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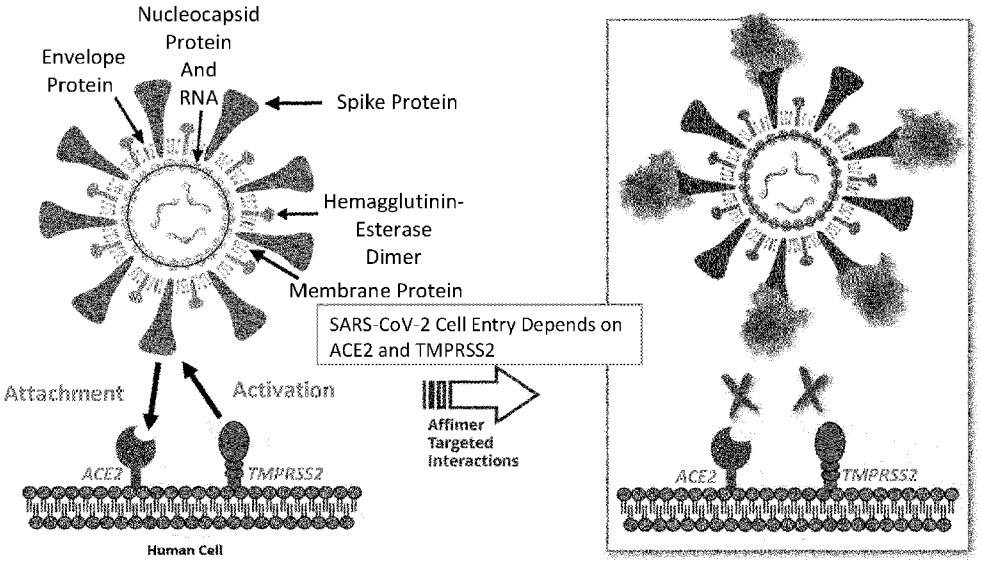
(54) Title: SARS-COV2 DIAGNOSTIC POLYPEPTIDES AND METHODS

FIGURE 1



(57) Abstract: The invention relates to a polypeptide comprising a) amino acid sequence having at least 80% sequence identity to SEQ ID NO: 3, or b) amino acid sequence having at least 80% sequence identity to SEQ ID NO: 2; wherein said polypeptide further comprises a first heterologous peptide insertion having a first amino acid sequence, and wherein said polypeptide further comprises a second heterologous peptide insertion having a second amino acid sequence, wherein said first and second amino acid sequences are selected from the group as defined. The invention also relates to methods, systems, solid phase substrates, and magnetic agarose beads comprising same.

[Continued on next page]

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SARS-COV2 DIAGNOSTIC POLYPEPTIDES AND METHODS

BACKGROUND TO THE INVENTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiological agent of the coronavirus induced disease 19 (COVID-19) that emerged in China late 2019 and is causing a pandemic. As of 19 April 2020, 2,241,778 cases have been reported worldwide, of which 152,551 (6.8%) succumbed to the infection. SARS-CoV-2 belongs to the Sarbecovirus subgenus (genus Betacoronavirus, family Coronaviridae) together with SARS-CoV that emerged in 2002 causing ~8000 infections with a lethality of 10%. Both viruses crossed species barriers from an animal reservoir and can cause a life-threatening respiratory illness in humans.

The surfaces of coronaviruses are decorated with a spike (S) glycoprotein, a large class I fusion protein. Mutated viruses with the D614G substitution in the S protein were isolated early in the pandemic and are now the most dominant form of SARS-CoV-2 worldwide. Further, mutated viruses with other mutations have recently been isolated. One example of such a mutation is the Y453F substitution in the S protein. Another example of a mutated SARS-CoV-2 virus that has recently been isolated is the B.1.1.7 variant. The spike protein forms a trimeric complex that can be functionally categorized into two distinct subunits, S1 and S2, that are separated by a protease cleavage site. The S1 subunit contains the receptor-binding domain (RBD), which interacts with a host-cell receptor protein to trigger membrane fusion. The S2 subunit contains the membrane fusion machinery, including the hydrophobic fusion peptide and the α-helical heptad repeats. The functional host cell receptors for SARS-CoV-1 and MERS-CoV are angiotensin converting enzyme 2 (ACE2) and dipeptidyl peptidase 4 (DPP4), respectively. The interactions between these receptors and their respective RBDs have been thoroughly characterized, both structurally and biophysically. Recently, it has been reported that SARS-CoV-2 spike protein also makes use of ACE2 as a functional host-cell receptor, and several structures of this complex have already been reported.

The World Health Organisation recently highlighted the need for the development of rapid tests to quickly diagnose COVID-19 at point-of-care to assist in limiting and tracking infections. Serology tests ('antibody tests') are not suitable because they can only indicate an exposure to virus which may be historic, and so they do not reliably indicate current infection. Existing antigen tests are not suitable for screening large numbers of people for the infection as they are laboratory based and/or it can take up to several days to get the results.

Thus there is a need in the art for an effective test to determine presence of the SARS-CoV-2 virus.

Presently, no approved targeted therapeutics are available for SARS-CoV-2. Monoclonal antibodies targeting vulnerable sites on viral surface proteins are increasingly recognized as a promising class of drugs against infectious diseases and have shown therapeutic efficacy for a number of viruses. Those coronavirus-neutralizing antibodies being tauted in the art primarily target the SPIKE glycoprotein on the viral surface and are thought to inhibit entry into host cells by that mechanism. However, the physiochemical features of antibodies, such as the multiple chain format and post-translational modification, along with the potential function of the Fc portion of those proteins makes impractical a variety of targeted delivery techniques, including pulmonary delivery or delivery of nucleic acid encoding the virus neutralizing antibodies when expressed and secreted in vivo.

Moreover, the SARS-CoV-2 virus is certainly only the next virus in what has been and will certainly be a continuing succession of virus that produce life threatening infections, including localized inflammation in vital organs – such as immune cell infiltration into the lungs of patients that can advance to acute respiratory distress syndrome and pulmonary fibrosis.

The present invention seeks to overcome problem(s) associated with the prior art.

SUMMARY OF THE INVENTION

Multiple highly specific polypeptides have now been generated that bind to the spike protein of the SARS-CoV-2 virus. Advantageously these do not cross-react with other very closely related viruses, such as SARS and MERS. Thus, the invention provides polypeptides (reagents) that detect the COVID-19 virus (i.e. detect the SARS-CoV-2 virus), including variants thereof including but not limited to variants comprising a mutation at position 614 and/or at position 453, such as the D614G and/or the Y453F substitution. The invention further provides polypeptides that detect the SARS-CoV-2 virus, including variants thereof such as the B.1.1.7 variant. The invention further provides polypeptides that detect variants of the SARS-CoV-2 virus comprising mutations at positions including but not limited to 69, 70, 144, 501, 570 and/or 681, such as the HV 69-70 deletion, the Y144 deletion, the N501Y substitution, the A570D substitution and/or the P681H substitution. The invention encompasses all polypeptides as defined in the claims that can bind to SARS-CoV-2 virus and variant strains thereof. The invention is based on these surprisingly effective polypeptides. The

invention also provides tests incorporating these polypeptides. These tests include ELISA tests and BAMS tests. These tests also advantageously include portable/point-of-care tests such as test strips/lateral flow devices (LFD) which incorporate these polypeptides. Further, the polypeptides according to the invention incorporated into an LFD show excellent clinical performance, achieving clinical sensitivity (Ct ≤27) of 100.0%, clinical sensitivity (Ct < 31) of 98.0% and clinical specificity of 99.0%. The polypeptides according to the invention have excellent cross-reactivity profiles and excellent microbial interference profiles. Specifically, no cross-reactivity was detected with 22 common respiratory pathogens on the LFD platform Avacta® AffiDX® SARS-CoV-2 Lateral Flow Antigen Test. No microbial interference was detected when merging 22 common respiratory pathogens with SARS-CoV-2 on the LFD platform Avacta® AffiDX® SARS-CoV-2 Lateral Flow Antigen Test. Thus, the invention provides highly specific polypeptides that bind to SARS-CoV-2 virus and variant strains thereof that are SARS-CoV-2 strains but comprise mutations relative to a SARS-CoV-2 sequence referenced herein. The invention also provides tests that incorporate these polypeptides, which demonstrate excellent clinical performance, excellent cross-reactivity profiles and excellent microbial interference profiles.

Thus in one aspect the invention provides a polypeptide comprising

a) amino acid sequence having at least 80% sequence identity to SEQ ID NO: 3, or

b) amino acid sequence having at least 80% sequence identity to SEQ ID NO: 2;

wherein said polypeptide further comprises a first heterologous peptide insertion having a first amino acid sequence, and

wherein said polypeptide further comprises a second heterologous peptide insertion having a second amino acid sequence,

wherein said first and second amino acid sequences are selected from the group consisting of: SEQ ID NO: 5 and SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9, SEQ ID NO: 11 and SEQ ID NO: 12, SEQ ID NO: 14 and SEQ ID NO: 15, SEQ ID NO: 17 and SEQ ID NO: 18, SEQ ID NO: 20 and SEQ ID NO: 21, SEQ ID NO: 23 and SEQ ID NO: 24, SEQ ID NO: 26 and SEQ ID NO: 27, SEQ ID NO: 29 and SEQ ID NO: 30, SEQ ID NO: 32 and SEQ ID NO: 33, SEQ ID NO: 35 and SEQ ID NO: 36, SEQ ID NO: 38 and SEQ ID NO: 39, SEQ ID NO: 41 and SEQ ID NO: 42, SEQ ID NO: 44 and SEQ ID NO: 45, SEQ ID NO: 47 and SEQ ID NO: 48, SEQ ID NO: 50 and SEQ ID NO: 51, SEQ ID NO: 53 and SEQ ID NO: 54, SEQ ID NO: 56 and SEQ ID NO: 57, SEQ ID NO: 59 and SEQ ID NO: 60, SEQ ID NO: 62 and SEQ ID NO: 63, SEQ ID NO: 65 and SEQ ID NO: 66, SEQ ID NO: 68 and SEQ ID NO: 69, SEQ ID NO: 71 and SEQ ID NO: 72, SEQ ID

NO: 74 and SEQ ID NO: 75, SEQ ID NO: 77 and SEQ ID NO: 78, SEQ ID NO: 80 and SEQ ID NO: 81, SEQ ID NO: 83 and SEQ ID NO: 84, SEQ ID NO: 86 and SEQ ID NO: 87, SEQ ID NO: 89 and SEQ ID NO: 90, SEQ ID NO: 92 and SEQ ID NO: 93, SEQ ID NO: 95 and SEQ ID NO: 96, SEQ ID NO: 98 and SEQ ID NO: 99, SEQ ID NO: 101 and SEQ ID NO: 102, SEQ ID NO: 104 and SEQ ID NO: 105, SEQ ID NO: 107 and SEQ ID NO: 108, SEQ ID NO: 110 and SEQ ID NO: 111, SEQ ID NO: 113 and SEQ ID NO: 114, SEQ ID NO: 116 and SEQ ID NO: 117, SEQ ID NO: 119 and SEQ ID NO: 120, SEQ ID NO: 122 and SEQ ID NO: 123, SEQ ID NO: 125 and SEQ ID NO: 126, SEQ ID NO: 128 and SEQ ID NO: 129, SEQ ID NO: 131 and SEQ ID NO: 132, SEQ ID NO: 134 and SEQ ID NO: 135, SEQ ID NO: 137 and SEQ ID NO: 138, SEQ ID NO: 140 and SEQ ID NO: 141, SEQ ID NO: 143 and SEQ ID NO: 144, SEQ ID NO: 146 and SEQ ID NO: 147, SEQ ID NO: 149 and SEQ ID NO: 150, SEQ ID NO: 152 and SEQ ID NO: 153, SEQ ID NO: 155 and SEQ ID NO: 156, SEQ ID NO: 158 and SEQ ID NO: 159, SEQ ID NO: 161 and SEQ ID NO: 162, SEQ ID NO: 164 and SEQ ID NO: 165, SEQ ID NO: 167 and SEQ ID NO: 168, SEQ ID NO: 170 and SEQ ID NO: 171, or SEQ ID NO: 173 and SEQ ID NO: 174.

For the avoidance of doubt, suitably each pair is selected simultaneously (i.e. together). In other words suitably said first and second amino acid sequences comprise a pair of amino acid sequences, each pair being selected from the pairs disclosed in said group.

Suitably said polypeptide as described above comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 7 (620_826257), SEQ ID NO: 67 (620_825425), and SEQ ID NO: 70 (620_825436).

In one embodiment suitably said polypeptide as described above comprises

a) amino acid sequence having at least 80% sequence identity to SEQ ID NO: 3,

and wherein said first and second amino acid sequences are selected from the group consisting of: SEQ ID NO: 5 and SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9, SEQ ID NO: 11 and SEQ ID NO: 12, SEQ ID NO: 14 and SEQ ID NO: 15, SEQ ID NO: 17 and SEQ ID NO: 18, SEQ ID NO: 20 and SEQ ID NO: 21, SEQ ID NO: 23 and SEQ ID NO: 24, SEQ ID NO: 26 and SEQ ID NO: 27, SEQ ID NO: 29 and SEQ ID NO: 30, SEQ ID NO: 32 and SEQ ID NO: 33, SEQ ID NO: 35 and SEQ ID NO: 36, SEQ ID NO: 38 and SEQ ID NO: 39, SEQ ID NO: 41 and SEQ ID NO: 42, SEQ ID NO: 44 and SEQ ID NO: 45, SEQ ID NO: 47 and SEQ ID NO: 48, SEQ ID NO: 50 and SEQ ID NO: 51, SEQ ID NO: 53 and SEQ ID NO: 54, SEQ ID NO: 56 and SEQ ID NO: 57, SEQ ID NO: 59 and SEQ ID NO: 60, SEQ ID NO: 62 and SEQ ID NO: 63.

Suitably said polypeptide comprises the amino acid sequence of SEQ ID NO: 7 (620_826257).

Suitably said polypeptide is biotinylated.
Suitably said first heterologous peptide is inserted into the polypeptide at position 48-<heterologous peptide>-50
and said second heterologous peptide is inserted into the polypeptide at position 73-<heterologous peptide>-78 relative to SEQ ID NO: 1.
These polypeptides comprise mammalian based scaffold backbone.

In one embodiment suitably said polypeptide as described above comprises
b) amino acid sequence having at least 80% sequence identity to SEQ ID NO: 2,
and wherein said first and second amino acid sequences are selected from the group consisting of: SEQ ID NO: 65 and SEQ ID NO: 66, SEQ ID NO: 68 and SEQ ID NO: 69, SEQ ID NO: 71 and SEQ ID NO: 72, SEQ ID NO: 74 and SEQ ID NO: 75, SEQ ID NO: 77 and SEQ ID NO: 78, SEQ ID NO: 80 and SEQ ID NO: 81, SEQ ID NO: 83 and SEQ ID NO: 84, SEQ ID NO: 86 and SEQ ID NO: 87, SEQ ID NO: 89 and SEQ ID NO: 90, SEQ ID NO: 92 and SEQ ID NO: 93, SEQ ID NO: 95 and SEQ ID NO: 96, SEQ ID NO: 98 and SEQ ID NO: 99, SEQ ID NO: 101 and SEQ ID NO: 102, SEQ ID NO: 104 and SEQ ID NO: 105, SEQ ID NO: 107 and SEQ ID NO: 108, SEQ ID NO: 110 and SEQ ID NO: 111, SEQ ID NO: 113 and SEQ ID NO: 114, SEQ ID NO: 116 and SEQ ID NO: 117, SEQ ID NO: 119 and SEQ ID NO: 120, SEQ ID NO: 122 and SEQ ID NO: 123, SEQ ID NO: 125 and SEQ ID NO: 126, SEQ ID NO: 128 and SEQ ID NO: 129, SEQ ID NO: 131 and SEQ ID NO: 132, SEQ ID NO: 134 and SEQ ID NO: 135, SEQ ID NO: 137 and SEQ ID NO: 138, SEQ ID NO: 140 and SEQ ID NO: 141, SEQ ID NO: 143 and SEQ ID NO: 144, SEQ ID NO: 146 and SEQ ID NO: 147, SEQ ID NO: 149 and SEQ ID NO: 150, SEQ ID NO: 152 and SEQ ID NO: 153, SEQ ID NO: 155 and SEQ ID NO: 156, SEQ ID NO: 158 and SEQ ID NO: 159, SEQ ID NO: 161 and SEQ ID NO: 162, SEQ ID NO: 164 and SEQ ID NO: 165, SEQ ID NO: 167 and SEQ ID NO: 168, SEQ ID NO: 170 and SEQ ID NO: 171, or SEQ ID NO: 173 and SEQ ID NO: 174.
Suitably said polypeptide comprises SEQ ID NO: 67 (620_825425) or SEQ ID NO: 70 (620_825436).
Suitably said first heterologous peptide is inserted into the polypeptide at position 46-<heterologous peptide>-47 and said second heterologous peptide is inserted into the polypeptide at position 71-<heterologous peptide>-72 relative to SEQ ID NO: 2.
These polypeptides comprise plant based scaffold backbone.

Suitably the polypeptide as described above comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 7, SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 25, SEQ ID NO: 28, SEQ ID NO:

31, SEQ ID NO: 34, SEQ ID NO: 37, SEQ ID NO: 40, SEQ ID NO: 43, SEQ ID NO: 46, SEQ ID NO: 49, SEQ ID NO: 52, SEQ ID NO: 55, SEQ ID NO: 58, SEQ ID NO: 61, SEQ ID NO: 64, SEQ ID NO: 67, SEQ ID NO: 70, SEQ ID NO: 73, SEQ ID NO: 76, SEQ ID NO: 79, SEQ ID NO: 82, SEQ ID NO: 85, SEQ ID NO: 88, SEQ ID NO: 91, SEQ ID NO: 94, SEQ ID NO: 97, SEQ ID NO: 100, SEQ ID NO: 103, SEQ ID NO: 106, SEQ ID NO: 109, SEQ ID NO: 112, SEQ ID NO: 115, SEQ ID NO: 118, SEQ ID NO: 121, SEQ ID NO: 124, SEQ ID NO: 127, SEQ ID NO: 130, SEQ ID NO: 133, SEQ ID NO: 136, SEQ ID NO: 139, SEQ ID NO: 142, SEQ ID NO: 145, SEQ ID NO: 148, SEQ ID NO: 151, SEQ ID NO: 154, SEQ ID NO: 157, SEQ ID NO: 160, SEQ ID NO: 163, SEQ ID NO: 166, SEQ ID NO: 169, SEQ ID NO: 172, or SEQ ID NO: 175.

These are sequences of exemplary polypeptides according to the present invention, each comprising two heterologous peptide insertions as is apparent from the sequences.

In another embodiment the invention relates to a polypeptide as described above further comprising at the C-terminal end of the polypeptide one of more of:

i) purification tag suitably HHHHHH (SEQ ID NO: 176);
ii) conjugation tag suitably C;
iii) detection tag suitably DYKDDDDK (SEQ ID NO: 177) and/or HA tag suitably YPYDVPDYA (SEQ ID NO: 179) and/or AviTag™ suitably GLNDIFEAQKIEWHE (SEQ ID NO: 189); and/or
iv) linker sequence suitably GGGGS (SEQ ID NO: 178).

More suitably these elements are in the order N-terminus-<polypeptide as described above>-<(detection tag suitably DYKDDDDK)>-<(linker sequence suitably GGGGS)>-<(purification tag suitably HHHHHH)>-<(conjugation tag suitably C)>-C-terminus

In another embodiment the invention relates to a polypeptide as described above further comprising an N-terminal Methionine (M).

In another embodiment the invention relates to a polypeptide as described above which binds SARS-CoV-2 S1 spike protein and variants thereof comprising mutations with an Equilibrium dissociation constant (K_D) value < 100 nM.

In another embodiment, the invention relates to a polypeptide as described above, which binds to SARS-CoV-2 virus variant B.1.1.7.

In some embodiments, the S1 spike protein of the SARS-CoV-2 virus comprises a mutation at position 614 and/or at position 453. In preferred embodiments, the SARS-CoV-2 S1 spike protein comprises a mutation at position 614 and at position 453. For example, the SARS-CoV-2 S1 spike protein may comprise the D614G substitution and/or the Y453F substitution. In preferred embodiments, the SARS-CoV-2 S1 spike protein comprises the Y453F substitution or both the D614G substitution and the Y453F substitution.

Thus, in some embodiments, a polypeptide according to the invention binds to a SARS-CoV-2 S1 spike protein comprising a mutation at position 614 and/or at position 453, preferably at position 453 or at both position 614 and at position 453.

For example, a polypeptide according to the invention may bind to a SARS-CoV-2 S1 spike protein comprising the D614G substitution and/or the Y453F substitution, preferably the Y453F substitution or both the D614G substitution and the Y453F substitution.

In some embodiments, the S1 spike protein comprises a mutation at one or more of the positions selected from H69, V70, Y144, N501, A570, and/or P681. In preferred embodiments, the SARS-CoV-2 S1 spike protein comprises mutations at positions H69, V70, Y144, N501, A570, and P681. For example, the SARS-CoV-2 S1 spike protein may comprise one or more of the HV 69-70 deletion, Y144 deletion, N501Y substitution, A570D substitution, and/or the P681H substitution. In preferred embodiments, the SARS-CoV-2 S1 spike protein comprises the HV 69-70 deletion, Y144 deletion, N501Y substitution, A570D substitution, and the P681H substitution.

Thus, in some embodiments, a polypeptide according to the invention binds to a SARS-CoV-2 S1 spike protein comprising a mutation at one or more of the positions selected from H69, V70, Y144, N501, A570, and/or P681, preferably at all of the positions H69, V70, Y144, N501, A570, and P681.

For example, a polypeptide according to the present invention may bind to a SARS-CoV-2 S1 spike protein comprising one or more of the HV 69-70 deletion, Y144 deletion, N501Y substitution, A570D substitution, and/or the P681H substitution. Preferably, a polypeptide according to the present invention may bind to a SARS-CoV-2 S1 spike protein comprising the HV 69-70 deletion, Y144 deletion, N501Y substitution, A570D substitution, and the P681H substitution.

In another embodiment the invention relates to a nucleic acid comprising nucleotide sequence encoding a polypeptide as described above.
Also provided is a vector comprising a nucleic acid as described above.
Also provided is a host cell comprising a nucleic acid as described above.

In another embodiment the invention relates to a method comprising the steps of:
i) contacting a sample with a polypeptide as described above;
ii) incubating to allow binding of said polypeptide to any SARS-CoV-2 virus present in said sample
iii) assaying for the presence of any polypeptide – SARS-CoV-2 bound complexes, wherein presence of any such complexes indicates a presence of SARS-CoV-2 virus in said sample.

Suitably said method is a method for detecting the presence of the SARS-CoV-2 virus in a sample.

In another embodiment the invention relates to a lateral flow assay device, wherein the lateral flow assay device comprises: (i) a sample receiving region; and (ii) a capture membrane operably connected to said sample receiving region downstream of said sample receiving region,
wherein said capture membrane comprises a test region comprising an immobilised first polypeptide as described above.

In another embodiment the invention relates to a lateral flow assay device as described above wherein said immobilised first polypeptide comprises one or more amino acid sequences selected from the group consisting of SEQ ID NO: 70 (620_825436) and SEQ ID NO: 67 (620_825425).

In another embodiment the invention relates to a lateral flow assay device as described above further comprising a conjugate pad located between said sample receiving region and said capture membrane, wherein said conjugate pad comprises a second polypeptide as described above joined to an indicator agent, and wherein said first and second polypeptides are different.

In another embodiment the invention relates to a lateral flow assay device as described above wherein said second polypeptide comprises SEQ ID NO: 7 (620_826257).

Suitably said second polypeptide is biotinylated.

In another embodiment the invention relates to a lateral flow assay device as described above, further comprising a control region comprising an immobilised reagent capable of specifically binding said second polypeptide as described above joined to an indicator agent.

Suitably said control region is positioned downstream of said test region

In another embodiment the invention relates to a system for detecting a presence of a component of interest in a sample, comprising:
(a) a lateral flow device as described above, and
(b) a liquid formulation of a second polypeptide as described above joined to an indicator agent, wherein said first and second polypeptides are different.

In another embodiment the invention relates to a lateral flow assay device as described above or a system as described above, wherein the lateral flow assay device further comprises a reservoir region positioned downstream of the capture membrane for absorbing an excess of fluid.

In another embodiment the invention relates to a method for detecting a presence of any SARS-CoV-2 virus in a liquid test sample, comprising the steps of:
(a) providing a lateral flow assay device as described above;
(b) applying the test sample to the sample receiving region
(c) either when the lateral flow assay device comprises a conjugate pad comprising a second polypeptide as described above joined to an indicator agent wherein said first and second polypeptides are different, allowing said sample to migrate through said conjugate pad to the capture membrane and incubating to allow virus-indicator complex to form; or when the lateral flow assay device does not comprise a conjugate pad, applying a liquid formulation of a second polypeptide as described above joined to an indicator agent, wherein said first and second polypeptides are different, to the sample receiving region and allowing said liquid formulation to migrate to the capture membrane and incubating to allow virus-indicator complex to form;
(d) allowing the virus-indicator complex to migrate through the capture membrane to the test region and contact the immobilised first polypeptide thereby immobilising the virus-indicator complex and forming a detectable signal, wherein the presence of the SARS-CoV-2 virus in the sample is indicated by the signal.

In another embodiment the invention relates to a method as described above wherein said first polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 70 (620_825436) and SEQ ID NO: 67 (620_825425), and wherein said second polypeptide comprises the amino acid sequence of SEQ ID NO: 7 (620_826257).
Suitably, said second polypeptide is biotinylated.

In another embodiment the invention relates to a magnetic agarose bead comprising a polypeptide as described above attached thereto.

In one embodiment the invention relates to a magnetic agarose bead comprising a polypeptide as described above attached thereto, wherein said polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 82 (620_825336), SEQ ID NO: 70 (620_825436), SEQ ID NO: 67 (620_825425) and SEQ ID NO: 7 (620_826257).
Suitably, said polypeptide comprises the amino acid sequence of SEQ ID NO: 82 (620_825336).

In one embodiment suitably said bead comprises two or more polypeptides, more suitably three or more polypeptides, more suitably four or more polypeptides, more suitably five or more polypeptides, or even more. Most suitably said bead comprises one polypeptide or two polypeptides or three polypeptides.
In one embodiment suitably the attachment is by passive absorption. In one embodiment suitably attachment of a polypeptide of the invention to the bead is covalent attachment. In one embodiment suitably the attachment is by a malemide-functionalised surface on the bead which captures polypeptide(s) of the invention (Affimer® Agent) by the free cysteines. In one embodiment suitably the attachment is by use of biotinylated polypeptide(s) of the invention (Affimer® Agents) and capturing those onto Streptavidin/Neutravidin functionalised beads.
Most suitably attachment to the bead is by binding to amine(s), most suitably primary amine(s), on the polypeptide(s) of the invention (Affimer® Agents). In this embodiment suitably the bead comprises an amine-reactive moiety for coupling of the polypeptide(s) of the invention thereto.

Suitably the magnetic agarose bead is about 375μm in diameter.

In another embodiment the invention relates to a method comprising:
a) contacting a magnetic agarose bead as described above with a sample from a subject
b) incubating to allow binding of the polypeptide to any SARS-CoV-2 virus in the sample
c) washing the magnetic agarose bead to remove unbound material
d) eluting bound material from the washed bead of (c)
e) analysing the eluted material of (d) by mass spectrometry (MS) to detect protein(s) or fragment(s) or ion(s) thereof originating from SARS-CoV-2 virus wherein detection of any such protein(s) or fragment(s) or ion(s) indicates presence of SARS-CoV-2 virus in said sample.

Suitably said mass spectrometry (MS) comprises matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

In another embodiment the invention relates to a solid phase substrate comprising immobilised polypeptide as described above.
Suitably the solid phase substrate may comprise one or more immobilised polypeptides.
Suitably said solid phase substrate is an ELISA plate.

In another embodiment the invention relates to a method comprising
i) providing an ELISA plate as described above,
ii) contacting said immobilised polypeptide with a test sample,
iii) incubating to allow binding of any SARS-CoV-2 virus to said immobilised polypeptide,
iv) washing to remove unbound material,
v) contacting the immobilised polypeptide – SARS-CoV-2 bound complex with a second polypeptide as described above joined to an indicator agent, wherein said immobilised polypeptide and said second polypeptide are different
vi) incubating to allow binding of said second polypeptide to the immobilised polypeptide – SARS-CoV-2 bound complex,
vii) washing to remove unbound material, and
viii) detecting presence of said second polypeptide joined to an indicator agent, wherein presence of same indicates presence of SARS-CoV-2 virus in the test sample.

Suitably more than one polypeptide may be immobilised.
Suitably more than one second polypeptide joined to an indicator agent may be used.

Suitably said method is an ELISA assay.

In another embodiment the invention relates to a solid phase substrate as described above, or a method as described above, wherein said immobilised polypeptide comprises amino acid sequence selected from the group consisting of SEQ ID NO: 70 (620_825436) and SEQ ID NO: 67 (620_825425).

In another embodiment the invention relates to a solid phase substrate as described above, or a method as described above, wherein said second polypeptide comprises amino acid sequence of SEQ ID NO: 7 (620_826257).
Suitably, said second polypeptide is biotinylated.

Suitably the sample comprises, or consists of, saliva, or comprises, is derived from, or consists of, an anterior nasal swab.

Suitably the polypeptide is an Affimer® Agent or an Affimer® polypeptide. Affimer® Agents/ Affimer® polypeptides are described in more detail below.

Suitably, in any method of the present invention, the S1 spike protein of the SARS-CoV-2 virus comprises a mutation at position 614 and/or at position 453, for example the D614G substitution and/or the Y453F substitution.

Suitably, in any method of the present invention, the polypeptide binds to SARS-CoV-2 virus variant B.1.1.7.

Suitably, in any method of the present invention, the S1 spike protein of the SARS-CoV-2 virus comprises a mutation at one or more of the positions selected from H69, V70, Y144, N501, A570, and/or P681, for example one or more of the HV 69-70 deletion, Y144 deletion, N501Y substitution, A570D substitution, and/or P681H substitution.

In some aspects, the present disclosure provides a protein comprising an Affimer® Agent having a COVID (SARS-CoV-2) binding Affimer® polypeptide sequence which binds to SPIKE with a Kd of 1×10^{-6}M or less and inhibits infection of human cells by the coronaviridae particle to which the Affimer® Agent is bound. In certain embodiments, the Affimer® Agent binds to the viral particle and inhibits or reduces the rate of infection of cells by the virus, preferably human cells. In certain embodiments, the coronaviridae particle is a SARS-CoV-2 viral particle.

In some aspects, the present disclosure provides a protein comprising an Affimer® Agent having a COVID (SARS-CoV-2) binding Affimer® polypeptide sequence which binds to SPIKE with a Kd of 1×10^{-6}M or less and inhibits interaction of the SPIKE protein to which it is bound with ACE2.

In some aspects, the present disclosure provides a protein comprising an Affimer® Agent having a COVID (SARS-CoV-2) binding Affimer® polypeptide sequence which binds to SPIKE with a Kd of 1×10^{-6}M or less and inhibits interaction of the SPIKE protein to which it is bound with TMPRSS2.

In some aspects, the present disclosure provides a protein comprising an Affimer® Agent having a COVID (SARS-CoV-2) binding Affimer® polypeptide sequence which binds to SPIKE with a Kd of 1×10^{-6}M or less and inhibits interaction of the SPIKE protein to which it is bound with dipeptidylpeptidase 4 (DPP4).

In certain embodiments, the Affimer® Agent binds to the SPIKE protein on coronaviridae particles and inhibits or reduces the rate of infection of cells by the virus, preferably human cells. In certain embodiments, the viral particle is a SARS-CoV-2 viral particle.

In some embodiments, the Affimer® Agent binds viral SPIKE and blocks interactions with human ACE2.

In some embodiments, the Affimer® Agent binds to viral SPIKE with a Kd of 1×10^{-7}M or less, Kd of 1×10^{-8}M or less, Kd of 1×10^{-9}M or less, or even a Kd of 1×10^{-10}M or less. In some embodiments, the COVID (SARS-CoV-2) binding Affimer® polypeptide binds to viral SPIKE with a K_{off} of 10^{-3} s^{-1} or slower, 10^{-4} s^{-1} or slower, or even 10^{-5} s^{-1} or slower. In some embodiments, the Affimer® Agent binds to viral SPIKE with a K_{on} of 10^{3} $M^{-1}s^{-1}$ or faster, 10^{4} $M^{-1}s^{-1}$ or faster, 10^{5} $M^{-1}s^{-1}$ or faster, or even 10^{6} $M^{-1}s^{-1}$ or faster. In some embodiments, the Affimer® Agent binds to viral SPIKE with an IC50 in a competitive binding assay with human ACE2 of 1 μM or less, 100 nM or less, 40 nM or less, 20 nM or less, 10 nM or less, 1 nM or less, or even 0.1 nM or less.

In certain embodiments, the Affimer® Agent has a single COVID (SARS-CoV-2) binding Affimer® sequence.

In other embodiments, the Affimer® Agent includes at least two or even three COVID (SARS-CoV-2) binding Affimer® sequence, which may bind the same or different sites on the viral SPIKE protein. In certain embodiments, Affimer® Agent is mutliparatopic, such as biparatopic or triparatopic, having indepdently selected COVID (SARS-CoV-2) binding Affimer® sequences that can simulataneous bind two or more different sites on the same SPIKE protein. In certain embodiments, Affimer® Agent can simulataneous bind two or more different SPIKE proteins on the same viral particle. In some embodiments, the Affimer® Agent has one or more COVID (SARS-CoV-2) binding Affimer® sequence each independently having an amino acid sequence represented in general formula (I)

$FR1\text{-}(Xaa)_n\text{-}FR2\text{-}(Xaa)_m\text{-}FR3$ **(I)**

wherein

FR1 is a polypeptide sequence represented by MIPGGLSEAK PATPEIQEIV DKVKPQLEEK TNETYGKLEA VQYKTQVLA **(SEQ ID NO: 186)** or a polypeptide sequence having at least 70% homology thereto;

FR2 is a polypeptide sequence represented by GTNYYIKVRA GDNKYMHLKV FKSL **(SEQ ID NO: 187)** or a polypeptide sequence having at least 70% homology thereto;

FR3 is a polypeptide sequence represented by EDLVLTGYQV DKNKDDELTG F **(SEQ ID NO: 188)** or a polypeptide sequence having at least 70% homology thereto; and

Xaa, individually for each occurrence, is an amino acid residue; and

n and m are each, independently, an integer from 3 to 20.

For some embodiments, the FR1 may a polypeptide sequence having at least 80%, 85%, 90%, 95% or even 98% homology with SEQ ID NO: 186. For some embodiments, FR2 is a polypeptide sequence having at least 80%, 85%, 90%, 95% or even 98% homology with SEQ ID NO: 187. For some embodiments, FR3 is a polypeptide sequence having at least 80%, 85%, 90%, 95% or even 98% homology with SEQ ID NO: 188.

In some embodiments, the anti-SPIKE Affimer® Agent has an amino acid sequence represented in the general formula:

$MIP\text{-}Xaa1\text{-}GLSEAKPATPEIQEIVDKVKPQLEEKTNETYGKLEAVQYKTQVLA\text{-}(Xaa)_n\text{-}Xaa2\text{-}TNYYIKVRAGDNKYMHLKVF\text{-}Xaa3\text{-}Xaa4\text{-}Xaa5\text{-}(Xaa)_m\text{-}Xaa6\text{-}D\text{-}Xaa7\text{-}VLTGYQVDKNKDDELTGF$ **(SEQ ID NO:195)**

wherein

Xaa, individually for each occurrence, is an amino acid residue; n and m are each, independently, an integer from 3 to 20; Xaa1 is Gly, Ala, Val, Arg, Lys, Asp, or Glu, more preferably Gly, Ala, Arg or Lys, and more even more preferably Gly or Arg; Xaa2 is Gly, Ala, Val, Ser or Thr, more preferably Gly or Ser; Xaa3 is Arg, Lys, Asn, Gln, Ser,

Thr, more preferably Arg, Lys, Asn or Gln, and even more preferably Lys or Asn; Xaa4 is Gly, Ala, Val, Ser or Thr, more preferably Gly or Ser; Xaa5 is Ala, Val, Ile, Leu, Gly or Pro, more preferably Ile, Leu or Pro, and even more preferably Leu or Pro; Xaa6 is Gly, Ala, Val, Asp or Glu, more preferably Ala, Val, Asp or Glu, and even more preferably Ala or Glu; and Xaa7 is Ala, Val, Ile, Leu, Arg or Lys, more preferably Ile, Leu or Arg, and even more preferably Leu or Arg.

For instance, the anti-SPIKE Affimer® Agent can have an amino acid sequence represented in the general formula:
MIPRGLSEAKPATPEIQEIVDKVKPQLEEKTNETYGKLEAVQYKTQVLA-$(Xaa)_n$-STNYYIKVRAGDNKYMHLKVFNGP-$(Xaa)_m$-ADRVLTGYQVDKNKDDELTGF **(SEQ ID NO: 196),** wherein Xaa, individually for each occurrence, is an amino acid residue; n and m are each, independently, an integer from 3 to 20.
In some embodiments, n is 3 to 15, 3 to 12, 3 to 9, 3 to 7, 5 to 7, 5 to 9, 5 to 12, 5 to 15, 7 to 12 or 7 to 9.
In some embodiments, m is 3 to 15, 3 to 12, 3 to 9, 3 to 7, 5 to 7, 5 to 9, 5 to 12, 5 to 15, 7 to 12 or 7 to 9.
In some embodiments, Xaa, independently for each occurrence, is an amino acid that can be added to a polypeptide by recombinant expression in a prokaryotic or eukaryotic cell, and even more preferably one of the 20 naturally occurring amino acids.

In certain other embodiments, the Affimer® Agent includes one or more COVID (SARS-CoV-2) binding Affimer® sequence represented in following sequences (which may omit the N-terminal methionine if the Affimer® sequence is not presented on the N-terminus of the Affimer® Agent:

Clone	Amino Acid Sequence (with methione N-terminus)
825436	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQHQPKWPGFTTMYYL TLEAKDGGKKKLYEAKVWVKTFHQTEPSPNFKELQEFKPVGDAGDYKDHDGDYKDHDIDYK DDDDKGGGGS (SEQ ID NO: 197)
825456	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQHIDFKLNQHTMYYL TLEAKDGGKKKLYEAKVWVKYKNRINHVINFKELQEFKPVGDAGDYKDHDGDYKDHDIDYK DDDDKGGGGS (SEQ ID NO: 198)
825478	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQAIGQINYPDTMYYL TLEAKDGGKKKLYEAKVWVKAQSKYRHLFNFKELQEFKPVGDAGDYKDHDGDYKDHDIDYK DDDDKGGGGS (SEQ ID NO: 199)
826005	MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDRPNFLTEFIGLNY YIKVRVNGKYIHLKVFKSLHSSHGQEILEDLVLTGYQVDKNKDDELTGFGYPYDVPDYACG

	GGGS (SEQ ID NO: 200)
825392	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQDAQNDFFPETMYYL TLEAKDGGKKKLYEAKVWVKEEGVQKHLFNFKELQEFKPVGDAGYPYDVPDYACGGGGS (SEQ ID NO: 201)
825336	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQKDDVGVRYLTMYYL TLEAKDGGKKKLYEAKVWVKVGWQNDPHPNFKELQEFKPVGDAGYPYDVPDYACGGGGS (SEQ ID NO: 202)
825317	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQRWTYLVDSNTMYYL TLEAKDGGKKKLYEAKVWVKLRNQLNRSNNFKELQEFKPVGDAGYPYDVPDYACGGGGS (SEQ ID NO: 203)
825499	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQYEPDSVPSQTMYYL TLEAKDGGKKKLYEAKVWVKWFLGFKGPYNFKELQEFKPVGDAGDYKDHDGDYKDHDIDYK DDDDKGGGGS (SEQ ID NO: 204)
825513	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQNGLHPLYDETMYYL TLEAKDGGKKKLYEAKVWVKADAPFQYNNNFKELQEFKPVGDAGDYKDHDGDYKDHDIDYK DDDDKGGGGS (SEQ ID NO: 205)
825515	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQQGFHPIIVETMYYL TLEAKDGGKKKLYEAKVWVKAAENFKELQEFKPVGDAGDYKDHDGDYKDHDIDYKDDDDKG GGGS (SEQ ID NO: 206)
826049	MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDHVPVHSYFIGLNY YIKVRVNGKYIHLKVFKSLHNDVDDVVWEDLVLTGYQVDKNKDDELTGFGYPYDVPDYACG GGGS (SEQ ID NO: 207)
825564	MATGVRAIPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQLFKYEHAKVTMYYL TLEAKDGGKKKLYEAKVWVKNWDPNFKELQEFKPVGDAGDYKDHDGDYKDHDIDYKDDDDK GGGGS (SEQ ID NO: 208)
829511	MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDGDWPNSNFRGLNY YIKVRVNGKYIHLKVFKSLHHHEKFDFLEDLVLTGYQVDKNKDDELTGFGYPYDVPDYACG GGGS (SEQ ID NO: 209)
830214	MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDYSVGSTGYRGLNY YIKVRVNGKYIHLKVFKSLHSGVGHDTVEDLVLTGYQVDKNKDDELTGFGYPYDVPDYACG GGGS (SEQ ID NO: 210)
829494	MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDWKQRQSGFIGLNY YIKVRVNGKYIHLKVFKSLHSWERLHVVEDLVLTGYQVDKNKDDELTGFGYPYDVPDYACG GGGS (SEQ ID NO: 211)
829566	MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDDYNQEQYEHGLNY YIKVRVNGKYIHLKVFKSLWKKRLFPWTEDLVLTGYQVDKNKDDELTGFGYPYDVPDYACG GGGS (SEQ ID NO: 212)
829515	MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDFWPAPTGFRGLNY

	YIKVRVNGKYIHLKVFKSLHGSTNHRTAEDLVLTGYQVDKNKDDELTGFGYPYDVPDYACG GGGS (SEQ ID NO: 213)
829519	MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDRQGLGSFYIGLNY YIKVRVNGKYIHLKVFKSLHSNEGGHYREDLVLTGYQVDKNKDDELTGFGYPYDVPDYACG GGGS (SEQ ID NO: 214)
830162	MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDEPRIGYGFIGLNY YIKVRVNGKYIHLKVFKSLHTHHYDDYYEDLVLTGYQVDKNKDDELTGFGYPYDVPDYACG GGGS (SEQ ID NO: 215)
829638	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQTTEYQDHVWTMYYL TLEAKDGGKKKLYEAKVWVKPRFAPILLINFKELQEFKPVGDAGYPYDVPDYACGGGGS (SEQ ID NO: 216)
829568	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQTHNLDVVFHTMYYL TLEAKDGGKKKLYEAKVWVKTIYYIAAEHNFKELQEFKPVGDAGYPYDVPDYACGGGGS (SEQ ID NO: 217)
829610	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQYGSHPLYLETMYYL TLEAKDGGKKKLYEAKVWVKAAENFKELQEFKPVGDAGYPYDVPDYACGGGGS (SEQ ID NO: 218)
829592	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQNGEHPLYVETMYYL TLEAKDGGKKKLYEAKVWVKAAENFKELQEFKPVGDAGYPYDVPDYACGGGGS (SEQ ID NO: 219)
829432	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQQNPVKSFYTTMYYL TLEAKDGGKKKLYEAKVWVKTITDPWNQYNFKELQEFKPVGDAGYPYDVPDYACGGGGS (SEQ ID NO: 220)
829641	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQDELFVDLGVTMYYL TLEAKDGGKKKLYEAKVWVKSWRPYNPNNFKELQEFKPVGDAGYPYDVPDYACGGGGS (SEQ ID NO: 221)
825425	MATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQEWFIVPIPQTMYYL TLEAKDGGKKKLYEAKVWIDWNFKELQEFKPVGDAGDYKDHDGDYKDHDIDYKDDDDKGGG GS (SEQ ID NO: 222)

In some embodiments, the Affimer® Agent is a fusion protein which may include in addition to the COVID (SARS-CoV-2) binding Affimer® polypeptide, to illustrate, one or more additional amino acid sequences selected from the group consisting of: secretion signal sequences, peptide linker sequences, affinity tags, transmembrane domains, cell surface retention sequence, substrate recognition sequences for post-translational modifications, multimerization domains to create multimeric structures of the protein aggregating through protein-protein interactions, half-life extending

polypeptide moieties, polypeptide sequences for altering tissue localization and antigen binding site of an antibody, and one or more additional Affimer® polypeptide sequences binding the viral SPIKE protein (either the same or a different epitope) or a different protein on the viral particle.

In certain embodiments, the Affimer® Agent includes (in addition to COVID (SARS-CoV-2) binding Affimer® sequence(s)) the extracellular SPIKE binding domains of one or more of ACE2, TMPRSS2 and/or DPP4, which are able to bind to the SPIKE protein simultaneously with COVID (SARS-CoV-2) binding Affimer® sequence(s) of the protein. In certain preferred embodiments, the Affimer® Agent includes an Affimer® Agent which binds the SPIKE protein at a site other than at the ACE2 binding site (such as the TMPRSS2 cleavage site) and an extracellular fragment of ACE2 that binds to the SPIKE protein -i.e., the Affimer® sequence binding to SPIKE is not competitive with the ACE2 fragment binding to SPIKE. For example, the Affimer® Agent can include a SPIKE binding domain of ACE2 represented in the sequence (SEQ ID NO: 223):
QSTIEEQAKT FLDKFNHEAE DLFYQSSLAS WNYNTNITEE NVQNMNNAGD
KWSAFLKEQS TLAQMYPLQE IQNLTVKLQL QALQQNGSSV LSEDKSKRLN
TILNTMSTIY STGKVCNPDN PQECLLLEPG LNEIMANSLD YNERLWAWES
WRSEVGKQLR PLYEEYVVLK NEMARANHYE DYGDYWRGDY EVNGVDGYDY
SRGQLIEDVE HTFEEIKPLY EHLHAYVRAK LMNAYPSYIS PIGCLPAHLL
GDMWGRFWTN LYSLTVPFGQ KPNIDVTDAM VDQAWDAQRI FKEAEKFFVS
VGLPNMTQGF WENSMLTDPG NVQKAVCHPT AWDLGKGDFR ILMCTKVTMD
DFLTAHHEMG HIQYDMAYAA QPFLLRNGAN EGFHEAVGEI MSLSAATPKH
LKSIGLLSPD FQEDNETEIN FLLKQALTIV GTLPFTYMLE KWRWMVFKGE
IPKDQWMKKW WEMKREIVGV VEPVPHDETY CDPASLFHVS NDYSFIRYYT
RTLYQFQFQE ALCQAAKHEG PLHKCDISNS TEAGQKLFNM LRLGKSEPWT
LALENVVGAK NMNVRPLLNY FEPLFTWLKD QNKNSFVGWS TDWSPYAD;

or a sequence in which one or more mutations have been introduced to reduce or abrogate the protease activity of ACE2.

Furthermore, minor modifications may also include small deletions or additions – beyond the loop 2 and loop 4 inserts described above – to the Stefin A or Stefin A derived sequences disclosed herein, such as addition or deletion of up to 10 amino acids relative to Stefin A or the Stefin A derived Affimer® polypeptide.

In some embodiments, the Affimer® Agent is a SARS-CoV-2 binding Affimer® Agent having an Affimer® polypeptide portion that binds viral SPIKE as a monomer with a dissociation constant (K_D) of about 1 μM or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, about 10 nM or less, about 1 nM or less, or about 0.1 nM or less.

In some embodiments, the Affimer® Agent is a SARS-CoV-2 binding Affimer® Agent having an Affimer® polypeptide portion that binds viral SPIKE as a monomer with an off rate constant (K_{off}), such as measured by Biacore, of about 10^{-3} s^{-1} (i.e., unit of 1/second) or slower; of about 10^{-4} s^{-1} or slower or even of about 10^{-5} s^{-1} or slower.

In some embodiments, the Affimer® Agent is a SARS-CoV-2 binding Affimer® Agent having an Affimer® polypeptide portion that binds viral SPIKE as a monomer with an association constant (K_{on}), such as measured by Biacore, of at least about 10^3 $M^{-1}s^{-1}$ or faster; at least about 10^4 $M^{-1}s^{-1}$ or faster; at least about 10^5 $M^{-1}s^{-1}$ or faster; or even at least about 10^6 $M^{-1}s^{-1}$ or faster.

In some embodiments, the Affimer® Agent is a SARS-CoV-2 binding Affimer® Agent having an Affimer® polypeptide portion that binds viral SPIKE as a monomer with an IC50 in a competitive binding assay with human ACE2 of 1 μM or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, about 10 nM or less, about 1 nM or less, or about 0.1 nM or less.

In some embodiments, the Affimer® Agent has a melting temperature (Tm, i.e., temperature at which both the folded and unfolded states are equally populated) of 65°C or higher, and preferably at least 70°C, 75°C, 80°C or even 85°C or higher. Melting temperature is a particularly useful indicator of protein stability. The relative proportions of folded and unfolded proteins can be determined by many techniques known to the skilled person, including differential scanning calorimetry, UV difference spectroscopy, fluorescence, circular dichroism (CD), and NMR (Pace et al. (1997) "Measuring the conformational stability of a protein" in Protein structure: A practical approach 2: 299-321).

In some embodiments, the fusion protein includes a half-life extending polypeptide moiety such as selected from the group consisting of an Fc domain or portion thereof, an albumin protein or portion thereof, an albumin-binding polypeptide moiety,

transferrin or portion thereof, a transferrin-binding polypeptide moiety, fibronectin or portion thereof, or a fibronectin-binding polypeptide moiety.

Where the fusion protein includes an Fc domain or a portion thereof, in some embodiments it is an Fc domain that retains FcRn binding.

Where the fusion protein includes an Fc domain or a portion thereof, in some embodiments the Fc domain or a portion thereof is from IgA, IgD, IgE, IgG, and IgM or a subclass (isotype) thereof such as IgG1, IgG2, IgG3, IgG4, IgA1 or IgA2.

In some embodiments, where the fusion protein includes a half-life extending polypeptide moiety, that moiety increases the serum half-life of the protein by at least 5-fold relative to its absence from the protein, for example, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, 200-fold, 500-fold or even 1000-fold.

In some embodiments, the fusion protein of the disclosure is provided as a pharmaceutical preparation suitable for therapeutic use in a human patient, further comprising one or more pharmaceutically acceptable excipients, buffers, salts or the like.

In certain embodiments, the invention provides inhaled formulations for pulmonary delivery of the subject Affimer® Agents. Topical drug delivery to or systemic delivery through the lung can be achieved using propellants, non-aqueous inhalers, dry powder inhalers, and jet or ultrasonic nebulizers as examples. These include metered dose inhalers, nebulizers, and dry powder inhalers.

Yet another aspect of the invention provides a drug eluting device, such as an airway stent or nanofibers, for sustained delivery of an Affimer® Agent to lung tissue, which device comprises drug release means including an Affimer® Agent, which device when deployed in a patient positions the drug release means proximal to the surface of the lung (such as bronchial or bronchiole placement) and releases the Affimer® Agent in an amount sufficient to achieve a therapeutically effective exposure of the luminal surface to the agent. Examples of drug eluting devices are drug eluting stents, drug eluting collars and drug eluting balloons.

In other embodiments, there are provided drug eluting devices that can be implanted proximal to the diseased portion of the luminal surface of the pulmonary tract, such as implanted extraluminally (i.e., submucosally or in or on the circular muscle or longitudinal muscle) rather than intraluminally.

Still another aspect of the disclosure, there is provided a multispecific APC-cell engaging fusion protein (APCE) comprising (i) a SARS-CoV-2 binding polypeptide sequence(s), such as an antibody, antibody fragment or antibody mimetic, which binds to SPIKE or other coronaviridae coat protein with a Kd of 1×10−6M or less, and (ii) an APC binding polypeptide that binds to receptor on the surface of an antigen presenting cell and promotes internalization of the fusion protein in a manner that causes antigenic presentation of coronaviridae antigens by the antigen presenting cell.

In another aspect of the disclosure, there is provided a bispecific dendritic cell engaging (BiDE) fusion protein comprising (i) a SARS-CoV-2 binding Affimer® polypeptide sequence(s) which binds to SPIKE or other coronaviridae coat protein with a Kd of 1×10−6M or less, and (ii) an dendritic cell binding polypeptide sequence that binds to receptor on the surface of an antigen presenting cell and promotes internalization of the fusion protein in a manner that causes antigenic presentation of coronaviridae antigens by the antigen presenting cell.

For each of the APCE and BiDE embodiments, specific non-limiting examples of antibody fragments and antibody mimetics that can be utilized for the COVID (SARS-CoV-2) binding polypeptide sequence include Fab, Fab', F(ab')2, Fv, Fd, single-chain Fv (scFv), disulfide-linked Fvs (sdFv), VL, VH, Camel Ig, V-NAR, VHH, trispecific (Fab3), bispecific (Fab2), diabody ((VL-VH)2 or (VH-VL)2), triabody (trivalent), tetrabody (tetravalent), minibody ((scFv-CH3)2), bispecific single-chain Fv (Bis-scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (cysteine knot protein, knottin), affibody, aptamer, avimer, nanobody, unibody, a domain antibody, affilin, affitin, adnectin, atrimer, evasin, DARPin, anticalin, avimer, fynomer, versabody or a duocalin. In certain preferred embodiments, the COVID (SARS-CoV-2) binding polypeptide sequence is one or more Affimer® polypeptide sequences.

Dendritic cells take up antigens through phagocytosis, micro- or macro-pinocytosis, and endocytosis using Fc receptors (Fcγ receptor type I or CD64 and Fcγ receptor type II or CD32), integrins (αvβ3 or αvβ5), C-type lectin receptors (CLRs, including mannose receptor and DEC205), apoptotic cell receptors, and scavenger receptors.

Exemplary dendritic cell binding polypeptide sequences include polypeptides that bind to DC-specific ICAM-3-grabbing integrin (DC-SIGN), DEC-205 (CD205, Lymphocyte antigen 75), macrophage mannose receptor or C-type lectin macrophage galactose-type lectin (MGL), Dectin-1, Mannose R, BST-2, Siglec-H, LOX-1 and Langerin. In certain preferred embodiments, the dendritic cell binding polypeptide binds to DEC205, DNGR1 (Clec9A) or DC-SIGN.

In certain embodiments, the dendritic cell binding polypeptide is an antibody, antibody fragment or antibody mimeticsuch as Fab, Fab', F(ab')2, Fv, Fd, single-chain Fv (scFv), disulfide-linked Fvs (sdFv), VL, VH, Camel Ig, V-NAR, VHH, trispecific (Fab3), bispecific (Fab2), diabody ((VL-VH)2 or (VH-VL)2), triabody (trivalent), tetrabody (tetravalent), minibody ((scFv-CH3)2), bispecific single-chain Fv (Bis-scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (cysteine knot protein, knottin), affibody, aptamer, avimer, nanobody, unibody, a domain antibody, affilin, affitin, adnectin, atrimer, evasin, DARPin, anticalin, avimer, fynomer, versabody or a duocalin. In certain preferred embodiments, the dendritic cell binding polypeptide sequence is one or more Affimer® polypeptide sequences.

In certain embodiments, the multispecific APC-cell engaging fusion proteins of the disclosure are provided as a pharmaceutical preparation suitable for therapeutic use in a human patient, further comprising one or more pharmaceutically acceptable excipients, buffers, salts or the like.

In another aspect of the disclosure, the disclosure provides an antibody or antigen binding fragment thereof further comprising a COVID (SARS-CoV-2) binding Affimer® polypeptide conjugated thereto, including as fusion protein. For instance, the present invention provides a bispecific antibody including COVID (SARS-CoV-2) binding Affimer® polypeptide(s).

In another aspect of the disclosure, the disclosure provides a soluble receptor or ligand binding domain thereof further comprising a COVID (SARS-CoV-2) binding Affimer® polypeptide conjugated thereto, including as a fusion protein.

In another aspect of the disclosure, the disclosure provides a growth factor, cytokine or chemokine biologically active polypeptide fragment thereof further comprising a COVID (SARS-CoV-2) binding Affimer® polypeptide conjugated thereto, including as a fusion protein.

In another aspect of the disclosure, the disclosure provides a costimulatory agonist polypeptide further comprising a COVID (SARS-CoV-2) binding Affimer® polypeptide conjugated thereto, including as a fusion protein.

In another aspect of the disclosure, the disclosure provides a checkpoint inhibitory polypeptide further comprising a COVID (SARS-CoV-2) binding Affimer® polypeptide conjugated thereto, including as a fusion protein.

In another aspect of the disclosure, there is provided nucleic acids comprising a coding sequence encoding a Coronviradae Neutralizing Agent, such as protein described above (and herein).

In some embodiments, the coding sequence is operably linked to one or more transcriptional regulatory sequences, such as a promoter and/or enhancer.

In some embodiments, the nucleic acid includes one or more origins of replication, minichromosome maintenance elements (MME) and/or nuclear localization elements.

In some embodiments, the nucleic acid includes a polyadenylation signal sequence which is operably linked and transcribed with the coding sequence.

In some embodiments, the coding sequence includes one or more intronic sequences.

In some embodiments, the nucleic acid includes one or more ribosome binding sites which are transcribed with the coding sequence.

In some embodiments, the nucleic acid is DNA.

In some embodiments, the nucleic acid is RNA, such as an mRNA.

In another aspect of the disclosure, there is provided viral vectors including a coding sequence encoding a Affimer® Agent, such as protein described above (and herein).

In another aspect of the disclosure, there is provided plasmid DNA, plasmid Vectors or minicircles including a coding sequence encoding an Affimer® Agent, such as protein described above (and herein).

Also provided herein is a pharmaceutical preparation suitable for therapeutic gene delivery in a human patient, comprising a nucleic acid, a viral vector, a plasmid DNA, plasmid Vector or minicircle of the present disclosure, and (ii) one or more pharmaceutically acceptable excipients, buffers, salts, transfection enhancers, electroporation enhancers or the like.

In certain embodiments, the present disclosure also provides recombinantly engineered cells, preferably mesenchymal stem cells (MSCs) or regulatory T-cells (Treg cells), that have been engineered with one or more genes encoding one or more polypeptide binders which bind to and neutralize a viral particle, which gene(s) when expressed results in the secretion of the neutralizing polypeptide(s) and the inhibition or reduction of infection by the virus, particularly in the tissue into which the engineered cells has been introduced or into which it infiltrates/extravasates. For example, the encoded neutralizing polypeptide can be an antibody, antibody fragment or antibody mimetic such as Fab, Fab', F(ab')2, Fv, Fd, single-chain Fv (scFv), disulfide-linked Fvs (sdFv), VL, VH, Camel Ig, V-NAR, VHH, trispecific (Fab3), bispecific (Fab2), diabody ((VL-VH)2 or (VH-VL)2), triabody (trivalent), tetrabody (tetravalent), minibody ((scFv-CH3)2), bispecific single-chain Fv (Bis-scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (cysteine knot protein, knottin), affibody, aptamer, avimer, nanobody, unibody, a domain antibody, affilin, affitin, adnectin, atrimer, evasin, DARPin, anticalin, avimer, fynomer, versabody or a duocalin. In certain preferred embodiments, the neutralizing polypeptide is a viral coat protein binding Affimer® polypeptide.

In certain embodiments, the present disclosure also provides recombinantly engineered cells, preferably mesenchymal stem cells (MSCs) or regulatory T-cells (Treg cells), that have been engineered with a gene encoding a polypeptide of the present invention, which gene when expressed results in the secretion of the polypeptide and the inhibition or reduction of coronaviradae infection, particularly in the tissue into which the engineered cells has been introduced or into which it infiltrates/extravasates.

Further provided herein are methods comprising administering to a subject the protein, recombinant antibody, nucleic acid or engineered cells described herein (such as a COVID (SARS-CoV-2) binding Affimer® polypeptide).

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

To facilitate an understanding of the present disclosure, a number of terms and phrases are defined below.

a. Affimer® Agent, Affimer® Scaffold, Affimer® Polypeptide

The term "Stefin Polypeptide" refers to a sub-group of proteins in the cystatin superfamily, a family which encompasses proteins that contain multiple cystatin-like sequences.

The Stefin sub-group of the cystatin family is relatively small (around 100 amino acids) single domain proteins. They receive no known post-translational modification, and lack disulphide bonds, suggesting that they will be able to fold identically in a wide range of extra- and intracellular environments. Stefin A itself is a monomeric, single chain, single domain protein of 98 amino acids. The structure of Stefin A has been solved, facilitating the rational mutation of Stefin A into the Affimer® Scaffold. The only known biological activity of cystatins is the inhibition of cathepsin activity, which allowed us to exhaustively test for residual biological activity of our engineered proteins.

The term "Affimer® Agent" (or "Affimer® Scaffold" or "Affimer® Polypeptide") refers to small, highly stable polypeptides that are recombinantly engineered variants of Stefin A or Cystatin C polypeptides. Affimers® Agents display heterologous peptide(s) ('loop sequence'(s)) (typically one to three such heterologous peptides, most suitably two such heterologous peptides). These can each be randomised to bind to desired target proteins with high affinity and specificity, in a similar manner to monoclonal antibodies. Stabilisation of the peptides by the Affimer® Agent protein scaffold constrains the possible conformations that the peptides can take, increasing the binding affinity and specificity compared to libraries of free peptides. These engineered non-antibody binding proteins are designed to mimic the molecular recognition characteristics of monoclonal antibodies in different applications. Variations to other parts of the polypeptide sequence can be carried out, with such variations improving the properties of these affinity reagents, such as increasing stability (such as increasing Tm), such as making them more robust across a range of temperatures or pH or other environments.

It will be apparent to a person skilled in the art that modifications (such as mutations to the amino acid sequence) may be made to the scaffold polypeptide without departing from the disclosure, wherein the modifications do not adversely affect the ability of the

polypeptide to bind to the desired target (such as the SARS-CoV-2 Spike protein). More suitably any such modifications do not restore or generate biological functions such as those which are possessed by wild type Stefin A / Cystatin C but which are abolished in the polypeptides described by the mutational change(s) disclosed below (for example suitably the mutation corresponding to V48D which abolished domain-swap dimerisation should suitably not be mutated back to wild-type to restore domain-swap dimerisation).

An "Affimer® Agent" refers to a polypeptide comprising an Affimer® Polypeptide sequence and having one or more further modification(s) (e.g., non-mutational modification(s) such as conjugation, post-translational modification, etc). An Affimer® Polypeptide sequence may have one or more of such further modifications so as to represent the therapeutically active protein intended for delivery to a patient.

An "anti-SPIKE Affimer® Agent" refers to an Affimer® Agent having at least one Affimer® Polypeptide that binds to a coronaviradae SPIKE protein, particularly SARS-CoV-2 SPIKE protein, with a dissociation constant (Kd) of at least 10^{-6}M.

An "Encoded Affimer® [Agent]" refers to a nucleic acid construct which, when expressed by cells in a patient's body through a gene delivery process, produces an intended Affimer® Agent *in vivo*.

An "Affimer®-Linked Conjugate" refers to an Affimer® Agent having one or more moieties conjugated thereto through a chemical conjugation other than through the formation of a continuous peptide bond through the C-terminus or N-terminus of the polypeptide portion of the Affimer® Agent containing Affimer® Polypeptide sequence.

b. Polypeptides

The terms "polypeptide" and "peptide" and "protein" are used interchangeably herein and refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and/or it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labelling component. Also included within the definition are, for example, polypeptides containing one or more amino acid analogues (for example, unnatural amino acids), as well as other modification(s) known in the art.

The terms "amino acid residue" and "amino acid" are used interchangeably and means, in the context of a polypeptide, an amino acid that is participating in one more peptide bonds of the polypeptide. In general, the abbreviations used herein for designating the amino acids are based on recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (see Biochemistry (1972) 11:1726-1732). For instance, Met, Ile, Leu, Ala and Gly represent "residues" of methionine, isoleucine, leucine, alanine and glycine, respectively. By 'amino acid residue' is meant a radical derived from the corresponding alpha amino acid (or amino acid analogue) by eliminating the OH portion of the carboxyl group and the H portion of the alpha-amino group. The term "amino acid side chain" is that part of an amino acid exclusive of the --CH(NH2)COOH portion, as defined by K. D. Kopple, "Peptides and Amino Acids", W. A. Benjamin Inc., New York and Amsterdam, 1966, pages 2 and 33.

For the most part, the amino acids used in the application of this disclosure are those naturally occurring amino acids found in proteins, or the naturally occurring anabolic or catabolic products of such amino acids which contain amino and carboxyl groups. Particularly suitable amino acid side chains include side chains selected from those of the following amino acids: glycine, alanine, valine, cysteine, leucine, isoleucine, serine, threonine, methionine, glutamic acid, aspartic acid, glutamine, asparagine, lysine, arginine, proline, histidine, phenylalanine, tyrosine, and tryptophan, and those amino acids and amino acid analogs which have been identified as constituents of peptidylglycan bacterial cell walls.

Amino acid residues having "basic sidechains" include Arg, Lys and His. Amino acid residues having "acidic sidechains" include Glu and Asp. Amino acid residues having "neutral polar sidechains" include Ser, Thr, Asn, Gln, Cys and Tyr. Amino acid residues having "neutral non-polar sidechains" include Gly, Ala, Val, Ile, Leu, Met, Pro, Trp and Phe. Amino acid residues having "non-polar aliphatic sidechains" include Gly, Ala, Val, Ile and Leu. Amino acid residues having "hydrophobic sidechains" include Ala, Val, Ile, Leu, Met, Phe, Tyr and Trp. Amino acid residues having "small hydrophobic sidechains" include Ala and Val. Amino acid residues having "aromatic sidechains" include Tyr, Trp and Phe.

The term amino acid residue further includes analogues, derivatives and congeners of an amino acid; thus the polypeptides described herein may include one or more amino acid analogue(s), for example cyanoalanine, canavanine, djenkolic acid, norleucine, 3-phosphoserine, homoserine, dihydroxy-phenylalanine, 5-hydroxytryptophan, 1-

methylhistidine, 3-methylhistidine, diaminiopimelic acid, ornithine, or diaminobutyric acid. Other naturally occurring amino acid metabolites or precursors having side chains which are suitable herein will be recognized by those skilled in the art and are included in the scope of the present disclosure.

Also included are the (D) and (L) stereoisomers of such amino acids when the structure of the amino acid admits of stereoisomeric forms. The configuration of the amino acids and amino acid residues herein are designated by the appropriate symbols (D), (L) or (DL), furthermore when the configuration is not designated the amino acid or residue can have the configuration (D), (L) or (DL). It will be noted that the structure of some of the compounds of this disclosure includes asymmetric carbon atoms. It is to be understood accordingly that the isomers arising from such asymmetry are included within the scope of this disclosure. Such isomers can be obtained in substantially pure form by classical separation techniques and by sterically controlled synthesis. For the purposes of this application, unless expressly noted to the contrary, a named amino acid shall be construed to include both the (D) or (L) stereoisomers.

The polypeptide of the invention may be PEGylated.
The polypeptide of the invention may be biotinylated.

COVID19 is the disease caused by the SARS-CoV-2 virus in humans. Thus, as used herein the term 'COVID binding Affimer® polypeptide', in particular the term 'COVID binding', means 'SARS-CoV-2 binding' i.e. refers to binding to the SARS-CoV-2 virus, more suitably binding to the SARS-CoV-2 coat, most suitably binding to the SARS-CoV-2 spike protein.

Similarly, 'viral spike' and/or 'spike' as used herein suitably means the Spike protein of the SARS-CoV-2 virus. Most suitably Spike protein is as in GenBank Accession # QHD43416.1.

A polypeptide, soluble protein, antibody, polynucleotide, vector, cell, or composition which is "isolated" is a polypeptide, soluble protein, antibody, polynucleotide, vector, cell, or composition which is in a form not found in nature. Isolated polypeptides, soluble proteins, antibodies, polynucleotides, vectors, cells, or compositions include those which have been purified to a degree that they are no longer in a form in which they are found in nature. In some embodiments, a polypeptide, soluble protein, antibody, polynucleotide, vector, cell, or composition which is isolated is substantially pure.

The term "substantially pure" as used herein refers to material which is at least 50% pure (i.e., free from contaminants), at least 90% pure, at least 95% pure, at least 98% pure, or at least 99% pure.

The term "fusion protein" or "fusion polypeptide" as used herein refers to a hybrid protein expressed by a nucleic acid molecule comprising nucleotide sequences of at least two genes.

The term "linker" or "linker region" as used herein refers to a linker inserted between a first polypeptide (e.g., copies of an Affimer® polypeptide) and a second polypeptide (e.g., another Affimer® polypeptide, an Fc domain, a ligand binding domain, etc). In some embodiments, the linker is a peptide linker. Linkers should not adversely affect the expression, secretion, or bioactivity of the polypeptides. Preferably, linkers are not antigenic and do not elicit an immune response.

An "Affimer®-Antibody fusion" refers to a fusion protein including an Affimer® polypeptide portion and a variable region of an antibody. Affimer®-Antibody fusions include full length antibodies having, for example, one or more Affimer® polypeptide sequences appended to the C-terminus or N-terminus of one or more of its VH and/or VL chains, i.e., at least one chain of the assembled antibody is a fusion protein with an Affimer® polypeptide. Affimer®-Antibody fusions also include embodiments wherein one or more Affimer® polypeptide sequences are provided as part of a fusion protein with an antigen binding site or variable region of an antibody fragment.

The term "antibody" as used herein refers to an immunoglobulin molecule that recognizes and specifically binds a target, such as a protein, polypeptide, peptide, carbohydrate, polynucleotide, lipid, or a combination of any of the foregoing, through at least one antigen-binding site wherein the antigen-binding site is usually within the variable region of the immunoglobulin molecule. As used herein, the term encompasses intact polyclonal antibodies, intact monoclonal antibodies, antibody fragments (such as Fab, Fab', F(ab')2, and Fv fragments), single chain Fv (scFv) antibodies provided those fragments have been formatted to include an Fc or other FcγRIII binding domain, multispecific antibodies, bispecific antibodies, monospecific antibodies, monovalent antibodies, chimeric antibodies, humanized antibodies, human antibodies, fusion proteins comprising an antigen-binding site of an antibody (formatted to include an Fc or other FcγRIII binding domain), and any other modified immunoglobulin molecule comprising an antigen-binding site as long as the antibodies exhibit the desired biological activity.

While the antibody can be any of the five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, or subclasses (isotypes) thereof (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), based on the identity of their heavy-chain constant domains referred to as alpha, delta, epsilon, gamma, and mu.

The term "variable region" of an antibody refers to the variable region of an antibody light chain, or the variable region of an antibody heavy chain, either alone or in combination. Generally, the variable region of heavy and light chains each consist of

four framework regions (FR) and three complementarity determining regions (CDRs), also known as "hypervariable regions". The CDRs in each chain are held together in close proximity by the framework regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding sites of the antibody. There are at least two techniques for determining CDRs: (1) an approach based on cross-species sequence variability (i.e., Kabat et al., 1991, Sequences of Proteins of Immunological Interest, 5th Edition, National Institutes of Health, Bethesda Md.), and (2) an approach based on crystallographic studies of antigen-antibody complexes (Al Lazikani et al., 1997, J. Mol. Biol., 273:927-948). In addition, combinations of these two approaches are sometimes used in the art to determine CDRs.

The term "humanized antibody" as used herein refers to forms of non-human (e.g., murine) antibodies that are specific immunoglobulin chains, chimeric immunoglobulins, or fragments thereof that contain minimal non-human sequences. Typically, humanized antibodies are human immunoglobulins in which residues of the CDRs are replaced by residues from the CDRs of a non-human species (e.g., mouse, rat, rabbit, or hamster) that have the desired specificity, affinity, and/or binding capability. In some instances, the Fv framework region residues of a human immunoglobulin are replaced with the corresponding residues in an antibody from a non-human species. The humanized antibody can be further modified by the substitution of additional residues either in the Fv framework region and/or within the replaced non-human residues to refine and optimize antibody specificity, affinity, and/or binding capability. The humanized antibody may comprise variable domains containing all or substantially all of the CDRs that correspond to the non-human immunoglobulin whereas all or substantially all of the framework regions are those of a human immunoglobulin sequence. In some embodiments, the variable domains comprise the framework regions of a human immunoglobulin sequence. In some embodiments, the variable domains comprise the framework regions of a human immunoglobulin consensus sequence. The humanized antibody can also comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. A humanized antibody is usually considered distinct from a chimeric antibody.

The terms "epitope" and "antigenic determinant" are used interchangeably herein and refer to that portion of an antigen capable of being recognized and specifically bound by a particular antibody, a particular Affimer® Agent or other particular binding domain. When the antigen is a polypeptide, epitopes can be formed both from contiguous amino acids and noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids (also referred to as linear epitopes) are typically retained upon protein denaturing, whereas epitopes formed by tertiary folding (also referred to as conformational epitopes) are typically lost upon protein denaturing. An epitope typically includes at least 3, and more usually, at least 5, 6, 7, or 8-10 amino acids in a unique spatial conformation.

As used herein, the term "specifically binds to" or is "specific for" refers to measurable and reproducible interactions such as binding between a target and an Affimer® Agent,

antibody or other binding partner, which is determinative of the presence of the target in the presence of a heterogeneous population of molecules including biological molecules. For example, an Affimer® Agent that specifically binds to a target is an Affimer® Agent that binds this target with greater affinity, avidity (if multimeric formatted), more readily, and/or with greater duration than it binds to other targets.

As used herein, "conjugate," "conjugation" or grammatical variations thereof refers the joining or linking together of two or more compounds resulting in the formation of another compound, by any joining or linking methods known in the art. It can also refer to a compound which is generated by the joining or linking together two or more compounds. For example, an anti-SPIKE Affimer® Agent linked directly or indirectly to one or more chemical moieties or polypeptide is an exemplary conjugate. Such conjugates include fusion proteins, those produced by chemical conjugates and those produced by any other methods.

c. Nucleic Acids

The terms "polynucleotide" and "nucleic acid" and "nucleic acid molecule" are used interchangeably herein and refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase.

As used herein, the term "nucleic acid molecule encoding", "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of nucleotides along a strand of deoxyribonucleic acid deoxyribonucleotides. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. Thus, a nucleic acid sequence encoding the amino acid sequence.

When used in reference to nucleotide sequences, "sequence" as used herein, the term grammatical and other forms may comprise DNA or RNA, and may be single or double stranded. Nucleic acid sequences may be mutated. Nucleic acid sequence may have any length, for example 2 to 1,000,000 or more nucleotides (or any integral value above or between) a nucleic acid, for example a length of from about 100 to about 10,000, or from about 200 nucleotides to about 500 nucleotides.

The term "vector" as used herein means a construct, which is capable of delivering, and usually expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid, or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, and DNA or RNA expression vectors encapsulated in liposomes.

As used herein, the term "transfection" refers to an exogenous nucleic acid into a eukaryotic cell. Transfection can be achieved by various means known in the art, including calcium phosphate -DNA co-precipitation, DEAE- dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics technology (biolistics).

The term "carrier" as used herein is an isolated nucleic acid comprising the isolated nucleic acid can be used to deliver a composition to the interior of the cell. It is known in the art a number of carriers including, but not limited to the linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or virus. The term should also be construed to include facilitate transfer of nucleic acid into cells of the non-plasmid and non-viral compounds, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to adenoviral vectors, adeno-associated virus vectors, retroviral vectors and the like.

As used herein, the term "expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequence and a nucleotide sequence to be expressed operably linked. The expression vector comprises sufficient cis-acting elements (cis-acting elements) used for expression; other elements for expression can be supplied by the host cell or in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses (e.g., lentivirus, retroviruses, adenoviruses and adeno-associated viruses).

As used herein, the term "operably linked" refers to functional linkage between the regulatory sequence and a heterologous nucleic acid sequence is connected to a connection results in the expression of the latter. For example, when the first nucleic acid sequence and a second nucleic acid sequence is a functional relationship between the first nucleic acid sequence and the second nucleic acid sequence is operably linked. For example, if the promoter affects the transcription or expression of the coding sequence, the promoter is operably linked to a coding sequence. Typically, DNA sequencing operably linked are contiguous, and to join two protein coding regions in the same reading frame as necessary.

As used herein, the term "promoter" is defined as a promoter DNA sequence recognized by the synthetic machinery required for the synthesis machinery of the cell specific transcription of a polynucleotide sequence or introduced.

The term "constitutive expression" as used herein refers to all expressed under physiological conditions.

The term "inducible expression" as used herein refers to expression under certain conditions, such as activation (or inactivation) of an intracellular signaling pathway or the contacting of the cells harboring the expression construct with a small molecule that regulates the expression (or degree of expression) of a gene operably linked to an inducible promoter sensitive to the concentration of the small molecule.

The term "electroporation" refers to the use of a transmembrane electric field pulse to induce microscopic pathways (pores) in a bio-membrane; their presence allows biomolecules such as plasmids or other oligonucleotide to pass from one side of the cellular membrane to the other.

d. Miscellaneous

The term "treatment" as used herein refers to the individual trying to change the process or treatment of a clinical disease caused by intervention of a cell, may be either preventive intervention course of clinical pathology. Including but not limited to treatment to prevent the occurrence or recurrence of disease, alleviation of symptoms, reducing the direct or indirect pathological consequences of any disease, preventing metastasis, slow the rate of disease progression, amelioration or remission of disease remission or improved prognosis.

The term "subject" refers to any animal (e.g., a mammal), including, but not limited to, humans, non-human primates, canines, felines, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

The term "pharmaceutically acceptable" refers to a substance approved or approvable by a regulatory agency of the Federal government or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, including humans.

The terms "pharmaceutically acceptable excipient, carrier or adjuvant" or "acceptable pharmaceutical carrier" refer to an excipient, carrier or adjuvant that can be administered to a subject, together with at least one agent of the present disclosure, and which does not destroy the pharmacological activity thereof and is nontoxic when administered in doses sufficient to deliver a therapeutic effect. In general, those of skill in the art and the U.S. FDA consider a pharmaceutically acceptable excipient, carrier, or adjuvant to be an inactive ingredient of any formulation.

The terms "effective amount" or "therapeutically effective amount" or "therapeutic effect" refer to an amount of an Affimer® Agent described herein effective to "treat" a disease or disorder in a subject such as, a mammal. In the case of infection, or the risk of being infected, with a coronavirade virus (for example), a therapeutically effective

amount of a COVID (SARS-CoV-2) binding Affimer® Agent has a therapeutic effect if it reduces the risk of becoming infected, or if infected, if it reduces one or more of the severity of infection, spread of infection in the body, severity of the effects of infection, duration of infection, the extent one or more of the symptoms associated with the infection, or morbidity and mortality from infection, or a combination of such effects. The terms "treating" or "treatment" or "to treat" or "alleviating" or "to alleviate" refer to both (1) therapeutic measures that cure, slow down, lessen symptoms of, and/or halt progression of a diagnosed pathologic condition or disorder and (2) prophylactic or preventative measures that prevent or slow the development of a targeted pathologic condition or disorder. Thus those in need of treatment include those already with the disorder; those prone to have the disorder; and those in whom the disorder is to be prevented. In the case of viral infection, a subject is successfully "treated" according to the methods of the present disclosure if the patient shows one or more of the following: reduction in the severity of infection, reduction in the spread of infection in the body, reduction in the severity of the effects of infection, reduction in the duration of infection, reduction in the symptoms associated with the infection, reduction in the morbidity and mortality from infection, improvement in quality of life during or after infection, or some combination of effects.

