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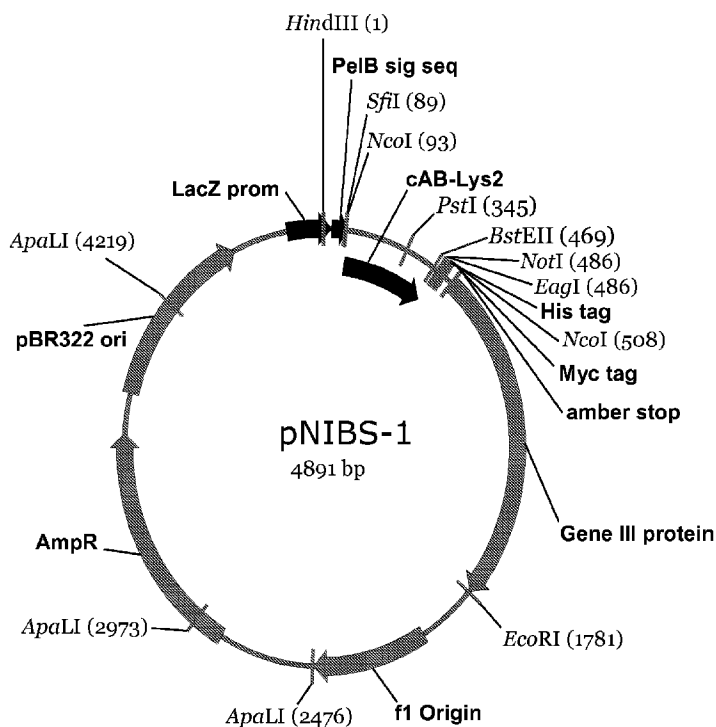
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(54) Title: INFLUENZA VIRUS ANTIBODY COMPOSITIONS



(57) Abrégé/Abstract:

The present invention relates to unique CDR3 amino acid sequences, and to antibodies comprising said sequences. Also provided are therapeutic applications of said antibodies for use in treating or preventing influenza infection, diagnostic applications of said antibodies for use in detecting influenza virus, potency testing of influenza virus vaccines, and screening applications of said antibodies for use in designing a universal influenza vaccine.

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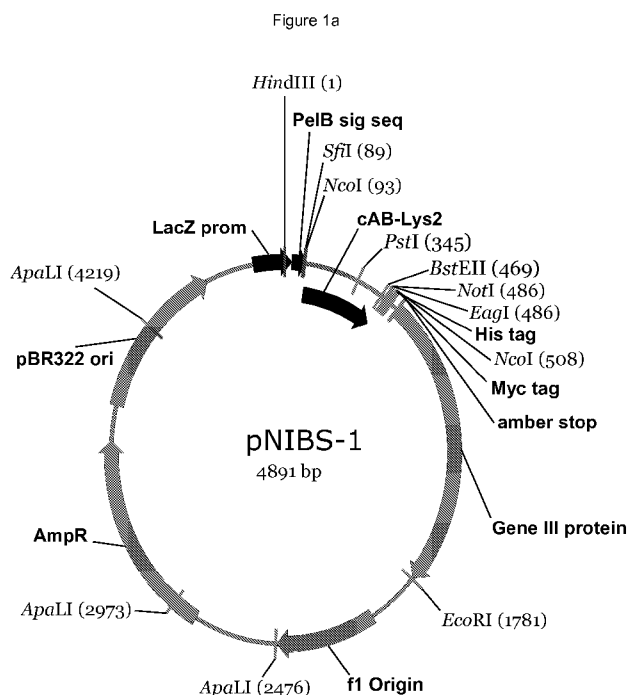
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(54) Title: INFLUENZA VIRUS ANTIBODY COMPOSITIONS



(57) **Abstract:** The present invention relates to unique CDR3 amino acid sequences, and to antibodies comprising said sequences. Also provided are therapeutic applications of said antibodies for use in treating or preventing influenza infection, diagnostic applications of said antibodies for use in detecting influenza virus, potency testing of influenza virus vaccines, and screening applications of said antibodies for use in designing a universal influenza vaccine.

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Influenza virus antibody compositions

The present invention relates to antibody compositions, and to the use thereof for preventing or treating influenza infection. The present invention also relates to the use of said antibody compositions for assessing cross-reactivity and haemagglutinin antigen content of influenza vaccines in vaccine potency assays.

The emergence of highly pathogenic influenza viral strains poses a serious threat to public health on a global scale. Although vaccines are the mainstay of infection, controlling the gap between the emergence of new viral strains and a clinically approved vaccine presents a serious concern particularly as resistance to existing anti-viral drugs like oseltamavirTM and zanamivirTM is increasing.

One current approach is passive immunotherapy with serum from convalescent patients. This approach, however, has major limitations to population-wide implementation, such as prohibitive cost, high risk of toxicity, uncertain dosing, and difficulty in administration.

Another approach is to stockpile recombinant monoclonal antibodies for immediate deployment on emergence of a new pandemic threat. By way of example, recent reports have described the isolation of functional human antibodies from survivors of the 1918 influenza pandemic (Kashyap *et al.*, 2008), from seasonal vaccinated individuals (Ekiert *et al.*, 2009), and from naive human antibody libraries (Sui *et al.*, 2009). However, although conventional monoclonal antibodies are generally regarded as a successful class of drug, their high cost and complexity is a major limitation. Also, in view of the rapidly changing epidemiology of influenza virus, such monoclonal antibody approach would necessitate the provision of a monoclonal antibody that demonstrates reactivity across different viral subtypes. This problem currently presents a major challenge for monoclonal antibody technology.

Pandemic influenza occurs when a new virus emerges and infects a global human population that has little or no pre-existing immunity. The most recent H1N1 2009 pandemic, although a considerable economic burden, did not result in the same rates of mortality as previous pandemics like the 1918 'Spanish flu' pandemic, which resulted in an estimated 50 million deaths worldwide. Highly pathogenic avian H5N1 influenza viruses have demonstrated mortality rates of 60% in infected humans and clearly highlight the risk that pandemic influenza presents.

- Current treatment options fall into two categories; either anti-viral drugs or vaccines. Current anti-viral drugs target either the viral neuraminidase (e.g. Oseltamavir™) or the M2 ion channel (e.g. Amantadine™); both of which may become ineffective in time due to increasing resistance. Vaccines are the corner stone of infection control, but have limitations in that it
- 5 takes significant time to develop a vaccine upon emergence of a new viral strain, they do not act quickly, they are not effective in all population groups (e.g. immune-compromised patients), protection is largely strain-specific, and there is limited world-wide production capacity. Thus, there is clearly a need for additional treatment options.
- 10 Another problem in the art is a need for the standardisation and control of both seasonal and pandemic influenza vaccines. Currently, the single radial immunodiffusion assay (SRD) is considered a 'gold-standard' test to determine the content of HA antigen in influenza vaccines. This assay requires the development of reference antiserum to each new viral strain, which may typically add 2-3 months to the vaccine development pipeline. Naturally,
- 15 the elimination of this delay would be invaluable in mounting a rapid response to a pandemic emergency. To this end, there is a need for a 'universal antibody' reagent, which comprises cross-reactive antibodies that may be used in a SRD assay or in other related assay formats based on for example, ELISA, Surface Plasmon Resonance (SPR) or mass spectrometry.
- 20 There is therefore an urgent need for alternative influenza virus treatment options, for a robust influenza virus detection assay, and/ or for an influenza virus antigen-based vaccine potency assay.

Figures 1a and 1b of the present invention illustrate pNIBS-1 generated by cloning a

25 Hind111/ EcoR1 fragment into the standard phagemid vector pUC119.

Figure 2 of the present invention illustrates phage antibody library selections designed to recover both H1 specific antibodies and also H1/ H5 cross reactive antibodies.

30 Figure 3 of the present invention illustrates a primary ELISA screen using unpurified soluble VHH antibodies in a 96 well ELISA format.

The present invention addresses one or more of the above problems by providing an antibody as described herein.

A principal feature of the antibodies of the present invention is that they possess a complementary determining region (CDR), for example a CDR3 amino acid sequence, from an alpaca V_{HH} single-domain antibody.

- 5 In one embodiment, the present invention comprises or consists of a single-domain camelid (e.g. an alpaca single-domain antibody) - said antibodies have a very simple and robust protein scaffold, and demonstrate several advantages over conventional monoclonal antibodies, such as: (i) size – single-domain antibodies are approximately 1/10th the size of monoclonal antibodies. As a result, said antibodies have the ability to access buried or
- 10 hidden epitopes that would normally be silent to the human immune system (eg. accessing more conserved epitopes buried in the haemagglutinin stalk region); (ii) production at

significantly lower cost than conventional monoclonal antibodies; (iii) simpler methods to engineer into bi- or multi-specific antibodies; (iv) longer CDR3 loops than conventional antibodies, which facilitates more adept accessing of clefts and grooves on antigenic surfaces; and/ or (v) high structural stability.

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In one embodiment, an antibody of the invention binds to a H1 influenza virus (e.g. a H1N1 influenza virus), for example a pandemic H1 (e.g. H1N1) influenza virus. In one embodiment, the antibody binds to strain A/California/06/09 and/ or A/California/07/2009 and/ or A/Brisbane/59/2007.

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In one embodiment an antibody of the invention has a CDR3, which comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 1-19; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 1-19. In this regard, SEQ ID NOs 1-19 are defined according to the conventional Kabat nomenclature.

15

In another embodiment an antibody of the invention has a CDR3, which comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 20-38; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 20-38. In this regard, SEQ ID NOs 20-38 are defined according to the conventional international ImMunoGeneTics information system for immunoglobulins or antibodies (IMGT) nomenclature.

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An antibody of the invention may further include a CDR2, wherein said CDR2 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 39-57; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 39-57. In this regard, SEQ ID NOs 39-57 are defined according to conventional Kabat nomenclature. Alternatively, said CDR2 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 58-76; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 58-76. In this regard, SEQ ID NOs 58-78 are defined according to conventional IMGT nomenclature.

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Simply by way of illustration, an antibody of the invention may comprise CDR 3 and CDR2 combinations as hereinbefore defined based on the following combinations of SEQ ID NOs:

- 1 plus 39 or 58; 20 plus 39 or 58;
- 2 plus 40 or 59; 21 plus 40 or 59;
- 5 3 plus 41 or 60; 22 plus 41 or 60;
- 4 plus 42 or 61; 23 plus 42 or 61;
- 5 plus 43 or 62; 24 plus 43 or 62;
- 6 plus 44 or 63; 25 plus 44 or 63;
- 7 plus 45 or 64; 26 plus 45 or 64;
- 10 8 plus 46 or 65; 27 plus 46 or 65;
- 9 plus 47 or 66; 28 plus 47 or 66;
- 10 plus 48 or 67; 29 plus 48 or 67;
- 11 plus 49 or 68; 30 plus 49 or 68;
- 12 plus 50 or 69; 31 plus 50 or 69;
- 15 13 plus 51 or 70; 32 plus 51 or 70;
- 14 plus 52 or 71; 33 plus 52 or 71;
- 15 plus 53 or 72; 34 plus 53 or 72;
- 16 plus 54 or 73; 35 plus 54 or 73;
- 17 plus 55 or 74; 36 plus 55 or 74;
- 20 18 plus 56 or 75; 37 plus 56 or 75;
- 19 plus 57 or 76; 38 plus 57 or 76.

- In addition to the above-mentioned CDR3 feature (and optionally in addition to the above-mentioned CDR2 feature), an antibody of the invention may further include a CDR1, wherein
- 25 said CDR1 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 77-95; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 77-95. In this regard, SEQ ID NOs 77-95 are defined according to conventional Kabat nomenclature.
 - 30 Alternatively, said CDR1 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 96-114; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 96-114. In this regard, SEQ ID NOs 96-114 are defined according to conventional IMGT
 - 35 nomenclature.

Simply by way of illustration, an antibody of the invention may comprise CDR3 and CDR1 combinations as hereinbefore defined based on the following combinations of SEQ ID NOs:

- 1 plus 77 or 96; 20 plus 77 or 96;
- 2 plus 78 or 97; 21 plus 78 or 97;
- 5 3 plus 79 or 98; 22 plus 79 or 98;
- 4 plus 80 or 99; 23 plus 80 or 99;
- 5 plus 81 or 100; 24 plus 81 or 100;
- 6 plus 82 or 101; 25 plus 82 or 101;
- 7 plus 83 or 102; 26 plus 83 or 102;
- 10 8 plus 84 or 103; 27 plus 84 or 103;
- 9 plus 85 or 104; 28 plus 85 or 104;
- 10 plus 86 or 105; 29 plus 86 or 105;
- 11 plus 87 or 106; 30 plus 87 or 106;
- 12 plus 88 or 107; 31 plus 88 or 107;
- 15 13 plus 89 or 108; 32 plus 89 or 108;
- 14 plus 90 or 109; 33 plus 90 or 109;
- 15 plus 91 or 110; 34 plus 91 or 110;
- 16 plus 92 or 111; 35 plus 92 or 111;
- 17 plus 93 or 112; 36 plus 93 or 112;
- 20 18 plus 94 or 113; 37 plus 94 or 113;
- 19 plus 95 or 114; 38 plus 95 or 114.

Again, simply by way of illustration, an antibody of the invention may comprise CDR3 plus CDR2 and CDR1 combinations as hereinbefore defined based on the following combinations

- 25 of SEQ ID NOs:
- 1 plus 39 plus 77; 20 plus 58 plus 96;
- 2 plus 40 plus 78; 21 plus 59 plus 97;
- 3 plus 41 plus 79; 22 plus 60 plus 98;
- 4 plus 42 plus 80; 23 plus 61 plus 99;
- 30 5 plus 43 plus 81; 24 plus 62 plus 100;
- 6 plus 44 plus 82; 25 plus 63 plus 101;
- 7 plus 45 plus 83; 26 plus 64 plus 102;
- 8 plus 46 plus 84; 27 plus 65 plus 103;
- 9 plus 47 plus 85; 28 plus 66 plus 104;
- 35 10 plus 48 plus 86; 29 plus 67 plus 105;
- 11 plus 49 plus 87; 30 plus 68 plus 106;
- 12 plus 50 plus 88; 31 plus 69 plus 107;

- 13 plus 51 plus 89; 32 plus 70 plus 108;
 14 plus 52 plus 90; 33 plus 71 plus 109;
 15 plus 53 plus 91; 34 plus 72 plus 110;
 16 plus 54 plus 92; 35 plus 73 plus 111;
 5 17 plus 55 plus 93; 36 plus 74 plus 112;
 18 plus 56 plus 94; 37 plus 75 plus 113;
 19 plus 57 plus 95; 38 plus 76 plus 114.

In one embodiment, an antibody of the invention comprises an amino acid sequence having
 10 at least 80% sequence identity to an amino acid sequence selected from the group
 consisting of SEQ ID NOs: 115-133. In a related embodiment, said antibody retains the
 precise CDR3 amino acid sequence as herein before defined, and optionally the precise
 CDR1 amino acid sequence and/ or precise CDR2 amino acid sequence as hereinbefore
 defined.

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In a related second aspect, an antibody of the invention binds not only to H1 influenza virus
 (as hereinbefore described) but also to a H5 influenza virus (e.g. H5N1 or H5N3), for
 example a pandemic H5 influenza virus. In one embodiment, the antibody binds to strain
 A/Vietnam/1203/2004 and/ or A/Vietnam/1194/2004 and/ or A/Vietnam/119/2004 and/ or
 20 A/HongKong/213/2003 and/ or A/turkey/Turkey/1/2005 and/ or A/Indonesia/05/2005 and/ or
 A/Duck/Singapore-Q/119-3/97. In one embodiment of this aspect, an antibody of the
 invention has a CDR3, which comprises an amino acid sequence selected from the group
 consisting of: SEQ ID NO: 3-4, 6-7, 10, 13 or 15; or a variant amino acid sequence thereof,
 wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid
 25 substitution, deletion or insertion when compared with the corresponding amino acid
 sequence of SEQ ID NO: 3-4, 6-7, 10, 13 or 15. Alternatively, said CDR3 comprises an
 amino acid sequence selected from the group consisting of: SEQ ID NO: 22-23, 25-26, 29,
 32 or 34; or a variant amino acid sequence thereof, wherein said variant amino acid
 sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion
 30 when compared with the corresponding amino acid sequence of SEQ ID NO: 22-23, 25-26,
 29, 32 or 34.

