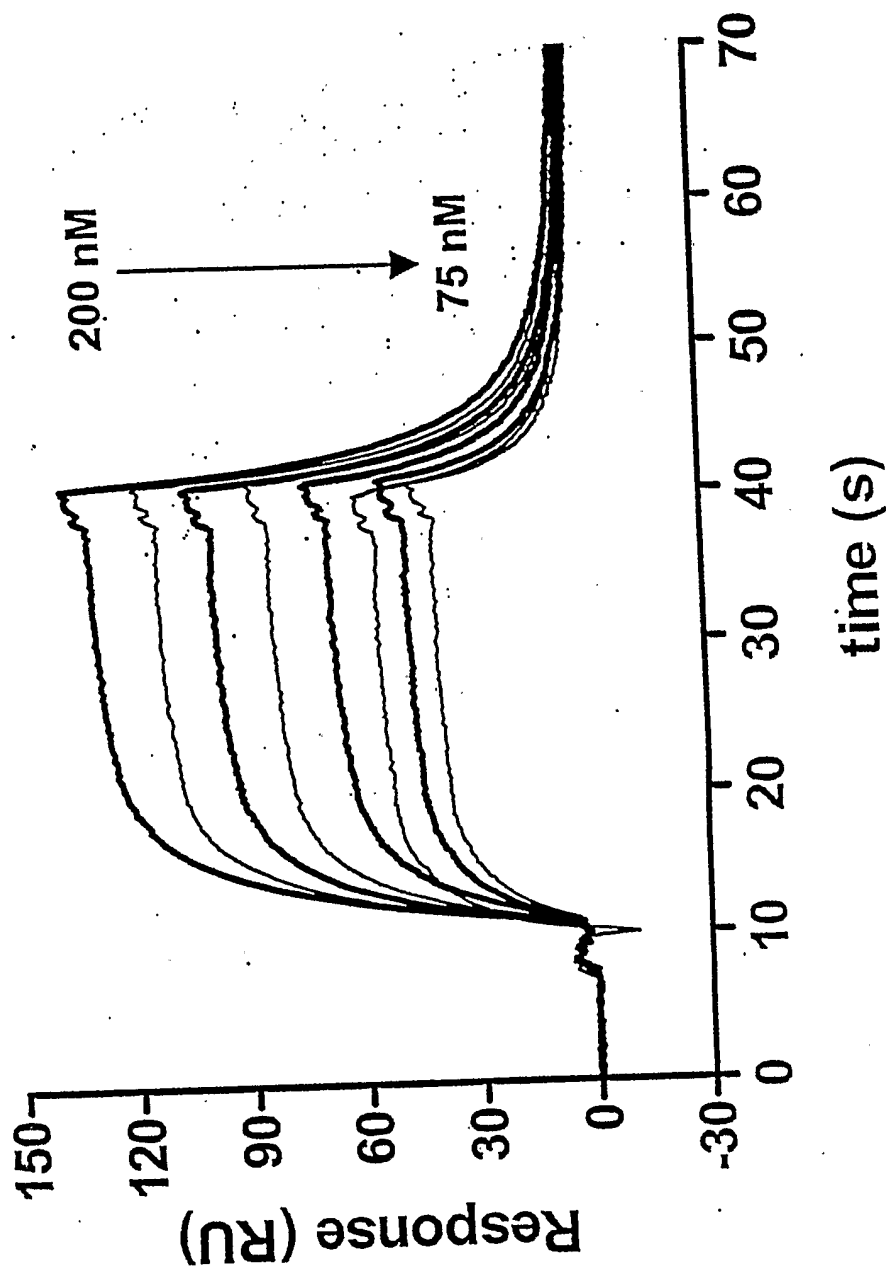
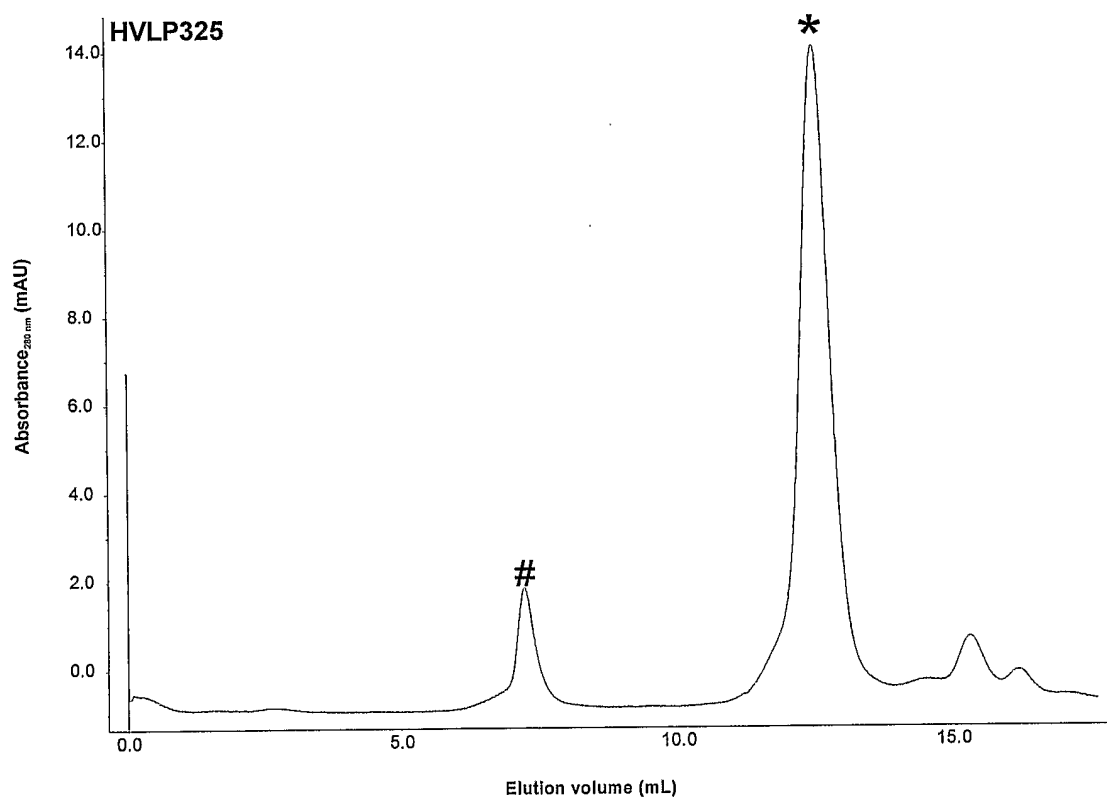