It is understood that wherever embodiments are described herein with the language "comprising" otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided. It is also understood that wherever embodiments are described herein with the language "consisting essentially of" otherwise analogous embodiments described in terms of "consisting of" are also provided.

REFERENCE SEQUENCE

Suitably sequences herein, in particular mammalian derived sequences herein, are discussed with reference to human wild-type Stefin A (Cystatin A) having the sequence Uniprot P01040 (most suitably Uniprot P01040-1). For the avoidance of doubt, this sequence is presented below:

SEQ ID NO: 1 - Uniprot P01040 - wild type human Cystatin A

```
        10         20         30         40         50
MIPGGLSEAK PATPEIQEIV DKVKPQLEEK TNETYGKLEA VQYKTQVVAG
        60         70         80         90
TNYYIKVRAG DNKYMHLKVF KSLPGQNEDL VLTGYQVDKN KDDELTGF
```

When particular amino acid residues are referred to herein using numeric addresses, the numbering is taken with reference to the wild type Stefin A (Cystatin A) amino acid sequence (or to the polynucleotide sequence encoding same if referring to nucleic acid). An exemplary nucleic acid encoding wild type Stefin A (Cystatin A) is:

SEQ ID NO: 4 (DNA sequence; artificial (not natural); the amino acid sequence SEQ ID NO: 1 was used to generate a codon-optimised DNA sequence suitable for expression in *E. coli*):

ATGATTCCTGGTGGTTTGTCGGAAGCCAAACCGGCTACTCCGGAAATCCAGGAGATT
GTGGACAAAGTCAAACCGCAACTGGAGGAAAAGACCAATGAAACCTATGGCAAACTC
GAAGCGGTACAGTACAAAACCCAAGTCGTTGCGGGTACGAACTACTACATCAAAGTA
CGCGCAGGAGATAACAAGTATATGCATCTGAAAGTGTTCAAAAGCTTACCAGGGCAG
AATGAGGATCTGGTTCTTACGGGCTATCAGGTGGATAAGAACAAAGACGATGAACTG
ACAGGCTTT

Similarly the reference sequence for discussion of plant consensus scaffold based polypeptides is SEQ ID NO: 2 (plant T2 consensus – backbone sequence without loops).

Suitably the current version of sequence database(s) are relied upon. Alternatively, the release in force at the date of filing is relied upon. For the avoidance of doubt, UniProt release 2020_04 is relied upon. In more detail, the UniProt consortium European Bioinformatics Institute (EBI), SIB Swiss Institute of Bioinformatics and Protein Information Resource (PIR)'s UniProt Knowledgebase (UniProtKB) Release 2020_04 published 12 August 2020 is relied upon. UniProt (Universal Protein Resource) is a comprehensive catalogue of information on proteins ("UniProt: the universal protein knowledgebase" Nucleic Acids Res. 45: D158-D169 (2017)).

This is to be used as is well understood in the art to locate the residue of interest. This is not always a strict counting exercise - attention must be paid to the context. For example, if the protein of interest is of a slightly different length, then location of the correct residue in that sequence may require the sequences to be aligned and the equivalent or corresponding residue picked. This is well within the ambit of the skilled reader. In case any further guidance is required, below is an alignment of the human wild-type Stefin A (Cystatin A) reference sequence (SEQ ID NO: 1) with the 3r2 scaffold as disclosed as SEQ ID NO: 19 in WO2019/008335. This 3r2 scaffold sequence is 97 aa in length (i.e. this sequence includes the loops of 3r2 which are suitably replaced by heterologous peptide insertions in the polypeptides of the invention (SEQ ID NO: 3 presents the 3r2 backbone sequence without those loops)).

Alignment:

```
# Length: 98
# Identity:      85/98 (86.7%)
# Similarity:    92/98 (93.9%)
# Gaps:           1/98 ( 1.0%)
# Score: 434.0

Human SEQ ID NO:1  1 MIPGGLSEAKPATPEIQEIVDKVKPQLEEKTNETYGKLEAVQYKTQVVAG     50
                     ||||||||||||||||||||||||||||::|.:|:|||||||:||||.||
T3r2               1 MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDAG     50

Human SEQ ID NO:1 51 TNYYIKVRAGDNKYMHLKVFKSLPGQNEDLVLTGYQVDKNKDDELTGF     98
                       .|||||||. :.||:||||||||||||||||||||||||||||||
T3r2               51 LNYYIKVRV-NGKYIHLKVFKSLPGQNEDLVLTGYQVDKNKDDELTGF     97
```

This illustrates how the amino acids of the 3r2 can be numbered/discussed using the reference sequence as is usual in the art.

Mutating has its normal meaning in the art and may refer to the substitution or truncation or deletion of one or more residues, motifs or domains. Mutation may be effected at the polypeptide level, for example, by synthesis of a polypeptide having the mutated sequence, or may be effected at the nucleotide level, for example, by making a polynucleotide encoding the mutated sequence, which polynucleotide may be subsequently translated to produce the mutated polypeptide. Suitably, the mutations to be used are as set out herein. Unless otherwise apparent from the context, mutations mentioned herein are substitutions. For example 'V48D' means that the residue corresponding to 'V48' in the wild type Stefin A (SEQ ID NO: 1) is substituted with D.

Sequence Variation

The polypeptides described herein may comprise sequence changes relative to the reference sequence in addition to the key mutations present in (e.g.) 3r2 relative to SEQ ID NO: 1. Specifically the polypeptides described herein may comprise additional sequence changes at sites which do not significantly compromise the function or operation of the polypeptides described herein. The sequence changes may be made at the polypeptide or the nucleotide level.

Polypeptide function may be easily tested using the methods as set out in the examples section, for example in order to verify that the peptide structure or conformation has not been significantly altered. Thus, provided that the polypeptide retains its structure or conformation which can be easily tested as set out herein, sequence variations may be made in the polypeptide relative to the wild type reference sequence. By function is suitably meant binding to SARS-CoV-2 spike protein as described herein.

Suitably binds means selectively binds. In the context of the polypeptides disclosed herein, selectively binds means that the polypeptide of interest binds SARS-CoV-2 spike protein. Kinetic binding assays for assessing this are known in the art. For guidance we refer to example 3.

Scaffold Mutations – Biological Neutrality/Stability

Exemplary scaffolds provided herein to illustrate the invention comprise certain known functional mutations. These may be employed to achieve/maintain biological neutrality. For example the 3r2 scaffold backbone comprises V48D (i.e. comprising amino acid D at position 48 relative to SEQ ID NO: 1). V48 mutation such as V48D, inhibits domain swap dimerisation and so is extremely useful in providing biological neutrality.

Thus the scaffold may comprise a V48 mutation such as V48D, which inhibits domain swap dimerisation.

Similarly the scaffold may comprise a Q42E mutation, which provides an increase in thermal stability of +3.2 °C

Similarly the scaffold may comprise a T51L mutation, which provides a stronger increase in thermal stability of +4.6 °C

Similarly the scaffold may comprise a M65I mutation, which provides an especially strong increase in thermal stability of +6 °C

Similarly the scaffold may comprise a N32G mutation, which removes a potential glycosylation site, removes the risk of deamidation and offers improved stability of the helix.

Similarly the scaffold may comprise a Y35W mutation, which provides an aromatic residue for fluorescence/absorbance.

Details of these and other useful mutations which the skilled worker may incorporate into the polypeptides of the invention may be found in WO2019/008335 (Type 3 scaffold - in particular for mutations useful in manipulation of the melting point (Tm), as well as for biological neutrality), as well as in WO2006/131749 (Type 1 scaffold – STM), WO2009/136182 (Type 1 scaffold – SQT) and/or WO2014/125290 (Type 2 scaffold – plant consensus.)

The skilled worker may select these or other mutations depending on the desired application(s) of the invention.

ADDITIONAL AMINO ACID SEQUENCES

In one embodiment the polypeptide as described above may further comprise one of more of:

i) purification tag, suitably HHHHHH;
ii) conjugation tag, suitably C;
iii) detection tag, suitably DYKDDDDK;
iv) linker sequence, suitably GGGGS.

Suitably said purification tag, and/or conjugation tag, and/or detection tag, and/or linker sequence(s) may be at any suitable location in the polypeptide. Any suitable location means any location which retains function of the polypeptide (e.g. retains the ability to bind, suitably selectively bind, SARS-CoV-2 spike protein as detailed herein). More suitably said purification tag, and/or conjugation tag, and/or detection tag, and/or linker sequence(s) may be located at the C-terminal end of the polypeptide or at the N-terminal end of the polypeptide.
Most suitably said purification tag, and/or conjugation tag, and/or detection tag, and/or linker sequence(s) may be located at the C-terminal end of the polypeptide. 'N-terminal end of the polypeptide' means N-terminal to (i.e. before) the amino acid corresponding to the first amino acid of SEQ ID NO: 3 or the amino acid corresponding to the first amino acid of SEQ ID NO: 2.
'C-terminal end of the polypeptide' means C-terminal to (i.e. after or distal to) the amino acid corresponding to the last amino acid of SEQ ID NO: 3 or the amino acid corresponding to the last amino acid of SEQ ID NO: 2.

Examples of especially suitable additional sequences which the skilled worker may append to the polypeptide of the invention are disclosed herein.

LEADING METHIONINE

Amino acid sequences for the exemplary polypeptides described herein based on the plant cystatin C scaffold (i.e. scaffolds having at least 80% sequence identity to SEQ ID NO: 2) are shown without a leading Methionine (Met or M) at the N-terminus. As the skilled reader will be aware, production of certain polypeptides by recombinant methods leads to cleavage of certain amino acid sequence(s) from precursor polypeptide(s) produced by translation of the nucleic acid encoding said polypeptide. This cleavage of the precursor polypeptide leaves a 'mature' polypeptide with a particular amino acid sequence. Most commonly this process cleaves off the lead methionine from the polypeptide after translation. Therefore the amino acid sequences

provided herein reflect the amino acid sequence of the 'mature' polypeptide for various exemplary polypeptides, i.e. without the lead methionine.

Of course the polypeptide may be produced by translation of a nucleic acid with a nucleotide sequence comprising the necessary start codon and so the precursor polypeptide comprises a lead methionine for these sequences (this is then cleaved off and so does not appear on the final mature polypeptide). Therefore in one embodiment suitably the invention relates to a polypeptide as described above further comprising a N-terminal Methionine. Most suitably in one embodiment the invention relates to a polypeptide as described above comprising amino acid sequence having at least 80% sequence identity to SEQ ID NO: 2, said polypeptide further comprising a N-terminal Methionine.

It will be noted that the invention embraces the polypeptides described herein as monomers – i.e. 'on their own' rather than as part of a larger fusion protein – in those embodiments it is of course helpful to add a lead methionine (initiation methionine) to the sequence for efficient translation (production) of the polypeptide (i.e. to add a codon for methionine at the start of the nucleotide sequence encoding the polypeptide of interest). Thus in one embodiment the invention relates to a polypeptide as described above further comprising a N-terminal Methionine (M). Thus in one embodiment the invention relates to a nucleic acid comprising nucleotide sequence encoding a polypeptide as described above, further comprising an initiation codon (ATG) at the start of the coding sequence.

As with any polypeptide production system, there can be variation in behaviour regarding this cleavage event. In particular the polypeptides disclosed herein based on the mammalian scaffold (i.e. having at least 80% sequence identity to SEQ ID NO: 3) typically retain the lead Methionine when produced. Therefore amino acid sequences for the exemplary polypeptides described herein based on the mammalian Stefin A scaffold (i.e. scaffolds having at least 80% sequence identity to SEQ ID NO: 3) are shown with a leading Methionine (Met or M) at the N-terminus.

As is well known to the skilled worker, if a polypeptide sequence is incorporated into a larger protein sequence, for example when making a fusion protein such as a multi-domain protein, any lead methionine in the polypeptide sequence being incorporated internally into the larger protein sequence is usually omitted. In other words, when producing a polypeptide in a laboratory or industrial setting, lead methionine(s) other than at the initiation position (i.e. the first amino acid in the polypeptide chain –

encoded by the initiation codon i.e. Methionine) are usually omitted. In practice this means that the nucleotide sequence used to direct expression of the polypeptide of interest usually has any codons for 'lead' methionines which would become 'internal' methionines upon fusion (e.g. methionine(s) at the start of the respective polypeptide(s) being added internally into the single fusion protein) removed (deleted). In other words, a start codon is not included in the coding sequence of part of a fusion protein if it is not the first domain of the fusion protein. Suitably a start codon is only included at the start of the first domain of the protein.

FURTHER SCAFFOLD MUTATIONS

Without wishing to be bound by theory, it is believed that the deletion of amino acids VK relative to SEQ ID NO: 2 from the scaffold in polypeptide 825425 may contribute to the 3-dimensional structure of that polypeptide. Therefore, if the loop sequences of this polypeptide are transplanted onto a different scaffold background, it is preferred to make a deletion of the two amino acids corresponding to the VK deletion in that different scaffold sequence (i.e. the destination scaffold - the scaffold backbone/scaffold background into which the loop sequence (i.e. heterologous peptide) is transferred).

Without wishing to be bound by theory, the L to P mutation relative to SEQ ID NO: 3 in polypeptide 826257 is thought to produce a "kink" in the polypeptide chain. Thus, it is believed that this mutation may contribute to the structure of that polypeptide. Therefore, if the heterologous peptide sequences ("loop sequences") of this polypeptide are transplanted into a different scaffold sequence, it is preferred that the corresponding L to P mutation is made in that different scaffold sequence.

Without wishing to be bound by theory, the D to Y mutation relative to SEQ ID NO: 3 in polypeptide 826110 is regarded as a "loop extension". In other words, it is believed that this amino acid may contribute to the 3-dimensional structure of the polypeptide. Therefore, if the heterologous peptide sequence ("loop sequence") of this polypeptide is transplanted from this polypeptide into a different scaffold backbone, it is preferred that the corresponding D to Y mutation is made in that different scaffold sequence.

As used herein, "loop sequence" means a heterologous peptide insertion.

Loop Nomenclature

The secondary structure of the polypeptide / scaffold protein may be modelled using open source software, most suitably using "PPopen" available from the Technical University of Munich, Germany. The numbering of structures such as loops in the polypeptide / scaffold protein of the invention adheres to the systematic naming given using the PPopen software.

It should be noted that prior art documents have used an informal nomenclature. Therefore, disclosures in prior art documents such as WO 2006/131749 and/or WO 2009/136182 and/or WO2014/125290 might mention the informal name "loop 1", which would actually relate to "loop 2" using the systematic naming via PPopen software. The table below sets out the nomenclature of the different structural parts of the protein, and this will be adhered to throughout the text unless otherwise indicated. Unless otherwise apparent from the context, the following loop nomenclature is adhered to. Amino acid residue numbers are as in, or corresponding to, SEQ ID NO: 1 but are equally used to refer to corresponding loops in polypeptide sequences based on plant scaffolds (e.g. such as based on SEQ ID NO: 2):

Name	Amino Acid address (SEQ ID NO: 1)
N-terminus	1 – 13
Helix 1	14 – 30
Loop 1	31 – 37
Strand 1	38 – 48
Loop 2	49 – 51
Strand 2	52 – 59
Loop 3	60 – 62
Strand 3	63 – 70
Loop 4	71 – 78
Strand 4	79 – 85
C-terminus	86 – 98 (not inc. GH6 tag)

As used here, the word "loop" means "connecting regular secondary structure" (i.e. alpha/beta).

'Heterologous' has its natural meaning i.e. the inserted polypeptide has an amino acid sequence which is heterologous to the scaffold sequence which it comprises e.g. the sequence derived from or corresponding to SEQ ID NO: 3; e.g. the sequence derived from or corresponding to SEQ ID NO: 2. Therefore heterologous may mean from another species, and/or may mean from a polypeptide other than SEQ ID NO: 3 or SEQ ID NO: 2. Most suitably the heterologous polypeptide insertion comprises artificial

amino acid sequence, most suitably artificial amino acid sequence designed by the operator.

ALTERNATE SCAFFOLD BACKBONES

In some embodiments, heterologous peptide sequences ("loop sequences") may be transplanted between alternate scaffold backbones. In other words, heterologous peptide sequences may be captured/identified in a particular scaffold backbone ('scaffold background') (e.g. from a library based on the scaffold backbone of SEQ ID NO: 3), and it may then be desired to utilise those heterologous peptide sequences in a different scaffold background. This may be sometimes be referred to as 'transplanting' heterologous peptide sequences between scaffolds or as 'transferring' heterologous peptide sequences into a different scaffold backbone.

This is easily accomplished using the information disclosed herein – a new polypeptide sequence is simply created using the amino acid sequence from the scaffold of interest (i.e. the scaffold into which it is desired to transfer or transplant the heterologous peptide sequences), and the heterologous peptide sequences are inserted into the appropriate points in the scaffold of interest, and the resulting polypeptide is synthesised in a conventional manner.

In one embodiment when the original polypeptide comprises two or more heterologous peptide insertions, suitably each of those heterologous peptides is transplanted onto an alternate scaffold background together i.e. the new polypeptide onto which the heterologous peptides are transplanted will comprise each of the same heterologous peptide insertions as the original polypeptide.

In one embodiment heterologous peptide sequences from more than one different original polypeptide(s) may be mixed when transplanting onto a different scaffold background e.g. a first heterologous peptide from a first original polypeptide and a second heterologous peptide from a second original polypeptide may be taken and transplanted into a scaffold background to create a new combination of first and second heterologous peptides. The scaffold background may be the same as the original scaffold background(s) or may be different from the original scaffold background(s).

In the unlikely event of any difficulty choosing where in the scaffold of interest the heterologous peptide sequences should be inserted, the amino acid sequence of the scaffold of interest can be aligned with the amino acid sequence of the original scaffold from which the heterologous peptide sequences were captured/identified. The heterologous peptide sequences may then simply be inserted into the amino acid

sequence of the scaffold of interest at those locations corresponding to the insertion points in the original scaffold.

Such transplanting of heterologous peptide sequences is particularly suitable between scaffolds showing high degrees of sequence identity e.g. scaffolds showing at least about 80% sequence identity of the scaffold backbone. For example, transplanting of heterologous peptide sequences may be carried out between Type 1 scaffolds (SQT type scaffolds e.g. as disclosed in WO 2009/136182 - suitably the SQT scaffold is a scaffold having a sequence of SEQ ID NO:24 of WO 2009/136182 (SQT): MIPRGLSEAK PATPEIQEIV DKVKPQLEEK TNETYGKLEA VQYKTQVLAS TNYYIKVRAG DNKYMHLKVF NGPPGQNADR VLTGYQVDKN KDDELTGF) and Type 3 scaffolds (i.e. mammalian scaffolds such as SEQ ID NO: 3 disclosed herein).

Without wishing to be bound by theory, transplanting heterologous peptide sequences between Type 2 scaffolds (plant/adhiron type scaffolds e.g. as disclosed in WO2014/125290 i.e. plant cystatin C based scaffolds such as SEQ ID NO: 2 disclosed herein) and mammalian scaffolds such as Type 1 scaffolds (i.e. human Stefin A based scaffold SQT – see above) or between Type 2 scaffolds and mammalian scaffolds such as Type 3 scaffolds (i.e. mammalian scaffolds such as human Stefin A based scaffold e.g. SEQ ID NO: 3 disclosed herein) is considered less advantageous. This is because the plant scaffold backbone and mammalian scaffold backbone sequences have very low levels of sequence identity (e.g. as low as 16% sequence identity overall). Therefore, suitably if heterologous peptide sequences are transferred/transplanted between different scaffold backbones, suitably those scaffold backbones are derived from the same phylogenetic group e.g. derived from mammalian Stefin A scaffolds; more suitably those different scaffold backbones have at least 70% sequence identity to each other, suitably at least 75%; more suitably at least 80%, more suitably at least 85%, more suitably at least 88%, more suitably at least 90%, more suitably at least 92%, more suitably at least 94%, more suitably at least 95%, more suitably at least 96%, more suitably at least 97%, more suitably at least 98%, more suitably at least 99% identity.

For the avoidance of doubt, 'scaffold backbone' or 'scaffold background' refers to the part of the polypeptide of the invention which corresponds to the scaffold protein sequence e.g. corresponds to the sequence of SEQ ID NO: 3 or SEQ ID NO: 2. The terms 'scaffold backbone' or 'scaffold background' specifically do not include (i.e. specifically exclude) the heterologous peptide insertion(s) present in the polypeptides of the invention. A polypeptide of the invention is typically made up of a scaffold

backbone sequence and one or more heterologous peptide insertion(s) into said scaffold backbone sequence as described herein.

It should be noted that the 57 exemplary polypeptide sequences presented herein comprise loop sequences inserted into either Type 2 (plant/adhiron) scaffold backbones or inserted into Type 3 (mammalian) scaffold backbones.

Suitably the polypeptide comprises at least 80 amino acids; suitably at least 81 amino acids, suitably at least 82 amino acids, suitably at least 83 amino acids, suitably at least 84 amino acids, suitably at least 85 amino acids, suitably at least 86 amino acids, suitably at least 87 amino acids, suitably at least 88 amino acids, suitably at least 89 amino acids, suitably at least 90 amino acids, suitably at least 91 amino acids, suitably at least 92 amino acids, suitably at least 93 amino acids, suitably at least 94 amino acids, suitably at least 95 amino acids, suitably at least 96 amino acids; more suitably at least 97 amino acids; most suitably 98 amino acids (excluding any heterologous peptide insertions).
In one embodiment suitably the polypeptide comprises amino acid sequence derived from or corresponding to full length SEQ ID NO: 2 (85 amino acids). In this embodiment suitably the polypeptide comprises at least 85 amino acids (excluding any heterologous peptide insertions). In this embodiment, optionally if a lead methionine is retained, suitably the polypeptide comprises at least 86 amino acids (excluding any heterologous peptide insertions).
In one embodiment suitably the polypeptide comprises amino acid sequence derived from or corresponding to full length SEQ ID NO: 3 (92 amino acids). In this embodiment suitably the polypeptide comprises at least 92 amino acids (excluding any heterologous peptide insertions). In this embodiment, optionally if the lead methionine is removed, suitably the polypeptide comprises at least 91 amino acids (excluding any heterologous peptide insertions).

Heterologous Peptide Insertion

Suitably the heterologous peptide comprises 36 amino acids or fewer, more suitably 20 amino acids or fewer, more suitably 12 amino acids or fewer.
Suitably the heterologous peptide comprises 3 amino acids or more, more suitably 12 amino acids or more, more suitably 20 amino acids or more, more suitably up to 36 amino acids.

In one aspect suitably each heterologous peptide comprises 6 to 36 amino acids, more suitably 6 to 20 amino acids, more suitably 6 to 12 amino acids, more suitably 6 to 9 amino acids, most suitably 9 amino acids.

Suitably each heterologous peptide comprises 3 to 36 amino acids, more suitably 3 to 20 amino acids, more suitably 3 to 12 amino acids, most suitably 3 to 9 amino acids.

In one embodiment suitably each heterologous peptide comprises 9 amino acids.

When there is more than one heterologous peptide in a single polypeptide, each heterologous peptide may be the same length or each heterologous peptide may be a different length. For example in one embodiment a first heterologous peptide may be 9 amino acids and a second heterologous peptide may be 3 amino acids. For example in one embodiment a first heterologous peptide may be 9 amino acids and a second heterologous peptide may be 9 amino acids.

When the polypeptide is based on mammalian scaffold sequence (such as SEQ ID NO: 3), the heterologous peptide insertion(s) may be made at one of the following positions relative to SEQ ID NO: 1:

a) 47-<heterologous peptide>-55
b) 46-<heterologous peptide>-54
c) 46-<heterologous peptide>-50
d) 48-<heterologous peptide>-50
e) 49-<heterologous peptide>-51
f) 50-<heterologous peptide>-52
g) 66-<heterologous peptide>-85
h) 67-<heterologous peptide>-84
i) 70-<heterologous peptide>-74
j) 72-<heterologous peptide>-74
k) 71-<heterologous peptide>-73
l) 72-<heterologous peptide>-81
m) 73-<heterologous peptide>-80
n) 79-<heterologous peptide>-81
o) 80-<heterologous peptide>-81
p) 82-<heterologous peptide>-83
q) 72-<heterologous peptide>-77

r) 73-<heterologous peptide>-78
s) 74-<heterologous peptide>-79
t) 4-<heterologous peptide>-5

Optionally said polypeptide based on mammalian scaffold sequence may comprise two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (a) to (f), and a second heterologous peptide insertion at position (t).

More suitably said polypeptide based on mammalian scaffold sequence comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (a) to (f), and a second heterologous peptide insertion at any of positions (g) to (s).

Most suitably said polypeptide based on mammalian scaffold sequence comprises two heterologous peptide insertions, a first heterologous peptide insertion at position (d), and a second heterologous peptide insertion at position (r).

Thus most suitably said polypeptide based on mammalian scaffold sequence comprises two heterologous peptide insertions, wherein said first heterologous peptide is inserted into the polypeptide at position 48-<heterologous peptide>-50 and said second heterologous peptide is inserted into the polypeptide at position 73-<heterologous peptide>-78 relative to SEQ ID NO: 1.

When the polypeptide is based on plant consensus scaffold sequence (such as SEQ ID NO: 2), the heterologous peptide insertion(s) may be made at any of the following positions relative to SEQ ID NO: 2:

(i) 43-<heterologous peptide>-44
(ii) 44-<heterologous peptide>-45
(iii) 45-<heterologous peptide>-46
(iv) 46-<heterologous peptide>-47
(v) 47-<heterologous peptide>-48
(vi) 48-<heterologous peptide>-49
(vii) 49-<heterologous peptide>-50
(viii) 68-<heterologous peptide>-69
(ix) 69-<heterologous peptide>-70
(x) 70-<heterologous peptide>-71
(xi) 71-<heterologous peptide>-72

(xii) 72-<heterologous peptide>-73
(xiii) 73-<heterologous peptide>-74; or
(xiv) 74-<heterologous peptide>-75.

Most suitably said polypeptide based on plant consensus scaffold sequence comprises two heterologous peptide insertions, a first heterologous peptide insertion at position 46-<heterologous peptide>-47, and a second heterologous peptide insertion at position 71-<heterologous peptide>-72 relative to SEQ ID NO: 2.

Thus in one embodiment when the polypeptide comprises amino acid sequence derived from or corresponding to full length SEQ ID NO: 2 (Type 2 /Plant consensus) the polypeptide may comprise 95-104 amino acids (aa), (or 96-105 amino acids (aa) if a lead methionine is retained). This includes the heterologous peptide insertions, but excludes any N- or C- terminal extension(s) (e.g. "vector-derived terminus" sequence). The shortest, at 95 aa, is a result of a combination of a 3 amino acid Loop 4, and the deletion of two amino acids (VK) from the scaffold immediately prior to this loop, as explained above in connection with exemplary polypeptide (620_825425). This polypeptide is especially suitable for use as a capture reagent.

Thus in one embodiment when the polypeptide comprises amino acid sequence derived from or corresponding to full length SEQ ID NO: 3 (Type 3 /Mammalian (3r2)) the polypeptide may comprise 110 amino acids (aa), (or 109 amino acids (aa) if a lead methionine is removed). This includes the heterologous peptide insertions, but excludes any N- or C- terminal extension(s) (e.g. "vector-derived terminus" sequence).

<u>Scaffold Mutations - Amino Acids Flanking Heterologous Peptide Insertions</u>

Whether the scaffold backbone sequence is derived from plant (such as T2 (SEQ ID NO: 2)) or from mammalian (such as T3r2 (e.g. SEQ ID NO: 3)) sequence, the scaffold backbone amino acids immediately flanking the heterologous peptide insertions may be mutated (e.g. deleted or inserted or substituted, more suitably deleted or substituted, most suitably substituted) relative to the wild-type scaffold backbone sequence (e.g. SEQ ID NO: 1) or relative to the reference scaffold backbone sequence (e.g. SEQ ID NO: 2 or SEQ ID NO: 3) if desired. Suitably any such mutations are of conservative amino acids.

Conservative substitutions may be made, for example according to the table below. Amino acids in the same block in the second column and suitably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Most suitably any such mutations maintain the Beta strand polypeptide structure. Suitably any such mutations are 1, 2 or 3 amino acids from the heterologous peptide insertion site.

In one embodiment, the mutations are 1, 2 or 3 amino acids after the heterologous peptide insertion i.e. C-Terminal relative to heterologous peptide insertion. This is sometimes referred to as "after the loop".

In one embodiment, the mutations are 1, 2 or 3 amino acids prior to the heterologous peptide insertion i.e. N-Terminal relative to the heterologous peptide insertion. This is sometimes referred to as "before the loop".

Tags/Vector Derived Terminus Sequence

The polypeptides described herein, such as Affimer® polypeptides, may comprise N-terminal extension(s) or C-terminal extension(s) to the scaffold backbone sequence, most suitably C-terminal extension(s). An N-terminal extension refers to one or more amino acids occurring before the first amino acid of the scaffold sequence i.e. before the amino acid corresponding to the first amino acid of SEQ ID NO: 2 or SEQ ID NO: 3; a C-Terminal extension refers to one or more amino acids occurring after the final amino acid of the scaffold sequence i.e. after the amino acid corresponding to the last amino acid of SEQ ID NO: 2 or SEQ ID NO: 3.

One or more amino acid sequence tags may be added to polypeptides of the invention for various reason(s) for example, detection (e.g. epitopes such as the flag tag, e.g. epitopes such as the HA (haemagglutinin) tag) or purification (e.g. a "6His tag", e.g. a cysteine residue at the extreme C-terminus). Occasionally notation may indicate the location of a particular tag e.g. cHis6 means His6 (6His) located at the C-terminal end of the polypeptide.

Various tags are disclosed herein within the amino acid sequence labelled "Vector Derived Terminus" in the polypeptide sequences provided herein. Unless otherwise apparent, "Vector Derived Terminus" means a C-terminal extension i.e. the "Vector Derived Terminus" is added to the polypeptide sequence (Affimer® polypeptide sequence) immediately after the last amino acid of the polypeptide sequence. Suitably the tag may comprise a detection tag such as the FLAG tag (amino acid sequence DYKDDDDK (SEQ ID NO: 177)) and/or HA tag (amino acid sequence YPYDVPDYA (SEQ ID NO: 179)). More suitably the tag may comprise multiple repeats of a detection tag such as FLAG tag e.g. 3xFLAG (amino acid sequence DYKDHDGDYKDHDIDYKDDDDK (SEQ ID NO: 180)).
Exemplary extension sequences, such as C-terminal extension sequences, are disclosed herein such as the 'vector derived terminus' sequences in Example 5.
For the avoidance of doubt, data provided in this patent application are typically generated using polypeptide sequences comprising one or more C-Terminal tags, in particular data provided may be for polypeptide sequences comprising the particular Vector Derived Terminus disclosed for each said respective polypeptide sequence.

It is expected that the properties disclosed are the same for tagged and for untagged versions of the polypeptides. Clearly there may be some variation in the precise absolute values of the measurements if tagged polypeptides were compared to untagged polypeptides. However, this is normal and routine in the art. It is important to note that key properties of the peptides would be expected not to vary between tagged and untagged versions for example, binding affinities should not be affected by the presence or absence of a C-terminal tag. Typically data provided in this patent application are understood not to be dependent on the presence or absence of tags such as C-terminal tags.

Combinations

For certain detection or diagnostic applications, the polypeptides described herein may be used in combinations. This means that a particular assay may comprise the use of a combination of two or more polypeptides as disclosed herein, and/or that a particular assay may comprise the use of a combination of one or more polypeptides as disclosed herein with one or more antibodies. Thus, in one embodiment a particular assay may comprise the use of a combination of two or more polypeptides as disclosed herein, suitably three or more, suitably four or more, suitably five or more, suitably six or more polypeptides as disclosed herein, or even more. In another embodiment, a particular assay may comprise the use of a combination of on polypeptide as disclosed herein with

one antibody. In another embodiment, a particular assay may comprise the use of a combination of one or more polypeptides as disclosed herein with one or more antibodies, suitably two or more polypeptides as disclosed herein with one or more antibodies, suitably two or more polypeptides as disclosed herein with two or more antibodies, suitably three or more polypeptides as disclosed herein with one or more antibodies, suitably three or more polypeptides as disclosed herein with two or more antibodies, suitably three or more polypeptides as disclosed herein with three or more antibodies, suitably four or more polypeptides as disclosed herein with one or more antibodies, suitably four or more polypeptides as disclosed herein with two or more antibodies, suitably four or more polypeptides as disclosed herein with three or more antibodies, suitably four or more polypeptides as disclosed herein with four or more antibodies.

Particularly suitable examples of combinations of polypeptides are provided herein.

Suitably, the first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') is selected from the group consisting of polypeptides 620_825594, 620_825456, 620_825586, 620_825317, 620_826257, 620_825436, 620_825425, 620_825543 and 620_825478. In one embodiment suitably the first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') is a combination of two or more polypeptides selected from polypeptides 620_825594, 620_825456, 620_825586, 620_825317, 620_826257, 620_825436, 620_825425, 620_825543 and 620_825478. In one embodiment suitably the first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') is a combination of three or more polypeptides selected from the polypeptides 620_825594, 620_825456, 620_825586, 620_825317, 620_826257, 620_825436, 620_825425, 620_825543 and 620_825478. In one embodiment suitably the first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') is a combination of four or more polypeptides selected from polypeptides 620_825594, 620_825456, 620_825586, 620_825317, 620_826257, 620_825436, 620_825425, 620_825543 and 620_825478. In one embodiment suitably the first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') is a combination of five or more polypeptides selected from polypeptides 620_825594, 620_825456, 620_825586, 620_825317, 620_826257, 620_825436, 620_825425, 620_825543 and 620_825478.

Suitably, 620_826257 is combined with any polypeptide selected from the group consisting of 620_825425, 620_825436, 620_825456, 620_825336, 620_825317,

620_825594 and 620_826478. Suitably a test according to the present invention comprises a first polypeptide which is a detection polypeptide (suitably 620_826257) and comprises a second polypeptide which is selected from the group consisting of 620_825425, 620_825436, 620_825456, 620_825336, 620_825317, 620_825594 and 620_826478.

Different combinations may be especially advantageous in different detection settings such as LFD (Lateral Flow Device), such as MS (Mass Spectrometry), or such as ELISA (Enzyme Linked Immunosorbent Assay).

Combinations especially suitable for ELISA:
Polypeptide 620_826257 is the most suitable detection polypeptide for this assay format.
Suitably, 620_826257 is combined with any polypeptide marked as '1' or '2' in the 'ELISA Ranking' column in Figure 5. More suitably 620_826257 is combined with any polypeptide marked as '1' in the 'ELISA Ranking' column in Figure 5. Suitably an ELISA test according to the present invention comprises a first polypeptide which is a detection polypeptide (suitably 620_826257) and comprises a second polypeptide which is selected from the group consisting of any polypeptide marked as '1' or '2' in the 'ELISA Ranking' column in Figure 5, more suitably comprises a second polypeptide which is selected from the group consisting of any polypeptide marked as '1' in the 'ELISA Ranking' column in Figure 5.

In one embodiment an ELISA test according to the present invention a first polypeptide which is a detection polypeptide (suitably 620_826257) and comprises polypeptide 620_825436 and polypeptide 620_825425 – this combination has the advantage of being a preferred multivalent capture surface; this combination has the advantage of providing an avidity effect and assay sensitivity increase.

In one embodiment an ELISA test according to the present invention comprises a first polypeptide which is a detection polypeptide (suitably 620_826257) and comprises polypeptide 620_825436 and polypeptide 620_825594 – this combination has the advantage of being a very effective multivalent capture surface; this combination has the advantage of providing an avidity effect and assay sensitivity increase.

In one embodiment, an ELISA test according to the present invention comprises one or more antibodies and one or more polypeptides as disclosed herein. In some

embodiments, an ELISA test according to the present invention comprises multiple (more than one) antibodies (preferably two antibodies) and a single polypeptide as disclosed herein. Embodiments with three or more antibodies and a single polypeptide are also disclosed. In some embodiments, an ELISA test according to the present invention comprises a single antibody and multiple (more than one) polypeptides as disclosed herein, preferably, two polypeptides. Embodiments with a single antibody and three or more polypeptides are also disclosed. In some embodiments, an ELISA test according to the present invention comprises two or more antibodies and one or two polypeptides according to the present invention. In some embodiments, an ELISA test according to the present invention comprises one or two antibodies and two or more polypeptides according to the present invention.

Combinations especially suitable for LFD:

In LFD applications, suitably the first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') is selected from the group consisting of polypeptides 620_825436, 620_825425 and 620_825478. In one embodiment suitably the first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') is a combination of two or more polypeptides selected from polypeptides 620_825436, 620_825425 and 620_825478. In one embodiment suitably the first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') is a combination of three polypeptides: 620_825436, 620_825425 and 620_825478.

In LFD applications, suitably the second polypeptide (the polypeptide joined to an indicator agent i.e. 'detection polypeptide') is selected from the group consisting of polypeptides 620_826257, 620_825543 and 620_825411.

In LFD applications, suitably the first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') is selected from the group consisting of polypeptides 620_825436, 620_825425 and 620_825478 and suitably the second polypeptide (the polypeptide joined to an indicator agent i.e. 'detection polypeptide') is selected from the group consisting of polypeptides 620_826257, 620_825543 and 620_825411.

In LFD applications, suitably the first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') comprises one or two or three polypeptides selected from the group consisting of polypeptides 620_825436, 620_825425 and 620_825478 and suitably the second polypeptide (the polypeptide joined to an indicator agent i.e. 'detection polypeptide') comprises one or two or three polypeptides selected from the group consisting of polypeptides 620_826257, 620_825543 and 620_825411.

In one embodiment a LFD test (e.g. LFD device) according to the present invention comprises a first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') which is a combination consisting of polypeptides 620_825436 and 620_825425, and comprises a second polypeptide (the polypeptide joined to an indicator agent i.e. 'detection polypeptide') comprising, or consisting of, polypeptide 620_825411. This embodiment provides the advantage of an excellent multivalent capture surface, and provides the advantage of an avidity effect and assay sensitivity increase.

In one embodiment a LFD test (e.g. LFD device) according to the present invention comprises a first polypeptide (the immobilised first polypeptide i.e. 'capture polypeptide') which is a combination of polypeptides 620_825436 and 620_825425, and comprises a second polypeptide (the polypeptide joined to an indicator agent i.e. 'detection polypeptide') comprising, or consisting of, polypeptide 620_826257. This embodiment provides the advantage of being a preferred multivalent capture surface; this combination has the advantage of providing an avidity effect and assay sensitivity increase.

In one embodiment, a LFD test according to the present invention comprises one or more antibodies and one or more polypeptides as disclosed herein. In some embodiments, an LFD test according to the present invention comprises multiple (more than one) antibodies (preferably two antibodies) and a single polypeptide as disclosed herein. Embodiments with three or more antibodies and a single polypeptide are also disclosed. In some embodiments, an LFD test according to the present invention comprises a single antibody and multiple (more than one) polypeptides as disclosed herein, preferably, two polypeptides. Embodiments with a single antibody and three or more polypeptides are also disclosed. In some embodiments, an LFD test according to the present invention comprises two or more antibodies and one or two polypeptides according to the present invention. In some embodiments, an LFD test according to the present invention comprises one or two antibodies and two or more polypeptides according to the present invention.

It is important to note that all of the polypeptide sequences such as Affimer® polypeptide sequences disclosed herein all share the common property of binding to the S1 spike protein of SARS-CoV-2.

Certain combinations of polypeptides disclosed herein are especially suitable across different assay formats. These combinations are especially preferred. The polypeptides described in these combinations share the common property of cross-platform performance, for example for detection of SARS-CoV-2 S1 spike protein across different detection platforms.

We refer to Figure 5 which presents rankings of the polypeptides of the invention. These are ranked by application (ELISA, LFD, BAMS). These are also provided in a separate disclosure of combined ranking (first table on left side of Figure 5).

Most preferred are polypeptide(s) selected from the group consisting of 620_826257, 620_825425, 620_825436 and 620_825411.
In more detail, preferred are polypeptide(s) selected from the group consisting of 620_826257, 620_825425 and 620_825436.

In more detail, polypeptide 620_825411 is especially preferred as a detection polypeptide.
In one embodiment polypeptide 620_825411 is especially preferred as a detection polypeptide in LFD assays.
In another embodiment, polypeptide 620_826257 is especially preferred as a detection polypeptide in LFD assays.

Fusions

In certain embodiments, the polypeptide of the invention has a single spike protein binding amino acid sequence. In other embodiments, the polypeptide may include at least two or even three spike binding amino acid sequences, which may bind the same or different site(s) on the spike protein. In certain embodiments the polypeptide may comprise a fusion protein containing the amino acid sequences of two or more individual polypeptides as presented herein on a single polypeptide chain (fusion protein). In certain embodiments, polypeptide of the invention may be multiparatopic, such as biparatopic or triparatopic, having spike binding amino acid sequences that can simultaneously bind two or more different sites on the same spike protein. In certain embodiments, the polypeptide of the invention can simultaneously bind two or more different spike proteins on the same viral particle.

In some embodiments, the polypeptide of the invention is a fusion protein which may include, in addition to the spike binding polypeptide, one or more additional amino

acid sequences selected from the group consisting of: secretion signal sequences, peptide linker sequences, affinity tags, transmembrane domains, cell surface retention sequence, substrate recognition sequences for post-translational modifications, multimerization domains to create multimeric structures of the protein aggregating through protein-protein interactions, half-life extending polypeptide moieties, polypeptide sequences for altering tissue localization and antigen binding site of an antibody, and one or more additional polypeptide sequence(s) binding the viral spike protein (either the same or a different epitope) or a different protein on the viral particle.

In some embodiments the polypeptide of the invention is a fusion protein which may include two or more spike binding polypeptides as disclosed herein. These fusion proteins are discussed using standard notation – for example "620_825436-620_825436-cHis6" means a polypeptide comprising amino acid sequence (SEQ ID NO: 70>-< SEQ ID NO: 70>-<His6>) (cHis6 means His6 at the c-terminus); similarly 620_825425-620_825425-cHis6 means a polypeptide comprising amino acid sequence (SEQ ID NO: 67>-< SEQ ID NO: 67>-<His6>) etc. The notation may not be exhaustive – e.g. see Example 9 where an additional Gly is present as a linking amino acid as described for certain exemplary polypeptides.

SEQUENCE IDENTITY

Sequence comparisons can be conducted by eye or, more usually, with the aid of readily available sequence comparison programs. These publicly and commercially available computer programs can calculate percent homology (such as percent identity) between two or more sequences. A suitable computer program for carrying out such analysis is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A; Devereux et al., 1984, *Nucleic Acids Research* 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package, FASTA (Altschul et al., 1990, *J. Mol. Biol.* 215:403-410) and the GENEWORKS suite of comparison tools.

Suitably any heterologous peptide insertions are excluded from percent identity calculations.

Suitably any N-terminal extensions are excluded from percent identity calculations.

Suitably any C-terminal extensions are excluded from percent identity calculations.

Suitably sequence identity is judged against the 'background' or 'backbone' scaffold sequence of the polypeptide compared to the corresponding residues of SEQ ID NO: 3

or SEQ ID NO: 2. Suitably sequence identity is judged against the full length of SEQ ID NO: 3 or SEQ ID NO: 2.

In one embodiment, suitably the polypeptides are based on a Type 3 scaffold i.e. a mammalian scaffold sequence as described in WO2019/008335.

Thus in one embodiment suitably the polypeptide of the invention comprises amino acid sequence having at least 70% sequence identity to SEQ ID NO: 3, suitably at least 75%; more suitably at least 80%, more suitably at least 85%, more suitably at least 88%, more suitably at least 90%, more suitably at least 92%, more suitably at least 94%, more suitably at least 95%, more suitably at least 96%, more suitably at least 97%, more suitably at least 98%, more suitably at least 99% identity to SEQ ID NO: 3. In one embodiment the polypeptide of the invention comprises amino acid sequence having 100% sequence identity to SEQ ID NO: 3.

In one embodiment, suitably the polypeptides are based on a Type 2 scaffold i.e. a plant cystatin scaffold sequence as described in WO2014/125290.

Thus in one embodiment suitably the polypeptide of the invention comprises amino acid sequence having at least 70% sequence identity to SEQ ID NO: 2, suitably at least 75%; more suitably at least 80%, more suitably at least 85%, more suitably at least 88%, more suitably at least 90%, more suitably at least 92%, more suitably at least 94%, more suitably at least 95%, more suitably at least 96%, more suitably at least 97%, more suitably at least 98%, more suitably at least 99% identity to SEQ ID NO: 2. In one embodiment the polypeptide of the invention comprises amino acid sequence having 100% sequence identity to SEQ ID NO: 2.

Suitably 'based on' means that the scaffold backbone sequence of a polypeptide of the invention comprises amino acid sequence derived from a Stefin A/Cystatin scaffold backbone sequence as specified.

In all discussions of sequence identity, it will be noted that neither SEQ ID NO: 3 nor SEQ ID NO: 2 is 100 amino acids in length. Therefore each single substitution is equivalent to slightly more than 1% or slightly less than 1% change in identity respectively when all amino acids of SEQ ID NO: 3 or SEQ ID NO: 2 are considered. The above values are given to nearest whole percentage point and should be understood accordingly given that it is not possible to substitute partial amino acids within a polypeptide sequence.

Unless otherwise apparent from the context, the same sequence identity levels as noted above for amino acid sequences also apply to nucleotide sequences herein.

Exemplary Mammalian Scaffold Sequence

An exemplary mammalian scaffold sequence is the 3r2 scaffold as disclosed as SEQ ID NO: 19 in WO2019/008335. This scaffold is 97 aa in length and has the following mutations relative to WT hSteA (SEQ ID NO: 1):

3r2 – hSteA Y35W N32G V48D M65I Q42E T51L (A59V G60N ΔD61 N62G) (E29K K30E E33K)

Insertion of heterologous peptides is most suitably accomplished by insertion between residues D48 and G50, deleting residue A49 (first heterologous peptide (loop 2)), and/or insertion between residues L73 and E78, deleting residues P74, G75, Q76 and N77 (second heterologous peptide (loop 4)) (all numbering with reference to SEQ ID NO: 1).

Thus, once the heterologous peptide insertions are carried out as illustrated above, the result is an exemplary polypeptide of the invention based on Mammalian scaffold 3r2 showing insertion of two 9mer heterologous peptides (underlined - one in 'loop 2' and one in 'loop 4' – where X is any amino acid): T3r2[9_9]:

MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD**XXXXXXXXX**GLNYYIK
VRVNGKYIHLKVFKSL**XXXXXXXXX**EDLVLTGYQVDKNKDDELTGF (SEQ ID NO: 181)

Thus the background/backbone scaffold sequence of polypeptides of the invention based on mammalian scaffold proteins such as 3r2 is suitably as below (SEQ ID NO: 3). This comprises 92 aa since 3r2 has a deletion of D61 (ΔD61), since insertion at loop 2 deletes A49 and since insertion at loop 4 deletes P74-N77, leaving 92 aa in total (compared to 98 aa for WT hSteA):

SEQ ID NO: 3 – Exemplary Mammalian Backbone Scaffold Sequence: Type 3r2 (92aa)
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDGLNYYIKVRVNGKYIH
LKVFKSLEDLVLTGYQVDKNKDDELTGF

Exemplary Plant Scaffold Sequence

An exemplary plant scaffold sequence is the T2 scaffold. This is based on a consensus sequence derived from plant Cystatin C sequences as disclosed in WO2014/125290.

An exemplary plant Cystatin C consensus sequence is:

ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ**VVAG**TMYYLTLEAKDGGK
KKLYEAKVWVK**PWE**NFKELQEFKPVGDA (SEQ ID NO: 182)

The VVAG (loop 2) and PWE (loop 4) sequences are underlined to aid understanding since these are suitably deleted by insertion of heterologous peptides.

Thus, once the heterologous peptide insertions are carried out as illustrated above, the result is an exemplary polypeptide of the invention based on Cystatin C consensus sequence T2 Scaffold showing insertion of two 9mer heterologous peptides (underlined - one in 'loop 2' and one in 'loop 4' – where X is any amino acid): T2[9_9]:

ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ**XXXXXXXXX**TMYYLTLEA
KDGGKKKLYEAKVWVK**XXXXXXXXX**NFKELQEFKPVGDA (SEQ ID NO: 183)

In another embodiment, the heterologous peptide insertions may be carried out as illustrated above, but using a constant 3mer insertion at 'loop 4' whilst varying the heterologous peptide insertion at 'loop 2'. The result is an exemplary polypeptide of the invention based on Cystatin C consensus sequence T2 Scaffold showing insertion of one 9mer heterologous peptide at loop 2 and insertion of one constant 3mer (AAE) heterologous peptide at loop 4 (each underlined - where X is any amino acid): T2[9_AAE]:

ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ**XXXXXXXXX**TMYYLTLEA
KDGGKKKLYEAKVWVK**AAE**NFKELQEFKPVGDA (SEQ ID NO: 184)

Thus the background/backbone scaffold sequence of polypeptides of the invention based on the plant Cystatin C consensus derived scaffold backbone such as T2 is suitably as below (SEQ ID NO: 2). This comprises 85 aa, since insertion of the first heterologous peptide (loop 2) deletes VVAG and insertion of the second heterologous peptide (loop 4) deletes PWE, leaving 85 aa in total:

SEQ ID NO: 2 – Exemplary Plant Backbone Scaffold Sequence: Type 2 (T2) (85aa):

ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQTMYYLTLEAKDGGKKKLY
EAKVWVKNFKELQEFKPVGDA

BINDING

Spike Protein

Suitably spike protein (sometimes referred to as 'viral spike' or 'spike') means polypeptide having the amino acid sequence as in GenBank Accession # QHD43416.1.

In some aspects, spike protein means polypeptide having an amino acid sequence as in GenBank Accession # QHD43416.1, but comprising one or more amino acid substitutions, deletions or additions. For example, the spike protein may comprise mutations (i.e., substitutions, deletions or additions) at positions including but not limited to position 614 and/or at position 453, such as amino acid substitutions including but not limited to D614G and/or Y453F. For example, the spike protein may be the spike protein of the B.1.1.7 variant of SARS-CoV-2 virus. For example, the spike protein may comprise a mutation at positions including but not limited to one or more of the positions selected from H69, V70, Y144, N501, A570, and/or P681, such as one or more of the HV 69-70 deletion, Y144 deletion, N501Y substitution, A570D substitution, and/or the P681H substitution. The spike protein may also comprise mutations at other positions, or different mutations at the positions explicitly mentioned herein.

In some aspects, the polypeptide of the invention binds to SARS-CoV-2 spike protein with a Kd of 1×10^{-6}M or less.

Suitably binding of the polypeptide of the invention to the spike protein is when said spike protein is present on the viral particle.

Suitably the viral particle is a SARS-CoV-2 viral particle.

In some aspects, suitably binding of the polypeptide of the invention to the spike protein may inhibit interaction of the spike protein to which it is bound with ACE2, suitably human ACE2.

In some aspects, suitably binding of the polypeptide of the invention to the spike protein may inhibit interaction of the spike protein to which it is bound with TMPRSS2 (Transmembrane Protease, serine 2), suitably human TMPRSS2. Suitably TMPRSS2 means a polypeptide having the amino acid sequence of UniProt accession number O15393.

In some aspects, suitably binding of the polypeptide of the invention to the spike protein may inhibit interaction of the spike protein to which it is bound with dipeptidylpeptidase 4 (DPP4), suitably human DPP4. Suitably DPP4 means a polypeptide having the amino acid sequence of UniProt accession number P27487.

In some embodiments, the polypeptide of the invention binds to viral spike with a Kd of 1×10^{-7}M or less, Kd of 1×10^{-8}M or less, Kd of 1×10^{-9}M or less, or even a Kd of 1×10^{-10}M or less.

In some embodiments, the polypeptide of the invention binds to viral spike as a monomer with a dissociation constant (K_D) of about 1 μM or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, about 10 nM or less, about 1 nM or less, or about 0.1 nM or less.

In some embodiments, the polypeptide of the invention binds to viral spike with a K_{off} (off rate constant (K_{off}), such as measured by Biacore), of 10^{-3} s^{-1} or slower, 10^{-4} s^{-1} or slower, or even 10^{-5} s^{-1} or slower.

In some embodiments, the polypeptide of the invention binds to viral spike with a K_{on} (association constant (K_{on}), such as measured by Biacore), of 10^{3} $M^{-1}s^{-1}$ or faster, 10^{4} $M^{-1}s^{-1}$ or faster, 10^{5} $M^{-1}s^{-1}$ or faster, or even 10^{6} $M^{-1}s^{-1}$ or faster.

In some embodiments, the polypeptide of the invention binds to viral spike with an IC50 in a competitive binding assay with human ACE2 of 1 μM or less, 100 nM or less, 40 nM or less, 20 nM or less, 10 nM or less, 1 nM or less, or even 0.1 nM or less.

Suitably competitive binding assay with human ACE2 is carried out as in Example 4 below.

The terms used herein to describe binding/association/dissociation characteristics are well known in the art and such properties (for example dissociation constant (K_D), off rate constant (K_{off}), association constant (K_{on}), half maximal inhibitory concentration (IC50) in a competitive binding assay, etc.) may be determined by any suitable method known in the art.

Unless otherwise apparent from the context, most suitably K_D is assessed using SARS-CoV-2 S1 protein (ACRO Biosystems, S1N-C52H3) in the "Protocol for determination of SARS-CoV-2 S1 binding kinetics constants by SPRi" disclosed herein below.

Midpoint transition temperature (T_m) may be determined by any suitable method known in the art. In case any further guidance is required, we refer to WO2019/008335 which deals in depth with the assessment of T_m.

When the polypeptide of the invention and the SARS-CoV-2 spike protein associate ('bind') with one another, the resulting collection of associated molecules ('bound' molecules) is described as 'a complex' or 'a bound complex'.

In some embodiments, the polypeptide of the invention has a melting temperature (Tm, i.e., temperature at which both the folded and unfolded states are equally populated) of 65°C or higher, suitably at least 70°C, suitably at least 75°C, suitably at least 80°C, suitably at least 85°C or higher. Melting temperature (Tm) is a particularly useful indicator of protein stability. The relative proportions of folded and unfolded proteins can be determined by any suitable technique known to the skilled person, including differential scanning calorimetry, UV difference spectroscopy, fluorescence, circular dichroism (CD), and/or NMR (Pace et al. (1997) "Measuring the conformational stability of a protein" in Protein structure: A practical approach 2: 299-321).

TESTS

In some aspects the invention relates to a test for detecting Coronavirus SARS-CoV-2 (the causative agent for COVID-19).

In some aspects the invention relates to a test for detecting infection with Coronavirus SARS-CoV-2 (the causative agent for COVID-19).

Suitably the test is an antigen test.

Suitably the test is a single use test.

Suitably the test in the format of an enclosed absorbent material for receiving the sample such as a saliva sample, or anterior nasal swab sample, coupled to a visual indicator window. Suitably the absorbent material comprises reagents capable of visualising the presence of the SARS-CoV-2 virus. Suitably said reagents comprise one or more polypeptides as described above (e.g. Affimer® Agents) capable of binding the SARS-CoV-2 spike protein.

Spike Protein

Suitably the antigen is the SARS-CoV-2 spike protein.

Suitably the SARS-CoV-2 spike protein comprises polypeptide having the amino acid sequence as in GenBank Accession # QHD43416.1, more suitably consisting of the amino acid sequence as in GenBank Accession # QHD43416.1.

In some aspects, spike protein means polypeptide having an amino acid sequence as in GenBank Accession # QHD43416.1, but comprising one or more amino acid substitutions, deletions or additions. For example, the spike protein may comprise a mutation (e.g., a substitution, deletion or addition) at positions including but not limited to position 614 and/or at position 453, such as amino acid substitutions including but not limited to D614G and/or Y453F. For example, the spike protein may be the spike protein of the B.1.1.7 variant of SARS-CoV-2 virus. For example, the spike protein may comprise a mutation (e.g., a substitution, deletion or addition) at positions including but

not limited to one or more of the positions selected from H69, V70, Y144, N501, A570, and/or P681, such as one or more of the HV 69-70 deletion, Y144 deletion, N501Y substitution, A570D substitution, and/or the P681H substitution. For example, the spike protein may comprise mutations at other positions, or different mutations at the positions explicitly mentioned herein, including but not limited to mutations of variants known at the time of writing.

GenBank is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences (National Center for Biotechnology Information, U.S. National Library of Medicine 8600 Rockville Pike, Bethesda MD, 20894 USA; Nucleic Acids Research, 2013 Jan;41(D1):D36-42) and accession numbers provided relate to this unless otherwise apparent. Suitably the current release is relied upon. More suitably the release available at the effective filing date is relied upon. Most suitably the GenBank database release referred to is NCBI-GenBank Release 236: 15 February 2020.

Suitably S1 Spike protein is as in GenBank Accession # QHD43416.1.

Suitably S1 Spike protein is as obtained from Acro Biosystems, 1 Innovation Way, Newark, DE 19711, USA.

Suitably SARS-CoV-2 means the virus having the genome sequence, from which the Spike protein is taken (Spike protein Accession # QHD43416.1) i.e. the viral genome of Genbank Accession number MN908947.3.

For the avoidance of doubt the amino acid sequence of S1 Spike protein (SEQ ID NO: 185) as presented in GenBank Accession # QHD43416.1 is below. The S1 Fragment is marked in **bold.** The **S1 fragment** means **Val 16 - Arg 685 (v16-r685).**

1 mfvflvllpl vssqc**vnltt rtqlppaytn sftrgvyypd kvfrssvlhs tqdlflpffs**

61 **nvtwfhaihv sgtngtkrfd npvlpfndgv yfasteksni irgwifgttl dsktqslliv**

121 **nnatnvvikv cefqfcndpf lgvyyhknnk swmesefrvy ssannctfey vsqpflmdle**

181 **gkqgnfknlr efvfknidgy fkiyskhtpi nlvrdlpqgf saleplvdlp iginitrfqt**

241 **llalhrsylt pgdsssgwta gaaayyvgyl qprtfllkyn engtitdavd caldplsetk**

301 **ctlksftvek giyqtsnfrv qptesivrfp nitnlcpfge vfnatrfasv yawnrkrisn**

361 **cvadysvlyn sasfstfkcy gvsptklndl cftnvyadsf virgdevrqi apgqtgkiad**

421 **ynyklpddft gcviawnsnn ldskvggnyn ylyrlfrksn lkpferdist eiyqagstpc**

481 **ngvegfncyf plqsygfqpt ngvgyqpyrv vvlsfellha patvcgpkks tnlvknkcvn**

541 fnfngltgtg vltesnkkfl pfqqfgrdia dttdavrdpq tleilditpc sfggvsvitp
601 gtntsnqvav lyqdvnctev pvaihadqlt ptwrvystgs nvfqtragcl igaehvnnsy
661 ecdipigagi casyqtqtns prrarsvasq siiaytmslg aensvaysnn siaiptnfti
721 svtteilpvs mtktsvdctm yicgdstecs nlllqygsfc tqlnraltgi aveqdkntqe
781 vfaqvkqiyk tppikdfggf nfsqilpdps kpskrsfied llfnkvtlad agfikqygdc
841 lgdiaardli caqkfngltv lpplltdemi aqytsallag titsgwtfga gaalqipfam
901 qmayrfngig vtqnvlyenq klianqfnsa igkiqdslss tasalgklqd vvnqnaqaln
961 tlvkqlssnf gaissvlndi lsrldkveae vqidrlitgr lqslqtyvtq qliraaeira
1021 sanlaatkms ecvlgqskrv dfcgkgyhlm sfpqsaphgv vflhvtyvpa qeknfttapa
1081 ichdgkahfp regvfvsngt hwfvtqrnfy epqiittdnt fvsgncdvvi givnntvydp
1141 lqpeldsfke eldkyfknht spdvdlgdis ginasvvniq keidrlneva knlneslidl
1201 qelgkyeqyi kwpwyiwlgf iagliaivmv timlccmtsc csclkgccsc gscckfdedd
1261 sepvlkgvkl hyt

Sample

Suitably the test is carried out on a sample. Suitably the sample is from a subject of interest. Suitably the sample is, or is derived from, a biological fluid. Suitably the sample is a respiratory sample. Suitably the biological fluid is from, or is derived from, a part of the body associated with respiratory function and/or airflow. Suitably the biological fluid is, or is derived from, saliva or lung wash (e.g. bronchoalveolar lavage) or mucus or buccal wash (e.g. gargle wash) or nasal wash or an anterior nasal swab; more suitably the fluid is or is derived from saliva or buccal wash or nasal wash or an anterior nasal swab; most suitably the biological fluid is, or is derived from, saliva or an anterior nasal swab.

Suitably the sample is a body fluid. Suitably the sample is from the subject of interest. Suitably the sample is previously obtained from the subject of interest. Suitably the sample is an *in vitro* sample. Suitably the method does not involve collection of the sample. Suitably the sample is previously collected. Suitably the method is an *in vitro* method. Suitably the test is an *in vitro* test.

Suitably the sample comprises saliva.

Suitably the sample comprises an anterior nasal swab.

Suitably the sample consists essentially of saliva.

Suitably the sample consists essentially of an anterior nasal swab.

Suitably the sample consists of saliva.

Suitably the sample consists of an anterior nasal swab.

It is an advantage of the invention that using saliva as a sample facilitates easy collection, without any invasive procedure, from the subject of interest.

It is an advantage of using saliva or an anterior nasal swab as the sample that the test is very sensitive.

Saliva or an anterior nasal swab contains a significant number of viral particles when a patient is infected with SARS-CoV-2.

Families

Within the population of polypeptides described herein, there are several distinct families.

For example, some polypeptides interfere with the ACE2-spike binding, whereas some do not interfere with this binding. Thus, at least two epitopes on the spike protein are represented amongst the polypeptides described.

In more detail, it is disclosed that a number of the polypeptides (e.g. Affimer® Agents) described herein can abolish or inhibit (e.g. reduce) the binding of the SARS-CoV-2 spike protein to the ACE2 receptor protein. Suitably polypeptides described herein can abolish or inhibit (e.g. reduce) the binding of the SARS-CoV-2 spike protein to the ACE2 receptor protein.

In one embodiment suitably the polypeptides of the invention share the further property of interfering with binding between ACE2 and SARS-CoV-2 spike protein.

In one embodiment suitably the polypeptides of the invention share the property of not interfering in binding between ACE2 and SARS-CoV-2 spike protein.

In one embodiment suitably the polypeptides of the invention share the further property of competing for binding of the ACE2 receptor.

In one embodiment suitably the polypeptides of the invention share the further property of not competing for binding of the ACE2 receptor.

Some polypeptides of the invention described herein share the property of binding to the same epitope of the SARS-CoV-2 spike protein.

Some polypeptides of the invention described herein share the property of being clustered in terms of their sequence relationship.

In one embodiment suitably the test may be a laboratory test. Thus in one embodiment suitably the test comprises an ELISA test. Thus in one embodiment suitably the test comprises a mass-spectrometry test.

More suitably in one embodiment the test is a portable test which has no need for laboratory equipment. Thus in one embodiment suitably the test comprises a point-of-care test strip. Thus in one embodiment suitably the test comprises a rapid test strip. Thus in one embodiment suitably the test comprises a lateral flow device (LFD). Thus in one embodiment suitably the test comprises a lateral flow test strip.

Lateral Flow Device

Immunochromatographic assays, also called lateral flow tests or simply strip tests are useful in field testing, point of care testing e.g. doctors' surgeries, or home testing. Lateral flow assays can be used to detect any ligand that can be bound to a visually detectable solid support, such as dyed microspheres, both qualitatively and, in many cases, semi-quantitatively.

These offer advantages such as ease of manufacture, long term stability, ease of interpretation, and rapid result. They are also easy to use and non-invasive (especially when the sample is, or comprises, saliva).

Thus in one embodiment the invention provides a strip test (lateral flow test or lateral flow device) for detection of the SARS-CoV-2 virus. Suitably the ligand being detected is the SARS-CoV-2 spike protein.

Suitably a lateral flow device (LFD) according to the present invention comprises a system of overlapping porous materials containing the dried components needed to perform the test. These porous materials may be membranes. These are suitably assembled in small strips, which may be placed into a plastic housing for ease in handling.

A typical LFD is described in published patent US8,399,261B2. Figures 4A and 4B herein are adapted from Figure 2 and Figure 1 of US8,399,261B2 respectively.

Referring to Figure 4B, The sample to be tested, such as saliva or a sample derived from an anterior nasal swab, is loaded onto sample application pad 10. The liquid fraction of the sample then moves through a conjugate release pad 12 onto which a conjugate has been dried. The conjugate may comprise detection molecules specifically directed against the analyte of interest, i.e. a polypeptide of the invention capable of specifically binding SARS-CoV-2 spike protein, and indicator particles, such as colloidal gold or gold Sol. Alternatively, the conjugate may comprise detection molecules specifically

directed against the analyte of interest, i.e. a polypeptide of the invention capable of specifically binding SARS-CoV-2 spike protein, and a further moiety enabling detection/immobilisation by a further molecule which can be placed at a defined location (e.g. capture line, or region) on the membrane as below. Upon contact with the liquid sample, the conjugate re-dissolves and specifically binds to any SARS-CoV-2 viral particles present in the sample to form an analyte-conjugate complex. This complex flows through a membrane such as a nitrocellulose membrane 14, also referred to as the analytical membrane, on which test and control reagents have been immobilized. More specifically, membrane 14 is provided with two capture lines, or regions, arranged sequentially and positioned perpendicularly to the flow direction, each containing bound reagents. Test line 16 contains analyte-specific molecules which are able to bind to and immobilize the analyte-conjugate complex, resulting in a visible coloured line. Control line 18 does not contain analyte-specific molecules but is able to fix non-bound conjugate containing particles. The formation of a coloured line at control line 18 indicates that the test sample has flowed past test line 16. The colour intensity observed at test line 16 is directly proportional to the analyte concentration, i.e. concentration of SARS-CoV-2 viral particles, in the sample and therefore enables semi-quantitative interpretation of the test result. If the analyte of interest, i.e. SARS-CoV-2 viral particles, is present at a level above the detection limit, test line 16 and control line 18 both become clearly visible. If the analyte, i.e. SARS-CoV-2 viral particles, is absent or is present at a level below the detection limit, only control line 18 becomes visible during the test.

Optionally the lateral flow device (LFD) may further comprise an absorbent pad 20 (also known as a wicking or sink pad) which collects the fluid which has flowed through the test system and prevents any backflow of fluid. Absorbent pad 20 allows the use of samples whose volume exceeds the wicking capacity of membrane such as nitrocellulose membrane 14.

Referring to Figure 4A, the lateral flow device (LFD) may optionally omit a conjugate pad. In this implementation, a pre-incubation of sample and liquid conjugated indicator (e.g. liquid conjugated gold) precedes lateral flow on a test strip, which can lead to better sensitivity. No conjugate pad is needed, however a separate mixing between the sample and the conjugated indicator is needed.

Alternatively in this implementation (Figure 4A), a liquid formulation of a conjugated indicator, such as gold conjugate, is applied to the lateral flow test device following application of a test sample and a chase buffer. A second application of chase buffer is made following application of the liquid conjugated indicator. The absence of a conjugate pad (i.e. a separate pad comprising a dried conjugate such as dried gold

conjugate) in this implementation also simplifies manufacture of the device, leading to further reduced costs.

Optionally one or more stabilisers are included with the conjugate to improve shelf life.

In one aspect, the invention provides a system for detecting the presence of SARS-CoV-2 viral particles in a sample, preferably a biological sample. Suitably the system comprises a lateral flow assay device and a liquid formulation of a conjugated indicator, such as gold conjugate. The lateral flow assay device comprises: (a) a sample receiving region; and (b) a capture membrane including a test region comprising an immobilized detection agent specific for the analyte such as a polypeptide as described above, and a control region including an immobilized reagent that binds to the detection agent. The lateral flow assay device may also include a reservoir region positioned downstream of the capture membrane for absorbing excess fluid, such as excess of the biological sample, liquid formulation of a conjugated indicator or an excess of a chase buffer that may be used with the assay device as further described below.
The lateral flow assay device may also include a conjugate pad positioned downstream of the sample receiving region and upstream of the capture membrane. Suitably this conjugate pad comprises conjugated indicator, such as detection molecules specifically directed against the analyte of interest (i.e. a polypeptide of the invention capable of specifically binding SARS-CoV-2 spike protein), conjugated to indicator molecules such as gold conjugate.

In another aspect, a lateral flow assay device for detecting the presence of an analyte of interest (e.g. SARS-CoV-2 viral particles) in a test sample is provided, the device consisting essentially of: (a) a sample receiving region; (b) a capture membrane comprising a test region including an immobilized detection agent specific for the analyte of interest (e.g. a polypeptide of the invention capable of specifically binding SARS-CoV-2 spike protein), and a control region positioned downstream of the test region and including an immobilized reagent that binds to the detection agent; and (c) a reservoir region positioned downstream of the capture membrane for absorbing excess fluid.
In a related aspect, kits for the detection of an analyte, or component, (e.g. SARS-CoV-2 viral particles) are provided. Such kits comprising a lateral flow assay device disclosed herein, and a container of a liquid gold conjugate formulation, packaged together with instructions for using the device and liquid gold conjugate to detect the presence of the analyte in a sample such as a biological sample. In a further aspect, methods for

detecting the presence of an analyte of interest (e.g. SARS-CoV-2 viral particles) in a liquid test sample are provided. In certain embodiments, such methods comprise: (a) providing a lateral flow assay device described herein; (b) applying the test sample to the sample receiving region; (c) applying a first volume of a chase buffer to the sample receiving region; (d) applying a liquid formulation of a gold conjugate to the sample receiving region to form an analyte-gold conjugate complex; (e) applying a second volume of a chase buffer to the sample receiving region; and (f) allowing the analyte-gold conjugate complex to migrate through the capture membrane to the test region and contact the detection agent thereby immobilizing the analyte-gold conjugate complex and forming a detectable signal, where information of the signal indicates the presence of the analyte in the sample.

Indicator conjugate such as gold conjugate is readily made by following published protocols (see, for example, Bioconjugate Techniques; Chapter 14, pp 593-604: Greg T Hermanson; Academic Press). The conjugate is formulated in a buffer (pH range between 6 and 10) containing stabilizer such as detergent, sugar, protein solutions and/or other relevant blocking components. The conjugate is preferably stable for at least one year. The chase buffer aids in the movement of any complex formed between the conjugate and the analyte laterally along the device. The volume of chase buffer shall vary depending on the system, and may be between 5-500 ul, for example between 10-100 ul. In certain embodiments, the chase buffer comprises a buffer system such as phosphate, Tris-carbonate, bicarbonate, etc., mixed with a detergent such as Tween® 20, Triton X-100 or other non-ionic detergent, CHAPS, non interfering protein blocking substances, such as bovine serum albumin, gelatin or other animal serum- or milk-derived proteins, such as casein, and anti-microbial and anti-fungal substances, such as sodium azide. Considering the needs for product shelf life and ease of evaluation, phosphate based buffers may be preferred.
An exemplary lateral flow assay device (LFD) according to the present invention is shown in Figure 14.

Enzyme-Linked-Immunosorbent Assay (ELISA)

ELISAs are a class of common bioanalytical tests that are able to detect and quantify a specific, soluble analyte (known as an antigen). These cover a whole range of targets from hormones to drugs or pathogens. At least one aspect of the assay set up is immobilised on a solid surface such as a plastic microplate well, with the immunoreagent conveying specificity to the target and the reporter enzyme giving a visual indication of binding. These reagents are typically added stepwise to the assay to

build up layers, with washing in between steps removing any weakly or non-specifically bound immunoreagent(s), analyte or reporter enzyme. This is important to reduce any background signal and enhance the limit of detection.

To be certain that the signal generated was due to the formation of the immunoreagent:antigen complex, the reporter enzyme was chemically conjugated (linked) to the immunoreagent, hence giving the tests name. More recent ELISA-like techniques no longer rely on colourimetric changes to indicate the presence of the desired analyte; instead fluorescence (FLISA) and electrochemiluminescence (ECLIA) are used to generate a measurable signal that can allow higher sensitivities to be reached. Whilst these are not strictly ELISAs due to no reporter enzyme being conjugated to the immunoreagent, these assays are typically called ELISAs as well.

It is the immunoreagent(s) that provide the crucial element of the ELISA: without the confidence in the specificity of the immunoreagent to the desired target, any results cannot be relied upon for a clinical diagnosis or a myriad of other uses that ELISAs are currently wielded for. For many decades, antibodies were the gold standard of immunoreagents as they were well understood and any flaws could be accounted for. Recently however, a number of non-antibody immunoreagents have been developed, with polypeptides as described herein (e.g. Affimer® reagents) addressing many of the concerns faced with antibodies.

There are multiple formats that ELISAs can take. All must have suitable positive and negative controls to show all layers are binding as expected, and no non-specific binding is occurring. Calibration curves should be determined by the developer to allow quantitative analysis from single point ELISAs.

Figure 13 shows schematic representation of ELISA formats. A. Direct ELISA: target passively adsorbed and reporter enzyme directly conjugated to immunoreagent. B. Indirect ELISA: target passively adsorbed and reporter enzyme conjugated to a secondary immunoreagent. C. Sandwich (Pair) ELISA: in this example a capture immunoreagent is immobilised, binding the target and detected with a detection immunoreagent with the reporter enzyme conjugated. D) Competition ELISA: in this example the immunoreagent competes with the target's ligand for binding with the labelled target protein, therefore, the more competitive the immunoreagent, the lower the signal.

Direct ELISA

The antigen is either directly adsorbed onto the surface, with an immunoreagent chemically linked to the reporter enzyme, giving the signal. The advantages of this

assay set up include the fact that it requires minimal steps and is therefore faster and has less room for error introduced by the addition of required reagents. However, because the reporter enzyme is directly linked to the specific immunoreagent, the assay has less flexibility and no amplification. Additionally, because the antigen is directly adsorbed onto the surface, this may deform the target somehow (see immobilisation below). If the antigen is within a complex mixture such as saliva or serum, there may be significant background caused by non-specific binding.

Indirect ELISA

This has a similar assay set up as the Direct ELISA with the exception that the primary immunoreagent is not labelled; rather it is a secondary immunoreagent that recognises the primary immunoreagent. This can increase sensitivity, if multiple secondary detection reagents are able to bind to the primary detection reagent, whilst also allowing greater efficiencies if less labelled immunoreagent is required. It also allows more flexibility with multiple primary detection reagents able to be detected using a singular secondary detection reagent. However, there is a risk of higher assay background if the secondary detection reagent cross-reacts or binds non-specifically to the antigen or the complex mixture adsorbed to the surface.

Sandwich (Pair) ELISA

A Sandwich ELISA requires two (or more) immunoreagents that are able to bind to different epitopes on the same antigen. One immunoreagent is immobilised on the surface (the capture reagent) and is able to capture the antigen in solution. The other immunoreagent(s) detects the captured antigen and generates a signal with its directly linked reporter enzyme. Alternatively, a secondary detection reagent could be used which has the reporter enzyme conjugated to it (an Indirect Sandwich ELISA). This assay format allows for higher specificity and sensitivity, as both immunoreagents need to have bound the antigen to give a signal, and is commonly used for identifying targets within a complex mixture.

Competition ELISA

Whilst the other three forms of ELISA highlighted have identified binding by a measurable signal being generated, the results of a Competition ELISA are measured by the reduction in signal. An antigen (or immunoreagent known to bind antigen) is immobilised on the surface. An unknown amount of antigen (pure or in a complex mixture) is then mixed with a known, limited amount of immunoreagent and allowed time to reach an equilibrium. This mixture of antigen and immunoreagent is added to the assay, with the immobilised analyte competing with the analyte in solution for binding to the limited amount of detection immunoreagent. After washing, any target bound by immunoreagent on the same epitope as the immobilised analyte would bind

is removed. The addition of the reporter enzyme and its substrate gives an inverse signal proportional to the unknown concentration of antigen in solution: the more antigen available in solution, the greater the reduction in signal. All ELISA formats can be converted into a competitive format, with the advantages of minimal sample processing and higher assay robustness and consistency. It is commonly used for smaller antigens where there is too much steric hindrance and limited epitopes available for two immunoreagents to bind simultaneously.

All forms of ELISA depend on building up layers on an immobilised surface and ensuring that the assay is only detecting the desired antigen. This requires optimisation of all aspects of the assay to achieve the desired dynamic range and sensitivity:

Immunoreagent(s)

The reagents that underpins any form of ELISA are the immunoreagents that bind to the target of interest. These have historically been antibodies – proteins produced by B lymphocytes as part of mammal's adaptive immune system to bind to and neutralise any pathogens infecting the animal. Recently many non-antibody immunoreagents have been developed that share many underlying features of antibodies (stable structure, vast ability for differentiation, ability for high specificity), but work around their disadvantages (slower production, batch to batch variation due to glycosylation, cannot work against toxic targets due to death of host mammal before immune response raised). Examples of non-antibody immunoreagents include Affimer® reagents, Nanobodies®, DARPin® proteins (all protein based) and Aptamers (DNA or RNA based), which all use a stable scaffold with at least one variable region to allow binding to the analyte.

To be a useful immunoreagent, there are a number of properties that should be considered:

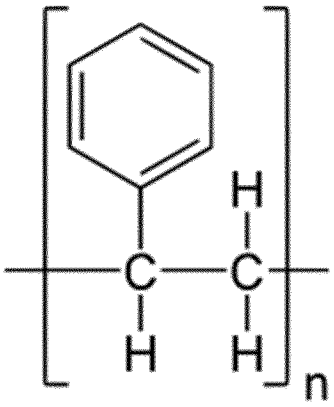
- *Specificity* is whether the immunoreagent binds to an epitope found only on that particular target and not to closely related targets.
- *Affinity* is an indication of the strength of binding between the immunoreagent and the epitope it binds. As binding is reversible, affinity looks at how quickly the immunoreagent binds the analyte (association rate constant, k_a) and how quickly the complex falls apart (dissociation rate constant, k_d). Due to the incubation times of ELISAs, those immunoreagents with high affinity that can form the greatest number of stable immunoreagent:analyte complexes will give the highest sensitivity.
- *Avidity* is a measure of the overall strength of multiple affinities between an immunoreagent and an analyte, also known as function affinity. Avidity can

occur if there are multiple copies of the epitope present on the analyte the immunoreagent can bind to, or the immunoreagent has multiple binding domains that each bind to a different epitope on the analyte simultaneously. The enhanced local concentration of analyte caused upon initial immunoreagent binding can allow other nearby binding sites to bind, ensuring the analyte or immunoreagent doesn't diffuse away.

The concentrations of all immunoreagents in an ELISA are advantageously optimised for a particular set of conditions (e.g. a particular dilution of patient sample in a particular diluent)

Immobilisation

Typically, an ELISA is carried out in a standard 96-well (or larger) polystyrene microplate to allow high throughput and/or automation of the test. Polyvinyl Chloride-based microplates are also available, but due to their flexibility are not suitable for automation. Plates for ELISA are manufactured such that there is a low coefficient of variation between wells and plates within the same production batch, and that any 'edge effect' error is avoided. The surface of the well has a key function to ensure that immunoreagents or antigens (dependent on test type) attach or do not attach to the well as appropriate, by a mixture of chemical and physical properties of the surface and the solution within the well. Most ELISAs are based on biomolecules (proteins, nucleic acids etc.), all of which denature to some degree at interfaces. During an ELISA, there are typically 2 interfaces to consider: the solid:liquid interface between the plastic and the assay solution; and the liquid:gas interface between the assay solution and the atmosphere. Additionally, if the assay is dried to the surface for long term storage, then this also introduces solid:dry film and dry film:gas interfaces to consider. During washing steps and the removal of liquid, any denaturing effect of the solid:liquid:gas interfaces is enhanced. Denaturing carries with it the risk of any binding activity being severely reduced. Chemical structure of polystyrene is shown below. The benzene ring contributes towards polystyrene hydrophobicity.