An antibody of the second aspect may further include a CDR2, wherein said CDR2
 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 41-
 35 42, 44-45, 48, 51 or 53; or a variant amino acid sequence thereof, wherein said variant
 amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or
 insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 41-

42, 44-45, 48, 51 or 53. Alternatively, said CDR2 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 60-61, 63-64, 67, 70 or 72; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 60-61, 63-64, 67, 70 or 72.

Simply by way of illustration, an antibody of the second aspect may comprise CDR 3 and CDR2 combinations as hereinbefore defined for the second aspect based on the following combinations of SEQ ID NOs:

- 10 3 plus 41 or 60; 22 plus 41 or 60;
- 4 plus 42 or 61; 23 plus 42 or 61;
- 6 plus 44 or 63; 25 plus 44 or 63;
- 7 plus 45 or 64; 26 plus 45 or 64;
- 10 plus 48 or 67; 29 plus 48 or 67;
- 15 13 plus 51 or 70; 32 plus 51 or 70;
- 15 plus 53 or 72; 34 plus 53 or 72.

An antibody of the second aspect, in addition to the above-mentioned CDR3 feature (and optionally in addition to the above-mentioned CDR2 feature), may further include a CDR1, wherein said CDR1 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 79-80, 82-83, 86, 89 or 91; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 79-80, 82-83, 86, 89 or 91. Alternatively, said CDR1 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 98-99, 101-102, 105, 108 or 110; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 98-99, 101-102, 105, 108 or 110.

Simply by way of illustration, an antibody of the second aspect may comprise CDR3 and CDR1 combinations as hereinbefore defined for the second aspect based on the following combinations of SEQ ID NOs:

- 3 plus 79 or 98; 22 plus 79 or 98;
- 35 4 plus 80 or 99; 23 plus 80 or 99;
- 6 plus 82 or 101; 25 plus 82 or 101;
- 7 plus 83 or 102; 26 plus 83 or 102;

10 plus 86 or 105; 29 plus 86 or 105;
 13 plus 89 or 108; 32 plus 89 or 108;
 15 plus 91 or 110; 34 plus 91 or 110.

5 Again, simply by way of illustration, an antibody of the second aspect may comprise CDR3 plus CDR2 and CDR1 combinations as hereinbefore defined for the second aspect based on the following combinations of SEQ ID NOs:

3 plus 41 plus 79; 22 plus 60 plus 98;
 4 plus 42 plus 80; 23 plus 61 plus 99;
 10 6 plus 44 plus 82; 25 plus 63 plus 101;
 7 plus 45 plus 83; 26 plus 64 plus 102;
 10 plus 48 plus 86; 29 plus 67 plus 105;
 13 plus 51 plus 89; 32 plus 70 plus 108
 15 plus 53 plus 91; 34 plus 72 plus 110.

15

An antibody of the second aspect may comprise an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 117-118, 120-121, 124, 127 or 129. In a related embodiment, said antibody retains the precise CDR3 amino acid sequence as herein before defined, and optionally the
 20 precise CDR1 amino acid sequence and/ or precise CDR2 amino acid sequence as hereinbefore defined.

In a related third aspect, an antibody of the invention binds not only to a H1 influenza virus and to a H5 influenza virus (as hereinbefore described), but also to 1) a H2 influenza virus
 25 (e.g. H2N2 or H2N3) such as to a pandemic H2 influenza virus (e.g. H2N2 or H2N3), and to 2) a H9 influenza virus (e.g. H9N2) such as to a pandemic H9 influenza virus (e.g. H9N2). For example, the antibody binds to H2 influenza virus strain A/Japan/305/1957 and/ or to A/Mallard/Eng/727/06. For example, the antibody binds to H9 influenza virus strain A/HongKong/1073/99. In one embodiment of this aspect, an antibody of the invention has a
 30 CDR3, which comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 3, 4, 7 or 10; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 3, 4, 7 or 10. Alternatively, said CDR3 comprises an amino acid sequence selected from the
 35 group consisting of: SEQ ID NO: 22, 23, 26 or 29; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid

substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 22, 23, 26 or 29.

- An antibody of the third aspect may further include a CDR2, wherein said CDR2 comprises
- 5 an amino acid sequence selected from the group consisting of: SEQ ID NO: 41, 42, 45 or 48; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 41, 42, 45 or 48. Alternatively, said CDR2 comprises an amino acid sequence selected from the group
- 10 consisting of: SEQ ID NO: 60, 61, 64 or 67; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 60, 61, 64 or 67.
- 15 Simply by way of illustration, an antibody of the third aspect may comprise CDR 3 and CDR2 combinations as hereinbefore defined for the third aspect based on the following combinations of SEQ ID NOs:
- 3 plus 41 or 60; 22 plus 41 or 60;
- 4 plus 42 or 61; 23 plus 42 or 61;
- 20 7 plus 45 or 64; 26 plus 45 or 64;
- 10 plus 48 or 67; 29 plus 48 or 67.

- An antibody of the third aspect, in addition to the above-mentioned CDR3 feature (and optionally in addition to the above-mentioned CDR2 feature), may further include a CDR1,
- 25 wherein said CDR1 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 79, 80, 83 or 86; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 79, 80, 83 or 86. Alternatively, said CDR1 comprises an amino acid sequence selected
- 30 from the group consisting of: SEQ ID NO: 98, 99, 102 or 105; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 98, 99, 102 or 105.

- 35 Simply by way of illustration, an antibody of the third aspect may comprise CDR3 and CDR1 combinations as hereinbefore defined for the third aspect based on the following combinations of SEQ ID NOs:

3 plus 79 or 98; 22 plus 79 or 98;
 4 plus 80 or 99; 23 plus 80 or 99;
 7 plus 83 or 102; 26 plus 83 or 102;
 10 plus 86 or 105; 29 plus 86 or 105.

5

Again, simply by way of illustration, an antibody of the third aspect may comprise CDR3 plus CDR2 and CDR1 combinations as hereinbefore defined for the third aspect based on the following combinations of SEQ ID NOs:

3 plus 41 plus 79; 22 plus 60 plus 98;
 10 4 plus 42 plus 80; 23 plus 61 plus 99;
 7 plus 45 plus 83; 26 plus 64 plus 102;
 10 plus 48 plus 86; 29 plus 67 plus 105;
 15 plus 53 plus 91; 34 plus 72 plus 110.

15 An antibody of the third aspect may comprise an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 117, 118, 121 or 124. In a related embodiment, said antibody retains the precise CDR3 amino acid sequence as herein before defined, and optionally the precise CDR1 amino acid sequence and/ or precise CDR2 amino acid sequence as hereinbefore defined.

20

In a related fourth aspect, an antibody of the invention binds not only to a H1 influenza virus and to a H5 influenza virus (as hereinbefore described), but also to a H2 influenza virus (e.g. H2N2 or H2N3) for example to a pandemic H2 influenza virus (e.g. H2N2 or H2N3). By way of a specific H2 influenza virus example, the antibody binds to strain A/Japan/305/1957 and/
 25 or to A/Mallard/Eng/727/06. In one embodiment of this aspect, an antibody of the invention has a CDR3, which comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 6 or 15; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 6 or
 30 15. Alternatively, said CDR3 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 25 or 34; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 25 or 34.

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An antibody of the fourth aspect may further include a CDR2, wherein said CDR2 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 44 or 53; or a

variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 44 or 53. Alternatively, said CDR2 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 63 or 72; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 63 or 72.

Simply by way of illustration, an antibody of the fourth aspect may comprise CDR3 and CDR2 combinations as hereinbefore defined for the fourth aspect based on the following combinations of SEQ ID NOs:

6 plus 44 or 63; 25 plus 44 or 63;
15 plus 53 or 61; 34 plus 53 or 61.

An antibody of the fourth aspect, in addition to the above-mentioned CDR3 feature (and optionally in addition to the above-mentioned CDR2 feature), may further include a CDR1, wherein said CDR1 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 82 or 91; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 62 or 91. Alternatively, said CDR1 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 101 or 110; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 101 or 110.

Simply by way of illustration, an antibody of the fourth aspect may comprise CDR3 and CDR1 combinations as hereinbefore defined for the fourth aspect based on the following combinations of SEQ ID NOs:

6 plus 82 or 101; 25 plus 82 or 101;
15 plus 91 or 110; 34 plus 91 or 110.

Again, simply by way of illustration, an antibody of the fourth aspect may comprise CDR3 plus CDR2 and CDR1 combinations as hereinbefore defined for the fourth aspect based on the following combinations of SEQ ID NOs:

6 plus 44 plus 82; 25 plus 63 plus 101;
15 plus 53 plus 91; 34 plus 72 plus 110.

An antibody of the fourth aspect may comprise an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 120 or 129. In a related embodiment, said antibody retains the precise CDR3 amino acid sequence as herein before defined, and optionally the precise CDR1 amino acid sequence and/ or precise CDR2 amino acid sequence as hereinbefore defined.

In a related fifth aspect, an antibody of the invention binds not only to a H1 influenza virus and to a H5 influenza virus (as hereinbefore described), but also to a H9 influenza virus (e.g. H9N2) for example to a pandemic H9 influenza virus (e.g. H9N2). By way of a specific H9 influenza virus example, the antibody binds to strain A/HongKong/1073/99. In one embodiment of this aspect, an antibody of the invention has a CDR3, which comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 13; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 13. Alternatively, said CDR3 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 32; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 32.

An antibody of the fifth aspect may further include a CDR2, wherein said CDR2 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 51 or 70; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 51 or 70.

Simply by way of illustration, an antibody of the fifth aspect may comprise CDR3 and CDR2 combinations as hereinbefore defined for the fifth aspect based on the following combinations of SEQ ID NOs: 13 plus 51 or 70; 32 plus 51 or 70.

An antibody of the fifth aspect, in addition to the above-mention CDR3 feature (and optionally in addition to the above-mentioned CDR2 feature), may further include a CDR1, wherein said CDR1 comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 89 or 108; or a variant amino acid sequence thereof, wherein said variant amino acid sequence possesses a maximum of 1 or 2 amino acid substitution, deletion or

insertion when compared with the corresponding amino acid sequence of SEQ ID NO: 89 or 108.

Simply by way of illustration, an antibody of the fifth aspect may comprise CDR3 and CDR1 combinations as hereinbefore defined for the fifth aspect based on the following combinations of SEQ ID NOs: 13 plus 89 or 108; 32 plus 89 or 108.

Again, simply by way of illustration, an antibody of the fifth aspect may comprise CDR3 plus CDR2 and CDR1 combinations as hereinbefore defined for the fifth aspect based on the following combinations of SEQ ID NOs: 13 plus 51 plus 89; 32 plus 70 plus 108.

An antibody of the fifth aspect may comprise an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 127. In a related embodiment, said antibody retains the precise CDR3 amino acid sequence as herein before defined, and optionally the precise CDR1 amino acid sequence and/ or precise CDR2 amino acid sequence as hereinbefore defined.

In one embodiment, the antibodies of the invention bind and preferably neutralise all Group I haemagglutinin influenza viruses (e.g. H1, H2, H5, H6, H11, H13, H16, H9, H8 and/ or H12 influenza viruses). In one embodiment, the antibodies of the invention bind and preferably neutralise A/California/07/09 (H1N1) and/ or A/Vietnam/1194/04 (H5N1).

In one embodiment, the antibody may be humanized. Examples of conventional humanization techniques are described in Jones P.T. *et al.* (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse; Nature, 321 (6069): pp. 522-525. In one embodiment, the CDR3 V_{HH} amino acid sequence of the present invention may be inserted (e.g. replace) a corresponding CDR3 V_{HH} amino acid sequence present on a human antibody.

Alternatively, the entire V_{HH} amino acid sequence of an antibody of the present invention may be sequence aligned with a corresponding human antibody and amino acid differences mapped. Following said mapping, amino acid differences identified on the antibody sequence of the present invention present may then be modified to mimic the corresponding human sequence. In this regard, CDR regions are preferably not modified. For example, differences identified in the FR regions of an antibody of the present invention, optionally subject to 'Hallmark' residues (Vincke *et al.*, 2008, J. Biol. Chem., 284(5), pp. 3273-3284), may be

modified to mimic the corresponding human sequence. Simply for illustrative purposes, one humanization protocol is described by way of Example 10.

5 Purely for illustrative purposes, a humanized antibody of the invention may comprise an amino acid sequence having at least 80% sequence identity to a human VH antibody, for example a human VH3 antibody such as is encoded by the VH3 germline genes DP51 or DP53.

10 In one embodiment, an antibody (optionally humanized) of the present invention consists or comprises a single-domain antibody.

In another embodiment, an antibody (optionally humanized) of the present invention may comprise two or more single-domains as hereinbefore defined. Said single-domains may be the same or different. When first and second (or more) different single-domains are
15 employed, the first single-domain typically has a binding affinity, avidity and/ or specificity that is different from that of the second single-domain.

In one embodiment, an antibody (optionally humanized) of the present invention consists or comprises a bi-specific or multi-specific antibody.

20

According to a further aspect of the present invention, there is provided an antibody as hereinbefore defined, for use in preventing or treating influenza virus infection. In a related aspect, the present invention provides a method comprising administering a prophylactically effective or therapeutically effective amount of an antibody as hereinbefore defined to a
25 patient. These aspects include the use of two or more of said hereinbefore defined antibodies.

Typical patients benefiting from the above 'passive immunotherapy' aspect include 'at risk' individuals on emergence of a new influenza virus strain (eg. a new pandemic strain such as
30 a new Group 1 influenza strain). Examples of such 'at risk' patients include: vaccine non-responders; immune-compromised patients; and health professionals. A particular advantage to the passive immunotherapy approach of the present invention is that it does not add to the growing problems associated with anti-viral drug resistance.

35 In one embodiment, the antibody or antibody combinations of the present invention provide heterosubtypic cross-reactivity and/ or neutralisation against influenza H1, H1 & H5

subtypes, and/ or H1 & H2 & H5 & H9 subtypes, and/ or all Group I haemagglutinin subtypes (e.g. H1, H2, H5, H6, H11, H13, H16, H9, H8 and/ or H12 influenza viruses).

5 A further aspect of the present invention provides method for designing “universal vaccine” to elicit heterosubtypic immune response. Simply for illustrative purposes, suitable protocols are described in Example 14.

A yet further aspect of the present invention provides robust ‘field’ diagnostic assays and reagents. Simply for illustrative purposes, suitable protocols are described in Example 13.

10

A further aspect of the present invention provides cross-reactive antibodies for vaccine potency determination. Simply for illustrative purposes suitable protocols are described in Example 13.

15 **Antibody delivery**

In use, the present invention employs a pharmaceutical composition, comprising the antibody composition of the present invention in a form suitable for parenteral, usually intravenous administration. The purified intact antibodies, or their fragments, are formulated for such delivery. For example, antibody, or its fragment, at a concentration between 5-50 or 15-50 or 20 25-50 g/litre may be formulated in buffer. Examples of suitable buffer components include physiological salts such as sodium citrate and/ or citric acid. Preferred buffers contain 100-200 or 125-175 or approximately 150 (eg. 153) mM physiological salts such as sodium chloride. Preferred buffers maintain the pharmaceutical composition at a pH that is close to the physiological pH of the patient – for example, at a pH of 5.5-6.4, or at a pH of 5.6-6.3, or at a pH 25 of 5.7-6.2, or at a pH of 5.8-6.2. The antibody-containing compositions of the present invention preferably exclude adjuvant(s) as it is undesirable to stimulate an immune response against said antibodies in the patient.

Antibodies of the invention may be formulated for, but not limited to intramuscular, 30 subcutaneous, intranasal or intravenous delivery. Compositions suitable for intramuscular, subcutaneous or intravenous injection include sterile aqueous solutions. Such aqueous solutions should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. The antibody compositions of the present invention may separately be formulated as oral formulations.

35

Compositions suitable for injection may be in the form of solutions, suspensions or dry powders which are dissolved or suspended in a suitable vehicle prior to use.

In preparing solutions of the antibodies or their fragments can be dissolved in the vehicle, the solution being made isotonic if necessary by addition of sodium chloride and sterilised by filtration through a sterile filter using aseptic techniques before filling into suitable sterile vials or ampoules and sealing. Advantageously additives such as buffering, solubilising, stabilising, preservative or bactericidal or suspending and/or local anaesthetic agents may be dissolved in the vehicle.

Dry powders, which are dissolved or suspended in a suitable vehicle prior to use, may be prepared by filling pre-sterilised ingredients into a sterile container using aseptic technique in a sterile area. Alternatively the ingredients may be dissolved into suitable containers using aseptic technique in a sterile area. The product is then freeze dried and the containers are sealed aseptically.