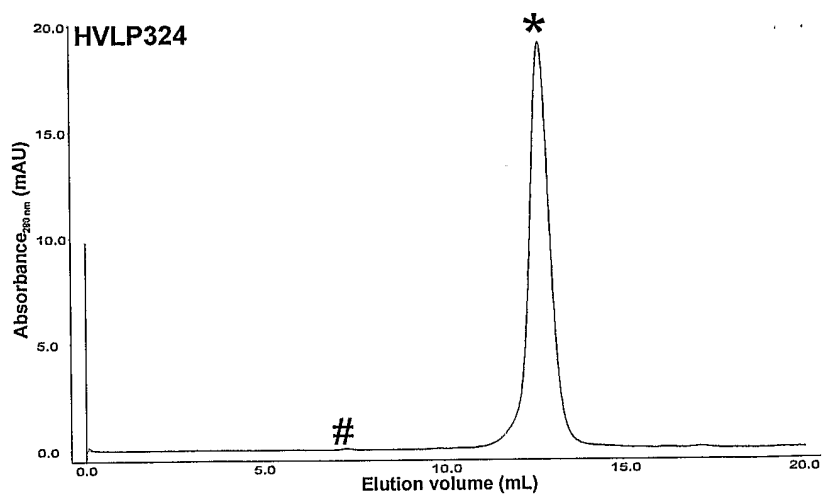