This denaturing partially unfolds the biomolecule to expose previously hidden hydrophobic regions. As polystyrene is naturally a hydrophobic compound, the partially denatured antigen or immunoreagent passively adsorb onto the surface via hydrophobic interactions (known as medium binding polystyrene). These surfaces are suitable for antigens or immunoreagents with larger hydrophobic segments. An alkaline immobilisation buffer (PBS pH 7.4, or carbonate-bicarbonate buffer pH 9.6) ensures any amino acids of a protein-based antigen or immunoreagent are neutrally charged, enhancing the hydrophobic interactions required for immobilisation. The benzene ring of polystyrene can be chemically modified to change its properties with the incorporation of carboxyl making the binding surface slightly ionic, with hydrophobic elements, allowing interactions with positively charged groups on antigens or immunoreagents. This surface has a higher capacity (known as high binding polystyrene) to bind antigens/immunoreagents and requires a smaller proportion of the antigen/immunoreagent to be in contact with the surface and hence a reduced denaturation effect. Aminated polystyrene has had amine groups added to the benzene ring ensuring that, with the appropriate assay buffer, only ionic/hydrophilic interactions are responsible for immobilisation to the surface. Additionally, surfaces that are aminated or carboxylated can have the antigen/immunoreagent covalently immobilised on the surface in a highly specific manner. Passive adsorption is affected by the time, temperature, pH of the immobilisation buffer and concentration of the reagents being immobilised.

The physical properties of the well can also have an impact on the ELISA carried out. The size of the well and the assay volume can affect the surface area to volume ratio and diffusion distance, which in turn affect the rate of adsorption to the surface, and the time required to carry out the assay. The shape of the well used depends on the format of the assay. A flat bottom allows for excellent optical transmission and low background absorbance when measuring the signal obtained, as well as retaining a small residual amount of fluid that can negate some denaturing effects of excessive drying.

Alternatively, round-bottomed wells can remove any steric hindrance from large molecules becoming stuck in the sharp corners of a flat-bottomed well and more fluid can be removed from the well if required. However, the curvature does mean that the well does not have as good optical qualities as a flat-bottomed well.

The optimal ELISA microplates typically have more immunoreagent or antigen immobilised than can be bound during an assay, to allow as large a dynamic range as possible, with the exception of competition ELISAs. Often it is best to coat reagents at concentrations lower than the maximum binding capacity to avoid introducing the 'hook effect' error into an ELISA. Hooking can occur as a result of assay components becoming trapped between the immobilised reagents on the plate surface, which cannot be removed efficiently during later washing steps. This can result in non-specific binding by detection reagents (the detection reagent is binding the target, but that target is not specifically captured on the surface) and higher signal background, reducing the signal to noise ratio. Alternatively, excess immunoreagent or analyte can prevent both the capture and detection reagents binding simultaneously to the same analyte, giving false negatives.

Whilst passive adsorption usually works well, issues can arise due to denaturation (discussed above); improper orientation so any epitopes or binding regions are inaccessible; or if using a complex mixture, contaminants being immobilised.

Commercial ELISA microplates can be purchased pre-coated with a variety of capture immunoreagents immobilised on the surface to get around these issues. Examples include Protein A, Protein G or a combination of the 2 that bind the Fc region of antibodies; glutathione that is bound by Glutathione-S-Transferases (can be generated as fusion proteins with reagent of interest); or streptavidin/neutravidin that bind biotin with high affinity (biomolecules can have biotin chemically conjugated to them in a myriad of different ways).

Blocking

The quoted binding capacity of each microplate well (usually given in ng/cm^2) is typically larger than the amount of antigen/immunoreagent immobilised on the surface. This means that the remaining surface must be 'blocked' to prevent other components of the assay binding non-specifically to the well during any subsequent steps. A 'blocking buffer' is usually made up a mixture of irrelevant proteins and/or non-ionic detergents preventing passive absorbance to the plate. Protein-based blocks (e.g. milk powder, BSA or casein) are described as permanent blocks (won't be removed during washing) whilst detergents (e.g. Polysorbate 20) are described as temporary (removed by washing and must be replaced in each step). This will enhance the signal to noise ratio by lowering any background signal seen and will ideally be done without

the desired signal being dampened by obscuring the epitope on the antigen. Protein-based blocks can be included in all steps after immobilisation as this can help stabilise any immunoreagent or antigen immobilised on the plate.

Washing

Between each step of the ELISA, the well must be washed (typically 3-5 times) to remove any non-specifically bound material such as the antigen or immunoreagent. A detergent such as Polysorbate-20 is typically included at low concentrations (between 0.01 and 0.1%), with the addition of salts (such as NaCl) available if more stringent washing is required to reduce any background. Conversely, excessive washing can result in decreased sensitivity with immobilised layers of immunoreagent, antigen or detection reagent washed from the well. After the final repeat of each washing step, excess liquid must be removed from wells to avoid dilution of the reagents in the subsequent step.

To allow high throughput and increased consistency, automated plate washers are typically used to carry out these washing steps. Assuming it is maintained and calibrated at an appropriate frequency, this ensures all wells within an ELISA are washed with the same physical force and same volumes, minimising analyst error.

Detection methods

Detection reagents (whether a primary or secondary immunoreagent) are conjugated to a reporter enzyme in the ELISA format, which gives a measurable signal that should be directly proportional to the amount of the target in question. Commonly, a substrate is used that when exposed to the reporter enzyme, is converted to a chromogenic, precipitating product. When the required signal intensity is reached or a designated length of time has passed, the light absorbance is measured at a particular wavelength by spectrophotometric methods. A 'stop' solution can be added to stop the colour signal developing any further e.g. a strong acid to denature the enzyme's active site.

Two common reporter enzymes used in ELISAs are Horse Radish Peroxidase (HRP) and Alkaline Phosphatase (AP). These are both cheap, stable and light insensitive proteins with relatively high enzyme turnover rates, resulting in higher signal sooner at the location of the enzyme i.e. the target the immunoreagent has specifically bound to. Both have known inhibitors which should be avoided from the assay at the point the substrate is added to avoid knock-down in signal. It is possible for multiple copies of reporter enzymes to be conjugated to a single immunoreagent, with the enzyme's molecular size and the availability of suitable amino acids being limiting factors to the number linked. This would allow signal to be visible faster than if a single copy of the reporter enzyme was conjugated and amplify any signal. Substrate choice can affect the signal outcome. Some enzyme:substrate combinations may result in a much faster

signal but lack the higher sensitivity or broader dynamic range of other enzyme:substrate combinations.

HRP catalyses substrates such as 3,3-diaminobenzidine (DAB) and 3,3,5,5-tetramethylbenzidine (TMB) in the presence of the oxidising hydrogen peroxide to brown and blue precipitates respectively at the location. As HRP is relatively small compared to other enzyme conjugates, it reduces steric hindrance and the chance of its conjugation interfering with the immunoreagent it is linked to. However, if testing material that may have endogenous peroxidases present, the addition of the substrate may result in non-specific signal if the assay has not been optimised enough. The products typically fade when exposed to light.

As the name suggests, AP is an enzyme which works optimally at an alkaline pH and catalyses the removal of phosphate groups from proteins and nucleotides such as *p*-Nitrophenyl Phosphate (pNPP). AP is approximately 3.5 times the size as HRP with the attached increased risk of steric hinderance. AP-based detection systems are often able to give higher sensitivities compared to HRP-based assays.

To allow additional flexibility in ELISA development, there are common secondary detection reagents with reporter enzymes conjugated to them. These include streptavidin or neutravidin, which bind biotin moieties with an extremely high affinity. Multiple biotin molecules can be conjugated to a single primary immunoreagent or analyte, allowing multiple streptavidin-reporter enzyme conjugates to bind to a single analyte or immunoreagent. This amplifies any available signal. Another example are antibodies raised against a particular species immunoglobulin and are able to bind an epitope present in all subtypes of a particular class of immunoglobulin e.g. human IgG1, IgG2, IgG3 and IgG4. These 'anti-species, enzyme conjugates' allow a single immunoreagent- enzyme conjugate to be used for detecting a species antibodies Fc region. Amplification can also occur in this form of Indirect ELISA as each primary immunoreagent contains multiple epitopes that can be bound by the secondary immunoreagent.

In case any further detail is required, suitably Example 9 provides an exemplary protocol (Protocol of anti-SARS-CoV-2 S1 protein ELISA) of an ELISA according to the present invention, and Example 10 provides an exemplary protocol for a competition ELISA according to the present invention.

BAMS test

Mass spectrometry can be used to identify SARS-CoV-2 proteins, even from highly diluted samples such as buccal wash or gargle samples. The principle is as with known MS approaches – identification of proteins (i.e. observing MS fragments identifying

proteins) originating from SARS-CoV-2 indicates that the virus was present in the sample analysed.

See for example Ihling et al Journal of Proteome Research 2020 report "Mass Spectrometric Identification of SARS-CoV-2 Proteins from Gargle Solution Samples of COVID-19 Patients" (https://dx.doi.org/10.1021/acs.jproteome.0c00280). Similarly Nachtigall et al Nature Biotechnology 2020 report "Detection of SARS-CoV-2 in nasal swabs using MALDI-MS" (https://doi.org/10.1038/s41587-020-0644-7). This MS approach has even been extended to extracellular vesicles as reported by Kreimer et al 2015 Journal of Proteome Research, vol 14, 2367–2384 "Mass-Spectrometry-Based Molecular Characterization of Extracellular Vesicles: Lipidomics and Proteomics" (DOI: 10.1021/pr501279t).

Introduction to BAMS™ Single Bead Immunoassay Platform

Bead-Assisted Mass Spectrometry™ (BAMS™) is an open platform for developing singleplex and multiplex analytical assays for mass spectrometry (MS). It is based on a "single bead immunocapture" concept, which uses large (375 µm) magnetic agarose beads as reactive sites. The high binding capacity of a single bead enables capture and detection of multiple targets, quantitation within a dynamic range spanning about 3 orders of magnitude and detailed MS analysis, including target identification via MS-MS sequencing. There are two ways to take advantage of the multiplexing capability of BAMS™. The on-bead multiplexing is achieved using an antibody, more suitably a polypeptide as described herein, which recognizes an epitope that is present in several proteoforms, more suitably present on/in the SARS-CoV-2 virus. Using this approach, target analytes, e.g. the SARS-CoV-2 virus or proteins therefrom, are cocaptured on a same bead and subsequently resolved by MS based on their distinct molecular weights and/or other properties, such as MS-MS fragmentation profiles (tandem MS) or collision cross section values (ion mobility MS).

The second approach to multiplexing is to combine beads with different specificities, i.e. beads conjugated to different capture antibodies, more suitably different polypeptides as described herein. Unlike sandwich immunoassays, BAMS™ does not utilize detection antibodies and therefore does not suffer from the effects of antibody cross-reactivity.

BAMS™ provides significant increase in analytical sensitivity and precision by capturing and enriching low abundance target analytes on single beads and eluting the captured analytes into miniature (500 µm) spots, which are measured using Matrix-Assisted Laser Desorption Ionization (MALDI) MS. An entire spot is sampled in

seconds, providing an accurate measurement of relative abundances of multiple analytes within the spot.

2. Single Bead Immunoaffinity Capture

The immunoprecipitation (IP) protocol utilized by BAMS™ differs from conventional magnetic bead-based enrichment methods in two aspects: (1) it is adapted for use with larger BAMS™ beads, which have different binding kinetics than regular beads, and (2) it is designed to prevent the loss of individual beads during the assay. As magnetic particles, BAMS™ beads can be easily manipulated, e.g. collected, transferred and released using a hand-held bead picker or automated benchtop magnetic platform such as Kingfisher Flex. Moving particles with a bead picker facilitates liquid exchange during the bead incubation, wash and rinse steps. If a bead picker is not available, BAMS™ beads may be moved as bead suspensions using a 200 µL pipette with a wide orifice pipette tip to prevent bead clumping at the tip. When using the pipetting method, ensure that no beads are lost during the transfer steps and avoid crushing the beads with the tip. BAMS™ protocols provided below are designed for manual bead handling using a 1- channel magnetic QuicPick™ tool ("bead picker") available from Bio-Nobile (Finland), or a similar device. The protocols can be adapted for parallel handling of several samples using an 8-channel QuicPick™. Automated processing of up to 96 samples can be achieved using a robotic workstation, such as MagRo™ 96-M from Bio-Nobile or Kingfisher Flex from Thermo Fisher Scientific. When using a bead picker always collect and release the beads into a solution, without touching a surrounding surface, such as a sidewall or a bottom of a microwell.

BAMS™ assay kits contain a number of pre-aliquoted beads (typically 3 replicate beads for each target analyte) distributed in individual wells of a 96-well plate. The contents of one plate are sufficient for performing 96 IP reactions. The plate may be opened and sealed multiple times using the provided sealing mat. The beads are supplied in a storage buffer and should be stored at 4°C unless indicated otherwise. BAMS™ IP reactions may be performed from a wide range of sample types, including biofluids (both intact and digested), cell and tissue lysates, *in vitro* reactions, etc. Specific experimental details, including assay volumes, buffer compositions, incubation times and bead wash conditions are provided with a particular BAMS™ assay. The IP reactions are typically carried out at 4°C. BAMS™ IP reactions are conveniently performed in a 96-well plate. It is recommended that the plate has V-shaped wells (rather than flat bottom) and holds a volume, which is at least 2-fold greater than the reaction volume. A typical incubation time is several hours to overnight, depending on the assay. An overnight incubation is recommended for capturing low abundance analytes. The incubation reaction should be performed using a temperature-controlled

plate shaker with mixing and vortexing capabilities, such as Eppendorf ThermoMixer®. The mixing speed should be selected to allow beads to continuously circulate through the sample solution for optimal binding, without ejecting them from the solution. The recommended speed setting for ThermoMixer® is 1,200 RPM. Basic microplate shakers without the vortexing capability are not recommended for BAMS™ assays, as they are not designed to keep beads in suspension. Smaller scale IP reactions (up to 300 µL volume) may be performed in a microcentrifuge tube. The tube is inserted into a tube revolver/rotator, which gently rotates the beads inside the tube. The beads are subsequently washed to remove non-specifically bound compounds. A recommended protocol is provided with each BAMS™ assay and typically includes incubation of beads in a high salt buffer (1 M KCl, 100 mM TrisHCl, pH 8.0) for 10 minutes on a microplate shaker, followed by short (2 minutes) sequential incubations in a low salt buffer (100 mM KCl, 100 mM TrisHCl, pH 8.0), ammonium bicarbonate buffer (10 mM) and finally, deionized water (twice). The volume of each wash solution is 0.7 mL. The wash may be performed by sequentially transferring the beads between individual wells of a 48-well plate filled with the corresponding solutions.

3. Arraying Reacted Beads

The captured target analytes are subsequently transferred onto a MALDI compatible surface. This is accomplished by placing a suspension of reacted beads on a microwell array, which causes the beads to sink into microwells, one bead per well, and exposing the bead array to an aerosol containing MALDI matrix solution. The entire protocol is completed in less than 1 hour, with ~10 minutes hands-on time. BAMS™ STARTER KIT contains all necessary parts for arraying beads, including several microwell arrays, 8-, 16- and 24-chamber frames, stainless steel clips, slide trays and a bead retaining magnet. A microwell array is made of a MALDI-compatible gold-coated microscope slide attached to a silicone gasket that contains a square grid array of 88x26 through-holes (microwells). Dimensions of individual microwells are slightly larger than a diameter of a single bead. The number of microwells (>2,000) is much greater than the number of beads in a typical BAMS™ assay to allow screening several samples on a single array. The gasket also contains a chamfer located in the upper left corner. The microwell array may be subdivided into several regions using a multi-chamber frame. Multi-chamber frames included in the starter kit contain 8, 16 or 24 chambers. The number of microwells contained within a single chamber are approximately (8 chamber frame), (16 chamber frame) and (24 chamber frame). 1, 2, 4 and 64 chamber frames are also available. It is recommended to select a chamber size that provides about 2:1 microwell-to-bead ratio. This ensures that every bead will be placed into a microwell without leaving a large number of empty microwells. After selecting a suitable multi-

chamber frame, position it on the microwell array and secure using the stainless steel clips by first inserting the hooked edge of the clip into the groove of the chamber frame, then sliding the rounded edge of the clip over the bottom of the microscope slide. Dispense deionized water into individual chambers to cover the entire surface of the microwell array. Place the slide tray and a properly weighed counterbalance, such as an MTP plate filled with deionized water into a centrifuge with a swing bucket rotor for MTP buckets. Centrifuge at 2,000 RPM for 5 minutes to ensure the microwells are hydrated.

Remove the chambered microwell array from the slide tray be gently pressing on the round section of one of the stainless steel clips while pushing the clip upwards. Using the bead picker, remove washed beads from the wash container and release them into a designated chamber. Make sure the bead picker does not touch any surface to avoid crushing the beads. Repeat the procedure for additional chambers / bead samples. Make a record of which sample is located in a particular chamber. Disperse the bead pellet(s) by tapping several times on a side of the chamber frame, which will cause the beads to sink into individual microwells and form a bead array, in which each microwell is either empty or contains a single bead. Remove bulk water from individual chambers. To do so, position the retaining magnet underneath the microwell array between the stainless steel clips. Invert the chambered microwell array and gently tap several times against a paper towel. The beads will be retained inside microwells by the magnet. Remove the magnet. Remove the stainless steel clips by sliding them out of their grooves or by pulling them away from the slide, being careful not to bend the clips. Separate the chamber frame from the microwell array.

It is natural for small (the size of a single microwell) droplets of water to remain on the surface of the microwell array after the chamber frame is removed. Such droplets do not affect the quality of elution and do not need to be removed. Pat drying of the slide is not recommended, as this will pull water out of the microwells leading to rapid drying of the beads. It is recommended to take a photograph of the bead array so that locations of beads can be recorded and used for subsequent MS data acquisition.

Immediately proceed to the next step (Eluting analytes from bead arrays). A delay longer than ~3 minutes may cause water to start evaporating from the microwells and beads to dry, especially under conditions of low humidity. This will result in incomplete elution and a dramatically reduced MS signal. It is advised that the matrix sprayer is set up beforehand. If a delay cannot be avoided the bead array should be placed inside a humidified chamber, such as a capped slide mailer lined with a wet paper towel.

4. Eluting Analytes from Bead Arrays

EQUIPMENT: MALDI matrix sprayer

The matrix sprayer generates an aerosol containing microdroplets of MALDI matrix solution over the entire surface of a microwell array. Elution occurs as the acidic matrix solution gradually replaces deionized water in the microwells, causing the analytes to dissociate from their respective beads. Upon solvent evaporation, the eluted analytes become incorporated into the MALDI matrix and localized in discrete spots, which are formed at a bottom of each microwell. Due to varying performance of different sprayers, a working elution protocol must be created and tested before running an actual BAMS™ assay. An optimal elution protocol ensures reproducible, consistent delivery of the matrix solution into every microwell, which is neither too fast nor too slow. The former may overfill the microwells, causing delocalization of the analytes and cross-talk between adjacent beads, while the latter may result in an incomplete elution and weak or absent signal. A good visual indicator of a working elution protocol is that the microwell slide appears moderately wet but without large surface droplets immediately after the matrix application cycle stops. Once the working protocol is established, it may be used consistently, provided that the ambient humidity and/or temperature do not change significantly. Larger changes in relative humidity, e.g. by more than 5%, may require adjusting one of the parameters (usually it is the speed of matrix application, which is increased/decreased to match the faster/slower rate of the solvent evaporation from the microwells). The protocol below is provided for elution from a single microwell array using BAMS™ Matrix Sprayer operating at typical ambient humidity and temperature. The provided values are only exemplary and should be tested before performing an actual BAMS™ assay. Changing the spray area to perform simultaneous elution from 2 or 3 microwell arrays will require re-calibration of the protocol. The elution protocol has been validated for the common MALDI matrices α-cyano-4- hydroxycinnamic acid (CHCA) and sinapinic acid (SA). It has not yet been tested with other MALDI matrices, which may have different crystallization patterns. The composition of the matrix solution is 5 mg/mL of CHCA, 50% (v/v) acetonitrile, 0.4% (v/v) trifluoroacetic acid (TFA), 10 mM diammonium citrate. To improve ionization of peptides containing 2 or more phosphorylated sites, up to 1% phosphoric acid may be included in the solution. Note that matrix solutions containing phosphoric acid are not compatible with Indium Tin Oxide (ITO) coated slides, as they dissolve the surface layer.

The following parameters are validated for room temperature (25°C) and 30% relative humidity: Height: 60 mm; Line Distance: 0.5 mm; Speed: 60 mm/s; Density: 5 µL/cm2; Number of cycles: 10; Delay: 0 sec; Spray area width: 80 mm; Spray area depth: 30 mm. Note that the spray area (30x80 mm) is set to be slightly larger than the

microwell array area (25x75 mm) to ensure uniform coating across the entire array. The gas pressure is set to be slightly below 0.1 MPa (about 0.09 MPa). To minimize the external air flow, which may cause uneven matrix coating, the front, back and sides of the sprayer may be blocked with sheets of paper. Due to the presence of organic solvents in the matrix solution, the sprayer should be always operated inside a chemical hood. Position the microwell array within the spray area (it is helpful to mark boundaries of the spray area beforehand) and immediately initiate the matrix application cycle. A delay will cause drying of the beads resulting in a loss of analyte signal! After the matrix application cycle is complete, the residual solvent should be allowed to evaporate, which may take between 10 and 45 minutes, depending upon ambient humidity. At this point the matrix will crystallize and beads shrink due to desiccation. Accelerating the drying process by heating or vacuum has not been tested. It is recommended that all beads are removed from the microarray prior to MS analysis. Using compressed gas ("duster can") eject beads from the microwells. This procedure will not displace matrix crystals. Lift the silicone gasket from any corner and peel off. Any remaining beads on a surface of the microarray should be removed by compressed gas. The gasket may be saved and reused. The microarray contains 2288 matrix spots (88 columns and 26 rows). Typically, only a fraction of the spots will contain bead-eluted analytes. A fiducial is adjacent to spot X01Y01. Place 2 or 4 calibration standards (0.5-1.0 µL per spot) at the corners of the microarray. The calibration spots may be placed directly over peripheral microarray spots located within two outer rows and columns, as these do not contain bead-eluted analytes. Allow the calibration spots to dry. Using a smartphone camera, take a photograph of the microarray with the flash ON. The microarray image is useful for identifying analyte-containing spots (corresponding to locations of microwells that previously contained a bead) and distinguishing those from blank spots, which only contain MALDI matrix (corresponding to locations of empty microwells). The former spots exhibit characteristic donut or crescent-like shape with an inner region devoid of the matrix (original location of a bead), while the latter spots exhibit substantially uniform matrix coverage throughout the spot. The analyte-containing spots may be subsequently selected for MS analysis, while the empty spots may be excluded from the analysis. Note that this approach has been validated with CHCA and SA matrices. Testing with other MALDI matrices is within the ambit of the skilled worker.

5. Measuring Microarrays of Bead-Eluted Analytes

This step allows measurement of individual microarray spots by MALDI MS in either manual or automated data acquisition mode. EQUIPMENT: MALDI mass spectrometer that accepts 25x75x1 mm microscope slides. The protocol described below is adapted

for Bruker® Flex series MALDI TOF MS such as autofleX, ultraflextreme and rapiflex. It should be modified for other MALDI MS instruments. Insert the microarray slide into the MTP II slide adapter, such that the fiducial is adjacent to the A1 and secure using the clips. Load the slide adapter into the mass spectrometer and allow vacuum to stabilize. Load the microarray template. The custom for microarray containing 88x26. Perform instrument teaching by aligning positions. The default teaching positions are X01Y01 (upper left), X88Y01 (upper right) and X88Y26 (lower right). If desired, other spots may used instead. Select the data acquisition method and perform instrument calibration using the calibration spots. Load the microarray template and record positions of spots that previously contained beads. For manual data acquisition, select the spot by record. The spot (with CHCA and, to a lesser extent, SA matrix) is visibly containing an area that is devoid of matrix. For automated data acquisition using AutoExecute, enter the spot coordinates into. The data will be saved with XY coordinates of individual spots. About 1,000 to 2,000 laser shots (1 sec acquisition time) per spot is typically sufficient. For 20,000 laser shots (10 sec acquisition time) for detection of low abundance analytes and accurate quantification of non isotope-labeled compounds. Greater than 20,000 shots may be collected for where depletion is required, for label-free quantification. Note that the spots typically do not get depleted until several hundred thousand shots. The amount material allows collecting multiple spectra even for MS-MS analysis.

6. Analyzing MS Data

The selected spots containing mass spectra. With appended coordinates. The through proprietary BAMS™ software. If desired, the same spots may be interrogated further, for example by MS-MS sequencing.

7. Storing and Reusing BAMS™ Assay Components

Multi-chamber frames, stainless steel clips and sample trays are reusable. These parts should be stored in a dry environment and protected from dust and other types of contamination. Multi-chamber frames may be rinsed with deionized water. The use of organic solvents is not recommended as they may dissolve the adhesive. Silicone microwell gaskets may be cleaned and reused. A previously used gasket must be thoroughly washed with ethanol to remove traces of MALDI matrix and carryover analytes. Cleaned gaskets should be stored dry and protected from dust. Gold-coated microscope slides may be reused by removing a previously spotted array of matrix spots using ethanol or isopropanol wash, followed by deep surface cleaning using a detergent, such as Decon 90, in an ultrasonic cleaning bath. Note that the previously spotted compounds may become a potential source of contamination unless the slide is extensively cleaned. Alternatively, single use gold-coated microscope slides are

available for purchase. To assemble a microwell array, attach the silicone microwell gasket to the gold-coated microscope slide. First, locate the bonding surface of the gasket using the chamfer as a visual marking. To remove residual dust particles, put a piece of Scotch® tape over an entire bonding surface, gently press the tape into the gasket and lift the tape. Place the gasket, the bonding surface facing up, on a Kimwipes® sheet positioned on a flat surface. Note that the chamfer should be located in the upper right corner. Lower the microscope slide, the gold-coated surface facing down, onto the gasket and gently press down across the entire slide to form a fluidic seal between the slide and the gasket. Visually inspect the microwell array to ensure proper positioning of the gasket. The fluidic seal should be visible throughout an entire area and the gasket should be located entirely within the slide, without overhangs. If the gasket is misaligned, peel it off and repeat the bonding procedure. Small variations in the gasket orientation on the slide are acceptable as these will be corrected by the microarray teaching procedure.

8. Custom Applications

Single bead Open architecture allows BAMS™ allows multiple customizations. Multiple bead surface chemistries. For example, different types of MALDI matrix can be tested. Different solvents. Further experimentations include in-well processing such as on-bead digestions by spraying the bead array with a solution of a digestive enzyme (trypsin or other). The use of also allows the use of photochemistry, such as cleavage of photo-labile chemical linkers

Nucleic Acids, Promoters, Vectors, Host Cells

Manufacture/production of recombinant polypeptides and/or nucleic acids according to the present invention is well known to the person skilled in the art and requires only routine knowledge such as how to synthesise polypeptide or polynucleotide, and/or how to express a polynucleotide to produce a polypeptide in the laboratory or a scaled-up commercial bioreactor. Numerous companies around the world offer such routine production services and require only an indication of the sequence(s) to be produced. Host cells, vectors for expression of polypeptide(s) according to the invention, promoters for use in such systems and the codon optimisation (if any) of the nucleic acid(s) encoding them are all well known to the person skilled in the art. Choice of particular vectors such as phage, phagemids, plasmids, or of promoters or host cells or other such 'tools' for production of the polypeptides or libraries described herein is a matter for the skilled person working the invention. Exemplary choices may be taken from the examples section below. Similarly, PCR or cloning strategies, ligations,

transformation/electroporation techniques are all routine and do not form part of the invention but are determined by the operator.
In case further guidance is needed, general molecular biological techniques are well known in the art, for example as in (2000 Current Protocols in Molecular Biology F.M. Ausubel et al, Eds. ISBN: 978-0-471-50338-5 published by John Wiley & Sons Ltd, Oldlands Way, Bognor Regis, West Sussex, PO22 9NQ, UK).

Exemplary cell strains:
TG1 (Lucigen, catalogue number 60502-2)
ER2738 (New England Biolabs, catalogue number E4104)

Exemplary phage strain:
M13KO7 (New England Biolabs, catalogue number N0315)

Exemplary phagemid vector:
pUC119 (Clontech, catalogue number 3319), which contains the lac promoter

Exemplary promoter:
lac promoter (see above)

FURTHER ADVANTAGES

Suitably the test indicates if a person has the virus.

Suitably the test, such as LFD test, delivers a result in minutes e.g. 60 minutes or less.
Suitably the test, such as LFD test, may be carried out *in-situ*.
Suitably the test, such as LFD test, has no need for laboratory equipment.

Multiple highly specific polypeptides (e.g. Affimer® Agents) are disclosed that bind to the spike protein of the SARS-CoV-2 virus. It is an advantage that the polypeptides do not cross-react with other very closely related viruses, such as SARS (SARS-CoV virus) and MERS.

In one embodiment the invention provides a polypeptide-based (e.g. Affimer® Agent-based) point-of-care rapid test intended for population screening to diagnose the COVID-19 coronavirus infection.

There is a known "sensitest" lateral flow device. This lateral flow device is essentially an antibody test. The invention provides advantages over this known test.

It is an advantage of the invention that the polypeptides described are resistant to freeze thaw cycles. For example the polypeptides are resistant to 5 or 6 freeze thaw cycles, or even more.

It is an advantage of the invention that the biotinylated polypeptides are stable at 4°C for at least a year.

It is an advantage of the invention that polypeptides with free cysteine(s) are stable at 4°C for at least a few weeks; it is a further advantage of the invention that once the Cysteine(s) is/are reacted, the polypeptide is back to being stable at 4°C for at least a year.

The skilled worker will be aware that stability may be affected by what is conjugated to the polypeptide.

It is to be noted that the inventors generated polypeptides capable of recognising the target antigen in an extremely rapid timescale. The inventors assert that polypeptide (Affimer® Agent) production was the fastest they have ever achieved starting from a target antigen to obtaining candidate binders, which illustrates how their innovative approach was surprisingly effective.

It is advantage that a high proportion of the polypeptides described herein do not cross-react with either MERS virus spike protein, or SARS-CoV-1 spike protein. Suitably the polypeptide of the invention does not cross-react with MERS spike protein. Suitably the polypeptide of the invention does not cross-react with SARS-CoV-1 spike protein.

It is an advantage of the invention that the inventors specifically designed their screening assays to use the native spike protein. This helps to generate excellent performance of the screening and therefore to obtain the best performing polypeptides (Affimer® Agents).

The viral spike protein antigen binds the angiotensin converting enzyme 2 (ACE2) receptor, which is part of the angiotensin family involved in homeostasis. The inventors made the intellectual choice to verify that their batch(es) of recombinant spike protein did indeed bind the ACE2 receptor before using that spike protein in their screening procedures. This ensured the excellent results described herein.

The selection strategy increased stringency as the screening proceeded, which helped to isolate the tightest and most specific polypeptides binding to spike protein. Moreover the selection strategy involved 6 selections, each having 3 panning rounds, which represents an unusually complex strategy. In addition, carefully designed deselection rounds against related proteins such as SARS spike and/or MERS spike were used to enhance selectivity for SARS-CoV-2 spike protein as described herein. Moreover the use of biotinylated reagents and the inclusion of a range of reagents from alternate suppliers were designed into the strategy, as well as programmed 'target switching' during the procedural steps, all combined to make it an unusually complex scheme.

This itself is indicative of inventive step, but also it will be apparent that the urgent need to generate tests and reagents as described herein would be expected to motivate a rapid and streamlined selection i.e. the simplest conceivable scheme to lead most rapidly to candidate binder polypeptides. However, the inventors went against this conventional thinking and instead created the highly complicated, more expensive and more time consuming schemes set out herein which is further indication of non-obviousness.

The inventors describe at least 57 exemplary polypeptides (Affimer® Agents) with the capability to bind the SARS-CoV-2 spike protein herein. These at least 57 polypeptides possess unity of invention (i.e. represent a single invention) since each of them was obtained from exactly the same screening protocol, and each binds exactly the same viral antigen target. These technical properties of the polypeptides are demonstrated with data in this application.

THERAPEUTIC ASPECTS

In some aspects the present disclosure relates to the generation of Affimer® Agents that bind to SPIKE and inhibit the interaction of that molecule with ACE2, and consequently represent coronaviridae neutralizing inhibitors that have utility in the treatment or prevention of coronaviradae infections.

Based on naturally occurring proteins (cystatins) and engineered to stably display two loops which create a binding surface, the COVID (SARS-CoV-2) binding Affimer® polypeptides of the present disclosure provide a number of advantages over antibodies, antibody fragments and other non-antibody binding proteins.

One is the small size of the Affimer® polypeptide itself. In its monomeric form it is about 14 kDa, or 1/10th the size of an antibody. This small size gives greater potential for increased tissue penetration, particularly in poorly vascularized and/or fibrotic target tissues (like infected lung tissues).

Affimer® polypeptides have a simple protein structure (versus multi-domain antibodies), and as the Affimer® polypeptides do not require disulfide bonds or other post-translational modifications for function, many of the format embodiments including these polypeptides can be manufactured in prokaryotic and eukaryotic systems.

The ability to utilize libraries of Affimer® Agents (such as the phage display techniques described in the appended examples) as well as site directed mutagenesis, the Affimer® Agents can be generated with tunable binding kinetics with ideal ranges for

therapeutic uses. For instance, the Affimer® Agents can have high affinity for SPIKE, such as single digit nanomolar or lower K_D for monomeric Affimer® Agents and picomolar K_D and avidity in multi-valent formats. The Affimer® Agents can be generated with tight binding kinetics for SPIKE, such as slow K_{off} rates in the 10^{-4} to 10^{-5} (s-1) range which benefits target tissue localization.

The COVID (SARS-CoV-2) binding Affimer® Agents of the present disclosure include Affimer® Agents with exquisite selectivity.

Moreover, the COVID (SARS-CoV-2) binding Affimer® Agents can be readily formatted, allowing formats such as fusion proteins, both with other polypeptide domains as well as in-line multimers of Affimer® Agents, to be generated and manufactured with ease.

The lack of need for disulfide bonds and post-translational modifications also permit many embodiments of proteins including the COVID (SARS-CoV-2) binding Affimer® Agents (or monomeric Affimer® Agents) to be delivered therapeutically by expression of gene delivery constructs that are introduced in vivo into the tissues of the patient or ex vivo into cells administered to the patient, including formats where the protein is delivered systemically (such as expression from muscle tissue) or delivered locally (such as through intratumoral gene delivery).

In some embodiments, the invention relates to a polypeptide such as an Affimer® Agent which has one or more COVID (SARS-CoV-2) binding Affimer® sequence each independently having an amino acid sequence represented in general formula (I)

FR1-$(Xaa)_n$-FR2-$(Xaa)_m$-FR3 **(I)**

wherein

FR1 is a polypeptide sequence represented by MIPGGLSEAK PATPEIQEIV DKVKPQLEEK TNETYGKLEA VQYKTQVLA (SEQ ID NO: 186) or a polypeptide sequence having at least 70% homology thereto;

FR2 is a polypeptide sequence represented by GTNYYIKVRA GDNKYMHLKV FKSL (SEQ ID NO: 187) or a polypeptide sequence having at least 70% homology thereto;

FR3 is a polypeptide sequence represented by EDLVLTGYQV DKNKDDELTG F (SEQ ID NO: 188) or a polypeptide sequence having at least 70% homology thereto; and

Xaa, individually for each occurrence, is an amino acid residue; and

n and m are each, independently, an integer from 3 to 20.

For some embodiments, the FR1 may a polypeptide sequence having at least 80%, 85%, 90%, 95% or even 98% homology with MIPGGLSEAK PATPEIQEIV DKVKPQLEEK TNETYGKLEA VQYKTQVLA (SEQ ID NO: 186). For some embodiments, FR2 is a polypeptide sequence having at least 80%, 85%, 90%, 95% or even 98% homology with GTNYYIKVRA GDNKYMHLKV FKSL (SEQ ID NO: 187). For some embodiments, FR3 is a polypeptide sequence having at least 80%, 85%, 90%, 95% or even 98% homology with EDLVLTGYQV DKNKDDELTG F (SEQ ID NO: 188).
In certain other embodiments, the Affimer® Agent includes one or more COVID (SARS-CoV-2) binding Affimer® sequence represented in SEQ ID No:s 5 to 175 (which may omit the N-terminal methionine if the Affimer® sequence is not presented on the N-terminus of the Affimer® Agent.

Further particular and preferred aspects are set out in the accompanying independent and dependent claims. Features of the dependent claims may be combined with features of the independent claims as appropriate, and in combinations other than those explicitly set out in the claims.

Where an apparatus feature is described as being operable to provide a function, it will be appreciated that this includes an apparatus feature which provides that function or which is adapted or configured to provide that function.

a. Fusion Proteins – General

In some embodiments, the Affimer® polypeptides may further comprise an additional insertion, substitution or deletion that modulates biological activity of the Affimer® polypeptide. For example, the additions, substitutions or deletions may modulate one or more properties or activities of modified Affimer® polypeptides. For example, the additions, substitutions or deletions may modulate affinity for the Affimer® polypeptide, e.g., for binding to SPIKE protein and inhibiting interaction with ACE2, modulate the circulating half-life, modulate the therapeutic half-life, modulate the stability of the Affimer® polypeptide, modulate cleavage by proteases, modulate dose, modulate release or bio-availability, facilitate purification, decrease deamidation, improve shelf-life, or improve or alter a particular route of administration. Similarly, Affimer® polypeptides may comprise protease cleavage sequences, reactive groups, antibody-binding domains (including but not limited to, FLAG or poly-His) or other affinity based sequences (including but not limited to, FLAG, poly-His, GST, etc.) or linked molecules (including but not limited to, biotin) that improve detection, purification or other traits of the polypeptide.

In some instances, these additional sequences are added to one end and/or the other of the Affimer® polypeptide in the form of a fusion protein. Accordingly, in certain aspects of the disclosure the Affimer® Agent is a fusion protein having at least one Affimer® polypeptide sequence and one or more heterologous polypeptide sequences ("fusion domain" herein). A fusion domain may be selected so as to confer a desired property, such as secretion from a cell or retention on the cell surface (i.e., for Encoded Affimer® Agents), to serve as substrate or other recognition sequences for post-translational modifications, to create multimeric structures aggregating through protein-protein interactions, to alter (often to extend) serum half-life, or to alter tissue localization or tissue exclusion and other ADME properties – merely as examples.

For example, some fusion domains are particularly useful for isolation and/or purification of the fusion proteins, such as by affinity chromatography. Well known examples of such fusion domains that facilitate expression or purification include, merely to illustrate, affinity tags such as polyhistidine (i.e., a His_6 tag), Strep II tag, streptavidin-binding peptide (SBP) tag, calmodulin-binding peptide (CBP), glutathione S-transferase (GST), maltose-binding protein (MBP), S-tag, HA tag, c-Myc tag, thioredoxin, protein A and protein G.

In order for the Affimer® Agent to be secreted, it will generally contain a signal sequence that directs the transport of the protein to the lumen of the endoplasmic reticulum and ultimately to be secreted (or retained on the cell surface if a transmembrane domain or other cell surface retention signal). Signal sequences (also referred to as signal peptides or leader sequences) are located at the N-terminus of nascent polypeptides. They target the polypeptide to the endoplasmic reticulum and the proteins are sorted to their destinations, for example, to the inner space of an organelle, to an interior membrane, to the cell outer membrane, or to the cell exterior via secretion. Most signal sequences are cleaved from the protein by a signal peptidase after the proteins are transported to the endoplasmic reticulum. The cleavage of the signal sequence from the polypeptide usually occurs at a specific site in the amino acid sequence and is dependent upon amino acid residues within the signal sequence.

In some embodiments, the signal peptide is about 5 to about 40 amino acids in length (such as about 5 to about 7, about 7 to about 10, about 10 to about 15, about 15 to about 20, about 20 to about 25, or about 25 to about 30, about 30 to about 35, or about 35 to about 40 amino acids in length).

In some embodiments, the signal peptide is a native signal peptide from a human protein. In other embodiments, the signal peptide is a non-native signal peptide. For example, in some embodiments, the non-native signal peptide is a mutant native signal peptide from the corresponding native secreted human protein, and can include one or more (such as 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more) substitutions insertions or deletions.

In some embodiments, the signal peptide is a signal peptide or mutant thereof from a non-IgSF protein family, such as a signal peptide from an immunoglobulin (such as IgG heavy chain or IgG-kappa light chain), a cytokine (such as interleukin-2 (IL-2), or CD33), a serum albumin protein (e.g. HSA or albumin), a human azurocidin preprotein signal sequence, a luciferase, a trypsinogen (e.g. chymotrypsinogen or trypsinogen) or other signal peptide able to efficiently secrete a protein from a cell. Exemplary signal peptides include, but are not limited to:

Native Protein	**Signal Sequence**
HSA	MKWVTFISLLFLFSSAYS (SEQ ID NO: 224)
Ig kappa light chain	MDMRAPAGIFGFLLVLFPGYRS (SEQ ID NO: 225)
Human azurocidin preprotein	MTRLTVLALLAGLLASSRA (SEQ ID NO: 226)
IgG heavy chain	MELGLSWIFLLAILKGVQC (SEQ ID NO: 227)
IgG heavy chain	MELGLRWVFLVAILEGVQC (SEQ ID NO: 228)
IgG heavy chain	MKHLWFFLLLVAAPRWVLS (SEQ ID NO: 229)
IgG heavy chain	MDWTWRILFLVAAATGAHS (SEQ ID NO: 230)
IgG heavy chain	MDWTWRFLFVVAAATGVQS (SEQ ID NO: 231)
IgG heavy chain	MEFGLSWLFLVAILKGVQC (SEQ ID NO: 232)
IgG heavy chain	MEFGLSWVFLVALFRGVQC (SEQ ID NO: 233)
IgG heavy chain	MDLLHKNMKHLWFFLLLVAAPRWVLS (SEQ ID NO: 234)
IgG Kappa light	MDMRVPAQLLGLLLLWLSGARC (SEQ ID NO: 235)
IgG Kappa light	MKYLLPTAAAGLLLLAAQPAMA (SEQ ID NO: 236)
Gaussia luciferase	MGVKVLFALICIAVAEA (SEQ ID NO: 237)
Human albumin	MKWVTFISLLFLFSSAYS (SEQ ID NO: 224)
Human chymotrypsinogen	MAFLWLLSCWALLGTTFG (SEQ ID NO: 238)
Human interleukin-2	MQLLSCIALILALV (SEQ ID NO: 239)
Human trypsinogen-2	MNLLLILTFVAAAVA (SEQ ID NO: 240)
Human CD33	MPLLLLLPLLWAGALA (SEQ ID NO: 241)
Prolactin	MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS (SEQ ID NO: 242)
Human tPA	MDAMKRGLCCVLLLCGAVFVSPS (SEQ ID NO: 243)

Synthetic/Consensus	MLLLLLLLLLLALALA (SEQ ID NO: 244)
Synthetic/Consensus	MWWRLWWLLLLLLLLWPMVWA (SEQ ID NO: 245)

In some embodiments of a secreted Affimer® Agent, the recombinant polypeptide comprises a signal peptide when expressed, and the signal peptide (or a portion thereof) is cleaved from the Affimer® Agent upon secretion.

The subject fusion proteins may also include one or more linkers separating heterologous protein sequences or domains. As used herein, the term "linker" refers to a linker amino acid sequence inserted between a first polypeptide (e.g., an Affimer® polypeptide) and a second polypeptide (e.g., a second Affimer® polypeptide, an Fc region, a receptor trap, albumin, etc). Empirical linkers designed by researchers are generally classified into 3 categories according to their structures: flexible linkers, rigid linkers, and *in vivo* cleavable linkers. Besides the basic role in linking the functional domains together (as in flexible and rigid linkers) or releasing free functional domain *in vivo* (as in *in vivo* cleavable linkers), linkers may offer many other advantages for the production of fusion proteins, such as improving biological activity, increasing expression yield, and achieving desirable pharmacokinetic profiles. Linkers should not adversely affect the expression, secretion, or bioactivity of the fusion protein. Linkers should not be antigenic and should not elicit an immune response.

Suitable linkers are known to those of skill in the art and often include mixtures of glycine and serine residues and often include amino acids that are sterically unhindered. Other amino acids that can be incorporated into useful linkers include threonine and alanine residues. Linkers can range in length, for example from 1-50 amino acids in length, 1-22 amino acids in length, 1-10 amino acids in length, 1-5 amino acids in length, or 1-3 amino acids in length. In some embodiments, the linker may comprise a cleavage site. In some embodiments, the linker may comprise an enzyme cleavage site, so that the second polypeptide may be separated from the first polypeptide.

In some embodiments, the linker can be characterized as flexible. Flexible linkers are usually applied when the joined domains require a certain degree of movement or interaction. They are generally composed of small, non-polar (e.g. Gly) or polar (e.g. Ser or Thr) amino acids. See, for example, Argos P. (1990) “An investigation of oligopeptides linking domains in protein tertiary structures and possible candidates for general gene fusion” J Mol Biol. 211:943–958. The small size of these amino acids

provides flexibility and allows for mobility of the connecting functional domains. The incorporation of Ser or Thr can maintain the stability of the linker in aqueous solutions by forming hydrogen bonds with the water molecules, and therefore reduces the unfavorable interaction between the linker and the protein moieties. The most commonly used flexible linkers have sequences consisting primarily of stretches of Gly and Ser residues ("GS" linker). An example of the most widely used flexible linker has the sequence of (Gly-Gly-Gly-Gly-Ser)n (SEQ ID NO: 246). By adjusting the copy number "n", the length of this GS linker can be optimized to achieve appropriate separation of the functional domains, or to maintain necessary inter-domain interactions. Besides the GS linkers, many other flexible linkers have been designed for recombinant fusion proteins. As These flexible linkers are also rich in small or polar amino acids such as Gly and Ser, but can contain additional amino acids such as Thr and Ala to maintain flexibility, as well as polar amino acids such as Lys and Glu to improve solubility.

In some embodiments, the linker can be characterized as rigid. While flexible linkers have the advantage to connect the functional domains passively and permitting certain degree of movements, the lack of rigidity of these linkers can be a limitation in certain fusion protein embodiments, such as in expression yield or biological activity. The ineffectiveness of flexible linkers in these instances was attributed to an inefficient separation of the protein domains or insufficient reduction of their interference with each other. Under these situations, rigid linkers have been successfully applied to keep a fixed distance between the domains and to maintain their independent functions.

Many natural linkers exhibited α-helical structures. The α-helical structure was rigid and stable, with intra-segment hydrogen bonds and a closely packed backbone. Therefore, the stiff α-helical linkers can act as rigid spacers between protein domains. George et al. (2002) "An analysis of protein domain linkers: their classification and role in protein folding" Protein Eng. 15(11):871-9. In general, rigid linkers exhibit relatively stiff structures by adopting α-helical structures or by containing multiple Pro residues. Under many circumstances, they separate the functional domains more efficiently than the flexible linkers. The length of the linkers can be easily adjusted by changing the copy number to achieve an optimal distance between domains. As a result, rigid linkers are chosen when the spatial separation of the domains is critical to preserve the stability or bioactivity of the fusion proteins. In this regard, alpha helix-forming linkers with the sequence of (EAAAK)n (SEQ ID NO: 247) have been applied to the construction of many recombinant fusion proteins. Another type of rigid linkers has a

Pro-rich sequence, (XP)n, with X designating any amino acid, preferably Ala, Lys, or Glu.

Merely to illustrate, exemplary linkers include:

• Flexible	• $(GGGGS)_n$ (i.e., n = 1-6) (SEQ ID NO: 246)
• Flexible	• $(Gly)_8$ (SEQ ID NO: 248)
• Flexible	• $(Gly)_6$ (SEQ ID NO: 249)
• Flexible	• KESGSVSSEQLAQFRSLD (SEQ ID NO: 250)
• Flexible	• EGKSSGSGSESKST (SEQ ID NO: 251)
• Flexible	• GSAGSAAGSGEF (SEQ ID NO: 252)
• Rigid	• $(EAAAK)_n$ (i.e., n = 1-6) (SEQ ID NO: 247)
• Rigid	• $A(EAAAK)_4ALEA(EAAAK)_4A$ (SEQ ID NO: 253)
• Rigid	• PAPAP (SEQ ID NO: 254)
• Rigid	• AEAAAKEAAAKA (SEQ ID NO: 255)
• Rigid	• (Ala-Pro)n (10 to 34 aa)

Other linkers that may be used in the subject fusion proteins include, but are not limited to, SerGly, GGSG (SEQ ID NO: 256), GSGS (SEQ ID NO: 257), GGGS (SEQ ID NO: 258), S(GGS)n where n is 1-7 (SEQ ID NO: 259), GRA, poly(Gly), poly(Ala), GGGSGGG (SEQ ID NO: 260), ESGGGGVT (SEQ ID NO: 261), LESGGGGVT (SEQ ID NO: 262), GRAQVT (SEQ ID NO: 263), WRAQVT (SEQ ID NO: 264), and ARGRAQVT (SEQ ID NO: 265). The hinge regions of the Fc fusions described below may also be considered linkers.

Various elements can be employed to anchor proteins on the plasma membrane of cells. For example, the transmembrane domains (TM) of type-I (oriented with the N-terminus outside the cell) and type-II (oriented with the N-terminus in the cytosol) integral membrane proteins can be used to target chimeric proteins to the plasma membrane. Proteins can also be attached to the cell surface by fusion of a GPI (glycophosphatidylinositol lipid) signal to the 3' end of genes. Cleavage of the short carboxy-terminal peptide allows attachment of a glycolipid to the newly exposed C-terminus through an amide linkage. See Udenfriend et al. (1995) "How Glycosylphoshpatidylinositol Anchored Membrane Proteins are Made" Annu Rev Biochem 64:563–591.

In some embodiments, the fusion protein includes a transmembrane polypeptide sequence (a transmembrane domain). The distinguishing features of appropriate transmembrane polypeptides comprise the ability to be expressed at the surface of the cell on which the Affimer® Agent is to be displayed. In some embodiments, that may be an immune cell, in particular lymphocyte cells or Natural killer (NK) cells, and once there to interact with viral SPIKE protein so as to directing cellular response of the immune cell against virus infected cells displaying viral SPIKE protein or viral particles. The transmembrane domain can be derived either from a natural or from a synthetic source. The transmembrane domain can be derived from any membrane-bound or transmembrane protein. As non limiting examples, the transmembrane polypeptide can be a subunit of the T cell receptor such as α, β, γ or δ, polypeptide constituting CD3 complex, IL2 receptor p55 (a chain), p75 (β chain) or γ chain, subunit chain of Fc receptors, in particular Fey receptor III or CD proteins. Alternatively the transmembrane domain can be synthetic and can comprise predominantly hydrophobic residues such as leucine and valine.

In certain other embodiments, the Affimer® Agent is a fusion protein including, in addition to an Affimer® polypeptide, a sequence that signals for the posttranslational addition of a glycosylphosphatidylinositol (GPI) anchor. GPI anchors are glycolipid structures that are added post-translationally to the C-terminus of many eukaryotic proteins. This modification to the Affimer® Agent will cause it to be anchored (attached) on the extracellular surface of the cell membrane of the cell in which the Affimer® Agent is re-expressed as a recombinant protein (i.e., an Encoded Affimer® Agent as described below). In these embodiments, the GPI anchor domain is C-terminal to the Affimer® polypeptide sequence, and preferably occurs at the C-terminus of the fusion protein.

In some embodiments, the GPI anchor domain is a polypeptide that signals for the posttranslational addition of a GPI anchor when the fusion protein of which it is a part is expressed in a eukaryotic system. The GPI anchor signal sequence consists of a set of small amino acids at the site of anchor addition (the ω site) followed by a hydrophilic spacer and ending in a hydrophobic stretch (Low, (1989) FASEB J. 3:1600-1608). Cleavage of this signal sequence occurs in the ER before the addition of an anchor with conserved central components but with variable peripheral moieties (Homans et al., Nature, 333:269-272 (1988)). The C-terminus of a GPI-anchored protein is linked through a phosphoethanolamine bridge to the highly conserved core glycan,

mannose(α1-2)mannose(α1-6)mannose(α1-4)glucosamine(α1-6)myo-inositol. A phospholipid tail attaches the GPI anchor to the cell membrane.

Exemplary GPI anchor domains that can be used in the subject Affimer® polypeptide-containing fusion proteins include:

SGTTSGTTRLLSGHTCFTLTGLLGTLVTMGLLT (SEQ ID NO: 266)
SGTSPGLSAGATVGIMIGVLVGVALI (SEQ ID NO: 267)
SAPVLSAVATVGITIGVLARVALI (SEQ ID NO: 268)
SSPDLSAGTAVSIMIGVLAGMALI (SEQ ID NO: 269)
TLGGNSASYTFVSLLFSAVTLLLLC (SEQ ID NO: 270)
SGTSPGLSAGATVGIMIGVLVGVALI (SEQ ID NO: 267)

GPI anchor attachment can be achieved by expression of the Affimer® fusion protein containing the GPI anchor domain in a eukaryotic system capable of carrying out GPI posttranslational modifications. As with the transmembrane domain fusion proteins, human cells, including lymphocytes and other cells involved in initiating or promoting an antiviral activities are so capable and can be engineered to express and Encoded Affimer® Agent including a GPI anchor domain in order retain the expressed Affimer® polypeptide-containing fusion on the surface of the engineered cell.

Still other modifications that can be made to the Affimer® polypeptide sequence itself or to a flanking polypeptide moiety provided as part of a fusion protein is one or more sequences that are sites for post-translational modifications by enzymes. These can include, but are not limited to, glycosylation, acetylation, acylation, lipid-modification, palmitoylation, palmitate addition, phosphorylation, glycolipid-linkage modification, and the like.

b. Engineering PK and ADME Properties

In some embodiment, the Affimer® Agent may not have a half-life and/or PK profile that is optimal for the route of administration, such as parenteral therapeutic dosing. The term “half-life” refers to the amount of time it takes for a substance, such as an Affimer® Agent of the present disclosure, to lose half of its pharmacologic or physiologic activity or concentration. Biological half-life can be affected by elimination, excretion, degradation (e.g., enzymatic) of the substance, or absorption and concentration in certain organs or tissues of the body. In some embodiments, biological half-life can be assessed by determining the time it takes for the blood plasma

concentration of the substance to reach half its steady state level ("plasma half-life"). To address this shortcoming, there are a variety of general strategies for prolongation of half-life that have been used in the case of other protein therapeutics, including the incorporation of half-life extending moieties as part of the Affimer® Agent.

The term "half-life extending moiety" refers to a pharmaceutically acceptable moiety, domain, or molecule covalently linked ("conjugated" or "fused") to the Affimer® polypeptide to form the Affimer® Agents described herein, optionally via a non-naturally encoded amino acid, directly or via a linker, that prevents or mitigates *in vivo* proteolytic degradation or other activity-diminishing modification of the Affimer® polypeptide, increases half-life, and/or improves or alters other pharmacokinetic or biophysical properties including but not limited to increasing the rate of absorption, reducing toxicity, improving solubility, reducing protein aggregation, increasing biological activity and/or target selectivity of the modified Affimer® polypeptide, increasing manufacturability, and/or reducing immunogenicity of the modified Affimer® polypeptide, compared to a comparator such as an unconjugated form of the modified Affimer® polypeptide. The term "half-life extending moiety" includes non-proteinaceous, half-life extending moieties, such as a water soluble polymer such as polyethylene glycol (PEG) or discrete PEG, hydroxyethyl starch (HES), a lipid, a branched or unbranched acyl group, a branched or unbranched C8-C30 acyl group, a branched or unbranched alkyl group, and a branched or unbranched C8-C30 alkyl group; and proteinaceous half-life extending moieties, such as serum albumin, transferrin, adnectins (e.g., albumin-binding or pharmacokinetics extending (PKE) adnectins), Fc domain, and unstructured polypeptide, such as XTEN and PAS polypeptide (e.g. conformationally disordered polypeptide sequences composed of the amino acids Pro, Ala, and/or Ser), and a fragment of any of the foregoing. An examination of the crystal structure of an Affimer® polypeptide and its interaction with its target, such as the anti-SPIKE Affimer® Agent complex with ACE2, can indicate which certain amino acid residues have side chains that are fully or partially accessible to solvent.

In some embodiments, the half-life extending moiety extends the half-life of the resulting Affimer® Agent circulating in mammalian blood serum compared to the half-life of the protein that is not so conjugated to the moiety (such as relative to the Affimer® polypeptide alone). In some embodiments, half-life is extended by greater than or greater than about 1.2-fold, 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold., 5.0-fold, or 6.0-fold. In some embodiments, half-life is extended by more than 6 hours, more than

12 hours, more than 24 hours, more than 48 hours, more than 72 hours, more than 96 hours or more than 1 week after in vivo administration compared to the protein without the half-life extending moiety.

As means for further exemplification, half-life extending moieties that can be used in the generation of Affimer® Agents of the disclosure include:

- Genetic fusion of the pharmacologically Affimer® sequence to a naturally long-half-life protein or protein domain (e.g., Fc fusion, transferrin [Tf] fusion, or albumin fusion. See, for example, Beck et al. (2011) "Therapeutic Fc-fusion proteins and peptides as successful alternatives to antibodies. MAbs. 3:1–2; Czajkowsky et al. (2012) "Fc-fusion proteins: new developments and future perspectives. EMBO Mol Med. 4:1015–28; Huang et al. (2009) "Receptor-Fc fusion therapeutics, traps, and Mimetibody technology" Curr Opin Biotechnol. 2009;20:692–9; Keefe et al. (2013) "Transferrin fusion protein therapies: acetylcholine receptor-transferrin fusion protein as a model. In: Schmidt S, editor. Fusion protein technologies for biopharmaceuticals: applications and challenges. Hoboken: Wiley; p. 345–56; Weimer et al. (2013) "Recombinant albumin fusion proteins. In: Schmidt S, editor. Fusion protein technologies for biopharmaceuticals: applications and challenges. Hoboken: Wiley; 2013. p. 297–323; Walker et al. (2013) "Albumin-binding fusion proteins in the development of novel long-acting therapeutics. In: Schmidt S, editor. Fusion protein technologies for biopharmaceuticals: applications and challenges. Hoboken: Wiley; 2013. p. 325–43.
- Genetic fusion of the pharmacologically Affimer® sequence to an inert polypeptide, e.g., XTEN (also known as recombinant PEG or "rPEG"), a homoamino acid polymer (HAP; HAPylation), a proline-alanine-serine polymer (PAS; PASylation), or an elastin-like peptide (ELP; ELPylation). See, for example, Schellenberger et al. (2009) "A recombinant polypeptide extends the *in vivo* half-life of peptides and proteins in a tunable manner. Nat Biotechnol. 2009;27:1186–90; Schlapschy et al. Fusion of a recombinant antibody fragment with a homo-amino-acid polymer: effects on biophysical properties and prolonged plasma half-life. Protein Eng Des Sel. 2007;20:273–84; Schlapschy (2013) PASylation: a biological alternative to PEGylation for extending the plasma halflife of pharmaceutically active proteins. Protein Eng Des Sel. 26:489–501. Floss et al. (2012) "Elastin-like polypeptides revolutionize recombinant protein expression and their biomedical application. Trends

Biotechnol. 28:37–45. Floss et al. "ELP-fusion technology for biopharmaceuticals. In: Schmidt S, editor. Fusion protein technologies for biopharmaceuticals: application and challenges. Hoboken: Wiley; 2013. p. 372–98.

- Increasing the hydrodynamic radius by chemical conjugation of the pharmacologically active peptide or protein to repeat chemical moieties, e.g., to PEG (PEGylation) or hyaluronic acid. See, for example, Caliceti et al. (2003) "Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates" Adv Drug Delivery Rev. 55:1261–77; Jevsevar et al. (2010) PEGylation of therapeutic proteins. Biotechnol J 5:113–28; Kontermann (2009) "Strategies to extend plasma half-lives of recombinant antibodies" BioDrugs. 23:93–109; Kang et al. (2009) "Emerging PEGylated drugs" Expert Opin Emerg Drugs. 14:363–80; and Mero et al. (2013) "Conjugation of hyaluronan to proteins" Carb Polymers. 92:2163–70.
- Significantly increasing the negative charge of fusing the pharmacologically active peptide or protein by polysialylation; or, alternatively, (b) fusing a negatively charged, highly sialylated peptide (e.g., carboxy-terminal peptide [CTP; of chorionic gonadotropin (CG) b-chain]), known to extend the half-life of natural proteins such as human CG b-subunit, to the biological drug candidate. See, for example, Gregoriadis et al. (2005) "Improving the therapeutic efficacy of peptides and proteins: a role for polysialic acids" Int J Pharm. 2005; 300:125–30; Duijkers et al. "Single dose pharmacokinetics and effects on follicular growth and serum hormones of a long-acting recombinant FSH preparation (FSHCTP) in healthy pituitary-suppressed females" (2002) Hum Reprod. 17:1987–93; and Fares et al. "Design of a longacting follitropin agonist by fusing the C-terminal sequence of the chorionic gonadotropin beta subunit to the follitropin beta subunit" (1992) Proc Natl Acad Sci USA. 89:4304–8. 35; and Fares "Half-life extension through O-glycosylation".
- Binding non-covalently, via attachment of a peptide or protein-binding domain to the bioactive protein, to normally long-half-life proteins such as HSA, human IgG, transferrin or fibronectin. See, for example, Andersen et al. (2011) "Extending half-life by indirect targeting of the neonatal Fc receptor (FcRn) using a minimal albumin binding domain" J Biol Chem. 286:5234–41; O'Connor-Semmes et al. (2014) "GSK2374697, a novel albumin-binding domain antibody (albudAb), extends systemic exposure of extendin-4: first study in humans—PK/PD and safety" Clin Pharmacol Ther. 2014;96:704–12. Sockolosky et al. (2014) "Fusion of a short peptide that binds immunoglobulin G to a

recombinant protein substantially increases its plasma half-life in mice" PLoS One. 2014;9:e102566.

Classical genetic fusions to long-lived serum proteins offer an alternative method of half-life extension distinct from chemical conjugation to PEG or lipids. Two major proteins have traditionally been used as fusion partners: antibody Fc domains and human serum albumin (HSA). Fc fusions involve the fusion of peptides, proteins or receptor exodomains to the Fc portion of an antibody. Both Fc and albumin fusions achieve extended half-lives not only by increasing the size of the peptide drug, but both also take advantage of the body's natural recycling mechanism: the neonatal Fc receptor, FcRn. The pH-dependent binding of these proteins to FcRn prevents degradation of the fusion protein in the endosome. Fusions based on these proteins can have half-lives in the range of 3-16 days, much longer than typical PEGylated or lipidated peptides. Fusion to antibody Fc domains can improve the solubility and stability of the peptide or protein drug. An example of a peptide Fc fusion is dulaglutide, a GLP-1 receptor agonist currently in late-stage clinical trials. Human serum albumin, the same protein exploited by the fatty acylated peptides is the other popular fusion partner. Albiglutide is a GLP-1 receptor agonist based on this platform. A major difference between Fc and albumin is the dimeric nature of Fc versus the monomeric structure of HSA leading to presentation of a fused peptide as a dimer or a monomer depending on the choice of fusion partner. The dimeric nature of an Affimer® Agent-Fc fusion can produce an avidity effect if the Affimer®Agent target, such as SPIKE protein on a viral particle, are spaced closely enough together or are themselves dimers. This may be desirable or not depending on the target.

(i) Fc Fusions

In some embodiments, the Affimer® polypeptide may be part of a fusion protein with an immunoglobulin Fc domain ("Fc domain"), or a fragment or variant thereof, such as a functional Fc region. In this context, an Fc fusion ("Fc-fusion"), such as an Affimer® Agent created as an Affimer® Agent-Fc fusion protein, is a polypeptide comprising one or more Affimer® sequences covalently linked through a peptide backbone (directly or indirectly) to an Fc region of an immunoglobulin. An Fc-fusion may comprise, for example, the Fc region of an antibody (which facilitates effector functions and pharmacokinetics) and an Affimer® sequence as part of the same polypeptide. An immunoglobulin Fc region may also be linked indirectly to one or more Affimer® Agents. Various linkers are known in the art and can optionally be used to link an Fc to a polypeptide including an Affimer® sequence to generate an Fc-fusion. In some

embodiments, Fc-fusions can be dimerized to form Fc-fusion homodimers, or using non-identical Fc domains, to form Fc-fusion heterodimers.

There are several reasons for choosing the Fc region of human antibodies for use in generating the subject Affimer® Agents as Affimer® fusion proteins. The principle rationale is to produce a stable protein, large enough to demonstrate a similar pharmacokinetic profile compared with those of antibodies, and to take advantage of the properties imparted by the Fc region; this includes the salvage neonatal FcRn receptor pathway involving FcRn-mediated recycling of the fusion protein to the cell surface post endocytosis, avoiding lysosomal degradation and resulting in release back into the bloodstream, thus contributing to an extended serum half-life. Another obvious advantage is the Fc domain's binding to Protein A, which can simplify downstream processing during production of the Affimer® Agent and permit generation of highly pure preparation of the Affimer® Agent.

In general, an Fc domain will include the constant region of an antibody excluding the first constant region immunoglobulin domain. Thus, Fc domain refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM Fc may include the J chain. For IgG, Fc comprises immunoglobulin domains Cγ2 and Cγ3 and the hinge between Cγ1 and Cγ2. Although the boundaries of the Fc domain may vary, the human IgG heavy chain Fc region is usually defined to comprise residues C226 or P230 to its carboxyl-terminus, wherein the numbering is according to the EU index as set forth in Kabat (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, NIH, Bethesda, Md. (1991)). Fc may refer to this region in isolation, or this region in the context of a whole antibody, antibody fragment, or Fc fusion protein. Polymorphisms have been observed at a number of different Fc positions and are also included as Fc domains as used herein.

In some embodiments, the Fc As used herein, a "functional Fc region" refers to an Fc domain or fragment thereof which retains the ability to bind FcRn. A functional Fc region binds to FcRn, but does not possess effector function. The ability of the Fc region or fragment thereof to bind to FcRn can be determined by standard binding assays known in the art. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface

receptors (e.g., B cell receptor; BCR), etc. Such effector functions can be assessed using various assays known in the art for evaluating such antibody effector functions.

In an exemplary embodiment, the Fc domain is derived from an IgG1 subclass, however, other subclasses (e.g., IgG2, IgG3, and IgG4) may also be used. An exemplary sequence of a human IgG1 immunoglobulin Fc domain which can be used is:

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT
ISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO: 271)

In some embodiments, the Fc region used in the fusion protein may comprise the hinge region of an Fc molecule. An exemplary hinge region comprises the core hinge residues spanning positions 1-16 (i.e., DKTHTCPPCPAPELLG (SEQ ID NO: 272)) of the exemplary human IgG1 immunoglobulin Fc domain sequence provided above. In some embodiments, the Affimer® Agent-containing fusion protein may adopt a multimeric structure (e.g., dimer) owing, in part, to the cysteine residues at positions 6 and 9 within the hinge region of the exemplary human IgG1 immunoglobulin Fc domain sequence provided above. In other embodiments, the hinge region as used herein, may further include residues derived from the CH1 and CH2 regions that flank the core hinge sequence of the exemplary human IgG1 immunoglobulin Fc domain sequence provided above. In yet other embodiments, the hinge sequence may comprise or consist of GSTHTCPPCPAPELLG (SEQ ID NO: 273) or EPKSCDKTHTCPPCPAPELLG (SEQ ID NO: 274).

In some embodiments, the hinge sequence may include one or more substitutions that confer desirable pharmacokinetic, biophysical, and/or biological properties. Some exemplary hinge sequences include:

EPKSCDKTHTCPPCPAPELLGGPS (SEQ ID NO: 275);
EPKSSDKTHTCPPCPAPELLGGPS (SEQ ID NO: 276);
EPKSSDKTHTCPPCPAPELLGGSS (SEQ ID NO: 277);
EPKSSGSTHTCPPCPAPELLGGSS (SEQ ID NO: 278);
DKTHTCPPCPAPELLGGPS (SEQ ID NO: 279) and
DKTHTCPPCPAPELLGGSS (SEQ ID NO: 280).

In some embodiments, the residue P at position 18 of the exemplary human IgG1 immunoglobulin Fc domain sequence provided above may be replaced with S to ablate Fc effector function; this replacement is exemplified in hinges having the sequences EPKSSDKTHTCPPCPAPELLGGSS (SEQ ID NO: 277), EPKSSGSTHTCPPCPAPELLGGSS (SEQ ID NO: 278), and DKTHTCPPCPAPELLGGSS (SEQ ID NO: 280). In another embodiment, the residues DK at positions 1-2 of the exemplary human IgG1 immunoglobulin Fc domain sequence provided above may be replaced with GS to remove a potential clip site; this replacement is exemplified in the sequence EPKSSGSTHTCPPCPAPELLGGSS (SEQ ID NO: 278). In another embodiment, the C at the position 103 of the heavy chain constant region of human IgG1 (i.e., domains CH_1-CH_3), may be replaced with S to prevent improper cysteine bond formation in the absence of a light chain; this replacement is exemplified by EPKSSDKTHTCPPCPAPELLGGPS (SEQ ID NO: 276), EPKSSDKTHTCPPCPAPELLGGSS (SEQ ID NO: 277), and EPKSSGSTHTCPPCPAPELLGGSS (SEQ ID NO: 278).

In some embodiments, the Fc is a mammalian Fc such as a human Fc, including Fc domains derived from IgG1, IgG2, IgG3 or IgG4. The Fc region may possess at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity with a native Fc region and/or with an Fc region of a parent polypeptide. In some embodiments, the Fc region may have at least about 90% sequence identity with a native Fc region and/or with an Fc region of a parent polypeptide.

In some embodiments, the Fc domain comprises an amino acid sequence selected from SEQ ID NOs: 271, or an Fc sequence from the examples provided by SEQ ID Nos. 281-294. It should be understood that the C-terminal lysine of an Fc domain is an optional component of a fusion protein comprising an Fc domain. In some embodiments, the Fc domain comprises an amino acid sequence selected from SEQ ID NOs: 271 and 281 - 294, except that the C-terminal lysine thereof is omitted. In some embodiments, the Fc domain comprises the amino acid sequence of SEQ ID NO: 271. In some embodiments, the Fc domain comprises the amino acid sequence of SEQ ID NOs: 271 except the C-terminal lysine thereof is omitted.

hIgG1a_191 [A subtype]	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN

(SEQ ID NO: 281)	KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
hIgG1a_189 [hIgG1a_191 sans "GK" on C term; A subtype] **(SEQ ID NO: 282)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSP
hIgG1a_191b [A/F subtype] **(SEQ ID NO: 283)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSRDEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA LHNHYTQKSLSLSPGK
hIgG1f_1.1_191 [Contains 5 point mutations to alter ADCC function, F subtype] **(SEQ ID NO: 284)**	DKTHTCPPCPAPEAEGAPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPSSIEKTISKAKGQPREPQVYTLPPSREEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
hIgG1f_1.1_186 [Contains 5 point mutations to alter ADCC function and C225S (Edlemen numbering); F subtype] **(SEQ ID NO: 285)**	EPKSSDKTHTCPPCPAPEAEGAPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS VMHEALHNHYTQKSLSLSPGK
hIgG1a_(N297G)_191 [A subtype] **(SEQ ID NO: 286)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYGSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH

	NHYTQKSLSLSPGK
hIgG1a_190 [hIgG1a_190 sans "K" on C term; A subtype] **(SEQ ID NO: 287)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPG
hIgG1a_(N297Q)_191 [A subtype] **(SEQ ID NO: 288)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
hIgG1a_(N297S)_191 [A subtype] **(SEQ ID NO: 289)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYSSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
hIgG1a_(N297A)_191 [A subtype] **(SEQ ID NO: 290)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
hIgG1a_(N297H)_191 [A subtype] **(SEQ ID NO: 291)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYHSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
hIgG4 **(SEQ ID NO: 292)**	DKRVESKYGPPCPSCPAPEFLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVH

	NAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSV MHEALHNHYTQKSLSLSLGK
hIgG4_(S241P) **(SEQ ID NO: 293)**	DKRVESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVH NAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSV MHEALHNHYTQKSLSLSLGK
hIgG1 (Contain 2 point mutations to alter ADCC function L20A, L21A) **SEQ ID NO: 294**	SEPKSCDKTHTCPPCPAPE**AA**GGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS VMHEALHNHYTQKSLSLSPGK

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., Natural Killer (NK) cells, neutrophils, and macrophages) enables these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins.

In some embodiments, the fusion protein includes an Fc domain sequence for which the resulting Affimer® Agent has no (or reduced) ADCC and/or complement activation or effector functionality. For example, the Fc domain may comprise a naturally disabled constant region of IgG2 or IgG4 isotype or a mutated IgG1 constant region. Examples of suitable modifications are described in EP0307434. One example comprises the substitutions of alanine residues at positions 235 and 237 (EU index numbering).

In other embodiments, the fusion protein includes an Fc domain sequence for which the resulting Affimer® Agent will retain some or all Fc functionality for example will be capable of one or both of ADCC and CDC activity, as for example if the fusion protein comprises the Fc domain from human IgG1 or IgG3. Levels of effector function can be varied according to known techniques, for example by mutations in the CH2 domain,

for example wherein the IgG1 CH2 domain has one or more mutations at positions selected from 239 and 332 and 330, for example the mutations are selected from S239D and I332E and A330L such that the antibody has enhanced effector function, and/or for example altering the glycosylation profile of the antigen-binding protein of the disclosure such that there is a reduction in fucosylation of the Fc region.

(ii) Albumin fusion

In other embodiments, the Affimer® Agent is a fusion protein comprising, in addition to at least one Affimer® sequence, an albumin sequence or an albumin fragment. In other embodiments, the Affimer® Agent is conjugated to the albumin sequence or an albumin fragment through chemical linkage other than incorporation into the polypeptide sequence including the Affimer® Agent. In some embodiments, the albumin, albumin variant, or albumin fragment is human serum albumin (HSA), a human serum albumin variant, or a human serum albumin fragment. Albumin serum proteins comparable to HSA are found in, for example, cynomolgus monkeys, cows, dogs, rabbits and rats. Of the non-human species, bovine serum albumin (BSA) is the most structurally similar to HSA. See, e.g., Kosa et al., (2007) J Pharm Sci. 96(11):3117-24. The present disclosure contemplates the use of albumin from non-human species, including, but not limited to, albumin sequence derived from cyno serum albumin or bovine serum albumin.

Mature HSA, a 585 amino acid polypeptide (approx. 67 kDa) having a serum half-life of about 20 days, is primarily responsible for the maintenance of colloidal osmotic blood pressure, blood pH, and transport and distribution of numerous endogenous and exogenous ligands. The protein has three structurally homologous domains (domains I, II and III), is almost entirely in the alpha-helical conformation, and is highly stabilized by 17 disulphide bridges. In some embodiments, the Affimer® Agent can be an albumin fusion protein including one or more Affimer® polypeptide sequences and the sequence for mature human serum albumin (SEQ ID NO: 295) or a variant or fragment thereof which maintains the PK and/or biodistribution properties of mature albumin to the extent desired in the fusion protein.

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADES
AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRL
VRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAAD
KAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEV
SKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI

AEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLL
RLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQN
ALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVL
HEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQ
IKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAAS
QAALGL

(SEQ ID NO: 295)

The albumin sequence can be set off from the Affimer® polypeptide sequence or other flanking sequences in the Affimer® Agent by use of linker sequences as described above.

While unless otherwise indicated, reference herein to "albumin" or to "mature albumin" is meant to refer to HSA. However, it is noted that full-length HSA has a signal peptide of 18 amino acids (MKWVTFISLLFLFSSAYS (SEQ ID NO: 224)) followed by a pro-domain of 6 amino acids (RGVFRR (SEQ ID NO: 296)); this 24 amino acid residue peptide may be referred to as the pre-pro domain. The Affimer® Agent-HSA fusion proteins can be expressed and secreted using the HSA pre-pro-domain in the recombinant proteins coding sequence. Alternatively, the Affimer® Agent-HSA fusion can be expressed and secreted through inclusion of other secretion signal sequences, such as described above.

In alternative embodiments, rather than provided as part of a fusion protein with the Affimer® polypeptide, the serum albumin polypeptide can be covalently coupled to the Affimer® Agent-containing polypeptide through a bond other than a backbone amide bond, such as cross-linked through chemical conjugation between amino acid sidechains on each of the albumin polypeptide and the Affimer® Agent-containing polypeptide.

(iii) Albumin binding domain

In some embodiments, the Affimer® Agent can include a serum-binding moiety – either as part of a fusion protein (if also a polypeptide) with the Affimer® polypeptide sequence or chemically conjugated through a site other than being part of a contiguous polypeptide chain.

In some embodiments, the serum-binding polypeptide is an albumin binding moiety. Albumin contains multiple hydrophobic binding pockets and naturally serves as a

transporter of a variety of different ligands such as fatty acids and steroids as well as different drugs. Furthermore, the surface of albumin is negatively charged making it highly water-soluble.

The term "albumin binding moiety" as used herein refers to any chemical group capable of binding to albumin, i.e. has albumin binding affinity. Albumin binds to endogenous ligands such as fatty acids; however, it also interacts with exogenous ligands such as warfarin, penicillin and diazepam. As the binding of these drugs to albumin is reversible the albumin-drug complex serves as a drug reservoir that can enhance the drug biodistribution and bioavailability. Incorporation of components that mimic endogenous albumin-binding ligands, such as fatty acids, has been used to potentiate albumin association and increase drug efficacy.

In some embodiments, a chemical modification method that can be applied in the generation of the subject Affimer® Agents to increase protein half-life is lipidation, which involves the covalent binding of fatty acids to peptide side chains. Originally conceived of and developed as a method for extending the half-life of insulin, lipidation shares the same basic mechanism of half-life extension as PEGylation, namely increasing the hydrodynamic radius to reduce renal filtration. However, the lipid moiety is itself relatively small and the effect is mediated indirectly through the non-covalent binding of the lipid moiety to circulating albumin. One consequence of lipidation is that it reduces the water-solubility of the peptide but engineering of the linker between the peptide and the fatty acid can modulate this, for example by the use of glutamate or mini PEGs within the linker. Linker engineering and variation of the lipid moeity can affect self-aggregation which can contribute to increased half-life by slowing down biodistribution, independent of albumin. See, for example, Jonassen et al. (2012) Pharm Res. 29(8):2104-14.

Other examples of albumin binding moieties for use in the generation of certain Affimer® Agents include albumin-binding (PKE2) adnectins (See WO2011140086 "Serum Albumin Binding Molecules", WO2015143199 "Serum albumin-binding Fibronectin Type III Domains" and WO2017053617 "Fast-off rate serum albumin binding fibronectin type iii domains"), the albumin binding domain 3 (ABD3) of protein G of Streptococcus strain G148, and the albumin binding domain antibody GSK2374697 ("AlbudAb") or albumin binding nanobody portion of ATN-103 (Ozoralizumab).