The dosage ranges for administration of the antibodies of the present invention are those to produce the desired therapeutic effect. It will be appreciated that the dosage range required depends on the precise nature of the antibody or composition, the route of administration, the nature of the formulation, the age of the patient, the nature, extent or severity of the patient's condition, contraindications, if any, and the judgement of the attending physician. Variations in these dosage levels can be adjusted using standard empirical routines for optimisation.

Suitable daily dosages are in the range of 5-20mg per kg of body weight. The unit dosage can vary from less than 100mg, but typically will be in the region of 250-2000 mg per dose, which may be administered daily or less frequently (e.g. on alternative days for up to 1 week)

It is also within the scope of the invention to use the antibodies of the invention in therapeutic methods for the prevention or treatment of influenza virus infection in combination with one another, or as an adjunct to, or in conjunction with, other established therapies normally used in the prevention or treatment of influenza virus infection. For example, the antibodies of the present invention may be administered in conjunction with a suitable antiviral (e.g. OseltamavirTM, RimantadineTM, ZanamavirTM, and/or AmantadineTM).

The combination treatment may be carried out in any way as deemed necessary or convenient by the person skilled in the art and for the purpose of this specification, no limitations with regard to the order, amount, repetition or relative amount of the compounds to be used in combination is contemplated.

Definitions Section

An antibody that binds to an influenza virus of interest is one capable of binding said virus with sufficient affinity such that the antibody is useful as a therapeutic agent and/ or vaccine potency reagent. By way of example, an antibody that binds to an influenza virus of interest is one that may bind to an influenza virus with an affinity (K_a) of at least 10^4 M.

A therapeutically effective amount refers to the amount of the antibody, which when administered alone or in combination to a patient for treating or preventing influenza infection, or at least one of the clinical symptoms of influenza infection, is sufficient to affect such treatment or prevention of the disease, or symptom. The therapeutically effective amount can vary depending, for example, on the antibody, the infection, and/or symptoms of the infection, severity of the infection, and/or symptoms of the infection, the age, weight, and/or health of the patient to be treated, and the judgment of the prescribing physician. An appropriate therapeutically effective amount in any given instance may be ascertained by those skilled in the art or capable of determination by routine experimentation. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody are outweighed by the beneficial effects.

A prophylactically effective amount is any amount of the antibody that, when administered alone or in combination to a patient, inhibits or delays the onset or reoccurrence of the influenza virus infection, or at least one of the clinical symptoms of influenza virus infection. In some embodiments, the prophylactically effective amount prevents the onset or reoccurrence of the influenza virus infection entirely. Inhibiting the onset means either lessening the likelihood of the infection's onset, or preventing the onset entirely.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences may be compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequent coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percentage sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Throughout this specification, reference to at least 80% sequence identity is used interchangeably with one or more of at least 81, at least 82, at least 83, at least 84, at least 85, at least 86, at least 87, at least 88, at least 89, at least 90, at least 91, at least 92, at

least 93, at least 94, at least 95, at least 96, at least 97, at least 98, at least 99 and/ or 100% sequence identity.

Optimal alignment of sequences for comparison may be conducted, for example, by the local
5 homology alignment algorithm of Smith and Waterman [Adv. Appl. Math. 2: 484 (1981)], by
the algorithm of Needleman & Wunsch [J. Mol. Biol. 48: 443 (1970)] by the search for
similarity method of Pearson & Lipman [Proc. Nat'l. Acad. Sci. USA 85: 2444 (1988)], by
computer implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA -
Sequence Analysis Software Package of the Genetics Computer Group, University of
10 Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705), or by
visual inspection [see Current Protocols in Molecular Biology, F.M. Ausbel et al, eds, Current
Protocols, a joint venture between Greene Publishing Associates, Inc. And John Wiley &
Sons, Inc. (1995 Supplement) Ausubel].

15 Examples of algorithms suitable for determining percent sequence similarity are the BLAST
and BLAST 2.0 algorithms [see Altschul (1990) J. Mol. Biol. 215: pp. 403-410; and
the National Center for Biotechnology Information].

In a preferred homology comparison, the identity exists over a region of the sequences that
20 is at least 5 contiguous amino acid residues, or at least 10 contiguous amino acid residues,
or at least 15 contiguous amino acid residues, or at least 20 contiguous amino acid residues,
or at least 25 contiguous amino acid residues, or at least 30 contiguous amino acid residues
in length.

25 An "antibody" is used in the broadest sense and specifically covers antibodies and antibody
fragments so long as they exhibit the desired biological activity. For example, an antibody is
a protein including at least one or two, heavy (H) chain variable regions (abbreviated herein
as VHC), and at least one or two light (L) chain variable regions (abbreviated herein as
VLC). The VHC and VLC regions can be further subdivided into regions of hypervariability,
30 termed "complementarity determining regions" ("CDR"), interspersed with regions that are
more conserved, termed "framework regions" (FR). The extent of the framework region and
CDRs has been precisely defined (see, Kabat, E.A., et al. Sequences of Proteins of
Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH
Publication No. 91-3242, 1991, and Chothia, C. et al, J. Mol. Biol. 196:901-917, 1987) .

35 Preferably, each VHC and VLC is composed of three CDRs and four FRs, arranged from
amino-terminus to carboxy-terminus in the following order: FR1, CDRI, FR2, CDR2, FR3,
CDR3, FR4.

- The VHC or VLC chain of the antibody can further include all or part of a heavy or light chain constant region. In one embodiment, the antibody is a tetramer of two heavy immunoglobulin chains and two light immunoglobulin chains, wherein the heavy and light immunoglobulin chains are inter-connected by, e.g., disulfide bonds. The heavy chain constant region includes three domains, CH2 and CH3, and optionally CH1. The light chain constant region is comprised of one domain, CL. The variable region of the heavy and light chains contains a binding domain that interacts with an antigen. The constant regions of the antibodies typically mediate the binding of the antibody to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system. The term "antibody" includes intact immunoglobulins of types IgA, IgG, IgE, IgD, IgM (as well as subtypes thereof), wherein the light chains of the immunoglobulin may be of types kappa or lambda.
- The term antibody, as used herein, also refers to a portion of an antibody that binds to an influenza virus, for example a molecule in which one or more immunoglobulin chains is not full length, but which binds to an influenza virus. Examples of binding portions encompassed within the term antibody include (i) a Fab fragment, a monovalent fragment consisting of the VLC, VHC, CL and CHI domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fc fragment consisting of the VHC and CHI domains; (iv) a Fv fragment consisting of the VLC and VHC domains of a single arm of an antibody, (v) a dAb fragment (Ward et al, Nature 341:544-546, 1989), which consists of a VHC domain; and (vi) an isolated complementarity determining region (CDR) having sufficient framework to bind, e.g. an antigen binding portion of a variable region. An antigen binding portion of a light chain variable region and an antigen binding portion of a heavy chain variable region, e.g., the two domains of the Fv fragment, VLC and VHC, can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VLC and VHC regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:1527-1533; and Huston et al. (1988) Proc. Natl. Acad. Sci USA 85:5879-5883). Such single chain antibodies are also encompassed within the term antibody. These are obtained using conventional techniques known to those with skill in the art, and the portions are screened for utility in the same manner as are intact antibodies.
- Likewise, the term antibody embraces protein scaffolds.

Protein scaffolds provide universal binding frameworks to complement the expanding repertoire of therapeutic monoclonal antibodies and derivatives such as scFvs, Fab molecules, dAbs (single-domain antibodies), diabodies and minibodies. Scaffold systems create or modify known protein recognition domains either through creation of novel scaffolds or modification of known protein binding domains. Such scaffolds include but are not limited to:

- (i) protein A based scaffolds - affibodies (Nord, K. *et al* 1997 "Binding proteins selected from combinatorial libraries of an alpha-helical bacterial receptor domain". *Nat Biotechnol* 15, 772-777);
- (ii) lipocalin based scaffolds – anticalins (Skerra 2008 "Alternative binding proteins: anticalins - harnessing the structural plasticity of the lipocalin ligand pocket to engineer novel binding activities". *FEBS J.* 275:2677-83);
- (iii) fibronectin based scaffolds – adnectin (Dineen *et al* 2008 "The Adnectin CT-322 is a novel VEGF receptor 2 inhibitor that decreases tumor burden in an orthotopic mouse model of pancreatic cancer". *BMC Cancer* 8:352);
- (iv) avimers (Silverman *et al* 2005 "Multivalent avimer proteins evolved by exon shuffling of a family of human receptor domains". *Nat Biotechnol* 23:1556-61);
- (v) ankyrin based scaffolds – darpins (Zahnd *et al* 2006 "Selection and characterization of Her2 binding-designed ankyrin repeat proteins". *J Biol Chem.* 281:35167-75); and
- (vi) centyrin scaffolds – based on a protein fold that has significant structural homology to Ig domains with loops that are analogous to CDRs. Ig domains are a common module in human proteins and have been widely applied as alternative scaffold proteins.

Reference to the term preventing or treating may be used synonymously with the term suppressing.

The amino acid sequences described herein may include one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, for example conservative amino acid substitutions (see below) and other substitutions that do not significantly affect the folding or activity of the antibody.

Conservative amino acid substitutions

Basic: arginine
lysine
histidine

	Acidic:	glutamic acid
		aspartic acid
	Polar:	glutamine
		asparagine
5	Hydrophobic:	leucine
		isoleucine
		valine
	Aromatic:	phenylalanine
		tryptophan
10		tyrosine
	Small:	glycine
		alanine
		serine
		threonine
15		methionine

- In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-*N*-methyl lysine, 2-aminoisobutyric acid, isovaline and α -methyl serine) may be substituted for amino acid residues of the polypeptides of the present invention. A
- 20 limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for natural polypeptide amino acid residues. The polypeptides of the present invention can also comprise non-naturally occurring amino acid residues.
- 25 Non-naturally occurring amino acids include, without limitation, trans-3-methylproline, 2,4-methano-proline, cis-4-hydroxyproline, trans-4-hydroxy-proline, N-methylglycine, allo-threonine, methyl-threonine, hydroxy-ethylcysteine, hydroxyethylhomo-cysteine, nitro-glutamine, homoglutamine, pipecolic acid, tert-leucine, norvaline, 2-azaphenylalanine, 3-azaphenyl-alanine, 4-azaphenyl-alanine, β -naphthylalanine, β -pyridylalanine, N-guanidino-
- 30 (butyl)-homoarginine, N, N'-guanidino-(dimethyl)-homoarginine, N, N'-guanidino-bis-(2,2,2-trifluoroethyl)-homoarginine, N, N'-guanidino-(methyl, hexyl)-homoarginine, N⁶-methyllysine, N⁶-isopropyllysine, aminomethylphenylalanine, aminocyclohexylalanine, α -aminobutyric acid, 4-thiaproline, N-methylleucine, ornithine, norleucine, 5-bromo-tryptophan, 5-fluoro-tryptophan, 5-nitro-tryptophan, γ -aminobutyric acid, J-mercaptopropionyl, acetyl,
- 35 pencillamine, and 4-fluorophenylalanine.

Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is carried out in a cell free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., J. Am. Chem. Soc. 113:2722, 1991; Ellman et al., Methods Enzymol. 202:301, 1991; Chung et al., Science 259:806-9, 1993; and Chung et al., Proc. Natl. Acad. Sci. USA 90:10145-9, 1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., J. Biol. Chem. 271:19991-8, 1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the polypeptide in place of its natural counterpart. See, Koide et al., Biochem. 33:7470-6, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, Protein Sci. 2:395-403, 1993).

A number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for amino acid residues of polypeptides of the present invention.

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244: 1081-5, 1989). Sites of biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., Science 255:306-12, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992. The identities of essential amino acids can also be inferred from analysis of homologies with related antibody sequences.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer

- (Science 241:53-7, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-6, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each
- 5 position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-7, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).
- 10 Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-7, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-6, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more
- 15 positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-7, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

20

SEQ ID NOs

CDR3 (Kabat)

SEQ ID NO: 1 = STTTPPYEY

SEQ ID NO: 2 = RRDWRDY

25 SEQ ID NO: 3 = NPPGNLY

SEQ ID NO: 4 = DPLSTGWGQYSY

SEQ ID NO: 5 = STTTPPHEF

SEQ ID NO: 6 = RDGFFNRYDY

SEQ ID NO: 7 = SGPGGLNV

30 SEQ ID NO: 8 = TRWVPTMKADEYNY

SEQ ID NO: 9 = ASWVASLWSPSEYDY

SEQ ID NO: 10 = DPPGILY

SEQ ID NO: 11 = DPVCTAGWYRPSRFDL

SEQ ID NO: 12 = STLTTPHEY

35 SEQ ID NO: 13 = GNTGSSDRSSSYVH

SEQ ID NO: 14 = KSPLVDNEY

SEQ ID NO: 15 = KGFAPFLIGCPWGKAEYDY

SEQ ID NO: 16 = TKAFGIATITADYEL

SEQ ID NO: 17 = SSTVAPHEY

SEQ ID NO: 18 = SGPGGVEV

SEQ ID NO: 19 = SGPGGVIL

5 CDR3 (IMGT)

SEQ ID NO: 20 = KTSTTTPPYEY

SEQ ID NO: 21 = NTRRDWRDY

SEQ ID NO: 22 = NLNPPGNLY

SEQ ID NO: 23 = NADPLSTGWGQYSY

10 SEQ ID NO: 24 = KCSTTTPPHEF

SEQ ID NO: 25 = NARDGFFNRYDY

SEQ ID NO: 26 = MASGPGGLNV

SEQ ID NO: 27 = AGTRWVPTMKADEYNY

SEQ ID NO: 28 = AAASWVASLWSPSEYDY

15 SEQ ID NO: 29 = NLDPPGILY

SEQ ID NO: 30 = AADPVCTAGWYRPSRFDL

SEQ ID NO: 31 = KISTLTPPHEY

SEQ ID NO: 32 = GAGNTGSSDRSSSYVH

SEQ ID NO: 33 = HAKSPLVDNEY

20 SEQ ID NO: 34 = AVKGFAPFLIGCPWGKAEYDY

SEQ ID NO: 35 = AATKAFGIATITADYEL

SEQ ID NO: 36 = KFSSTVAPHEY

SEQ ID NO: 37 = KASGPGGVEV

SEQ ID NO: 38 = KASGPGGVIL

25

CDR2 (Kabat)

SEQ ID NO: 39 = VIGNYGNTNYADSVKR

SEQ ID NO: 40 = DIASTRGTTNYADSVKG

SEQ ID NO: 41 = GITYDDSTNYAGSVKG

30 SEQ ID NO: 42 = AITSGESTNYADSVKG

SEQ ID NO: 43 = VIGNYGNTNYADSVKG

SEQ ID NO: 44 = AVTTDGSTSYADYAKG

SEQ ID NO: 45 = VIGNGGNTNYAESVKG

SEQ ID NO: 46 = FITSTSAVTKYADSVKG

35 SEQ ID NO: 47 = CRASDGNTYYAESLKG

SEQ ID NO: 48 = SIAYDGSTSYADPVKG

SEQ ID NO: 49 = CISPDSFTEYGDSVKG

SEQ ID NO: 50 = VIGNNDNTVYGDSVQG

SEQ ID NO: 51 = AIDWGDGPTTYADSVKG

40 SEQ ID NO: 52 = SIDGRGTPMYADSVKG

SEQ ID NO: 53 = CMNSRDGTTNYADSVKG

SEQ ID NO: 54 = AITAGGNTYYADSAKA

SEQ ID NO: 55 = VIGNYGNTNYADSVKG

SEQ ID NO: 56 = VINGGGNTNYADSVKG

5 SEQ ID NO: 57 = VINGGGTNYADSVKG

CDR2 (IMGT)

SEQ ID NO: 58 = IGNYGNT

SEQ ID NO: 59 = IASTRGTT

10 SEQ ID NO: 60 = ITYDDST

SEQ ID NO: 61 = ITSGEST

SEQ ID NO: 62 = IGNYGNT

SEQ ID NO: 63 = VTTDGST

SEQ ID NO: 64 = IGNGGNT

15 SEQ ID NO: 65 = ITSTSAVT

SEQ ID NO: 66 = RASDGNT

SEQ ID NO: 67 = IAYDGST

SEQ ID NO: 68 = ISPSDSFT

SEQ ID NO: 69 = IGNNNT

20 SEQ ID NO: 70 = IDWGDGPT

SEQ ID NO: 71 = IDGRGTP

SEQ ID NO: 72 = MNSRDGTT

SEQ ID NO: 73 = ITAGGNT

SEQ ID NO: 74 = IGNYGNT

25 SEQ ID NO: 75 = IGNGGNT

SEQ ID NO: 76 = IGNGGNT

CDR1 (Kabat)