Figure 5

A. V _H III subgroup	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
(i) L8	10 20	30 32	40	50	60 70	80 85 86	100
HVLP333(Jc1)	EIVMTQSPATLSLSPGERATLSC	RASQSVSS--YLA	WYQKPGQASRLIIY	ΔASNRAT	GIPARFSGSGSGTDFTLTISRLEPEDFAVYCC	QYGGSSP--AT	FGQGTKVTVL
HVLP368(Jc1)						Y.	
HVLP368(Jc1)	..L.....	S.	P.		S.....	Y.	..RSNW--P.
HVLP371(Jc1)	..L.....Q.	..R..GT--	P.			Y.	..S.....K.
HVLP381(Jc1)	..L.....		P.	G..S...	..D.....	Y.	..S.....K.
HVLP384(Jc1)	..L.....	..R..Y--H.	PK.		S.....	Y.	..RSNW--P.
HVLP3104(Jc1)			P.	S	S.....	S.I.Y.	..RSGW--K.
HVLP3110(Jc1)	..L.....	T--	P.		S.....	Y.	..H.T.--K.
HVLP356(Jc4)	..L.....		P.		S.....	Y.	..RSNW--T.
HVLP325(Jc4)	..L.....T.	GR--	..R...P...VF	.T...P.V.	..R...L...	S...S...F.	..RS.G--L.
HVLP349(Jc4)	G.....G.	--H.	TP.	..K...	S.....	SGI.Y.	..RSNR--LS
HVLP351(Jc4)	V.....	GT--S.	P.		S.....	Y.	..RYNW--
HVLP389(Jc4)	P.....	--H.	P.	..H...	S.....	Y.	..RSYW--L.
HVLP3109(Jc4)	..L.....	..I.G--S.	..T...P...	GT.	S.....	S...S...F.	..R.DW--S.
HVLP3109(Jc4)	..L.....T.	GR--	..R...P...VF	.T...P.V.	..R...L...	S...S...F.	..KRS.G--L.
HVLP393(Jc2)	..L.....	GR--S.V	..H...P...		S.....	Y.	..RSNW.HMY.
(ii) A27							
HVLP334(Jc1)	.TTL...G.....	S-S.	P.	G..S...	..D.....	G...V...Y.	..--E.
HVLP335(Jc1)		S-S.	P.	GT.	..D.....	H...N...Y.	
HVLP3106(Jc1)	.TTL...G.....	..H.....NV.	F...PP.	G..S...	..D.....	E.....Y.	R..DK...K.
HVLP382(Jc1)	G.....D.	DI.T--	P.	S..R...	..D.....	Y.	..S.....K.
(iii) L2							
HVLP383(Jc2)	.TTL...G.....	K--N.	..K...SP.	H SI.T...	.V.....G.	Y.	...N...Q.
HVLP3103(Jc2)	.TTL...G.....	RN--N.	..R...P...	G..T...	S.QV..V...	Y.	..YTT--K.
HVLP323(Jc1)	.TTL.....V.....	N.--N.	P.	G..T...	E.....S.QS.	Y.	..NNW.--HS
(iv) L16							
HVLP394(Jc2)	.TTL.....V.....F.	N--N.	P.	G..S.T.-	..D...A.....	Y.	...DF.--
B. V _H I subgroup							
(i) O2/O12							
HVLP320(Jc1)	D.QL...SS..A.V.D.V.IT.	I.T--.N	K.PK.	A..SLES	.V.S...R.....V.S.Q.	...T.F.	..GYTT--
HVLP324(Jc1)	D.Q.....SS..A.V.D.V.IT.	I.T--.N	K.PK.	F A..TLQS	.V.S.....N.Q.	...T.Y.	..SY.T.--
HVLP397(Jc1)	SS..A.V.D.V.IT.	I.....N	F...K.PK.	A..SLQS	.V.S.....S.Q.	...T.Y.	..SYTT--
HVLP322(NF)	D.Q.....SS..A.V.D.V.IP.	I.....N	K.PK.	A..SLQS	.V.S.....S.Q.	...T.Y.	..SY.T.--
HVLP330(NF)	D.Q.....PS..AFV.D.V.IT.	E..GN--S.S	..L...KNE--VS	GG.FLQS	.VS.....A..L...TG.RLD.S.T.Y.	..SDAV--	..H...S..
(ii) A30							
HVLP342(Jc1)	D.Q.....SS..A.V.D.V.IT.	DIRT--D.D	F..R..R.PHR.	G..SLQG	.V.S.....E.....G.Q.	...T.Y.	L.HHTY.--
HVLP350(Jc1)	D.Q.....SS..A.V.D.V.IT.	DIRN--D.G	M.PKR.	G..RLQS	.V.S.....E.....S.Q.	...T.Y.	..SY.T.--
C. V _H I subgroup							
(i) 1b							
HVLP389(JL3b)	QS.V..P.S-V.AA..Q.V.I..	SG.SYNIgensVS	...L..T.PK.	GNDK.PS	...D....K...SA..G.TG.QTG.E.D.Y.	GTWD.NLRASV	..G.....