(iv) PEGylation, XTEN, PAS and Other Polymers

A wide variety of macromolecular polymers and other molecules can be linked to the Affimer® Agent containing polypeptides of the present disclosure to modulate biological properties of the resulting Affimer® Agent, and/or provide new biological properties to the Affimer® Agent. These macromolecular polymers can be linked to the Affimer® Agent containing polypeptide via a naturally encoded amino acid, via a non-naturally encoded amino acid, or any functional substituent of a natural or non-natural amino acid, or any substituent or functional group added to a natural or non-natural amino acid. The molecular weight of the polymer may be of a wide range, including but not limited to, between about 100 Da and about 100,000 Da or more. The molecular weight of the polymer may be between about 100 Da and about 100,000 Da, including but not limited to, 100,000 Da, 95,000 Da, 90,000 Da, 85,000 Da, 80,000 Da, 75,000 Da, 70,000 Da, 65,000 Da, 60,000 Da, 55,000 Da, 50,000 Da, 45,000 Da, 40,000 Da, 35,000 Da, 30,000 Da, 25,000 Da, 20,000 Da, 15,000 Da, 10,000 Da, 9,000 Da, 8,000 Da, 7,000 Da, 6,000 Da, 5,000 Da, 4,000 Da, 3,000 Da, 2,000 Da, 1,000 Da, 900 Da, 800 Da, 700 Da, 600 Da, 500 Da, 400 Da, 300 Da, 200 Da, and 100 Da. In some embodiments, the molecular weight of the polymer is between about 100 Da and about 50,000 Da. In some embodiments, the molecular weight of the polymer is between about 100 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 1,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 5,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 10,000 Da and about 40,000 Da.

For this purpose, various methods including pegylation, polysialylation, HESylation, glycosylation, or recombinant PEG analogue fused to flexible and hydrophilic amino acid chain (500 to 600 amino acids) have been developed (See Chapman, (2002) Adv Drug Deliv Rev. 54. 531-545; Schlapschy et al., (2007) Prot Eng Des Sel. 20, 273-283; Contermann (2011) Curr Op Biotechnol. 22, 868-876; Jevsevar et al., (2012) Methods Mol Biol. 901, 233-246).

Examples of polymers include but are not limited to polyalkyl ethers and alkoxy-capped analogs thereof (e.g., polyoxyethylene glycol, polyoxyethylene/propylene glycol, and methoxy or ethoxy-capped analogs thereof, especially polyoxyethylene glycol, the latter is also known as polyethylene glycol or PEG); discrete PEG (dPEG); polyvinylpyrrolidones; polyvinylalkyl ethers; polyoxazolines, polyalkyl oxazolines and polyhydroxyalkyl oxazolines; polyacrylamides, polyalkyl acrylamides, and

polyhydroxyalkyl acrylamides (e.g., polyhydroxypropylmethacrylamide and derivatives thereof); polyhydroxyalkyl acrylates; polysialic acids and analogs thereof; hydrophilic peptide sequences; polysaccharides and their derivatives, including dextran and dextran derivatives, e.g., carboxymethyldextran, dextran sulfates, aminodextran; cellulose and its derivatives, e.g., carboxymethyl cellulose, hydroxyalkyl celluloses; chitin and its derivatives, e.g., chitosan, succinyl chitosan, carboxymethylchitin, carboxymethylchitosan; hyaluronic acid and its derivatives; starches; alginates; chondroitin sulfate; albumin; pullulan and carboxymethyl pullulan; polyaminoacids and derivatives thereof, e.g., polyglutamic acids, polylysines, polyaspartic acids, polyaspartamides; maleic anhydride copolymers such as: styrene maleic anhydride copolymer, divinylethyl ether maleic anhydride copolymer; polyvinyl alcohols; copolymers thereof; terpolymers thereof; mixtures thereof; and derivatives of the foregoing.

The polymer selected may be water soluble so that the Affimer® Agent to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. The water soluble polymer may be any structural form including but not limited to linear, forked or branched. Typically, the water soluble polymer is a poly(alkylene glycol), such as poly(ethylene glycol) (PEG), but other water soluble polymers can also be employed. By way of example, PEG is used to describe some embodiments of this disclosure. For therapeutic use of the Affimer® Agent, the polymer may be pharmaceutically acceptable.

The term "PEG" is used broadly to encompass any polyethylene glycol molecule, without regard to size or to modification at an end of the PEG, and can be represented as linked to the Affimer® Agent containing polypeptide by the formula:

$XO—(CH_2CH_2O)_n—CH_2CH_2—$

or

$XO—(CH_2CH_2O)_n—$

where n is 2 to 10,000 and X is H or a terminal modification, including but not limited to, a C1-4 alkyl, a protecting group, or a terminal functional group. In some cases, a PEG used in the polypeptides of the disclosure terminates on one end with hydroxy or methoxy, i.e., X is H or CH_3 ("methoxy PEG").

It is noted that the other end of the PEG, which is shown in the above formulas by a terminal "—", may attach to the Affimer® Agent containing polypeptide via a naturally-occurring or non-naturally encoded amino acid. For instance, the attachment may be through an amide, carbamate or urea linkage to an amine group (including but not limited to, the epsilon amine of lysine or the N-terminus) of the polypeptide. Alternatively, the polymer is linked by a maleimide linkage to a thiol group (including but not limited to, the thiol group of cysteine) – which in the case of attachment to the Affimer® polypeptide sequence per se requires altering a residue in the Affimer® sequence to a cysteine.

The number of water soluble polymers linked to the Affimer® Agent-containing polypeptide (i.e., the extent of PEGylation or glycosylation) can be adjusted to provide an altered (including but not limited to, increased or decreased) pharmacologic, pharmacokinetic or pharmacodynamic characteristic such as *in vivo* half-life in the resulting Affimer® Agent. In some embodiments, the half-life of the resulting Affimer® Agent is increased at least about 10, 20, 30, 40, 50, 60, 70, 80, 90 percent, 2-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold, 20-fold, 25-fold, 30-fold, 35-fold, 40-fold, 50-fold, or at least about 100-fold over an unmodified polypeptide.

Another variation of polymer system useful to modify the PK or other biological properties of the resulting Affimer® Agent are the use of unstructured, hydrophilic amino acid polymers that are functional analogs of PEG, particularly as part of a fusion protein with the Affimer® polypeptide sequence. The inherent biodegradability of the polypeptide platform makes it attractive as a potentially more benign alternative to PEG. Another advantage is the precise molecular structure of the recombinant molecule in contrast to the polydispersity of PEG. Unlike HSA and Fc peptide fusions, in which the three-dimensional folding of the fusion partner needs to be maintained, the recombinant fusions to unstructured partners can, in many cases, be subjected to higher temperatures or harsh conditions such as HPLC purification.

One of the more advanced of this class of polypeptides is termed XTEN (Amunix) and is 864 amino acids long and comprised of six amino acids (A, E, G, P, S and T). See Schellenberger et al. "A recombinant polypeptide extends the *in vivo* half-life of peptides and proteins in a tunable manner" 2009 Nat Biotechnol. 27(12):1186-90. Enabled by the biodegradable nature of the polymer, this is much larger than the 40 KDa PEGs typically used and confers a concomitantly greater half-life extension. The

fusion of XTEN to the Affimer® Agent containing polypeptide should result in halflife extension of the final Affimer® Agent by 60- to 130-fold over the unmodified polypeptide.

c. Multispecific Fusion Proteins

In some embodiments, the Affimer® Agent is a multi-specific polypeptide including, for example, a first anti-SPIKE Affimer® polypeptide and at least one additional binding domain. The additional binding domain may be a polypeptide sequence selected from amongst, to illustrate, a second Affimer® polypeptide sequence (which may be the same or different than the first Affimer® polypeptide sequence), an antibody or fragment thereof or osther antigen binding polypeptide, a ligand binding portion of a receptor (such as a receptor trap polypeptide), a receptor-binding ligand (such as a cytokine, growth factor or the like), an enzyme or catalytic fragment thereof, or other polypeptide sequence that confers some additional function.

In some embodiments, the Affimer® Agent includes one or more additional Affimer® polypeptide sequence that are also directed to SPIKE. The additional anti-SPIKE Affimer® Agents may be the same or different (or a mixture thereof) as the first anti-SPIKE Affimer® polypeptide in order to create a multi-specific Affimer® fusion protein. The Affimer® Agents can bind the same or overlapping sites on SPIKE, or can bind two different sites such that the Affimer® Agent can simultaneously bind two sites on the same SPIKE protein (biparatopic) or more than two sites (multiparatopic).

In some embodiments, the Affimer® Agent includes one or more antigen binding sites from an antibody. The resulting Affimer® Agent can be a single chain including both the anti-SPIKE Affimer® Agent and the antigen binding site (such as in the case of an scFV), or can be a multimeric protein complex such as in antibody assembled with heavy and/or light chains to which the sequence of the anti-SPIKE antibody has also been fused.

d. Conjugates

The subject Affimer® Agents may also include one or more Functional Moieties intended to impart detectability or additional pharmacologic activity to the Affimer® Agent. Functional Moieties for detection are those which can be employed to detect association of the Affimer® Agent with a cell or tissue (such as an infected cell) *in vivo*. Functional Moieties with pharmacologic activity are those agents which are meant to be

delivered to the infected tissue expressing the target of the Affimer® Agent and in doing so have a pharmacologic consequence to the targeted tissues or cells.

The present disclosure provides Affimer® Agents including conjugates of substances having a wide variety of functional groups, substituents or moieties, with those Functional Moieties including but not limited to a label; a dye; an immunoadhesion molecule; a radionuclide; a cytotoxic compound; a drug; an affinity label; a photoaffinity label; a reactive compound; a resin; a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; a metal chelator; a cofactor; a fatty acid; a carbohydrate; a polynucleotide; a DNA; a RNA; an antisense polynucleotide; a saccharide; a water-soluble dendrimer; a cyclodextrin; an inhibitory ribonucleic acid; a biomaterial; a nanoparticle; a spin label; a fluorophore, a metal-containing moiety; a radioactive moiety; a novel functional group; a group that covalently or noncovalently interacts with other molecules; a photocaged moiety; an actinic radiation excitable moiety; a photoisomerizable moiety; biotin; a derivative of biotin; a biotin analogue; a moiety incorporating a heavy atom; a chemically cleavable group; a photocleavable group; an elongated side chain; a carbon-linked sugar; a redox-active agent; an amino thioacid; a toxic moiety; an isotopically labeled moiety; a biophysical probe; a phosphorescent group; a chemiluminescent group; an electron dense group; a magnetic group; an intercalating group; a chromophore; an energy transfer agent; a biologically active agent; a detectable label; a small molecule; a quantum dot; a nanotransmitter; a radionucleotide; a radiotransmitter; a neutron-capture agent; or any combination of the above, or any other desirable compound or substance.

Where the moiety is a detectable label, it can be a fluorescent label, radioactive label, enzymatic label or any other label known to the skilled person. In some embodiments, the Functional Moiety is a detectable label that can be included as part of a conjugate to form certain Affimer® Agents suitable for medical imaging. By "medical imaging" is meant any technique used to visualise an internal region of the human or animal body, for the purposes of diagnosis, research or therapeutic treatment. For instance, the Affimer® Agent can be detected (and quantitated) by radioscintigraphy, magnetic resonance imaging (MRI), computed tomography (CT scan), nuclear imaging, positron emission comprising a metal tomography (PET) contrast agent, optical imaging (such as fluorescence imaging including near-infrared fluorescence (NIRF) imaging), bioluminescence imaging, or combinations thereof. The Functional Moiety is optionally a contrast agent for X-ray imaging. Agents useful in enhancing such techniques are

those materials that enable visualization of a particular locus, organ or disease site within the body, and/or that lead to some improvement in the quality of the images generated by the imaging techniques, providing improved or easier interpretation of those images. Such agents are referred to herein as contrast agents, the use of which facilitates the differentiation of different parts of the image, by increasing the "contrast" between those different regions of the image. The term "contrast agents" thus encompasses agents that are used to enhance the quality of an image that may nonetheless be generated in the absence of such an agent (as is the case, for instance, in MRI), as well as agents that are prerequisites for the generation of an image (as is the case, for instance, in nuclear imaging).

In some embodiments, the detectable label includes a chelate moiety for chelating a metal, e.g., a chelator for a radiometal or paramagnetic ion. In some embodiments, the detectable label is a chelator for a radionuclide useful for radiotherapy or imaging procedures. Radionuclides useful within the present disclosure include gamma-emitters, positron-emitters, Auger electron-emitters, X-ray emitters and fluorescence-emitters, with beta- or alpha-emitters for therapeutic use. Examples of radionuclides useful as toxins in radiation therapy include: ^{43}K, ^{47}Sc, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{64}Cu, ^{67}Ga, ^{67}Cu, ^{68}Ga, ^{71}Ge, ^{75}Br, ^{76}Br, ^{77}Br, ^{77}As, ^{81}Rb, ^{90}Y, ^{97}Ru, ^{99m}Tc, ^{100}Pd, ^{101}Rh, ^{103}Pb, ^{105}Rh, ^{109}Pd, ^{111}Ag, ^{111}In, ^{113}In, ^{119}Sb ^{121}Sn, ^{123}I, ^{125}I, ^{127}Cs, ^{128}Ba, ^{129}Cs, ^{131}I, ^{131}Cs, ^{143}Pr, ^{153}Sm, ^{161}Tb, ^{166}Ho, ^{169}Eu, ^{177}Lu, ^{186}Re, ^{188}Re, ^{189}Re, ^{191}Os, ^{193}Pt, ^{194}Ir, ^{197}Hg, ^{199}Au, ^{203}Pb, ^{211}At, ^{212}Pb, ^{212}Bi and ^{213}Bi. Conditions under which a chelator will coordinate a metal are described, for example, by Gansow et al., U.S. Pat. Nos. 4,831,175, 4,454,106 and 4,472,509. Examples of chelators includes, merely to illustrate, 1,4,7-triazacyclononane-N,N',N"-triacetic acid (NOTA) 1,4,7,10-tetraazacyclododecane-N,N',N",N'"-tetraacetic acid (DOTA) 1 ,4,8,11-tetraazacyclotetradecane-N,N',N",N'"-tetraacetic acid (TETA).

Other detectable isotopes that can be incorporated directly into the amino acid residues of the Affimer® polypeptide or which otherwise do not require a chelator, include ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{36}Cl.

Paramagnetic ions, useful for diagnostic procedures, may also be administered. Examples of paramagnetic ions include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III), erbium (III), or combinations of these paramagnetic ions.

Examples of fluorescent labels include, but are not restricted to, organic dyes (e.g. cyanine, fluorescein, rhodamine, Alexa Fluors, DyLight fluors, ATTO Dyes, BODIPY Dyes, etc.), biological fluorophores (e.g. green fluorescent protein (GFP), R-Phycoerythrin, etc.), and quantum dots.

Non-limiting fluorescent compound that may be used in the present disclosure include, Cy5, Cy5.5 (also known as Cy5++), Cy2, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), phycoerythrin, Cy7, fluorescein (FAM), Cy3, Cy3.5 (also known as Cy3++), Texas Red, LightCycler-Red 640, LightCycler Red 705, tetramethylrhodamine (TMR), rhodamine, rhodamine derivative (ROX), hexachlorofluorescein (HEX), rhodamine 6G (R6G), the rhodamine derivative JA133, Alexa Fluorescent Dyes (such as Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 633, Alexa Fluor 555, and Alexa Fluor 647), 4′,6-diamidino-2-phenylindole (DAPI), Propidium iodide, AMCA, Spectrum Green, Spectrum Orange, Spectrum Aqua, Lissamine, and fluorescent transition metal complexes, such as europium. Fluorescent compound that can be used also include fluorescent proteins, such as GFP (green fluorescent protein), enhanced GFP (EGFP), blue fluorescent protein and derivatives (BFP, EBFP, EBFP2, Azurite, mKalama1), cyan fluorescent protein and derivatives (CFP, ECFP, Cerulean, CyPet) and yellow fluorescent protein and derivatives (YFP, Citrine, Venus, YPet). WO2008142571, WO2009056282, WO9922026.

Examples of enzymatic labels include, but are not restricted to, horseradish peroxidase (HRP), alkaline phosphatase (AP), glucose oxidase and β-galactosidase.

Another well-known label is biotin. Biotin labels are typically composed of the biotinyl group, a spacer arm and a reactive group that is responsible for attachment to target functional groups on proteins. Biotin can be useful for attaching the labelled protein to other moieties which comprise an avidin moiety.

EXPRESSION METHODS AND SYSTEMS

Recombinant Affimer® Agent proteins described herein can be produced by any suitable method known in the art. Such methods range from direct protein synthesis methods to constructing a DNA sequence encoding polypeptide sequences and expressing those sequences in a suitable host. For those recombinant Affimer® Agent proteins including further modifications, such as a chemical modifications or conjugation, the recombinant Affimer® Agent protein can be further manipulated chemically or enzymatically after isolation form the host cell or chemical synthesis.

The present disclosure includes recombinant methods and nucleic acids for recombinantly expressing the recombinant Affimer® Agent proteins of the present disclosure comprising (i) introducing into a host cell a polynucleotide encoding the amino acid sequence of said Affimer® Agent, for example, wherein the polynucleotide is in a vector and/or is operably linked to a promoter; (ii) culturing the host cell (e.g., eukaryotic or prokaryotic) under condition favorable to expression of the polynucleotide and, (iii) optionally, isolating the Affimer® Agent from the host cell and/or medium in which the host cell is grown. See e.g., WO 04/041862, WO 2006/122786, WO 2008/020079, WO 2008/142164 or WO 2009/068627.

In some embodiments, a DNA sequence encoding a recombinant Affimer® Agent protein of interest may be constructed by chemical synthesis using an oligonucleotide synthesizer. Oligonucleotides can be designed based on the amino acid sequence of the desired polypeptide and selecting those codons that are favored in the host cell in which the recombinant polypeptide of interest will be produced. Standard methods can be applied to synthesize a polynucleotide sequence encoding an isolated polypeptide of interest. For example, a complete amino acid sequence can be used to construct a back-translated gene. Further, a DNA oligomer containing a nucleotide sequence coding for the particular isolated polypeptide can be synthesized. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Once a nucleic acid sequence encoding a recombinant Affimer® Agent protein of the disclosure has been obtained, the vector for the production of the recombinant Affimer® Agent protein may be produced by recombinant DNA technology using techniques well known in the art. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the recombinant Affimer® Agent coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. (See, for example, the techniques described in Sambrook et al, 1990, MOLECULAR CLONING, A LABORATORY MANUAL, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. and Ausubel et al. eds., 1998, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, NY).

An expression vector comprising the nucleotide sequence of a recombinant Affimer® Agent protein can be transferred to a host cell by conventional techniques (e.g., electroporation, liposomal transfection, and calcium phosphate precipitation) and the transfected cells are then cultured by conventional techniques to produce the recombinant Affimer® Agent protein of the disclosure. In specific embodiments, the expression of the recombinant Affimer® Agent protein is regulated by a constitutive, an inducible or a tissue, specific promoter.

The expression vector may include an origin of replication, such as may be selected based upon the type of host cell being used for expression. By way of example, the origin of replication from the plasmid pBR322 (Product No. 303-3s, New England Biolabs, Beverly, Mass.) is useful for most Gram- negative bacteria while various origins from SV40, polyoma, adenovirus, vesicular stomatitus virus (VSV) or papillomaviruses (such as HPV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (for example, the SV40 origin is often used because it contains the early promoter).

The vector may include one or more selectable marker genes, e.g., genetic elements that encode a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, tetracycline, or kanamycin for prokaryotic host cells, (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex media. Preferred selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, and the tetracycline resistance gene. A neomycin resistance gene may also be used for selection in prokaryotic and eukaryotic host cells. Other selection genes may be used to amplify the gene which will be expressed. Amplification is a process where genes which are in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of selectable markers for mammalian cells include dihydrofolate reductase (DHFR) and thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of the marker present in the vector. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the recombinant Affimer® Agent protein. As

a result, increased quantities of the recombinant Affimer® Agent protein are synthesized from the amplified DNA.

The vector may also include one or more ribosome binding site, which will be transcribed into the mRNA including the coding sequence for the recombinant Affimer® Agent protein. For example, such a site is characterized by a Shine-Dalgarno sequence (prokaryotes) or a Kozak sequence (eukaryotes). The element is typically located 3' to the promoter and 5' to the coding sequence of the polypeptide to be expressed. The Shine-Dalgarno sequence is varied but is typically a polypurine (having a high A-G content). Many Shine-Dalgarno sequences have been identified, each of which can be readily synthesized using methods set forth above and used in a prokaryotic vector.

The expression vectors will typically contain a promoter that is recognized by the host organism and operably linked to a nucleic acid molecule encoding the recombinant Affimer® Agent protein. Either a native or heterologous promoter may be used depending the host cell used for expression and the yield desired.

Promoters for use with prokaryotic hosts include the beta-lactamase and lactose promoter systems; alkaline phosphatase, a tryptophan (trp) promoter system; and hybrid promoters such as the tac promoter. Other known bacterial promoters are also suitable. Their sequences have been published, and they can be ligated to a desired nucleic acid sequence(s), using linkers or adapters as desired to supply restriction sites.

Promoters for use with yeast hosts are also known in the art. Yeast enhancers are advantageously used with yeast promoters. Suitable promoters for use with mammalian host cells are well known and include those obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40). Other suitable mammalian promoters include heterologous mammalian promoters, e.g., heat-shock promoters and the actin promoter.

Additional promoters which may be used for expressing the selective binding agents of the disclosure include, but are not limited to: the SV40 early promoter region (Bernoist and Chambon, Nature, 290:304-310, 1981); the CMV promoter; the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al.

(1980), Cell 22: 787-97); the herpes thymidine kinase promoter (Wagner et al. (1981), Proc. Natl. Acad. Sci. U.S.A. 78: 1444-5); the regulatory sequences of the metallothionine gene (Brinster et al, Nature, 296; 39-42, 1982); prokaryotic expression vectors such as the beta- lactamase promoter (Villa-Kamaroff, et al., Proc. Natl. Acad. Sci. U.S.A., 75; 3727-3731, 1978); or the tac promoter (DeBoer, et al. (1983), Proc. Natl. Acad. Sci. U.S.A., 80: 21-5). Also of interest are the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: the elastase I gene control region which is active in pancreatic acinar cells (Swift et al. (1984), Cell 38: 639-46; Ornitz et al. (1986), Cold Spring Harbor Symp. Quant. Biol. 50: 399-409; MacDonald (1987), Hepatology 7: 425-515); the insulin gene control region which is active in pancreatic beta cells (Hanahan (1985), Nature 315: 115-22); the immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al. (1984), Cell 38; 647-58; Adames et al. (1985), Nature 318; 533-8; Alexander et al. (1987), Mol. Cell. Biol. 7: 1436-44); the mouse mammary tumour virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al. (1986), Cell 45: 485-95), albumin gene control region which is active in liver (Pinkert et al. (1987), Genes and Devel. 1: 268-76); the alphafetoprotein gene control region which is active in liver (Krumlauf et al. (1985), MoI. Cell. Biol. 5: 1639-48; Hammer et al. (1987), Science, 235: 53-8); the alpha 1- antitrypsin gene control region which is active in the liver (Kelsey et al. (1987), Genes and Devel. 1: 161-71); the beta-globin gene control region which is active in myeloid cells (Mogram et al., Nature, 315 338-340, 1985; Kollias et al. (1986), Cell 46: 89-94); the myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al. (1987), Cell, 48: 703-12); the myosin light chain-2 gene control region which is active in skeletal muscle (Sani (1985), Nature, 314: 283-6); and the gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al. (1986), Science 234: 1372-8).

An enhancer sequence may be inserted into the vector to increase transcription in eukaryotic host cells. Several enhancer sequences available from mammalian genes are known (e.g., globin, elastase, albumin, alpha-feto-protein and insulin). Typically, however, an enhancer from a virus will be used. The SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers are exemplary enhancing elements for the activation of eukaryotic promoters.

While an enhancer may be spliced into the vector at a position 5' or 3' to the polypeptide coding region, it is typically located at a site 5' from the promoter.

Vectors for expressing nucleic acids include those which are compatible with bacterial, insect, and mammalian host cells. Such vectors include, inter alia, pCRII, pCR3, and pcDNA3.1 (Invitrogen Company, San Diego, Calif.), pBSII (Stratagene Company, La Jolla, Calif.), pET15 (Novagen, Madison, Wis.), pGEX (Pharmacia Biotech, Piscataway, N.J.), pEGFP-N2 (Clontech, Palo Alto, Calif.), pETL (BlueBacII; Invitrogen), pDSR-alpha (PCT Publication No. WO90/14363) and pFastBacDual (Gibco/BRL, Grand Island, N.Y.).

Additional possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the selected host cell. Such vectors include, but are not limited to plasmids such as Bluescript® plasmid derivatives (a high copy number ColEl-based phagemid, Stratagene Cloning Systems Inc., La Jolla Calif.), PCR cloning plasmids designed for cloning Taq-amplified PCR products (e.g., TOPO™. TA Cloning® Kit, PCR2.1 plasmid derivatives, Invitrogen, Carlsbad, Calif.), and mammalian, yeast or virus vectors such as a baculovirus expression system (pBacPAK plasmid derivatives, Clontech, Palo Alto, Calif.). The recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, or other known techniques.

Eukaryotic and prokaryotic host cells, including mammalian cells as hosts for expression of the recombinant Affimer® Agent protein disclosed herein are well known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC). These include, inter alia, Chinese hamster ovary (CHO) cells, NSO, SP2 cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), A549 cells, 3T3 cells, HEK-293 cells and a number of other cell lines. Mammalian host cells include human, mouse, rat, dog, monkey, pig, goat, bovine, horse and hamster cells. Cell lines of particular preference are selected through determining which cell lines have high expression levels. Other cell lines that may be used are insect cell lines, such as Sf9 cells, amphibian cells, bacterial cells, plant cells and fungal cells. Fungal cells include yeast and filamentous fungus cells including, for example, Pichia pastoris, Pichia finlandica, Pichia trehalophila, Pichia koclamae, Pichia membranaefaciens, Pichia minuta (Ogataea minuta, Pichia lindneri), Pichia opuntiae, Pichia thermotolerans, Pichia salictaria, Pichia guercuum, Pichia pijperi, Pichia stiptis, Pichia methanolica,

Pichia sp., Saccharomyces cerevisiae, Saccharomyces sp., Hansenula polymorpha, Kluyveromyces sp., Kluyveromyces lactis, Candida albicans, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Trichoderma reesei, Chrysosporium lucknowense, Fusarium sp., Fusarium gramineum, Fusarium venenatum, Physcomitrella patens and Neurospora crassa. Pichia sp., any Saccharomyces sp., Hansenula polymorpha, any Kluyveromyces sp., Candida albicans, any Aspergillus sp., Trichoderma reesei, Chrysosporium lucknowense, any Fusarium sp., Yarrowia lipolytica, and Neurospora crassa.

A variety of host-expression vector systems may be utilized to express the recombinant Affimer® Agent protein of the disclosure. Such host-expression systems represent vehicles by which the coding sequences of the recombinant Affimer® Agent protein may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the recombinant Affimer® Agent protein of the disclosure in situ. These include, but are not limited to, microorganisms such as bacteria (e.g., E. coli and B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing Affimer® Agent protein coding sequences; yeast (e.g., Saccharomyces pichia) transformed with recombinant yeast expression vectors containing Affimer® Agent protein coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the Affimer® Agent protein coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus (CμMV) and tobacco mosaic virus (TMV)) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing Affimer® Agent protein coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 293T, 3T3 cells, lymphotic cells (see U.S. Pat. No. 5,807,715), Per C.6 cells (rat retinal cells developed by Crucell)) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the recombinant Affimer® Agent protein being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of the recombinant Affimer® Agent protein, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to

the E. coli expression vector pUR278 (Ruther et al. (1983) "Easy Identification Of cDNA Clones," EMBO J. 2:1791-1794), in which the Affimer® Agent protein coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye et al. (1985) "Up-Promoter Mutations In The Lpp Gene Of Escherichia coli," Nucleic Acids Res. 13:3101-3110; Van Heeke et al. (1989) "Expression Of Human Asparagine Synthetase In Escherichia coli," J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free gluta-thione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The Affimer® Agent protein coding sequence may be cloned individually into non-essential regions (e.g., the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (e.g., the polyhedrin promoter).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the Affimer® Agent protein coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the immunoglobulin molecule in infected hosts. (see e.g., see Logan et al. (1984) "Adenovirus Tripartite Leader Sequence Enhances Translation Of mRNAs Late After Infection," Proc. Natl. Acad. Sci. (U.S.A.) 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted Affimer® Agent protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription

terminators, etc. (see Bitter et al. (1987) "Expression And Secretion Vectors For Yeast," Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, Hela, COS, MDCK, 293, 293T, 3T3, WI38, BT483, Hs578T, HTB2, BT20 and T47D, CRL7030 and Hs578Bst.

For long-term, high-yield production of recombinant proteins, stable expression is contemplated. For example, cell lines which stably express an antibody of the disclosure may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the recombinant Affimer® Agent proteins of the disclosure. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the recombinant Affimer® Agent proteins.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al. (1977) "Transfer Of Purified Herpes Virus Thymidine Kinase Gene To Cultured Mouse Cells," Cell 11:223-232), hypoxanthine-guanine phosphoribosyltransferase (Szybalska et al. (1962) "Genetics Of Human Cess Line. IV. DNA-Mediated Heritable Transformation Of A Biochemical Trait," Proc. Natl.

Acad. Sci. (U.S.A.) 48:2026-2034), and adenine phosphoribosyltransferase (Lowy et al. (1980) "Isolation Of Transforming DNA: Cloning The Hamster Aprt Gene," Cell 22:817-823) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al. (1980) "Transformation Of Mammalian Cells With An Amplfiable Dominant-Acting Gene," Proc. Natl. Acad. Sci. (U.S.A.) 77:3567-3570; O'Hare et al. (1981) "Transformation Of Mouse Fibroblasts To Methotrexate Resistance By A Recombinant Plasmid Expressing A Prokaryotic Dihydrofolate Reductase," Proc. Natl. Acad. Sci. (U.S.A.) 78:1527-1531); gpt, which confers resistance to mycophenolic acid (Mulligan et al. (1981) "Selection For Animal Cells That Express The Escherichia coli Gene Coding For Xanthine-Guanine Phosphoribosyltransferase," Proc. Natl. Acad. Sci. (U.S.A.) 78:2072-2076); neo, which confers resistance to the aminoglycoside G-418 (Tachibana et al. (1991) "Altered Reactivity Of Immunoglobutin Produced By Human-Human Hybridoma Cells Transfected By pSV.2-Neo Gene," Cytotechnology 6(3):219-226; Tolstoshev (1993) "Gene Therapy, Concepts, Current Trials And Future Directions," Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan (1993) "The Basic Science Of Gene Therapy," Science 260:926-932; and Morgan et al. (1993) "Human gene therapy," Ann. Rev. Biochem. 62:191-217). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, NY; Kriegler, 1990, GENE TRANSFER AND EXPRESSION, A LABORATORY MANUAL, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli et al. (eds), 1994, CURRENT PROTOCOLS IN HUMAN GENETICS, John Wiley & Sons, NY.; Colbere-Garapin et al. (1981) "A New Dominant Hybrid Selective Marker For Higher Eukaryotic Cells," J. Mol. Biol. 150:1-14; and hygro, which confers resistance to hygromycin (Santerre et al. (1984) "Expression Of Prokaryotic Genes For Hygromycin B And G418 Resistance As Dominant-Selection Markers In Mouse L Cells," Gene 30:147-156).

The expression levels of a recombinant Affimer® Agent protein can be increased by vector amplification (for a review, see Bebbington and Hentschel, "The Use Of Vectors Based On Gene Amplification For The Expression Of Cloned Genes In Mammalian Cells," in DNA CLONING, Vol. 3. (Academic Press, New York, 1987)). When a marker in the vector system expressing a recombinant Affimer® Agent protein is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the nucleotide sequence of the recombinant Affimer® Agent protein, production of the

recombinant Affimer® Agent protein will also increase (Crouse et al. (1983) "Expression And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes," Mol. Cell. Biol. 3:257-266).

Where the Affimer® Agent is an Affimer® Agent antibody fusion or other multiprotein complex, the host cell may be co-transfected with two expression vectors, for instance the first vector encoding a heavy chain and the second vector encoding a light chain derived polypeptide, one or both of which includes an Affimer® polypeptide coding sequence. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot (1986) "Expression And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes," Nature 322:562-565; Kohler (1980) "Immunoglobulin Chain Loss In Hybridoma Lines," Proc. Natl. Acad. Sci. (U.S.A.) 77:2197-2199). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

In general, glycoproteins produced in a particular cell line or transgenic animal will have a glycosylation pattern that is characteristic for glycoproteins produced in the cell line or transgenic animal. Therefore, the particular glycosylation pattern of the recombinant Affimer® Agent protein will depend on the particular cell line or transgenic animal used to produce the protein. In some embodiments of Affimer® Agent/antibody fusions, a glycosylation pattern comprising only non-fucosylated N-glycans may be advantageous, because in the case of antibodies this has been shown to typically exhibit more potent efficacy than fucosylated counterparts both in vitro and *in vivo* (See for example, Shinkawa et al., J. Biol. Chem. 278: 3466-3473 (2003); U.S. Pat. Nos. 6,946,292 and 7,214,775).

Further, expression of an Affimer® Agent from production cell lines can be enhanced using a number of known techniques. For example, the glutamine synthetase gene expression system (the GS system) is a common approach for enhancing expression under certain conditions. The GS system is discussed in whole or part in connection with European Patent Nos. 0216846, 0256055, and 0323997 and European Patent Application No. 89303964.4. Thus, in some embodiments of the disclosure, the mammalian host cells (e.g., CHO) lack a glutamine synthetase gene and are grown in the absence of glutamine in the medium wherein, however, the polynucleotide

encoding the immunoglobulin chain comprises a glutamine synthetase gene which complements the lack of the gene in the host cell. Such host cells containing the binder or polynucleotide or vector as discussed herein as well as expression methods, as discussed herein, for making the binder using such a host cell are part of the present disclosure.

Expression of recombinant proteins in insect cell culture systems (e.g., baculovirus) also offers a robust method for producing correctly folded and biologically functional proteins. Baculovirus systems for production of heterologous proteins in insect cells are well-known to those of skill in the art.

The recombinant Affimer® Agent proteins produced by a transformed host can be purified according to any suitable method. Standard methods include chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. Affinity tags such as hexa-histidine, maltose binding domain, influenza coat sequence, and glutathione-S-transferase can be attached to the protein to allow easy purification by passage over an appropriate affinity column. Isolated proteins can also be physically characterized using such techniques as proteolysis, mass spectrometry (MS), nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC), and x-ray crystallography.

In some embodiments, recombinant Affimer® Agent proteins produced in bacterial culture can be isolated, for example, by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange, or size exclusion chromatography steps. HPLC can be employed for final purification steps. Microbial cells employed in expression of a recombinant protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

ENCODED AFFIMERS FOR *IN VIVO* DELIVERY

An alternative approach to the delivery of therapeutic Affimer® Agents protein, such as an anti-SPIKE Affimer® Agent, would be to leave the production of the therapeutic polypeptide to the body itself. A multitude of clinical studies have illustrated the utility of *in vivo* gene transfer into cells using a variety of different delivery systems. *In vivo* gene transfer seeks to administer to patients the Encoded Affimer® nucleotide sequence, rather than the Affimer® Agent. This allows the patient's body to produce

the therapeutic Affimer® Agent of interest for a prolonged period of time, and secrete it either systemically or locally, depending on the production site. Gene-based Encoded Affimer® Agents can present a labor- and cost-effective alternative to the conventional production, purification and administration of the polypeptide version of the Affimer® Agent. A number of antibody expression platforms have been pursued *in vivo* to which delivery of Encoded Affimer® Agents can be adapted: these include viral vectors, naked DNA and RNA. Encoded Affimer® Agent gene transfer can not only enable cost-savings by reducing the cost of goods and of production, but may also be able to reduce the frequency of drug administration. Overall, a prolonged *in vivo* production of the therapeutic Affimer® Agent by expression of the Encoded Affimer® Agent can contribute to (i) a broader therapeutic or prophylactic application of Affimer® Agents in price-sensitive conditions, (ii) an improved accessibility to therapy in both developed and developing countries, and (iii) more effective and affordable treatment modalities. In addition to *in vivo* gene transfer, cells can be harvested from the host (or a donor), engineered with Encoded Affimer® sequences to produce Affimer® Agents and re-administered to patients.

Intramuscular antibody gene administration has been most widely evaluated (reviewed in Deal et al. (2015) "Engineering humoral immunity as prophylaxis or therapy" Curr Opin Immunol. 35:113–22.), and also carries the highest clinical translatability and application when applied to Encoded Affimer® Agents. Indeed, the inherent anatomical, cellular and physiological properties of skeletal muscle make it a stable environment for long-term Encoded Affimer® Agent expression and systemic circulation. Skeletal muscle is easily accessible, allowing multiple or repeated administrations. The abundant blood vascular supply provides an efficient transport system for secreted therapeutic Affimer® Agents into the circulation. The syncytial nature of muscle fibers allows dispersal of nucleotides from a limited site of penetration to a large number of neighboring nuclei within the fiber. Skeletal muscle fibers are also terminally differentiated cells, and nuclei within the fibers are post-mitotic. Consequently, integration in the host genome is not a prerequisite to attain prolonged mAb expression. The liver is another site often used for pre-clinical antibody gene transfer, and is typically transfected via i.v. injection, and can also be a site of gene transfer for Encoded Affimer® Agents either for local delivery of Affimer® Agents (such as in the treatment of liver cancer and/or metaplasias) or for the generation of Affimer® Agents that are secreted into the vascular for systemic circulation. This organ has various physiological functions, including the synthesis of plasma proteins. This organ can be particularly well suited for *in vivo* Encoded Affimer® Agent expression.

The success of gene therapy has largely been driven by improvements in nonviral and viral gene transfer vectors. An array of physical and chemical nonviral methods have been used to transfer DNA and mRNA to mammalian cells and a substantial number of these have been developed as clinical stage technologies for gene therapy, both ex vivo and *in vivo*, and are readily adapted for delivery of the Encoded Affimer® Agents of the present disclosure. To illustrate, cationic liposome technology can be employed, which is based on the ability of amphipathic lipids, possessing a positively charged head group and a hydrophobic lipid tail, to bind to negatively charged DNA or RNA and form particles that generally enter cells by endocytosis. Some cationic liposomes also contain a neutral co-lipid, thought to enhance liposome uptake by mammalian cells. See, for example, Felgner et al. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. MNAS 84:7413–7417; San et al. (1983) "Safety and short term toxicity of a novel cationic lipid formulation for human gene therapy" Hum. Gene Ther. 4:781–788; Xu et al. (1996) "Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection" Biochemistry 35,:5616–5623; and Legendre et al. (1992) "Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes" Pharm. Res. 9, 1235-1242.

Similarly, other polycations, such as poly-l-lysine and polyethylene-imine, can be used to deliver Encoded Affimer® Agents. These polycations complex with nucleic acids via charge interaction and aid in the condensation of DNA or RNA into nanoparticles, which are then substrates for endosome-mediated uptake. Several of these cationic nucleic acid complex technologies have been developed as potential clinical products, including complexes with plasmid DNA, oligodeoxynucleotides, and various forms of synthetic RNA. Modified (and unmodified or "naked") DNA and RNA have also been shown to mediate successful gene transfer in a number of circumstances and can also be used as systems for delivery of Encoded Affimer® Agents. These include the use of plasmid DNA by direct intramuscular injection, as well as the use of intrapulmonary or intranasal injection of plasmid DNA. See, for example, Rodrigo et al. (2012) "De novo automated design of small RNA circuits for engineering synthetic riboregulation in living cells" PNAS 109:15271–15276; Oishi et al. (2005) "Smart polyion complex micelles for targeted intracellular delivery of PEGylated antisense oligonucleotides containing acid-labile linkages" Chembiochem. 6:718–725; Bhatt et al. (2015) "Microbeads mediated oral plasmid DNA delivery using polymethacrylate vectors: an effectual groundwork for colorectal cancer" Drug Deliv. 22:849–861; Ulmer et al. (1994) Protective immunity by intramuscular injection of low doses of influenza virus

DNA vaccines" Vaccine 12: 1541–1544; and Heinzerling et al. (2005) "Intratumoural injection of DNA encoding human interleukin 12 into patients with metastatic melanoma: clinical efficacy" Hum. Gene Ther. 16:35–48.

Viral vectors are currently used as a delivery vehicle in the vast majority of pre-clinical and clinical gene therapy trials and in the first to be approved directed gene therapy. See Gene Therapy Clinical Trials Worldwide 2017 (abedia.com/wiley/). The main driver thereto is their exceptional gene delivery efficiency, which reflects a natural evolutionary development; viral vector systems are attractive for gene delivery, because viruses have evolved the ability to cross through cellular membranes by infection, thereby delivering nucleic acids such as Encoded Affimer® Agents to target cells. Pioneered by adenoviral systems, the field of viral vector-mediated antibody gene transfer made significant strides in the past decades. The myriad of successfully evaluated administration routes, pre-clinical models and disease indications puts the capabilities of antibody gene transfer at full display through which the skilled artisan would readily be able to identify and adapt antibody gene transfer systems and techniques for *in vivo* delivery of Encoded Affimer® Agent constructs. Muscle has emerged as the administration site of choice for prolonged mAb expression and would similarly be a suitable target tissue for prolonged Affimer® Agent expression.

In vivo gene transfer of Encoded Affimer® Agents can also be accomplished by use of nonviral vectors, such as expression plasmids. Nonviral vectors are easily produced and do not seem to induce specific immune responses. Muscle tissue is most often used as target tissue for transfection, because muscle tissue is well vascularized and easily accessible, and myocytes are long-lived cells. Intramuscular injection of naked plasmid DNA results in transfection of a certain percentage of myocytes. Using this approach, plasmid DNA encoding cytokines and cytokine/IgG1 chimeric proteins has been introduced *in vivo* and has positively influenced (autoimmune) disease outcome.

In some instances, in order to increase transfection efficiency via so-called intravascular delivery in which increased gene delivery and expression levels are achieved by inducing a short-lived transient high pressure in the veins. Special blood-pressure cuffs that may facilitate localized uptake by temporarily increasing vascular pressure and can be adapted for use in human patients for this type of gene delivery. See, for example, Zhang et al. (2001) "Efficient expression of naked DNA delivered intraarterially to limb muscles of nonhuman primates" Hum. Gene Ther., 12:427-438.

Increased efficiency can also be gained through other techniques, such as in which delivery of the nucleic acid is improved by use of chemical carriers—cationic polymers or lipids—or via a physical approach—gene gun delivery or electroporation. See Tranchant et al. (2004) "Physicochemical optimisation of plasmid delivery by cationic lipids" J. Gene Med., 6 (Suppl. 1):S24-S35; and Niidome et al. (2002) "Gene therapy progress and prospects: nonviral vectors" Gene Ther., 9:1647-1652. Electroporation is especially regarded as an interesting technique for nonviral gene delivery. Somiari, et al. (2000) "Theory and *in vivo* application of electroporative gene delivery" Mol. Ther. 2:178-187; and Jaroszeski et al. (1999) "*In vivo* gene delivery by electroporation" Adv. Drug Delivery Rev., 35:131-137. With electroporation, pulsed electrical currents are applied to a local tissue area to enhance cell permeability, resulting in gene transfer across the membrane. Research has shown that *in vivo* gene delivery can be at least 10–100 times more efficient with electroporation than without. See, for example, Aihara et al. (1998) "Gene transfer into muscle by electroporation *in vivo*" Nat. Biotechnol. 16:867-870; Mir, et al. (1999) "High-efficiency gene transfer into skeletal muscle mediated by electric pulses" PNAS 96:4262-4267; Rizzuto, et al. (1999) "Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation" PNAS 96: 6417-6422; and Mathiesen (1999) "Electropermeabilization of skeletal muscle enhances gene transfer *in vivo*" Gene Ther., 6:508-514.

Encoded COVID (SARS-CoV-2) binding Affimer® Agents can be delivered by a wide range of gene delivery system commonly used for gene therapy including viral, non-viral, or physical. See, for example, Rosenberg et al., Science, 242:1575-1578, 1988, and Wolff et al., Proc. Natl. Acad. Sci. USA 86:9011-9014 (1989). Discussion of methods and compositions for use in gene therapy include Eck et al., in Goodman & Gilman's The Pharmacological Basis of Therapeutics, Ninth Edition, Hardman et al., eds., McGraw-Hill, New York, (1996), Chapter 5, pp. 77-101; Wilson, Clin. Exp. Immunol. 107 (Suppl. 1):31-32, 1997; Wivel et al., Hematology/Oncology Clinics of North America, Gene Therapy, S. L. Eck, ed., 12(3):483-501, 1998; Romano et al., Stem Cells, 18:19-39, 2000, and the references cited therein. U.S. Pat. No. 6,080,728 also provides a discussion of a wide variety of gene delivery methods and compositions. The routes of delivery include, for example, systemic administration and administration in situ.

An effective Encoded Affimer®Agent gene transfer approach must be directed to the specific tissues/cells where it is needed, and the resulting transgene expression should be at a level that is appropriate to the specific application. Promoters are a major cis-

acting element within the vector genome design that can dictate the overall strength of expression as well as cell-specificity.

Exemplary Ubiquitous and Cell-specific Promoters.

Promoter	Specificity	Relative Strength	Size (bps)	Reference(s)
CMV	Ubiquitous	+++	750–800	Xu et al. Gene Ther. 2001 8:1323–1332; Gray et al., Hum Gene Ther. 2011 22:1143–1153
CBA (including derivatives: CAG, CBh, etc.)	Ubiquitous	+++	248–1,600	Klein et al. Exp Neurol. 2002 176(1):66–74; Ohlfest et al. Blood. 2005 105:2691–2698; and Gray et al. Hum Gene Ther. 2011 22:1143–1153.
EF-1α	Ubiquitous	++	2,500	Gill et al. Gene Ther. 2001 8(20):1539–1546; Xu et al. Gene Ther. 2001 8:1323–1332; and Gilham et al. J Gene Med. 2010 12(2):129–136.
PGK	Ubiquitous	++	426	Gilham et al. J Gene Med. 2010 12(2):129–136.
UBC	Ubiquitous	+	403	Gill et al. Gene Ther. 2001 8(20):1539–1546; Qin et al. PLoS One. 2010 5(5):e10611.
GUSB (hGBp)	Ubiquitous	+	378	Husain et al. Gene Ther. 2009 16:927–932.
UCOE (Promoter of HNRPA2B1-CBX3)	Ubiquitous	++	600–2,500	Antoniou et al. Hum Gene Ther. 2013 24(4):363–374.
hAAT	Liver	++	347–1,500	Van Linthout et al. Hum Gene Ther. 2002 13(7):829–840; Cunningham et al. Mol Ther. 2008 16(6):1081–1088

Promoter	Specificity	Relative Strength	Size (bps)	Reference(s)
TBG	Liver	++	400	Yan et al. Gene. 2012 506(2):289–294.
Desmin	Skeletal muscle	+++	1,700	Talbot et al. Mol Ther. 2010 18:601–608.
MCK	Skeletal muscle	++	595–1,089	Talbot et al. Mol Ther. 2010 18:601–608; Wang et al. Gene Ther. 2008 15:1489–1499; Katwal et al. Gene Ther. 2013 20(9):930–938.
C5-12	Skeletal, cardiac, and diaphragm	++	312	Wang et al. Gene Ther. 2008 15:1489–1499
NSE	Neuron	+++	300–2,200	Xu et al. Gene Ther. 2001 8:1323–1332
Synapsin	Neuron	+	470	Kügler et al. Virology. 2003 311:89–95; Hioki et al. Gene Ther. 2007 14:872–882; Kuroda et al. J Gene Med. 2008 10:1163–1175.
PDGF	Neuron	+++	1,400	Patterna et al. Gene Ther. 2000 7(15):1304–1311; Hioki et al. Gene Ther. 2007 14:872–882
MecP2	Neuron	+	229	Rastegar et al. LoS One. 2009 4:e6810; Gray et al., Hum Gene Ther. 2011 22:1143–1153
CaMKII	Neuron	++	364–2,300	Hioki et al. Gene Ther. 2007 14:872–882; Kuroda et al. J Gene Med. 2008 10:1163–1175
mGluR2	Neuron	+	1,400	Brené et al. Eur J Neurosci. 2000 12:1525–1533; Kuroda et al. J Gene Med. 2008 10:1163–1175

Promoter	Specificity	Relative Strength	Size (bps)	Reference(s)
NFL	Neuron	+	650	Xu et al. Gene Ther. 2001 8:1323–1332
NFH	Neuron	+	920	Xu et al. Gene Ther. 2001 8:1323–1332
nβ2	Neuron	+	650	Xu et al. Gene Ther. 2001 8:1323–1332
PPE	Neuron	+	2700	Xu et al. Gene Ther. 2001 8:1323–1332
Enk	Neuron	+	412	Xu et al. Gene Ther. 2001 8:1323–1332
EAAT2	Neuron and astrocyte	++	966	Su et al. Proc Natl Acad Sci U S A. 2003 100:1955–1960; Kuroda et al. J Gene Med. 2008 10:1163–1175
GFAP	Astrocyte	++	681–2,200	Brenner et al. J Neurosci. 1994 14:1030–1037; Xu et al. Gene Ther. 2001 8:1323–1332; Lee et al. Glia. 2008 56:481–493; Dirren et al. Hum Gene Ther. 2014 25:109–120
MBP	Oligodendrocytes	++	1,900	Chen et al. Gene Ther. 1998 5(1):50–58

In some cases, ubiquitous expression of the Encoded Affimer® Agent construct in all cell types is desired. Constitutive promoters such as the human elongation factor 1α-subunit (EF1α), immediate-early cytomegalovirus (CMV), chicken β-actin (CBA) and its derivative CAG, the β glucuronidase (GUSB), or ubiquitin C (UBC) can be used to promote expression of the Encoded Affimer® Agent Construct in most tissues. Generally, CBA and CAG promote the larger expression among the constitutive promoters; however, their size of ~1.7 kbs in comparison to CMV (~0.8 kbs) or EF1α (~1.2 kbs) may limit use in vectors with packaging constraints such as AAV, particularly where Affimer® Agent produced by expression of the Encoded Affimer® Agent

construct is large. The GUSB or UBC promoters can provide ubiquitous gene expression with a smaller size of 378 bps and 403 bps, respectively, but they are considerably weaker than the CMV or CBA promoter. Thus, modifications to constitutive promoters in order to reduce the size without affecting its expression have been pursued and examples such as the CBh (~800 bps) and the miniCBA (~800 bps) can promote expression comparable and even higher in selected tissues (Gray et al., Hum Gene Ther. 2011 22:1143–1153).

When expression of the Encoded Affimer® Agent construct should be restricted to certain cell types within an organ, promoters can be used to mediate this specificity. For example, within the nervous system promoters have been used to restrict expression to neurons, astrocytes, or oligodendrocytes. In neurons, the neuron-specific enolase (NSE) promoter drives stronger expression than ubiquitous promoters. Additionally, the platelet-derived growth factor B-chain (PDGF-β), the synapsin (Syn), and the methyl-CpG binding protein 2 (MeCP2) promoters can drive neuron-specific expression at lower levels than NSE. In astrocytes, the 680 bps-long shortened version [gfaABC(1)D] of the glial fibrillary acidic protein (GFAP, 2.2 kbs) promoter can confer higher levels of expression with the same astrocyte-specificity as the GFAP promoter. Targeting oligodendrocytes can also be accomplished by the selection of the myelin basic protein (MBP) promoter, whose expression is restricted to this glial cell; however, its size of 1.9 kbs and low expression levels limit its use.

In the case of expressing the Encoded Affimer® Agent constructs in skeletal muscle cells, exemplary promoters based on muscle creatine kinase (MCK) and desmin (1.7 kbs) have showed a high rate of specificity (with minimal expression in the liver if desired). The promoter of the α-myosin heavy chain (α-MHC; 1.2 kbs) has shown significant cardiac specificity in comparison with other muscle promoters (Lee et al., 2011 J Cardiol. 57(1):115-22). In hematopoietic stem cells the synthetic MND promoter (Li et al., 2010 J Neurosci Methods. 189(1):56-64) and the promoter contained in the 2AUCOE (ubiquitous chromatin opening element) have shown to drive a higher transgene expression in all cell lineages when compared to the EF1α and CMV promoters, respectively (Zhang et al., 2007 Blood. 110(5):1448-57; Koldej 2013 Hum Gene Ther Clin Dev. 24(2):77-85; Dighe et al., 2014 PLoS One. 9(8):e104805.). Conversely, using promoters to restrict expression to only liver hepatocytes after vector-mediated gene transfer has been shown to reduce transgene-specific immune responses in systems where that is a risk, and to even induce immune tolerance to the expressed protein (Zhang et al., 2012 Hum Gene Ther. 23(5):460-72), which for certain

Affimer® Agents may be beneficial. The α1-antitrypsin (hAAT; 347 bps) and the thyroxine binding globulin (TBG; ~400 bps) promoters drive gene expression restricted to the liver with minimal invasion to other tissues (Yan et al., 2012 Gene. 506(2):289-94; Cunningham et al., 2008 Mol Ther. 16(6):1081-8).

In some embodiments, a mechanism to control the duration and amount of *in vivo* Encoded Affimer® Agent expression will typically be desired. There are a variety of inducible promoters which can be adapted for use with viral vectored- and plasmid DNA-based Encoded Affimer® Agent gene transfer. See Fang et al. (2007) "An antibody delivery system for regulated expression of therapeutic levels of monoclonal antibodies *in vivo*" Mol Ther. 5(6):1153–9; and Perez et al. (2004) "Regulatable systemic production of monoclonal antibodies by *in vivo* muscle electroporation" Genet Vaccines Ther. 2(1):2. An exemplary a regulatable mechanism currently under clinical evaluation is an ecdysone-based gene switch activated by a small molecule ligand. Cai et al. (2016) "Plasma pharmacokinetics of veledimex, a small-molecule activator ligand for a proprietary gene therapy promoter system, in healthy subjects" Clin Pharmacol Drug Dev. 2016.

In some embodiments of the Encoded Affimer® Agent constructs, viral post-transcriptional regulatory elements (PREs) may be used; these cis-acting elements are required for nuclear export of intronless viral RNA (Huang and Yen, 1994 J Virol. 68(5):3193-9; and 1995 Mol Cell Biol. 15(7):3864-9). Examples include HPRE (Hepatitis B Virus PRE, 533 bps) and WPRE (Woodchuck Hepatitis Virus PRE, 600 bps), which can increase the level of transgene expression by almost 10-fold in certain instances (Donello et al., 1998 J Virol. 72(6):5085-92). To further illustrate, using lentiviral and AAV vectors, WPRE was found to increase CMV promoter driven transgene expression, as well as increase PPE, PDGF and NSE promoter-driven transgene expression. Another effect of the WPRE can be to protect Encoded Affimer® Agent constructs transgenes from silencing (Paterna et al., 2000 Gene Ther. 7(15):1304-11; Xia et al., 2007 Stem Cells Dev. 2007 Feb; 16(1):167-76).

The polyadenylation of a transcribed Encoded Affimer® Agent transcript can also be important for nuclear export, translation, and mRNA stability. Therefore, in some embodiments, the Encoded Affimer® Agent construct will include a polyadenylation signal sequence. A variety of studies are available that have determined the effects of different polyA signals on gene expression and mRNA stability. Exemplary polyadenylation signal sequences include SV40 late or bovine growth hormone polyA

(bGHpA) signal sequences, as well as minimal synthetic polyA (SPA) signal (Levitt et al., 1989 Genes Dev. 3(7):1019-25; Yew et al., 1997 Hum Gene Ther. 1997 8(5):575-84). The efficiency of polyadenylation is increased by the SV40 late polyA signal upstream enhancer (USE) placed upstream of other polyA signals (Schek et al., 1992 Mol Cell Biol. 12(12):5386-93). In some embodiments, merely to illustrate, the Encoded Affimer® Agent construct will include an SV40 late + 2xUSE polyA signal.

Exemplary Polyadenylation Signals

PolyA Signal and USE	**Relative Strength**	**Size (bps)**	**Source**	**Reference(s)**
hGH	+	624	Human growth hormone	Ostedgaard et al. Proc Natl Acad Sci U S A. 2005 102(8):2952–2957
SV40 late	+++	135	Simian virus 40	Choi et al. Mol Brain. 2014 7:17
SPA (synthetic polyA)	+	49	Rabbit β-globin	Levitt et al. Genes Dev. 3(7):1019–1025; Yew et al. Hum Gene Ther. 1997 8(5):575–584; Ostedgaard et al. Proc Natl Acad Sci U S A. 2005 102(8):2952–2957; Choi et al. Mol Brain. 2014 7:17
bGH	++	250	Bovine growth hormone	Yew et al. Hum Gene Ther. 1997 8(5):575–584; Xu et al. Gene Ther. 2001 8:1323–1332; Wu et al. Mol Ther. 2008 16(2):280–289; Gray et al., Hum Gene Ther. 2011 22:1143–1153; Choi et al. Mol Brain. 2014 7:17
SV40 late 2xUSE	++	100	Simian virus 40	Schambach et al. Mol Ther. 2007 15(6):1167–1173; Choi et al. Mol Brain. 2014 7:17
HIV-1 USE	+	35	Human immunodeficiency virus 1	Schambach et al. Mol Ther. 2007 15(6):1167–1173
GHV USE	+	39	Ground squirrel hepatitis virus	Schambach et al. Mol Ther. 2007 15(6):1167–1173

PolyA Signal and USE	Relative Strength	Size (bps)	Source	Reference(s)
Adenovirus (L3) USE	+	21	Adenovirus	Schambach et al. Mol Ther. 2007 15(6):1167–1173
hTHGB USE	+	21	Human prothrombin	Schambach et al. Mol Ther. 2007 15(6):1167–1173
hC2 USE	+	53	Human C2 complement gene	Schambach et al. Mol Ther. 2007 15(6):1167–1173

In some embodiments, it may be desirable for the Encoded Affimer® Agent construct to include one or more regulatory enhancers, i.e., in addition to any promoter sequences. The CMV enhancer is upstream of the CMV promoter at –598 to –68 (Boshart et al., 1985 Cell. 41(2):521-30) (~600 bps) and contains transcription binding sites. In some embodiments, a CMV enhancer can be included in the construct to increase tissue-specific promoter-driven transgene expression, such as using the ANF (atrial natriuretic factor) promoter, the CC10 (club cell 10) promoter, SP-C (surfactant protein C) promoter, or the PDGF-β (platelet-derived growth factor-β) promoter (merely as examples). Altogether, the CMV enhancer increases transgene expression under different cell-specific promoters and different cell types making it a broadly applicable tool to increase transgene expression levels. In muscle, for example, in AAV expression systems transgene expression using the CMV enhancer with a muscle-specific promoter can increase expression levels of the protein encoded by the transgene, so would be particularly useful in the current disclosure for expressing Affimer® Agents from Encoded Affimer® Agent constructs introduced into muscle cells of a patient.

The subject Encoded Affimer® Agent constructs may also include one or more intronic sequences. The presence of an intron or intervening sequence in mRNA was first described, in vitro, to be important for mRNA processing and increased transgene expression (Huang and Gorman, 1990 Mol Cell Biol. 10(4):1805-10; Niwa et al., 1990 Genes Dev. 4(9):1552-9). The intron(s) can be placed within the coding sequence for the Affimer® Agent and/or can be placed between the promoter and transgene. A variety of introns (Table “Exemplary Introns”) placed between the promoter and transgene were compared, in mice using AAV2, for liver transgene expression (Wu et al., 2008). The MVM (minute virus of mice) intron increased transgene expression

more than any other intron tested and more than 80-fold over no intron (Wu et al., 2008). However, in cultured neurons using AAV expression cassettes, transgene expression was less under a CaMPKII promoter with a chimeric intron (human β-globin donor and immunoglobulin heavy chain acceptor) between the transgene and polyA signal compared to a WPRE (Choi et al., 2014). Together, an intron can be a valuable element to include in an expression cassette to increase transgene expression.

Exemplary Introns

Itron	**Relative Strength**	**Size (bps)**	**Source**	**Reference(s)**
MVM	+++	67–97	Minute virus of mice	Wu et al. Mol Ther. 2008 16(2):280–289
F.IX truncated intron 1	+	300	Human factor IX	Wu et al. Mol Ther. 2008 16(2):280–289; Kurachi et al. J Biol Chem. 1995 270(10):5276–5281
β-globin SD / immunoglobin heavy chain SA	+	250	Human, pZac2.1	Wu et al. Mol Ther. 2008 16(2):280–289; Choi et al. Mol Brain. 2014;7:17
Adenovirus SD# / immunoglobulin SA*	++	500	pAdβ	Wong et al. Chromosoma. 1985 92(2):124–135; Yew et al. Hum Gene Ther. 1997 8(5):575–584
SV40 late SD# / SA* (19S/16S)	+	180	pCMVβ	Yew et al. Hum Gene Ther. 1997 8(5):575–584
Hybrid adenovirus SD# / IgG SA*	+++	230	Adenovirus	Choi et al. Mol Brain. 2014;7:17; Huang et al. Mol Cell Biol. 1990 10(4):1805–1810

In the case of episomal vectors, the subject Encoded Affimer® Agent constructs may also include one or more origins of replication, minichromosome maintenance elements (MME) and/or nuclear localization elements. Episomal vectors of the disclosure comprise a portion of a virus genomic DNA that encodes an origin of replication (ori) which is required for such vectors to be self- replicating and, thus, to persist in a host cell over several generations. In addition, an episomal vector of the

disclosure also may contain one or more genes encoding viral proteins that are required for replication, i.e., replicator protein (s). Optionally, the replicator protein(s) which help initiate replication may be expressed in trans on another DNA molecule, such as on another vector or on the host genomic DNA, in the host cell containing a self-replicating episomal expression vector of this disclosure. Preferred self-replicating episomal LCR-containing expression vectors of the disclosure do not contain viral sequences that are not required for long-term stable maintenance in a eukaryotic host cell such as regions of a viral genome DNA encoding core or capsid proteins that would produce infectious viral particles or viral oncogenic sequences which may be present in the full-length viral genomic DNA molecule. The term "stable maintenance" herein, refers to the ability of a self-replicating episomal expression vector of this disclosure to persist or be maintained in non-dividing cells or in progeny cells of dividing cells in the absence of continuous selection without a significant loss (e.g., >50%) in copy number of the vector for two, three, four, or five or more generations. In some embodiments, the vectors will be maintained over 10-15 or more cell generations. In contrast, "transient" or "short-term" persistence of a plasmid in a host cell refers to the inability of a vector to replicate and segregate in a host cell in a stable manner; that is, the vector will be lost after one or two generations, or will undergo a loss of >51% of its copy number between successive generations.

Several representative self-replicating, LCR-containing, episomal vectors useful in the context of the present disclosure are described further below. The self-replicating function may alternatively be provided by one or more mammalian sequences such as described by Wohlge uth et al., 1996, Gene Therapy 3:503; Vos et al., 1995, Jour. Cell. Biol., Supp. 21A, 433; and Sun et al., 1994, Nature Genetics 8:33, optionally in combination with one or more sequence which may be required for nuclear retention. The advantage of using mammalian, especially human sequences for providing the self-replicating function is that no extraneous activation factors are required which could have toxic or oncogenic properties. It will be understood by one of skill in the art that the disclosure is not limited to any one origin of replication or any one episomal vector, but encompasses the combination of the tissue-restricted control of an LCR in an episomal vector. See also WO1998007876 “Self-replicating episomal expression vectors conferring tissue-specific gene expression” and US Patent 7790446 “Vectors, cell lines and their use in obtaining extended episomal maintenance replication of hybrid plasmids and expression of gene products”.

Epstein-Barr Virus-Based Self-Replicating Episomal Expression Vectors. The latent origin oriP from Epstein-Barr Virus (EBV) is described in Yates et. al., Proc . Natl . Acad . Sci . USA 81:3806-3810 (1984); Yates et al., Nature 313:812-815 (1985); Krysan et al., Mol . Cell . Biol . 9:1026-1033 (1989); James et al.. Gene 86: 233-239 (1990), Peterson and Legerski, Gene 107:279-284 (1991); and Pan et al., Som . Cell Molec. Genet . 18:163-177 (1992)). An EBV-based episomal vector useful according to the disclosure can contain the oriP region of EBV which is carried on a 2.61 kb fragment of EBV and the EBNA-1 gene which is carried on a 2.18 kb fragment of EBV. The EBNA-1 protein, which is the only viral gene product required to support in trans episomal replication of vectors containing oriP, may be provided on the same episomal expression vector containing oriP. It is also understood, that as with any protein such as EBNA-1 known to be required to support replication of viral plasmid in trans, the gene also may be expressed on another DNA molecule, such as a different DNA vector.

Papilloma Virus-Based, Self-Replicating, Episomal Expression Vectors. The episomal expression vectors of the disclosure also may be based on replication functions of the papilloma family of virus, including but not limited to Bovine Papilloma Virus (BPV) and Human Papilloma Viruses (HPVs) . BPV and HPVs persist as stably maintained plasmids in mammalian cells. -S trans-acting factors encoded by BPV and HPVs, namely El and E2, have also been identified which are necessary and sufficient for mediate replication in many cell types via minimal origin of replication (Ustav et al., EMBO J. 10: 449-457 (1991); Ustavet al., EMBO J . 10:4231-4329, (1991); Ustav et al., Proc . Natl . Acad . Sci . USA 90: 898-902 (1993)).

An episomal vector useful according to the disclosure is the BPV-I vector system described in Piirsoo et al., EMBO J. , 15:1 (1996) and in WO 94/12629. The BPV-1 vector system described in Piirsoo et al. comprises a plasmid harboring the BPV-1 origin of replication (minimal origin plus extrachromosomal maintenance element) and optionally the El and E2 genes. The BPV-l El and E2 genes are required for stable maintenance of a BPV episomal vector. These factors ensure that the plasmid is replicated to a stable copy number of up to thirty copies per cell independent of cell cycle status. The gene construct therefore persists stably in both dividing and non-dividing cells. This allows the maintenance of the gene construct in cells such as hemopoietic stem cells and more committed precursor cells.

The BPV origin of replication has been located at the 31 end of the upstream regulatory region within a 60 base pair (bp) DNA fragment (nucleotides (nt) 7914 - 7927) which

includes binding sites for the E1 and E2 replication factors. The minimal origin of replication of HPV has also been characterized and located in the URR fragment (nt 7022- 7927) of HPV (see, for example, Chiang et al., Proc . Natl . Acad. Sci . USA 89:5799-5803 (1992)). As used herein, "E1" refers to the protein encoded by nucleotides (nt) 849-2663 of BPV subtype 1 or by nt 832- 2779 of HPV of subtype 11, to equivalent E1 proteins of other papilloma viruses, or to functional fragments or mutants of a papilloma virus E1 protein, i.e., fragments or mutants of E1 which possess the replicating properties of E1.

As used herein, "E2H refers to the protein encoded by nt 2594-3837 of BPV subtype 1 or by nt 2723-3823 of HPV subtype 11, to equivalent E2 proteins of other papilloma viruses, or to functional fragments or mutants of a papilloma virus E2 protein, i.e., fragments or mutants of E2 which possess the replicating properties of E2. "Minichromosomal maintenance element" (MME) refers to the extrachromosomal maintenance element of the papilloma viral genome to which viral or human proteins essential for papilloma viral replication bind, which region is essential for stable episomal maintenance of the papilloma viral MO in a host cell, as described in Piirsoo et al. (supra). Preferably, the MME is a sequence containing multiple binding sites for the transcriptional activator E2. The MME in BPV is herein defined as the region of BPV located within the upstream regulatory region which includes a minimum of about six sequential E2 binding sites, and which gives optimum stable maintenance with about ten sequential E2 binding sites. E2 binding site 9 is an example sequence for this site, as described hereinbelow, wherein the sequential sites are separated by a spacer of about 4-10 nucleotides, and optimally 6 nucleotides. El and E2 can be provided to the plasmid either in cis or in trans, also as described in WO 94/12629 and in Piirsoo et al. (supra).

"E2 binding site" refers to the minimum sequence of papillomavirus double-stranded DNA to which the E2 protein binds. An E2 binding site may include the sequence 5* ACCGTTGCCGGT 3' (SEQ ID NO: 297), which is high affinity E2 binding site 9 of the BPV-1 URR; alternatively, an E2 binding site may include permutations of binding site 9, which permutations are found within the URR, and fall within the generic E2 binding sequence 5' ACCN6GGT 3' (SEQ ID NO: 298). One or more transcriptional activator E2 binding sites are, in most papillomaviruses, located in the upstream regulatory region, as in BPV and HPV. A vector which also is useful according to the disclosure may include a region of BPV between 6959 - 7945/1 - 470 on the BPV genetic map (as described in WO 94/12629) , which region includes an origin of replication, a

first promoter operatively associated with a gene of interest, the BPV El gene operatively associated with a second promoter to drive transcription of the El gene; and the BPV E2 gene operatively associated with a third promoter to drive transcription of the E2 gene.

E1 and E2 from BPV will replicate vectors containing the BPV origin or the origin of many HPV subtypes (Chiang et al., supra). E1 and E2 from HPV will replicate vectors via the BPV origin and via the origin of many HPV subtypes (Chiang et al., supra). As with all vectors of the disclosure, the BPV-based episomal expression vectors of the disclosure must persist through 2-5 or more divisions of the host cell.

See also US Patent 7790446 and Abroi et al. (2004) "Analysis of chromatin attachment and partitioning functions of bovine papillomavirus type 1 E2 protein. Journal of Virology 78:2100-13 which have shown that the BPV1 E2 protein dependent MME and EBV EBNA1 dependent FR segregation/partitioning activities function independently from replication of the plasmids. The stable-maintenance function of EBNA1/FR and E2/MME can be used to ensure long-time episomal maintenance for cellular replication origins.

Papovavirus-Based, Self-Replicating, Episomal Expression Vectors. The vectors of the disclosure also may be derived from a human papovavirus BK genomic DNA molecule. For example, the BK viral genome can be digested with restriction enzymes EcoRI and BamHI to produce a 5 kilobase (kb) fragment that contains the BK viral origin of replication sequences that can confer stable maintenance on vectors (see, for example, De Benedetti and Rhoads, Nucleic Acids Res . 19:1925 (1991), as can a 3.2 kb fragment of the BK virus (Cooper and Miron, Human Gene Therapy 4:557 (1993)).

The Encoded Affimer® Agent constructs of the present disclosure can be provided as circular or linear nucleic acids. The circular and linear nucleic acids are capable of directing expression of the Affimer® Agent coding sequence in an appropriate subject cell. The one or more nucleic acid systems for expressing an Affimer® Agent may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components.

a. Viral vectors

Exemplary viral gene therapy system that are readily adapted for use in the present disclosure include plasmid, adenovirus, adeno-associated virus (AAV), retrovirus,

lentivirus, herpes simplex virus, vaccinia virus, poxvirus, reovirus, measles virus, Semliki Forest virus, and the like. Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the nucleic acid construct carrying the nucleic acid sequences encoding the epitopes and targeting sequences of interest.

To further illustrate, encoded Affimer® Agents can be delivered *in vivo* using adenoviruses and adeno-associated (AAV) viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy.

Adenovirus Vectors

One illustrative method for *in vivo* delivery of one or more nucleic acid sequences involves the use of an adenovirus ("AdV") expression vector. AdVs are non-enveloped, double-stranded DNA viruses that neither integrate in the host genome nor replicate during cell division. AdV-mediated antibody gene transfer has shown therapeutic efficacy in a variety of different disease models advancing towards the clinic. Systemic mAb expression has mostly been pursued, via s.c. and especially i.v. and intramuscular AdV injection. See Wold et al. (2013) "Adenovirus vectors for gene therapy, vaccination and cancer gene therapy" Curr Gene Ther. 13(6):421–33; and Deal et al. "Engineering humoral immunity as prophylaxis or therapy" 2015 Curr Opin Immunol. 35:113–22. Other routes of delivery have focused on more local mAb production, such as via intranasal, intratracheal or intrapleural administration of the encoding AdV. The use of AdVs as oncolytic vectors is a popular approach particularly for generation of encoded antibodies at the site of vector incorporation. Foreign genes delivered by current adenoviral gene delivery system are episomal, and therefore, have low genotoxicity to host cells. Therefore, gene therapy using adenoviral gene delivery systems may be considerably safe. The present disclosure specifically contemplates the delivery of Affimer® Agents by expression of Encoded Affimer® Agent constructs delivered in the form of an adenoviral vector and delivery system.

Adenovirus has been usually employed as a gene delivery vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contains 100-200 bp ITRs (inverted terminal repeats), which are cis elements necessary for viral DNA replication and packaging. The E1 region (E1A and E1B) of genome encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The E2 region (E2A and E2B) encodes proteins responsible for viral DNA replication. Of adenoviral vectors

developed so far, the replication incompetent adenovirus having the deleted E1 region is usually used and represent one exemplary choice of AdV for generating the Encoded Affimer® Agent constructs of the present disclosure. The deleted E3 region in adenoviral vectors may provide an insertion site for transgenes (Thimmappaya, B. et al., Cell, 31:543-551(1982); and Riordan, J. R. et al., Science, 245:1066-1073(1989)).

An "adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express a polynucleotide that encodes a polypeptide including an Affimer® Agent such as a COVID (SARS-CoV-2) binding Affimer® Agent (the Encoded Affimer® sequence). In some embodiments, the sequence for an Encoded Affimer® Agent may be inserted into the DA promoter region. According to an exemplary embodiment, the recombinant adenovirus comprises deleted E1B and E3 region and the nucleotide sequence for an encoded Affimer® Agent is inserted into the deleted E1B and E3 region.

Adeno-Associated Virus Vectors (AAV)

AAVs (or "rAAV" for recombinant AAV) are non-enveloped small, single-stranded DNA viruses capable of infecting both dividing and non-dividing cells. Similar to AdV, AAV-based vectors remain in an episomal state in the nucleus and display a limited risk of integration. In contrast to the generally limited durability of AdV-mediated gene transfer, transgene expression can persist for years following intramuscular recombinant AAV (rAAV) vector delivery.

Alipogene tiparvovec (Glybera™), an rAAV encoding the human lipoprotein lipase gene, was approved in 2012 as the first gene therapy product in Europe. Since then, various rAAV-based gene therapy products are currently under clinical evaluation. In the context of antibody gene transfer, a variety of reports have demonstrated *in vivo* production of an anti-human immune deficiency virus (HIV) mAb in mice following intramuscular injection of the mAb-encoding rAAV. The rAAV vector's potential for combination therapy has also been demonstrated, i.e. by expressing two mAbs. Similar to AdV, intramuscular and i.v. rAAV administration have been most often pursued. Reviewed in Deal et al. "Engineering humoral immunity as prophylaxis or therapy" 2015 Curr Opin Immunol. 35:113–22. A variety of additional delivery sites have also been demonstrated to achieve more local therapeutic effects, including intracranial, intranasal, intravitreal, intrathecal, intrapleural, and intraperitoneal routes. With the utility of rAAV demonstrated for antibody gene transfer, the present disclosure also specifically contemplates the use of rAAV systems for the delivery of Encoded Affimer®

sequences *in vivo* and the production of Affimer® Agents in the body of a patient as a consequence to expression of the rAAV construct.

One important feature to AAV is that these gene transfer viruses are capable of infecting non-dividing cells and various types of cells, making them useful in constructing the Encoded Affimer® Agent delivery system of this disclosure. The detailed descriptions for use and preparation of exemplary AAV vectors are found in, for example, U.S. Pat. Nos. 5,139,941 and 4,797,368, as well as LaFace et al, Viology, 162:483486 (1988), Zhou et al., Exp. Hematol. (NY), 21:928-933 (1993), Walsh et al, J. Clin. Invest., 94:1440-1448(1994) and Flotte et al., Gene Therapy, 2:29-37(1995). AAV is a good choice of delivery vehicles due to its safety, i.e., genetically engineered (recombinant) does not integrate into the host genome. Likewise, AAV is not pathogenic and not associated with any disease. The removal of viral coding sequences minimizes immune reactions to viral gene expression, and therefore, recombinant AAV does not evoke an inflammatory response.

Typically, a recombinant AAV virus is made by co-transfecting a plasmid containing the gene of interest (i.e., the coding sequence for an Affimer® Agent) flanked by the two AAV terminal repeats (McLaughlin et al., J. Virol., 62:1963-1973(1988); Samulski et al., J. Virol., 63:3822-3828(1989)) and an expression plasmid containing the wild type AAV coding sequences without the terminal repeats (McCarty et al., J. Virol., 65:2936-2945(1991)). Typically, viral vectors containing an Encoded Affimer® Agent construct are assembled from polynucleotides encoding the Affimer® Agent containing polypeptide, suitable regulatory elements and elements necessary for expression of the encoded Affimer® Agent which mediate cell transduction. In some embodiments, adeno-associated viral (AAV) vectors are employed. In a more specific embodiment, the AAV vector is an AAV1, AAV6, or AAV8.

The AAV expression vector which harbors the Encoded Affimer® sequence bounded by AAV ITRs, can be constructed by directly inserting the selected sequence(s) into an AAV genome which has had the major AAV open reading frames ("ORFs") excised therefrom.

For eukaryotic cells, expression control sequences typically include a promoter, an enhancer, such as one derived from an immunoglobulin gene, SV40, cytomegalovirus, etc. (see above), and a polyadenylation sequence which may include splice donor and

acceptor sites. The polyadenylation sequence generally is inserted following the transgene sequences and before the 3′ ITR sequence.

Selection of these and other common vector and regulatory elements are conventional, and many such sequences are available. See, e.g., Sambrook et al., and references cited therein at, for example, pages 3.18-3.26 and 16.17-16.27 and Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1989). Of course, not all vectors and expression control sequences will function equally well to express all of the transgenes of this disclosure. However, one of skill in the art may make a selection among these expression control sequences without departing from the scope of this disclosure. Suitable promoter/enhancer sequences may be selected by one of skill in the art using the guidance provided by this application. Such selection is a routine matter and is not a limitation of the molecule or construct.

Retrovirus Vectors

Non-cytopathic viruses useful in the context of delivery of Encoded Affimer® Agent constructs include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are known to those of skill in the art.

In order to construct a retroviral vector, the Affimer® Agent coding sequence is inserted into the viral genome in the place of certain viral sequences to produce a replication-defective virus. To produce virions, a packaging cell line containing the gag, pol and env genes but without the LTR (long terminal repeat) and psi (ψ) components is constructed (Mann et al., Cell, 33:153-159(1983)). When a recombinant plasmid containing the cytokine gene, LTR and psi is introduced into this cell line, the psi sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubinstein

"Retroviral vectors," In: Vectors: A survey of molecular cloning vectors and their uses, Rodriguez and Denhardt (eds.), Stoneham: Butterworth, 494-513(1988)). The media containing the recombinant retroviruses is then collected, optionally concentrated and used for gene delivery system.

Successful gene transfer using such second-generation retroviral vectors has been reported. Kasahara et al. (Science, 266:1373-1376(1994)) prepared variants of moloney murine leukemia virus in which the EPO (erythropoietin) sequence is inserted in the place of the envelope region, consequently, producing chimeric proteins having novel binding properties. Likely, the present gene delivery system can be constructed in accordance with the construction strategies for the second-generation retroviral vector.

In some embodiments, the retrovirus is a "gammaretroviruses", which refers to a genus of the retroviridae family. Exemplary gammaretroviruses include mouse stem cell virus, murine leukemia virus, feline leukemia virus, feline sarcoma virus, and avian reticuloendotheliosis viruses.

In some embodiments, the retroviral vector for use in the present disclosure is a lentiviral vector, which refers to a genus of retroviruses that are capable of infecting dividing and non-dividing cells and typically produce high viral titers. Several examples of lentiviruses include HIV (human immunodeficiency virus: including HIV type 1, and HIV type 2); equine infectious anemia virus; feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV).