SEQ ID NO: 77 = IVTMG

30 SEQ ID NO: 78 = WYDVG

SEQ ID NO: 79 = RYRMG

SEQ ID NO: 80 = LYTMG

SEQ ID NO: 81 = IVTMG

SEQ ID NO: 82 = TYPMS

35 SEQ ID NO: 83 = FYTMG

SEQ ID NO: 84 = NYAIG

SEQ ID NO: 85 = GYAIA

SEQ ID NO: 86 = RYRMG

SEQ ID NO: 87 = AYAIA

40 SEQ ID NO: 88 = IITMG

- SEQ ID NO: 89 = LYRVG
 SEQ ID NO: 90 = MYMID
 SEQ ID NO: 91 = NNAIG
 SEQ ID NO: 92 = INAMG
 5 SEQ ID NO: 93 = IVTMG
 SEQ ID NO: 94 = FYTMG
 SEQ ID NO: 95 = FYTMG
- CDR1 (IMGT)
- 10 SEQ ID NO: 96 = GSISRIVT
 SEQ ID NO: 97 = GFTFSWYD
 SEQ ID NO: 98 = GSFFSRYR
 SEQ ID NO: 99 = GSAFSLYT
 SEQ ID NO: 100 = GSISRIVT
 15 SEQ ID NO: 101 = GSAVLSTYP
 SEQ ID NO: 102 = NDIFSFYT
 SEQ ID NO: 103 = GFSLDNYA
 SEQ ID NO: 104 = GPFDDGYA
 SEQ ID NO: 105 = GSFFSRYR
 20 SEQ ID NO: 106 = GFTLGAYA
 SEQ ID NO: 107 = GSMSRIIT
 SEQ ID NO: 108 = VLTFSLYR
 SEQ ID NO: 109 = GDIFVMYM
 SEQ ID NO: 110 = GSTLNNNA
 25 SEQ ID NO: 111 = GSAFSINA
 SEQ ID NO: 112 = GSISSIVT
 SEQ ID NO: 113 = DNIFSFYT
 SEQ ID NO: 114 = TNIASFYT
- 30 Full length VHH
 SEQ ID NO: 115 =
 QLQLVESGGGLVQPGGSLRLSCAASGSISRIVTMGWYRQASGKERELVAVIGNYGNTNYADSVKGR
 FTVSRDPAKDTAYLQMNLNVEDTAIYYCKTSTTTPPYEYWGQGTQVTVSS
 SEQ ID NO: 116 =
 35 QVQLVESGGGLVQPGGSLRLSCAASGFTFSWYDVGWYRRAPGKERELVADIASTRGTTNYADSVK
 GRFTISRDNKNSVYLQMNSLKPEDTAVYYCNTRRDWRDYGWGQGIQVTVSS
 SEQ ID NO: 117 =
 QVQLVESGGGLVQPGGSLRLSCAASGSFFSRYRMGWYRQAPGEQRELVAGITYDDSTNYAGSVKG
 RFTISRDNKNTVHLQMNSLKPEDTAVYYCNLNPPGNLYWGQGTQVTVSS

SEQ ID NO: 118 =

QVQLVESGGGLVQPGGSLRLSCLASGSFAFLYTMGWYRQAPGNQRELVAAITSGESTNYADSVKG
RFTISRDNANTVYLMNSLKPEDTAVYYCNADPLSTGWGQYSYWGQGTQVTSS

SEQ ID NO: 119 =

5 QVQLVESGGGLVQPGGSLRLSCAASGSISRIVTMGWYRQGPGERELVAVIGNYGNNTNYADSVKGR
FTVSRDNADTAYLQMNINLVEDTAMYYCKCSTTPPHEFWGQGTQVTSS

SEQ ID NO: 120 =

10 QVQLVESGGGLVQPGGSLRLSCSASGSALFSTYPMWYRQAPGKQRELVAAVTTDGSTSYADYA
KGRFTISRDNANTVYLMNSLKPEDTAVYYCNARDGFFNRYDYWGQGTQVTSS

SEQ ID NO: 121 =

15 QVQLVESGGGLVQPGGSLRLSCAASNDIFSFTMGWYLQAIGKQREPVAVIGNGGNTNYAESVKGR
FTISRDAKNTAYLQMNSLKPEDTAVYYCMASGPGLNVWGQGTQVTSS

SEQ ID NO: 122 =

QLQLVESGGGLVQPGGSLRLSCTASGFSLDNYAIGWFRRAPGEEREVSFITSTSAVTKYADSVKGR
FTISRDNANTVYLMNTSLKPEDTAVYYCAGTRWVPTMKADEYNYWGQGTQVTSS

20 SEQ ID NO: 123 =

QLQLVESGGGLVQVGGSLRLSCAASGFPPFDGYAIAWFRQAPGKEREGVSCRASDGNTYYAESLKG
RLTMSTDNAKNTVYLMNSLKPEDTAVYYCAAASWVASLWSPSEYDYWGQGTQVTSS

SEQ ID NO: 124 =

25 QVQLVESGGGLVQPGGSLRLSCAASGSFFSRYRMGWYRQAPGEQRELVASIAYDGSTSYADPVKG
RFTISRDNANTVHLQMYSLKPDDTAVYYCNLDPPGILYWGQGTQVTSS

SEQ ID NO: 125 =

30 QVQLVESGGGLVQPGGSLRLSCAASGFTLGAYIAWFRQAPGKEREGVSCISPDSFTEYGDSVKG
RFTVSRDNANTVYLMNSLQPEDTAVYYCAADPVCTAGWYRPSRFDLWGQGTQVTSS

SEQ ID NO: 126 =

35 QVQLVESGGGLVQSGGSLRLSCAASGSMSRIITMGWYRQAPGMERELVAVIGNNDNTVYGDSVQG
RFTVSRDNANTAYLQMNSLNAEDTAMYYCKISTLTPPHEYWGQGTQVTSS

SEQ ID NO: 127 =

QVQLVESGGGLVQAGDSLRLSCTASVLTFSLYRVGWFRQAPGKEREFVAIDWGDGPTTYADSVKG
RFTISRDNAAERTAYLQINSLKPEDTAVYYCGAGNTGSSDRSSSYVHWGQGTQVTSS

40 SEQ ID NO: 128 =

QLQLVESGGGLVQPGGSLRLSCTASGDIFVMYMWYRQAPGKQRELVASIDGRGTPMYADSVKGR
FTISRDNANTVYLMNSLKPEDTAVYSSCHAKSPLVDNEYWGQGTQVTSS

SEQ ID NO: 129 =

QLQLAESGGGLVQAGGSLRLSCAASGSTLNNNAIGWFRQAPGKEREGVSCMNSRDGTTNYADSVK
GRFTISRDAKNTVYQLMNSLKPEDTAVYFCVKGKGFAPFLIGCPWKGAEYDYWGQGTQVTVSS

5 SEQ ID NO: 130 =

QVQLVESGGGLVQPGGSLRLSCAASGSFAFSINAMGWYRQAPGEEREFAAITAGGNTYYADSAKAR
FTISRDNAMNLYQLMNSLKPEDTAMYYCAATKAFGIATITADYELWGQGTQVTVSS

SEQ ID NO: 131 =

10 QVQLVESGGGLVQPGGSLRLSCSASGSISSIVTMGWYRQAPGKERELVAVIGNYGNTNYADSVKGR
FTVSRDNAMNTVYQLMNSLNVEDTAMVYCKFSSTVAPHEYWGQGTQVTVSS

SEQ ID NO: 132 =

QVQLVESGGGLVQPGGSLRLSCAASDNIFSFTMGWYRQSPGKERELVAVIGNGGNTNYADSVKG
15 RFTISRDNKGKNAHLQMNSLKPEDTAVYYCKASGPGGVEVWGQGTQVTVSS

SEQ ID NO: 133 =

QVQLVESGGGLVQPGGSLRLSCAASSTNIASFYTMGWYRQTPGQQRDVAVIGNGGNTNYADSVKG
RFTISRDKAKNTAHLQMNSLKPEDTDVYYCKASGPGGVILWGQGTQVTVSS

20

Examples

Example 1: Immunization procedure and analysis of immune response to influenza

Juvenile male alpacas were maintained at the Royal Veterinary College, Hertfordshire, UK. All animal experiments were performed in accordance with Home Office licence 80/2117. A
25 blood sample prior to immunisation was obtained from the jugular vein and this was followed by 4 intramuscularly injections at 0 day, 21 day, 43 day and 71 day intervals divided between 4 legs. The primary immunisation consisted of 50µg of recombinant H1 (A/California/07/09 (H1N1pdm) (Protein SciencesTM) in 400µl of sterile PBS and emulsified with 800 µl of complete Freund's adjuvant (Sigma) just prior to immunisation. Similarly three separate
30 booster injections of 50µg of recombinant H1 (A/California/07/09 (H1N1pdm) (Protein SciencesTM) in incomplete Freund's adjuvant (Sigma) were administered. Approximately 3 days after each injection a 10 ml blood sample was collected from which serum was prepared after allowing the blood to clot overnight at 4°C. The serological response to A/California/07/09 at each stage of the immunisation schedule was evaluated by ELISA,
35 haemagglutination inhibition assay (HI assay) on a panel of inactivated viral strains and microneutralisation assays (MN assays) using the pandemic H1N1 viral strain A/California/07/09 (Table 1). A clear response to the immunogen (H1N1 pdm) was seen which increased with each subsequent immunisation.

Table 1. Assessment of the serological immune response in immunised alpacas.

Immunisation strategy				HI titre ²				MN titre ³	
Antigen	Immuniza tion	Blood	Day	A/California/ 07/09 (H1N1) pdm	B/Brisbane/ 60/08 Control	A/Uruguay/ 716/07 (H3N2)	A/HongKong/ 213/03 (H5N1)	A/Vietnam/ 1194/04 (H5N1)	A/California/07/09 (H1N1) pdm
	Primary	Pre - immune	0	<8	<8	<8	<8	<8	<10
Rec HA ¹ (H1)	Boost 1	First bleed	21	64	<8	<8	ND ⁺	ND	ND
Rec HA (H1)	Boost 2	Second bleed	43	2048	<8	<8	ND	ND	ND
Rec HA (H1)	Boost 3	Third bleed	71	8192	<8	<8	<8	<8	>1280

¹Rec HA (H1), recombinant haemagglutinin derived from A/California/07/09 pandemic H1N1 viral strain.

²HI titre, haemagglutination inhibition assay and titres are indicated as the reciprocal of the minimum serum dilution at which inhibition of agglutination of turkey erythrocytes is seen.

³MN titre, microneutralisation titre is given as the reciprocal of the minimum dilution at which lysis inhibition is seen of MDCK cells by influenza strain A/California/07/09 (H1N1 pdm).

⁴ND is not determined.

Example 2: Phage displayed single domain VHH antibody library construction

For antibody library construction approximately 10 ml samples of blood were collected from an immunized alpaca (Example 1) into heparin tubes. Peripheral blood lymphocytes were purified using a ficol hypaque centrifugation procedure (Sigma). RNA was extracted using RiboPure™ RNA extraction kit (Novagen) according to manufacturer's instructions and stored at -70°C prior to cDNA synthesis. First strand cDNA synthesis was performed according to manufacturer's instructions (Invitrogen). In short, up to 200ng of total RNA was used per oligo dT primed cDNA synthesis reaction which was allowed to proceed for 50 minutes at 50°C after which time the reaction was terminated by incubation at 85°C for 5 minutes followed by placing the reaction on ice. The reactions were collected by brief centrifugation and 1µl of RNase H was added to each tube and incubated for 20 minutes at 37°C. The resulting cDNA was either stored at -20°C or used immediately in primary PCR reaction to amplify antibody gene sequences.

Primary PCR was performed using two oligonucleotide primers designed to universally prime mammalian immunoglobulin genes in the CH2 domain and also at the 5' end of alpaca VHH genes (Harmsen *et al.*, 2000) within the signal sequence. High Fidelity taq polymerase (Roche) was used for all DNA amplification.

Alp-CH2_Rev

5' CGC CAT CAA GGT ACC AGT TGA

AlpL-Fw_VHH

5' GGT GGT CCT GGC TGC

This resulted in the amplification of both a 600bp and 900bp antibody gene products. The 600bp product corresponds to the heavy chain only antibody population and does not contain a CH1 domain. The 900bp gene product corresponds to the conventional antibody heavy chain population and is not required. The 600bp heavy chain only gene product was gel purified (QIAGEN) and then subjected to a secondary PCR step to amplify just the VHH antibody gene population (~ 450bp) and to append appropriate restrictions sites for cloning into the phage display vector pNIBS-1 (Example 3). Primers for secondary PCR were designed to amplify all VHH genes by annealing to the antibody FR1 and FR4 regions. The *Sfi*1 and *Not*1 restriction sites were chosen for cloning as they do not cut frequently within antibody genes.

Alp_FR1_Sfi1

5' CTG CAG GGA TCC GTT AGC AAG GCC CAG CCG GCC ATG GCA CAG KTG CAG
CTC GTG GAG TCN

Alp_FR4back_*Not*1

5' GCT AGT GCA TGG AGC TCA TGC GGC C

Approximately 5µg of VHH antibody DNA was digested with *Sfi*I restriction enzyme (New England Biolabs) in a 200µl reaction overnight at 50°C. After further purification (QIAGEN) the VHH genes were digested with *Not*I in a 100µl reaction at 37°C for 6 hours. The digested VHH genes were then ligated into the phage display vector pNIBS-1 (Example 3) which was similarly digested with *Sfi*I and *Not*I restriction enzymes. Ligations were set up in a ratio of 4:1 insert to vector in a 20µl reaction with each reaction containing approximately 100ng of vector. After the addition of T4 DNA ligase (New England Biolabs) the reactions were incubated overnight at 16°C. The individual ligations were pooled and purified (QIAGEN) according to manufacturer's instructions. The purified ligation mix was then transformed into TG1 electrocompetent cells (Agilent) using electroporation (BIO-RAD). Cells were recovered from electroporation cuvettes using 1ml SOC medium (Sigma) pre-warmed to 37°C and then incubated at 37°C for 1 hour with shaking to allow expression of antibiotic resistance. Libraries were spread on 22 cm dishes containing 2 TY agar ampicillin (100µg/ml) and 20% v/v glucose. The library size was determined by preparing a dilution series counting viable colonies and was 5×10^7 independent clones. After overnight incubation of the 22cm dishes at 37°C the libraries were harvested by flooding the plates 2 TY agar carbenicillin (100µg/ml), 20% v/v glucose and scraping off the colonies. The resuspended colonies representing the antibody library were then stored at -70°C in 20% v/v glycerol.

Example 3: Construction of phage display vector pNIBS-1

A polylinker sequence of 1782bp was designed comprising the key features below:

- (i) A *pelB* signal sequence which drives the secretion of expressed antibody into the periplasm and whose c terminal amino acid and DNA sequence is compatible with encoding a *Sfi*I restriction site.
- (ii) A hexa-histidine tag which is fused to the C-terminal end of a cloned VHH antibody and facilitates both purification and detection of expressed antibodies.
- (iii) A c-Myc epitope tag attached to the C-terminal end of the cloned antibody after the hexa-histidine tag and facilitates detection of expressed antibody
- (iv) Gene III sequence which encodes the M13 bacteriophage coat protein 3 and is in frame with antibody cassette to facilitate the production of antibody-gene III fusion products which can be displayed on the surface of phage.

- (v) An amber DNA stop codon between the c-Myc epitope tag and the gene III phage coat protein. This codon is read as a stop codon in *Escherichia coli* but in strains carrying a mutant suppressor tRNA gene it is frequently read as an encoding amino acid and read through to produce a antibody-gene 3 fusion product. This allows display of the antibody-gene 3 fusion on phage. Suppression of the amber codon is generally of low efficiency and this has the advantage that soluble antibody not fused to gene 3 can be produced from the same *E.coli* clone cell without sub-cloning to remove the gene 3 sequence.
- (vi) *Sfi*1 and *Not*1 restriction sites for cloning antibody genes were included as they cut infrequently in antibody genes and can be placed within DNA sequence whilst maintaining a functional expression cassette. Other restriction site like *Mfe*1 and *Bst*EII were also incorporated as these are highly conserved in antibody germline genes and can be useful for cloning selected antibodies into other formats

The polylinker sequence was synthesised from oligonucleotides (Geneart™) as a *Hind*111/*Eco*R1 fragment and cloned into the standard phagemid vector pUC119 to generate pNIBS-1 (Figure 1a and Figure 1b).