Figure 6

**Figure 7A**

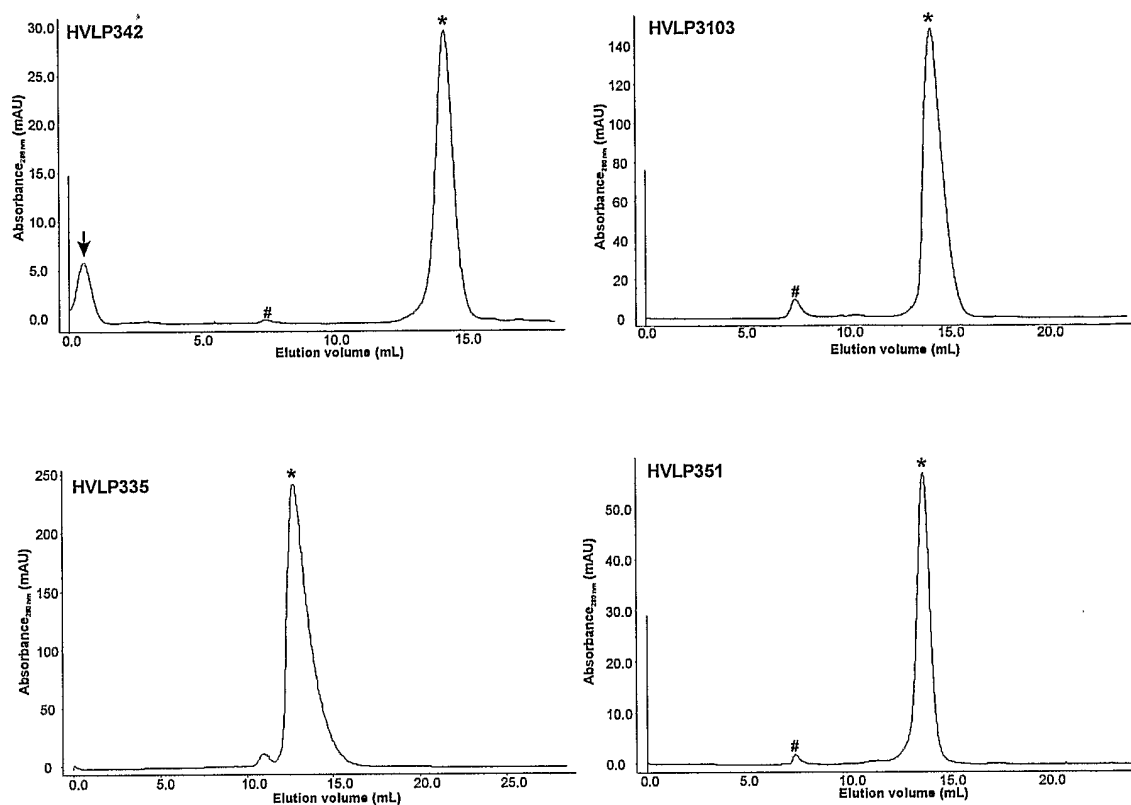


Figure 7B