Another class of widely used retroviral vectors that can be used for the delivery and expression of an Encoded Affimer® Agent include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV) and combinations thereof (see, e.g., Buchscher et al., J. Virol. 66:2731-2739, 1992; Johann et al., J. Virol. 66: 1635-1640, 1992; Sommerfelt et al., Virol. 176:58-59, 1990; Wilson et al., J. Virol. 63:2374-2378, 1989; Miller et al., J. Virol. 65:2220-2224, 1991 ; and PCT/US94/05700).

Still other retroviral vectors that can be also be used in the present disclosure include, e.g., vectors based on human foamy virus (HFV) or other viruses in the Spumavirus genera. Foamy viruses (FVes) are the largest retroviruses known today and are widespread among different mammals, including all non-human primate species, however are absent in humans. This complete apathogenicity qualifies FV vectors as ideal gene transfer vehicles for genetic therapies in humans and clearly distinguishes

FV vectors as gene delivery system from HIV-derived and also gammaretrovirus-derived vectors.

Suitable retroviral vectors for use herein are described, for example, in U.S. Pat. Nos. 5,399,346 and 5,252,479; and in WIPO publications WO 92/07573, WO 90/06997, WO 89/05345, WO 92/05266 and WO 92/14829, which provide a description of methods for efficiently introducing nucleic acids into human cells using such retroviral vectors. Other retroviral vectors include, for example, mouse mammary tumour virus vectors (e.g., Shackleford et al., Proc. Natl. Acad. Sci. U.S.A. 85:9655-9659, 1998), lentiviruses, and the like.

Additional retroviral viral delivery systems that can be readily adapted for delivery of a transgene encoding an anti-SPIKE Affimer® Agent include, merely to illustrate Published PCT Applications WO/2010/045002, WO/2010/148203, WO/2011/126864, WO/2012/058673, WO/2014/066700, WO/2015/021077, WO/2015/148683, WO/2017/040815 - the specifications and FIGS. of each of which are incorporated by reference herein.

In some embodiments, a retroviral vector contains all of the cis-acting sequences necessary for the packaging and integration of the viral genome, i.e., (a) a long terminal repeat (LTR), or portions thereof, at each end of the vector; (b) primer binding sites for negative and positive strand DNA synthesis; and (c) a packaging signal, necessary for the incorporation of genomic RNA into virions. More detail regarding retroviral vectors can be found in Boesen, et al., 1994, Biotherapy 6:291-302; Clowes, et ai, 1994, J. Clin. Invest. 93:644-651 ; Kiem, et al., 1994, Blood 83: 1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4: 129-141 ; Miller, et al., 1993, Meth. Enzymol. 217:581-599; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3: 110-1 14.

In some embodiments, the retrovirus is a recombinant replication competent retrovirus comprising: a nucleic acid sequence encoding a retroviral GAG protein; a nucleic acid sequence encoding a retroviral POL protein; a nucleic acid sequence encoding a retroviral envelope; an oncoretroviral polynucleotide sequence comprising Long-Terminal Repeat (LTR) sequences at the 5' and 3' end of the oncoretroviral polynucleotide sequence; a cassette comprising an internal ribosome entry site (IRES) operably linked to a coding sequence for an Affime®r Agent, such as for an anti-SPIKE Affimer® Agent, wherein the cassette is positioned 5' to the U3 region of the 3' LTR

and 3' to the sequence encoding the retroviral envelope; and cis-acting sequences for reverse transcription, packaging and integration in a target cell.

In some embodiments, the retrovirus is a recombinant replication competent retrovirus comprising: a retroviral GAG protein; a retroviral POL protein; a retroviral envelope; a retroviral polynucleotide comprising Long-Terminal Repeat (LTR) sequences at the 3' end of the retroviral polynucleotide sequence, a promoter sequence at the 5' end of the retroviral polynucleotide, the promoter being suitable for expression in a mammalian cell, a gag nucleic acid domain, a pol nucleic acid domain and an env nucleic acid domain; a cassette comprising an Encoded Affimer® sequence, wherein the cassette is positioned 5' to the 3' LTR and is operably linked and 3' to the env nucleic acid domain encoding the retroviral envelope; and cis-acting sequences necessary for reverse transcription, packaging and integration in a target cell.

In some embodiments of the recombinant replication competent retrovirus, the envelope is chosen from one of amphotropic, polytropic, xenotropic, 10A1, GALV, Baboon endogenous virus, RD114, rhabdovirus, alphavirus, measles or influenza virus envelopes.

In some embodiments of the recombinant replication competent retrovirus, the retroviral polynucleotide sequence is engineered from a virus selected from the group consisting of murine leukemia virus (MLV) , Moloney murine leukemia virus (MoMLV) , Feline leukemia virus (FeLV) , Baboon endogenous retrovirus (BEV) , porcine endogenous virus (PERV) , the cat derived retrovirus RD114, squirrel monkey retrovirus, Xenotropic murine leukemia virus-related virus (XMRV) , avian reticuloendotheliosis virus (REV) , or Gibbon ape leukemia virus (GALV).

In some embodiments of the recombinant replication competent retrovirus, retrovirus is a gammaretrovirus.

In some embodiments of the recombinant replication competent retrovirus, there is a second cassette comprising a coding sequence for a second therapeutic protein, such as another checkpoint inhibitor polypeptide, a co-stimulatory polypeptide and/or a immunostimulatory cytokine (merely as examples), e.g., downstream of the cassette. In certain instances, the second cassette can include an internal ribosome entry site (IRES) or a minipromoter or a polIII promoter operably linked to the coding sequence for the second therapeutic protein.

In some embodiments of the recombinant replication competent retrovirus, it is a nonlytic, amphotropic retroviral replicating vector which, preferably, selectively infects and replicates in the cells of mucosal tissues such as of the sinus or gastrointestinal tract, of in pulmonary tissue such as in the lung microenvironment.

Other Viral Vectors as Expression Constructs

Other viral vectors may be employed as a gene delivery system in the present disclosure. Vectors derived from viruses such as vaccinia virus (Puhlmann M. et al., Human Gene Therapy, 10:649-657(1999); Ridgeway, "Mammalian expression vectors," In: Vectors: A survey of molecular cloning vectors and their uses. Rodriguez and Denhardt, eds. Stoneham: Butterworth, 467-492(1988); Baichwal and Sugden, "Vectors for gene transfer derived from animal DNA viruses: Transient and stable expression of transferred genes," In: Kucherlapati R, ed. Gene transfer. New York: Plenum Press, 117-148(1986) and Coupar et al., Gene, 68:1-10(1988)), lentivirus (Wang G. et al., J. Clin. Invest., 104(11):R55-62(1999)), herpes simplex virus (Chamber R., et al., Proc. Natl. Acad. Sci USA, 92:1411-1415(1995)), poxvirus (GCE, NJL, Krupa M, Esteban M., The poxvirus vectors MVA and NYVAC as gene delivery systems for vaccination against infectious diseases and cancer Curr Gene Ther 8(2):97-120(2008)), reovirus, measles virus, Semliki Forest virus, and polioviruses may be used in the present delivery systems for transferring the gene of interest into cells. They offer several attractive features for various mammalian cells. Also included are hepatitis B viruses.

b. Non-Viral Vectors

In 1990, Wolff et al. showed how injection of naked plasmid DNA (pDNA) into the skeletal muscle of mice led to the local expression of the encoded protein, kick-starting the field of DNA-based therapeutics. See Wolff et al. 1990 “Direct gene transfer into mouse muscle *in vivo*” Science. 247(4949 Pt 1):1465–8. The use of “pDNA” for delivering Encoded Affimer® Agents of the present disclosure waives the need for a virus as biological vector, and presents an appealing platform for Encoded Affimer® Agent gene transfer. Compared to viral vectors, pDNA is considered low-immunogenic (allowing e.g. repeated dosing), is cheaper to produce, ship, and store, and has a much longer shelf-life. After entry in the nucleus, pDNA remains in a non-replicating non-integrating episomal state, and is lost during the breakdown of the nuclear envelope at mitosis. pDNA has no defined restrictions regarding the size of the transgene compared to viral vectors, and its modular nature allows for straightforward molecular cloning, making them easy to manipulate and design for therapeutic use. Hardee et al. 2017

"Advances in non-viral DNA vectors for gene therapy" Genes. 8(2):65. Plasmids are used in about 17% of the ongoing or completed gene therapy clinical trials, and showed to be well-tolerated and safe.

The method of DNA administration can greatly impact transgene expression. *In vivo* DNA-mediated Encoded Affimer® Agent gene transfer can utilize such physical methods of transfection used for antibody gene transfer, such as electroporation or hydrodynamic injection. Electroporation presents the propagation of electrical fields within tissues, which induces a transient increase in cell membrane permeability. Electrotransfer of DNA is a multistep process, involving (i) electrophoretic migration of DNA towards the plasma membrane, (ii) DNA accumulation and interaction with the plasma membrane, and (iii) intracellular trafficking of the DNA to the nucleus, after which gene expression can commence. Heller LC. 2015 "Gene electrotransfer clinical trials" Adv Genet. 89:235–62. Intramuscular, intrapulomonary, intramucosal tissue and intradermal administration have been evaluated in clinical trials and are also suitable target tissues for electroporation of Encoded Affimer® Agent constructs.

Hydrodynamic-based transfection utilizes the i.v. injection of high volumes of pDNA, driving DNA molecules out of the blood circulation and into tissue. Other potentially less invasive physical delivery methods include sonoporation and magnetofection. DNA uptake can also be improved by complexing the molecules with chemical delivery vehicles (e.g. cationic lipids or polymers and lipid nanoparticles). Such techniques can also be applied to *in vivo* DNA-mediated Encoded Affimer® Agent gene transfer.

In addition to the choice of delivery method, Encoded Affimer® Agent transgene expression can be improved by modifying the make-up of pDNA constructs. See, for example, Hardee et al. 2017 "Advances in non-viral DNA vectors for gene therapy" Genes 8(2):65; and Simcikova et al. 2015 "Towards effective non-viral gene delivery vector" Biotechnol Genet Eng Rev. 31(1–2):82–107. Conventional pDNA consists of a transcription unit and bacterial backbone. The transcription unit carries the Encoded Affimer® sequence along with regulatory elements. The bacterial backbone includes elements like an antibiotic resistance gene, an origin of replication, unmethylated CpG motifs, and potentially cryptic expression signals. Some of these sequences are required for the production of plasmid DNA. However, in general, for therapeutic Encoded Affimer® Agent gene therapy the presence of a bacterial backbone will likely be counterproductive. However, there are a variety of different types of available minimal vectors that can be selected, including minicircle DNA (mcDNA) which already been

used for antibody gene transfer and can be readily adapted for Encoded Affimer® Agent gene transfer. Minicircles are plasmid molecules devoid of bacterial sequences, generated via a process of recombination, restriction and/or purification. Simcikova et al. 2015 *supra*. Elimination of the bacterial backbone has shown higher transfection efficiency and prolonged transgene expression in a variety of tissues.

Also provided herein is a linear nucleic acid, or linear expression cassette ("LEC"), that is capable of being efficiently delivered to a subject via electroporation and expressing the Encoded Affimer® sequence included therein. The LEC may be any linear DNA devoid of any phosphate backbone. The LEC may contain a promoter, an intron, a stop codon, and/or a polyadenylation signal. The expression of the Encoded Affimer® coding sequence may be controlled by the promoter.

Plasmid Vectors

In some embodiments, the subject Encoded Affimer® Agents constructs are delivered as plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g. Sambrook et al., 1989, cited above. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells *in vivo*. They are particularly advantageous for this because they reduced safety concerns relative to other vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide epitope encoded by nucleic acid within the plasmid. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

To expand the application and efficiency of using plasmid DNA to deliver an Encoded Affimer® Agent construct to tissue *in vivo*, different approaches can be pursued based on principles producing higher mAb expression or overall efficacy in prior art reports. A first strategy simply relies on giving multiple or repeated pDNA doses. Kitaguchi et al. 2005 “Immune deficiency enhances expression of recombinant human antibody in

mice after nonviral *in vivo* gene transfer" Int J Mol Med 16(4):683–8; and Yamazaki et al. 2011 "Passive immune-prophylaxis against influenza virus infection by the expression of neutralizing anti-hemagglutinin monoclonal antibodies from plasmids" Jpn J Infect Dis. 64(1):40–9. Another approach relates to the use of a delivery adjuvant. pDNA electrotransfer can be enhanced by pre-treating the muscle with hyaluronidase, an enzyme that transiently breaks down hyaluronic acid, decreasing the viscosity of the extracellular matrix and facilitating DNA diffusion. Yamazaki et al. 2011, supra; and McMahon et al. 2001 "Optimisation of electrotransfer of plasmid into skeletal muscle by pretreatment with hyaluronidase: increased expression with reduced muscle damage" Gene Ther. 8(16):1264–70. For antibody gene transfer, this led to an increase in mAb expression by approximately 3.5-fold, achieving plasma peak titers of 3.5 μg/ml with 30 μg pDNA, and can be adapted by one skilled in the art for Encoded Affimer® Agent gene transfer. Still another strategy focuses on antibody or cassette engineering. Following codon-, RNA- and leader sequence-optimization, peak serum mAb or Fab titers have been attained with intramuscular electrotransfer of 'optimized' pDNA. See, for example, Flingai et al. 2015 "Protection against dengue disease by synthetic nucleic acid antibody prophylaxis/immunotherapy" Sci Rep. 5:12616.

The purpose of the plasmid is the efficient delivery of nucleic acid sequences to and expression of therapeutic Affimer® Agents in a cell or tissue. In particular, the purpose of the plasmid may be to achieve high copy number, avoid potential causes of plasmid instability and provide a means for plasmid selection. As for expression, the nucleic acid cassette contains the necessary elements for expression of the Encoded Affimer® Agent within the cassette. Expression includes the efficient transcription of an inserted gene, nucleic acid sequence, or nucleic acid cassette with the plasmid. Thus, in some aspects, a plasmid is provided for expression of Encoded Affimer® Agent construct which includes an expression cassette comprising the coding sequence for the Affimer Agent®; also referred to as a transcription unit. When a plasmid is placed in an environment suitable for epitope expression, the transcriptional unit will express the Affimer® Agent and anything else encoded in the construct. The transcription unit includes a transcriptional control sequence, which is transcriptionally linked with a cellular immune response element coding sequence. Transcriptional control sequence may include promoter/enhancer sequences such as cytomegalovirus (CMV) promoter/enhancer sequences, such as described above. However, those skilled in the art will recognize that a variety of other promoter sequences suitable for expression in mammalian cells, including human patient cells, are known and can similarly be used in the constructs disclosed herein. The level of expression of the Affimer® Agent will

depend on the associated promoter and the presence and activation of an associated enhancer element.

In some embodiments, the Encoded Affimer® sequence (encoding the desired Affimer® Agent) can be cloned into an expression plasmid which contains the regulatory elements for transcription, translation, RNA stability and replication (i.e., including a transcriptional control sequence). Such expression plasmids are well known in the art and one of ordinary skill would be capable of designing an appropriate expression construct for producing a recombinant Affimer® Agent *in vivo*.

Minicircle

Minicircle (mcDNA) -based antibody gene transfer can also be adapted for delivery of Encoded Affimer® Agents to tissues *in vivo*. Under certain circumstances, plasmid DNA used for non-viral gene delivery can cause unacceptable inflammatory responses. Where this happens, immunotoxic responses are largely due to the presence of unmethylated CpG motifs and their associated stimulatory sequences on plasmids following bacterial propagation of plasmid DNA. Simple methylation of DNA in vitro may be enough to reduce an inflammatory response, but can result in reduced gene expression. The removal of CpG islands by cloning out, or elimination of non-essential sequences has been a successful technique for reducing inflammatory responses. Yew et al. 2000 “Reduced inflammatory response to plasmid DNA vectors by elimination and inhibition of immunostimulatory CpG motifs” Mol Ther 1(3), 255-62.

Since bacterial DNA contains on average 4 times more CpG islands than mammalian DNA, a good solution is to eliminate entirely the bacterial control regions, such as the origin of replication and antibiotic resistance genes, from gene delivery vectors during the process of plasmid production. Thus, the "parent" plasmid is recombined into a "minicircle" which generally comprises the gene to be delivered (in this case, the Encoded Affimer® coding sequence) and suitable control regions for its expression, and a miniplasmid which generally comprises the remainder of the parent plasmid.

Removal of bacterial sequences needs to be efficient, using the smallest possible excision site, whilst creating supercoiled DNA minicircles which consist solely of gene expression elements under appropriate--preferably mammalian--control regions. Some techniques for minicircle production use bacterial phage lambda (λ) integrase mediated recombination to produce minicircle DNA. See, for example, Darquet, et al. 1997 Gene

Ther 4(12): 1341-9; Darquet et al. 1999 Gene Ther 6(2): 209-18; and Kreiss, et al. 1998 Appl Micbiol Biotechnol 49(5):560-7).

Therefore, embodiments of nucleic acid constructs described herein may be processed in the form of minicircle DNA. Minicircle DNA pertains to small (2-4 kb) circular plasmid derivatives that have been freed from all prokaryotic vector parts. Since minicircle DNA vectors contain no bacterial DNA sequences, they are less likely to be perceived as foreign and destroyed. As a result, these vectors can be expressed for longer periods of time compared to certain conventional plasmids. The smaller size of minicircles also extends their cloning capacity and facilitates their delivery into cells. Kits for producing minicircle DNA are known in the art and are commercially available (System Biosciences, Inc., Palo Alto, Calif.). Information on minicircle DNA is provided in Dietz et al., Vector Engineering and Delivery Molecular Therapy (2013); 21 8, 1526-1535 and Hou et al., Molecular Therapy—Methods & Clinical Development, Article number: 14062 (2015) doi:10.1038/mtm.2014.62. More information on Minicircles is provided in Chen Z Y, He C Y, Ehrhardt A, Kay M A. Mol Ther. 2003 September; 8(3):495-500 and Minicircle DNA vectors achieve sustained expression reflected by active chromatin and transcriptional level. Gracey Maniar L E, Maniar J M, Chen Z Y, Lu J, Fire A Z, Kay M A. Mol Ther. 2013 January; 21(1):131-8.

As a nonlimiting example, a minicircle DNA vector may be produced as follows. An expression cassette, which comprises the Encoded Affimer® coding sequence along with regulatory elements for its expression, is flanked by attachment sites for a recombinase. A sequence encoding the recombinase is located outside of the expression cassette and includes elements for inducible expression (such as, for example, an inducible promoter). Upon induction of recombinase expression, the vector DNA is recombined, resulting in two distinct circular DNA molecules. One of the circular DNA molecules is relatively small, forming a minicircle that comprises the expression cassette for the Encoded Affimer® Agent; this minicircle DNA vector is devoid of any bacterial DNA sequences. The second circular DNA sequence contains the remaining vector sequence, including the bacterial sequences and the sequence encoding the recombinase. The minicircle DNA containing the Encoded Affimer® sequence can then be separately isolated and purified. In some embodiments, a minicircle DNA vector may be produced using plasmids similar to pBAD.ϕ.C31.hFIX and pBAD.ϕ.C31.RHB. See, e.g., Chen et al. (2003) Mol. Ther. 8:495-500.

Exemplary recombinases that may be used for creating a minicircle DNA vector include, but are not limited to, Streptomyces bacteriophage ϕ31 integrase, Cre recombinase, and the λ integrase/DNA topoisomerase IV complex. Each of these recombinases catalyzes recombination between distinct sites. For example, ϕ31 integrase catalyzes recombination between corresponding attP and attB sites, Cre recombinase catalyzes recombination between loxP sites, and the λ integrase/DNA topoisomerase IV complex catalyzes recombination between bacteriophage λ attP and attB sites. In some embodiments, such as, for example, with ϕ31 integrase or with λ integrase in the absence of the λ is protein, the recombinase mediates an irreversible reaction to yield a unique population of circular products and thus high yields. In other embodiments, such as, for example, with Cre recombinase or with λ integrase in the presence of the λ protein, the recombinase mediates a reversible reaction to yield a mixture of circular products and thus lower yields. The reversible reaction by Cre recombinase can be manipulated by employing mutant loxP71 and loxP66 sites, which recombine with high efficiency to yield a functionally impaired P71/66 site on the minicircle molecule and a wild-type loxP site on the minicircle molecule, thereby shifting the equilibrium towards the production of the minicircle DNA product.

Published US Application 20170342424 also describes a system making use of a parent plasmid which is exposed to an enzyme which causes recombination at recombination sites, thereby forming a (i) minicircle including the Encoded Affimer® sequence and (ii) a miniplasmid comprising the remainder of the parent plasmid. One recombination site is modified at the 5' end such that its reaction with the enzyme is less efficient than the wild type site, and the other recombination site is modified at the 3' end such that its reaction with the enzyme is less efficient than the wild type site, and the other recombination site is modified at the 3' end such that its reaction with the enzyme is less efficient than the wild type site, both modified sites being located in the minicircle after recombination. This favors the formation of minicircle.

c. RNA-mediated Encoded Affimer® Agent Gene Transfer

Exemplary nucleic acids or polynucleotides for the encoded anti-SPIKE Affimer® Agents of the present disclosure include, but are not limited to, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β- D-ribo configuration, a-LNA having an a-L-ribo configuration (a diastereomer of LNA), 2'-amino-LNA having a 2 '-amino functionalization, and 2'-

amino- a-LNA having a 2'-amino functionalization), ethylene nucleic acids (ENA), cyclohexenyl nucleic acids (CeNA) or hybrids or combinations thereof.

mRNA presents an emerging platform for antibody gene transfer that can be adapted by those skilled in the art for delivery of Encoded Affimer® Agent constructs of the present disclosure. Although current results differ considerably, in certain instances the mRNA constructs appear to be able to rival viral vectors in terms of generated serum mAb titers. Levels were in therapeutically relevant ranges within hours after mRNA administration, a marked shift in speed compared to DNA. The use of lipid nanoparticles (LNP) for mRNA transfection, rather than the physical methods typically required for DNA, can provide significant advantages in some embodiments towards application range.

In their 1990 study, Wolff et al. (1990, *supra*) found that, in addition to pDNA, intramuscular injection of in vitro transcribed (IVT) mRNA also led to local expression of the encoded protein. mRNA was not pursued as actively as DNA at that time because of its low stability. Progress over the past years allowed mRNA to catch up with DNA and viral vectors as a tool for gene transfer. Reviewed in Sahin et al. (2014) "mRNA-based therapeutics: developing a new class of drugs" Nat Rev Drug Discov. 13(10):759–80. Conceptually, there are several differences with these expression platforms. mRNA does not need to enter into the nucleus to be functional. Once it reaches the cytoplasm, mRNA is translated instantly. mRNA-based therapeutics are expressed more transiently compared to DNA- or viral vector-mediated gene transfer, and do not pose the risk of insertional mutagenesis in the host genome. mRNA production is relatively simple and inexpensive. In terms of administration, mRNA uptake can be enhanced using electroporation. Broderick et al. 2017 "Enhanced delivery of DNA or RNA vaccines by electroporation" Methods Mol Biol. 2017;1499:193–200. Most focus, however, has gone to non-physical transfection methods. Indeed, a variety of mRNA complexing formulations have been developed, including lipid nanoparticles (LNP), which have proven to be safe and very efficient mRNA carriers for administration in a variety of tissues and i.v. Pardi et al. 2015 "Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes" J Control Release 217:345–51. In line with this progress, IVT mRNA has reached the stage of clinical evaluation.

Beissert et al. WO2017162266 "RNA Replicon for Versatile and Efficient Gene Expression" describes agents and methods suitable for efficient expression of Affimer®

Agents of the present disclosure, such as suitable for antiviral treatment for the prevention and therapy of viral infections. For instance, the Affimer® Agent coding sequence can be provided as an RNA replicon comprising a 5' replication recognition sequence such as from an alphavirus 5' replication recognition sequence. In some embodiments, the RNA replicon comprises a (modified) 5' replication recognition sequence and an open reading frame encoding the Affimer® Agent, in particular located downstream from the 5' replication recognition sequence such as that the 5' replication recognition sequence and the open reading frame do not overlap, e.g. the 5' replication recognition sequence does not contain a functional initiation codon and in some embodiments does not contain any initiation codon. Most preferably, the initiation codon of the open reading frame encoding the Affimer® Agent is in the 5'→ 3' direction of the RNA replicon.

In some embodiments, to prevent immune activation, modified nucleosides can be incorporated into the in vitro–transcribed mRNA. In some embodiments, the IVT RNA can be 5' capped, such an m7G5′ppp5′G2´-O-Met-capped IVT. Efficient translation of the modified mRNA can be ensured by removing double-stranded RNA. Moreover, the 5′ and 3′ UTRs and the poly(A) tail can be optimized for improved intracellular stability and translational efficiency. See, for example, Stadler et al. (2017) Nature Medicine 23:815–817 and Kariko et al. WO/2017/036889 "Method for Reducing Immunogenicity of RNA".

In some embodiments, the mRNA that encodes the anti-SPIKE Affimer® Agent may include at least one chemical modification described herein. As a non-limiting example, the chemical modification may be 1 -methylpseudouridine, 5-methylcytosine or 1 -methylpseudouridine and 5-methylcytosine. In some embodiments, linear polynucleotides encoding one or more anti-SPIKE Affimer® Agents of the present disclosure which are made using only in vitro transcription (IVT) enzymatic synthesis methods are referred to as "IVT polynucleotides." Methods of making IVT polynucleotides are known in the art and are described in PCT Application WO2013/151666, the contents of which are incorporated herein by reference in their entirety.

In another embodiment, the polynucleotides that encode the anti-SPIKE Affimer® Agent of the present disclosure have portions or regions which differ in size and/or chemical modification pattern, chemical modification position, chemical modification percent or chemical modification population and combinations of the foregoing are

known as "chimeric polynucleotides." A "chimera" according to the present disclosure is an entity having two or more incongruous or heterogeneous parts or regions. As used herein a "part" or "region" of a polynucleotide is defined as any portion of the polynucleotide which is less than the entire length of the polynucleotide. Such constructs are taught in for example PCT Application WO2015/034928.

In yet another embodiment, the polynucleotides of the present disclosure that are circular are known as "circular polynucleotides" or "circP." As used herein, "circular polynucleotides" or "circP" means a single stranded circular polynucleotide which acts substantially like, and has the properties of, an RNA. The term "circular" is also meant to encompass any secondary or tertiary configuration of the circP. Such constructs are taught in for example PCT Application WO2015/034925 and WO2015/034928, the contents of each of which are incorporated herein by reference in their entirety.

Exemplary mRNA (and other polynucleotides) that can be used to encode anti-SPIKE Affimer® Agents of the present disclosure include those which can be adapted from the specifications and FIGS. of, for example, PCT Publications WO2017/049275, WO2016/118724, WO2016/118725, WO2016/011226, WO2015/196128, WO/2015/196130, WO/2015/196118, WO/2015/089511, with WO2015/105926 (the later titled “Polynucleotides for the *In vivo* Production Of Antibodies”), each of which is incorporated by reference herein.

Electroporation, as described below, is one exemplary method for introducing mRNA or other polynucleotides into a cell.

Lipid-containing nanoparticle compositions have proven effective as transport vehicles into cells and/or intracellular compartments for a variety of RNAs (and related polynucleotides described herein). These compositions generally include one or more "cationic" and/or ionizable lipids, phospholipids including polyunsaturated lipids, structural lipids (e.g., sterols), and lipids containing polyethylene glycol (PEG lipids). Cationic and/or ionizable lipids include, for example, amine-containing lipids that can be readily protonated.

d. Delivery of Encoded Affimer® Agent Constructs into Target Cells

The introduction into host cell of the gene delivery system can be performed through various methods known to those skilled in the art.

Where the present gene delivery system is constructed on the basis of viral vector construction, delivery can be performed as conventional infection methods known in the art.

Physical methods to enhance delivery both viral and non-viral Encoded Affimer® Agent constructs include electroporation (Neumann, E. et al., EMBO J., 1:841(1982); and Tur-Kaspa et al., Mol. Cell Biol., 6:716-718(1986)), gene bombardment (Yang et al., Proc. Natl. Acad. Sci., 87:9568-9572 (1990) where DNA is loaded onto (e.g., gold) particles and forced to achieve penetration of the DNA into the cells, sonoporation, magnetofection, hydrodynamic delivery and the like, all of which are known to those of skill in the art.

Electroporation

In the past several years, there has been a great advance in the plasmid DNA delivery technology that is utilized for *in vivo* production of proteins. This included codon optimization for expression in human cells, RNA optimization to improve mRNA stability as well as more efficient translation at the ribosomal level, the addition of specific leader sequences to enhance translation efficiency, the creation of synthetic inserts to further enhance production *in vivo* and the use of improved adaptive electroporation (EP) delivery protocols to improve *in vivo* delivery. EP assists in the delivery of plasmid DNA by generating an electrical field that allows the DNA to pass into the cell more efficiently. *In vivo* electroporation is a gene delivery technique that has been used successfully for efficient delivery of plasmid DNA to many different tissues. Kim et al. "Gene therapy using plasmid DNA-encoded anti-HER2 antibody for cancers that overexpress HER2" (2016) Cancer Gene Ther. 23(10): 341–347 teaches a vector and electroporation system for intramuscular injection and *in vivo* electroporation of the plasmids that results in high and sustained antibody expression in sera; the plasmid and electroporation system of Kim et al. can be readily adapted for the *in vivo* delivery of a plasmid for expressing an encoded COVID (SARS-CoV-2) binding Affimer® Agent of the present disclosure.

Accordingly, in certain some embodiments of the present disclosure, the Encoded Affimer® Agent construct is introduced into target cells via electroporation.

Administration of the composition via electroporation may be accomplished using electroporation devices that can be configured to deliver to a desired tissue of a mammal, a pulse of energy effective to cause reversible pores to form in cell

membranes, and preferable the pulse of energy is a constant current similar to a preset current input by a user. The electroporation device may comprise an electroporation component and an electrode assembly or handle assembly. The electroporation component may include and incorporate one or more of the various elements of the electroporation devices, including: controller, current waveform generator, impedance tester, waveform logger, input element, status reporting element, communication port, memory component, power source, and power switch. The electroporation may be accomplished using an *in vivo* electroporation device, for example CELLECTRA EP system (VGX Pharmaceuticals, Blue Bell, Pa.) or Elgen electroporator (Genetronics, San Diego, Calif.) to facilitate transfection of cells by the plasmid.

The electroporation component may function as one element of the electroporation devices, and the other elements are separate elements (or components) in communication with the electroporation component. The electroporation component may function as more than one element of the electroporation devices, which may be in communication with still other elements of the electroporation devices separate from the electroporation component. The elements of the electroporation devices existing as parts of one electromechanical or mechanical device may not limited as the elements can function as one device or as separate elements in communication with one another. The electroporation component may be capable of delivering the pulse of energy that produces the constant current in the desired tissue, and includes a feedback mechanism. The electrode assembly may include an electrode array having a plurality of electrodes in a spatial arrangement, wherein the electrode assembly receives the pulse of energy from the electroporation component and delivers same to the desired tissue through the electrodes. At least one of the plurality of electrodes is neutral during delivery of the pulse of energy and measures impedance in the desired tissue and communicates the impedance to the electroporation component. The feedback mechanism may receive the measured impedance and can adjust the pulse of energy delivered by the electroporation component to maintain the constant current.

A plurality of electrodes may deliver the pulse of energy in a decentralized pattern. The plurality of electrodes may deliver the pulse of energy in the decentralized pattern through the control of the electrodes under a programmed sequence, and the programmed sequence is input by a user to the electroporation component. The programmed sequence may comprise a plurality of pulses delivered in sequence, wherein each pulse of the plurality of pulses is delivered by at least two active electrodes with one neutral electrode that measures impedance, and wherein a

subsequent pulse of the plurality of pulses is delivered by a different one of at least two active electrodes with one neutral electrode that measures impedance.

The feedback mechanism may be performed by either hardware or software. The feedback mechanism may be performed by an analog closed-loop circuit. The feedback occurs every 50 μs, 20 μs, 10 μs or 1 μs, but in some embodiments is a real-time feedback or instantaneous (i.e., substantially instantaneous as determined by available techniques for determining response time). The neutral electrode may measure the impedance in the desired tissue and communicates the impedance to the feedback mechanism, and the feedback mechanism responds to the impedance and adjusts the pulse of energy to maintain the constant current at a value similar to the preset current. The feedback mechanism may maintain the constant current continuously and instantaneously during the delivery of the pulse of energy.

Examples of electroporation devices and electroporation methods that may facilitate delivery of the Encoded Affimer® Agent constructs of the present disclosure, include those described in U.S. Pat. Nos. 7,245,963; 6,302,874; 5,676,646; 6,241,701; 6,233,482; 6,216,034; 6,208,893; 6,192,270; 6,181,964; 6,150,148; 6,120,493; 6,096,020; 6,068,650; and 5,702,359, the contents of which are incorporated herein by reference in their entirety. The electroporation may be carried out via a minimally invasive device.

In some embodiments, the electroporation is carried using a minimally invasive electroporation device ("MID"). The device may comprise a hollow needle, DNA cassette, and fluid delivery means, wherein the device is adapted to actuate the fluid delivery means in use so as to concurrently (for example, automatically) inject the Encoded Affimer® Agent nucleic acid construct into body tissue during insertion of the needle into the body tissue. This has the advantage that the ability to inject the DNA and associated fluid gradually while the needle is being inserted leads to a more even distribution of the fluid through the body tissue. The pain experienced during injection may be reduced due to the distribution of the DNA being injected over a larger area.

The MID may inject the Encoded Affimer® Agent nucleic acid construct into tissue without the use of a needle. The MID may inject the Encoded Affimer® Agent nucleic acid construct as a small stream or jet with such force that the nucleic acid pierces the surface of the tissue and enters the underlying tissue and/or muscle. The force behind the small stream or jet may be provided by expansion of a compressed gas, such as

carbon dioxide through a micro-orifice within a fraction of a second. Examples of minimally invasive electroporation devices, and methods of using them, are described in published U.S. Patent Application No. 20080234655; U.S. Pat. No. 6,520,950; U.S. Pat. No. 7,171,264; U.S. Pat. No. 6,208,893; U.S. Pat. No. 6,009,347; U.S. Pat. No. 6,120,493; U.S. Pat. No. 7,245,963; U.S. Pat. No. 7,328,064; and U.S. Pat. No. 6,763,264, the contents of each of which are herein incorporated by reference.

The MID may comprise an injector that creates a high-speed jet of liquid that painlessly pierces the tissue. Such needle-free injectors are commercially available. Examples of needle-free injectors that can be utilized herein include those described in U.S. Pat. Nos. 3,805,783; 4,447,223; 5,505,697; and 4,342,310, the contents of each of which are herein incorporated by reference.

A desired Encoded Affimer® Agent nucleic acid construct in a form suitable for direct or indirect electrotransport may be introduced (e.g., injected) using a needle-free injector into the tissue to be treated, usually by contacting the tissue surface with the injector so as to actuate delivery of a jet of the agent, with sufficient force to cause penetration of the nucleic acid into the tissue. For example, if the tissue to be treated is a mucosa, skin or muscle, the agent is projected towards the mucosal or skin surface with sufficient force to cause the agent to penetrate through the stratum corneum and into dermal layers, or into underlying tissue and muscle, respectively. Needle-free injectors are well suited to deliver Encoded Affimer® Agent nucleic acid construct to all types of tissues, including into gastrointestinal, nasal and lung.

The MID may have needle electrodes that electroporate the tissue. By pulsing between multiple pairs of electrodes in a multiple electrode array, for example set up in rectangular or square patterns, provides improved results over that of pulsing between a pair of electrodes. Disclosed, for example, in U.S. Pat. No. 5,702,359 entitled "Needle Electrodes for Mediated Delivery of Drugs and Genes" is an array of needles wherein a plurality of pairs of needles may be pulsed during the therapeutic treatment. In that application, which is incorporated herein by reference as though fully set forth, needles were disposed in a circular array, but have connectors and switching apparatus enabling a pulsing between opposing pairs of needle electrodes. A pair of needle electrodes for delivering the Encoded Affimer® Agent nucleic acid construct to cells may be used. Such a device and system is described in U.S. Pat. No. 6,763,264, the contents of which are herein incorporated by reference. Alternatively, a single needle device may be used that allows injection of the DNA and electroporation with a single

needle resembling a normal injection needle and applies pulses of lower voltage than those delivered by presently used devices, thus reducing the electrical sensation experienced by the patient.

The MID may comprise one or more electrode arrays. The arrays may comprise two or more needles of the same diameter or different diameters. The needles may be evenly or unevenly spaced apart. The needles may be between 0.005 inches and 0.03 inches, between 0.01 inches and 0.025 inches; or between 0.015 inches and 0.020 inches. The needle may be 0.0175 inches in diameter. The needles may be 0.5 mm, 1.0 mm, 1.5 mm, 2.0 mm, 2.5 mm, 3.0 mm, 3.5 mm, 4.0 mm, or more spaced apart.

The MID may consist of a pulse generator and a two or more-needle vaccine injectors that deliver the Encoded Affimer® Agent nucleic acid construct and electroporation pulses in a single step. The pulse generator may allow for flexible programming of pulse and injection parameters via a flash card operated personal computer, as well as comprehensive recording and storage of electroporation and patient data. The pulse generator may deliver a variety of volt pulses during short periods of time. For example, the pulse generator may deliver three 15 volt pulses of 100 ms in duration. An example of such a MID is the Elgen 1000 system by Inovio Biomedical Corporation, which is described in U.S. Pat. No. 7,328,064, the contents of which are herein incorporated by reference.

The MID may be a CELLECTRA (Inovio Pharmaceuticals, Plymouth Meeting, Pa.) device and system, which is a modular electrode system, that facilitates the introduction of a macromolecule, such as an Encoded Affimer® Agent nucleic acid construct, into cells of a selected tissue in a body. The modular electrode system may comprise a plurality of needle electrodes; a hypodermic needle; an electrical connector that provides a conductive link from a programmable constant-current pulse controller to the plurality of needle electrodes; and a power source. An operator can grasp the plurality of needle electrodes that are mounted on a support structure and firmly insert them into the selected tissue in a body or plant. The nucleic acid is then delivered via the hypodermic needle into the selected tissue. The programmable constant-current pulse controller is activated and constant-current electrical pulse is applied to the plurality of needle electrodes. The applied constant-current electrical pulse facilitates the introduction of the nucleic acid into the cell between the plurality of electrodes. Cell death due to overheating of cells is minimized by limiting the power dissipation in the

tissue by virtue of constant-current pulses. The Cellectra device and system is described in U.S. Pat. No. 7,245,963, the contents of which are herein incorporated by reference.

The MID may be an Elgen 1000 system (Inovio Pharmaceuticals). The Elgen 1000 system may comprise device that provides a hollow needle; and fluid delivery means, wherein the apparatus is adapted to actuate the fluid delivery means in use so as to concurrently (for example automatically) inject fluid, the described Encoded Affimer® Agent nucleic acid construct herein, into body tissue during insertion of the needle into the said body tissue. The advantage is the ability to inject the fluid gradually while the needle is being inserted leads to a more even distribution of the fluid through the body tissue. It is also believed that the pain experienced during injection is reduced due to the distribution of the volume of fluid being injected over a larger area.

In addition, the automatic injection of fluid facilitates automatic monitoring and registration of an actual dose of fluid injected. This data can be stored by a control unit for documentation purposes if desired.

It will be appreciated that the rate of injection could be either linear or non-linear and that the injection may be carried out after the needles have been inserted through the skin of the subject to be treated and while they are inserted further into the body tissue.

Suitable tissues into which fluid may be injected by the apparatus of the present disclosure include lung, nasal, gastrointestinal, skin and other epithelial tissues, liver tissue and muscle tissue, merely as examples.

The apparatus further comprises needle insertion means for guiding insertion of the needle into the body tissue. The rate of fluid injection is controlled by the rate of needle insertion. This has the advantage that both the needle insertion and injection of fluid can be controlled such that the rate of insertion can be matched to the rate of injection as desired. It also makes the apparatus easier for a user to operate. If desired means for automatically inserting the needle into body tissue could be provided.

A user could choose when to commence injection of fluid. Ideally however, injection is commenced when the tip of the needle has reached the target tissue and the apparatus may include means for sensing when the needle has been inserted to a sufficient depth for injection of the fluid to commence. This means that injection of fluid can be prompted to commence automatically when the needle has reached a desired depth

(which will normally be the depth at which muscle tissue begins). The depth at which muscle tissue begins could for example be taken to be a preset needle insertion depth such as a value of 4 mm which would be deemed sufficient for the needle to get through the skin layer.

The sensing means may comprise an ultrasound probe. The sensing means may comprise a means for sensing a change in impedance or resistance. In this case, the means may not as such record the depth of the needle in the body tissue but will rather be adapted to sense a change in impedance or resistance as the needle moves from a different type of body tissue into muscle. Either of these alternatives provides a relatively accurate and simple to operate means of sensing that injection may commence. The depth of insertion of the needle can further be recorded if desired and could be used to control injection of fluid such that the volume of fluid to be injected is determined as the depth of needle insertion is being recorded.

The apparatus may further comprise: a base for supporting the needle; and a housing for receiving the base therein, wherein the base is moveable relative to the housing such that the needle is retracted within the housing when the base is in a first rearward position relative to the housing and the needle extends out of the housing when the base is in a second forward position within the housing. This is advantageous for a user as the housing can be lined up on the skin of a patient, and the needles can then be inserted into the patient's skin by moving the housing relative to the base.

As stated above, it is desirable to achieve a controlled rate of fluid injection such that the fluid is evenly distributed over the length of the needle as it is inserted into the skin. The fluid delivery means may comprise piston driving means adapted to inject fluid at a controlled rate. The piston driving means could for example be activated by a servo motor. However, the piston driving means may be actuated by the base being moved in the axial direction relative to the housing. It will be appreciated that alternative means for fluid delivery could be provided. Thus, for example, a closed container which can be squeezed for fluid delivery at a controlled or non-controlled rate could be provided in the place of a syringe and piston system.

The apparatus described above could be used for any type of injection. It is however envisaged to be particularly useful in the field of electroporation and so it may further comprises means for applying a voltage to the needle. This allows the needle to be used not only for injection but also as an electrode during, electroporation. This is

particularly advantageous as it means that the electric field is applied to the same area as the injected fluid. There has traditionally been a problem with electroporation in that it is very difficult to accurately align an electrode with previously injected fluid and so users have tended to inject a larger volume of fluid than is required over a larger area and to apply an electric field over a higher area to attempt to guarantee an overlap between the injected substance and the electric field. Using the present disclosure, both the volume of fluid injected and the size of electric field applied may be reduced while achieving a good fit between the electric field and the fluid.

U.S. Pat. No. 7,245,963 by Draghia-Akli, et al. describes modular electrode systems and their use for facilitating the introduction of a biomolecule into cells of a selected tissue in a body or plant. The modular electrode systems may comprise a plurality of needle electrodes; a hypodermic needle; an electrical connector that provides a conductive link from a programmable constant-current pulse controller to the plurality of needle electrodes; and a power source. An operator can grasp the plurality of needle electrodes that are mounted on a support structure and firmly insert them into the selected tissue in a body or plant. The biomolecules are then delivered via the hypodermic needle into the selected tissue. The programmable constant-current pulse controller is activated and constant-current electrical pulse is applied to the plurality of needle electrodes. The applied constant-current electrical pulse facilitates the introduction of the biomolecule into the cell between the plurality of electrodes. The entire content of U.S. Pat. No. 7,245,963 is hereby incorporated by reference.

U.S. Patent Pub. 2005/0052630 submitted by Smith, et al. describes an electroporation device which may be used to effectively facilitate the introduction of a biomolecule into cells of a selected tissue in a body or plant. The electroporation device comprises an electro-kinetic device ("EKD device") whose operation is specified by software or firmware. The EKD device produces a series of programmable constant-current pulse patterns between electrodes in an array based on user control and input of the pulse parameters, and allows the storage and acquisition of current waveform data. The electroporation device also comprises a replaceable electrode disk having an array of needle electrodes, a central injection channel for an injection needle, and a removable guide disk. The entire content of U.S. Patent Pub. 2005/0052630 is hereby incorporated by reference.

The electrode arrays and methods described in U.S. Pat. No. 7,245,963 and U.S. Patent Pub. 2005/0052630 may be adapted for deep penetration into not only tissues such as

muscle, but also other tissues or organs. Because of the configuration of the electrode array, the injection needle (to deliver the biomolecule of choice) is also inserted completely into the target organ, and the injection is administered perpendicular to the target issue, in the area that is pre-delineated by the electrodes. The electrodes described in U.S. Pat. No. 7,245,963 and U.S. Patent Pub. 2005/005263 are, for example, 20 mm long and 21 gauge.

Use of *in vivo* electroporation enhances plasmid DNA uptake in infected (or infectible) tissue, resulting in expression of anti-SPIKE Affimer® Agents within that tissue, which can either be designed to accumulate locally or result in systemic exposure to the secreted Affimer® Agents, Additional exemplary techniques, vectors and devices for electroporating anti-SPIKE Affimer® Agent transgenes into cells *in vivo* include PCT Publications WO/2017/106795, WO/2016/161201, WO/2016/154473, WO/2016/112359 and WO/2014/066655.

Typically, the electric fields needed for *in vivo* cell electroporation are generally similar in magnitude to the fields required for cells in vitro. In some embodiments, the magnitude of the electric field range from approximately, 10 V/cm to about 1500 V/cm, 300 V/cm to 1500 V/cm, or 1000 V/cm to 1500 V/cm. Alternatively, lower field strengths (from about 10 V/cm to 100 V/cm, and more preferably from about 25 V/cm to 75 V/cm) the pulse length is long. For example, when the nominal electric field is about 25-75 V/cm, if is preferred that the pulse length is about 10 msec.

The pulse length can be about 10 s to about 100 ms. There can be any desired number of pulses, typically one to 100 pulses per second. The delay between pulses sets can be any desired time, such as one second. The waveform, electric field strength and pulse duration may also depend upon the type of cells and the type of molecules that are to enter the cells via electroporation.

Also encompassed are electroporation devices incorporating electrochemical impedance spectroscopy ("EIS"). Such devices provide real-time information on *in vivo*, in particular, intra-target tissue electroporation efficiency, allowing for the the optimization of conditions. Examples of electroporation devices incorporating EIS can be found, e.g., in WO2016/161201 , which is hereby incorporated by reference.

Uptake of the Encoded Affimer® Agent nucleic acid constructs of the present disclosure may also be enhanced by plasma electroporation also termed avalanche

transfection. Briefly, microsecond discharges create cavitation microbubbles at electrode surface. The mechanical force created by the collapsing microbubbles combined with the magnetic field serve to increase transport efficiency across the cell membrane as compared with the diffusion mediated transport associated with conventional electroporation. The technique of plasma electroporation is described in United States Patent Nos. 7,923,251 and 8,283,171. This technique may also be employed *in vivo* for the transformation of cells. Chaiberg, et al (2006) Investigative Ophthalmology & Visual Science 47:4083-4090; Chaiberg, et al United States Patent No 8, 101 169 Issued January 24, 2012.

Other alternative electroporation technologies are also contemplated. *In vivo* nucleic acid delivery can also be performed using cold plasma. Plasma is one of the four fundamental states of matter, the others being solid, liquid, and gas. Plasma is an electrically neutral medium of unbound positive and negative particles (i.e. the overall charge of a plasma is roughly zero). A plasma can be created by heating a gas or subjecting it to a strong electromagnetic field, applied with a laser or microwave generator. This decreases or increases the number of electrons, creating positive or negative charged particles called ions (Luo, et al. (1998) Phys. Plasma 5:2868-2870) and is accompanied by the dissociation of molecular bonds, if present.

Cold plasmas (i.e., non-thermal plasmas) are produced by the delivery of pulsed high voltage signals to a suitable electrode. Cold plasma devices may take the form of a gas jet device or a dielectric barrier discharge (DBD) device. Cold temperature plasmas have attracted a great deal of enthusiasm and interest by virtue of their provision of plasmas at relatively low gas temperatures. The provision of plasmas at such a temperature is of interest to a variety of applications, including wound healing, anti-bacterial processes, various other medical therapies and sterilization. As noted earlier, cold plasmas (i.e., non-thermal plasmas) are produced by the delivery of pulsed high voltage signals to a suitable electrode. Cold plasma devices may take the form of a gas jet device, a dielectric barrier discharge (DBD) device or multi-frequency harmonic-rich power supply.

Dielectric barrier discharge device, relies on a different process to generate the cold plasma. A dielectric barrier discharge (DBD) device contains at least one conductive electrode covered by a dielectric layer. The electrical return path is formed by the ground that can be provided by the target substrate undergoing the cold plasma treatment or by providing an in-built ground for the electrode. Energy for the dielectric

barrier discharge device can be provided by a high voltage power supply, such as that mentioned above. More generally, energy is input to the dielectric barrier discharge device in the form of pulsed DC electrical voltage to form the plasma discharge. By virtue of the dielectric layer, the discharge is separated from the conductive electrode and electrode etching and gas heating is reduced. The pulsed DC electrical voltage can be varied in amplitude and frequency to achieve varying regimes of operation. Any device incorporating such a principle of cold plasma generation (e.g., a DBD electrode device) falls within the scope of various embodiments of the present disclosure.

Cold plasma has been employed to transfect cells with foreign nucleic acids (see, e.g., Connolly, et al. (2012) Human Vaccines & Immune-therapeutics 8: 1729-1733; and Connolly et al (2015) Bioelectrochemistry 103: 15-21).

In certain illustrative embodiments, the transgene construct encoding the anti-SPIKE Affimer® Agent of the present disclosure is delivered using an electroporation device comprising: an applicator; a plurality of electrodes extending from the applicator, the electrodes being associated with a cover area; a power supply in electrical communication with the electrodes, the power supply configured to generate one or more electroporating signals to cells within the cover area; and a guide member coupled to the electrodes, wherein the guide member is configured to adjust the cover area of the electrodes. At least a portion of the electrodes can be positioned within the applicator in a conical arrangement. The one or more electroporating signals may be each associated with an electric field. The device may further comprise a potentiometer coupled to the power supply and electrodes. The potentiometer may be configured to maintain the electric field substantially within a predetermined range.

The one or more electroporating signals may be each associated with an electric field. The device may further comprise a potentiometer coupled to the power supply and the electrodes. The potentiometer may be configured to maintain the electric field within a predetermined range so as to substantially prevent permanent damage in the cells within the cover area and /or substantially minimize pain. For instance, potentiometer may be configured to maintain the electric field to about 1300 V/cm.

The power supply may provide a first electrical signal to a first electrode and a second electrical signal to a second electrode. The first and second electrical signals may combine to produce a wave having a beat frequency. The first and second electrical signals may each have at least one of a unipolar waveform and a bipolar waveform. The

first electrical signal may have a first frequency and a first amplitude. The second electrical signal may have a second frequency and a second amplitude. The first frequency may be different from or the same as the second frequency. The first amplitude may be different from or the same as the second amplitude.

Transfection Enhancing Formulations

Encoded Affimer® Agent nucleic acid constructs can also be encapsulated in liposomes, preferably cationic liposomes (Wong, T. K. et al., Gene, 10:87(1980); Nicolau and Sene, Biochim. Biophys. Acta, 721:185-190 (1982); and Nicolau et al., Methods Enzymol., 149:157-176 (1987)) or polymersomes (synthetic liposomes) which can interact with the cell membrane and fuse or undergo endocytosis to effect nucleic acid transfer into the cell. The DNA also can be formed into complexes with polymers (polyplexes) or with dendrimers which can directly release their load into the cytoplasm of a cell.

Illustrative carriers useful in this regard include microparticles of poly(lactide-co-glycolide), polyacrylate, latex, starch, cellulose, dextran and the like. Other illustrative carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (e.g., a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound, such as a phospholipid (see e.g., U.S. Pat. No. 5,151,254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active agent contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

Biodegradable microspheres (e.g., polylactate polyglycolate) may be employed as carriers for compositions. Suitable biodegradable microspheres are disclosed, for example, in U.S. Pat. Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344, 5,407,609 and 5,942,252. Modified hepatitis B core protein carrier systems such as described in WO/99 40934, and references cited therein, will also be useful for many applications. Another illustrative carrier/delivery system employs a carrier comprising particulate-protein complexes, such as those described in U.S. Pat. No. 5,928,647, which can have the added benefit when used intra-target tissue to deliver the coding sequence for an anti-SPIKE Affimer® Agent.

Biodegradable polymeric nanoparticles facilitate nonviral nucleic acid transfer to cells. Small (approximately 200 nm), positively charged (approximately 10 mV) particles are

formed by the self-assembly of cationic, hydrolytically degradable poly(beta-amino esters) and plasmid DNA.

Polynucleotides may also be administered to cells by direct microinjection, temporary cell permeabilizations (e.g., co-administration of repressor and/or activator with a cell permeabilizing agent), fusion to membrane translocating peptides, and the like.

Lipid-mediated nucleic acid delivery and expression of foreign nucleic acids, including mRNA, in vitro and *in vivo* has been very successful. Lipid based non-viral formulations provide an alternative to viral gene therapies. Current *in vivo* lipid delivery methods use subcutaneous, intradermal, intrapulmonary, intra-gastrointestinal, intranasal or intracranial injection. Advances in lipid formulations have improved the efficiency of gene transfer *in vivo* (see PCT Application WO 98/07408). For instance, a lipid formulation composed of an equimolar ratio of l,2-bis(oleoyloxy)-3-(trimethyl ammonio)propane (DOTAP) and cholesterol can significantly enhances systemic *in vivo* gene transfer. The DOTAP:cholesterol lipid formulation forms unique structure termed a "sandwich liposome". This formulation is reported to "sandwich" DNA between an invaginated bi-layer or 'vase' structure. Beneficial characteristics of these lipid structures include a positive p, colloidal stabilization by cholesterol, two dimensional nucleic acid packing and increased serum stability.

Cationic liposome technology is based on the ability of amphipathic lipids, possessing a positively charged head group and a hydrophobic lipid tail, to bind to negatively charged DNA or RNA and form particles that generally enter cells by endocytosis. Some cationic liposomes also contain a neutral co-lipid, thought to enhance liposome uptake by mammalian cells. Similarly, other polycations, such as poly-l-lysine and polyethylene-imine, complex with nucleic acids via charge interaction and aid in the condensation of DNA or RNA into nanoparticles, which are then substrates for endosome-mediated uptake. Several of these cationic-nucleic acid complex technologies have been developed as potential clinical products, including complexes with plasmid DNA (pDNA), oligodeoxynucleotides, and various forms of synthetic RNA, and be used as part of the delivery system for the Encoded Affimer® Agent nucleic acid constructs of the present disclosure.

The Encoded Affimer® Agent nucleic acid constructs disclosed herein may be associated with polycationic molecules that serve to enhance uptake into cells.

Complexing the nucleic acid construct with polycationic molecules also helps in packaging the construct such their size is reduced, which is believed to assist with cellular uptake. Once in the endosome, the complex dissociates due to the lower pH, and the polycationic molecules can disrupt the endosome's membrane to facilitate DNA escape into the cytoplasm before it can be degraded. Preliminary data shows that the nucleic acid construct embodiments had enhanced uptake into SCs over DCs when complexed with the polycationic molecules polylysine or polyethyleneimine.

One example of polycationic molecules useful for complexing with nucleic acid constructs includes cell penetrating peptides (CPP), examples include polylysine (described above), polyarginine and Tat peptides. Cell penetrating peptides (CPP) are small peptides which can bind to DNA and, once released, penetrate cell membranes to facilitate escape of the DNA from the endosome to the cytoplasm. Another example of a CPP pertains to a 27 residue chimeric peptide, termed MPG, was shown some time ago to bind ss- and ds-oligonucleotides in a stable manner, resulting in a non-covalent complex that protected the nucleic acids from degradation by DNase and effectively delivered oligonucleotides to cells in vitro (Mahapatro A, et al., J Nanobiotechnol, 2011, 9:55). The complex formed small particles of approximately 150 nm to 1 um when different peptide:DNA ratios were examined, and the 10:1 and 5:1 ratios (150 nm and 1 um respectively). Another CPP pertains to a modified tetrapeptide [tetralysine containing guanidinocarbonylpyrrole (GCP) groups (TL-GCP)], which was reported to bind with high affinity to a 6.2 kb plasmid DNA resulting in a positive charged aggregate of 700-900 nm Li et al., Agnew Chem Int Ed Enl 2015; 54(10):2941-4). RNA can also be complexed by such polycationic molecules for *in vivo* delivery.

Other examples of polycationic molecules that may be complexed with the nucleic acid constructs described herein include polycationic polymers commercially available as JETPRIME® and *In vivo* JET (Polypus-transfection, S.A., Illkirch, France).

In some embodiments, the present disclosure contemplates a method of delivering an mRNA (or other polynucleotide)f encoding an anti-SPIKE Affimer® Agent to a patient's cells by administering a nanoparticle composition comprising (i) a lipid component comprising a compound of formula (I), a phospholipid, a structural lipid, and a PEG lipid; and (ii) an mRNA (or other polynucleotide)f, said administering comprising contacting said mammalian cell with said nanoparticle composition, whereby said mRNA (or other polynucleotide)f is delivered to said cell.

In exemplary embodiments, the PEG lipid is selected from the group consisting of a PEG-modified phosphatidylethanolamine, a PEG-modified phosphatide acid, a PEG-modified ceramide, a PEG-modified dialkylamine, a PEG-modified diacylglycerol and a PEG-modified dialkylglycerol. In exemplary embodiments, the structural lipid is selected from the group consisting of cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, ursolic acid, and alpha-tocopherol. In some embodiments, the structural lipid is cholesterol.

In exemplary embodiments, the phospholipid includes a moiety selected from the group consisting of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl serine, phosphatidic acid, 2-lysophosphatidyl choline, and a sphingomyelin. In some embodiments, the phospholipid includes one or more fatty acid moieties selected from the group consisting of lauric acid, myristic acid, myristoleic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alpha-linolenic acid, erucic acid, arachidic acid, arachidonic acid, phytanoic acid, eicosapentaenoic acid, behenic acid, docosapentaenoic acid, and docosahexaenoic acid. In some embodiments, the phospholipid is selected from the group consisting of 1 ,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1 ,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1 ,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1 ,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1 ,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1 ,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1 -palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1 ,2-di-0-octadecenyl-sn-glycero-3-phosphocholine (1 8:0 Diether PC), 1 -oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1 -hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1 ,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1 ,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1 ,2-didocosahexaenoyl-sn-glycero-3-phosphocholine,1 ,2-dioleoyl-sn-glycero-3-phosphoethanola mine (DOPE), 1 ,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 1 6.0 PE), 1 ,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1 ,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1 ,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1 ,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1 ,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1 ,2-dioleoyl-sn-glycero-3-phospho-rac-(1 -glycerol) sodium salt (DOPG), and sphingomyelin In some embodiments, the phospholipid is DOPE or DSPC.

To further illustrate, the phospholipid can be DOPE and said the component can comprise about 35 mol % to about 45 mol % said compound, about 1 0 mol % to about 20 mol % DOPE, about 38.5 mol % to about 48.5 mol % structural lipid, and about 1 .5

mol % PEG lipid. The lipid component can be about 40 mol % said compound, about 15 mol % phospholipid, about 43.5 mol % structural lipid, and about 1 .5 mol % PEG lipid.

In some embodiments, the wt/wt ratio of lipid component to anti-SPIKE Affimer® Agent encoding mRNA (or other polynucleotide) is from about 5:1 to about 50:1, or about 10:1 to about 40:1.

In some embodiments, the mean size of said nanoparticle composition is from about 50 nm to about 150 nm, or from about 80 nm to about 120 nm.

In some embodiments, the polydispersity index of said nanoparticle composition is from about 0 to about 0.18, or from about 0.13 to about 0.17.

In some embodiments, the nanoparticle composition has a zeta potential of about -10 to about +20 mV.

In some embodiments, the nanoparticle composition further comprises a cationic and/or ionizable lipid selected from the group consisting of 3-(didodecylamino)-N1 ,N 1 ,4-tridodecyl-1 -piperazineethanamine (KL1 0), 14,25-ditridecyl-1 5, 1 8,21 ,24-tetraaza-octatriacontane (KL25), 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLin-DMA), 2,2-dilinoleyl-4-dimethylaminomethyl-[1 ,3]-dioxolane (DLin-K-DMA), heptatriaconta-6, 9,28,31 -tetraen-1 9-yl 4-(dimethylamino)butanoate (DLin-MC3-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1 ,3]-dioxolane (DLin-KC2-DMA), 1 ,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), and (2R)-2-({8-[(3P)-cholest-5-en-3-yloxy]octyl}oxy)-N,N-dimethyl-3-[(9Z,1 2Z)-octadeca-9, 12-dien-1 -yl oxy]propan-1 -amine (Octyl-CLinDMA (2R)).

METHODS OF USE AND PHARMACEUTICAL COMPOSITIONS

The present disclosure also provides pharmaceutical compositions comprising an Affimer® Agent described herein and a pharmaceutically acceptable vehicle.

Formulations are prepared for storage and use by combining a purified Affimer® Agent of the present disclosure with a pharmaceutically acceptable vehicle (e.g., a carrier or excipient). Those of skill in the art generally consider pharmaceutically acceptable carriers, excipients, and/or stabilizers to be inactive ingredients of a formulation or pharmaceutical composition.

In some embodiments, an Affimer® Agent described herein is lyophilized and/or stored in a lyophilized form. In some embodiments, a formulation comprising an Affimer® Agent described herein is lyophilized.

Suitable pharmaceutically acceptable vehicles include, but are not limited to, nontoxic buffers such as phosphate, citrate, and other organic acids; salts such as sodium chloride; antioxidants including ascorbic acid and methionine; preservatives such as octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride, benzethonium chloride, phenol, butyl or benzyl alcohol, alkyl parabens, such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol; low molecular weight polypeptides (e.g., less than about 10 amino acid residues); proteins such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; carbohydrates such as monosaccharides, disaccharides, glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes such as Zn-protein complexes; and non-ionic surfactants such as TWEEN or polyethylene glycol (PEG). (Remington: The Science and Practice of Pharmacy, 22.sup.nd Edition, 2012, Pharmaceutical Press, London.).

The pharmaceutical compositions of the present disclosure can be administered in any number of ways for either local or systemic treatment. Administration can be topical by epidermal or transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders; pulmonary by inhalation or insufflation of powders or aerosols, including by nebulizer, intratracheal, and intranasal; oral; or parenteral including intravenous, intraarterial, pulomonary, nasal, gastrointestinal, subcutaneous, intraperitoneal, intramuscular (e.g., injection or infusion), or intracranial (e.g., intrathecal or intraventricular).

The therapeutic formulation can be in unit dosage form. Such formulations include tablets, pills, capsules, powders, granules, solutions or suspensions in water or non-aqueous media, or suppositories. In solid compositions such as tablets the principal active ingredient is mixed with a pharmaceutical carrier. Conventional tableting ingredients include corn starch, lactose, sucrose, sorbitol, talc, stearic acid, magnesium stearate, dicalcium phosphate or gums, and diluents (e.g., water). These can be used to form a solid preformulation composition containing a homogeneous mixture of a compound of the present disclosure, or a non-toxic pharmaceutically acceptable salt

thereof. The solid preformulation composition is then subdivided into unit dosage forms of a type described above. The tablets, pills, etc. of the formulation or composition can be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner composition covered by an outer component. Furthermore, the two components can be separated by an enteric layer that serves to resist disintegration and permits the inner component to pass intact through the stomach or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials include a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol and cellulose acetate.

The Affimer® Agents described herein can also be entrapped in microcapsules. Such microcapsules are prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in macroemulsions as described in Remington: The Science and Practice of Pharmacy, 22.sup.nd Edition, 2012, Pharmaceutical Press, London.

In some embodiments, pharmaceutical formulations include an Affimer® Agent of the present disclosure complexed with liposomes. Methods to produce liposomes are known to those of skill in the art. For example, some liposomes can be generated by reverse phase evaporation with a lipid composition comprising phosphatidylcholine, cholesterol, and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes can be extruded through filters of defined pore size to yield liposomes with the desired diameter.

In some embodiments, sustained-release preparations comprising Affimer® Agents described herein can be produced. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing an Affimer® Agent, where the matrices are in the form of shaped articles (e.g., films or microcapsules). Examples of sustained-release matrices include polyesters, hydrogels such as poly(2-hydroxyethyl-methacrylate) or poly(vinyl alcohol), polylactides, copolymers of L-glutamic acid and 7 ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT.TM. (injectable microspheres composed of lactic acid-glycolic acid copolymer

and leuprolide acetate), sucrose acetate isobutyrate, and poly-D-(-)-3-hydroxybutyric acid.

In some embodiments, in addition to administering an Affimer® Agent described herein, the method or treatment further comprises administering at least one additional therapeutic agent. An additional therapeutic agent can be administered prior to, concurrently with, and/or subsequently to, administration of the Affimer® Agent. Pharmaceutical compositions comprising an Affimer® Agent and the additional therapeutic agent(s) are also provided. In some embodiments, the at least one additional therapeutic agent comprises 1, 2, 3, or more additional therapeutic agents.

The Affimer® Agents of the invention can be administered by inhalation, typically in the form of a dry powder (either alone, as a mixture, for example, in a dry blend with lactose, or as a mixed component particle, for example, mixed with phospholipids, such as phosphatidylcholine) from a dry powder inhaler or as an aerosol spray from a pressurised container, pump, spray, atomiser (preferably an atomiser using electrohydrodynamics to produce a fine mist), or nebuliser, with or without the use of a suitable propellant, such as 1,1,1,2-tetrafluoroethane or 1,1,1,2,3,3,3-heptafluoropropane. For intranasal use, the powder may comprise a bioadhesive agent, for example, chitosan or cyclodextrin.

The pressurised container, pump, spray, atomizer, or nebuliser contains a solution or suspension of the compound(s) of the invention comprising, for example, ethanol, aqueous ethanol, or a suitable alternative agent for dispersing, solubilising, or extending release of the active, a propellant(s) as solvent and an optional surfactant, such as sorbitan trioleate, oleic acid, or an oligolactic acid.

Prior to use in a dry powder or suspension formulation, the drug product (i.e., including the Affimer® Agent) is micronised to a size suitable for delivery by inhalation (typically less than 5 microns). This may be achieved by any appropriate comminuting method, such as spiral jet milling, fluid bed jet milling, supercritical fluid processing to form nanoparticles, high pressure homogenisation, or spray drying.

In the case of dry powder inhalers and aerosols, the dosage unit is determined by means of a valve which delivers a metered amount. Units in accordance with the invention are typically arranged to administer a metered dose or "puff" containing from 0.001 mg to 10mg of the compound of the drug moiety. The overall daily dose will

typically be in the range 0.001 mg to 40mg which may be administered in a single dose or, more usually, as divided doses throughout the day.

Many conventional techniques have been reported to produce dry powder inhalers (DPI) formulations. However, these methods have number of limitations, such as particle size, size distribution, shape and poor control over powder crystallinity. These problems can be rectified by specialized milling techniques. Jet-milling of drug under nitrogen gas with new nanojet milling instrument is the most suitable method for creating nanoparticles meant for pulmonary drug delivery. Below are some of the illustrative techniques.

The powdered formulation may be prepared starting from a dry product comprising an Affimer® Agent, its salt or mixtures thereof, by altering the particle size of the agent to form a dry formulation of particle size about 0.01 μm to about 500 μm in diameter; and selecting particles of the formulation comprising at least or greater than about 80%, about 85%, about 90%, about 95%, or about 100% particles of about 0.01 μm, 0.1 μm or 0.5 μm to about 100 μm or 200 μm in diameter. The particle size is desirably less than about 200 μm, preferably in the range about 0.05 μm, about 0.1 μm, about 1 μm, about 2 μm to about 5 μm, about 6 μm, about 8 μm, about 10 μm, about 20 μm, about 50 μm, about 100 μm. Preferably, the selected particles of the formulation of about 0.1 to about 200 μm in diameter. More preferably, the selected particles of the formulation of about 0.1 to about 100 μm in diameter. Even more preferably, the selected particles of the formulation of about 0.1 to about 10 μm in diameter. Even much more preferably, the selected particles of the formulation of about 0.1 to about 8 μm in diameter. Even further much more preferably, the selected particles of the formulation of about 0.1 to about 5 μm in diameter.

The particle size of the dry agent may be then altered so as to permit the absorption of a substantial amount of the agent into the lungs upon inhalation of the formulation. The particle size of the medicament may be reduced by any known means, for example by milling or micronization. Typically, the particle size for the agent is altered by milling the dry agent either alone or in combination with a formulation ingredient to a suitable average particle size, preferably in the about 0.05 μm, about 5 μm range (inhalation) or about 10 μm, to about 50 μm (nasal delivery or lung instillation). Jet milling, also known as fluid energy milling, may be employed and are preferred among the procedures to give the particle size of interest using known devices. Jet milling is the preferred process. It should be understood that although a large percentage of the

particles will be in the narrow range desired, this will not generally be true for all particles. Thus, it is expected that the overall particle range may be broader than the preferred range as stated above. The proportion of particles within the preferred range may be greater than about 80%, about 85%, about 90%, about 95%, and so on, depending on the needs of a specific formulation.

The particle size may be also altered by sieving, homogenization, and/or granulation, amongst others. These techniques are used either separately or in combination with one another. Typically, milling, homogenization and granulation are applied, followed by sieving to obtain the dry altered particle size formulation. These procedures may be applied separately to each ingredient, or the ingredients added together and then formulated.

Examples of the formulation ingredients that may be employed are not limited to, but include, an excipient, preservatives, stabilizers, powder flowability improving agents, a cohesiveness improving agent, a surfactant, other bioactive agents, a coloring agent, an aromatic agent, anti-oxidants, fillers, volatile oils, dispersants, flavoring agents, buffering agents, bulking agents, propellants or preservatives. One preferred formulation comprises the active agent and an excipient(s) and/or a propellant(s).

The particle size may be altered not only in a dry atmosphere but also by placing the active agent in solution, suspension or emulsion in inter-mediate steps. The active agent may be placed in solution, suspension, or emulsion, either prior to, or after, altering the particle size of the agent. An example of this embodiment that may be performed by dissolving the agent in a suitable solvent solution, and heating to an appropriate temperature. The temperature may be maintained in the vicinity of the appropriate temperature for a predetermined period of time to allow for crystals to form. The solution and the fledgling crystals then are cooled to a second lower temperature to grow the crystals by maintaining them at the second temperature for a period of time as is known in the art. The crystals are then allowed to reach room temperature when recrystalization is completed and the crystals of the agent have grown sufficiently. The particle size of the agent may also be altered by sample precipitation, which is conducted from solution, suspension or emulsion in an adequate solvent(s).

Spray drying is useful in altering the particle size, as well. By “spray dried or spray drying” what is meant is that the agent or composition is prepared by a process in

which a homogeneous mixture of the agent in a solvent or composition termed herein the "pre-spray formulation", is introduced via an atomizer, e.g. a two-fluid nozzle, spinning disk or an equivalent device into a heated atmosphere or a cold fluid as fine droplets. The solution may be an aqueous solution, suspension, emulsion, slurry or the like, as long as it is homogeneous to ensure uniform distribution of the material in the solution and, ultimately, in the powdered formulation. When sprayed into a stream of heated gas or air, each droplet dries into a solid particle. Spraying of the agent into the cold fluid results in a rapid formation of atomized droplets that form particles upon evaporation of the solvent. The particles are collected, and then any remaining solvent may be removed, generally through sublimation (lyophilization), in a vacuum. As discussed below, the particles may be grown, e.g. by raising the temperature prior to drying. This produces a fine dry powder with particles of a specified size and characteristics, that are more fully discussed below. Suitable spray drying methodologies are also described below. See, for example U.S. Pat. Nos. 3,963,559; 6,451,349; and, 6,458,738, the relevant portions of which are incorporated herein by reference.

As used herein, the term "powder" means a composition that consists of finely dispersed solid particles that are relatively free flowing and capable of being readily dispersed in an inhalation or dry powder device and subsequently inhaled by a patient so that the particles can reach the intended region of the lung. Thus, the powder is "respirable" and suitable for pulmonary delivery. When the particle size of the next agent or the formulation is above about 10 μm, the particles are of such size that a good proportion of them will deposit in the nasal cavities, and will be absorbed there through.

The term "dispersibility" means the degree to which a dry powder formulation may be dispersed, i.e. suspended, in a current of air so that the dispersed particles may be respired or inhaled into the lungs or absorbed through the walls of the nasal cavities of a subject. Thus, a powder that is only 20% dispersible means that only 20% of the mass of particles may be suspended for inhalation into the lungs. The present formulation preferably has a dispersibility of about 1 to 99%, although others are also suitable.

The dry powder formulation may be characterized on the basis of a number of parameters, including, but not limited to, the average particle size, the range of particle size, the fine powder fraction (FPF), the average particle density, and the mass median aerodynamic diameter (MMAD), as is known in the art.

In a preferred embodiment, the agent is DHEA-S in a dihydrate crystalline form. The DHEA-S is first crystallized into the dihydrate crystalline form. The crystals are then put through the jet mill to produce it into a powder form. The preparation can further comprise lactose that is separately sieved or milled and mixed with the powdered crystalline dihydrate DHEA-S.

In a preferred embodiment, the dry powder formulation of this invention is characterized on the basis of their average particle size that was described above. The average particle size of the powdered agent or formulation may be measured as the mass mean diameter (MMD) by conventional techniques. The term, "about" means the numerical values could have an error in the range of about 10% of the numerical value. The dry powdered formulation of this invention may also be characterized on the basis of its fine particle fraction (FPF). The FPF is a measure of the aerosol performance of a powder, where the higher the fraction value, the better. The FPF is defined as a powder with an aerodynamic mass median diameter of less than 6.8 μm as determined using a multiple-stage liquid impinger with a glass throat (MLSI, Astra, Copley Instrument, Nottingham, UK) through a dry powder inhaler (Dryhalter™, Dura Pharmaceuticals). Accordingly, the dry powder formulation of the invention preferably has a FPF of at least about 10%, with at least about 20% being preferred, and at least about 30% being especially preferred. Some systems may enable very high FPFs, of the order of 40 to 50%.

The dry powdered formulation may be characterized also on the basis of the density of the particles containing the agent of the invention. In a preferred embodiment, the particles have a tap density of less than about 0.8 g/cm3, with tap densities of less than about 0.4 g/cm3 being preferred, and a tap density of less than about 0.1 g/cm3 being especially preferred. The tap density of dry powder particles may be measured using a GeoPyc™ (Micrometrics Instruments Corp), as is known in the art. Tap density is a standard measure of the envelope mass density, which is defined generally as the mass of the particle divided by the minimum sphere envelope volume within which it may be enclosed.

In another preferred embodiment, the aerodynamic particle size of the dry powdered formulation may be characterized as is generally outlined in the Examples. Similarly, the mass median aerodynamic diameter (MMAD) of the particles may be evaluated,

using techniques well known in the art. The particles may be characterized on the basis of their general morphology as well.

The term "dry" means that the formulation has a moisture content such that the particles are readily dispersible in an inhalation device to form an aerosol. The dry powdered formulation in the invention comprises preferably substantially active compound, although some aggregation may occur, particularly upon long storage periods. As is known for many dry powder formulation, some percentage of the material in a powder formulation may aggregate, this resulting in some loss of activity. Accordingly, the dry powdered formulation has at least about 70% w/w active compound, i.e. % of total compound present, with at least about 80% w/w active compound being preferred, and at least about 90% w/w active compound being especially preferred. More highly active compound or agent is also contemplated, and may be prepared by the present method, i.e., an activity greater than about 95% and higher. The measurement of the total compound present will depend on the compound and, generally, will be done as is known in the art, on the basis of activity assays, etc. The measurement of the activity of the agent will be dependent on the compound and will be done on suitable bioactivity assays as will be appreciated by those in the art.

In spray drying, an individual stress event may arise due to atomization (shear stress and air-liquid interfacial stress), cold or heat denaturation, optionally freezing (ice-water interfacial stress and shear stress), and/or dehydration. Cryoprotectants and lyoprotectants have been used during lyophilization to counter freezing destabilization, and dehydration and long-term storage destabilization, respectively. Cryoprotectant molecules, e.g., sugars, amino acids, polyols, etc., have been widely used to stabilize active compounds in highly concentrated unfrozen liquids associated with ice crystallization. These are not required in the formulation.

The dry powdered formulations comprising an active compound may or not contain an excipient. "Excipients" or "protectants" including cryoprotectants and lyoprotectants generally refers to compounds or materials that are added as diluents or to ensure or increase flowability and aerosol dispersibility of the active compounds during the spray drying step and afterwards, and for long-term flowability of the powdered product. Suitable excipients are generally relatively free flowing particulate solids, do not thicken or polymerize upon contact with water, are basically innocuous when placed in the respiratory tract of a patient and do not substantially interact with the active compound in a manner that alters its biological activity.

Suitable excipients include, but are not limited to, proteins such as human and bovine serum albumin, gelatin, immunoglobulins, carbohydrates including monosaccharides (galactose, D-mannose, sorbose, etc.), disaccharides (lactose, trehalose, sucrose, etc.), cyclodextrins, and polysaccharides (raffinose, maltodextrins, dextrans, etc.); an amino acid such as monosodium glutamate, glycine, alanine, arginine or histidine, as well as hydrophobic amino acids (tryptophan, tyrosine, leucine, phenylalanine, etc.); a lubricant such as magnesium stearate; a methylamine such as betaine; an excipient salt such as magnesium sulfate; a polyol such as trihydric or higher sugar alcohols, e.g. glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; pluronics; surfactants; (lipid and non-lipid. surfactants) and combinations thereof. Preferred excipients are trehalose, sucrose, sorbitol, and lactose,: as well as mixtures thereof. When excipients are used, they are used generally in amounts ranging from about 0.1, about 1, about 2, about 5, about 10 to about 15, about 10, about 15, about 20, about 40, about 60, about 99% w/w composition. Preferred are formulations containing lactose, or low amounts of excipient or other ingredients.

In another preferred embodiment, the dry powdered formulation of this invention is substantially free of excipients. “Substantially free” in this case generally means that the formulation contains less than about 10%, w/w preferably less than about 5%, w/w more preferably less than about 2-3% w/w, still more preferably less than about 1% w/w of any components other than the agent. Generally, for the purposes of this invention, the formulation may include a propellant and a co-solvent, buffers or salts, and residual water. In one preferred embodiment the dry powdered formulation (prior to the addition of bulking agent, discussed below) consists of the agent and protein as a major component, with small amounts of buffer(s), salt(s) and residual water. Generally, in this embodiment, the spray drying process comprises a temperature raising step prior to drying, as is more fully outlined below.

In another preferred embodiment, the pre-spray dried formulation, i.e. the soiution formulation used in the spray drying process comprises the active agent in solution, e.g. aqueous solution, with only negligible amounts of buffers or other compounds. The pre-spray dried formulation containing little or no excipient may not be highly stable over a long period of time. It is, thus, desirable to perform the spray drying process within a reasonable short time after the pre-spray dried formulation is produced. Although, the pre-spray dried formulation utilizing little or no excipient may not be

highly stable, the dry powder made from it may, and generally is both surprisingly stable and highly dispersible, as shown in the Examples.

The agents that are spray dried to form the formulations of the invention comprise the agent and optionally a buffer, and may or may not contain additional salts. The suitable range of the pH of the buffer in solution can be readily ascertained by those in the art. Generally, this will be in the range of physiological pH, although the agent of the invention may flowable at a wider range of pHs, for example acidic pH. Thus, preferred pH ranges of the pre-spray dry formulation are about 1, about 3, about 5, about 6 to about 7, about 8, about 10, and a pH about 7 being especially preferred. As will be appreciated by those in the art, there are a large number of suitable buffers that may be used. Suitable buffers include, but are not limited to, sodium acetate, sodium citrate, sodium succinate, sodium phosphate, ammonium bicarbonate and carbonate. Generally, buffers are used at molarities from about 1 mM, about 2 mM to about 200 mM about 10 mM, about 0.5 M, about 1 M, about 2 M, about 50 M being particularly preferred.