Example 4: Phage antibody library selection strategy

The phage displayed antibody library (Example 2) was inoculated into 50 mls of 2 x TY media supplemented with carbenicillin at 100µg/ml (w/v) and 2% v/v glucose (2xTY AG). An inoculation of greater than 10 x library size was used to ensure library diversity is maintained during the phage rescue procedure [Library size 5×10^7 in size therefore 5×10^8 cells were used as a starter inoculation from a glycerol stock]. The culture was grown at 37°C to an OD600 of 0.5 and 5mls of culture (corresponding to approximately 7.5×10^8 bacteria) were transferred to a 50 ml sterile tube containing M13KO7 helper phage (New England Biolabs). The multiplicity of infection (MOI) was approximately 20:1 phage to bacteria so approximately 1.5×10^{10} phage were used to infect the bacterial culture. After the bacterial culture was added to the appropriate amount of M13KO7 helper phage it was incubated in a water bath at 37°C for 30 minutes. The infected cells were then centrifuged for 10 minutes at 4000 x g, the supernatant removed and the bacterial pellet resuspended in 25mls of pre-warmed 2 x TY supplemented with 25µg/ml kanamycin and 100µg/ml ampicillin (2 x TY AK) and incubated overnight at 30°C with shaking. The next day cells were pelleted by centrifugation for 15 minutes at 4,000 x g and the supernatant containing the phage displayed antibody library collected. To purify the phage 1/5 volume of 20% polyethylene glycol 6000, 2.5M NaCl (PEG) and incubated on ice for 1 hour. The phage were then pelleted in a microfuge and resuspended in 1 ml of sterile PBS. The remaining bacteria were

then removed by a brief centrifugation and the phage supernatant transferred to a new tube. A second purification was performed by adding 200µl of PEG and incubating on ice for 20mins. The phage were collected by centrifugation for 5 minutes at 4°C. The pelleted phage were then resuspended in 1ml sterile PBS and any further contaminating bacteria removed by a further brief centrifugation. The purified phage were then used for selection.

Phage antibody library selections were performed in immunotubes (Nunc) coated with 1ml of 10µg/ml recombinant haemagglutinin in PBS overnight at 4°C [recombinant H1 [A/California/07/2009 (pandemic H1N1), recombinant H5 (A/Vietnam/1203/2004) protein sciences™] (Figure 2). The selection strategy was designed to recover both H1 specific antibodies and also H1/ H5 cross reactive antibodies by alternating selection between immunotubes coated with H1 and H5 (Figure 2). A parallel selection with an empty immunotube was also used to monitor antigen specific enrichment of the phage antibody library. The next day the immunotubes tube and control tube were blocked by filling completely with 2% w/v milk powder in sterile PBS (M-PBS) followed by a 2 hour incubation at room temperature. 500µl of purified concentrated phage ($10^{12} - 10^{13}$ phage) were added to 0.5mls 4% MPBS and the phage blocked for 1 hour at room temperature. The immunotube was then washed with PBS Tween 20 (0.1% v/v) and 2 x PBS and the phage mix transferred to the immunotubes covered with parafilm and incubated for 30 minutes on rotator and then 1.5 hours standing at room temperature. The immunotube was then washed 20 times with PBS Tween (0.1% v/v) and then 20 times with PBS [10 washes were used for the first round of selection to maximise the percentage recovery of antigen specific phage antibodies]. Specific phage antibodies were eluted by adding 1 ml of 100mM triethylamine to the tube and covering with fresh parafilm followed by incubation for 10min on rotator at room temperature. The eluted phage were then neutralised by transferring to a fresh microtube containing 0.5ml 1M Tris HCl pH7.5. The eluted phage were then infected into *E.coli* to both prepare phage for a second round of selection and also to titrate the amount of phage recovered between sequential rounds of selection.

A culture of *E. coli* ER2378 cells was grown to an OD 600 of approximately 0.5. To amplify the selected phage for a next round of selection 1ml of eluted phage were mixed with 5mls of ER2378 culture and 4 mls of 2 x TY media. This was then incubated in a water bath at 37°C for 30 minutes. This was then spread onto 22cm bioassay dishes 2 x TY AG plus tetracycline (20µg/ml) and grown overnight at 30°C. The bioassay dishes were then scraped and the recovered cells used for a second round of selection as above. In order to monitor the progress of selections between sequential round of selection the titre of input phage and output phage were determined. A serial dilution of phage were made in 500µl 2TY to which

500µl of log phage ER2738 culture was added and incubated in a water bath for 30mins at 37°C. The infected cells were spread on 2 x TY AG tetracycline (20µg/ml) agar plates and the colonies counted after overnight incubation at 30°C. The phage titre before and after selection for three rounds was calculated as pfu/ml and showed an increase in the recovery of phage indicative of antigen selective enrichment of specific phage antibodies (Table 2).

Table 2. Selection and screening of phage displayed single domain antibody library

Round	Antigen ¹	Input ² Titre (pfu/ml)	Output ² Titre (pfu/ml)	Number binders H5 ³	Number binders H1 ³
R1a	H1	2×10^{13}	5.6×10^8	5/24	10/24
R2a	H1	4×10^{13}	4×10^{10}	-	12/24
R2b	H5	4×10^{13}	2×10^{10}	16/24	-
R3a	H1	2.3×10^{13}	8×10^{10}	-	12/24
R3b	H5	2.3×10^{13}	4.8×10^{10}	19/24	-

¹ recombinant antigen is either H1 derived from A/California/07/09 (H1N1 pdm) or H5 derived from A/Vietnam/1203/04 (H5N1)

² phage titres before and after selection are given as plaque forming units per ml (pfu/ml)

³ number of antibodies binding to either recombinant H1 (A/California/07/09) or recombinant H5 (A/Vietnam/1203/04) out of total number screened

Example 5: Screening of selected library for antibody clones binding to recombinant H1 (A/California/07/09) and recombinant H5 (A/Vietnam/1203/2004)

Primary screening of selected antibodies was done using unpurified soluble VHH antibodies induced in a 96 well format. Individual colonies from each round of selection were inoculated into 100µl of 2 x TY AG (2%) in 96 well flat bottom plate (Costar) using sterile toothpicks. The plates were incubated overnight at 30°C rotating at 255 rpm. The next day 35ml of 60% v/v glycerol was then added to each well and the master plate stored at -70°C. From this master plate a new 96 well round bottom plate (Costar) containing 120µl of 2 x TY ampicillin 0.1% w/v glucose was inoculated and grown for 6 hours at 37°C until OD 600 is approximately 0.9. After which 30µl 2 x TY ampicillin 100µg/ml plus 5mM IPTG (1mM final concentration) was added and continue shaking at 37°C overnight. The plates were then centrifuged 600 x g for 10 minutes and the supernatant containing soluble VHH antibodies was used to test antigen specific reactivity in ELISA.

A 96 well plate (Nunc) was coated with recombinant haemagglutinin at 1µg/ml overnight in PBS at 4°C. The plates were washed three times with PBS and then blocked with 120µl/well 2% w/v milk powder PBS (M-PBS) for 1 hour at room temperature. The plates were then washed twice each with PBS 0.1% (v/v) Tween and PBS. To each well 50µl of 4% (w/v) milk powder PBS was added to each well and then 50µl of culture supernatant containing soluble VHH. The ELISA plate was then incubated for 1.5 hours on a shaking platform at room temperature. After this incubation the plates were washed three times each with PBS 0.1% (v/v) Tween 20 and three times with PBS. To detect antibody binding 100µl of anti myc 9E10- HRP (1/1000 dilution) (Roche) in 2% MPBS was added to each well and incubated for 1 hour at room temperature on a shaking platform. Plates were then washed three times each with PBS 0.1% (v/v) Tween 20 and three times with PBS and antibody binding developed using standard TMB staining. After stopping the reaction with 50µl of 1M sulphuric acid the plates were read at 450nm. An example of a primary ELISA screen using unpurified soluble VHH antibodies in a 96 well ELISA format is shown in Figure 3. Positive clones were scored as having at least twice background signal and were further characterised to analyse sequence diversity in particular the number of times a particular clone was recovered in the clones screened (Table 3). Using sequence analysis and evaluation by ELISA, nineteen antibodies with a unique VHH CDR3 sequence with specificity for recombinant H1 and/ or recombinant H5 were isolated and progressed for more detailed secondary screening (Example 6).

Table 3. CDR sequences of unique antibodies specific to pandemic H1N1

Clone ¹	SEQ ID ²	Number ³	V _H H CDR1 ⁴	V _H H CDR2 ⁴	V _H H CDR3 ⁴
R1a G5	1	4	IVTMG	VIGNYGNNTNYADSVKR	STTTPPYEY
R2b D8	2	2	WYDVG	DIASRGTTNYADSVKG	RRDWRDY
R2b E8	3	4	RYRMG	GITYDDSTNYAGSVKG	NPFGNLY
R2b D9	4	1	LYTMG	AITSGESTNYADSVKG	DPLSTGWGQYSY
R1a F4	5	2	IVTMG	VIGNYGNNTNYADSVKG	STTTPPHEF
R1a A5	6	34	TYPMS	AVTTDGSTSYADYAKG	RDGFNRYDY
R1a C5	7	1	FYTMG	VIGNGGNTNYAESVKG	SGPGGLNV
R1a E5	8	3	NYAIG	FITSTSAVTKYADSVKG	TRWVPTMKADEYNY
R1a F5	9	1	GYAIA	CRASDGNTYYAESLKG	ASWVASLWSPSEYDY
R1a B6	10	1	RYRMG	SIAYDGSTSYADPVGK	DPFGILY
R1a E6	11	1	AYAIA	CISPSDSFTEYGDVSKG	DPVCTAGWYRPSRFDL
R1a G6	12	1	IITMG	VIGNNDNTVYGDSVQG	STLTPPHEY
R1a H6	13	1	LYRVG	AIDWGDGPTTYADSVKG	GNTGSSDRSSSYVH
R2a E8	14	1	MYMID	SIDGRGTPMYADSVKG	KSPLVDNEY
R2a G8	15	1	NNAIG	CMNSRDGTTNYADSVKG	KGFAPFLIGCPWGKAEYDY
R2a B9	16	1	INAMG	AITAGGNTYYADSAKA	TKAFGIATITADYEL
R2a F9	17	1	IVTMG	VIGNYGNNTNYADSVKG	SSTVAPHEY
R2a G9	18	1	FYTMG	VIGNGGNTNYADSVKG	SGPGGVEV
R2a H9	19	1	FYTMG	VIGNGGNTNYADSVKG	SGPGGVIL

¹ Clone identification number² Sequence identification³ Number of times a particular CDR3 sequence appeared in screening of 62 independently isolated clones⁴ CDR defined according to Kabat numbering scheme

Example 6: Analysis of purified single domain VHH antibodies on different influenza subtypes

For more detailed screening of antibody specificity, purified soluble VHH antibodies were purified from the *E.coli* periplasm. The nineteen different antibodies identified in Example 4 were each inoculated into 2ml of 2YT medium supplemented with 100µg/ml (w/v) ampicillin, 2 % (w/v) glucose, Tet (5µg/ml) in a 10ml tube and grown overnight at 37°C with shaking. From this starter a 50µl inoculation was taken into 50 mls of 2xTY medium supplemented with 100µg/ml ampicillin, 0.1% (v/v) glucose and grown to an OD of ~0.6 at 37°C. Soluble antibody expression was induced with the addition of 1 mM IPTG followed by further incubation overnight at 30°C. The 50 ml culture was centrifuged for 20 minutes and the bacterial pellet re-suspended in 2.5 mls of periplasmic buffer (200mM Tris HCl, pH 7.5, 20% (v/v) sucrose, 1mM EDTA) followed by incubation on a rotator for 1 hour at 4°C. Cell debris was removed by centrifugation twice and the supernatant corresponding to the soluble VHH antibodies recovered from the bacterial periplasm were dialysed (Pierce) into PBS with two changes of buffer. After removal of EDTA by dialysis the antibody was purified by metal chelate chromatography (Clontech) according to manufacturer's instructions. Approximately 1 ml of purification resin was used per antibody. After incubation of the resin with the periplasmic preparations it was transferred to a gravity column (Biorad) and washed according to manufacturers instructions. The purified antibody was eluted into two bed volumes of elution buffer containing 100mM imadazole. Antibody was further dialysed against PBS with two changes of buffer and antibody concentration determined using OD 280.

Inactivated viral preparations were reconstituted in 1ml PBS and a 96 well ELISA plate was incubated overnight at 4°C with 50µl of antigen resuspended in 10mls of 0.5M bicarbonate buffer pH9.6. Plates were washed and processed as in Example 4 except a serial dilution of purified antibody was used of 30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, 0.0003, 0.0001 µg/ml and assays were performed in duplicate. Dose response curves were plotted and EC50 values calculated using GraphPad Prism (Table 4). From this analysis cross-reactive antibodies were seen as R2b-E8, R2b-D9, R1a-A5, R1a-C5, R1a-B6, R1a-H6, and R2a-G8.

Table 4. Analysis of selected antibodies on different influenza viral strain sub-types

Clone	A/California/ 7/2009 (H1N1) EC50 ¹ (µg/ml)	A/Brisbane/ 9/2007 (H1N1-like) EC50 (µg/ml)	A/Brisbane 10/2007) (H3N2) EC50 (µg/ml)	B/Brisbane/ 60/2008 EC50 (µg/ml)	A/TurkTurk/ 1/2005 (H5N1) EC50 (µg/ml)	A/Vietnam/1 19/2004 (H5N1) EC50 (µg/ml)	A/HongKong /213/2003 (H5N1) EC50 (µg/ml)	A/Indonesia/ 05/2005 (H5N1) EC50 (µg/ml)	A/Duck/Sing- Q/97 (H5N3) EC50 (µg/ml)	A/HongKong /1073/99 (H9N2) EC50 (µg/ml)	A/mallard/E ng/727/06 (H2N3) EC50 (µg/ml)
R1a-G5	1.73	0.29	-	-	-	-	-	-	-	-	-
R2b-D8	-	14.8	-	-	-	-	-	-	-	-	-
R2b-E8	3.64	18.4	-	-	0.89	1.83	1.2	0.80	4.98	5.63	0.002
R2b-D9	0.90	0.80	-	-	0.76	1.81	2.8	0.58	5.64	-	0.06
R1a-F4	0.79	-	-	-	-	-	-	-	-	-	-
R1a-A5	0.31	0.38	-	-	0.13	0.25	0.27	0.15	0.27	-	0.006
R1a-C5	1.71	3.30	-	-	0.15	2.89	2.9	1.17	7.57	4.48	0.06
R1a-E5	3.23	-	-	-	-	-	-	-	-	-	-
R1a-F5	0.55	0.70	-	-	-	-	-	-	-	-	-
R1a-B6	0.39	0.58	-	-	0.44	0.82	0.37	0.42	1.32	2.3	0.102
R1a-E6	2.46	-	-	-	-	-	-	-	-	-	-
R1a-G6	0.30	-	-	-	-	-	-	-	-	-	-
R1a-H6	3.85	-	-	-	-	-	-	-	-	5.7	-
R2a-E8	0.51	1.42	-	-	-	-	-	-	-	-	-
R2a-G8	4.11	1.86	-	-	1.93	3.36	6.2	5.7	9.67	-	2.5
R2a-B9	1.00	9.84	-	-	-	-	-	-	-	-	-
R2a-F9	0.19	-	-	-	-	-	-	-	-	-	-
R2a-G9	0.30	-	-	-	-	-	-	-	-	-	-
R2a-H9	0.97	-	-	-	-	-	-	-	-	-	-
control	-	-	-	-	-	-	-	-	-	-	-

¹ EC50 is the concentration of antibody in µg/ml which gives 50% of the maximal binding effect of each purified VHH single domain antibody on a representative group of viral sub-types using ELISA. – No binding observed

Example 7: Affinity of single domain antibodies on recombinant haemagglutinin from different influenza viral sub-types

Affinity of purified VHH antibodies (Example 5) was determined using the single cycle kinetics procedure on a BIAcore T100 machine (Karlsson *et al.*, 2006). The procedure involves sequentially injecting an antibody concentration series over a haemagglutinin surface without regeneration between injections. Purified recombinant haemagglutinin of varying subtype (Protein Sciences, E enzymes) was immobilised onto a CM5 chip to approximately 3000 resonance units (RU). A series of antibody dilutions of 100nM, 50nM, 25nM, 10nM 5nM were sequentially run over the different antigen surfaces without regeneration. A reference surface was subtracted prior to evaluation of the sensograms. Antibody affinity was determined using single cycle kinetics procedure of BIA evaluation software (GE healthcare) using a 1:1 fitting model (Table 5). Analysis on recombinant haemagglutinin and viral preparations (Example 6) showed antibodies R2b-E8, R2b-D9, R1a-A5, R1a-C5, R1a-B6, R1a-H6, and R2a-G8 to be cross-reactive antibodies.