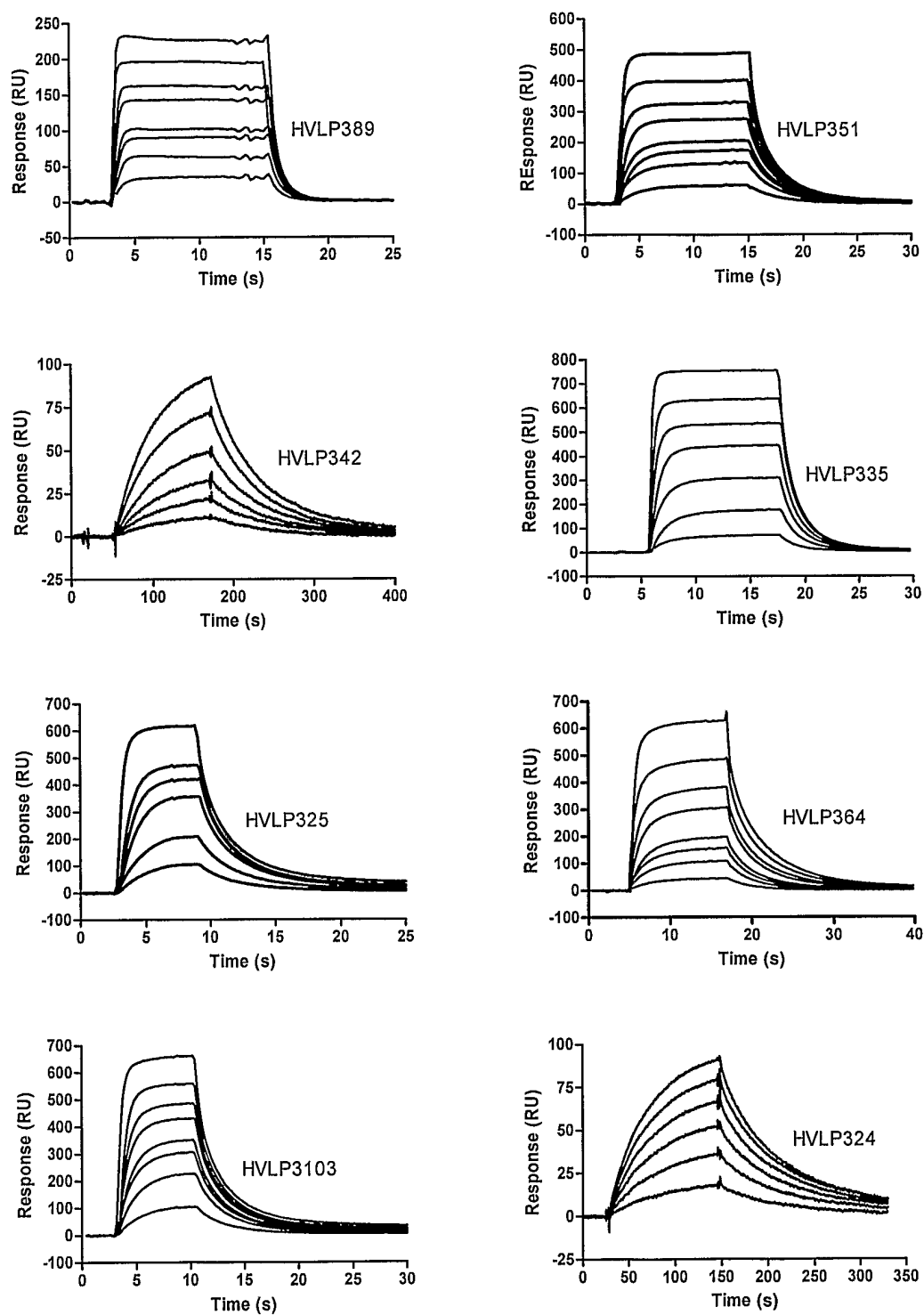
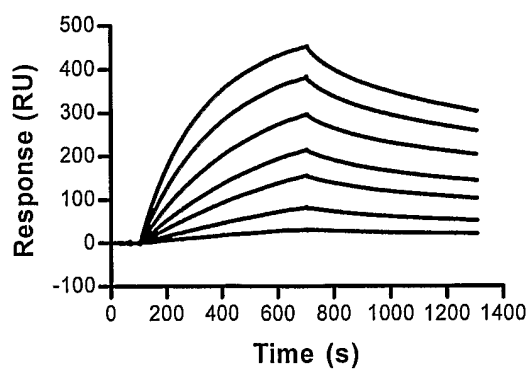


Figure 8

(A)



(B)