When water, buffers or solvents are used during the preparation process, they may additionally contain salts as already indicated.

In addition, the dry powdered formulation of the invention is generally substantially free of "stabilizers". The formulation may contain, however, an additional surfactant that has its own prophylactic or therapeutic effect on the respiratory system on the lungs. These active agents may compensate for loss of lung surfactant or generally act by other mechanisms. The dry powdered formulations of the invention is also generally substantially free of microsphere-forming polymers. See, e.g. WO 97/44013; U.S. Pat. No. 5,019,400. That is, the powders of the invention generally comprise the active agent(s) and excipient, and do not require the use of polymers for structural or other purposes. The dry powdered formulations of the invention is also preferably stable. "Stability" may mean one of two things, retention of biological activity and retention of dispersibility over time, with preferred embodiments showing stability in both areas.

The dry powdered formulation of the invention generally retains biological activity over time, e.g. physical and chemical stability and integrity upon storage. Losses of biological activity are generally due to aggregation, and/or oxidation of agent's particles. However, when the agent is agglomerate around particles of excipient, the resulting agglomerates are highly stable and active. As will be appreciated by those in

the art, there may be an initial loss of biological activity as a result of spray drying, due to the extreme temperatures used in the process. Once this has occurred, however, further loss of activity will be negligible, as measured from the time the powder is made. Moreover, the dry powdered formulation of the invention have been found to retain dispersibility over time, as quantified by the retention of a high FPF over time, the minimally aggregation, caking or clumping observed over time.

The agent(s) of the invention is (are) made by methods known in the art. See, for example, U.S. Pat. Nos. 6,087,351; 5,175,154; and, 6,284,750. The pre-spray drying composition may be formulated for stability as a liquid or solid formulation. For spray drying, the liquid formulations are subjected generally to diafiltration and/or ultrafiltration, as required, for buffer exchange (or removal) and/or concentration, as is known in the art. The pre-spray dry formulations comprise from about 1 mg/ml, about 5 mg/ml, about 10 mg/ml, about 20 mg/ml to about 60 mg/ml, about 75 mg/ml of the agent. Buffers and excipients, if present, are present at concentrations discussed above. The pre-spray drying formulation is then spray dried by dispersing the agent into hot air or gas, or by spraying it into a cold or freezing fluid, e.g. a liquid or gas. The pre-spray dry formulation may be atomized as is known in the art, for example via a two-fluid or ultrasonic nozzle using filtered pressurized air, into, for example, a fluid. Spray drying equipment may be used (Buchi; Niro Yamato; Okawara; Kakoki). It is generally preferable to slightly heat the nozzle, for example by wrapping the nozzle with heating tape to prevent the nozzle head from freezing when a cold fluid is used. The pre-spray dry formulation may be atomized into a cold fluid at a temperature of about −200° C. to about −100° C., about −80° C. The fluid may be a liquid such as liquid nitrogen or other inert fluids, or a gas such as air that is cooled. Dry ice in ethanol may be used as well as super-critical fluids. In one embodiment it is preferred to stir the liquid as the atomization process occurs, although this may not be required.

Micronization techniques involve placing bulk drug into a suitable mill. Such mills are commercially available from, for example, DT Industries, Bristol, Pa., under the tradename STOKES™. Briefly, the bulk drug is placed in an enclosed cavity and subjected to mechanical forces from moving internal parts, e.g., plates, blades, hammers, balls, pebbles, and so forth. Alternatively, or in addition to parts striking the bulk drug, the housing enclosing the cavity may turn or rotate such that the bulk drug is forced against the moving parts. Some mills, e.g., fluid energy or airjet mills, include a high-pressure air stream that forces the bulk powder into the air within the enclosed cavity for contact against internal parts. Once the size and shape of the drug is

achieved, the process may be stopped and drug having the appropriate size and shape is recovered. Generally, however, particles having the desired particle size range are recovered on a continuous basis by elutriation.

There are many different types of size reduction techniques that can be used to reduce to size of the particles. There is the cutting method employing the use of a cutter mill that can reduce the size of particles to about 100 μm. There is the compression method employing the use of an end-runner mill that can reduce the size of particles to less than about 50 μm. There is the impact method employing the use of a vibration mill that can reduce the size of particles to about 1 μm or a hammer mill that can reduce the size of particles to about 8 μm. There is the attrition method employing the use of a roller mill that can reduce the size of particles to about 1 μm. There is the combined impact and attrition method employing the use of a pin mill that can reduce the size of particles to about 10 μm, a ball mill that can reduce the size of particles to about 1 μm, a fluid energy mill (or jet mill) that can reduce the size of particles to about 1 μm. One of ordinary skill in the art is able through routine experimentation determine the particle size reduction method and means to produce the desired particle size of the composition.

Supercritical fluid processes may be used for altering the particle size of the agent. Supercritical fluid processes involve precipitation by rapid expansion of supercritical solvents, gas anti-solvent processes, and precipitation from gas-saturated solvents. A supercritical fluid is applied at a temperature and pressure that are greater than its critical temperature (Tc) and critical pressure (Pc), or compressed fluids in a liquid state. It is known that at near-critical temperatures, large variations in fluid density and transport properties from gas-like to liquid-like can result from relatively moderate pressure changes around the critical pressure (0.9-1.5 Pc). While liquids are nearly incompressible and have low diffusivity, gases have higher diffusivity and low solvent power. Supercritical fluids can be made to possess an optimum combination of these properties. The high compressibility of supercritical fluids (implying that large changes in fluid density can be brought about by relatively small changes in pressure, making solvent power highly controllable) coupled with their liquid-like solvent power and better-than-liquid transport properties (higher diffusivity, lower viscosity and lower surface tension compared with liquids), provide a means for controlling mass transfer (mixing) between the solvent containing the solutes (such as a drug) and the supercritical fluid.

The two processes that use supercritical fluids for particle formation and that have received attention in the recent past are: (1) Rapid Expansion of Supercritical Solutions (RESS) (Tom, J. W. Debenedetti, P. G., 1991, The formation of bioerodible polymeric microspheres and microparticles by rapid expansion of supercritical solutions. BioTechnol. Prog. 7:403-411), and (2) Gas Anti-Solvent (GAS) Recrystallization (Gallagher, P. M., Coffey, M. P., Krukonis, V. J., and Klasutis, N., 1989, GAS antisolvent recrystallization: new process to recrystallize compounds in soluble and supercritical fluids. Am. Chem. Sypm. Ser., No. 406; Yeo et al. (1993); U.S. Pat. No. 5,360,478 to Krukonis et al.; U.S. Pat. No. 5,389,263 to Gallagher et al.). In the RESS process, a solute (from which the particles are formed) is first solubilized in supercritical CO2 to form a solution. The solution is then, for example, sprayed through a nozzle into a lower pressure gaseous medium. Expansion of the solution across this nozzle at supersonic velocities causes rapid depressurization of the solution. This rapid expansion and reduction in CO2 density and solvent power leads to supersaturation of the solution and subsequent recrystallization of virtually contaminant-free particles. The RESS process, however, may not be suited for particle formation from polar compounds because such compounds, which include drugs, exhibit little solubility in supercritical CO2 Cosolvents (e.g., methanol) may be added to CO2 to enhance solubility of polar compounds; this, however, affects product purity and the otherwise environmentally benign nature of the RESS process. The RESS process also suffers from operational and scale-up problems associated with nozzle plugging due to particle accumulation in the nozzle and to freezing of CO2 caused by the Joule-Thompson effect accompanying the large pressure drop.

In the GAS process, a solute of interest (typically a drug) that is in solution or is dissolved in a conventional solvent to form a solution is sprayed, typically through conventional spray nozzles, such as an orifice or capillary tube, into supercritical CO2 which diffuses into the spray droplets causing expansion of the solvent. Because the CO2-expanded solvent has a lower solubilizing capacity than pure solvent, the mixture can become highly supersaturated and the solute is forced to precipitate or crystallize. The GAS process enjoys many advantages over the RESS process. The advantages include higher solute loading (throughput), flexibility of solvent choice, and fewer operational problems in comparison to the RESS process. In comparison to other conventional techniques, the GAS technique is more flexible in the setting of its process parameters, and has the potential to recycle many components, and is therefore more environmentally acceptable. Moreover, the high pressure used in this process (up to 2,500 psig) can also potentially provide a sterilizing medium for processed drug

particles; however, for this process to be viable, the selected supercritical fluid should be at least partially miscible with the organic solvent, and the solute should be preferably insoluble in the supercritical fluid.

Gallagher et al. (1989) teach the use of supercritical CO2 to expand a batch volume of a solution of nitroguanadine and recrystallize particles of the dissolved solute. Subsequent studies disclosed by Yeo et al. (1993) used laser-drilled, 25-30 μm capillary nozzles for spraying an organic solution into CO2. Use of 100 μm and 151 μm capillary nozzles also has been reported (Dixon, D. J. and Johnston, K. P., 1993, Formation of microporous polymer fibers and oriented fibrils by precipitation with a compressed fluid antisolvent. J. App. Polymer Sci. 50:1929-1942; Dixon, D. G., Luna-Barcenas, G., and Johnson K. P., 1994, Microcellular microspheres and microballoons by precipitation with a vapor-liquid compressed fluid antisolvent. Polymer 35:3998-4005).

Examples of solvents are selected from carbon dioxide (CO2), nitrogen (N2), Helium (He), oxygen (O2), ethane, ethylene, ethylene, ethane, methanol, ethanol, trifluoromethane, nitrous oxide, nitrogen dioxide, fluoroform (CHF3), dimethyl ether, propane, butane, isobutanes, propylene, chlorotrifluormethane (CClF3), sulfur hexafluoride (SF6), bromotrifluoromethane (CBrF3), chlorodifluoromethane (CHClF2), hexafluoroethane, carbon tetrafluoride carbon dioxide, 1,1,1,2-tetrafluoroethane, 1,1,1,2,3,3,3-heptafluoropropane, xenon, acetonitrile, dimethylsulfoxide (DMSO), dimethylformamide (DMF), and mixtures of two or more thereof.

The atomization conditions, including atomization gas flow rate, atomization gas pressure, liquid flow rate, etc., are generally controlled to produce liquid droplets having an average diameter of from about 0.5 μm, about 1 μm, about 5 μm to about 10 μm, about 30 μm, about 50 μm, about 100 μm, with droplets of average size about 10 μm and about 5 μm being preferred. Conventional spray drying equipment is generally used. (Buchi, Niro Yamato, Okawara, Kakoki, and the like). Once the droplets are produced, they are dried by removing the water and leaving the active agent, any excipient(s), and residual buffer(s), solvent(s) or salt(s). This may be done in a variety of ways, such as by lyophilization, as is known in the art. i.e. freezing as a cake rather than as droplets. Generally, and preferably, vacuum is applied, e.g. at about the same temperature as freezing occurred. However, it is possible to relieve some of the freezing stress on the agent by raising the temperature of the frozen particles slightly prior to or

during the application of vacuum. This process, termed “armealing”, reduces agent inactivation, and may be done in one or more steps, e.g. the temperature may be increased one or more times either before or during the drying step of the vacuum with a preferred mode utilizing at least two thermal increases. The particles may be incubated for a period of time, generally sufficient time for thermal equilibrium to be reached, i.e. depending on sample size and efficiency of heat exchange 1 to several hours, prior to the application of the vacuum, then vacuum is applied, and another annealing step is done. The particles may be lyophilized for a period of time sufficient to remove the majority of the water not associated with crystalline structure, the actual period of time depending on the temperature, vacuum strength, sample size, etc.

Spheronization involves the formation of substantially spherical particles and is well known in the art. Commercially available machines for spheronizing drugs are known and include, for example, Marumerizer™ from LCI Corp. (Charlotte, N.C.) and CF-Granulator from Vector Corp. (Marion, Iowa). Such machines include an enclosed cavity with a discharge port, a circular plate and a means to turn the plate, e.g., a motor. Bulk drug or moist granules of drug from a mixer/granulator are fed onto the spinning plate, which forces them against the inside wall of the enclosed cavity. The process results in particles with spherical shape. An alternative approach to spheronization that may be used includes the use of spray drying under controlled conditions. The conditions necessary to spheronize particles using spray-drying techniques are known to those skilled in the art and described in the relevant references and texts, e.g., Remington: The Science and Practice of Pharmacy, Twentieth Edition (Easton, Pa.: Mack Publishing Co., 2000).

In a preferred embodiment, a secondary lyophilization drying step is conducted to remove additional water at temperatures about 0° C., about 10° C., to about 20° C., to about 25° C., with about 20° C. being preferred. The powder is collected then by using conventional techniques, and bulking agents, if desirable, may be added although not required. Once made, the dry powder formulation of the invention may be being readily dispersed by a dry powder inhalation device and subsequently inhaled by a patient so that the particles penetrate into the target regions of the lungs. The powder of the invention may be formulated into unit dosages comprising therapeutically effective amounts of the active agent and used for delivery to a patient, for example, for the prevention and treatment of respiratory and lung disorders.

The dry powder formulation of this invention is formulated and dosed in a fashion consistent with good medical practice, taking into account, for example, the type of disorder being treated, the clinical condition of the individual patient, whether the active agent is administered for preventative or therapeutic purposes, its concentration in the dosage, previous therapy, the patient's clinical history and his/her response to the active agent, the method of administration, the scheduling of administration, the discretion of the attending physician, and other factors known to practitioners. The "effective amount" or "therapeutically effective amount" of the active compound for purposes of this patent include preventative and therapeutic administration, and will depend on the identity of the active agent and is, thus, determined by such considerations and is an amount that increases and maintains the relevant, favorable biological response of the subject being treated. The active agent is suitably administered to a patient at one time or over a series of treatments, preferably once a day, and may be administered to the patient at any time from diagnosis onwards. A "unit dosage" means herein a unit dosage receptacle containing a therapeutically effective amount of a micronized active agent. The dosage receptacle is one that fits within a suitable inhalation device to allow for the aerosolization of the dry powdered formulation by dispersion into a gas stream to form an aerosol. These can be capsules, foil pouches, blisters, vials, etc. The container may be formed from any number of different materials, including plastic, glass, foil, etc, and may be disposable or rechargeable by insertion of a filled capsule, pouch, blister etc. The container generally holds the dry powder formulation, and includes directions for use. The unit dosage containers may be associated with inhalers that will deliver the powder to the patient. These inhalers may optionally have chambers into which the powder is dispersed, suitable for inhalation by a patient.

The dry powdered formulations of the invention may be further formulated in other ways, e.g. as a sustained release composition, for example, for implants, patches, etc. Suitable examples of sustained-release compositions include semi-permeable polymer matrices in the form of shaped articles, e.g. films or microcapsules. Sustained-release matrices include for example polylactides. See for example, U.S. Pat. No. 3,773,919; EP 58,481. Copolymers of L-glutamic acid and gamma-ethyl-L-glutamate are also suitable. See, e.g. Sidman et al., Biopolymers 22: 547-556 (1983) as poly(2-hydroxyethyl methacrylate). See Langer et al., J. Biomed. Mater. Res. 15: 167-277 (1981); Langer, Chem. Tech., 12: 98-105 (1982). Also suitable are ethylene vinyl acetate and poly-D-(−)-3-hydroxybutyric acid. See, Langer et al, supra; (EP 133,988). Sustained-release compositions also include liposomally entrapped agent, that may be prepared by

known methods. See, for example, DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. USA 82: 3688-3692 (1985); Hwang et al., Proc. Natl. Acad. Sci. USA 77: 4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese Pat. Application 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; EP 102,324. The relevant sections of all referenced techniques are hereby incorporated by reference. Ordinarily, the liposomes are of the small unilamellar liposomes in about 200 to 800 Angstroms which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for optimal therapy.

In a preferred embodiment, the dry powdered formulation in the invention may not be inhaled but rather injected as a dry powder, using relatively new injection devices and methodologies for injecting powders. In this embodiment, the dispersibility and respirability of the powder is not important, and the particle size may be larger, for example in about 10 μm, about 20 μm to about 40 μm, about 50 μm to about 70 μm, about 100 μm. The dry powdered formulations in the invention may be reconstituted for injection as well. As the powder of the invention shows good stability, it may be reconstituted into liquid form using a diluent and then used in non-pulmonary routes of administration, e.g. by injection, subcutaneously, intravenously, etc. Known diluents may be used, including physiological saline, other buffers, salts, as well as non-aqueous liquids etc. It is also possible to reconstitute the dry powder of the invention and use it to form liquid aerosols for pulmonary delivery, either for nasal or intrapulmonary administration or for inhalation. As used herein, the term “treating” refers to therapeutic and maintenance treatment as well as prophylactic and preventative measures. Those in need of treatment include those already diagnosed with the disorder as well as those prone to having the disorder and those where the disorder is to be prevented. Consecutive treatment or administration refers to treatment on at least a daily basis without interruption in treatment for one or more days. Intermittent treatment or administration, or treatment or administration in an intermittent fashion, refers to treatment that is not consecutive, but rather cyclic in nature. The treatment regime herein may be consecutive or intermittent or of any other suitable mode. The dry powdered formulation may be obtained, for example, by sieving, lyophilization, spray-lyophilization, spray drying, and freeze drying, etc. These methods may be combined for improved effect. Filters may be employed for sieving, as will be known to a skilled artisan. The alteration and selection of the agent's particle size may be conducted in a single step, preferably, by micronizing under conditions effective to attain the desired particle size as previously described.

The dry powdered formulation may be then stored under controlled conditions of temperature, humidity, light, pressure etc., so long as the flowability of the agent is preserved. The agent's stability upon the storing may be measured at a selected temperature for a selected time period and for rapid screening a matrix of conditions are run, e.g. at 2-8° C., 30° C. and sometimes 40° C., for periods of 2, 4 and 24 weeks. The length of time and conditions under which a formulation should be stable will depend on a number of factors, including the above, amount made per batch, storage conditions, turnover of the product, etc. These tests are usually done at 38% (rh) relative humidity. Under these conditions, the agent generally loses less than about 30% biological activity over 18 months, sometimes less than about 20%, or less than about 10%. The dry powder of the invention loses less than about 50% FPF, in some cases less than about 30%, and in others less than about 20%.

The dry powder formulation of the invention may be combined with formulation ingredients, such as bulking agents or carriers, which are used to reduce the concentration of the agent in the dry powder being delivered to a patient. The addition of these ingredients to the formulation is not required, however, in some cases it may be desirable to have larger volumes of material per unit dose. Bulking agents may also be used to improve the flowability and dispersibility of the powder within a dispersion device, or to improve the handling characteristics of the powder. This is distinguishable from the use of bulking agents or carriers during certain particle size reduction processes (e.g. spraying drying). Suitable bulking agents or excipients are generally crystalline (to avoid water absorption) and include, but are not limited to, lactose and mannitol. If lactose, is added, for example, in amounts of about 99: about 1: about 5: active agent to bulking agent to about 1:99 being preferred, and from about 5 to about 5: and from about 1:10 to about 1:20.

The dry powder formulations of the invention may contain other drugs, e.g., combinations of therapeutic agents may be processed together, e.g. spray dried, or they may be processed separately and then combined, or one component may be spray dried and the other may not, while it is processed in one of the other manners enabled herein. The combination of drugs will depend on the disorder for which the drugs are given, as will be appreciated by those in the art. The dry powder formulation of the invention may also comprise, as formulation ingredients, excipients, preservatives, detergents, surfactants, antioxidants, etc, and may be administered by any means that transports the agent to the airways by any suitable means, but are preferably administered through the respiratory system as a respirable formulation, more

preferably in the form of an aerosol or spray comprising the agent's particles, and optionally, other therapeutic agents and formulation ingredients.

In another embodiment, the dry powdered formulations may comprise the dry pharmaceutical agent of this invention and one or more surfactants. Suitable surfactants or surfactant components for enhancing the uptake of the active compounds used in the invention include synthetic and natural as well as full and truncated forms of surfactant protein A, surfactant protein B, surfactant protein C, surfactant protein D and surfactant protein E, disaturated phosphatidylcholine (other than dipalmitoyl), dipalmitoylphosphatidylcholine, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, ubiquinones, lysophosphatidylethanolamine, lysophosphatidylcholine, palmitoyl-lysophosphatidylcholine, dehydroepiandrosterone, dolichols, sulfatidic acid, glycerol-3-phosphate, dihydroxyacetone phosphate, glycerol, glycero-3-phosphocholine, dihydroxyacetone, palmitate, cytidine diphosphate (CDP) diacylglycerol, CDP choline, choline, choline phosphate; as well as natural and artificial lamelar bodies which are the natural carrier vehicles for the components of surfactant, omega-3 fatty acids, polyenic acid, polyenoic acid, lecithin, palmitinic acid, non-ionic block copolymers of ethylene or propylene oxides, polyoxypropylene, monomeric and polymeric, polyoxyethylene, monomeric- and polymeric-, poly(vinylamine) with dextran and/or alkanoyl side chains, Brij 35®, Triton X-100®, and synthetic surfactants ALEC®, Exosurt®, Survan®, and Atovaquone®, among others. These surfactants may be used either as single or part of a multiple component surfactant in a formulation, or as covalently bound additions to the active compounds.

Examples of other therapeutic agents for use in the present formulation are analgesics such as Acetaminophen, Anilerdine, Aspirin, Buprenorphine, Butabital, Butorpphanol, Choline Salicylate, Codeine, Dezocine, Diclofenac, Diflunisal, Dihydrocodeine, Elcatoninin, Etodolac, Fenoprofen, Hydrocodone, Hydromorphone, Ibuprofen, Ketoprofen, Ketorolac, Levorphanol, Magnesium Salicylate, Meclofenamate, Mefenamic Acid, Meperidine, Methadone, Methotrimeprazine, Morphine, Nalbuphine, Naproxen, Opium, Oxycodone, Oxymorphone, Pentazocine, Phenobarbital, Propoxyphene, Salsalate, Sodium Salicylate, Tramadol and Narcotic analgesics in addition to those listed above. See, Mosby's Physician's GenRx.

Anti-anxiety agents are also useful including Alprazolam, Bromazepam, Buspirone, Chlordiazepoxide, Chlormezanone, Clorazepate, Diazepam, Halazepam, Hydroxyzine,

Ketaszolam, Lorazepam, Meprobamate, Oxazepam and Prazepam, among others. Anti-anxiety agents associated with mental depression, such as Chlordiazepoxide, Amitriptyline, Loxapine Maprotiline and Perphenazine, among others. Anti-inflammatory agents such as non-rheumatic Aspirin, Choline Salicylate, Diclofenac, Diflunisal, Etodolac, Fenoprofen, Floctafenine, Flurbiprofen, Ibuprofen, Indomethacin, Ketoprofen, Magnesium Salicylate, Meclofenamate, Mefenamic Acid, Nabumetone, Naproxen, Oxaprozin, Phenylbutazone, Piroxicam, Salsalate, Sodium Salicylate, Sulindac, Tenoxicam, Tiaprofenic Acid, Tolmetin, anti-inflammatories for ocular treatment such as Diclofenac, Flurbiprofen, Indomethacin, Ketorolac, Rimexolone (generally for post-operative treatment), anti-inflammatories for, non-infectious nasal applications such as Beclomethaxone, Budesonide, Dexamethasone, Flunisolide, Triamcinolone, and the like. Soporifics (anti-insomnia/sleep inducing agents) such as those utilized for treatment of insomnia, including Alprazolam, Bromazepam, Diazepam, Diphenhydramine, Doxylamine, treatments such as Tricyclic Antidepressants, including Amitriptyline HCl (Elavil), Amitriptyline HCl, Perphenazine (Triavil) and Doxepin HCl (Sinequan). Examples of tranquilizers Estazolam, Flurazepam, Halazepam, Ketazolam, Lorazepam, Nitrazepam, Prazepam Quazepam, Temazepam, Triazolam, Zolpidem and Sopiclone, among others. Sedatives including Diphenhydramine, Hydroxyzine, Methotrimeprazine, Promethazine, Propofol, Melatonin, Trimeprazine, and the like.

Sedatives and agents used for treatment of petit mal and tremors, among other conditions, such as Amitriptyline HCl; Chlordiazepoxide, Amobarbital; Secobarbital, Aprobarbital, Butabarbital, Ethchiorvynol, Glutethimide, L-Tryptophan, Mephobarbital, MethoHexital Na, Midazolam HCl, Oxazepam, Pentobarbital Na, Phenobarbital, Secobarbital Na, Thiamylal Na, and many others. Agents used in the treatment of head trauma (Brain Injury/Ischemia), such as Enadoline HCl (e.g. for treatment of severe head injury; orphan status, Warner Lambert), cytoprotective agents, and agents for the treatment of menopause, menopausal symptoms (treatment), e.g. Ergotamine, Belladonna Alkaloids and Phenobarbital, for the treatment of menopausal vasomotor symptoms, e.g. Clonidine, Conjugated Estrogens and Medroxyprogesterone, Estradiol, Estradiol Cypionate, Estradiol Valerate, Estrogens, conjugated Estrogens, esterified Estrone, Estropipate, and Ethinyl Estradiol. Examples of agents for treatment of pre-menstrual syndrome (PMS) are Progesterone, Progestin, Gonadotrophic Releasing Hormone, Oral contraceptives, Danazol, Luprolide Acetate, Vitamin B6. Examples of agents for treatment of emotional/psychiatric, anti-depressants and anti-anxiety agents are Diazepam (Valium), Lorazepam (Ativan),

Alprazolam (Xanax), SSRI's (selective Serotonin reuptake inhibitors), Fluoxetine HCl (Prozac), Sertaline HCl (Zoloft), Paroxetine HCl (Paxil), Fluvoxamine Maleate (Luvox), Venlafaxine HCl (Effexor), Serotonin, Serotonin Agonists (Fenfluramine), and other over the counter (OTC) medications.

Such combination therapeutic formulations can be manufactured using many conventional techniques. It may be necessary to micronize the active compounds and if appropriate (i.e. where an ordered mixture is not intended) any carrier in a suitable mill, for example in a jet mill at some point in the process, in order to produce primary particles in a size range appropriate for maximal deposition in the lower respiratory tract (i.e., from about 0.1 μm to about 10 μm). For example, one can dry mix DHEA and carrier, where appropriate, and then micronize the substances together; alternatively, the substances can be micronized separately, and then mixed. Where the compounds to be mixed have different physical properties such as hardness and brittleness, resistance to micronization varies and they may require different pressures to be broken down to suitable particle sizes. When micronized together, therefore, the obtained particle size of one of the components may be unsatisfactory. In such case it would be advantageous to first micronize the different components separately and then mix them.

It is also possible first to dissolve the active component including, where an ordered mixture is not intended, any carrier in a suitable solvent, e.g. water, to obtain mixing on the molecular level. This procedure also makes it possible to adjust the pH-value to a desired level. The pharmaceutically accepted limits of pH 3.0 to 8.5 for inhalation products must be taken into account, since products with a pH outside these limits may induce irritation and constriction of the airways. To obtain a powder, the solvent must be removed by a process which retains the biological activity of DHEA. Suitable drying methods include vacuum concentration, open drying, spray drying, freeze drying and use of supercritical fluids. Temperatures over 50° C. for more than a few minutes should generally be avoided, as some degradation of the DHEA may occur. After drying step the solid material can, if necessary, be ground to obtain a coarse powder, and then, if necessary, micronized.

If desired, the micronized powder can be processed to improve the flow properties, e.g., by dry granulation to form spherical agglomerates with superior handling characteristics, before it is incorporated into the intended inhaler device. In such a case, the device would be configured to ensure that the agglomerates are substantially deagglomerated prior to exiting the device, so that the particles entering the respiratory

tract of the patient are largely within the desired size range. Where an ordered mixture is desired, the active compound may be processed, for example by micronization, in order to obtain, if desired, particles within a particular size range. The carrier may also be processed, for example to obtain a desired size and desirable surface properties, such as a particular surface to weight ratio, or a certain texture, and to ensure optimal adhesion forces in the ordered mixture. Such physical requirements of an ordered mixture are well known, as are the various means of obtaining an ordered mixture which fulfils the said requirements, and may be determined easily by one skilled in the art.

The dry powder formulation of this invention may be administered into the respiratory tract as a formulation of respirable size particles i.e. particles of a size sufficiently small to pass through the nose, mouth, larynx or lungs upon inhalation, nasal administration or lung instillation, to the bronchi and alveoli of the lungs. In general, respirable particles range from about 0.1 μm to about 100 μm, and inhalable particles are about 0.1 μm to about 10 μm, to about 5 μm in size. Mostly, when inhaled, particles of non-respirable size that are included in the aerosol tend to deposit in the throat and be swallowed, which reduces the quantity of nonrespirable particles in the aerosol. For nasal administration, a particle size in the range of about 10 μm to about 20 μm, about 50 μm, about 60 μm, or about 100 μm, is preferred to ensure retention in the nasal cavity.

The size and shape of the particles may be analyzed using known techniques for determine and ensure proper particle morphology. For example, one skilled in the art can visually inspect the particles under a microscope and/or determine particle size by passing them through a mesh screen. Preferred techniques for visualization of particles include scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Particle size analysis may take place using laser diffraction methods. Commercially available systems for carrying out particle size analysis by laser diffraction are available from Clausthal-Zellerfeld, Germany (HELOS H1006).

The dry powdered formulation of the invention may be delivered with any device that generates solid particulate aerosols, such as aerosol or spray generators. These devices produce respirable particles, as explained above, and generate a volume of aerosol or spray containing a predetermined metered dose of a medicament at a rate suitable for human or animal administration. One illustrative type of solid particulate aerosol or spray generator is an insufflator, which are suitable for administration of finely

comminuted powders. The latter may be taken also into the nasal cavity in the manner of a snuff. In the insufflator, the powder, e.g. a metered dose of the agent effective to carry out the treatments described herein, is contained in a capsule or a cartridge. These capsules or cartridges are typically made of gelatin, foil or plastic, and may be pierced or opened in situ, and the powder delivered by air drawn through the device upon inhalation or by means of a manually-operated pump. The dry powder formulation employed in the insufflator may consist either solely of the agent or of a powder blend comprising the agent, and the agent typically comprises from 0.01 to 100% w/w of the formulation. The dry powdered formulation generally contains the active compound in an amount of about 0.01% w/w, about 1% w/w/, about 5% w/w, to about 20%, w/w, about 40% w/w, about 99.99% w/w. Other ingredients, and other amounts of the agent, however, are also suitable within the confines of this invention.

In a preferred embodiment, the dry powdered formulation is delivered by a nebulizer. This is means is especially useful for patients or subjects who are unable to inhale or respire the powder pharmaceutical composition under their own efforts. In serious cases, the patients or subjects are kept alive through artificial respirator. The nebulizer can use any pharmaceutically or veterinarily acceptable carrier, such as a weak saline solution. Preferably, the weak saline solution is less than about 1.0 or 0.5% NaCl. More preferably, the weak saline solution is less than about 0.2% or 0.15% NaCl. Even more preferably, the weak saline solution is less than about 0.12% NaCl. The nebulizer is the means by which the powder pharmaceutical composition is delivered to the target of the patients or subjects in the airways. The stability of anhydrous compounds, such as anhydrous DHEA-S, can be maintained or increased by eliminating or reducing the water content within the sealed container, e.g. vial, containing the compound. Preferably, besides the compound, it is a vacuum within the sealed container.

The formulation of the invention is also provided in various forms that are tailored for different methods of administration and routes of delivery. The formulations that are contemplated are, for example, a transdermal formulation also containing an excipient and other agents suitable for delivery through the skin, mouth, nose, vagina, anus, eyes, and other body cavities, intradermally, as a sustained release formulation, intrathecally, intravascularly, by inhalation, nasally, intrapulmonarily, into an organ, by implantation, by suppositories, as cremes, gels, and the like, all known in the art. In one embodiment, the dry powdered formulation comprises a respirable formulation, such as an aerosol or spray. The dry powder formulation of the invention is provided in bulk, and in unit form, as well as in the form of an implant, a capsule, blister or

cartridge, which may be openable or piercable as is known in the art. A kit is also provided, that comprises a delivery device, and in separate containers, the dry powdered formulation of the invention, and optionally other excipient and therapeutic agents, and instructions for the use of the kit components.

In one preferred embodiment, the agent is delivered using suspension metered dose inhalation (MDI) formulation. Such a MDI formulation can be delivered using a delivery device using a propellant such as hydrofluroalkane (HFA). Preferably, the HFA propellants contain 100 parts per million (PPM) or less of water. N. C. Miller (In: Respiratory Drug Delivery, P. R. Bryon (ed.), CRC Press, Boca Raton, 1990, pp. 249-257) reviewed the effect of water content on crystal growth in MDI suspensions. When exposed to water, anhydrous DHEA-S will hydrate and eventually form large particles. This hydration process can happen in a suspension of the anhydrous DHEA-S in an HFA propellant which has a water content. This hydration process would accelerate the crystal growth due to the formation of strong interparticle bonds and cause the formation of large particles. In contrast, the dihydrate form is already hydrated thus more stable, and thus more preferred, than the anyhydrous form in a MDI, as the dihydrate form will not further form larger particles. If DHEA-S forms a solvate with a HFA propellant that has a lower energy than the dihydrate form, then this DHEA-S solvate would be the most stable, and hence more preferred, form for an MDI.
In one preferred embodiment, the delivery device comprises a dry powder inhalator (DPI) that delivers single or multiple doses of the formulation. The single dose inhalator may be provided as a disposable kit which is sterilely preloaded with enough formulation for one application. The inhalator may be provided as a pressurized inhalator, and the formulation in a piercable or openable capsule or cartridge. The kit may optionally also comprise in a separate container an agent such as other therapeutic compounds, excipients, surfactants (intended as therapeutic agents as well as formulation ingredients), antioxidants, flavoring and coloring agents, fillers, volatile oils, buffering agents, dispersants, surfactants, antioxidants, flavoring agents, bulking agents, propellants and preservatives, among other suitable additives for the different formulations. The dry powdered formulation of this invention may be utilized by itself or in the form of a composition or various formulations in the treatment and/or prevention of a disease or condition associated with bronchoconstriction, allergy(ies), lung cancer and/or inflammation. Examples of diseases are airway inflammation, allergy(ies), asthma, impeded respiration, CF, COPD, AR,ARDS, pulmonary hypertension, lung inflammation, bronchitis, airway obstruction, bronchoconstriction, microbial infection, viral infection (such as SARS), among others. Clearly the present

formulation may be administered for treating any disease that afflicts a subject, with the above just being examples. Typically, the dry powdered formulation may be administered in an amount effective for the agent to reduce or improve the symptom of the disease or condition.

The dry powdered formulation may be administered directly to the lung(s), preferably as a respirable powder, aerosol or spray. Although an artisan will know how to titrate the amount of dry powdered formulation to be administered by the weight of the subject being treated in accordance with the teachings of this patent, the agent is preferably administered in an amount effective to attain an intracellular concentration of about 0.05 to about 10 μM agent, and more preferably up to about 5 μM. Propellants may be employed under pressure, and they may also carry co-solvents. The dry powdered formulation of the invention may be delivered in one of many ways, including a transdermal or systemic route, orally, intracavitarily, intranasally, intraanally, intravaginally, transdermally, intrabucally, intravenously, subcutaneously, intramuscularly, intratumorously, into a gland, by implantation, intradermally, and many others, including as an implant, slow release, transdermal release, sustained release formulation and coated with one or more macromolecules to avoid destruction of the agent prior to reaching the target tissue. Subject that may be treated by this agent include humans and other animals in general, and in particular vertebrates, and amongst these mammals, and more specifically and small and large, wild and domesticated, marine and farm animals, and preferably humans and domesticated and farm animals and pets.

Combination Therapies – Other agents

In certain embodiments, the Affimer® Agent can be administered conjointly with one or more agents that have other beneficial local activities in pulmonary tract.

In certain embodiments, the Affimer® Agent is conjointly administered with an anti-inflammatory agent selected from an IL-1 inhibitor, an IL-1 receptor (IL-1R) inhibitor, an IL-6 inhibitor, an IL-6 receptor (IL-6R) inhibitor, a NLRP3 inhibitor, a TNF inhibitor, an IL-8 inhibitor, an IL-18 inhibitor, an inhibitor of natural killer cells, or combinations thereof. In some embodiments, the anti-inflammatory agent is a nucleic acid, an aptamer, an antibody or antibody fragment, an inhibitory peptide, or a small molecule.

In certain embodiments, the Affimer® Agent is conjointly administered with an an NLRP3 inhibitor. In some embodiments, the NLPR3 inhibitor is an anti-sense oligonucleotide against NLPR3, colchicine, MCC950, CY-09, ketone metabolite beta-hydroxubutyrate (BHB), a type I interferon, resveratrol, arglabin, CB2R, Glybenclamide, Isoliquiritigenin, Z-VAD-FMK, or microRNA-223.

In certain embodiments, the Affimer® Agent is conjointly administered with a TNF inhibitor. In some embodiments, the TNF inhibitor is an anti-sense oligonucleotide against TNF, infliximab, adalimumab, certolizumab pegol, golimumab, etanercept (Enbrel), thalidomide, lenalidomide, pomalidomide, a xanthine derivative, bupropion, 5-HT2A agonist or a hallucinogen.

In certain embodiments, the Affimer® Agent is conjointly administered with an IL-18 inhibitor. In some embodiments, the IL-18 inhibitor is selected from the group consisting of: anti-sense oligonucleotides against IL-18, IL-18 binding protein, IL-18 antibody, NSC201631, NSC61610, and NSC80734.

In certain embodiments, the Affimer® Agent is conjointly administered with an inhibitor of natural killer cells. In some embodiments, the inhibitor of natural killer cells is an antibody targeting natural killer cells.

In certain embodiments, the Affimer® Agent is conjointly administered with methotrexate.

In certain embodiments, the Affimer® Agent is conjointly administered with arhalofenate.

In certain embodiments, the Affimer® Agent is conjointly administered with an IL-10 inhibitor.

FURTHER ASPECTS

In another aspect the invention relates to single domain binders, such as Affimer® Agents, capable of inhibiting viral infection.

The inventors describe the first examples of actual ability of small domains to interfere with viral infection where the person skilled in the art would not have been able to

predict that capability based on the potential surface area and the number of interactions between a single viral particle.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is now described by way of examples/embodiments, with reference to the accompanying drawings, in which:

Figure 1 shows diagrams - SARS-CoV-2 Binders May Block Viral Activation and Infectivity.

Figure 2 shows graphs - Affimer® Agent Validation Competition ELISA. ACE2 passively adsorbed to a MaxiSorp™ plate at 1 ug/mL to capture biotinylated SARS-CoV-2 (80 ng/mL) pre-incubated with Affimer® Agent candidates or ACE2 (titrated from 100 to 0.1 μg/mL, detected with streptavidin poly-HRP). Figure 2A: Three candidates inhibit the binding of SARS-CoV-2 to ACE2: 825436, 825425 and 825336; 826257 does not compete with ACE2 binding. Figure 2B: some Affimer® Agents bind to the RBD and are thus classified as ACE2 competing (e.g., 620_825436 and 620_825425), whereas other Affimer® Agents do not bind to RBD and are thus classified as ACE2 pairing (e.g., 620_826257). Figure 2C: exemplary Affimer® Agents 825425 and 825436 bind to the RBD comprising the Y453F mutation.

Figure 3 shows a flowchart illustrating the six phage display strategies that were employed with the aim of testing immobilised SARS-CoV-2 S1 protein against the single and double loop plant Stefin (Cystatin) derived type 2 Affimer® protein library (T2), as well as the human Stefin-A derived type 3r2 Affimer® protein libraries (T3r2) at Avacta® Life Sciences in Wetherby, as described in detail in Example 1.

Figure 4 shows lateral flow devices.

Figure 5 shows tables of polypeptides of the invention. These show ranked lists for each of the three most suitable assay types described herein, and a combined rank list (far left table). A ranking of 1 indicates a lead reagent (lead polypeptide) most suitable for inclusion in final assays, whereas a ranking of 5 indicates weaker performance (less advantageous for inclusion in a final assay). In a broad aspect all polypeptides shown ranked 1, 2, 3, 4 or 5 find application in the invention. More suitably the polypeptide(s) of the invention are those ranked 1, 2, 3 or 4. More suitably the polypeptide(s) of the invention are those ranked 1, 2 or 3. More suitably the polypeptide(s) of the invention are those ranked 1 or 2. More suitably the polypeptide(s) of the invention are those ranked 1 for a particular assay. More suitably the polypeptide(s) of the invention are those ranked 1 for two or more assays. Most suitably the polypeptide(s) of the invention are those ranked 1 for all three assays (i.e. ELISA and LFD and BAMS assays).

Figure 6 shows a graph of Purification of polypeptide of the invention (Affimer® Agent Candidate) 620_826257

Figure 7 shows a graph of Purification of polypeptide of the invention (Affimer® Agent Candidate) 620_825425

Figure 8 shows a graph of Purification of polypeptide of the invention (Affimer® Agent Candidate) 620_825436

Figure 9 shows a photograph of an SDS-PAGE 2 μg QC gel of Affimer® proteins: M = Marker; 1 = Control Affimer® protein 2μg mIgG$_{2b}$ G12 c3xFLAG-His$_6$-Cys; 2 -4 = Irrelevant Affimer® proteins; 5 = Sample ID 6353 Affimer® Candidate 620_826257 c3xFLAG-His$_6$-Cys; 6 = Sample ID 6342 Affimer® Candidate 620_825425 c3xFLAG-His$_6$-Cys; 7 = Sample ID 6343 Affimer® Candidate 620_825436 c3xFLAG-His$_6$-Cys; 8-10 = Irrelevant Affimer® proteins.

Figure 10 shows two bar charts. Exemplary ELISA results showing optical density data for a top concentration of 2.5 ng/mL of S1 spike protein reagents, specifically capture with 620_825436-620_825436-cHis6 and 620_825425-620_825425-cHis6; detection with 620_826257 c3xFLAG-His6-CysPEG$_2$Biotin; developed/visualised with Streptavidin, poly-HRP conjugate (Pierce, 21140)). The assay was challenged with several coronaviruses. This anti-SARS-CoV-2 S1 protein ELISA recognises SARS-CoV-2 S1 only. No interaction was observed with any of the other coronaviruses tested.

Figure 11 shows three bar charts of optical densities observed against biotinylated S1 proteins at 55 ng/mL for three exemplary polypeptides of the invention.

Figure 12 shows a graph showing exemplary accuracy and precision (A&P) curve data.

Figure 13 shows diagrams showing schematic representation of ELISA formats. A. Direct ELISA: target passively adsorbed and reporter enzyme directly conjugated to immunoreagent. B. Indirect ELISA: target passively adsorbed and reporter enzyme conjugated to a secondary immunoreagent. C. Sandwich (Pair) ELISA: in this example a capture immunoreagent is immobilised, binding the target and detected with a detection immunoreagent with the reporter enzyme conjugated. D. Competition ELISA: in this example the immunoreagent competes with the target's ligand for binding with the labelled target protein, therefore, the more competitive the immunoreagent, the lower the signal.

Figure 14 shows a photograph of an exemplary lateral flow assay device (LFD) according to the present invention.

Figure 15 shows graphs showing data from ELISA assays performed with PEG$_2$-biotinylated detection Affimer® Agent (Figure 15 A) and in vivo biotinylated detection Affimer® Agent (Figure 15 B), each assessed in both 50% saliva and in buffer.

Figure 16 shows graphs demonstrating that the anti-SARS-CoV-2 S1 protein ELISA produced comparable results for the detection of SARS-CoV-2 S1 spike protein or SARS-CoV-2 S1 (D614G) spike protein.

Figure 17 shows graphs demonstrating that the anti-SARS-CoV-2 S1 protein ELISA can detect both SARS-CoV-2-S1 spike protein or SAR-CoV-2 S spike protein trimer.

Figure 18 shows exemplary results achieved in the Affimer capture assay (ELISA) for nine of those Affimer® candidates that worked well as capture reagents for SARS-CoV-2 S1 spike protein (Figure 18A) and for seven of those Affimer® candidates that worked well as capture reagents in sandwich ELISA using the detection Affimer candidate 620_826257 (Figure 18B).

Figure 19 shows exemplary ELISA data using two or more capture Affimer® reagents, which demonstrates that the sensitivity of the assay is significantly increased by using multiple capture Affimer® reagents together.

EXAMPLES

EXAMPLE 1: Affimer® Agent Production Process

Target QC

Several different sources of SARS-CoV-2 S1 protein were procured from different suppliers and each with different tags. These included a his tagged version of the S1 protein from Sino biological, a Fc tagged form from Native Antigen Company and both a biotinylated avi-tagged form and an unbiotinylated his-tagged S1 protein supplied by Acro biosystems. Purchasing the target protein from multiple sources provided us with a measure of redundancy in the event that one of the targets failed to meet our required standards for phage display. With the downstream applications of these Affimer® proteins in mind we realized that the specificity of all identified binders was of the utmost importance. Therefore, several deselection targets were also purchased which could be used either to increase the stringency of the phage display, or to determine the specificity of any potential binders during primary screening. These included the S1 surface protein from the coronaviruses responsible for Severe Acute Respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS). The S1 proteins from three human coronavirus strains responsible for respiratory infections similar to and including the common cold were also purchased - HKU1, 229E and NL63. Finally, in order to test the functionality of the target proteins as well as determine ways that the Affimer® proteins could potentially be used in a diagnostic test, the angiotensin converting enzyme 2 (ACE2) was purchased. ACE2 is a human enzyme attached to the

surface of cells in the respiratory tract which is used by SARS-CoV-2 to gain entry into cells via the viral coat glycoprotein S1.

Initially the targets were analysed by SDS-PAGE in order to give us an idea of their purity. From here in order for them to be immobilised in several of the downstream assays (including the phage display) all of the targets were biotinylated using an NHS-Biotinylation reagent. Successful biotinylation was confirmed by western blotting. It was also a priority to ensure that the target used in phage display was still functional (i.e. still able to bind ACE2). Therefore, SPRI was carried out in both orientations (A chip coated with ACE2 and a chip coated with SARS-CoV-2 S1) with either binding partner immobilised on a chip and the other flowed over it in order to confirm binding. ELISAs were also carried out as a secondary assay to confirm S1's functionality. As before the assay was carried out in both orientations: the aspecific adsorption of ACE2 on a surface followed by biotinylated S1 protein and detection with streptavidin-HRP, and vice versa with the adsorption of S1 followed by the addition of biotinylated ACE2. This assay was particularly important as we needed to ensure that our biotinylation methods did not impair S1's function (and by doing so make it less representative of the native protein) in any way.

Phage Display

In order to take advantage of the vast diversity covered by our several Affimer® protein libraries, six phage display strategies were employed with the aim of testing immobilised SARS-CoV-2 S1 protein against the single and double loop plant Stefin (Cystatin) derived type 2 Affimer® protein library (T2), as well as the human Stefin-A derived type 3r2 Affimer® protein libraries (T3r2) at Avacta® life Sciences in Wetherby.

The typical phage display procedure carried out at Avacta® life sciences involves immobilizing a biotinylated target on a streptavidin coated surface and subjecting these targets to three panning rounds of phage display with the first (henceforth called P1) using purified naïve Affimer® protein phage library. Once unbound phage has been washed away, the desired target binding phage are eluted by incubating them in a low pH buffer followed by a high pH buffer and finally by the addition of trypsin. After each incubation the eluted phage is collected and kept on ice until it can be used to infect a suitable *E.coli* strain. The second and third panning rounds (henceforth called P2 and P3) use phage amplified bacterial supernatant from P1 and P2 respectively. In order to increase the specificity of the selection, the library can be deselected in each panning round against undesirable targets ranging from tags to protein homologs.

There are two alternative types of panning round that are routinely carried out at Avacta® life sciences, the first is denoted as 'standard' and involves carrying out the addition of phage to the immobilised target of interest, extensive washing to remove any unbound phage and the immediate elution of the bound phage ready for the infection of *E.coli*. The second is called 'Off-rate' and involves an extended incubation of the bound phage to the target after the extensive washing. This incubation ranges from 1 h (known as an off-rate selection with low stringency) to 24 h (anything above 22 h is known as a high stringency off rate selection). These alternative panning round types were designed to allow us to modulate the quality and characteristics of the generated phage after a selection. Phage derived from a standard selection tend to show a higher level of sequence diversity in binders to a single target, while phage derived from an off-rate selection tend to show a lower degree of diversity but a higher affinity for the target. For the six selection strategies against SARS-CoV-2 S1 protein, pan 1 was performed as a standard selection, while both P2 and P3 were high stringency off rate panning rounds with a minimum incubation time of 22 hours.

The first and second selections (henceforth called S01 and S02) used a single source of SARS-CoV-2 S1 (the biotinylated avi-tagged protein purchased from Acro Biosystems) with no deselection. S01 used an equal mix of the single and two-loop T2 Affimer® protein libraries while S02 used the T3r2 library alone. The third and fourth selections (S03 and S04) used the same SARS-CoV-2 S1 as before, but included deselections against the S1 glycoproteins from MERS in P2 and SARS in P3 with S03 using the mix of T2 libraries and S04 using the T3r2 library.

Keeping in mind the high degree of specificity Affimer® proteins typically show toward their targets, selections 5 and 6 (S05 and S06) switched the source of the S1 protein between panning rounds with SARS-CoV-2-S1 from Sino biological in P1 and P3, while P2 used the same S1 protein from Acro biosystems used in the other selections. S05 used the mixed T2 library while S06 used T3r2 and both selections included deselection against the MERS and SARS S1 proteins in P2 and P3 respectively. These selections aimed to develop Affimer® proteins able to recognize the SARS-CoV-2 S1 protein regardless of its source with a high degree of specificity.

The particular spike proteins used in these selection S01-S06 are as follows:

Commercially Available Spike 1 Target used for S01-S04 selections, screening and *in vitro* validation (from Acro Biosystems 1 Innovation Way, Newark, DE 19711, USA). (Spike protein with amino acid sequence of accession number Accession # QHD43416.1 *ibid.*)

Biotinylated SARS-CoV-2 (COVID-19) S1 protein, His,AviTag™ (MALS verified) – Catalogue number S1N-C82E8
SARS-CoV-2 (COVID-19) S1 protein, His Tag - Catalogue number S1N-C52H3

S05 and S06 selections used target from Sino Biological (in addition to Acro Biosystems)
SARS-CoV-2 (2019-nCoV) Spike S1-His Recombinant Protein (HPLC-verified) - Catalogue number 40591-V08H
GenBank: (NCBI Reference Sequence) accession number: YP_009724390.1
Identical sequence to above.
Figure 3 shows a flowchart outlining the selection procedures used in producing the exemplary polypeptides described herein.

High throughput cycle

Typically at Avacta® life sciences, the polyclonal DNA of phage display selection outputs are subcloned into expression vectors using a pETLECTRA plasmid system. This expression system was chosen as it enables the wholesale subcloning of polyclonal phagemid into pETLECTRA expression vectors via a single restriction endonuclease reaction followed by a ligation (both of which can be mixed in a single reaction tube). The ligated plasmids are then used to transform BL21 gold (DE3), plated on agar plates supplemented with kanamycin (the antibiotic to which the pETLECTRA vector confers resistance) and up to 96 single colonies are picked, grown in fresh cultures, subjected to IPTG expression and purified using nickel resin. Several of these processes utilise large automated liquid handling platforms in order to maximize efficiency and throughput. Once the Affimer® proteins are purified their purity and concentrations are checked using the GXII Labchip before they are placed back on an automated Hamilton liquid handling platform which normalises the concentrations of each of the Affimer® proteins to 25 μg/mL across a 96 well plate. A small sample of these normalized plates were then taken and diluted ten-fold (to 2.5 μg/mL) in a separate 386 well plate. These two normalized Affimer® protein plates were stored at 4°C ready for the primary screening.
As routine this process is carried out on panning rounds 2 and 3 of any selection, it was designed to provide the maximum amount of freedom in how the Affimer® proteins are tagged as various alternative pETLECTRA vectors are stored on site, each of which housing an alternative set of tags, with the exception of a His tag – which is universal. We know that for an effective SARS-CoV-2 diagnostic assay having two Affimer® proteins capable of working as a pair (i.e. one immobilised on a surface capable of

capturing the target and second used to detect the captured target) would be ideal. Therefore we decided to subclone the Pan 2 and 3 outputs from S01 and S02 into a pETLECTRA vector with a C-terminal 3x FLAG tag (upstream from the His6 tag and an accessible cystine); while S03 – S06 were subcloned into a vector housing a C-terminal HA tag (upstream from the His tag and cysteine as before). In this way HA tagged Affimer® proteins can be used to capture S1 in solution and subsequently FLAG tagged Affimer® proteins can be used to detect it (with final detection using an Anti-FLAG antibody) or vice versa. S01 and S02 were subcloned into the FLAG vector as their selections were less stringent than the other four and thus more likely to contain binders more suitable for detection reagents capable of pairing with other S1 binders.

Primary Screening and the identification of clones of interest

In order to confirm the binding activity of any Affimer® proteins of interest against the S1 protein, we designed several primary screening experiments on a homogenous bead-based flow cytometry system called the IQue (from Intellicyt). These experiments were designed to generate as much information regarding the Affimer® protein cohorts binding ability to S1 as possible, while minimizing the amount of time required. The information gleaned included insights into: their ability to bind SARS-CoV-2 S1 regardless of its source or the tags present, their ability to capture S1 protein when it is in solution and an Affimer® protein is immobilised on a surface, whether or not the Affimer® proteins are able to compete with S1 when it is in the presence of ACE2 and finally determining whether or not the Affimer® proteins are able to detect S1 after it has been captured by immobilised ACE2.

As previously mentioned, the IQue is a bead-based flow cytometry device which allows for multiple different conditions to be multiplexed and tested simultaneously. The initial primary screening experiments (cross-reactivity assay) carried out required SARS-CoV-2 S1 to be immobilised on beads (this is achieved by using streptavidin coated beads and biotinylated target) as well as the cross-reactive targets SARS/MERS and 229E S1 proteins. The nature of the beads under test is such that different bead sets emit a distinct base fluorescence from one another. This means by immobilizing each target under test to a different set of streptavidin coated beads; each of which fluoresce at a distinct point from one another when their signal is plotted on a graph; allows us to mix the different target beads, run them in the same assay and separate them during data analysis. We can then determine binding by using a specific detector such as a fluorophore conjugated to an antibody specific to the Affimer® protein. In the case of the SARS-CoV-2 S1 selection output, the immobilised target beads were treated with HA tagged Affimer® protein followed by anti-HA antibody conjugated to Alexa Flour

488 thus allowing us to determine the level of specific binding to each of the targets under test.

The second IQue assay performed with the S1 selection output was an ACE 2 competition assay. The experimental setup for this assay involved once again immobilizing SARS-CoV-2 S1 on beads. In this instance however ACE2-Fc was then added in solution and was subsequently captured by the immobilised S1. A forty-fold excess of Affimer® protein was then added to the beads in an attempt to displace the captured ACE2-Fc with final detection using Anti-Fc 488. We designed this assay to observe any reduction in signal that occurred as a result of the Affimer® proteins presence. The idea being, that Affimer® proteins capable of significantly reducing the signal could be of use as potential therapeutics.

The third IQue assay was an ACE2 'sandwich' (ACE2 sandwich assay) which involved immobilizing biotinylated ACE2 onto the bead surface and using it to capture SARS-CoV-2 S1 in solution. Affimer® protein was then introduced with the hope that it would bind to the ACE2/S1 complex prior to final detection with Anti-HA 488 antibody. We designed this assay to test the Affimer® proteins ability to pair with ACE2, as those capable of doing this would be well suited as the basis for a SARS-CoV-2 testing kit.

The final primary screening assay carried out on the IQue (Affimer® capture assay (IQue)) was a capture assay and used beads coated in Anti-HA antibody thereby allowing them to capture the HA tagged Affimer® protein in solution. The immobilised Affimer® protein was then used to capture biotinylated SARS-CoV-2 S1 in solution followed by final detection with Streptavidin conjugated to Alexa flour 488.

In parallel to the IQue assays a capture ELISA (Affimer® capture assay (ELISA) was also carried out. In these ELISAs, 2.5 μg/mL of individual Affimer® protein is adsorbed in a 96 well Maxisorp plate so that each well contains a single candidate. The ELISA was then blocked before the addition of biotinylated target and final detection using streptavidin conjugated to poly-HRP. An entire ELISA plate was used to test the Affimer® proteins against a single target, therefore when cross-reactive targets or a no target control plate are under test multiple Maxisorp plates are coated with Affimer® protein at the start of the ELISA. In this instance due to its high level of homology with the SARS-CoV-2 S1 protein, the SARS S1 and a no target control were included as the cross-reactive plates. Exemplary results achieved in the Affimer capture assay (ELISA) for nine of those Affimer® candidates that worked well as capture reagents for SARS-CoV-2 S1 spike protein and for seven of those Affimer® candidates that worked well as capture reagents for SARS-CoV-2 S1 spike protein in sandwich ELISA using the detection Affimer® candidate 620_826257 are shown in Figure 18A and 18B, respectively.

ELISA using dual Affimer® Capture

To further improve the sensitivity of detection, multiple capture Affimer® reagents were coated in one well. Affimer® reagents were coated separately or mixed to capture SARS-CoV-2 S1 spike protein titrated 2-fold from 100 ng/mL. Detection was performed using biotinylated 826257 detection Affimer® reagent and streptavidin poly-HRP as described herein. Exemplary results are shown in Figure 19A, 19B and 19C.

Some of the data resulting from these experiments is also summarised in the following table:

Capture surface	Accurate and precise range (ng/mL)
1 µg/mL 620_825436	100 to 0.8
0.5 µg/mL 620_825436	100 to 0.4
1 µg/mL 620_825594	100 to 0.8
0.5 µg/mL 620_825594	100 to 0.4
1 µg/mL 620_825425	100 to 0.4
0.5 µg/mL 620_825425	100 to 0.8
0.5 µg/mL each 620_825436 & 620_825594	25 to 0.1
0.5 µg/mL each 620_825436 & 620_825425	25 to 0.2

These data clearly demonstrate that the sensitivity of the assay is significantly increased by using multiple capture Affimer® reagents together.

Large scale expression and purification

Prior to their large-scale expression and purification, a way of reducing the number of successful Affimer® proteins identified by the primary screening was required. To this end, a set of preferred characteristics based upon the signal they produced in the primary screening were used to separate them into three distinct groups. The first was comprised of Affimer® proteins which bound the SARS-CoV-2 S1 protein regardless of its supplier and were able to pair with ACE2 with no cross reactivity to the various other homologous targets. The second group was made of binders which once again bound the S1 protein regardless of its supplier with little cross-reactivity, but did not pair with ACE2 and instead competed with it. Finally, the third group was filled with those capable of binding SARS-CoV-2 S1 regardless of its source and paired with ACE2, but showed varying degrees of cross-reactivity with the various homologous targets. Within these groups were a mix of FLAG tagged and HA tagged Affimer® proteins

which allowed for downstream assays to evaluate the potential for pairs between Affimer® proteins (i.e. an immobilised anti SARS-CoV-2 S1 Affimer® protein on a surface used to capture the target used in tandem with a second anti-SARS-CoV-2 S1 Affimer® protein used to detect the captured target) particularly from the first two groups. The benefit of the third group was that it offered options for detection reagents to be used with a more specific capture reagent.

Once the Affimer® proteins were separated into the three groups a further round of sorting was required in order to reduce the number for downstream processing. In this instance, factors such as their expression level (determined by the GXII), as well as their sequence (particularly the number of hydrophobic residues therein) were taken into consideration. This process resulted in 48 Affimer® proteins of particular interest, the majority of which were grown in 0.5 L cultures, expressed using IPTG and purified on Ni-SEC ÄKTAxpress columns. Exemplary graphs of purifications of polypeptides according to the invention (Affimer® Agent Candidates) are shown in Figures 6-8. Finally, in order to confirm that the Affimer® proteins were as expected they were analysed by Mass Spectroscopy.

Characterisation of Exemplary Clones

Now that the number of Affimer® proteins had been whittled down to the most promising and a larger amount of them were available for testing. A more thorough round of characterisation was performed on each of them with aim of confirming that they were specific for SARS-CoV-2 S1 protein, to investigate their potential to work in pairs with one another and to confirm if they were capable of working either in tandem or in competition with ACE2.

The first characterisation experiments looked to confirm that the increased purity of the Affimer® proteins (from the aforementioned large-scale expressions and purifications) did not have a detrimental effect on their ability to capture SARS-CoV-2 S1 in solution. To this end several IQue experiments were carried out. The first was a repeat of the initial primary screening experiment in which biotinylated SARS-CoV-2 S1 (as well as the various cross-reactive targets) was immobilised on streptavidin coated IQue beads, followed by the addition of the HA tagged Affimer® proteins under test and final detection with Anti-HA antibody conjugated to Alexa Fluor 488.

In order to maximise the versatility of the binders we can offer our collaborators, all of the Affimer® proteins were also biotinylated via the accessible cysteine residue present within their C-terminal HA or FLAG tags. The second IQue experiment was therefore designed to test if this biotinylation had affected binding and also if biotinylated Affimer® proteins could be paired with other Affimer® Agents. In order to test the

effects of biotinylation the biotinylated Affimer® proteins (either HA or FLAG) were captured on streptavidin coated IQue beads followed by SARS-CoV-2 S1 protein conjugated to Alexa Fluor 488 (prepared using a lightning link cross linking reagent). To test for pairs the biotinylated Affimer® proteins were captured on the beads like before, then nonconjugated target was added followed by an Affimer® protein with the opposite tag to the captured one (either HA or FLAG). Final detection depended on the detection reagent used, either anti-HA or anti-FLAG conjugated to Alexa Fluor 488. The IQue was also used to confirm whether or not some of the Affimer® proteins could pair with ACE2 when detecting SARS-CoV-2 S1 protein, a highly beneficial characteristic for many downstream applications. Two assay set ups were devised in order to determine which orientations the Affimer® proteins could be used with ACE2. The first involved once again immobilizing biotinylated Affimer® protein onto IQue beads, followed by unconjugated S1 protein and Fc tagged ACE2 and final detection with an anti-IgG-Fc antibody conjugated to Alexa Fluor 488. The alternative experimental set up required the immobilisation of biotinylated ACE2 on IQue beads followed by unlabeled S1 and either HA or FLAG tagged Affimer® protein, with final detection using either anti-HA or anti-FLAG antibody conjugated to Alexa Fluor 488 depending on the Affimer® protein used as in the previously described IQue experiment. All of these IQue experiments together confirmed the applicability of the Affimer® proteins as both capture and detection reagents in future SARS-CoV-2 S1 detection assays.

In parallel to the IQue, several ELISAs were carried out to characterize the Affimer® proteins. They were designed to highlight their versatility and show that their binding is not affected by the surface on which either they or the target are immobilised. Many of the ELISAs therefore had a similar experimental set up as the IQue experiments previously described. For example, just like the initial IQue experiment, the initial characterisation ELISA aimed to confirm the Affimer® proteins ability to capture the SARS-CoV-2 S1 in solution. Therefore, ELISA plates were coated with Affimer® protein prior to the addition of biotinylated target using a 3-fold titration down the plate and final detection with Streptavidin polyHRP. The various cross-reactive targets were also included in order to confirm their specificity to the S1 protein. The best capture Affimer® proteins were taken through to a secondary ELISA to identify pairs similar to before. In this instance, the Affimer® proteins were used to coat a Maxisorp plate, followed by the addition of unbiotinylated target (after washing and blocking the plate). Biotinylated Affimer® protein was then used to detect the Affimer® protein/SARS-CoV-2 S1 complex with final detection using Streptavidin polyHRP. This pair assay setup was also used to identify ACE2 pairing Affimer® proteins, by using

biotinylated ACE2 to detect captured SARS-CoV-2 S1 instead of biotinylated Affimer® protein. The specificity of the successful Affimer® protein pairs were tested using this experimental set up and the various cross-reactive targets.

ELISAs were also carried out to confirm the presence of Affimer® proteins capable of preventing the interaction between SARS-CoV-2 S1 and ACE2 which could be further developed into a therapeutic. In order to perform the competition assay the EC-50 for the S1/ACE2 interaction was required. It was determined by carrying out an ELISA with immobilised ACE2 at a single concentration and a titration of biotinylated S1 protein (using the same 3-fold range as in the previous assay), followed by final detection with streptavidin-polyHRP. Once the EC-50 was known a second ELISA was carried out in which ACE2 was once again adsorbed onto a plate, and the biotinylated SARS-CoV-2 S1 (at the EC-50) were mixed with a titration of Affimer® protein from 100 – 0.1 μg/mL prior to their addition to the plate. Final detection once again used streptavidin-polyHRP with a reduction in signal signifying a successful ACE2 competing reagent. Results obtained for exemplary Affimer® Agents are shown in Figure 2A. Three exemplary candidates shown here inhibit the binding of SARS-CoV-2 to ACE2, namely 825436, 825425 and 825336. In contrast, 826257 does not compete with ACE2 binding, thus indicating that this candidate Affimer® Agent binds to a different epitope on SARS-CoV-2 S1.

ELISAs were also carried out to further confirm whether or not Affimer® proteins are able to compete with S1 when it is in the presence of ACE2, using an alternative approach. Specifically, since the RBD portion of S1 is bound by ACE2, assays were carried out to determine whether Affimer® Agents bind to the RBD portion of SARS-CoV-2 S1 protein, and are thus capable of competing with ACE2. This assay used immobilized Affimer® Agents that were incubated with biotinylated SARS-CoV-2 S1 protein RBD (non-biotin construct comprises Arg319 – Lys537 and can be expressed using standard expression protocols known by the person skilled in the art, or can be purchased, for example, from ACRO Biosystems, SPD-C52H3, then biotinylated via lysines using methods known by the person skilled in the art), titrated 3-fold from 500 – 0.69 ng/mL in 1 x PBS Casein ELISA Reagent (PBS-C, Abcam, ab171532), streptavidin, poly-HRP conjugate (Pierce, 21140) diluted 1:10,000 in PBS-C, and TMB (Surmodics, TMBS-1000-01) prior to stopping the colour development with 0.5 M H_2SO_4 (Fisher, J/8430/15). Each plate's absorbance was read by a microplate reader at 450 nm and 630 nm. The background at 630 nm was subtracted from absorbance at 450 nm to give the optical density used for 4PL analysis using Prism version 8.4 software (GraphPad). Data obtained with three exemplary immobilized Affimer® Agents (620_825436; 620_825425; and 620_826257) is shown in Figure 2B. This data

demonstrates that some Affimer® Agents bind to the RBD and are thus classified as ACE2 competing (e.g., 620_825436 and 620_825425), whereas other Affimer® Agents do not bind to RBD and are thus classified as ACE2 pairing (e.g., 620_826257).
Finally, the Affimer® proteins were used in SPRi experiments in order to get an indication of some of the biophysical characteristics such as Kd's of the Affimer® proteins. To achieve this, 75 μg/mL of each candidate was printed onto an Epoxy surface using an SPRi-arrayer. Each of the SARS-CoV-2 S1 targets (supplied by Acro, Native Antigen Co. and Sino Biological) were then flowed over the chip on the XelPlex. This experiment allowed us to not only measure the level of binding to the target but to also compare this binding between the different S1 targets. The level of specificity of the Affimer® proteins were also tested as the cross-reactive targets (SARS S1, MERS S1, 229E S1 and HKU S1) were also flowed over the chips. This assay set up was also used to provide an indication of the overall stability of the Affimer® proteins, by exposing these printed candidates to the SARS-CoV-2 target before and after they have been treated with 100 mM NaOH over several days.
All of these characterisation experiments resulted in a cohort of Affimer® proteins which are highly specific to SARS-CoV-2 S1, some of which are well suited as capture reagents immobilising the target while in solution. While others are well suited as detection reagents, capable of binding immobilised SARS-CoV-2 S1 no matter the method by which this immobilisation is achieved, be it another Affimer® protein or ACE2. Some of the cohort were shown to successfully interrupt the interaction between SARS-CoV-2 S1 and ACE2, thereby showing the potential to neutralise the virus and act as a therapeutic. Finally, the versatility and robustness of the candidates was highlighted by the variety of tags to which they have been fused, the post purification modifications that were carried out on them and their stability and continued functionality in harsh buffers such as NaOH.

Mutated SARS-CoV-2 viruses comprising the Y453F substitution have recently been isolated.

ELISAs were carried out to further confirm whether or not Affimer® proteins are able to bind to the RBD portion of S1 comprising the Y453F mutation. This assay used immobilized Affimer® Agents that were incubated with wild-type biotinylated SARS-CoV-2 S1 spike protein (construct comprises Val16 – Arg685 and can be expressed using standard expression protocol known by the person skilled in the art, or can be purchased, for example, from ACRO Biosystems, S1N-C82E8) or immobilized Affimer® Agents that were incubated with biotinylated SARS-CoV-2 S protein RBD

comprising the Y453F substitution (construct comprises Arg319 – Lys537 and the Y453F mutation, and can be expressed using standard expression protocols known by the person skilled in the art, or can be purchased, for example, from ACRO Biosystems, SPD-C52H3, then biotinylated via lysines). The targets (i.e., biotinylated SARS-CoV-2 S1 spike protein or biotinylated SARS-CoV-2 S protein RBD comprising the Y453F mutation) were titrated 2-fold from 50 to 0.78 ng/mL in 1 x PBS Casein ELISA Reagent (PBS-C, Abcam, ab171532), streptavidin, poly-HRP conjugate (Pierce, 21140) diluted 1:10,000 in PBS-C, and TMB (Surmodics, TMBS-1000-01) prior to stopping the colour development with 0.5 M H_2SO_4 (Fisher, J/8430/15). Each plate's absorbance was read by a microplate reader at 450 nm and 630 nm. The background at 630 nm was subtracted from absorbance at 450 nm to give the optical density used for 4PL analysis using Prism version 8.4 software (GraphPad). Data obtained with two exemplary immobilized Affimer® Agents (620_825436 and 620_825425) with the wild-type SARS-CoV-2 S1 spike protein and the SARS-CoV-2 S protein RBD comprising the Y453F substitution is shown in Figure 2C. This data demonstrates that exemplary Affimer® Agents 825425 and 825436 bind to the RBD comprising the Y453F mutation.

EXAMPLE 2: BAMS Analysis

Exemplary BAMS™ Assay Protocol

This example may be used for Cultured Virus or Spiked Saliva (e.g. as test samples/reference samples). More importantly this example may be used, for example, for saliva samples or anterior nasal swab samples from a subject of interest e.g. to determine if the subject has SARS-CoV-2 virus.

This protocol has been adapted from a standard BAMS™ assay for capturing proteolytic peptides. Optimisation of the assay steps for use with virus particles or saliva samples or anterior nasal swab samples including incubation conditions, binding buffer composition and MALDI matrices may be required. This is within the ability of the skilled worker.

1. Add an equal volume of 2X Binding Buffer (200 mM Tris HCl, pH 8.0, 200 mM KCl) to an aliquot of cultured virus sample (or saliva sample or anterior nasal swab sample). Mix gently using pipette.

NOTE: KCl in concentration from 0.1 – 1.0 M is commonly used to adjust stringency and reduce nonspecific protein binding to beads.

2. Transfer the virus specimen sample in 1X binding buffer into a designated well of a 96-well plate (v-shape bottom).

3. Using a QuicPick (Bio-Nobile, #24001), transfer the magnetic BAMS assay beads to the well.

4. Place the 96-well sealing mat on the plate to seal the well.

5. Place the 96-well plate onto the Thermomixer C (Eppendorf) with the temperature set at 4°C and shake at 1200 RPM to maintain beads in suspension (1- 16 hours, typically overnight).

BAMS™ Bead Wash:

1. Prepare a 48 well plate with the following wash buffers: First Row = 700 µL of Wash Buffer A
(100 mM KCl, 50 mM Tris HCl, pH 8.0), Second Row = 700 µL of Wash Buffer B (100 mM KCl), Third Row = Wash Buffer C (10 mM Ammonium Bicarbonate), Fourth Row = 700 µL of Wash Buffer D (Milli Q Deionized Water).

2. Transfer the incubated beads into the Wash Buffer A and place on microplate shaker for 10 minutes (650 RPM)

3. Transfer the beads from Wash Buffer A to Wash Buffer B and place on microplate shaker for 2 minutes (650 RPM)

4. Transfer the beads from Wash Buffer B to Wash Buffer C and place on microplate shaker for 2 minutes (650 RPM)

5. Transfer the beads from Wash Buffer C to Wash Buffer D and place on microplate shaker for 2 minutes (650 RPM)

BAMS Bead Elution for Single-Plex Assay onto Standard MALDI Plate:

1. Transfer the beads from Wash Buffer D into a 650 µL Eppendorf tube containing 100 uL of deionized water.

2. Spin beads to settle beads and buffer in 650 µL Eppendorf tube (15 sec, ~ 100 RPM)

3. Place tube into a magnetic bead separation rack and remove any residual water.

4. Add 2 µL of Matrix Elution Buffer (50% Acetonitrile, 0.3% TFA, 4 mM diammonium citrate, 10 mg/mL CHCA) to beads with a 10 µL pipette tip. Avoid touching beads with the pipette tip, as it may cause beads to break apart.

5. Briefly spin tube to settle matrix elution buffer and beads. Allow beads to sit in matrix elution buffer at room temperature for 10 min. Viral components will be released from the affinity beads, into the matrix elution buffer.

6. Spot 0.5 µL of the matrix elution buffer (containing released analytes) onto a MALDI plate. Do not transfer the beads onto the MALDI plate. Allow spots to dry on the MALDI plate (on the bench or in hood).

7. If desired, add suitable calibration standard to MALDI plate.
8. Carry out MALDI-MS analysis.

Detection of protein(s) or fragment(s) or ion(s) thereof originating from SARS-CoV-2 virus indicates presence of SARS-CoV-2 virus in said sample.

EXAMPLE 3: Protocol for determination of SARS-CoV-2 S1 binding kinetics constants by SPRi

Polypeptides of the invention (sometimes referred to as 'Affimer® candidates'), and a negative reference polypeptide ('Affimer® Agent'), all containing a single cysteine in the vector-derived-terminal primary sequence, were diluted to 5 µM in 10 mM sodium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 (Sigma Aldrich, P4417) and were printed in triplicate to Epoxyde-functionalised SPRi-Biochips (HORIBA, CEp) using an SPRi-Arrayer (HORIBA) and 350 µm Xtend contact printing pin (HORIBA), at 20°C & 70% relative humidity (RH). The printed Biochip was incubated at 18 °C, 55% RH for 16 h in darkness, before the surface chemistry was quenched with 25 mM Ethanolamine (Sigma Aldrich, E9508), 10 mM sodium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 (Sigma Aldrich, P4417), for 30 minutes, 20°C, in darkness. The chip was then rinsed 4x using 18.2 MΩ.cm ultrapure water (Milli-Q®, Merk-millipore).
The printed Biochip was loaded to an XelPleX SPRi instrument (HORIBA) and the system was equilibrated with 10 mM sodium phosphate, 2.7 mM potassium chloride, 300 mM sodium chloride, 0.1% Tween®-20, pH 7.4 (Sigma Aldrich, P4417, S7653, P9416) – hereafter PBS-T, with a flow cell temperature of 25 °C (used throughout all analyte injections). After assigning spot definitions to the printed Affimer® spots using EZSuite (HORIBA), the biochip was blocked with 1 x Casein Blocking Buffer diluted from a 10x stock (Sigma Aldrich, B6429) in PBS-T, 200 µL injection, 25 µL/min, 10 minutes contact time. Reponses for each printed Affimer® spot were then normalised using an injection of 3 mg/mL Sucrose (Fisher Scientific, S/8600/53) diluted in PBS-T. Analyte injections of SARS-CoV-2 S1 protein (ACRO Biosystems, S1N-C52H3), diluted in PBS-T were performed at concentrations ranging from 200 nM to 3 nM, in addition to a PBS-T control injection, 400 µL injections, 100 µL/min. Regeneration of the chip surface between each analyte injection (removal of bound analyte following dissociation) was performed using 10 mM Glycine-HCl pH 2, diluted from a 100 mM stock (HORIBA, GLYCIN-HCL) in ultrapure water, 200 µL, 400 µL/min.

Response data for all SARS-CoV-2 S1 analyte injection concentrations was exported from EZSuite and analysed using Scrubber2 (HORIBA). Response data for injections were Y-axis referenced to a point immediately prior to injection start time, and all association phases were aligned. Further baseline referencing was performed against the negative reference Affimer® spots, and the PBS-T control analyte injection. Association rate constant (ka) (M-1 s-1), Dissociation rate constant (kd) (s-1), and Equilibrium dissociation constant (KD) (nM) were then determined by fitting using a 1:1 langmuir model.

EXAMPLE 4: Protocol for Competition ELISA

All pipetting and additions to the assay plate were carried out using calibrated multichannel pipettes and automated plate washers to minimise variation between wells.

Previous internal work had identified the half-maximal effective concentration (EC_{50}) of the Angiotensin Converting Enzyme 2 (ACE2) receptor to the SARS-CoV-2 S1 spike protein as approximately 80 ng/mL in Enzyme-Linked Immunosorbent Assay (ELISA) format. Briefly, 1 µg/mL ACE2 receptor (ACRO Biosystems, AC2-H5257) was coated on Maxisorp 96-well plates (Nunc, 442404) in 50 mM carbonate-bicarbonate buffer, pH 9.6 (Sigma Aldrich, C3041), incubated with biotinylated SARS-CoV-2 S1 spike protein (ACRO Biosystems, S1N-C82E8) titrated 3-fold from 500 – 0.69 ng/mL in 1 x PBS Casein ELISA Reagent (PBS-C, Abcam, ab171532), streptavidin, poly-HRP conjugate (Pierce, 21140) diluted 1:10,000 in PBS-C, and TMB (Surmodics, TMBS-1000-01) prior to stopping the colour development with 0.5 M H_2SO_4 (Fisher, J/8430/15). Each plate's absorbance was read by a microplate reader at 450 nm and 630 nm. The background at 630 nm was subtracted from absorbance at 450 nm to give the optical density used for 4PL analysis using Prism version 8.4 software (GraphPad).

Subsequent competition ELISAs used this EC_{50} value for the outlined conditions above to identify the half-maximal inhibitory concentration (IC_{50}) for each polypeptide of the invention (i.e. each anti-SARS-CoV-2 S1 protein Affimer® reagent described herein) tested in the following protocol:

Maxisorp 96-well plates were coated with 100 µL per well ACE2, diluted to 1 µg/mL in 50 mM carbonate-bicarbonate buffer, pH 9.6. This was left overnight for at least 15 hours at 4 °C, stationary. During this and each subsequent incubation, the plates were stacked vertically with the top plate having an unused Maxisorp 96-well plate placed on top to minimise evaporation. After the coating, and each subsequent step, plates were washed three times with 300 µL per well 1 x Phosphate Buffered Saline (1.06 mM

KH_2PO_4, 155.17 mM NaCl, 2.97 mM $Na_2HPO_4.7H_2O$), pH 7.4 (Gibco, 70011) containing 0.05 % (v/v) Tween®-20 (Sigma Aldrich, P9416) (PBS-T). Excess PBS-T was removed prior to subsequent additions.