Table 5 Binding analysis on different recombinant haemagglutinin using surface plasmon resonance

Clone	SEQ ID	H1 ^a (H1N1 pdm) Kd (nM)	H5 ^b (H5N1) Kd (nM)	H2 ^c (H2N2) Kd (nM)	H9 ^d (H9N2) Kd (nM)	H3 ^e (H3N2) Kd (nM)	H7 ^f (H7N7) Kd (nM)
R1a-G5	1	24	- ^g	-	-	-	-
R2b-D8	2	- ^g	-	-	-	-	-
R2b-E8	3	8.2	3.6	-	87	-	-
R2b-D9	4	0.4	1.6	-	59	-	-
R1a-F4	5	7.3	-	-	-	-	-
R1a-A5	6	3.3	0.7	186	-	-	-
R1a-C5	7	1.9	2.7	67	22	-	-
R1a-E5	8	0.6	-	-	-	-	-
R1a-F5	9	9.2	-	-	-	-	-
R1a-B6	10	0.7	1.7	43	7.6	-	-
R1a-E6	11	22.8	-	-	-	-	-
R1a-G6	12	4.4	-	-	-	-	-
R1a-H6	13	-	241	-	-	-	-
R2a-E8	14	0.8	-	34	-	-	-
R2a-G8	15	3.1	9	39	-	-	-
R2a-B9	16	0.2	-	-	-	-	-
R2a-F9	17	60	-	-	-	-	-
R2a-G9	18	62	-	-	-	-	-
R2a-H9	19	2.8	-	40	-	-	-
- control	-	-	-	-	-	-	-

^a Single cycle affinity determination on full length recombinant H1 residues 18-530 derived from A/California/06/09. Affinity is given in nM to 1 decimal place and is the average of at least two separate assays.

^b recombinant H5 A/Vietnam/1203/04 [H5N1]^c recombinant H2 A/Japan/305/1957 (H2N2)^d recombinant H9 A/HongKong/1073/99 (H9N2)^e recombinant H3

A/Brisbane/10/07 (H3N2)^f recombinant H7 A/Netherlands/219/03 (H7N7).^g - No Binding.

Example 8: Epitope mapping

Evaluation of whether the antibodies bound to the globular head of haemagglutinin was evaluated in BIAcore. Recombinant haemagglutinin HA1 domain (18-344) (A/California/06/09) H1N1 and whole haemagglutinin (18-530) (E Enzymes) was immobilised on a CM5 BIAcore chip (GE Healthcare). Affinity was determined using a single cycle kinetics procedure (Example 7) and the cross-reactive antibodies were shown not bind to the globular head domain HA1 and by inference likely bind to the HA2 stalk region. The HA2 stalk region is more conserved across viral sub-types and as such is consistent with the cross-reactivity of these antibodies.

Further evidence localising cross-reactive antibodies to the more conserved haemagglutinin stalk region was obtained using an acid treatment which triggers an irreversible conformational change in the HA molecule. A viral preparation of H1N1 (A/California/06/09) was resuspended in 1 ml of PBS and 200µl was diluted to 20 mls in PBS and the pH taken down to 2.83 with 1.5mls of 1M HCl. After incubation for 2.5 hours at room temperature the antigen was neutralised with 20mls of 0.5M bicarbonate buffer to give a final pH of ~9.0. This acid treated antigen was then used to coat ELISA plates overnight at 4°C. The next day ELISA plates were blocked and antigen binding was determined as in Example 6. A parallel assay with antigen that had not been treated with acid was also tested. All antibodies were shown to bind H1N1 which had not been treated with acid whereas several antibodies were shown to lose binding after including the cross-reactive antibodies R2b-E8, R2b-D9, R1a-A5, R1a-C5, R1a-B6, R1a-H6. Antibodies shown to bind to the globular head generally retained binding after acid treatment. This provided further evidence that the cross-reactive antibodies were binding to the more conserved stalk region of haemagglutinin.

Further evidence of cross reactive antibodies binding to the conserved stalk region was provided by assaying for the inhibition of heamagglutination of turkey erythrocytes. Heamagglutinin (HA) is the major viral coat protein which mediates binding to cell surface sialic acid via the globular head domain (HA1). Heamagglutination inhibition assays were performed as in Harmon et al., (1988) and were used to determine which antibodies could block attachment of influenza virus to turkey erythrocytes, and to indicate localisation of the antibody epitope to the globular head domain (HA1). Briefly serial dilutions of serum from immunised alpacas or purified VHH antibodies starting from 250µg/ml were prepared. Serial dilutions were incubated with 8 HA units of virus per well and turkey erythrocytes were added to a concentration of 0.5% and the plate was incubated at room temperature for 30 minutes. All antibodies which showed binding to the globular head (HA1) domain were also positive for inhibition of haeamgglutination, except R2a-G8. Of all the H1/H5 cross

neutralising antibodies identified only R1a-C5 showed any inhibition of heamagglutination (Table 6).

Table 6. Mapping of antibody epitopes to globular head or stalk region of haemagglutinin

Clone	HI (18-530) ¹ (pdm H1N1) Kd (nM)	HA1 (18-344) ² (pdm H1N1) Kd (nM)	HI titre (µg/ml) H1N1 (X-181) ³	Acid treatment ⁴	No acid treatment ⁴
R1a-G5	24	26	> 1.56	+	+
R2b-D8	- ⁵	-	>25	+	+
R2b-E8	8.2	-	>25	-	+
R2b-D9	0.4	-	>25	-	+
R1a-F4	7.3	4.7	> 2.08	+	+
R1a-A5	3.3	-	>25	-	+
R1a-C5	1.9	734	> 10.40	-	+
R1a-E5	0.6	-	>25	-	+
R1a-F5	9.2	6.1	>25	ND	+
R1a-B6	0.7	-	>25	-	+
R1a-E6	22.8	36	>25	ND	+
R1a-G6	4.4	4.6	> 1.04	+	+
R1a-H6	-	-	>25	-	+
R2a-E8	0.8	-	>25	-	+
R2a-G8	3.1	7.7	>25	ND ⁴	+
R2a-B9	0.2	-	>25	-	+
R2a-F9	60	79	> 1.04	+	+
R2a-G9	62	69	> 1.30	+	+
R2a-H9	2.8	4.7	>1.30	+	+
-control	-	-	>25	-	+

¹ Single cycle affinity determination on full length recombinant HI residues 18-530 from A/California/06/09. Affinity given to 1 decimal place in nM² Single cycle affinity determination on globular head HA1 domain residues 18-344 from A/California/06/09. Affinity given to 1 decimal place in nM³ Haemagglutination inhibition (HI) assay was given as the minimum dilution of antibody at which inhibition of agglutination of turkey erythrocytes in µg/ml of purified antibody. Values are given as an average of three independent assays. Laboratory adapted H1N1 strain (X-181) was used⁴ ELISA on viral preparation of A/California/07/09 (H1N1 pdm) treated with acid and corresponding control with no acid treatment⁵ ND not determined

Example 9 – humanisation of a V_{HH} antibody (exemplified by reference to camelid sequence VHH R2a-F9)

The framework regions (FR) of R2a-F9 were aligned with human germline sequence database using ClustalW and using sequences taken from V base — See Table 7. The closest human germline sequence was DP53.

The FR4 of R2a-F9 was aligned with the JH sequences and JH1 was chosen. The FR and CDR regions were defined according to Kabat *et al.*, (1991). The IMGT numbering system defines boundaries differently — for example cysteine 92 defines the start of CDR3 according to this nomenclature.

Framework residues in R2a-F9 different from germline VH-DP53 sequence are highlighted in blue (underline). The sequence identity in the framework regions is 79%. There are a total of 17 residues in the frameworks which are different between alpaca and human germline variable gene DP53.

The humanisation strategy involves replacing residues in FR that are not essential for antigen binding and stability. This involves choosing known typical camelid residues to be retained in the final sequence eg. 'Hallmark residues' (Vincke *et al.*, 2008, Journal of Biological Chemistry, 284(5), 3273-3284 .

In R2a-F9 five hallmark residues have been identified 4 of which are in the FR2. A series of variants are made and the importance of each lama residue to antigen binding and stability is assessed.

All 12 constructs are tested in BIAcore for binding to H1, expression yield and thermal stability – see Table 8. Mutations to human germline which do not affect antigen binding are combined in a second round of constructs and the testing is repeated until a final humanised construct with the minimum amount of alpaca antibody sequence in the framework regions is obtained.

Combine all mutations that do not affect function and stability in final constructs to create an antibody gene with maximum human sequence content in the framework regions. A total of 13 alpaca residues are converted to human germline are shown in Table 9.

Table 7

1	3C	CDR1- 3637	49	-----CDR2----- 66
R2a-F9-h	QVQLVESGGGLVQPGGSLRLSCAASGSISS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTVSRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF
DP53	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYWHH WYQAPGKGLVWYS	RINSDSSTSTSYADSVKNG	RTTISRDNNAKNTVY ₁ QMNSLNVEDTAMVYCKF
94	-CDR3----			
R2a-F9-h	KF SSTVAPHEY	WGQGTQVTVSS		
DP53 (96)	CAR	-----	WGQGTQVTVSS	

Table 8

First round Mutant constructs

R2a-F9_Q1E	QVQLVESGGGLVQPGGSLRLSCAASGSISS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTVSRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS
R2a-F9_S23A	EVQLVESGGGLVQPGGSLRLSCAASGSISS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTVSRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS
R2a-F9_S27F	EVQLVESGGGLVQPGGSLRLSCAASGFISS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTVSRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS
R2a-F9_I28T	EVQLVESGGGLVQPGGSLRLSCAASGTSIS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTVSRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS
R2a-F9_S29F	EVQLVESGGGLVQPGGSLRLSCAASGSIIS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTVSRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS
R2a-F9_E46V	EVQLVESGGGLVQPGGSLRLSCAASGSISS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTVSRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS
R2a-F9_A49S	EVQLVESGGGLVQPGGSLRLSCAASGSISS	IVTMG WYQAPGKERELVS	VIGNYGNTNYADSVKG	RTVSRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS
R2a-F9_V69I	EVQLVESGGGLVQPGGSLRLSCAASGSISS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTTISRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS
R2a-F9_N93R	EVQLVESGGGLVQPGGSLRLSCAASGSISS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTVSRDNAKNTLY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS
R2a-F9_V94A	EVQLVESGGGLVQPGGSLRLSCAASGSISS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTVSRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS
R2a-F9_N99V	EVQLVESGGGLVQPGGSLRLSCAASGSISS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTVSRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS
R2a-F9_N99V	EVQLVESGGGLVQPGGSLRLSCAASGSISS	IVTMG WYQAPGKERELVA	VIGNYGNTNYADSVKG	RTVSRDNAKNTVY ₁ QMNSLNVEDTAMVYCKF	SSTVAPHEY	WGQGTQVTVSS

Table 9

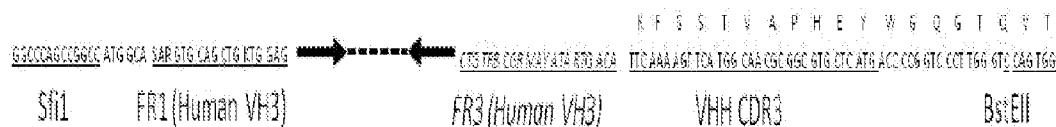
<i>R2a-F9 residue</i>	<i>Location</i>	<i>Hallmark diversity</i>	<i>VH-DP-53 germline</i>	<i>Comment</i>
Q1	FR1	-	E	Mutate Q1E
S23	FR1	-	A	Mutate S23A
S27	FR1	-	F	Mutate S27F
I28	FR1	-	T	Mutate I28T
S29	FR1	-	F	Mutate S29F
Y37	FR2	Yes (FYHILV)	V	Retain
E44	FR2	Yes (GEADQRSL)	G	Retain
R45	FR2	Yes (LRCILPQV)	L	Retain
E46	FR2	-	V	Mutate E46V
L47	FR2	Yes (WLFAGIMRS)	W	Retain
A49	FR2	-	S	Mutate A49S
V69	FR3	-	I	Mutate V69I
V78	FR3	-	L	Mutate V78L
N93	FR3	-	R	Mutate N93R
V94	FR3	-	A	Mutate V94A
M99	FR3	-	V	Mutate M99V
Q108	FR4	Yes (QLR)	L	Retain

Example 10: Introduction of a CDR3 VHH sequence into a human antibody

As the VHH CDR3 is the key determinant of binding specificity, this loop may be readily incorporated into an entirely human VH gene whilst preserving functionality. The resulting antibody is entirely human with a human CDR1 and CDR2 except for the VHH CDR3 loop which is derived from alpaca. Such an antibody has a lower risk of eliciting an adverse immune response when administered as a therapeutic monoclonal antibody due to the increased content of human sequence as compared to the antibody derived in Example 9.

By way of example, the VHH CDR3 loop is grafted into an appropriate human V gene by designing an oligonucleotide corresponding to the VHH CDR3 sequence bracketed by human antibody germline sequence corresponding to FR3 and FR4 of either an individual human VH or a collection of human VH sequences. An example is shown below which includes a 3' oligonucleotide corresponding to the VHH-CDR3 of R2a-F9 (SEQ ID 36) appended with a degenerate sequence designed to prime to all human VH3 gene family members. The VH3 gene was selected simply because camelid VHH antibodies show greatest homology to the human VH3 family. The corresponding 5' primer is a degenerate oligonucleotide designed to anneal to the FR1 of all human VH3 gene members. These two oligonucleotides are used in a PCR reaction with human VH genes derived from cDNA prepared for example from peripheral blood lymphocytes of naive donors using established

methods. The design shown below appends *Sfi*I and *Bst*EII restriction sites to facilitate cloning into the phage display vector pNIBS-1 (Example 3).



After construction of a phage display library of human VH genes grafted with the VHH-CDR3 of R2a-F9, the library is then selected on recombinant H1 as in Example 4 and humanised antibodies screened as in Example 5.

Example 11: Assessment of efficacy *in vivo*

The efficacy of cross-reactive antibodies for passive immunotherapy is assessed in a mouse challenge model. The seven H1/H5 cross neutralising antibodies (R2b-E8, R2b-D9, R1a-A5, R1a-C5, R1a-B6, R1a-H6, R2a-G8) are tested for their ability to protect BALB/c mice from challenge with H1N1 (A/California/07/09) and H5N1 (A/Vietnam/1203/2004) virus in a prophylactic setting. VHH antibodies are expressed, purified (Example 6) and administered intranasally as monovalent agents at 5mg/kg, 24 hours before challenge with a lethal dose of virus. A non-relevant VHH antibody and a PBS only arm of the study are included as controls. Survival, body weight and viral titres in lung and other tissues are monitored to assess the antibodies' ability to protect against lethal challenge with H1N1 and H5N1 virus. Said antibodies demonstrate protection against such lethal challenge. Antibodies are also evaluated in a therapeutic setting where antibody is administered 24 hours post-infection with H1N1 and H5N1. Again survival, body weight and post challenge viral titres in lung and other tissues are monitored. Said antibodies demonstrate protection against such lethal challenge.