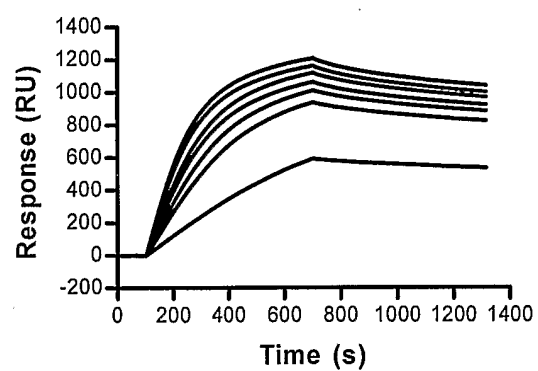


Figure 9

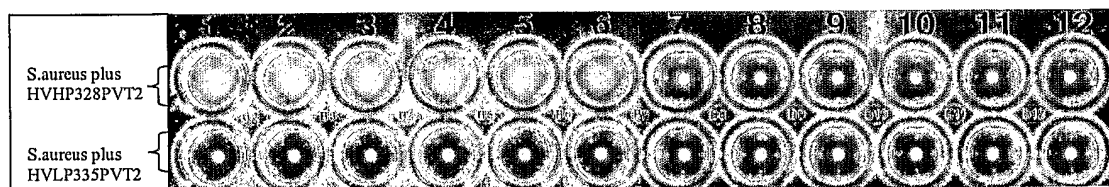


FIGURE 10

A. CLASSIFICATION OF SUBJECT MATTER

IPC: *C12N 15/13* (2006.01) , *C07K 1/00* (2006.01) , *A61K 39/395* (2006.01) , *C07K 14/52* (2006.01) ,
C07K 14/705 (2006.01) , *C07K 14/725* (2006.01) (more IPCs on the last page)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: *C12N 15/13* (2006.01) , *C07K 1/00* (2006.01) , *A61K 39/395* (2006.01) , *C07K 14/52* (2006.01) ,
C07K 14/705 (2006.01) , *C07K 14/725* (2006.01) (more IPCs on the last page)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Databases searched: DELPHION, MEDLINE, BIOSIS, CAlus and SCOPUS (search terms: phage, library, plaque(s), size, large, soluble, expression, stable, selection)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	TO R ET AL: "Isolation of Monomeric Human V _H S by a Phage Selection" THE JOURNAL OF BIOLOGICAL CHEMISTRY. December 16, 2005. Vol. 280, No. 50, pages 41395-41403 whole document	1-23
X	O'NEAL K ET AL: "Thermodynamic Genetics of the Folding of the B1 Immunoglobulin-Binding Domain From Streptococcal Protein G" PROTEINS: STRUCTURE, FUNCTION, AND GENETICS. 1995. Vol. 21, pages 11-21 whole document	1-23
A	JESPERS L ET AL: "Aggregation-resistant domain antibodies selected on phage by heat denaturation" NATURE BIOTECHNOLOGY. September 2004. Vol. 22, No. 9, pages 1161-1165	

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 June 2006 (30-06-2006)

Date of mailing of the international search report

13 July 2006 (13-07-2006)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001(819)953-2476

Authorized officer

Kristoffer Wilde (819) 953-0551

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2006/000451

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JUNG S ET AL: "Selection for Improved Protein Stability by Phage Display" JOURNAL OF MOLECULAR BIOLOGY. 1999. Vol. 294, No. 1, pages 163-180	

Continuation of Box No. III

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

Invention 1 - Claims 1-23 are directed to a method of identifying target polypeptides, comprising infecting a bacterial lawn with a phage display library and identifying the larger than average plaques.

Inventions 2-48 - Claims 24-79, 96 (partly 97-99) are directed to various VH and VL antibody fragments having an amino acid sequence that is selected from the group consisting of SEQ ID NO:8-54 (each sequence represents a separate invention); nucleic acids; multimers; and pharmaceutical compositions, recombinant vectors and host cells comprising said antibody fragments.

Invention 49 - Claims 80, 81 (partly 97-99) are directed to a polypeptide defined in terms of a process used to identify the polypeptide, wherein the polypeptide is not distinguished from other known polypeptides and pharmaceutical compositions, recombinant vectors and host cells comprising said polypeptides.

Invention 50 - Claims 82-88 are directed to display libraries.

Invention 51 - Claims 89-95 are directed to methods of producing polypeptides.

The technical feature of the method of claim 1 resides in the step of identifying phage which form larger than average plaques on a bacterial lawn. Neither the same nor a corresponding technical feature is present in any of the inventions 2-51. Thus, unity of invention is lacking between invention 1 and inventions 2-51. Furthermore, polypeptides encoding VH and VL domains are well known in the art. Thus, the technical feature linking the claimed polypeptides consisting of SEQ ID NO:8-54 is not special because the functional and structural similarity between the claimed molecules cannot form the contribution that the group of inventions as a whole makes over the prior art. Therefore, unity of invention is lacking between inventions 1-51.

C07K 16/00 (2006.01) , *C12N 1/11* (2006.01) , *C12N 15/70* (2006.01) , *C12Q 1/70* (2006.01) ,
C40B 30/00 (2006.01) , *C40B 40/02* (2006.01) , *C40B 40/10* (2006.01) , *G01N 33/50* (2006.01)

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

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

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

2. ☐ Claim Nos. :
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

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

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

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

See extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. : 1-23

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

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

☐ No protest accompanied the payment of additional search fees.