Unbound sites on the plate were blocked with 300 µL per well PBS-C, and incubated for 2 hours. During this and subsequent incubations, plates were shaken at 200 rpm at 25°C. During this blocking incubation, serial dilutions were prepared of the immobilised metal affinity chromatography and size exclusion chromatography (IMAC-SEC) purified polypeptide of the invention (anti-SARS-CoV-2 S2 spike protein Affimer® reagent) being tested, diluted in PBS-C, with the polypeptide of the invention titrated 2-fold from 100 µg/mL to 97 ng/mL. All polypeptide of the invention dilutions were incubated for 1 hour with 80 ng/mL biotinylated SARS-CoV-2 S1 spike protein diluted in PBS-C. 100 µL per well of titrated anti-SARS-CoV-2 S1 spike protein polypeptide of the invention (Affimer® reagent) or no anti-SARS-CoV-2 S1 spike protein polypeptide of the invention (Affimer® reagent) plus biotinylated SARS-CoV-2 S1 spike protein was transferred to the plate and incubated for 1 hour.

Within 20 minutes of addition to the plate, or when required, streptavidin, poly-HRP conjugate was diluted 1:10,000 in PBS-C. 100 µL per well was transferred to the plate and incubated for 1 hour. During this incubation TMB was warmed to room temperature. 100 µL per well TMB substrate was transferred to the plate with colour allowed to develop at room temperature, before the addition of 50 µL per well 0.5 M H_2SO_4 to the plate in order to cease the colorimetric reaction. Each plate's absorbance was read by a microplate reader at 450 nm and 630 nm. The background at 630 nm was subtracted from absorbance at 450 nm to give the optical density used for analysis. Further analysis was carried out using Prism version 8.4 software. Polypeptide of the invention (Affimer® reagent) concentrations were transformed using the X=Log(X) function, before carrying out a 4PL nonlinear regression (curve fit) analysis. This generated the IC_{50} values in µg/mL, which combined with known molecular weights of each polypeptide of the invention (Affimer® reagent) allowed the conversion to molar units (e.g. nM, µM etc).

EXAMPLE 5: Exemplary Polypeptides of the Invention

Each of the separate disclosures of exemplary polypeptides of the invention below also discloses beneficial technical properties of those polypeptides. In particular we refer to the 'reactivity' information in the tables headed "**Coronavirus S1 Protein - Reactivity**". This information represents the output of ELISA assay cross-reactivity assays as described below for each polypeptide (i.e. each Affimer® Agent (i.e. each

scaffold containing 1st and 2nd heterologous peptide insertions)) disclosed. The 'reactivity' column in each table for each polypeptide incorporates both ELISA and flow cytometry data.

In these tables:

-'Yes' equates to an observed ELISA optical density AND/OR flow cytometry fluorescence intensity of more than 3 fold higher than a reference Affimer® control known to be specific for a Biotinylated Mouse IgG2b, k Isotype Control (BD, 559531 from BD Biosciences (BD Pharmingen™), 1030 Eskdale Road, Winnersh Triangle, Wokingham, Berkshire RG41 5TS, England, UK) and more than 3 fold higher than a zero S1 control, when assayed with 0.5 μg/mL Biotinylated S1 protein by ELISA, or to beads coated with 1 μg/mL Biotinylated S1 protein by flow cytometry.

-'No' equates to an observed ELISA optical density OR flow cytometry fluorescence intensity of NOT more than 3 fold higher than a reference Affimer® control known to be specific for a Biotinylated Mouse IgG2b, k Isotype Control (BD, 559531) and NOT more than 3 fold higher than a zero S1 control, when assayed with 0.5 μg/mL Biotinylated S1 protein by ELISA, or to beads coated with 1 μg/mL Biotinylated S1 protein by flow cytometry.

Where 'Not Tested' means cross-reactivity toward this S1 protein has not been fully evaluated for this polypeptide.

Briefly, in case any further guidance is needed:

'Yes' means at least 3x background is seen either ELISA or flow cytometry, or more suitably both.

'No' means not more than 3x background is seen in either ELISA or flow cytometry

The data disclosed in the tables of Example 5 were produced using the protocol of Example 7 (**Protocol of S1 protein reactivity ELISA**), in particular using the following reagents:

SARS-CoV-2, ACRO Biosystems, S1N-C52H4

MERS-CoV, Sino Biological, V0069-V08H

SARS-CoV, Sino Biological, 40150-V08B1

hCoV-229E, Sino Biological, 40601-V08H

hCoV-HKU1, Sino Biological, 40602-V08H

hCoV-NL63, Sino Biological, 40600-V08H

hCoV-OC43, Sino Biological, 40607-V08B;

and/or using the protocol of Example 8 (Protocol of S1 protein reactivity flow cytometry).

Polypeptide: 620_826257

Scaffold: T3r2[9_9]*

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSP<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 5 HANWHPQLD

Second heterologous peptide (Loop 4): **SEQ ID NO:** 6 NSHKLFWPV

Polypeptide Sequence: **SEQ ID NO:** 7
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDHANWHPQLDGLNYYIKVRVNGKY
IHLKVFKSPNSHKLFWPVEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.67

Theoretical Isoelectric point (pI): 6.41

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 9.15E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 2.00E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 2.2

Midpoint transition temperature (T_m) (°C): 83.1

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No

hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_826285

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 8 EFTGLFSGK

Second heterologous peptide (Loop 4): **SEQ ID NO:** 9 VDWGFAPPT

Polypeptide Sequence: **SEQ ID NO:** 10
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDEFTGLFSGKGLNYYIKVRVNGKYIH
LKVFKSLVDWGFAPPTEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.46

Theoretical Isoelectric point (pI): 5.43

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 5.33E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 7.20E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 13.6

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	***Reactivity***
SARS-CoV-2	Yes
SARS-CoV	Yes
MERS-CoV	No

hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_826049

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 11 HVPVHSYFI

Second heterologous peptide (Loop 4): **SEQ ID NO:** 12 HNDVDDVVW

Polypeptide Sequence: **SEQ ID NO:** 13
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDHVPVHSYFIGLNYYIKVRVNGKYIH
LKVFKSLHNDVDDVVWEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.46

Theoretical Isoelectric point (pI): 5.51

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 980

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 3.24E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 5.20E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 16.1

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	***Reactivity***
SARS-CoV-2	Yes
SARS-CoV	No

MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_829515

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 14 FWPAPTGFR

Second heterologous peptide (Loop 4): **SEQ ID NO:** 15 HGSTNHRTA

Polypeptide Sequence: **SEQ ID NO:** 16
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDFWPAPTGFRGLNYYIKVRVNGKYI
HLKVFKSLHGSTNHRTAEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.62

Theoretical Isoelectric point (pI): 7.91

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 1021

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 7.32E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.65E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 4.4

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	***Reactivity***
SARS-CoV-2	Yes

SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_830214

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 17 YSVGSTGYR

Second heterologous peptide (Loop 4): **SEQ ID NO:** 18 HSGVGHDTV

Polypeptide Sequence: **SEQ ID NO:** 19
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDYSVGSTGYRGLNYYIKVRVNGKYIH
LKVFKSLHSGVGHDTVEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.55

Theoretical Isoelectric point (pI): 6.29

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 185

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 4.22E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 7.30E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 17.2

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein Reactivity

SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_826005

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 20 RPNFLTEFI

Second heterologous peptide (Loop 4): **SEQ ID NO:** 21 HSSHGQEIL

Polypeptide Sequence: **SEQ ID NO:** 22
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDRPNFLTEFIGLNYYIKVRVNGKYIHL
KVFKSLHSSHGQEILEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.53

Theoretical Isoelectric point (pI): 5.91

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 2703

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 8.90E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 9.20E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 10.3

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_829511

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 23 GDWPNSNFR

Second heterologous peptide (Loop 4): **SEQ ID NO:** 24 HHHEKFDFL

Polypeptide Sequence: **SEQ ID NO:** 25
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDGDWPNSNFRGLNYYIKVRVNGKYI
HLKVFKSLHHHEKFDFLEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.71

Theoretical Isoelectric point (pI): 6.07

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 253

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 5.66E+03

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 4.10E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 72.2

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_829519

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 26 RQGLGSFYI

Second heterologous peptide (Loop 4): **SEQ ID NO:** 27 HSNEGGHYR

Polypeptide Sequence: **SEQ ID NO:** 28
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDRQGLGSFYIGLNYYIKVRVNGKYIH
LKVFKSLHSNEGGHYREDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.65

Theoretical Isoelectric point (pI): 6.83

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 226

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 3.05E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 9.20E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 30.1

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_830162

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 29 EPRIGYGFI

Second heterologous peptide (Loop 4): **SEQ ID NO:** 30 HTHHYDDYY

Polypeptide Sequence: **SEQ ID NO:** 31
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDEPRIGYGFIGLNYYIKVRVNGKYIHL
KVFKSLHTHHYDDYYEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.65

Theoretical Isoelectric point (pI): 5.78

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 8

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 3.42E+03

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 3.00E-05

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 7.9

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_826047

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 32 QDYKIDVLL

Second heterologous peptide (Loop 4): **SEQ ID NO:** 33 GLDQWFTKT

Polypeptide Sequence: **SEQ ID NO:** 34
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDQDYKIDVLLGLNYYIKVRVNGKYIH
LKVFKSLGLDQWFTKTEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.52

Theoretical Isoelectric point (pI): 5.42

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 3.31E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 3.30E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 10.0

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_826101

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 35 LDVNFKWSG

Second heterologous peptide (Loop 4): **SEQ ID NO:** 36 IHSGKAWWH

Polypeptide Sequence: **SEQ ID NO:** 37
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDLDVNFKWSGGLNYYIKVRVNGKYI
HLKVFKSLIHSGKAWWHEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.55

Theoretical Isoelectric point (pI): 6.83

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 4.41E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 4.50E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 10.1

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_826110

Scaffold: T3r2[9_9]*

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EYLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 38 IWPAESEDF

Second heterologous peptide (Loop 4): **SEQ ID NO:** 39 SHWKRPWHV

Polypeptide Sequence: **SEQ ID NO:** 40
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDIWPAESEDFGLNYYIKVRVNGKYIH
LKVFKSLSHWKRPWHVEYLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.62

Theoretical Isoelectric point (pI): 6.30

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 4.31E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 9.90E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 22.9

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_826152

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 41 KEEVGAHLW

Second heterologous peptide (Loop 4): **SEQ ID NO:** 42 NLGPHFVKQ

Polypeptide Sequence: **SEQ ID NO:** 43
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDKEEVGAHLWGLNYYIKVRVNGKYI
HLKVFKSLNLGPHFVKQEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.56

Theoretical Isoelectric point (pI): 6.30

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 3.96E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.00E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 25.3

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_826024

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 44 FEKQNKWSG

Second heterologous peptide (Loop 4): **SEQ ID NO:** 45 VHFAKPYWQ

Polypeptide Sequence: **SEQ ID NO:** 46
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDFEKQNKWSGGLNYYIKVRVNGKYI
HLKVFKSLVHFAKPYWQEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.67

Theoretical Isoelectric point (pI): 7.85

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): Data Unavailable

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): Data Unavailable

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 0.0

Midpoint transition temperature (T_m) (°C): 87.9

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_829494

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 47 WKQRQSGFI

Second heterologous peptide (Loop 4): **SEQ ID NO:** 48 HSWERLHVV

Polypeptide Sequence: **SEQ ID NO:** 49
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDWKQRQSGFIGLNYYIKVRVNGKYI
HLKVFKSLHSWERLHVVEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.59

Theoretical Isoelectric point (pI): 7.90

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM):	514
SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1):	2.25E+04
SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1):	1.90E-03
SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM):	87.0
Midpoint transition temperature (T_m) (°C):	Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_829566

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 50 DYNQEQYEH

Second heterologous peptide (Loop 4): **SEQ ID NO:** 51 WKKRLFPWT

Polypeptide Sequence: **SEQ ID NO:** 52
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDDYNQEQYEHGLNYYIKVRVNGKYI
HLKVFKSLWKKRLFPWTEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.80

Theoretical Isoelectric point (pI): 6.13

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 785

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): Data Unavailable

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): Data Unavailable

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 0.0

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_826284

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 53 TLPWLHQPH

Second heterologous peptide (Loop 4): **SEQ ID NO:** 54 FVGSLFDVA

Polypeptide Sequence: **SEQ ID NO:** 55
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDTLPWLHQPHGLNYYIKVRVNGKYI
HLKVFKSLFVGSLFDVAEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.39

Theoretical Isoelectric point (pI): 5.89

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): Data Unavailable

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): Data Unavailable

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 0.0

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_826209

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 56 FHEGIQWNG

Second heterologous peptide (Loop 4): **SEQ ID NO:** 57 VYRQHHSQS

Polypeptide Sequence: **SEQ ID NO:** 58
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDFHEGIQWNGGLNYYIKVRVNGKYI
HLKVFKSLVYRQHHSQSEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.69

Theoretical Isoelectric point (pI): 6.41

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 2.59E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.02E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 3.9

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	Yes
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_826287

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 59 NQQYSASLS

Second heterologous peptide (Loop 4): **SEQ ID NO:** 60 NLGINFVLI

Polypeptide Sequence: **SEQ ID NO:** 61
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDNQQYSASLSGLNYYIKVRVNGKYI
HLKVFKSLNLGINFVLIEDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.39

Theoretical Isoelectric point (pI): 5.82

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 5.95E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.46E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 24.6

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_830177

Scaffold: T3r2[9_9]

Scaffold Sequence:
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD<LOOP_2>GLNYYIKVRVNGKYIHL
KVFKSL<LOOP_4>EDLVLTGYQVDKNKDDELTGF<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 62 WSQHLFGFR

Second heterologous peptide (Loop 4): **SEQ ID NO:** 63 HSEVGHSVR

Polypeptide Sequence: **SEQ ID NO:** 64
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDWSQHLFGFRGLNYYIKVRVNGKYI
HLKVFKSLHSEVGHSVREDLVLTGYQVDKNKDDELTGF

Polypeptide Amino Acid Length: 110

Theoretical GRAVY: -0.57

Theoretical Isoelectric point (pI): 6.91

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 5

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 3.38E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 6.50E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 19.2

Midpoint transition temperature (T_m) (°C): 86.3

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_825425

Scaffold: T2[9_9]*

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY EAKVW<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 65 EWFIVPIPQ

Second heterologous peptide (Loop 4): **SEQ ID NO:** 66 IDW

Polypeptide Sequence: **SEQ ID NO:** 67
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQEWFIVPIPQTMYYLTLEAKDGGKKKL YEAKVWIDWNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 95

Theoretical GRAVY: -0.49

Theoretical Isoelectric point (pI): 5.22

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 485

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 1.08E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.37E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 12.8

Midpoint transition temperature (T_m) (°C): 84.4

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825436

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 68 HQPKWPGFT

Second heterologous peptide (Loop 4): **SEQ ID NO:** 69 TFHQTEPSP

Polypeptide Sequence: **SEQ ID NO:** 70
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQHQPKWPGFTTMYYLTLEAKDGGKKK
LYEAKVWVKTFHQTEPSPNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.75

Theoretical Isoelectric point (pI): 7.08

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 390

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 1.24E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.02E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 8.3

Midpoint transition temperature (T_m) (°C): 84.6

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825411

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 71 SYFDTHLPQ

Second heterologous peptide (Loop 4): **SEQ ID NO:** 72 VGYVVNGFY

Polypeptide Sequence: **SEQ ID NO:** 73
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQSYFDTHLPQTMYYLTLEAKDGGKKKL
YEAKVWVKVGYVVNGFYNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.49

Theoretical Isoelectric point (pI): 6.17

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM):	Non-Competing
SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1):	4.42E+04
SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1):	1.13E-03
SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM):	25.6
Midpoint transition temperature (T_m) (°C):	87.1

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825478

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 74 AIGQINYPD

Second heterologous peptide (Loop 4): **SEQ ID NO:** 75 AQSKYRHLF

Polypeptide Sequence: **SEQ ID NO:** 76
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQAIGQINYPDTMYYLTLEAKDGGKKKL
YEAKVWVKAQSKYRHLFNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.61

Theoretical Isoelectric point (pI): 8.13

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 647

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 3.54E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.52E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 4.3

Midpoint transition temperature (T_m) (°C): 90.0

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825543

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 77 PIFAYEIGT

Second heterologous peptide (Loop 4): **SEQ ID NO:** 78 VKGVHGPFS

Polypeptide Sequence: **SEQ ID NO:** 79
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQPIFAYEIGTTMYYLTLEAKDGGKKKLY
EAKVWVKVKGVHGPFSNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.43

Theoretical Isoelectric point (pI): 6.99

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 1.34E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 5.00E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 3.7

Midpoint transition temperature (T_m) (°C): 80.3

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825336

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 80 KDDVGVRYL

Second heterologous peptide (Loop 4): **SEQ ID NO:** 81 VGWQNDPHP

Polypeptide Sequence: **SEQ ID NO:** 82
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQKDDVGVRYLTMYYLTLEAKDGGKKKL
YEAKVWVKVGWQNDPHPNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.68

Theoretical Isoelectric point (pI): 6.18

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 307

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 7.38E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 7.70E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 10.5

Midpoint transition temperature (T_m) (°C): 76.3

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825594

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 83 QELVDLWDA

Second heterologous peptide (Loop 4): **SEQ ID NO:** 84 ASYGQITFS

Polypeptide Sequence: **SEQ ID NO:** 85
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQQELVDLWDATMYYLTLEAKDGGKKK
LYEAKVWVKASYGQITFSNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.50

Theoretical Isoelectric point (pI): 5.24

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 7.54E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 3.90E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 5.2

Midpoint transition temperature (T_m) (°C): 86.6

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825317

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 86 RWTYLVDSN

Second heterologous peptide (Loop 4): **SEQ ID NO:** 87 LRNQLNRSN

Polypeptide Sequence: **SEQ ID NO:** 88
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQRWTYLVDSNTMYYLTLEAKDGGKKK
LYEAKVWVKLRNQLNRSNNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.73

Theoretical Isoelectric point (pI): 8.85

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 56

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 8.47E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 8.70E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 10.3

Midpoint transition temperature (T_m) (°C): 80.2

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825392

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 89 DAQNDFFPE

Second heterologous peptide (Loop 4): **SEQ ID NO:** 90 EEGVQKHLF

Polypeptide Sequence: **SEQ ID NO:** 91
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQDAQNDFFPETMYYLTLEAKDGGKKK
LYEAKVWVKEEGVQKHLFNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.68

Theoretical Isoelectric point (pI): 5.26

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 989

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 9.90E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.22E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 12.3

Midpoint transition temperature (T_m) (°C): 90.0

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_829592

Scaffold: T2[9_AAE]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVKAAENFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 92 NGEHPLYVE

Second heterologous peptide (Loop 4): **SEQ ID NO:** 93 AAE

Polypeptide Sequence: **SEQ ID NO:** 94
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQNGEHPLYVETMYYLTLEAKDGGKKKL
YEAKVWVKAAENFKELQEFKPVGDA

Polypeptide Amino Acid Length: 97

Theoretical GRAVY: -0.62

Theoretical Isoelectric point (pI): 5.46

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 830

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 9.66E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.40E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 14.8

Midpoint transition temperature (T_m) (°C): 89.8

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_829641

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 95 DELFVDLGV

Second heterologous peptide (Loop 4): **SEQ ID NO:** 96 SWRPYNPN

Polypeptide Sequence: **SEQ ID NO:** 97
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQDELFVDLGVTMYYLTLEAKDGGKKKL
YEAKVWVKSWRPYNPNNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 102

Theoretical GRAVY: -0.60

Theoretical Isoelectric point (pI): 5.50

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 195

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 2.64E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 5.50E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 21.0

Midpoint transition temperature (T_m) (°C): 73.2

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_825456

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 98 HIDFKLNQH

Second heterologous peptide (Loop 4): **SEQ ID NO:** 99 YKNRINHVI

Polypeptide Sequence: **SEQ ID NO:** 100
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQHIDFKLNQHTMYYLTLEAKDGGKKKL
YEAKVWVKYKNRINHVINFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.66

Theoretical Isoelectric point (pI): 8.80

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 2271

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 1.18E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.63E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 13.9

Midpoint transition temperature (T_m) (°C): 87.4

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825329

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 101 PIYAFTEIT

Second heterologous peptide (Loop 4): **SEQ ID NO:** 102 VAHHHFLLS

Polypeptide Sequence: **SEQ ID NO:** 103
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQPIYAFTEITTMYYLTLEAKDGGKKKLYE
AKVWVKVAHHHFLLSNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.38

Theoretical Isoelectric point (pI): 6.49

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM):	Non-Competing
SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1):	3.65E+04
SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1):	4.40E-04
SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM):	12.0
Midpoint transition temperature (T_m) (°C):	Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825586

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 104 AIFTYSYPT

Second heterologous peptide (Loop 4): **SEQ ID NO:** 105 VDNYPPWP

Polypeptide Sequence: **SEQ ID NO:** 106
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQAIFTYSYPTTMYYLTLEAKDGGKKKLY
EAKVWVKVDNYPPWPNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 102

Theoretical GRAVY: -0.57

Theoretical Isoelectric point (pI): 5.90

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM):	Non-Competing
SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1):	2.09E+05
SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1):	9.80E-04
SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM):	4.7
Midpoint transition temperature (T_m) (°C):	Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_829568

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 107 THNLDVVFH

Second heterologous peptide (Loop 4): **SEQ ID NO:** 108 TIYYIAAEH

Polypeptide Sequence: **SEQ ID NO:** 109
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQTHNLDVVFHTMYYLTLEAKDGGKKK LYEAKVWVKTIYYIAAEHNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.47

Theoretical Isoelectric point (pI): 6.10

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 148

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 3.34E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 3.30E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 10.0

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_829626

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 110 EFYPNSGGP

Second heterologous peptide (Loop 4): **SEQ ID NO:** 111 YYYQGDHNY

Polypeptide Sequence: **SEQ ID NO:** 112
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQEFYPNSGGPTMYYLTLEAKDGGKKKL
YEAKVWVKYYYQGDHNYNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.79

Theoretical Isoelectric point (pI): 5.73

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 90

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 6.67E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 7.20E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 10.8

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_829638

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 113 TTEYQDHVW

Second heterologous peptide (Loop 4): **SEQ ID NO:** 114 PRFAPILLI

Polypeptide Sequence: **SEQ ID NO:** 115
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQTTEYQDHVWTMYYLTLEAKDGGKKK
LYEAKVWVKPRFAPILLINFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.50

Theoretical Isoelectric point (pI): 6.18

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 10

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 5.17E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 6.10E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 11.8

Midpoint transition temperature (T_m) (°C): 88.1

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_825515

Scaffold: T2[9_AAE]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVKAAENFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 116 QGFHPIIVE

Second heterologous peptide (Loop 4): **SEQ ID NO:** 117 AAE

Polypeptide Sequence: **SEQ ID NO:** 118
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQQGFHPIIVETMYYLTLEAKDGGKKKLY
EAKVWVKAAENFKELQEFKPVGDA

Polypeptide Amino Acid Length: 97

Theoretical GRAVY: -0.49

Theoretical Isoelectric point (pI): 5.74

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Data Unavailable

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 1.46E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.70E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 11.7

Midpoint transition temperature (T_m) (°C): 87.1

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825499

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 119 YEPDSVPSQ

Second heterologous peptide (Loop 4): **SEQ ID NO:** 120 WFLGFKGPY

Polypeptide Sequence: **SEQ ID NO:** 121
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQYEPDSVPSQTMYYLTLEAKDGGKKKL
YEAKVWVKWFLGFKGPYNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.61

Theoretical Isoelectric point (pI): 5.92

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 2247

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 1.50E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 2.36E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 15.8

Midpoint transition temperature (T_m) (°C): 90.0

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825513

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 122 NGLHPLYDE

Second heterologous peptide (Loop 4): **SEQ ID NO:** 123 ADAPFQYNN

Polypeptide Sequence: **SEQ ID NO:** 124
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQNGLHPLYDETMYYLTLEAKDGGKKKL
YEAKVWVKADAPFQYNNNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.69

Theoretical Isoelectric point (pI): 5.43

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 2080

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 1.39E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 2.03E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 14.7

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825564

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 125 LFKYEHAKV

Second heterologous peptide (Loop 4): **SEQ ID NO:** 126 NWDP

Polypeptide Sequence: **SEQ ID NO:** 127
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQLFKYEHAKVTMYYLTLEAKDGGKKKL
YEAKVWVKNWDPNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 98

Theoretical GRAVY: -0.65

Theoretical Isoelectric point (pI): 6.99

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 2160

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 1.40E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 2.16E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 15.5

Midpoint transition temperature (T_m) (°C): 90.0

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825358

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 128 SEPYISNPE

Second heterologous peptide (Loop 4): **SEQ ID NO:** 129 WIDLGGYAF

Polypeptide Sequence: **SEQ ID NO:** 130
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQSEPYISNPETMYYLTLEAKDGGKKKLY
EAKVWVKWIDLGGYAFNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.55

Theoretical Isoelectric point (pI): 5.25

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM):	Non-Competing
SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1):	6.93E+04
SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1):	5.60E-04
SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM):	8.1
Midpoint transition temperature (T_m) (°C):	90.0

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825322

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 131 VQARNPQNDQ

Second heterologous peptide (Loop 4): **SEQ ID NO:** 132 VRNFLGAWI

Polypeptide Sequence: **SEQ ID NO:** 133
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQVQARNPQNDQTMYYLTLEAKDGGK
KKLYEAKVWVKVRNFLGAWINFKELQEFKPVGDA

Polypeptide Amino Acid Length: 104

Theoretical GRAVY: -0.62

Theoretical Isoelectric point (pI): 8.17

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM):	Non-Competing
SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1):	2.11E+05
SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1):	1.16E-03
SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM):	5.5
Midpoint transition temperature (T_m) (°C):	90.0

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	Yes
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_829610

Scaffold: T2[9_AAE]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVKAAENFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 134 YGSHPLYLE

Second heterologous peptide (Loop 4): **SEQ ID NO:** 135 AAE

Polypeptide Sequence: **SEQ ID NO:** 136
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQYGSHPLYLETMYYLTLEAKDGGKKKL
YEAKVWVKAAENFKELQEFKPVGDA

Polypeptide Amino Acid Length: 97

Theoretical GRAVY: -0.58

Theoretical Isoelectric point (pI): 5.74

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 1618

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 4.17E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.30E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 31.0

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_829432

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 137 QNPVKSFYT

Second heterologous peptide (Loop 4): **SEQ ID NO:** 138 TITDPWNQY

Polypeptide Sequence: **SEQ ID NO:** 139
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQQNPVKSFYTTMYYLTLEAKDGGKKKL YEAKVWVKTITDPWNQYNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.69

Theoretical Isoelectric point (pI): 6.86

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 299

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 5.78E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.20E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 21.2

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_825265

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 140 AIFSYVKHT

Second heterologous peptide (Loop 4): **SEQ ID NO:** 141 EHVNKQDIR

Polypeptide Sequence: **SEQ ID NO:** 142
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQAIFSYVKHTTMYYLTLEAKDGGKKKLY
EAKVWVKEHVNKQDIRNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.63

Theoretical Isoelectric point (pI): 8.14

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): Data Unavailable

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): Data Unavailable

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 0.0

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825302

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 143 AFQLWDTNP

Second heterologous peptide (Loop 4): **SEQ ID NO:** 144 SYNDGWAKF

Polypeptide Sequence: **SEQ ID NO:** 145
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQAFQLWDTNPTMYYLTLEAKDGGKKK
LYEAKVWVKSYNDGWAKFNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.65

Theoretical Isoelectric point (pI): 5.91

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): Data Unavailable

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): Data Unavailable

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 0.0

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825367

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 146 HVVHIIQVQ

Second heterologous peptide (Loop 4): **SEQ ID NO:** 147 NQFHYIDGI

Polypeptide Sequence: **SEQ ID NO:** 148
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQHVVHIIQVQTMYYLTLEAKDGGKKKL
YEAKVWVKNQFHYIDGINFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.45

Theoretical Isoelectric point (pI): 6.49

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 3.14E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 3.70E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 11.6

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_829410

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 149 PIYAFTEIT

Second heterologous peptide (Loop 4): **SEQ ID NO:** 150 GGPTPEWLT

Polypeptide Sequence: **SEQ ID NO:** 151
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQPIYAFTEITTMYYLTLEAKDGGKKKLYE
AKVWVKGGPTPEWLTNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.50

Theoretical Isoelectric point (pI): 5.50

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 3.39E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 5.10E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 15.1

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_829450

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 152 STQLAGQSQ

Second heterologous peptide (Loop 4): **SEQ ID NO:** 153 LGHVFNDLV

Polypeptide Sequence: **SEQ ID NO:** 154
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQSTQLAGQSQTMYYLTLEAKDGGKKK
LYEAKVWVKLGHVFNDLVNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.49

Theoretical Isoelectric point (pI): 6.17

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM):	Non-Competing
SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1):	6.83E+04
SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1):	1.70E-03
SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM):	24.0
Midpoint transition temperature (T_m) (°C):	Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_829453

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 155 VHVITSNVA

Second heterologous peptide (Loop 4): **SEQ ID NO:** 156 FGFIHHDVV

Polypeptide Sequence: **SEQ ID NO:** 157
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQVHVITSNVATMYYLTLEAKDGGKKKL
YEAKVWVKFGFIHHDVVNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.32

Theoretical Isoelectric point (pI): 6.49

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 4.59E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 6.30E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 13.8

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_829663

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 158 HIFAFAYPT

Second heterologous peptide (Loop 4): **SEQ ID NO:** 159 NHHPREEWA

Polypeptide Sequence: **SEQ ID NO:** 160
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQHIFAFAYPTTMYYLTLEAKDGGKKKLY
EAKVWVKNHHPREEWANFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.65

Theoretical Isoelectric point (pI): 6.49

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM):	Non-Competing
SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1):	2.84E+04
SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1):	1.60E-03
SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM):	57.0
Midpoint transition temperature (T_m) (°C):	Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_829428

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 161 IKRTFPLLF

Second heterologous peptide (Loop 4): **SEQ ID NO:** 162 HYEVHNASPF

Polypeptide Sequence: **SEQ ID NO:** 163
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQIKRTFPLLFTMYYLTLEAKDGGKKKLY
EAKVWVKHYEVHNASPFNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 104

Theoretical GRAVY: -0.51

Theoretical Isoelectric point (pI): 8.15

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GYPYDVPDYACGGGGSHHHHHH

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): 1557

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 2.06E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 7.80E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 38.0

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	No

Polypeptide: 620_825489

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 164 EIYAYSQGT

Second heterologous peptide (Loop 4): **SEQ ID NO:** 165 KVDDLFPFG

Polypeptide Sequence: **SEQ ID NO:** 166
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQEIYAYSQGTTMYYLTLEAKDGGKKKL
YEAKVWVKKVDDLFPFGNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.54

Theoretical Isoelectric point (pI): 5.50

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 2.07E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.18E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 5.7

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825601

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 167 SIYHNAVRS

Second heterologous peptide (Loop 4): **SEQ ID NO:** 168 YGFIGNTKL

Polypeptide Sequence: **SEQ ID NO:** 169
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQSIYHNAVRSTMYYLTLEAKDGGKKKL
YEAKVWVKYGFIGNTKLNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.53

Theoretical Isoelectric point (pI): 8.80

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 3.25E+05

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.82E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 5.6

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825423

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 170 DIHAFTVQT

Second heterologous peptide (Loop 4): **SEQ ID NO:** 171 NDRFLEDLD

Polypeptide Sequence: **SEQ ID NO:** 172
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQDIHAFTVQTTMYYLTLEAKDGGKKKL
YEAKVWVKNDRFLEDLDNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.60

Theoretical Isoelectric point (pI): 5.23

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 6.65E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 6.50E-04

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 9.8

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	No
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

Polypeptide: 620_825576

Scaffold: T2[9_9]

Scaffold Sequence:
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQ<LOOP_2>TMYYLTLEAKDGGKKKLY
EAKVWVK<LOOP_4>NFKELQEFKPVGDA<VECTOR-DERIVED TERMINUS>

First heterologous peptide (Loop 2): **SEQ ID NO:** 173 PQKPDYITI

Second heterologous peptide (Loop 4): **SEQ ID NO:** 174 NAEFLGGIV

Polypeptide Sequence: **SEQ ID NO:** 175
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQPQKPDYITITMYYLTLEAKDGGKKKLY
EAKVWVKNAEFLGGIVNFKELQEFKPVGDA

Polypeptide Amino Acid Length: 103

Theoretical GRAVY: -0.48

Theoretical Isoelectric point (pI): 5.92

Empirical data generated using <VECTOR-DERIVED TERMINUS>:

GDYKDHDGDYKDHDIDYKDDDDKGGGGSHHHHHHC

SARS-CoV-2 S1:ACE2 Competition ELISA IC_{50} (nM): Non-Competing

SARS-CoV-2 S1 Association rate constant (k_a) (M-1 s-1): 9.36E+04

SARS-CoV-2 S1 Dissociation rate constant (k_d) (s-1): 1.63E-03

SARS-CoV-2 S1 Equilibrium dissociation constant (K_D) (nM): 17.4

Midpoint transition temperature (T_m) (°C): Data Unavailable

Coronavirus S1 Protein	*Reactivity*
SARS-CoV-2	Yes
SARS-CoV	Yes
MERS-CoV	No
hCoV-229E	No
hCoV-HKU1	No
hCoV-NL63	Not tested

EXAMPLE 6: Exemplary Polypeptide Expression and Purification

In this example an exemplary method for expression and purification of anti-SARS-CoV-2 Spike protein polypeptide(s) (i.e. polypeptide(s) of the invention) is described. In this example, nucleic acid comprising nucleotide sequence encoding the polypeptide of the invention is placed into a pETLECTRA expression vector under control of IPTG-inducible promoter. In this example the expression vector is then introduced into a host cell for expression – in this example the host cell is BL21 gold (DE3).

Candidate polypeptide (Affimer® Agent) clones selected from phage display and high throughput screening are stored as glycerol stocks.

Expression

For expression 5µl of each Affimer® Agent candidate is inoculated from a partially thawed glycerol stock into 5ml LB (Lennox) EZ mix powder **Sigma L7658**, 1% (w/v) glucose **Sigma G8270**, 50µg/ml Kanamycin **Sigma K1377**, in a 14ml Snap Cap Falcon® tube, **Corning Product number 352051**. This is then cultured o/n 37°C 220rpm. The following morning the OD600 of the culture is determined and the volume of inoculum to transfer to the expression culture vessel is determined to give a final OD600 in the culture medium of 0.025. The expression is carried out in 2.2L baffled shake flasks containing 500ml of TB (Terrific Broth) medium, **Melford T15100-5000.0**, 0.4% (v/v) Glycerol, **Fisher G/0650/17**, with 50µg/ml Kanamycin, **Sigma K1377**. The expression culture is incubated 37°C 220rpm with the OD600 being followed until a value of 0.6-0.7 is reached at this point the temperature is lowered to 25°C and the expression is induced by the addition of IPTG, **Sigma I6758**, to final 1mM concentration. The cultures are harvested by centrifugation 20 minutes 9,000 x (g) at room temperature, roughly 24 hours post inoculation.

The harvested cell pellets are routinely frozen at -20°C – this is mainly for scheduling convenience but the added freeze thaw can aid in cell disruption.

Lysis

Lysis buffer: 100mM Sodium phosphate monobasic (Na2HPO4), **Sigma S8282-1KG**, 279mM Sodium Chloride, **Sigma S7653-1KG**, 30mM imidazole, **Fisher Scientific BP305-50**, 5mM TCEP, **Generon GEN-TCEP-10**, pH 8.0, with 10% (v/v) SoluLyse™, **Genlantis #L100500**, supplemented with Benzonase, **Novagen, Millipore #71205**, at 25u/ml of buffer.

Frozen cell pellets are retrieved from the freezer and allowed to thaw at room temperature.

25-30ml Lysis buffer is added to the thawed cell pellets. The pellets are resuspended into the lysis buffer with a 25ml Stripette and then transferred to 50ml CELLSTAR® polypropylene tubes, **Greiner BioOne International Item No.: 227261**. Once resuspended the cell slurry is left to incubate on a roller for half an hour at room temperature. The next step is sonication using a QSonica 700 disruptor, **Part #Q700** with 4-tip horn, **Part #4659**, **Qsonica L.L.C 53 Church Hill Rd. Newtown, CT 06470**, four samples being processed simultaneously; performed in a water/ice bath. Sonication settings amplitude 25µ, 5 minutes duration, duty cycle 10seconds on 10 seconds off, with a temperature cut out of 40°C. This generally delivers 10kJ of energy – if the sonication has not proceeded optimally it is run again until 10kJ is reached. The sonicated material is then clarified by centrifugation 20 minutes 16,000 x (g) at 4°C.

Purification

Clarified supernatants are purified by immobilised metal affinity chromatography (IMAC) followed by size exclusion chromatography (SEC) on ÄKTAxpress instruments, **GE Healthcare Product 18664501**. Using 4 x 1ml HisTrap FF crude columns, **Cytiva product 11000458** and one HiLoad Superdex 75 pg 16/60 column, **Cytiva Product 28989333**, one to four Affimer® protein candidates can be processed overnight. The clarified lysates are loaded sequentially on to the HisTrap columns. These columns are then eluted one at a time into 10ml holding loops prior to re-injection to the SEC column where monomeric and dimeric forms are separated. Fractionation of the eluted material is automated with peak calling based upon peak height or slope.

IMAC buffers:

NPI-10 TCEP pH8.0

- 100mM Sodium phosphate monobasic (Na2HPO4), **Sigma S8282-1KG**
- 300mM Sodium Chloride, **Sigma S7653-1KG**
- 10mM Imidazole, **Fisher Scientific BP305-50**

- 5mM TCEP, **Generon GEN-TCEP-10**

NPI-40 TCEP pH8.0

- 100mM Sodium phosphate monobasic (Na2HPO4), **Sigma S8282-1KG**
- 1M Sodium chloride, **Sigma S7653-1KG**
- 40mM Imidazole, **Fisher Scientific BP305-50**
- 5mM TCEP, **Generon GEN-TCEP-10**

NPI-400 TCEP pH8.0

- 100mM Sodium phosphate monobasic (Na2HPO4), **Sigma S8282-1KG**
- 500mM Sodium chloride, **Sigma S7653-1KG**
- 400mM Imidazole, **Fisher Scientific BP305-50**
- 5mM TCEP, **Generon GEN-TCEP-10**

SEC buffer (Affimer® Agent Storage CBS pH6.5):

- 100mM Sodium citrate tribasic dihydrate, **Sigma S4641**
- 150mM Sodium chloride, **Sigma S7653**
- 5mM TCEP, **Generon GEN-TCEP-10**
- 0.02% (w/v) Sodium azide, **Severn Biotech 40-2010-01**

The IMAC columns are pre-equilibrated with NPI-10 TCEP. Samples are loaded at 1ml/min until air is detected at which point the system switches back to NPI-10 TCEP buffer to finalise sample loading onto the column. The IMAC column is then washed with NPI-10 TCEP buffer for a further 10ml. The column is then washed for up to 20CV (20ml) with NPI-10 TCEP buffer or until a stable UV baseline is achieved. Next the column is washed for 10CV (10ml) with NPI-40 TCEP @ 2ml/min to remove non-specifically bound material from the column. The system is then flushed with 30ml NPI-10 TCEP prior to elution of the column with 5CV (5ml) NPI-400 TCEP @1ml/min. The eluted peak of Affimer® protein is automatically collected into a 10ml holding loop for re-injection over the SEC column.

The SEC column is pre-equilibrated in Affimer® Agent Storage CBS the re-injected Affimer® protein sample is run @1.5ml/min down the SEC column. Peak calling is automated and allows for fractions of up to 2ml to be collected from under the eluted peak materials. Monomeric polypeptides of the invention (Affimer® proteins) elute at around 80ml - 85ml.

We refer to Figures 6, 7 and 8 which show exemplary purification data.

Pooled Affimer® Candidate proteins are quantitated on the Perkin Elmer Droplet Quant instrument to allow a normalised 2µg loading for the final QC gel above.

We refer to Figure 9 which shows SDS-PAGE 2µg QC gel of Affimer® proteins, in which:

Marker:	SeeBlue® Plus2 Prestained Standard
1	Control Affimer® protein 2µg mIgG_{2b} G12 c3xFLAG-His_6-Cys
2. - 4.	Irrelevant Affimer® proteins
5.	Sample ID 6353 Affimer® Candidate 620_826257 c3xFLAG-His_6-Cys
6.	Sample ID 6342 Affimer® Candidate 620_825425 c3xFLAG-His_6-Cys
7.	Sample ID 6343 Affimer® Candidate 620_825436 c3xFLAG-His_6-Cys
8. - 10.	Irrelevant Affimer® proteins

EXAMPLE 7: Protocol of S1 protein reactivity ELISA

All pipetting carried out using calibrated single- and multi-channel pipettes and automated plate washers to minimise variation between wells.

Maxisorp 96-well plates were coated with 100 µL per well immobilised metal affinity chromatography and size exclusion chromatography (IMAC-SEC) purified polypeptide of the invention (i.e. anti-SARS-CoV-2 S1 spike protein Affimer® reagent) (as per example 5 above, each polypeptide including the relevant C-terminal extension (vector-derived terminus sequence) as disclosed above), diluted to 2 µg/mL in 50 mM carbonate-bicarbonate buffer, pH 9.6 (Sigma Aldrich, C3041; one capsule in 100 mM deionised water). This was left overnight for at least 15 hours at 4 °C, stationary. During this and each subsequent incubation, the plates were stacked vertically with the top plate having an unused Maxisorp 96-well plate placed on top to minimise evaporation. After the coating, and each subsequent step, plates were washed three times with 300 µL per well Phosphate Buffered Saline (PBS), pH 7.4 (Gibco, 70011; 10x solution diluted 1:10 in deionised water) containing 0.05 % (v/v) Tween®-20 (Sigma Aldrich, P9416) (PBS-T). Excess PBS-T was removed prior to subsequent additions.

Unbound sites on the plate were blocked with 300 µL per well 1 x PBS Casein ELISA Reagent (PBS-C) (Abcam, ab171532; 5x solution diluted 1:5 in deionised water), and incubated for 2 hours. During this and subsequent incubations, plates were shaken at 200 rpm at 25 °C. During the blocking step, serial dilutions were prepared of Biotinylated S1 spike proteins (Including: SARS-CoV-2, ACRO Biosystems, S1N-C82E8; SARS-CoV, Sino Biological, 40150-V08B1; MERS-CoV, Sino Biological, V0069-V08H; hCoV-229E, Sino Biological, 40601-V08H; hCoV-HKU1, Sino Biological,

40602-V08H, hCoV-NL63, Sino Biological, 40600-V08H) diluted in PBS-C, titrated 3-fold from 500 ng/mL to 0.7 ng/mL. 100 µL per well of the relevant assay condition containing titrated S1 spike protein or no S1 spike protein was transferred to the plate and incubated for 1 hour.

Streptavidin, poly-HRP conjugate (Pierce, 21140) was diluted 1:10,000 in PBS-C. 100 µL per well was transferred to the plate and incubated for 1 hour. During this incubation TMB was warmed to room temperature. 100 µL per well TMB substrate (Surmodics, TMBS-1000-01) was transferred to plate with colour allowed to develop, before the addition of 50 µL per well 0.5 M Sulphuric Acid (Fisher, J/8430/15) to the plate in order to cease the colorimetric reaction. Each plate's absorbance was read by a microplate reader at 450 nm and 630 nm. The background at 630 nm was subtracted from absorbance at 450 nm to give reported optical densities.

Exemplary S1-reactivity ELISA data

Optical densities observed against biotinylated S1 proteins at 55 ng/mL for three exemplary polypeptides of the invention are shown in Figure 11.

EXAMPLE 8: Protocol of S1 protein reactivity flow cytometry

All pipetting carried out using calibrated single- and multi-channel pipettes to minimise variation between wells.

Sulfhydryl QBeads DevScreen (Intellicyt, 90892-90921) beads were conjugated to Streptavidin (New England Biolabs, N7021S) according to the bead manufacturers protocol. Beads were washed with Phosphate Buffered Saline (PBS), pH 7.4 (Gibco, 70011; 10x solution diluted 1:10 in deionised water) containing 1 % (w/v) BSA (Sigma Aldrich, A3059) (1% BSA, 1 x PBS), sedimenting by centrifugation at 8000 x g, 3 minutes, at this and subsequent wash steps.
Biotinylated S1 spike proteins (Including: SARS-CoV-2, ACRO Biosystems, S1N-C82E8; SARS-CoV, Sino Biological, 40150-V08B1; MERS-CoV, Sino Biological, V0069-V08H; hCoV-229E, Sino Biological, 40601-V08H; hCoV-HKU1, Sino Biological, 40602-V08H, hCoV-NL63, Sino Biological, 40600-V08H) were then coated to beads at 1 µg/mL in (1% BSA, 1 x PBS), 30 minutes, end-over-end mixing, before addition of 1 µg/mL Biotin (Sigma, B4501) to quench unoccupied Streptavidin, 20 minutes, end-over-end mixing. Beads were washed twice as described above, before resuspension in

0.2% BSA, 1 x PBS (components as for 1% BSA, 1 x PBS), combination into a multi-plex bead set and addition of 10 µL per well to 96-well Microplates (Qiagen, 19581).

Immobilised metal affinity chromatography and size exclusion chromatography (IMAC-SEC) purified anti-SARS-CoV-2 S1 spike protein Affimer® reagent (as per disclosure, including listed vector-derived terminus) was diluted to 2.5 µg/mL in 0.2% BSA, 1 x PBS, and 10 µL was added to triplicate wells and incubated for 1 h. An Alexa-Fluor®488 tagged antibody specific for a peptide tag present in the vector derived terminus (Anti-HA: BioLegend, 901509, Anti-FLAG: BioLegend, 637318) was diluted to 1 µg/mL in 0.2% BSA, 1 x PBS and 10 µL added to the relevant wells, incubating for a further 1 hr, in darkness. The plate was analysed using an iQue Screener flow cytometer (Intellicyt), with each bead type within the multi-plex beadset gated using indexed fluorescent properties (using FL3-H/FL4-H channels in a 3:1 SDS configuration). The median fluorescence intensity of the Alexa-Fluor®488 reagent (FL1-A channel) was reported for each S1 spike protein-immobilised bead type.

EXAMPLE 9: Protocol of anti-SARS-CoV-2 S1 protein ELISA

All pipetting carried out using calibrated single- and multi-channel pipettes and automated plate washers to minimise variation between wells.
Maxisorp 96-well plates were coated with 100 µL per well immobilised metal affinity chromatography and size exclusion chromatography (IMAC-SEC) purified polypeptides of the invention (i.e. anti-SARS-CoV-2 S1 spike protein Affimer® reagents), 620_825436-620_825436-cHis6 and 620_825425-620_825425-cHis6, each diluted to 1 µg/mL in 50 mM carbonate-bicarbonate buffer, pH 9.6 (Sigma Aldrich, C3041; one capsule in 100 mM deionised water) to a combined total of 2 µg/mL polypeptide (Affimer® reagent) per well. This was left overnight for at least 15 hours at 4 °C, stationary. During this and each subsequent incubation, the plates were stacked vertically with the top plate having an unused Maxisorp 96-well plate placed on top to minimise evaporation. After the coating, and each subsequent step, plates were washed three times with 300 µL per well Phosphate Buffered Saline (PBS), pH 7.4 (Gibco, 70011; 10x solution diluted 1:10 in deionised water) containing 0.05 % (v/v) Tween®-20 (Sigma Aldrich, P9416) (PBS-T). Excess PBS-T was removed prior to subsequent additions.
Unbound sites on the plate were blocked with 300 µL per well 1 x PBS Casein ELISA Reagent (PBS-C) (Abcam, ab171532; 5x solution diluted 1:5 in deionised water), and incubated for 2 hours. During this and subsequent incubations, plates were shaken at

200 rpm at 25 °C. During the blocking step, serial dilutions were prepared of SARS-CoV-2 S1 spike protein (ACRO Biosystems, S1N-C52H4) diluted in PBS-C or PBS-C containing 50 % saliva from a pool of donors collected prior to the known SARS-CoV-2 outbreak (Dec 2019-Aug 2020 at time of writing) (Lee Bioscience, 991-05-P), with SARS-CoV-2 S1 spike protein titrated 2-fold from 2.5 ng/mL to 2 pg/mL. 100 µL per well of the relevant assay condition containing titrated SARS-CoV-2 S1 spike protein or no SARS-CoV-2 S1 spike protein was transferred to the plate and incubated for 1 hour. IMAC-SEC purified anti-SARS-CoV-2 S1 spike protein Affimer® reagent, 620_826257 c3xFLAG-His6-CysPEG$_2$Biotin (ThermoFisher Scientific, 21901BID biotin moiety conjugated to Affimer® reagent following manufacturer's protocol with 20-fold molar excess. This reagent includes PEG$_2$ linker.), was diluted to 1 µg/mL in PBS-C. 100 µL per well of 1 µg/mL 620_826257 c3xFLAG-His6-CysPEG$_2$Biotin was transferred to the plate and incubated for 1 hour.

Streptavidin, poly-HRP conjugate (Pierce, 21140) was diluted 1:10,000 in PBS-C. 100 µL per well was transferred to the plate and incubated for 1 hour. During this incubation TMB substrate was warmed to room temperature. 100 µL per well TMB substrate (Surmodics, TMBS-1000-01) was transferred to plate with colour allowed to develop, before the addition of 50 µL per well 0.5 M Sulphuric Acid (Fisher, J/8430/15) to the plate in order to cease the colorimetric reaction. Each plate's absorbance was read by a microplate reader at 450 nm and 630 nm. The background at 630 nm was subtracted from absorbance at 450 nm to give the reported optical density.

The OD values of the titrated target were then used to create a standard curve from which unknown sample concentrations, run alongside the curve, could be quantified. 4PL non-linear regression analysis was used to fit curves in GraphPad Prism software. The OD readings for the curve were then interpolated from the curve to provide calibration standard metrics (shown below). The inter-assay data was from duplicate measurements in 6 repeat assays.

		Buffer	50% saliva
Calibration range	ULOQ	0.625 ng/mL	1.25 ng/mL *
	LLOQ	5 pg/mL	20 pg/mL *
Inter-assay calibration standard metrics	% CV	0.3 – 9.3	0.4 – 4.9
	% Recovery	92.2 – 101.9	98.0 – 107.3

Intra-assay calibration standard metrics	% CV	0.0 – 16.6	0.3 – 17.0
	% Recovery	82.7 – 110.8	95.3 – 112.3

* physiological range in undiluted saliva

We refer to Figure 12 which shows exemplary accuracy and precision (A&P) curve data.

The biotinylation process for the detection Affimer® Agent was modified to an in vivo biotinylation process to allow improved manufacturability. In vivo biotinylation can be achieved, for example, with the co-expression of two different plasmids in E. coli: one plasmid encoding the Avi-tagged Affimer® protein of interest and one plasmid encoding the biotin ligase enzyme (BirA). Specifically, for the exemplary Affimer® Agent 620_826257, the nucleotide sequence encoding 620_826257 can be subcloned into a vector which contains a C-terminal 6xHistidine HisTag followed by a GGGGS linker and the 15 amino acid sequence known as an AviTag™ (GLNDIFEAQKIEWHE (SEQ ID NO: 189)). The plasmid containing an Avi-tagged Affimer® candidate sequence can then be transformed into a strain of E. coli which also contains the plasmid encoding the biotin ligase enzyme BirA (e.g. BL21 DE3 pBirA). Upon induction of Affimer® protein expression with Isopropyl β- d-1-thiogalactopyranoside (IPTG), excess biotin is added to the culture. The BirA enzyme catalyses formation of the amide linkage between biotin and the lysine within the AviTag™. The biotinylated Affimer® candidate can then be purified using standard IMAC-SEC processes known by the person skilled in the art.

The protocol of anti-SARS-CoV-2 S1 protein ELISA was used to determine the effect of the in vivo biotinylation process on assay performance. All steps were carried out as described above for the anti-SARS-CoV-2 S1 protein ELISA that generated the data shown in the Table above, but also using in vivo biotinylated IMAC-SEC purified anti-SARS-CoV-2 S1 spike protein Affimer® reagent, 620_826257_cHis6-AviTag™-Biotin.

The data obtained with the in vivo biotinylated anti-SARS-CoV-2 S1 spike protein Affimer® reagent, 620_826257_cHis6-AviTag™-Biotin is summarised in the following Table. The inter-assay data was from duplicate measurements in 4 repeat assays.

		Buffer	50% saliva
Calibration range	ULOQ	2.5 ng/mL	5.0 ng/mL *
	LLOQ	20 pg/mL	40 pg/mL *
Inter-assay calibration standard metrics	% CV	0.6 – 10.0	0.1 – 7.7
	% Recovery	98.7 – 106.0	97.8 – 102.8
Intra-assay calibration standard metrics	% CV	0.2 – 17.2	0.8 – 18.0
	% Recovery	89.4 – 114.1	91.4 – 110.1

* physiological range in undiluted saliva

Figure 15 further shows data from these assays performed with PEG_2-biotinylated detection Affimer® Agent (Figure 15 A) and in vivo biotinylated detection Affimer® Agent (Figure 15 B), each assessed in both 50% saliva and in buffer. When the in vivo biotinylated anti-SARS-CoV-2 S1 spike protein Affimer® reagent 620_826257_cHis6-AviTag™-Biotin (Figure 15 B) was used, sensitivity of the assay was slightly reduced, but the assay range was extended, relative to when the PEG_2 biotinylated anti-SARS-CoV-2 S1 spike protein Affimer® reagent, 620_826257 c3xFLAG-His6-CysPEG_2Biotin was used (Figure 15 A). Assay variation was similar. Sensitivity has been demonstrated at 40 pg/mL in 50% saliva and 20 pg/mL in buffer.

A similar calibration exercise can be carried out using virus, e.g. cultured live virus, to prepare serially diluted samples as described above for the spike protein. Suitably the assay may be used to detect viral titres in the range 10^3-10^4 viral particles/ml (vp/ml).

The polypeptides used in the above and below examples (e.g. to generate the corresponding data) are as follows:

620_825436-620_825436-cHis6 (SEQ ID NO: 190)
SEQ ID NO:70 (minus C-terminal A)|SEQ ID NO:70 **Glycine linker|6 x Histidine tag**
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQHQPKWPGFTTMYYLTLEAKDGGKKK
LYEAKVWVKTFHQTEPSPNFKELQEFKPVGD|ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRV
VKAKEQHQPKWPGFTTMYYLTLEAKDGGKKKLYEAKVWVKTFHQTEPSPNFKELQEFKPVGDA**GHHHHH**
H

620_825594-620_825594-cHis6 (SEQ ID NO: 191)
SEQ ID NO:85 (minus C-terminal A)|SEQ ID NO:85 **Glycine linker|6 x Histidine tag**
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQQELVDLWDATMYYLTLEAKDGGKKK LYEAKVWVKASYGQITFSNFKELQEFKPVGD|ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRV VKAKEQQELVDLWDATMYYLTLEAKDGGKKKLYEAKVWVKASYGQITFSNFKELQEFKPVGDA**G|HHHH HH**

620_825425-620_825425-cHis6 (SEQ ID NO: 192)
SEQ ID NO:67 (minus C-terminal A)|SEQ ID NO:67 **Glycine linker|6 x Histidine tag**
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQEWFIVPIPQTMYYLTLEAKDGGKKKL YEAKVWIDWNFKELQEFKPVGD|ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQE WFIVPIPQTMYYLTLEAKDGGKKKLYEAKVWIDWNFKELQEFKPVGDA**G|HHHHHH**

620_826257 c3xFLAG-His6-CysPEG2Biotin (SEQ ID NO: 193)
SEQ ID NO:7**Glycine linker|3xFLAG tag|poly-glycine/serine linker|6 x Histidine tag|Cysteine (Maleimide-PEG2-Biotin conjugated)**
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDHANWHPQLDGLNYYIKVRVNGKY IHLKVFKSPNSHKLFWPVEDLVLTGYQVDKNKDDELTGF**G|DYKDHDGDYKDHDIDYKDDDDK|GGGGS |HHHHHH|C**-Biotin

620_826257_cHis6-AviTag™ (SEQ ID NO: 194)
SEQ ID NO:7 **Glycine linker|6 x Histidine tag|poly-glycine/serine linker|AviTag™ (biotin conjugated on Lysine position 135)**
MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVDHANWHPQLDGLNYYIKVRVNGKY IHLKVFKSPNSHKLFWPVEDLVLTGYQVDKNKDDELTGF**G|**FGHHHHHHGGGGSGLNDIFEAQ**K-[Biotin]-**IEWHE

This exemplary ELISA protocol is tested and Figure 10 shows optical density data shown for a top concentration of 2.5 ng/mL of the S1 spike protein reagents as listed above (i.e. capture with 620_825436-620_825436-cHis6 and 620_825425-620_825425-cHis6; detection with 620_826257 c3xFLAG-His6-CysPEG$_2$Biotin; developed/visualised with Streptavidin, poly-HRP conjugate (Pierce, 21140)). The assay was challenged with several coronaviruses. This anti-SARS-CoV-2 S1 protein ELISA recognises SARS-CoV-2 S1 only. No interaction was observed with any of the other coronaviruses tested (Figure 10).

Exemplary anti-SARS-CoV-2 S1 protein ELISA data

The protocol of anti-SARS-CoV-2 S1 protein ELISA was also used to determine the ability of the polypeptides of the invention (i.e. anti-SARS-CoV-2 S1 spike protein

Affimer® reagents) to bind to constructs other than SARS-CoV-2 S1 spike protein (construct comprises Val16 – Arg685 and can be expressed using standard expression protocols known by the person skilled in the art, or can be purchased, for example, from ACRO Biosystems, S1N-C52H4), such as SARS-CoV-2 S1 spike protein with D614G mutation (the construct comprises Val16 – Arg685 and includes the D614G mutation and can be expressed using standard expression protocols known by the person skilled in the art, or can be purchased, for example, from ACRO Biosystems, S1N-C5256) and the homo-trimeric form of the SARS-CoV-2 spike protein (construct comprises Val16 – Pro1213 and includes the proline substitutions F817P, A892P, A899P, A942P, K986P, V987P and alanine substitutions R683A and R685A, which are introduced to stabilise the trimeric prefusion state of SARS-CoV-2 S protein and to abolish the furin cleavage site, respectively; this construct can be expressed using standard expression protocols known by the person skilled in the art, or can be purchased, for example, from ACRO Biosystems, SPN-C52H8).

Mutated viruses with D614G substitution in the S protein were isolated early in the pandemic and are now the most dominant form of SARS-CoV-2 worldwide.

The anti-SARS-CoV-2 S1 protein ELISA was challenged with SARS-CoV-2 S1 spike protein with the D614G mutation using the same protocol of anti-SARS-CoV-2 S1 protein ELISA described above with the following modifications. Experiments were carried out with two different sets of capture anti-SARS-CoV-2 S1 spike protein Affimer® reagents according to the invention. One set of experiments was carried out using 620_825436-c3xFLAG-His6-Cys and 620_825594-c3xFLAG-His6-Cys, each diluted to 0.5 µg/mL, in 50 mM carbonate-bicarbonate buffer, pH 9.6 (Sigma Aldrich, C3041; one capsule in 100 mM deionised water) to a combined total of 1 µg/mL polypeptide (Affimer® reagent) for coating each well of the Maxisorp 96-well plates. A second set of experiments was carried out using 620_825436-c3xFLAG-His6-Cys and 620_825425-c3xFLAG-His6-Cys, each diluted to 0.5 µg/mL in 50 mM carbonate-bicarbonate buffer, pH 9.6 (Sigma Aldrich, C3041; one capsule in 100 mM deionised water) to a combined total of 1 µg/mL polypeptide (Affimer® reagent) for coating each well of the Maxisorp 96-well plates. Maxisorp 96-well plates coated with mouse IgG2b G12 Affimer® Agents were used as a negative control. Plate incubation, washing and blocking was carried out as described in the above protocol of anti-SARS-CoV-2 S1 protein ELISA.

During the blocking step, serial dilutions were prepared of SARS-CoV-2 S1 spike protein (ACRO Biosystems, S1N-C52H3) and separately of SARS-CoV-2 S1 (D614G)

spike protein (ACRO Biosystems, S1N-C5256) diluted in PBS-C with SARS-CoV-2 S1 spike protein or SARS-CoV-2 S1 (D614G) spike protein titrated 2-fold from 2.5 ng/mL to 2.4 pg/mL. 100 µL per well of the relevant assay condition containing titrated SARS-CoV-2 S1 spike protein or SARS-CoV-2 S1 (D614G) spike protein was transferred to the plate and incubated for 1 hour. Detection and data analysis was carried out using IMAC-SEC purified anti-SARS-CoV-2 S1 spike protein Affimer® reagent, 620_826257_c3xFLAG-His6-CysPEG$_2$Biotin (ThermoFisher Scientific, 21901BID biotin moiety conjugated to Affimer® reagent following manufacturer's protocol with 20-fold molar excess. This reagent includes PEG$_2$ linker) as described in the above protocol of anti-SARS-CoV-2 S1 protein ELISA.

The anti-SARS-CoV-2 S1 protein ELISA produced comparable results for the detection of SARS-CoV-2 S1 spike protein or SARS-CoV-2 S1 (D614G) spike protein (data shown in Figure 16).

The surface of the SARS-CoV-2 virus has a spike (S) glycoprotein, which is a large homo-trimeric glycoprotein that forms a crown at the virus capsid surface. Each S protomer is further divided into two domains – S1 and S2 – that are demarcated by a furin cleavage site. The S1 domain is responsible for recognition and binding to the angiotensin-converting enzyme 2 (ACE2) receptor on the host cell.

The anti-SARS-CoV-2 S1 protein ELISA was carried out with SARS-CoV-2 S1 spike protein monomer (ACRO Biosystems, S1N-C52H3) or SARS-CoV-2 S spike protein trimer (ACRO Biosystems, SPN-C52H8) using the same protocol of anti-SARS-CoV-2 S1 protein ELISA described above.

During the blocking step, serial dilutions were prepared of SARS-CoV-2 S1 spike protein (ACRO Biosystems, S1N-C52H3) and separately of SARS-CoV-2 S spike protein trimer (ACRO Biosystems, SPN-C52H8), each diluted in PBS-C with SARS-CoV-2 S1 spike protein or SARS-CoV-2 S spike protein trimer titrated 2-fold from 2.5 ng/mL to 2.4 pg/mL. This was then converted to molarity for direct comparison (32.5 pM to 31.7 fM for the S1 and 6 pM to 5.9 fM for the trimer). 100 µL per well of the relevant assay condition containing titrated SARS-CoV-2 S1 spike protein or SARS-CoV-2 S spike protein trimer was transferred to the plate and incubated for 1 hour. Detection and data analysis was carried out using IMAC-SEC purified anti-SARS-CoV-2 S1 spike protein Affimer® reagent, 620_826257-c3xFLAG-His6-CysPEG$_2$Biotin (ThermoFisher Scientific, 21901BID biotin moiety conjugated to Affimer® reagent following

manufacturer's protocol with 20-fold molar excess. This reagent includes PEG_2 linker) as described in the above protocol of anti-SARS-CoV-2 S1 protein ELISA.

The anti-SARS-CoV-2 S1 protein ELISA can detect both SARS-CoV-2-S1 spike protein or SAR-CoV-2 S spike protein trimer (data shown in Figure 17).

EXAMPLE 10: Detection of variant SARS-CoV-2 S1 proteins

Anti-SARS-CoV-2 S1 spike protein Affimer® reagent (in-vivo biotinylated, 620_826257_cHis6-AviTag™-Biotin, obtained as described in Example 9) was used for detection in a lateral flow device suitable for use as a screening test to identify individuals with SARS-CoV-2 infection (see e.g. Figure 4) and was used to detect two different variants of SARS-CoV-2 antigen in human anterior nasal swabs.

All proteins were applied to the test at a concentration of 1 μg/mL and the results were read after 20 minutes.

Recombinant SARS-CoV-2 S1 proteins for two variants were tested. The wild type SARS-CoV-2 S1 recombinant protein used to develop the test was used as a positive control. The variants tested were (i) wild-type SARS-CoV-2 S1 (control), (ii) the D614G mutant, and (iii) the B.1.1.7 variant (also commonly referred to as the "Kent", "English" or "U.K." variant). The D614G mutant is the most prevalent variant globally at the time of writing. The B.1.1.7 variant is becoming more prevalent globally, and was found in many cases in the most recent peak in UK infections. It is of particular concern because it shows higher transmissibility than wild type SARS-CoV-2 virus.

The LFD assay showed positive results of comparable strength for each sample tested, i.e., the wild-type control and both variants. Thus, the results show that the test detects multiple variants of the SARS-CoV-2 virus, including the B.1.1.7 variant. The polypeptides of the invention as defined in the claims can bind to variant strains of SARS-CoV-2 virus.

EXAMPLE 11: Evaluation of Viral Neutralization Activity in Infectivity Assay

To evaluate the potential for raised Affimer® Agents to act as potent inhibitors of virus entry, we employed a pseudotype virus neutralisation assay. Lentivirus pseudotyped to bear the SARS-COV 2 S protein was incubated in the presence of COVID (SARS-CoV-2) binding Affimer® Agents over a series of known concentrations before being

innoculated onto HEK-293T cells engineered to express the cellular receptor ACE2. Readout of virus entry is by expression of a luciferase gene carried by the lentivirus. Most of the clones demonstrated the ability to inhibit viral infection of the HEK cells, with varying IC50s.

Although illustrative embodiments of the invention have been disclosed in detail herein, with reference to the accompanying drawings, the skilled reader will understand that the invention is not limited to the precise embodiments and that various changes and modifications can be effected by a person skilled in the art without departing from the scope of the invention as defined by the appended claims and their equivalents.

EXAMPLE 12: Cross Reactivity / Microbial Interference Study of Lateral Flow Antigen Test

Independent validation of the cross-reactivity and microbial interference was carried out for the exemplary lateral flow platform Avacta® AffiDX® SARS-CoV-2 Lateral Flow Antigen Test that detects S1 on a lateral flow device.

Cross reactivity studies were performed to demonstrate that the test does not react with related pathogens, high prevalence disease agents, and normal or pathogenic flora that are reasonably likely to be encountered in the clinical specimen. FDA recommends that the organisms are wet-tested in negative clinical matrix at concentrations of 10^6 CFU/ml or higher for bacteria and 10^5 pfu/ml or higher for viruses.

A microbial interference study demonstrates that false negatives will not occur when SARS-CoV-2 is present in a specimen with other microorganisms. Contrived specimens with SARS-CoV-2 and common organisms found in the matrix were used. Microbial interference was evaluated using samples spiked at a low (3 x LoD) SARS-CoV-2 concentration and a high interference level (preferably microorganisms) to represent the worst-case scenario. If interference is observed at the level tested, an additional titration study should be performed to determine the highest microorganism interference level the test can tolerate.

Cell culture supernatants containing enterovirus, rhinovirus, respiratory syncytial virus (RSV), influenza A virus H1N1, Influenza A virus H3N2, influenza B virus, parainfluenza 1 (PIV1), parainfluenza 2 (PIV2), parainfluenza 3 (PIV3), parainfluenza 4 (PIV4), human coronavirus 229E (hCoV) 229E and hCoV-OC-43, Human

metapneumovirus A (hMPV A), human metapneumovirus B (hMPV B) and) and human coronavirus NL63 (hCoV-NL63), human hCoV-HKU-1, Haemophilus influenza, Streptococcus pneumoniae, Streptococcus pyogenes, Candida albicans, Bordetella pertussis, Legionella pneumophila and Pneumocystis jirovecii were used to evaluate a COVID-19 Rapid Antigen Test from a different supplier cross-reactivity and microbial interference.

The organisms to be tested as recommended by the FDA were tested at a final concentration of 1.00 x 10^5 to 1.00 x 10^6 $TCID_{50}$/mL (see Table below) for both cross reactivity and microbial interference studies.

Organisms for testing and concentrations. ** 1 $TCID_{50}$/ml = 0.7 pfu/ml

Pathogen	**BSL**	**Concentration stock $TCID_{50}$ ** or CFU/mL**	**Concentration RDT**
Active			
hCoV-OC-43	BSL-2	4.17 x 10^5	1.00 x 10^5
hCoV-229-E	BSL-2	1.70 x 10^5	1.00 x 10^5
hCoV-NL-63	BSL-2	1.41 x 10^5	1.00 x 10^5
Adenovirus 5	BSL-2	1.30 x 10^5	1.00 x 10^5
hMPV-16	BSL-2	1.26 x 10^6	1.00 x 10^5
PIV1	BSL-2	5.01 x 10^5	1.00 x 10^5
PIV2	BSL-2	1.51 x 10^6	1.00 x 10^5
PIV3	BSL-2	1.70 x 10^5	1.00 x 10^5
PIV4A	BSL-2	4.17 x 10^5	1.00 x 10^5
H3N2	BSL-2	1.41 x 10^5	1.00 x 10^5
H1N1	BSL-2	1.41 x 10^5	1.00 x 10^5
Influenza B	BSL-2	1.26 x 10^6	1.00 x 10^5
Enterovirus type 68	BSL-2	1.26 x 10^6	1.00 x 10^5
RSV-A	BSL-2	5.01 x 10^5	1.00 x 10^5

Rhinovirus type 1A	BSL-2	1.41×10^5	1.00×10^5
Haemophilus influenza	BSL-2	5.43×10^5	1.00×10^5
Streptococcus pneumoniae	BSL-2	4.16×10^8	1.00×10^6
Streptococcus pyogenes	BSL-2	2.66×10^9	1.00×10^6
Candida albicans	BSL-2	4.50×10^8	1.00×10^6
Bordetella pertussis	BSL-2	6.43×10^9	1.00×10^6
Legionella pneumophila	BSL-2	1.42×10^{10}	1.00×10^6
Pneumocystis jiroveci	BSL-2	6.34×10^8	1.00×10^6
Mycobacterium tuberculosis	BSL-2	TBD	1.00×10^6
Pooled human nasal wash	BSL-2	N/A	14% v/v
Mycoplasma pneumoniae	BSL-2	TBD	1.00×10^6
Chlamydia pneumoniae	BSL-2	TBD	1.00×10^6
Staphylococcus aureus	BSL-2	TBD	1.00×10^6
Live			
MERS-CoV	BSL-2	1.17×10^5	1.00×10^5
SARS-Cov-1	BSL-2	TBC	1.00×10^5

Cross-reactivity testing procedure method

1) Pooled nasal swabs were collected from 30 volunteers using the protocol described (each swab were extracted into 350 µl of extraction buffer). After pooling, the final volume was approximately 8.75 ml, and the samples were

subsequently aliquoted into 1 ml vial and frozen at -20°C test to ensure that a negative signal is obtained before spiking.

2) Each organism was spiked into 350 µl of negative nasal fluid. — a volume will be sufficient to run 3 replicates of 100 µl per test. Cross reactivity organisms will be spiked into negative clinical matrix at displayed in the table summarising Organisms for testing and concentrations. Samples equilibrated at room temperature prior to use.

3) Negative controls were unspiked sample with no other microorganism added.

4) Three replicates were tested per spiked sample (100 µl added to the sample well). The cassette was then be incubated for 20 minutes. The presence of a test line indicated a positive test. For visual reading, correct diagnosis was considered to be 2/3 repetitions.

5) The results were interpreted by two independent operators, each blinded to the result of the other. Pictures were taken of all RDT results. Invalid results were repeated once.

6) If documented, pathogens giving a positive result were tested again using a 10-fold serial dilution to find the limit of detection (LOD). The serial dilution would be made with the negative clinical matrix.

Microbial interference method

1) Pooled nasal swabs were collected from 30 volunteers using the protocol described (each swab were extracted into 350 µl of extraction buffer). After pooling, the final volume was approximately 8.75 ml, and the samples were subsequently aliquoted into 1ml vial and frozen at -20°C test to ensure that a negative signal is obtained before spiking.

2) Negative clinical matrix were spiked at a low (3 x LoD) SARS-CoV-2 concentration. Samples equilibrated at room temperature prior to use.

3) Each organism was spiked into 300 µl of negative nasal fluid pre-spiked with SARS-CoV-2 — a volume sufficient to run 3 replicates of 100 µl per test.

Organisms were spiked into negative clinical matrix at displayed in the table summarising Organisms for testing and concentrations.

4) Negative controls were unspiked sample with no other microorganism added.

5) Three replicates were tested per spiked sample (100 µl added to the sample well). The cassette was incubated for 20 minutes. The presence of a test line indicated a positive test. For visual reading, correct diagnosis was considered to be 2/3 repetitions.

6) The results were interpreted by two independent operators, each blinded to the result of the other. Pictures were taken of all RDT results. Invalid results were repeated once.

7) If interference was observed at the level tested, an additional titration study was performed to determine the highest microorganism interferent level the test can tolerate. giving a positive result were tested again using a 10-fold serial dilution to find the limit of detection. The serial dilution would be made with the negative clinical matrix.

Acceptance Criteria: Cross-reactivity

Samples remain negative in the presence of microorganisms (2/3 for each sample tested should be obtained). Determine which organisms are cross reactive. Minimal cross reactivity should be obtained; <5% for it to be negative.

If cross reactivity was observed at the level tested, an additional titration study would be performed to determine the highest microorganism interference level the test can tolerate.

Acceptance Criteria: Microbial interference

Samples remain positive in the presence of microorganisms (2/3 for each sample tested should be obtained).

If interference was observed at the level tested, an additional titration study would be performed to determine the highest microorganism interference level the test can tolerate.

Results: Cross-reactivity

No cross-reactivity was detected to 22 common respiratory pathogens on the COVID-19 Rapid Antigen Test from a different supplier.

Cross-reactivity results. Visually-read line intensities 0-10 reported independently by two blinded operators

	Replicate 1		Replicate 2		Replicate 3	
Organism	**Reader 1**	**Reader 2**	**Reader 1**	**Reader 2**	**Reader 1**	**Reader 2**
Human coronavirus-OC-43	0	0	0	0	0	0
Human coronavirus-229-E	0	0	0	0	0	0
Human coronavirus-NL-63	0	0	0	0	0	0
Adenovirus 5	0	0	0	0	0	0
Human metapneumovirus -16	0	0	0	0	0	0
Parainfluenza virus 1 (PIV1)	0	0	0	0	0	0
PIV2	0	0	0	0	0	0
PIV3	0	0	0	0	0	0
PIV4A	0	0	0	0	0	0
Influenza A (H3N2)	0	0	0	0	0	0
Influenza A (H1N1)	0	0	0	0	0	0
Influenza B	0	0	0	0	0	0
Enterovirus type 68	0	0	0	0	0	0
Respiratory syncytial virus-A	0	0	0	0	0	0

Rhinovirus type 1a	0	0	0	0	0	0
Haemophilus influenzae	0	0	0	0	0	0
Streptococcus pneumoniae	0	0	0	0	0	0
Streptococcus pyogenes	0	0	0	0	0	0
Candida albicans	0	0	0	0	0	0
Bordatella pertussis	0	0	0	0	0	0
Legionella pneumophilia	0	0	0	0	0	0
Pneumocystis jiroveci	0	0	0	0	0	0
Pooled human nasal wash	0	0	0	0	0	0

Results: Microbial interference

No microbial interference was detected merging 22 common respiratory pathogens with SARS-CoV-2 on the Avacta® AffiDX® SARS-CoV-2 Lateral Flow Antigen Test.

Microbial interference results. Visually-read line intensities 0-10 reported independently by two blinded operators

	Replicate 1		**Replicate 2**		**Replicate 3**	
Organism	**Reader 1**	**Reader 2**	**Reader 1**	**Reader 2**	**Reader 1**	**Reader 2**
Human coronavirus-OC-43	3	3	4	4	3	4
Human coronavirus-229-E	3	3	4	4	3	3
Human coronavirus-NL-	4	4	4	4	4	4

63						
Adenovirus 5	3	3	3	3	3	3
Human metapneumovirus-16	3	3	3	3	3	3
Parainfluenza virus 1 (PIV1)	3	3	3	3	3	4
PIV2	3	3	4	4	3	3
PIV3	4	4	4	4	4	4
PIV4A	3	3	4	4	3	3
Influenza A (H3N2)	3	3	3	3	3	3
Influenza A (H1N1)	3	3	3	3	3	3
Influenza B	3	3	3	3	3	3
Enterovirus type 68	3	3	3	3	3	3
Respiratory syncytial virus-A	3	3	3	3	3	3
Rhinovirus type 1a	4	4	3	3	3	3
Haemophilus influenzae	3	3	3	3	3	3
Streptococcus pneumoniae	3	3	3	3	3	3
Streptococcus pyogenes	4	4	4	4	4	4
Candida albicans	3	3	3	3	3	3
Bordatella pertussis	4	4	3	3	3	3
Legionella pneumophilia	4	4	4	4	4	4
Pneumocystis jiroveci	3	3	3	3	3	3
Pooled human nasal wash	4	4	4	4	4	4

In silico analysis of potential cross-reactivity of SARS-CoV2 Surface Glycoprotein S1 subunit (S1 protein)

S1 protein sequence

Wuhan Hu1 isolate (reference sequence)
> QVQ56608 surface glycoprotein VAL 16-Arg 685 S1 subunit [Severe acute respiratory syndrome coronavirus 2]

VNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFSNVTWFHAIHVSGT
NGTKRFDNPVLPFNDGVYFASIEKSNIIRGWIFGTTLDSKTQSLLIVNNATNVVIKVCEF
QFCNDPFLGVYYHKNNKSWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQGNFKNL
REFVFKNIDGYFKIYSKHTPINLVRDLPQGFSALEPLVDLPIGINITRFQTLLALHRSYLT
PGGSSSGWTAGAAAYYVGYLQPRTFLLKYNENGTITDAVDCALDPLSETKCTLKSFTVE
KGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVL
YNSASFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKLPD
DFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQAGNTPCNGVEG
FNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNF
NGLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITPGT
NTSNQVAVLYQGVNCTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEHVNNS
YECDIPIGAGICASYQTQTNSPRRAR (SEQ ID NO: 299)

BlastP searched non-redundant protein sequences against organisms *Mycobacterium tuberculosis, Mycoplasma pneumoniae, Chlamydia pneumoniae* and *Staphylococcus aureus*.

Mycobacterium tuberculosis, Mycoplasma pneumoniae, Chlamydia pneumoniae and *Staphylococcus aureus* proteins showed no significant homology to the S1 protein.

Conservation with other human coronaviridae S1 proteins

Coronavirus	Coverage	Identity
HCoC-HKU1 (taxid:290028)	50%	30.81%
MERS-CoV (taxid:1335626)	63%	25.43%
SARS-CoV (urbani strain) (taxid:228330)	97%	68.20%

The potential for cross reactivity with the above Coronavirus types is unlikely, with the exception of SARS-CoV which is very closely related to SARS-CoV2. However, SARS-CoV is not circulating and therefore unlikely to be encountered.

Conclusions

Findings confirmed an excellent cross-reactivity profile and excellent microbial interference profile. No cross-reactivity was detected to 22 common respiratory pathogens on the Avacta® AffiDX® SARS-CoV-2 Lateral Flow Antigen Test. No microbial interference was detected when merging 22 common respiratory pathogens with SARS-CoV-2 on the Avacta® AffiDX® SARS-CoV-2 Lateral Flow Antigen Test.

No cross reactivity was detected for the outstanding pathogens not available for wet testing, such as *Mycobacterium tuberculosis, Mycoplasma pneumoniae, Chlamydia pneumoniae* and *Staphylococcus aureus*, HKU1 coronavirus, MERS-CoV, and SARS-CoV-1 as shown by *in silico* analysis using the Basic Local Alignment Search Tool (BLAST) managed by the National Centre for Biotechnology Information (NCBI) to assess the degree of protein homology.

These findings meet or exceed the target product profile requirements outlined by the WHO, FDA and MHRA.