Example 12: Neutralisation activity of recombinant VHH antibodies on H1N1 and H5N1

The influenza microneutralisation assay was performed essentially as in Harmon et al., (1988). In brief, either serum from a immunised alpaca or purified VHH antibodies (at a starting concentration of 5µg/ml) were serially diluted, two-fold, in a 50µl volume of assay diluent (DMEM with the addition of; 2mM glutamine, Pen/Strep 1/100, Amphotericin 1/100, non-essential amino acids and FCS 1/200) in a 96 well flat bottom plate (Costar) in duplicate columns 1-10. The reverse genetics reassortants NIBRG-14 (A/Vietnam/1194/04; H5N1) and the reassorted attenuated virus X-181 (A/California/07/09;H1N1) were used in viral

neutralisation assays. X-181 virus and NIBRG-14 viruses were used at 10^2 TCID₅₀ virus dose per 50µl of assay diluents. In all assays a 'virus only' (VC) control and a 'cells only' (CC) control was included. Plates were incubated at room temperature for 1 hour and 100µl of 1.5×10^5 /ml MDCK cells (Madin-Darby canine kidney cells) was added to all wells. Plates were incubated for 18 hours at 30°C in 5% CO₂. Cells were fixed with 100µl of 0.6% w/v methanol/hydrogen peroxide per well and left for 20 minutes at room temperature. Plates were then washed 3 times in PBS Tween-20 (0.05% w/v). Growth of influenza virus and release of nucleoprotein was with a primary anti-influenza A nucleoprotein (Serotec) mouse monoclonal antibody. 100µl of this antibody was added to each well at a 1:3000 dilution in 6 salt PBS/Tween-20 (0.1%) 5% w/v Marvel milk. 100µl of this was added to all wells and plates and incubated for 1 hour at 37°C. Rabbit anti-mouse IgG polyclonal HRP conjugate (DAKO) at a dilution of 1:5000 in 6 salt PBS/Tween-20 (0.1% v/v) , 5% (w/v) Marvel milk powder, 1% (v/v) bovine serum albumin was used and incubated for 1 hour at 37°C. Plates were washed 4 times and developed using TMB staining. The reactions were stopped by the addition of 100µl of 0.5M HCl to each well and read at 450nm & 620nm. The 620nm values were subtracted from the 450nm values to correct for plate absorption. The antibody concentration at which 50% viral neutralisation was seen was determined using the equation;

$$\frac{(\text{mean OD of VC}) - (\text{mean OD of CC}) + (\text{mean OD of CC})}{2} = x$$

Where **x** is the OD at which 50% of MDCK cells were infected.

All antibody clones except R2b-D8, R1a-F4, R1a-E5, R1a-F5, and R1a-E6 were able to neutralise X-181 which is a laboratory adapted strain of H1N1 (Table 10). Of the H1N1 neutralising antibodies, R2b-E8, R2b-D9, R1a-A5, R1a-C5, R1a-B6, R1a-H6 and R2a-G8 were also able to neutralise NIBRG-14 (H5N1) and were deemed to be cross neutralising antibodies (Table 10). This was consistent with the cross reactivity data shown in examples 5, 6 and 7. For antibodies R2b-E8, R2b-D9, R1a-A5, R1a-B6 and R1a-H6 this was consistent with absence of haemagglutination inhibition activity and specificity for acid sensitive epitopes in the conserved stalk region of haemagglutinin (Table 6).

Table 10. Antibody neutralisation of H1N1 and H5N1 viral infection of MDCK cells

Clone	Neutralisation X-181 A/California/07/09 (H1N1 pdm) EC50 nM	Neutralisation NIBRG-14 A/Vietnam/1194/04 (H5N1) EC50 nM
R1a-G5	30 nM	-
R2b-D8	-	-
R2b-E8	54 nM	29 nM
R2b-D9	55 nM	7.8 nM
R1a-F4	-	-
R1a-A5	23 nM	8.0 nM
R1a-C5	23 nM	55 nM
R1a-E5	-	-
R1a-F5	-	-
R1a-B6	31 nM	20 nM
R1a-E6	-	-
R1a-G6	12 nM	-
R1a-H6	53 nM	12 nM
R2a-E8	26 nM	-
R2a-G8	538 nM	> 1 μ M
R2a-B9	29 nM	-
R2a-F9	68 nM	-
R2a-G9	62 nM	-
R2a-H9	16 nM	-
control	-	-

Example 13: Evaluation as a 'Universal Antibody' for vaccine potency

Cross-reactive antibodies of the invention are used in potency assays for attenuated influenza vaccines. The cross-reactive antibodies of this application cross-react with (1) many/ all haemagglutinins of one subtype/type of influenza virus, or with (2) haemagglutinins of more than one subtype of influenza A virus with all of these subtypes belonging to one of the two recognised phylogenetic groups of influenza HA, or with (3) HAs from both phylogenetic groups, group 1 and group 2.

An antibody having cross-reactivity as in (1) is useful for the potency testing of multivalent, preferably trivalent and quadrivalent, influenza vaccines. An antibody having cross-reactivity as in (2) is useful for the potency testing of multivalent, preferably trivalent and quadrivalent, influenza vaccines; for the potency testing of pandemic or pre-pandemic influenza vaccines containing a single subtype of influenza A virus; and for the potency testing of vaccines

containing more than one subtype/type, with each virus component belonging to a different phylogenetic group. An antibody having cross-reactivity as in (3) is useful for the potency testing of monovalent influenza vaccines, preferable pandemic and pre-pandemic influenza vaccines.

The use of such cross-reactive antibodies in potency testing eliminates the need to frequently change the antibody reagent as is the case in currently used methods, for which specific sheep antisera have to be generated every time a strain change is announced and implemented; production of a new antiserum for every antigenically drifted virus that is used in influenza vaccines has been identified as a potential bottleneck in the pathway from virus strain change to vaccination with an updated influenza vaccine. To solve this problem, cross-reactive antibodies of this invention are produced in advance and are therefore available at all times; following a strain change in influenza vaccine production, (a) cross-reactive antibody/antibodies can be used immediately, with no lead time to availability of strain-specific antibodies.

By way of example, a cross-reactive antibody is used in the single radial immunodiffusion (SRID or SRD) assay. Following standard protocols for SRD, the cross-reactive antibody is incorporated into a gel matrix and then used in the assay. A precipitin ring is formed when influenza haemagglutinin is allowed to diffuse into the gel and can be measured following standard protocols for visualisation of the SRD ring/zone.

In another example, cross-reactive antibodies are used in ELISA assays to quantitatively measure the potency of vaccines and pre-vaccine samples, such as monovalent bulks. ELISA assays of various formats may be used. For instance, a sandwich ELISA using an antibody coated on a solid surface to capture haemagglutinin and an antibody to detect captured HA may be used. The capture and the detection antibody may be the same antibody, or may be different antibodies. Either of the two antibodies, or both, may be cross-reactive antibodies of this invention. In another format, a competition ELISA is used. HA is coated on a solid surface and reacted with cross-reactive antibody/antibodies of this invention. A reference antigen, or the sample to be measured, is used as a competitive reagent. Reduction of binding of the cross-reactive antibody to the coated HA can be used to quantify HA in solution, using a standard curve generated from the reference antigen reagent. Other formats of ELISAs may be employed to a similar effect; in all cases, a cross-reactive antibody, or a multitude of cross-reactive antibodies, are used as specific reagents to capture and/ or detect HA.

In another example, Surface Plasmon Resonance (SPR) technology is used to establish a potency assay for attenuated influenza vaccines. Cross-reactive antibodies are used to detect and quantify HA protein/ antigen. Various formats of SPR-based assays may be used. In a preferred embodiment, a competitive assay, with reference antigen and cross-reactive antibody competing for binding to HA coated on a chip, as appropriate for the specific device used, is employed.

In another example, cross-reactive antibody/ antibodies are used in a method using proteins (in particular, HA) immobilised on a membrane. The membrane may be a nitrocellulose membrane, a PVDF membrane, or another suitable membrane. Immobilisation of protein and HA on the membrane may occur through various processes, such as Western Blotting using various devices, slot blotting, spotting onto membrane, and others.

In another example, cross-reactive antibodies are used to enrich HA from samples, such as vaccines or monovalent bulks. Enrichment may be through use of cross-reactive antibodies in immune-precipitation, affinity chromatography, magnetic separation (with antibody being coupled by some means to magnetic substances, such as paramagnetic beads), or any other immunological method capable of binding and enriching HA out of a sample which will be, for the most part, a liquid containing HA in solution. HA adsorbed to substances, such as adjuvants, may also be subjected to such enrichment, either in untreated samples or following treatment to de-adsorb the HA. Enriched HA may then be quantified using any suitable method. Suitable methods include, but are not limited to, mass-spectrometry, in particular isotope dilution mass spectrometry as used in 'immunocapture isotope dilution mass spectrometry' (Pierce *et al.*, *Analytical Chemistry*, 2011 dx.doi.org/10.1021/ac2006526) and HPLC.

Example 14: Design of an influenza vaccine which elicits a cross-protective immune response against future Group 1 influenza strains

Cross-reactive antibodies are used to define epitopes on haemagglutinin, which are structurally conserved across different viral subtypes. Such epitopes are then used to design a vaccine, which when administered to patients elicits a protective immune response against future viral subtypes so eliminating the need for repeated seasonal vaccination against a constantly evolving influenza virus.

Mapping the epitope of a cross-reactive antibody of this invention is performed by co-crystallisation of the cross-reactive antibody in complex with haemagglutinin and subsequent X-ray crystallography. Alternatively, the cross-reactive antibody is constructed as a gene

fragment phage display library derived from the HA gene of an influenza virus such as a pandemic H1N1 (e.g. A/California/07/2009). The H1-HA gene is synthesised, digested with DNAase into fragments and cloned into the phage display vector pNIBS-1. This gene fragment phage-displayed library is then selected with purified cross-reactive single domain antibodies described in this invention. Sequence analysis of the selected gene fragments is then used to delineate the cross-neutralising epitope(s) and map them against available HA sequences in the public databases to determine the extent of conservation across all 16 HA subtypes. For more 'fine-tuned' epitope mapping, alanine scanning mutagenesis of the HA gene fragment displayed on phage is used followed by screening for loss of specific antibody binding whilst preserving display on phage. The minimal haemagglutinin gene fragment which can generate a correctly folded product corresponding to the cross-reactive antibody epitope is then expressed and purified in a heterologous gene expression system or synthesised as a peptide using established chemical procedures. This recombinant HA epitope is then used to immunise BALB/c mice and to protect from lethal challenge with different viral strains including H5N1 and H1N1 viral sub-types.

Alternatively, the cross-reactive antibodies of the invention can be used to isolate anti-idiotypic antibodies which represent a structural mimic of the relevant cross-reactive epitope on haemagglutinin. Such anti-idiotypic antibodies when used as a vaccine elicit an immune response which cross-reacts with relevant epitopes on viral haemagglutinin. An advantage of using the single domain VHH antibodies described in this invention is that they are structurally much simpler than a conventional antibody, which requires the stable association of both a VH and VL domain to form the paratope. By way of example, a cross-reactive single domain antibody (e.g. R1a-B6) is used to immunise an alpaca as in Example 1 and a phage display antibody library is constructed as in Example 2. The library is then selected on the initial cross-reactive antibody used for immunisations (Example 4). Anti-idiotypic antibodies are described as those which bind to the cross-reactive antibody (R1a-B6) used for immunisation and are negative for other antibodies carrying different CDR1, CDR2 and CDR3 sequences but having similar framework regions.

CLAIMS

1. A composition comprising a single domain camelid antibody and a pharmaceutically acceptable additive, wherein said single domain antibody binds to the conserved stalk region of haemagglutinin and is cross-reactive for multiple influenza virus subtypes, including H1N1, wherein said antibody comprises:

(a)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 3 or 22; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 41 or 60; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 79 or 98;

(b)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 4 or 23; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 42 or 61; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 80 or 99;

(c)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 6 or 25; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 44 or 63; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 82 or 101;

(d)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 10 or 29; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 48 or 67; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 86 or 105;
or

(e)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 15 or 34; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 53 or 72; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 91 or 110.

2. The composition of claim 1, wherein said single domain camelid antibody demonstrates antigenic cross-reactivity to H1 and H5 influenza virus subtypes.

3. The composition of claim 1, wherein said single domain camelid antibody demonstrates antigenic cross-reactivity to H2 influenza virus subtypes.

4. The composition of claim 1, wherein said single domain camelid antibody demonstrates antigenic cross-reactivity to H9 influenza virus subtypes, and wherein said antibody comprises:

(a)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 3 or 22; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 41 or 60; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 79 or 98;

- (b)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 4 or 23; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 42 or 61; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 80 or 99; or
- (c)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 10 or 29; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 48 or 67; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 86 or 105.
5. The composition of claim 1, wherein said single domain camelid antibody comprises an amino acid sequence selected from SEQ ID NOS: 124, 117, 118, 120 and 129.
6. A composition comprising two or three or four or more covalently linked single domain camelid antibodies and a pharmaceutically acceptable additive, wherein said two or three or four or more single domain camelid antibodies are the same or different, and each is a single domain antibody as defined in claim 1.
7. The composition of claim 1, wherein said single domain camelid antibody comprises a humanized framework region.
8. The composition of claim 1, wherein said single domain antibody is a human antibody that lacks one or more human V_H amino acid sequence, and in place thereof consists of or comprises one or more CDR3 V_{HH} amino acid sequence as defined in claim 1.
9. The composition of claim 1, wherein said single domain camelid antibody demonstrates neutralising activity against a H1 influenza virus.
10. The composition of claim 9, wherein the H1 influenza virus is H1N1.
11. The composition of claim 9 or 10, wherein the H1 influenza strain is one or more of A/California/06/09, A/California/7/2009 and A/Brisbane/59/2007.
12. The composition of claim 1, wherein said single domain camelid antibody demonstrates neutralising activity against a H5 influenza virus.
13. The composition of claim 12, wherein the H5 influenza virus is H5N1 or H5N3.

14. The composition of claim 12 or 13, wherein the H5 influenza virus strain is one or more of A/Vietnam/1203/2004, A/Vietnam/1194/04, A/Vietnam/119/2004, HongKong/213/2003, A/turkey/Turkey/1/2005, A/Indonesia/05/2005, and A/Duck/Singapore-Q/119-3/97.

15. The composition of claim 1, wherein said single domain camelid antibody demonstrates neutralising activity against a H2 influenza virus.

16. The composition of claim 15, wherein the H2 influenza virus is H2N2 or H2N3.

17. The composition of claim 15 or 16, wherein the H2 influenza virus strain is one or more of A/Japan/305/1957 and A/Mallard/Eng/727/06.

18. The composition of claim 1, wherein said single domain camelid antibody demonstrates neutralising activity against a H9 influenza virus, and wherein said antibody comprises:

- (a)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 3 or 22; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 41 or 60; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 79 or 98;
- (b)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 4 or 23; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 42 or 61; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 80 or 99; or
- (c)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 10 or 29; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 48 or 67; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 86 or 105.

19. The composition of claim 18, wherein the H9 influenza virus is H9N2.

20. The composition of claim 18 or 19, wherein the H9 influenza strain is A/HongKong/1073/99.

21. The composition of claim 1, wherein said single domain camelid antibody demonstrates neutralising activity against one, or more, or all Group I influenza viruses selected from:

- (A) the group consisting of H1, H2 and H5 influenza viruses; or

(B) the group consisting of H1, H2, H5 and H9 influenza viruses, wherein said antibody comprises:

(a)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 3 or 22; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 41 or 60; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 79 or 98;

(b)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 4 or 23; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 42 or 61; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 80 or 99; or

(c)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 10 or 29; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 48 or 67; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 86 or 105.

22. An composition according to any one of Claims 1 to 21, for use in preventing or treating infection with an influenza virus selected from:

(A) the group consisting of H1, H2 and H5 influenza viruses; or

(B) the group consisting of H1, H2, H5 and H9 influenza viruses, wherein said antibody comprises:

(a)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 3 or 22; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 41 or 60; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 79 or 98;

(b)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 4 or 23; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 42 or 61; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 80 or 99; or

(c)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 10 or 29; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 48 or 67; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 86 or 105.

23. Use of an antibody as defined in any one of Claims 1 to 21 as a reagent:

to detect the presence of an influenza virus subtype selected from:

- (A) H1 and H5 subtypes;
- (B) H1 and H5 and H2 subtypes; and
- (C) H1 and H5 and H2 and H9 subtypes, wherein said antibody comprises:
 - (a)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 3 or 22; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 41 or 60; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 79 or 98;
 - (b)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 4 or 23; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 42 or 61; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 80 or 99; or
 - (c)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 10 or 29; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 48 or 67; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 86 or 105.

24. Use of an antibody as defined in any one of Claims 1 to 21 as a reagent:

- (A) in an *in vitro* assay for assessing the potency of an influenza virus vaccine against an influenza virus subtype selected from H1, H5, H2 and/or H9;
- (B) in an assay for assessing the cross-reactivity of an influenza virus vaccine against influenza virus subtypes selected from H1, H5, H2 and/or H9;
- (C) to concentrate and/ or quantify the amount of H1, H5, H2 or H9 haemagglutinin present in a sample;
- (D) to isolate anti-idiotypic H1, H5, H2 or H9 haemagglutinin antibodies; or
- (E) *in vitro* to design a universal influenza vaccine against influenza virus subtypes H1, H5, H2 and H9;

wherein if the influenza virus subtype is H9, said antibody comprises:

- (a)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 3 or 22; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 41 or 60; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 79 or 98;

(b)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 4 or 23; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 42 or 61; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 80 or 99; or
(c)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 10 or 29; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 48 or 67; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 86 or 105.

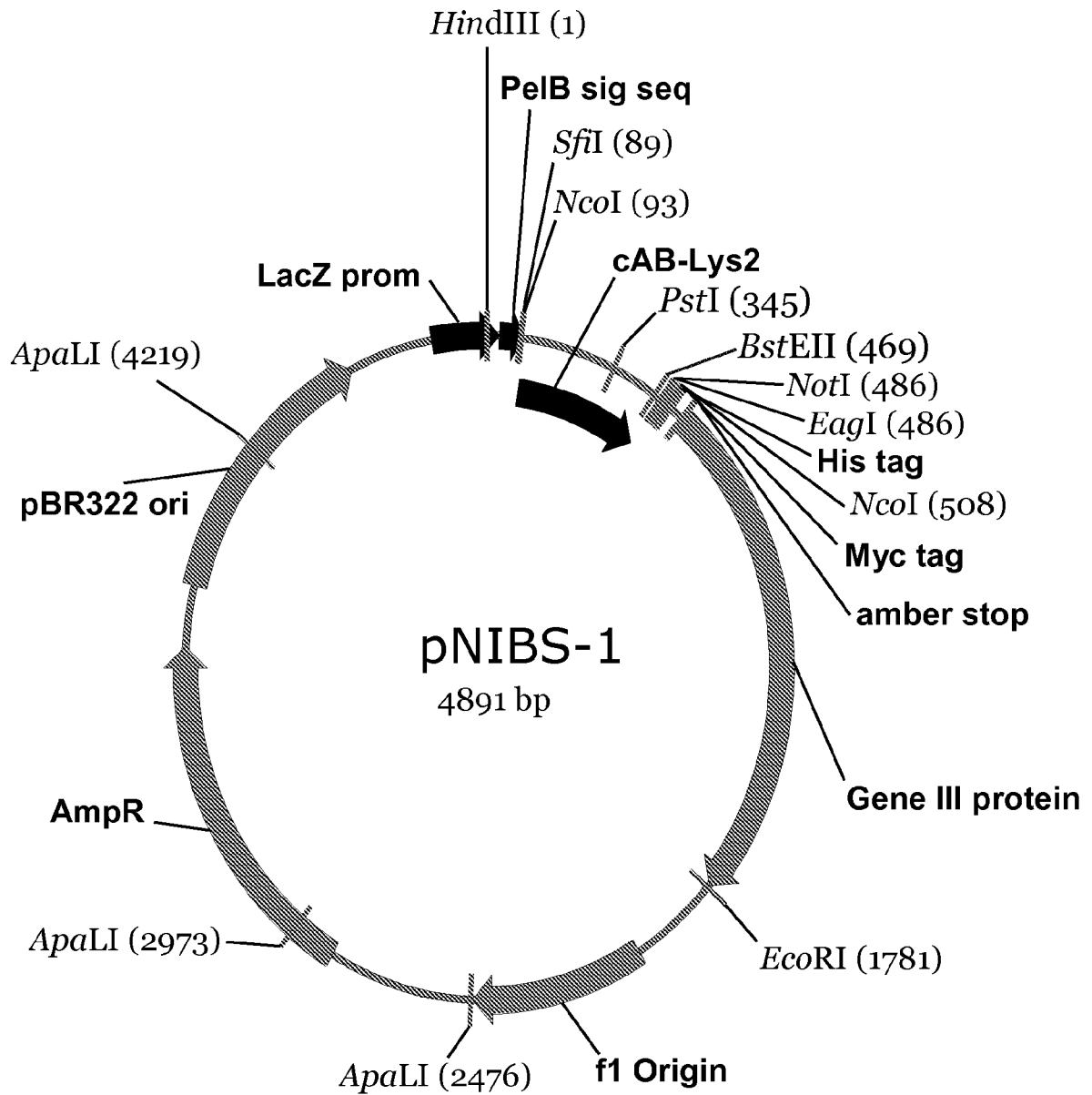
25. A composition comprising a single domain camelid antibody and a pharmaceutically acceptable additive, wherein said single domain antibody binds to the conserved stalk region of haemagglutinin and is cross-reactive for multiple influenza virus subtypes, including H1N1, wherein said antibody comprises:

(a)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 7 or 26; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 45 or 64; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 83 or 102;
(b)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 13 or 32; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 51 or 70; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 89 or 108;
or
(c)(i) a CDR3 which consists of the amino acid sequence of SEQ ID NO: 14 or 33; (ii) a CDR2 which consists of the amino acid sequence of SEQ ID NO: 52 or 71; and (iii) a CDR1 sequence which consists of the amino acid sequence of SEQ ID NO: 90 or 109;

wherein optionally said single domain camelid antibody comprises an amino acid sequence selected from SEQ ID NOS: 121, 127 and 128.

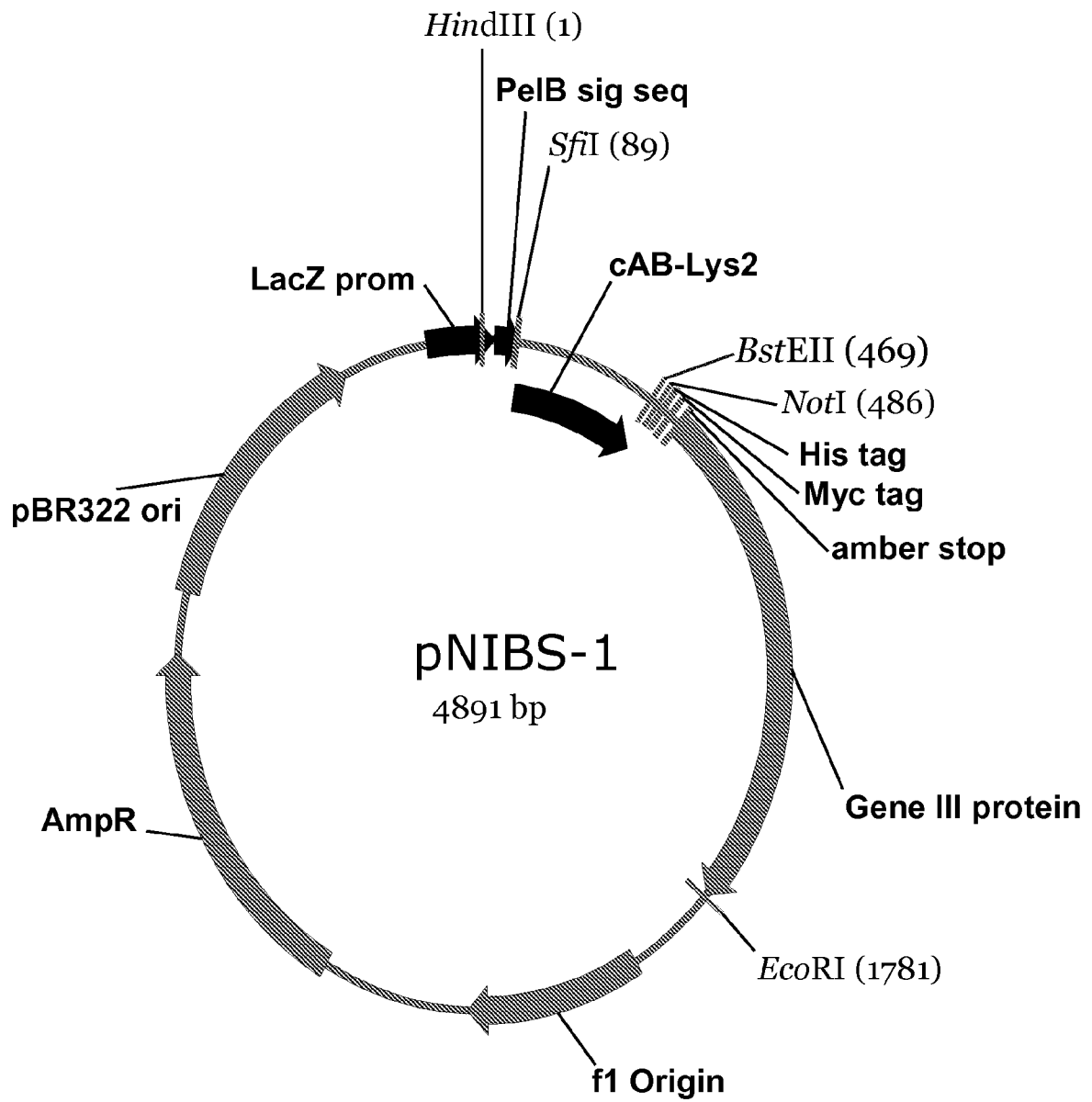
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Figure 1a



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Figure 1b



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Figure 2

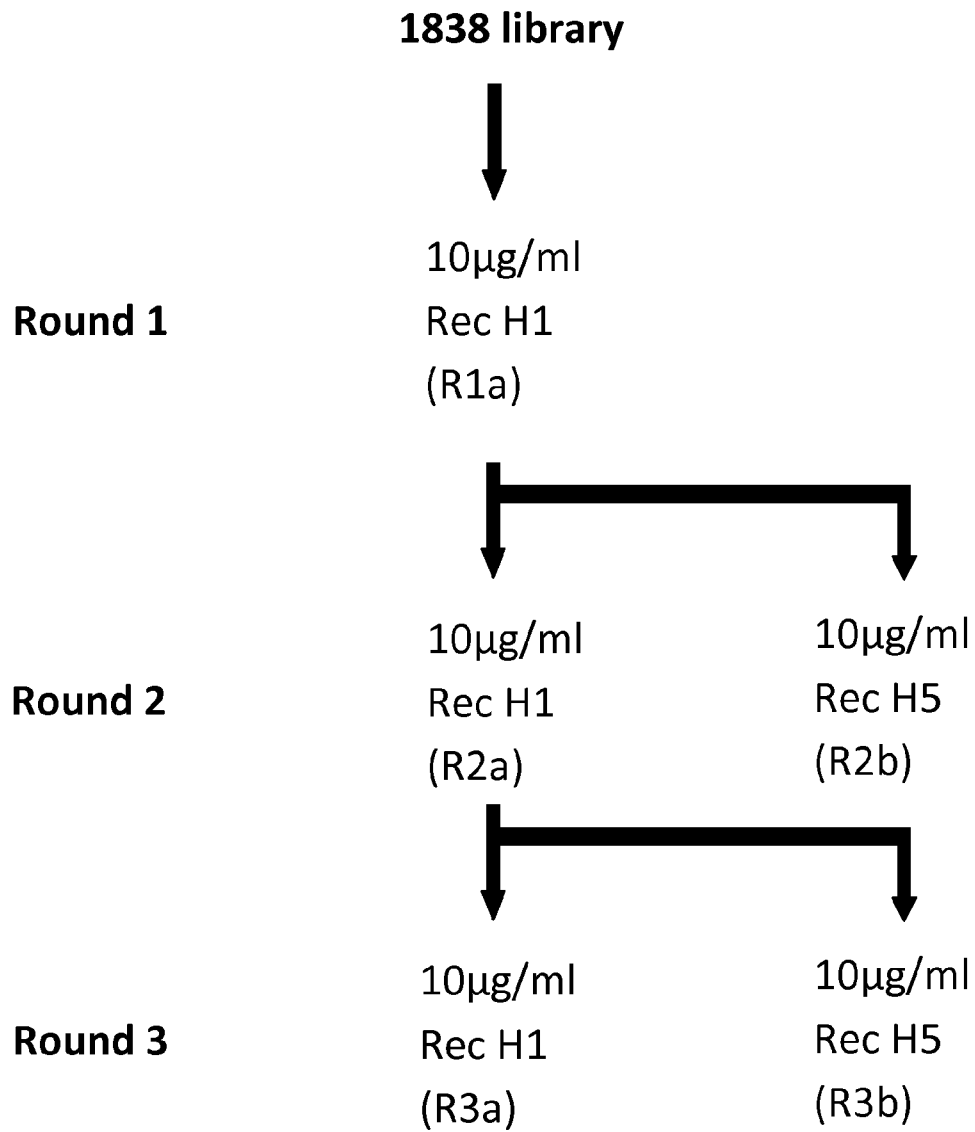
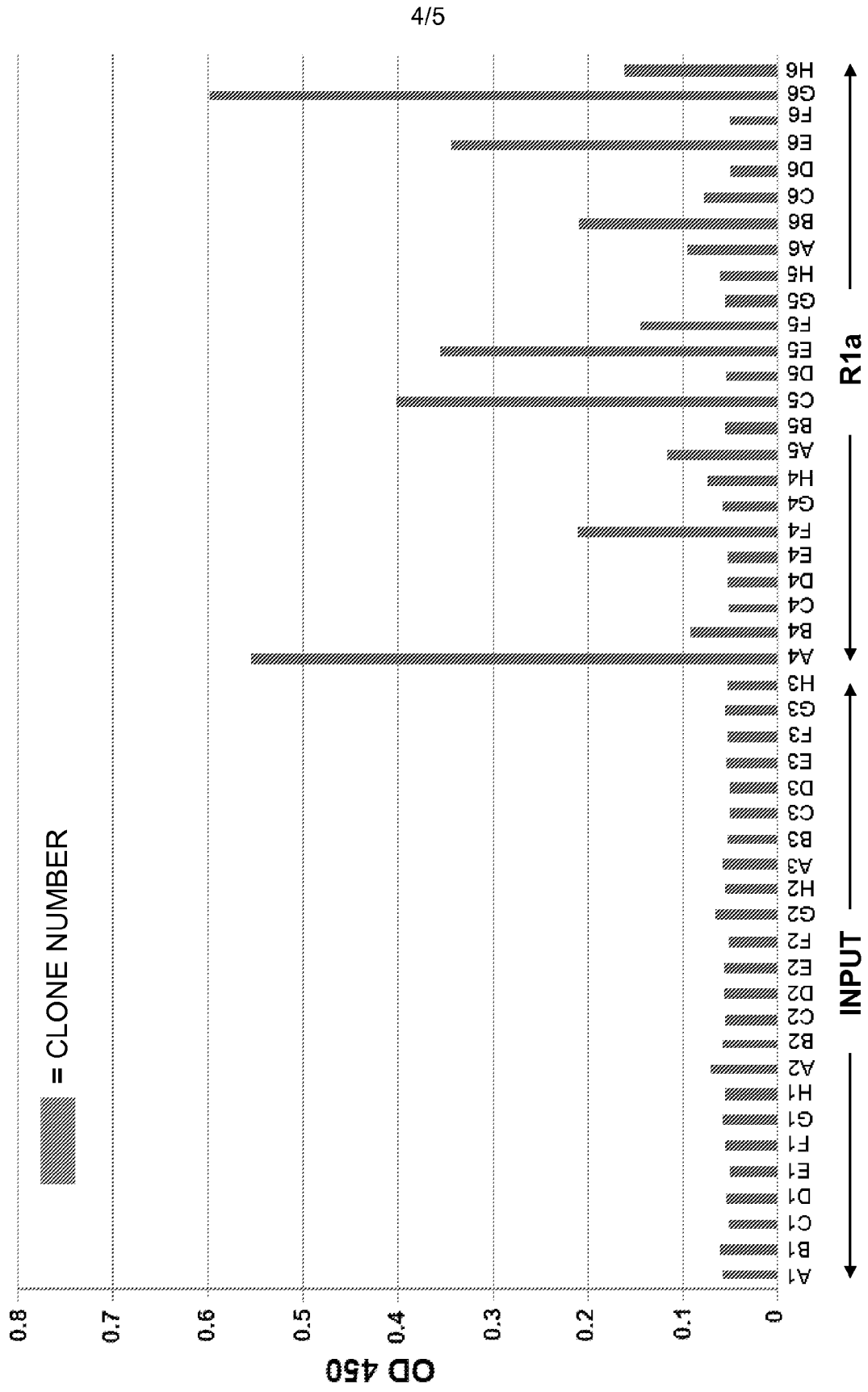


Figure 3 – Part 1



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Figure 3 - Part 2