EXAMPLE 13: Clinical performance

Clinical performance was determined by testing a total of 200 samples (98 pos., 102 neg., as determined by RT-qPCR) at a clinical study conducted in the EU. PCR Result (Ct change) was determined using the Applied Biosystems TaqPath COVID-19 CE-IVD RT-PCR Kit.

Clinical sensitivity (Ct ≤27):	**100.0%**
Clinical sensitivity (Ct<31):	**98.0%**
Clinical specificity:	**99.0%**
Analytical Limit of Detection (LoD):	**6 x 10^2 pfu/mL**

No cross reactivity of Affimer® reagent with MERS-CoV S1, SARS-CoV-1 S1, HCoV-229E S1, HCoV-HKU1 S1, HCoV-NL63 S1 or HCoV-OC43 S1.

Full clinical validation results:

PCR Result (Ct Range)	Number of Samples Tested in Ct Range	Avacta® AffiDX®		Clinical Sensitivity by Ct Range		
		Positive	Negative	Cumulative Ct Range	% Cumulative Sensitivity	95% CI
< 27	52	52	0	0-27	100.0%	(93.1 – 100.0%)
27-28	7	6	1	0-28	98.3%	(91.0 – 99.7%)
28-29	18	18	0	0-29	98.7%	(93.0 – 99.8%)
29-30	19	19	0	0-30	99.0%	(94.3 – 99.8%)
>30	2	1	1	All	98.0%	(92.9 – 99.4%)
TOTAL	98	96	2		98.0%	(92.9 – 99.4%)

PCR Result	Number of Negative Samples Tested	Avacta® AffiDX®		Clinical Specificity	
		Positive	Negative	% Specificity	95% CI
Negative	102	1	101	99.0%	(94.7 – 99.8%)

The Avacta® AffiDX® Lateral Flow Device shows excellent clinical performance, achieving clinical sensitivity (Ct ≤27) of 100.0%, clinical sensitivity (Ct < 31) of 98.0% and clinical specificity of 99.0%, as well as having an analytical limit of detection of 6 x 10^2 pfu/mL.

CLAIMS

1. A polypeptide comprising

a) amino acid sequence having at least 80% sequence identity to SEQ ID NO: 3, or

b) amino acid sequence having at least 80% sequence identity to SEQ ID NO: 2;

wherein said polypeptide further comprises a first heterologous peptide insertion having a first amino acid sequence, and

wherein said polypeptide further comprises a second heterologous peptide insertion having a second amino acid sequence,

wherein said first and second amino acid sequences are selected from the group consisting of: SEQ ID NO: 5 and SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9, SEQ ID NO: 11 and SEQ ID NO: 12, SEQ ID NO: 14 and SEQ ID NO: 15, SEQ ID NO: 17 and SEQ ID NO: 18, SEQ ID NO: 20 and SEQ ID NO: 21, SEQ ID NO: 23 and SEQ ID NO: 24, SEQ ID NO: 26 and SEQ ID NO: 27, SEQ ID NO: 29 and SEQ ID NO: 30, SEQ ID NO: 32 and SEQ ID NO: 33, SEQ ID NO: 35 and SEQ ID NO: 36, SEQ ID NO: 38 and SEQ ID NO: 39, SEQ ID NO: 41 and SEQ ID NO: 42, SEQ ID NO: 44 and SEQ ID NO: 45, SEQ ID NO: 47 and SEQ ID NO: 48, SEQ ID NO: 50 and SEQ ID NO: 51, SEQ ID NO: 53 and SEQ ID NO: 54, SEQ ID NO: 56 and SEQ ID NO: 57, SEQ ID NO: 59 and SEQ ID NO: 60, SEQ ID NO: 62 and SEQ ID NO: 63, SEQ ID NO: 65 and SEQ ID NO: 66, SEQ ID NO: 68 and SEQ ID NO: 69, SEQ ID NO: 71 and SEQ ID NO: 72, SEQ ID NO: 74 and SEQ ID NO: 75, SEQ ID NO: 77 and SEQ ID NO: 78, SEQ ID NO: 80 and SEQ ID NO: 81, SEQ ID NO: 83 and SEQ ID NO: 84, SEQ ID NO: 86 and SEQ ID NO: 87, SEQ ID NO: 89 and SEQ ID NO: 90, SEQ ID NO: 92 and SEQ ID NO: 93, SEQ ID NO: 95 and SEQ ID NO: 96, SEQ ID NO: 98 and SEQ ID NO: 99, SEQ ID NO: 101 and SEQ ID NO: 102, SEQ ID NO: 104 and SEQ ID NO: 105, SEQ ID NO: 107 and SEQ ID NO: 108, SEQ ID NO: 110 and SEQ ID NO: 111, SEQ ID NO: 113 and SEQ ID NO: 114, SEQ ID NO: 116 and SEQ ID NO: 117, SEQ ID NO: 119 and SEQ ID NO: 120, SEQ ID NO: 122 and SEQ ID NO: 123, SEQ ID NO: 125 and SEQ ID NO: 126, SEQ ID NO: 128 and SEQ ID NO: 129, SEQ ID NO: 131 and SEQ ID NO: 132, SEQ ID NO: 134 and SEQ ID NO: 135, SEQ ID NO: 137 and SEQ ID NO: 138, SEQ ID NO: 140 and SEQ ID NO: 141, SEQ ID NO: 143 and SEQ ID NO: 144, SEQ ID NO: 146 and SEQ ID NO: 147, SEQ ID NO: 149 and SEQ ID NO: 150, SEQ ID NO: 152 and SEQ ID NO: 153, SEQ ID NO: 155 and SEQ ID NO: 156, SEQ ID NO: 158 and SEQ ID NO: 159, SEQ ID NO: 161 and SEQ ID NO: 162, SEQ ID NO: 164 and SEQ ID NO: 165, SEQ ID NO: 167 and SEQ ID NO: 168, SEQ ID NO: 170 and SEQ ID NO: 171, and SEQ ID NO: 173 and SEQ ID NO: 174.

2. A polypeptide according to claim 1 wherein said polypeptide comprises a) amino acid sequence having at least 80% sequence identity to SEQ ID NO: 3, and wherein said first and second amino acid sequences are selected from the group consisting of: SEQ ID NO: 5 and SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9, SEQ ID NO: 11 and SEQ ID NO: 12, SEQ ID NO: 14 and SEQ ID NO: 15, SEQ ID NO: 17 and SEQ ID NO: 18, SEQ ID NO: 20 and SEQ ID NO: 21, SEQ ID NO: 23 and SEQ ID NO: 24, SEQ ID NO: 26 and SEQ ID NO: 27, SEQ ID NO: 29 and SEQ ID NO: 30, SEQ ID NO: 32 and SEQ ID NO: 33, SEQ ID NO: 35 and SEQ ID NO: 36, SEQ ID NO: 38 and SEQ ID NO: 39, SEQ ID NO: 41 and SEQ ID NO: 42, SEQ ID NO: 44 and SEQ ID NO: 45, SEQ ID NO: 47 and SEQ ID NO: 48, SEQ ID NO: 50 and SEQ ID NO: 51, SEQ ID NO: 53 and SEQ ID NO: 54, SEQ ID NO: 56 and SEQ ID NO: 57, SEQ ID NO: 59 and SEQ ID NO: 60, and SEQ ID NO: 62 and SEQ ID NO: 63.

3. A polypeptide according to claim 2 wherein said polypeptide comprises SEQ ID NO: 7 (620_826257).

4. A polypeptide according to any of claims 2 or 3 wherein said polypeptide is biotinylated.

5. A polypeptide according to any of claims 2 to 4 wherein said first heterologous peptide is inserted into the polypeptide at position 48-<heterologous peptide>-50 and said second heterologous peptide is inserted into the polypeptide at position 73-<heterologous peptide>-78 relative to SEQ ID NO: 1.

6. A polypeptide according to claim 1 wherein said polypeptide comprises b) amino acid sequence having at least 80% sequence identity to SEQ ID NO: 2, and wherein said first and second amino acid sequences are selected from the group consisting of: SEQ ID NO: 65 and SEQ ID NO: 66, SEQ ID NO: 68 and SEQ ID NO: 69, SEQ ID NO: 71 and SEQ ID NO: 72, SEQ ID NO: 74 and SEQ ID NO: 75, SEQ ID NO: 77 and SEQ ID NO: 78, SEQ ID NO: 80 and SEQ ID NO: 81, SEQ ID NO: 83 and SEQ ID NO: 84, SEQ ID NO: 86 and SEQ ID NO: 87, SEQ ID NO: 89 and SEQ ID NO: 90, SEQ ID NO: 92 and SEQ ID NO: 93, SEQ ID NO: 95 and SEQ ID NO: 96, SEQ ID NO: 98 and SEQ ID NO: 99, SEQ ID NO: 101 and SEQ ID NO: 102, SEQ ID NO: 104 and SEQ ID NO: 105, SEQ ID NO: 107 and SEQ ID NO: 108, SEQ ID NO: 110 and SEQ ID NO: 111, SEQ ID NO: 113 and SEQ ID NO: 114, SEQ ID NO: 116 and SEQ ID NO: 117, SEQ ID NO: 119 and SEQ ID NO: 120, SEQ ID NO: 122 and SEQ ID NO: 123, SEQ ID NO:

125 and SEQ ID NO: 126, SEQ ID NO: 128 and SEQ ID NO: 129, SEQ ID NO: 131 and SEQ ID NO: 132, SEQ ID NO: 134 and SEQ ID NO: 135, SEQ ID NO: 137 and SEQ ID NO: 138, SEQ ID NO: 140 and SEQ ID NO: 141, SEQ ID NO: 143 and SEQ ID NO: 144, SEQ ID NO: 146 and SEQ ID NO: 147, SEQ ID NO: 149 and SEQ ID NO: 150, SEQ ID NO: 152 and SEQ ID NO: 153, SEQ ID NO: 155 and SEQ ID NO: 156, SEQ ID NO: 158 and SEQ ID NO: 159, SEQ ID NO: 161 and SEQ ID NO: 162, SEQ ID NO: 164 and SEQ ID NO: 165, SEQ ID NO: 167 and SEQ ID NO: 168, SEQ ID NO: 170 and SEQ ID NO: 171, and SEQ ID NO: 173 and SEQ ID NO: 174.

7. A polypeptide according to claim 6 wherein said polypeptide comprises SEQ ID NO: 67 (620_825425) or SEQ ID NO: 70 (620_825436).

8. A polypeptide according to any of claims 6 or 7 wherein said first heterologous peptide is inserted into the polypeptide at position 46-<heterologous peptide>-47 and said second heterologous peptide is inserted into the polypeptide at position 71-<heterologous peptide>-72 relative to SEQ ID NO: 2.

9. A polypeptide according to claim 1 wherein said polypeptide comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 7, SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 25, SEQ ID NO: 28, SEQ ID NO: 31, SEQ ID NO: 34, SEQ ID NO: 37, SEQ ID NO: 40, SEQ ID NO: 43, SEQ ID NO: 46, SEQ ID NO: 49, SEQ ID NO: 52, SEQ ID NO: 55, SEQ ID NO: 58, SEQ ID NO: 61, SEQ ID NO: 64, SEQ ID NO: 67, SEQ ID NO: 70, SEQ ID NO: 73, SEQ ID NO: 76, SEQ ID NO: 79, SEQ ID NO: 82, SEQ ID NO: 85, SEQ ID NO: 88, SEQ ID NO: 91, SEQ ID NO: 94, SEQ ID NO: 97, SEQ ID NO: 100, SEQ ID NO: 103, SEQ ID NO: 106, SEQ ID NO: 109, SEQ ID NO: 112, SEQ ID NO: 115, SEQ ID NO: 118, SEQ ID NO: 121, SEQ ID NO: 124, SEQ ID NO: 127, SEQ ID NO: 130, SEQ ID NO: 133, SEQ ID NO: 136, SEQ ID NO: 139, SEQ ID NO: 142, SEQ ID NO: 145, SEQ ID NO: 148, SEQ ID NO: 151, SEQ ID NO: 154, SEQ ID NO: 157, SEQ ID NO: 160, SEQ ID NO: 163, SEQ ID NO: 166, SEQ ID NO: 169, SEQ ID NO: 172, and SEQ ID NO: 175.

10. A polypeptide according to any preceding claim further comprising at the C-terminal end of the polypeptide one of more of:

i) purification tag suitably HHHHHH;

ii) conjugation tag suitably C;

iii) detection tag suitably DYKDDDDK and/or HA tag suitably YPYDVPDYA and/or AviTag™ suitably GLNDIFEAQKIEWHE; and/or

iv) linker sequence suitably GGGGS.

11. A polypeptide according to any of claims 6 to 8 further comprising an N-terminal Methionine (M).

12. A polypeptide according to any preceding claim which binds SARS-CoV-2 S1 spike protein and variants thereof comprising mutations with an Equilibrium dissociation constant (K_D) value < 100 nM.

13. A polypeptide according to claim 12, wherein the S1 spike protein of the SARS-CoV-2 virus comprises a mutation at position 614 and/or at position 453.

14. A polypeptide according claim 13, wherein the SARS-CoV-2 S1 spike protein comprises the D614G substitution and/or the Y453F substitution.

15. A polypeptide according to claim 12, wherein the S1 spike protein of the SARS-CoV-2 virus comprises a mutation at one or more of the positions selected from H69, V70, Y144, N501, A570, and/or P681.

16. A polypeptide according to claim 15, wherein the S1 spike protein of the SARS-CoV-2 virus comprises one or more of the HV 69-70 deletion, Y144 deletion, N501Y substitution, A570D substitution, and/or P681H substitution.

17. A polypeptide according to claim 12, 15 or 16, wherein the polypeptide binds to SARS-CoV-2 virus variant B.1.1.7.

18. A polypeptide according to any preceding claim, wherein the polypeptide binds to a SARS-CoV-2 S1 spike protein comprising a mutation at position 614 and/or at position 453,

19. A polypeptide according to any preceding claim, wherein the polypeptide binds to a SARS-CoV-2 S1 spike protein comprising the D614G substitution and/or the Y453F substitution.

20. A polypeptide according to any preceding claim, wherein the polypeptide binds to a SARS-CoV-2 S1 spike protein comprising a mutation at one or more of the positions selected from H69, V70, Y144, N501, A570, and/or P681.

21. A polypeptide according to any preceding claim, wherein the polypeptide binds to a SARS-CoV-2 S1 spike protein comprising one or more of the HV 69-70 deletion, Y144 deletion, N501Y substitution, A570D substitution, and/or P681H substitution.

22. A polypeptide according to any preceding claim, wherein the polypeptide binds to SARS-CoV-2 virus variant B.1.1.7.

23. A nucleic acid comprising nucleotide sequence encoding a polypeptide according to any preceding claim.

24. A host cell comprising a nucleic acid according to claim 23.

25. A method comprising the steps of:
i) contacting a sample with a polypeptide according to any of claims 1 to 22;
ii) incubating to allow binding of said polypeptide to any SARS-CoV-2 virus present in said sample
iii) assaying for the presence of any polypeptide – SARS-CoV-2 bound complexes, wherein presence of any such complexes indicates a presence of SARS-CoV-2 virus in said sample.

26. A lateral flow assay device, comprising a polypeptide according to any of claims 1 to 22.

27. Use of a polypeptide according to any of claims 1 to 22 in a lateral flow assay device.

28. A lateral flow assay device according to claim 26 or a use according to claim 27, wherein the lateral flow assay device comprises: (i) a sample receiving region; and (ii) a capture membrane operably connected to said sample receiving region downstream of said sample receiving region,
wherein said capture membrane comprises a test region comprising an immobilised first polypeptide according to any of claims 1 to 22.

29. A lateral flow assay device according to claims 26 or 28 or a use according to claims 27 or 28 wherein said immobilised first polypeptide comprises one or more

amino acid sequences selected from the group consisting of SEQ ID NO: 70 (620_825436) and SEQ ID NO: 67 (620_825425).

30. A lateral flow assay device according to any of claims 26, 28 or 29 or a use according to any of claims 27-29, the lateral flow assay device further comprising a conjugate pad located between said sample receiving region and said capture membrane, wherein said conjugate pad comprises a second polypeptide according to any of claims 1 to 22 joined to an indicator agent, and wherein said first and second polypeptides are different.

31. A lateral flow assay device or use according to claim 30 wherein said second polypeptide comprises SEQ ID NO: 7 (620_826257).

32. A lateral flow assay device or use according to any of claims 30 or 31 wherein said second polypeptide is biotinylated.

33. A lateral flow assay device according to any of claims 26, or 28-32 or a use according to any of claims 27-32, the lateral flow assay device further comprising a control region comprising an immobilised reagent capable of specifically binding said second polypeptide according to any of claims 1 to 22 joined to an indicator agent.

34. A system for detecting a presence of a component of interest in a sample, comprising:
(a) a lateral flow device according to any of claims 26 to 33, and
(b) a liquid formulation of a second polypeptide according to any of claims 1 to 22 joined to an indicator agent, wherein said first and second polypeptides are different.

35. A lateral flow assay device according to any of claims 26 or 28-33, a use according to any of claims 27-33, or a system according to claim 34, wherein the lateral flow assay device further comprises a reservoir region positioned downstream of the capture membrane for absorbing an excess of fluid.

36. A lateral flow assay device according to any of claims 26, 28-33 or 35, a use according to any of claims 27-33 or 35, or a system according to claims 34 or 35, wherein the lateral flow assay device further comprises one or more antibodies.

37. A method for detecting a presence of any SARS-CoV-2 virus in a liquid test sample, comprising the steps of:
(a) providing a lateral flow assay device according to any of claims 26 to 36;
(b) applying the test sample to the sample receiving region
(c) either when the lateral flow assay device comprises a conjugate pad comprising a second polypeptide according to any of claims 1 to 22 joined to an indicator agent wherein said first and second polypeptides are different, allowing said sample to migrate through said conjugate pad to the capture membrane and incubating to allow virus-indicator complex to form; or when the lateral flow assay device does not comprise a conjugate pad, applying a liquid formulation of a second polypeptide according to any of claims 1 to 22 joined to an indicator agent, wherein said first and second polypeptides are different, to the sample receiving region and allowing said liquid formulation to migrate to the capture membrane and incubating to allow virus-indicator complex to form;
(d) allowing the virus-indicator complex to migrate through the capture membrane to the test region and contact the immobilised first polypeptide thereby immobilising the virus-indicator complex and forming a detectable signal, wherein the presence of the SARS-CoV-2 virus in the sample is indicated by the signal.

38. A method according to claim 37 wherein said first polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 70 (620_825436) and SEQ ID NO: 67 (620_825425), and wherein said second polypeptide comprises the amino acid sequence of SEQ ID NO: 7 (620_826257).

39. A method according to any of claims 36 to 38 wherein said second polypeptide is biotinylated.

40. A method according to any of claims 36 to 39 wherein the lateral flow assay device further comprises an antibody.

41. A magnetic agarose bead comprising a polypeptide according to any of claims 1 to 22 attached thereto.

42. A magnetic agarose bead according to claim 41 wherein said polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 82 (620_825336), SEQ ID NO: 70 (620_825436), SEQ ID NO: 67 (620_825425), and SEQ ID NO: 7 (620_826257).

43. A magnetic agarose bead according to any of claims 41 or 42 wherein said polypeptide comprises the amino acid sequence of SEQ ID NO: 82 (620_825336).

44. A magnetic agarose bead according to any of claims 41 to 43 which is about 375 μm in diameter.

45. A method comprising:
a) contacting a magnetic agarose bead according to any of claims 41 to 44 with a sample from a subject
b) incubating to allow binding of the polypeptide to any SARS-CoV-2 virus in the sample
c) washing the magnetic agarose bead to remove unbound material
d) eluting bound material from the washed bead of (c)
e) analysing the eluted material of (d) by mass spectrometry (MS) to detect protein(s) or fragment(s) or ion(s) thereof originating from SARS-CoV-2 virus wherein detection of any such protein(s) or fragment(s) or ion(s) indicates presence of SARS-CoV-2 virus in said sample.

46. A method according to claim 45 wherein said mass spectrometry (MS) comprises matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

47. A solid phase substrate comprising immobilised polypeptide according to any of claims 1 to 22.

48. A solid phase substrate comprising one or more immobilised polypeptides according to any of claims 1 to 22.

49. A solid phase substrate according to any of claims 47 or 48, which is an ELISA plate.

50. A method comprising
i) providing an ELISA plate according to claim 49,
ii) contacting said immobilised polypeptide with a test sample,
iii) incubating to allow binding of any SARS-CoV-2 virus to said immobilised polypeptide,

iv) washing to remove unbound material,

v) contacting the immobilised polypeptide – SARS-CoV-2 bound complex with a second polypeptide according to any of claims 1 to 22 joined to an indicator agent, wherein said immobilised polypeptide and said second polypeptide are different

vi) incubating to allow binding of said second polypeptide to the immobilised polypeptide – SARS-CoV-2 bound complex,

vii) washing to remove unbound material, and

viii) detecting presence of said second polypeptide joined to an indicator agent, wherein presence of same indicates presence of SARS-CoV-2 virus in the test sample.

51. A solid phase substrate according to any of claims 47 to 49, or a method according to claim 50, wherein said immobilised polypeptide comprises amino acid sequence selected from the group consisting of SEQ ID NO: 70 (620_825436) and SEQ ID NO: 67 (620_825425).

52. A solid phase substrate according to any of claims 47 to 49 or claim 51, or a method according to claim 50, wherein said second polypeptide comprises amino acid sequence of SEQ ID NO: 7 (620_826257).

53. A solid phase substrate according to any of claims 47 to 49 or claim 51 or 52, or a method according to claim 50 or 52, wherein said second polypeptide is biotinylated.

54. A method according to any of claims 25, 37 to 40, 45, 46, or 50 to 53, a device according to any of claims 26, 28-33, 35 or 36, a use according to any of claims 27-33, 35 or 36, or a system according to claims 34-36, wherein the sample comprises, or consists of, saliva.

55. A method according to any of claims 25, 37 to 40, 45, 46, or 50 to 54, a device according to any of claims 26, 28-33, 35 or 36, a use according to any of claims 27-33, 35 or 36, or a system according to claims 34-36, wherein the sample comprises, is derived from, or consists of an anterior nasal swab.

56. A method according to any of claims 25, 37 to 40, 45, 46, or 50 to 55, wherein the S1 spike protein of the SARS-CoV-2 virus comprises a mutation at position 614 and/or at position 453.

57. A method according to claim 56, wherein the SARS-CoV-2 S1 spike protein comprises the D614G substitution and/or the Y453F substitution.

58. A method according to any of claims 25, 37 to 40, 45, 46, or 50 to 55, wherein the S1 spike protein of the SARS-CoV-2 virus comprises a mutation at one or more of the positions selected from H69, V70, Y144, N501, A570, and/or P681.

59. A method according to claim 58, wherein the S1 spike protein of SARS-CoV-2 virus comprises one or more of the HV 69-70 deletion, Y144 deletion, N501Y substitution, A570D substitution, and/or P681H substitution.

60. A method according to any of claims 25, 37 to 40, 45, 46, or 50 to 55, wherein the polypeptide binds to SARS-CoV-2 virus variant B.1.1.7.

FIGURE 1

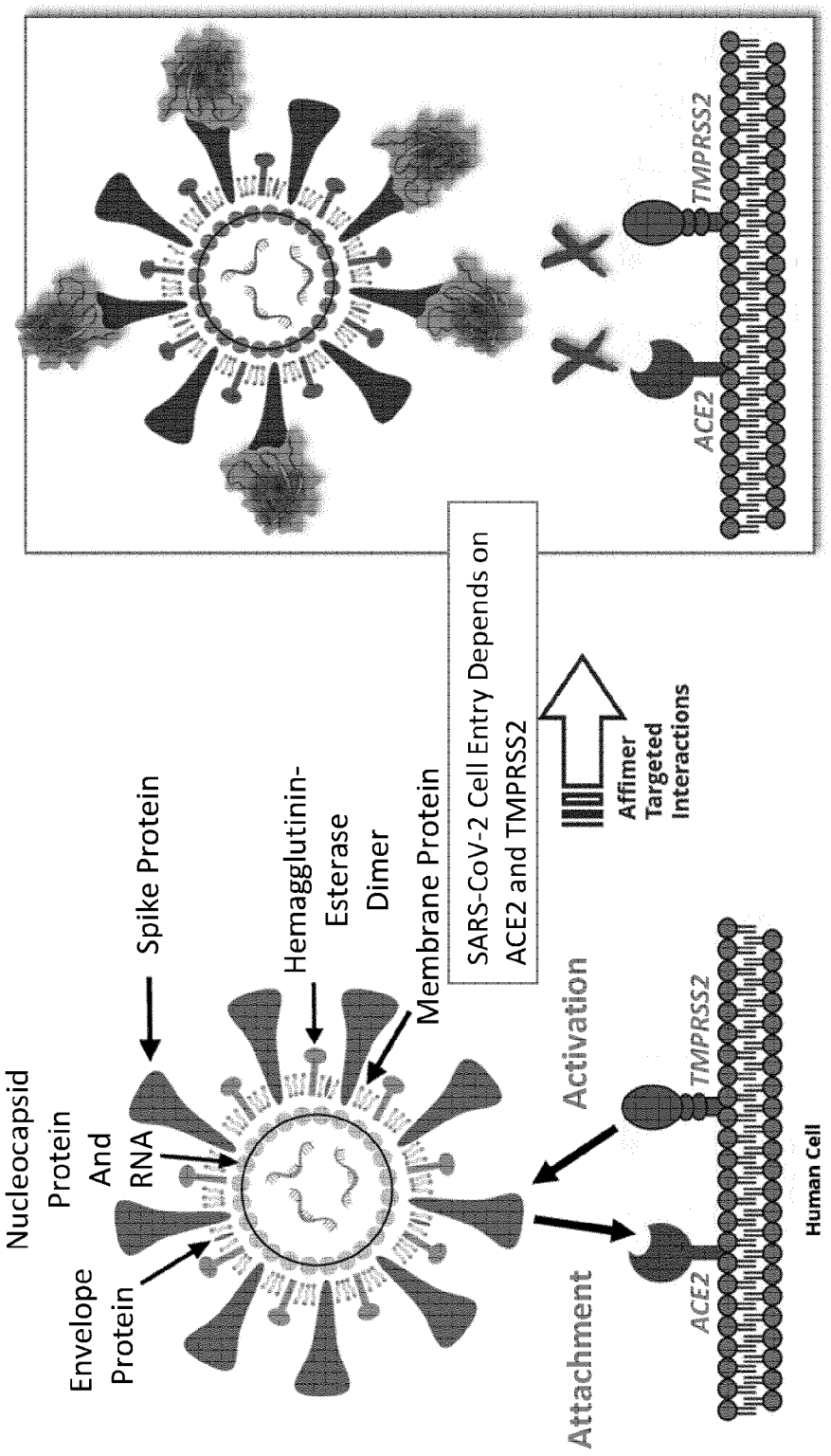

Nucleocapsid Protein And RNA
Envelope Protein
Spike Protein
Hemagglutinin-Esterase Dimer
Membrane Protein
Attachment
Activation
ACE2
TMPRSS2
Human Cell
SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2
Affimer Targeted Interactions
ACE2
TMPRSS2

FIGURE 2A

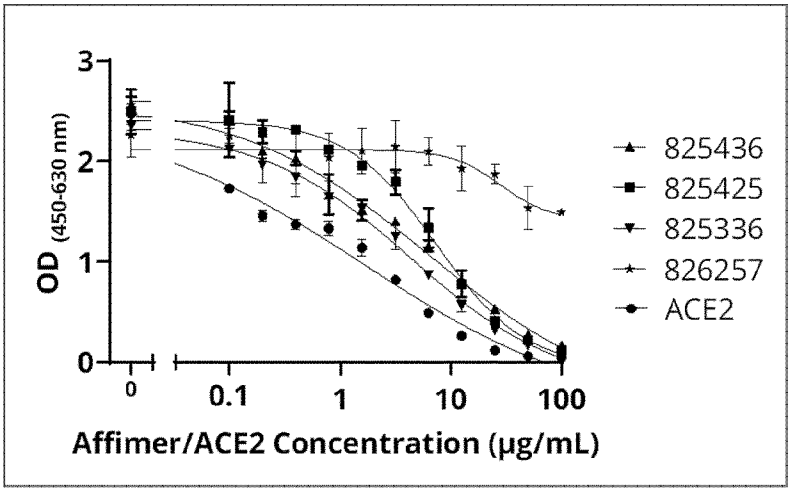


FIGURE 2B

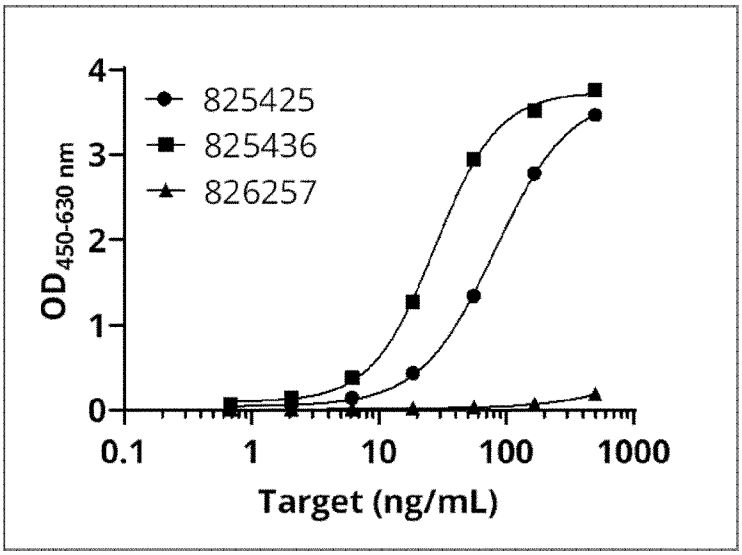


FIGURE 2C

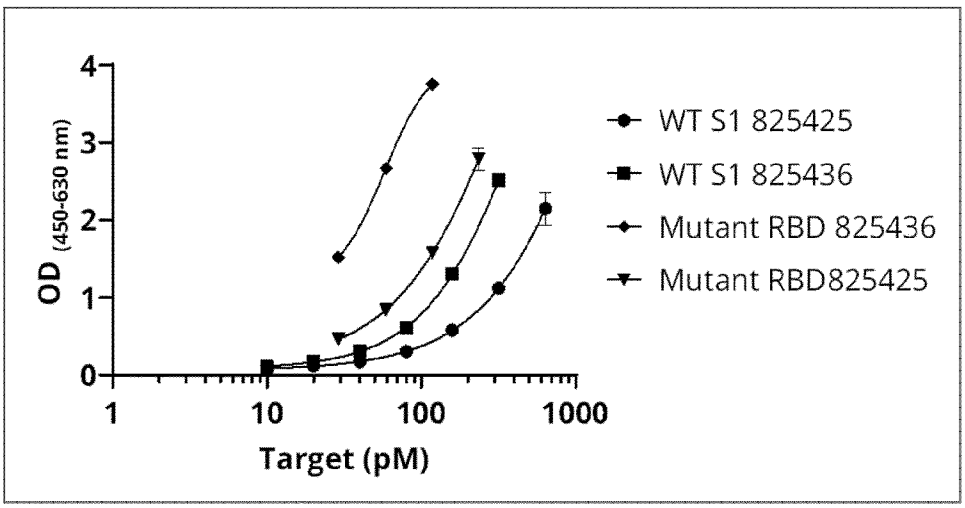


FIGURE 3

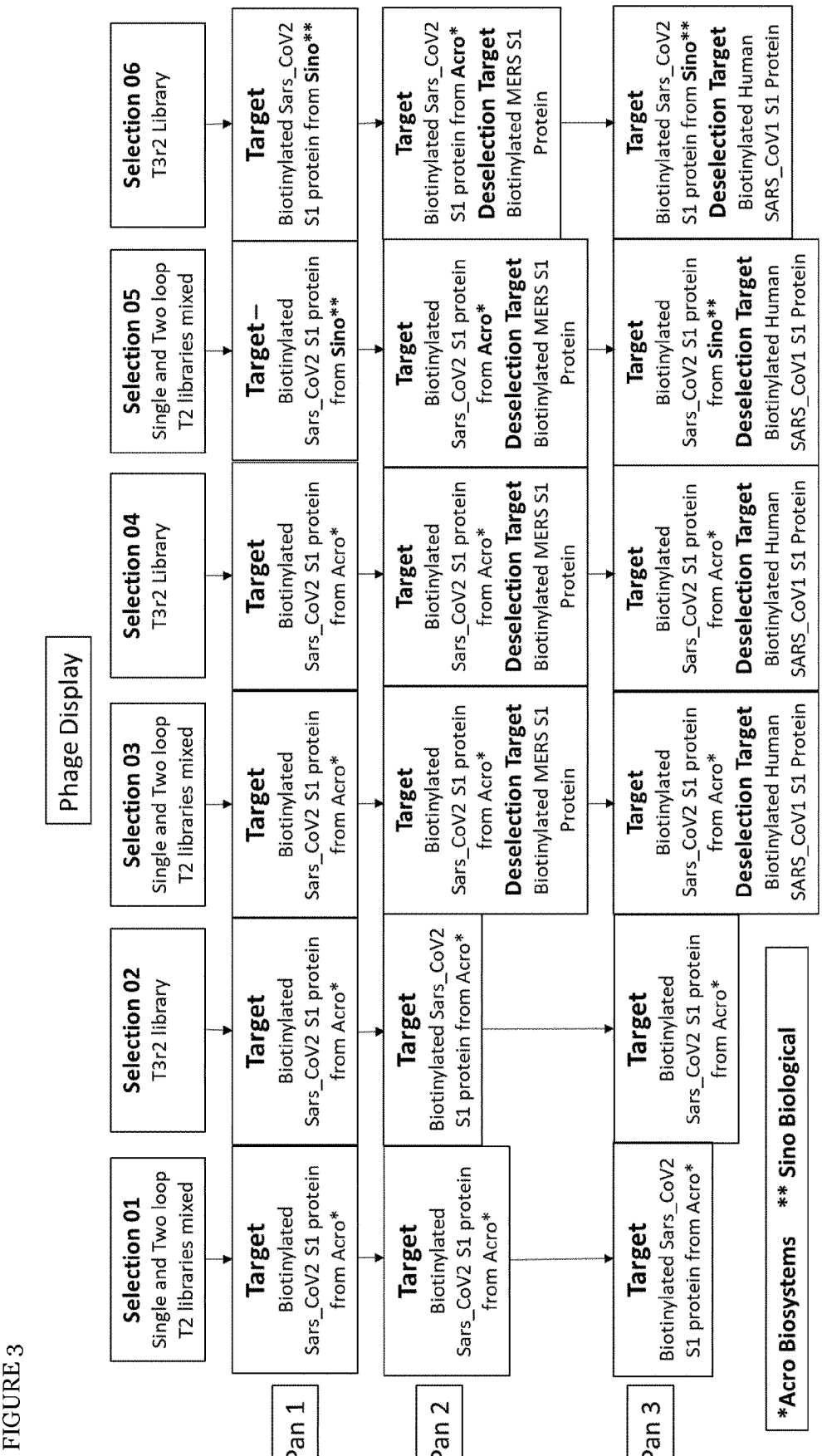

Phage Display
Selection 01
Single and Two loop T2 libraries mixed
Selection 02
T3r2 library
Selection 03
Single and Two loop T2 libraries mixed
Selection 04
T3r2 Library
Selection 05
Single and Two loop T2 libraries mixed
Selection 06
T3r2 Library
Target
Biotinylated Sars_CoV2 S1 protein from Acro*
Target
Biotinylated Sars_CoV2 S1 protein from Acro*
Target
Biotinylated Sars_CoV2 S1 protein from Acro*
Target
Biotinylated Sars_CoV2 S1 protein from Acro*
Target–
Biotinylated Sars_CoV2 S1 protein from Sino**
Target
Biotinylated Sars_CoV2 S1 protein from Sino**
Target
Biotinylated Sars_CoV2 S1 protein from Acro*
Deselection Target
Biotinylated MERS S1 Protein
Target
Biotinylated Sars_CoV2 S1 protein from Acro*
Deselection Target
Biotinylated MERS S1 Protein
Target
Biotinylated Sars_CoV2 S1 protein from Acro*
Deselection Target
Biotinylated MERS S1 Protein
Target
Biotinylated Sars_CoV2 S1 protein from Acro*
Deselection Target
Biotinylated Human SARS_CoV1 S1 Protein
Target
Biotinylated Sars_CoV2 S1 protein from Acro*
Deselection Target
Biotinylated Human SARS_CoV1 S1 Protein
Target
Biotinylated Sars_CoV2 S1 protein from Sino**
Deselection Target
Biotinylated Human SARS_CoV1 S1 Protein
Pan 1
Pan 2
Pan 3
*Acro Biosystems ** Sino Biological

FIGURE 4A

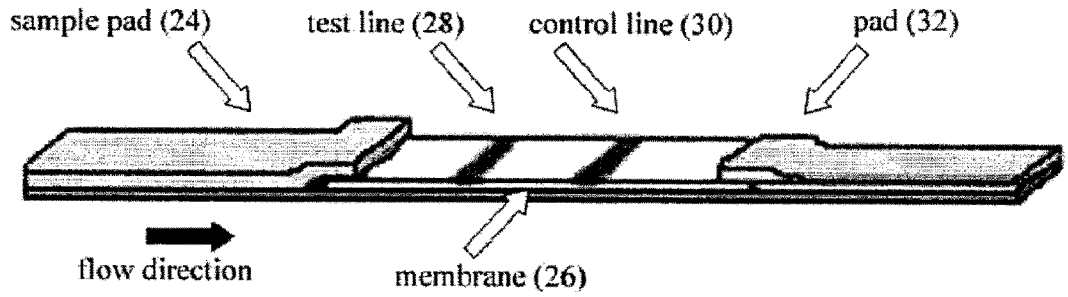


FIGURE 4B

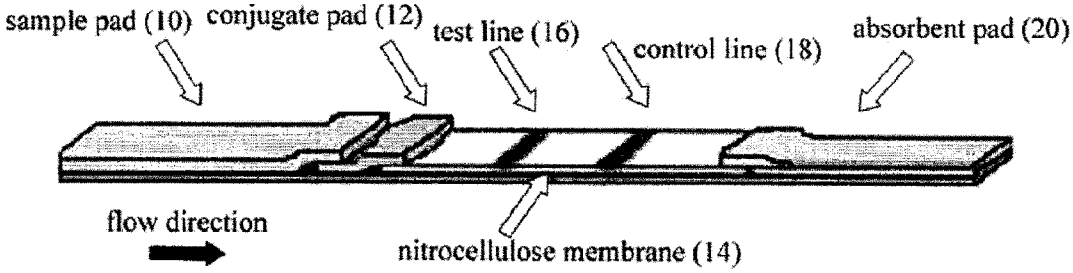


FIGURE 5

Polypeptide	ELISA Ranking	LFD Ranking	BAMS Ranking
620_826257	1	1	1
620_825425	1	1	1
620_825436	1	1	1
620_825478	2	1	N/A
620_825336	2	2	1
620_825317	2	2	2
620_825392	2	2	N/A
620_825594	2	2	N/A
620_825456	2	3	N/A
620_829592	2	N/A	N/A
620_829641	2	N/A	N/A
620_825543	3	1	2
620_826049	3	N/A	N/A
620_829515	3	N/A	N/A
620_830214	3	N/A	N/A
620_829568	3	N/A	N/A
620_829626	3	N/A	N/A
620_829638	3	N/A	N/A
620_825515	3	N/A	N/A
620_825499	3	N/A	N/A
620_825513	3	N/A	N/A
620_825564	3	N/A	N/A

Polypeptide	ELISA Ranking
620_826257	1
620_825425	1
620_825436	1
620_825478	2
620_825336	2
620_825317	2
620_825392	2
620_825594	2
620_829592	2
620_829641	2
620_825456	2
620_825543	3
620_826049	3
620_829515	3
620_830214	3
620_829568	3
620_829626	3
620_829638	3
620_825515	3
620_825499	3
620_825513	3
620_825564	3

Polypeptide	LFD Ranking
620_826257	1
620_825425	1
620_825436	1
620_825478	1
620_825543	1
620_825411	1
620_825336	2
620_825317	2
620_825392	2
620_825594	2
620_825329	2
620_825586	2
620_826285	3
620_825358	3
620_825456	3

Polypeptide	BAMS Ranking
620_826257	1
620_825425	1
620_825436	1
620_825336	1
620_825543	2
620_825317	2

Polypeptide	ELISA Ranking	LFD Ranking	BAMS Ranking
620_826005	4	N/A	N/A
620_829511	4	N/A	N/A
620_829519	4	N/A	N/A
620_830162	4	N/A	N/A
620_829610	4	N/A	N/A
620_829432	4	N/A	N/A
620_825411	5	1	N/A
620_825329	5	2	N/A
620_825586	5	2	N/A
620_826285	5	3	N/A
620_826047	5	N/A	N/A
620_826101	5	N/A	N/A
620_826152	5	N/A	N/A
620_826024	5	N/A	N/A
620_829494	5	N/A	N/A
620_829566	5	N/A	N/A
620_825265	5	N/A	N/A
620_825302	5	N/A	N/A
620_825358	5	N/A	N/A
620_825322	5	N/A	N/A
620_825367	5	N/A	N/A
620_829410	5	N/A	N/A
620_829450	5	N/A	N/A
620_829453	5	N/A	N/A

Polypeptide	ELISA Ranking
620_826005	4
620_829511	4
620_829519	4
620_830162	4
620_829610	4
620_829432	4
620_825411	5
620_825329	5
620_825586	5
620_826285	5
620_826047	5
620_826101	5
620_826152	5
620_826024	5
620_829494	5
620_829566	5
620_825265	5
620_825302	5
620_825358	5
620_825322	5
620_825367	5
620_829410	5
620_829450	5
620_829453	5

Fig. 5 (cont.)

Polypeptide	ELISA Ranking	LFD Ranking	BAMS Ranking
620_829663	5	N/A	N/A
620_829428	5	N/A	N/A
620_826110	5	N/A	N/A
620_826284	5	N/A	N/A
620_826209	5	N/A	N/A
620_826287	5	N/A	N/A
620_825489	5	N/A	N/A
620_825601	5	N/A	N/A
620_825423	5	N/A	N/A
620_825576	5	N/A	N/A
620_830177	N/A	N/A	N/A

Polypeptide	ELISA Ranking
620_829663	5
620_829428	5
620_826110	5
620_826284	5
620_826209	5
620_826287	5
620_825489	5
620_825601	5
620_825423	5
620_825576	5

Fig. 5 (cont.)

FIGURE 6

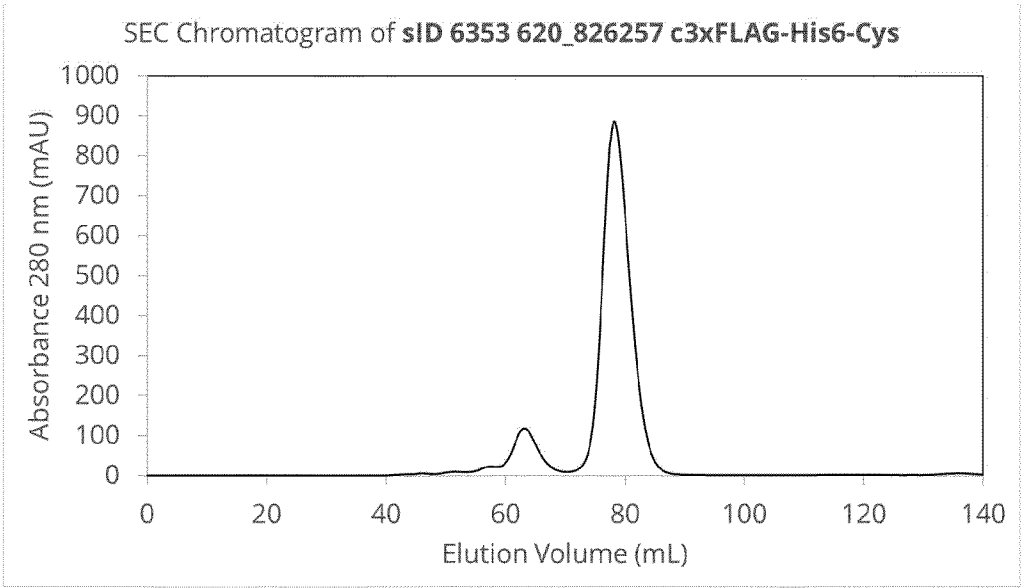


FIGURE 7

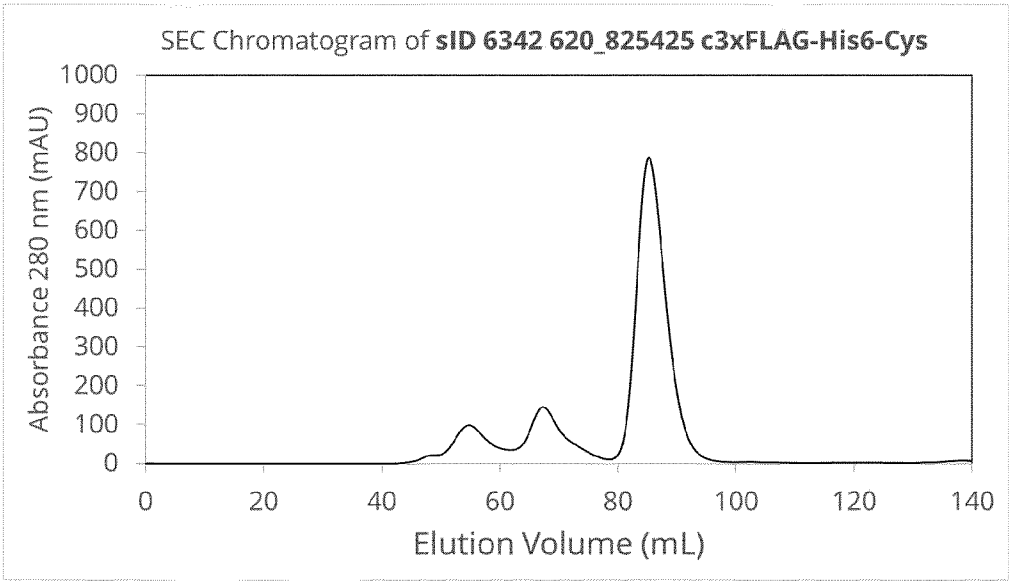


FIGURE 8

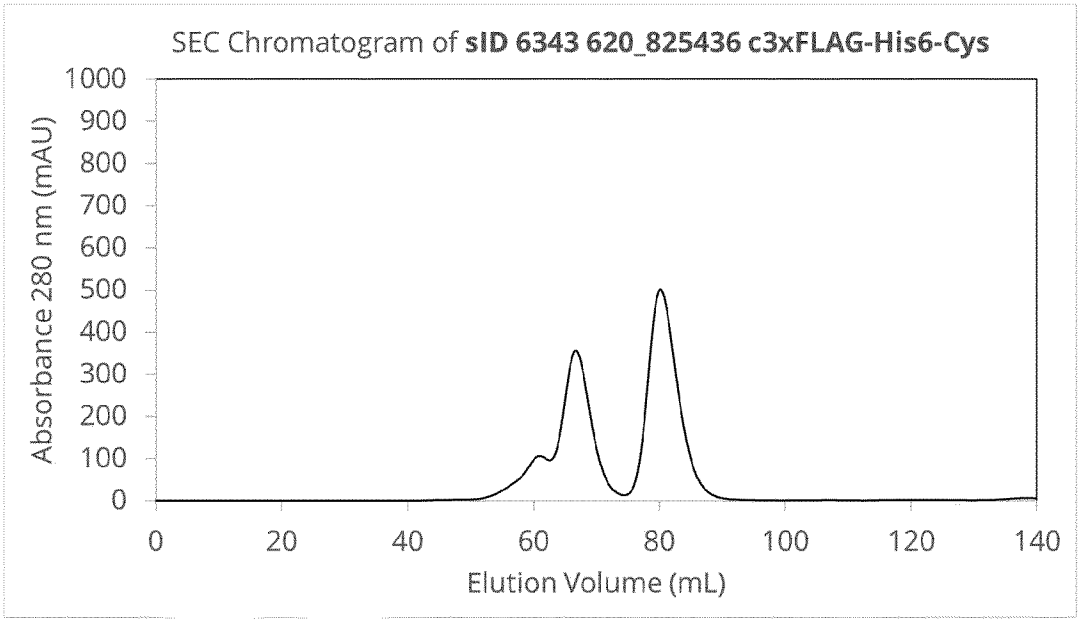


FIGURE 9

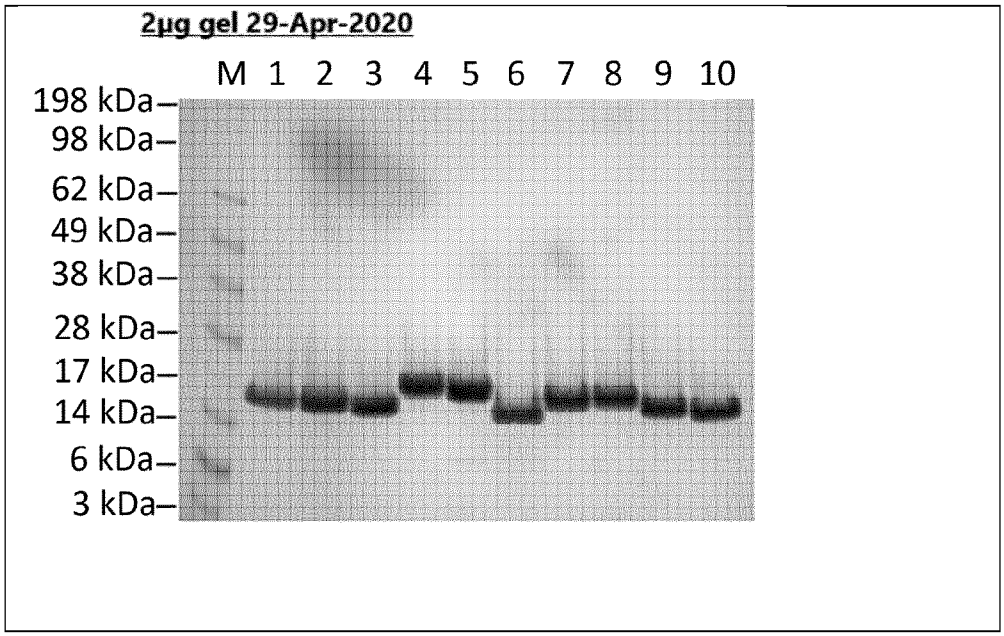


FIGURE 10

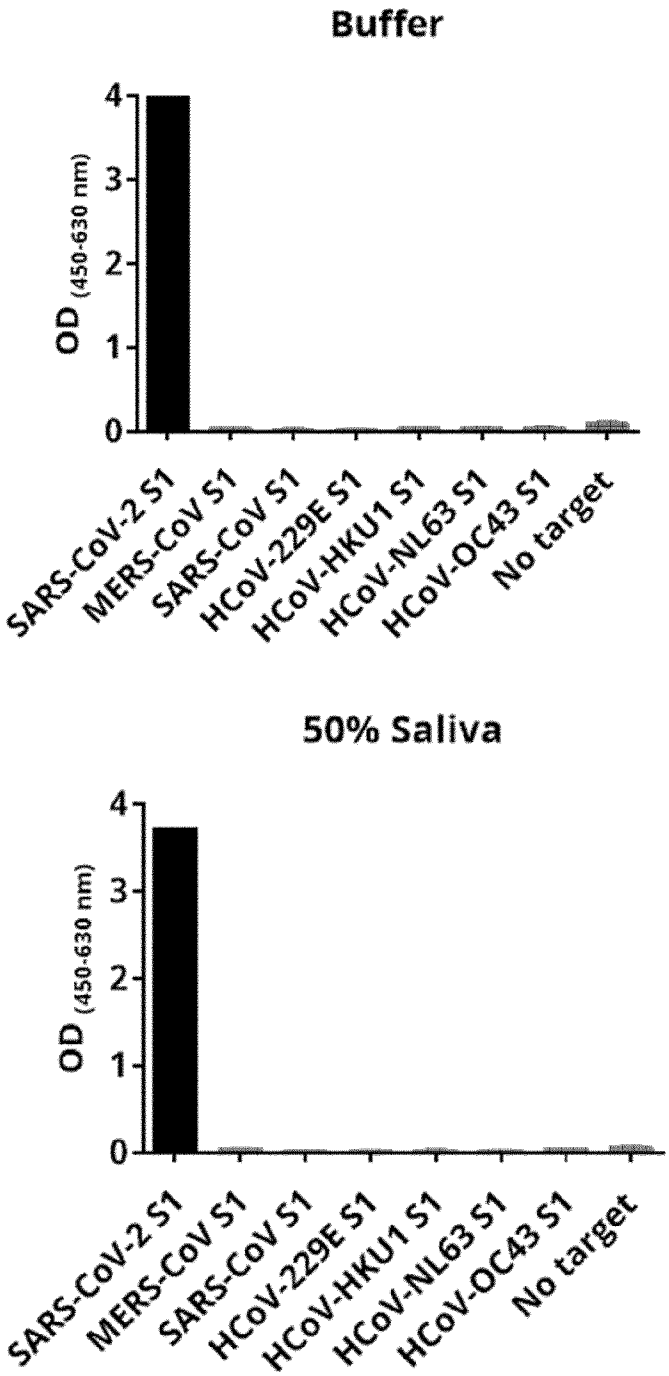

Buffer
OD (450-630 nm)
4
3
2
1
0
SARS-CoV-2 S1
MERS-CoV S1
SARS-CoV S1
HCoV-229E S1
HCoV-HKU1 S1
HCoV-NL63 S1
HCoV-OC43 S1
No target
50% Saliva
OD (450-630 nm)
4
3
2
1
0
SARS-CoV-2 S1
MERS-CoV S1
SARS-CoV S1
HCoV-229E S1
HCoV-HKU1 S1
HCoV-NL63 S1
HCoV-OC43 S1
No target

FIGURE 11A

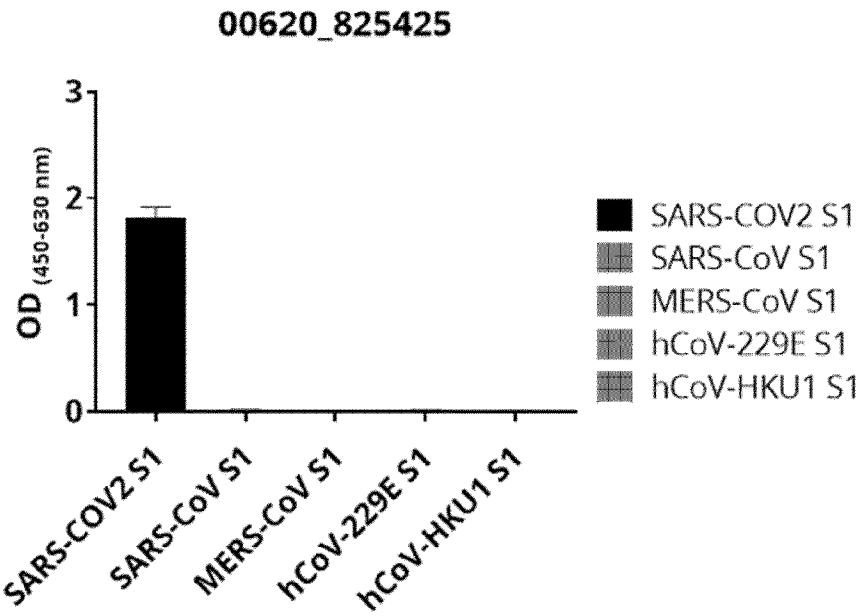


FIGURE 11B

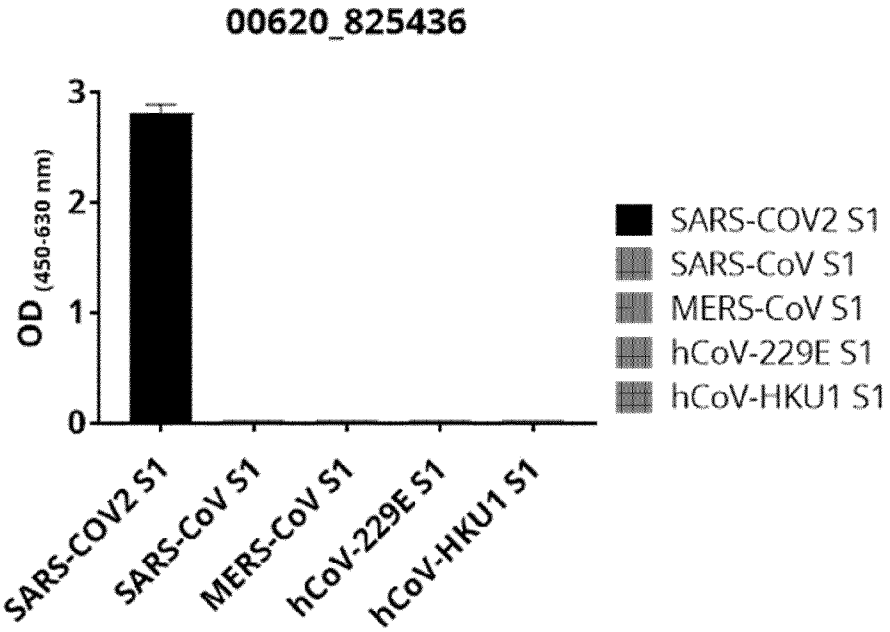


FIGURE 11C

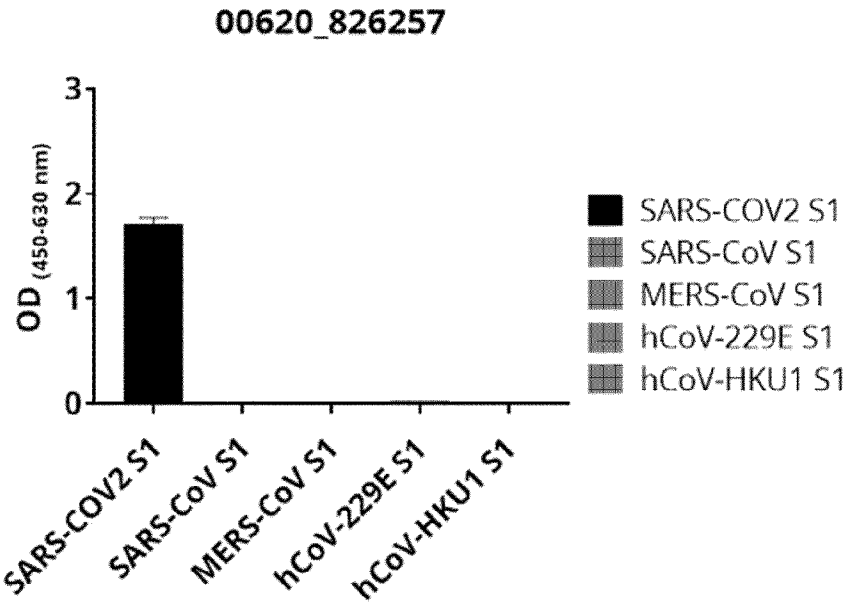


FIGURE 12

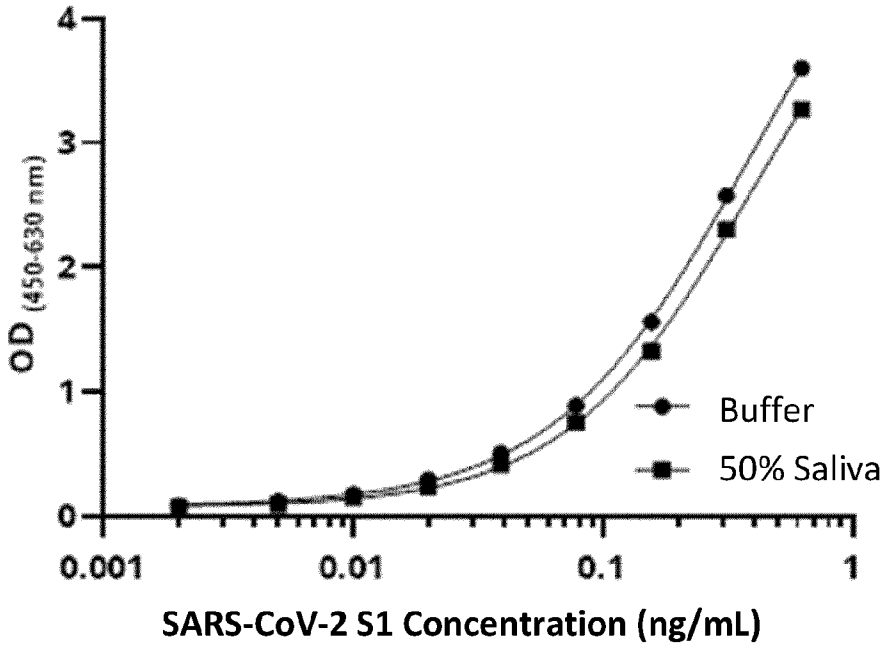


FIGURE 13A

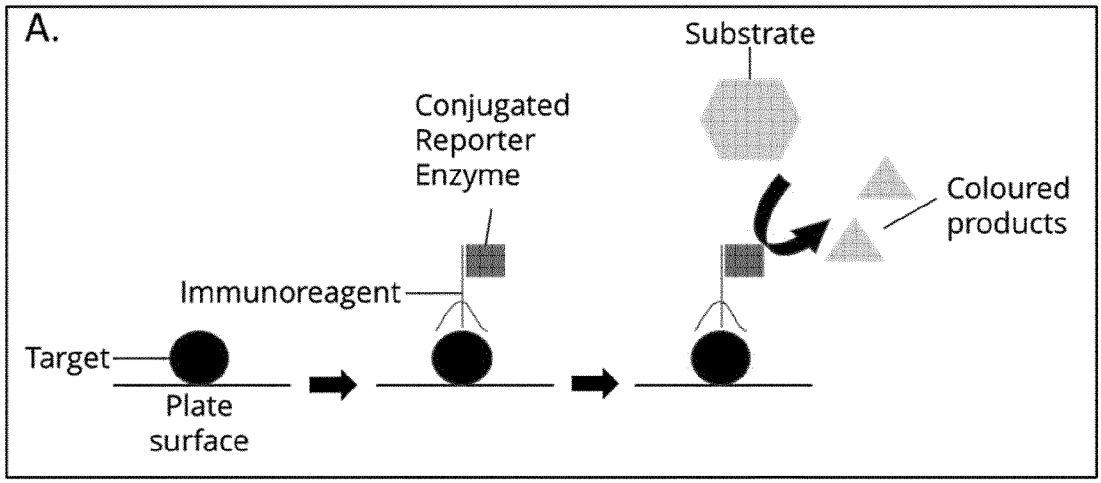


FIGURE 13B

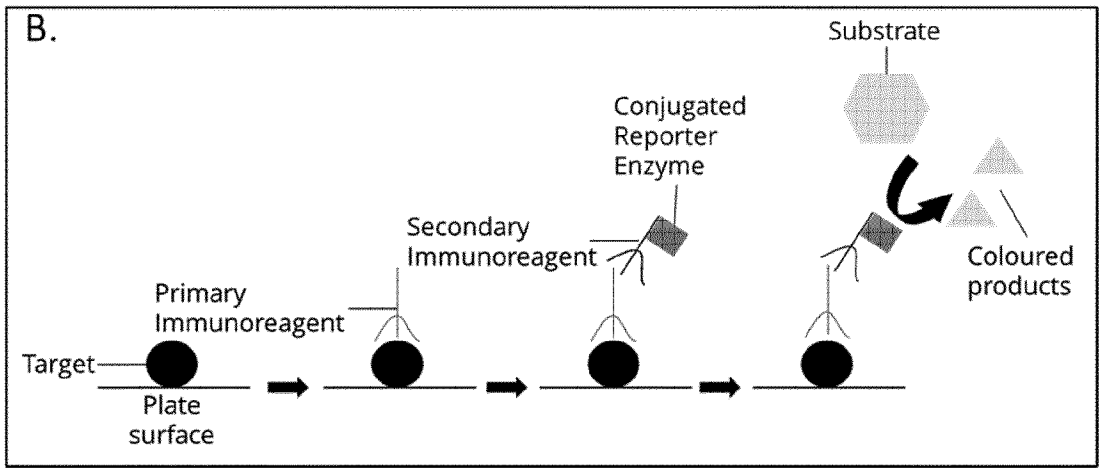


FIGURE 13C

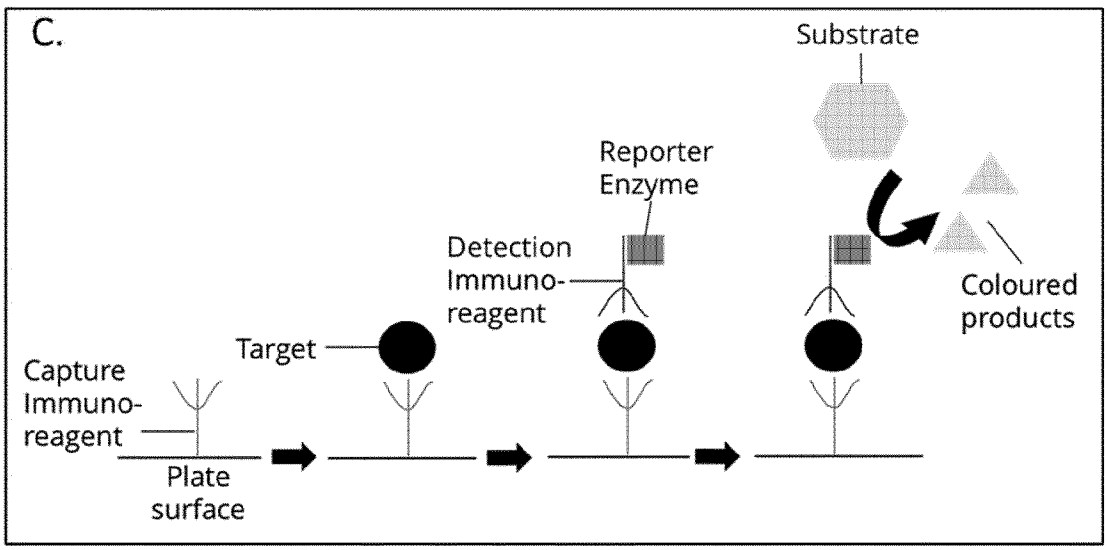


FIGURE 13D

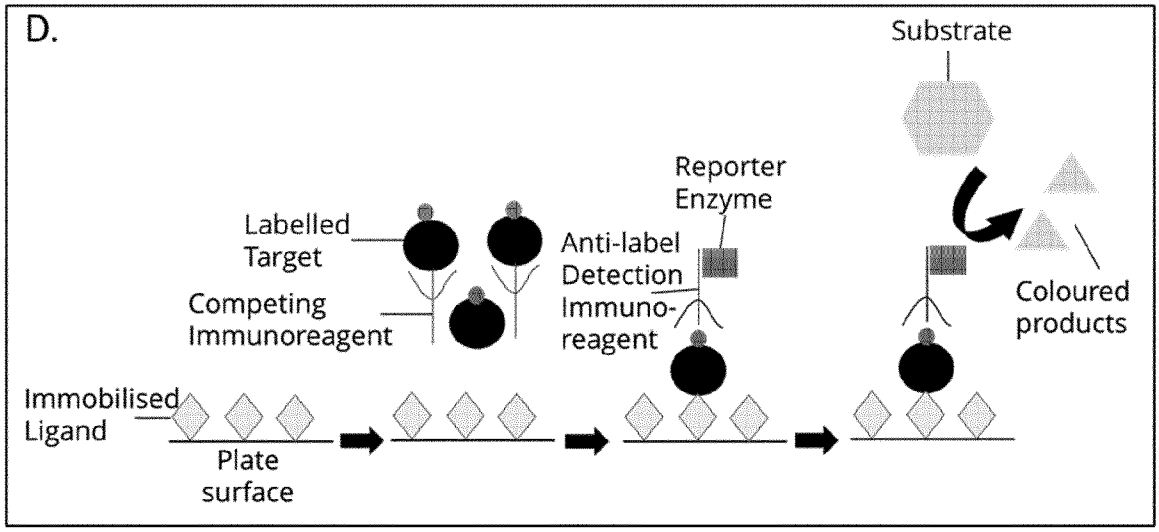


FIGURE 14

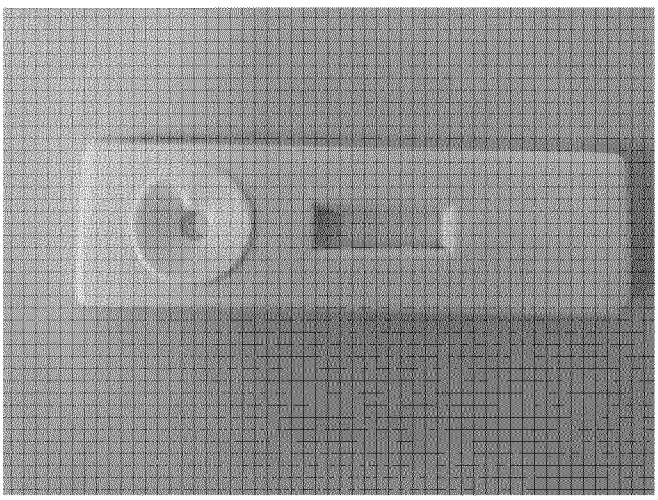


FIGURE 15A

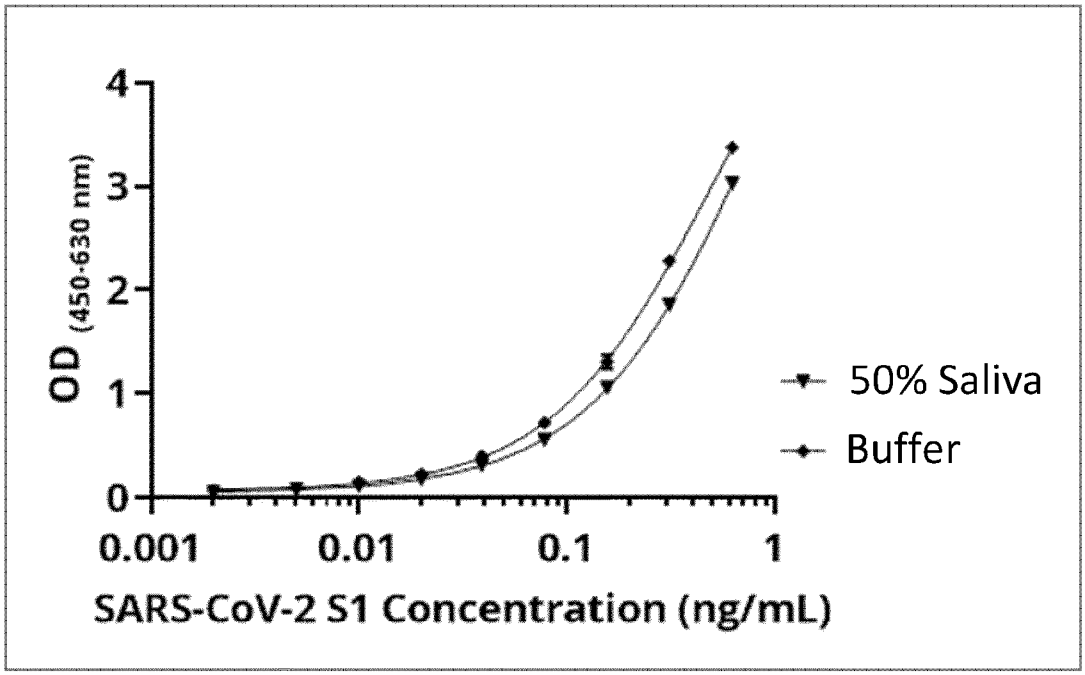


FIGURE 15B

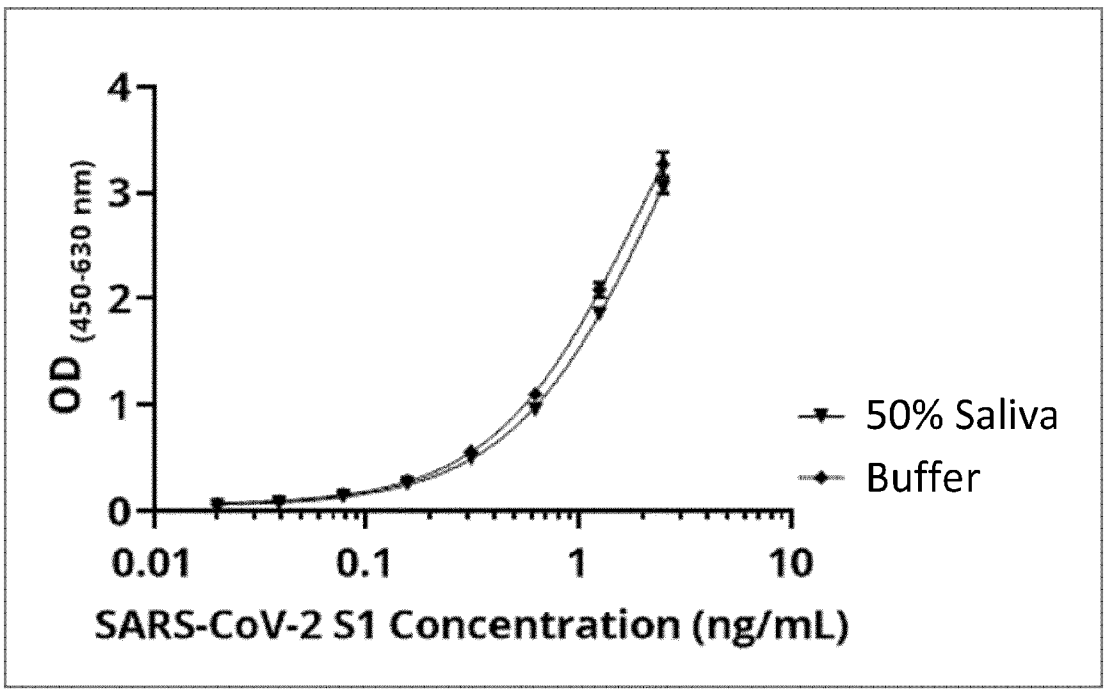
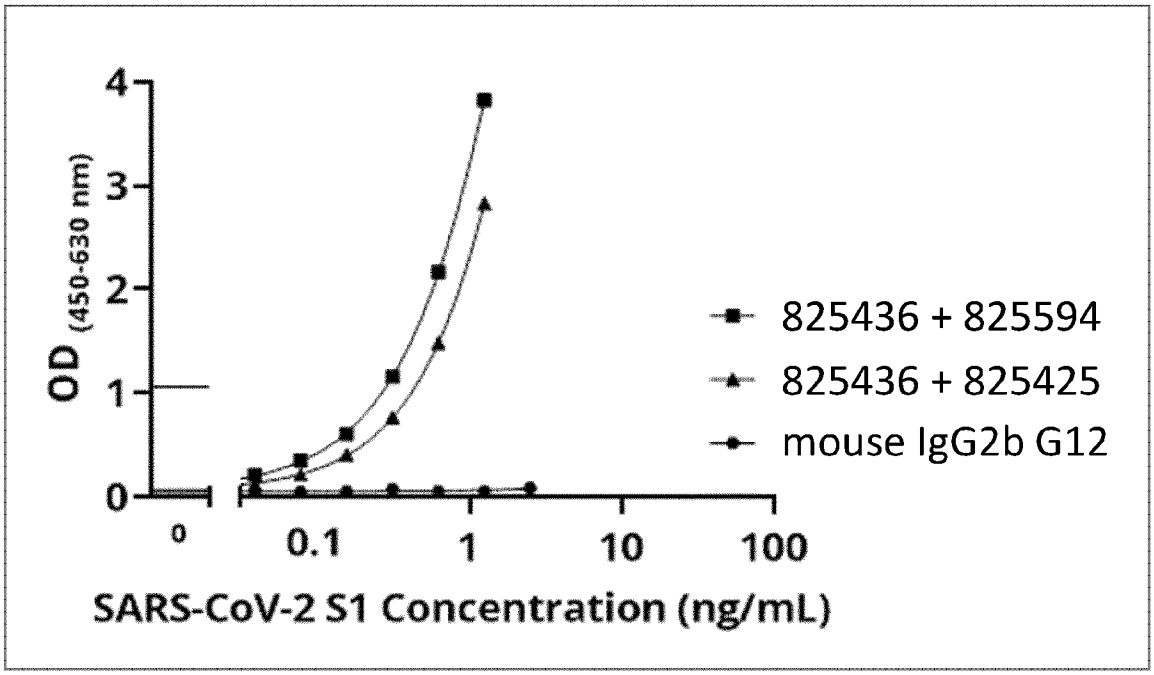


FIGURE 16


OD (450-630 nm)
4
3
2
1
0
0
0.1
1
10
100
SARS-CoV-2 S1 Concentration (ng/mL)
825436 + 825594
825436 + 825425
mouse IgG2b G12

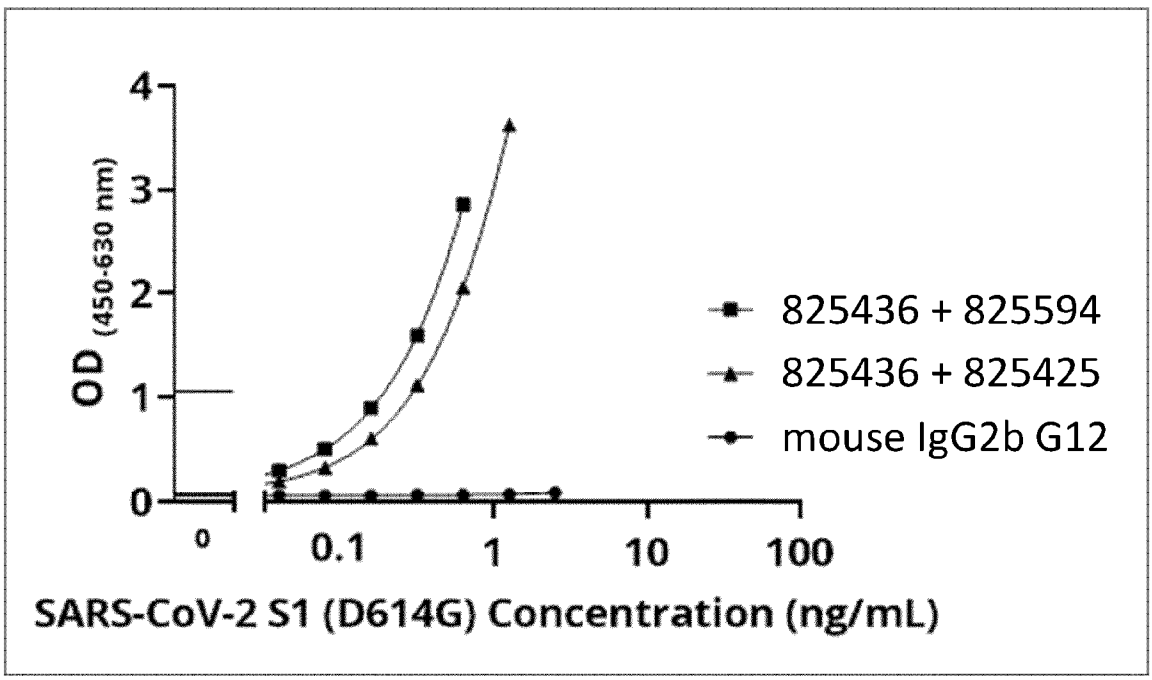

OD (450-630 nm)
4
3
2
1
0
0
0.1
1
10
100
SARS-CoV-2 S1 (D614G) Concentration (ng/mL)
825436 + 825594
825436 + 825425
mouse IgG2b G12

FIGURE 17

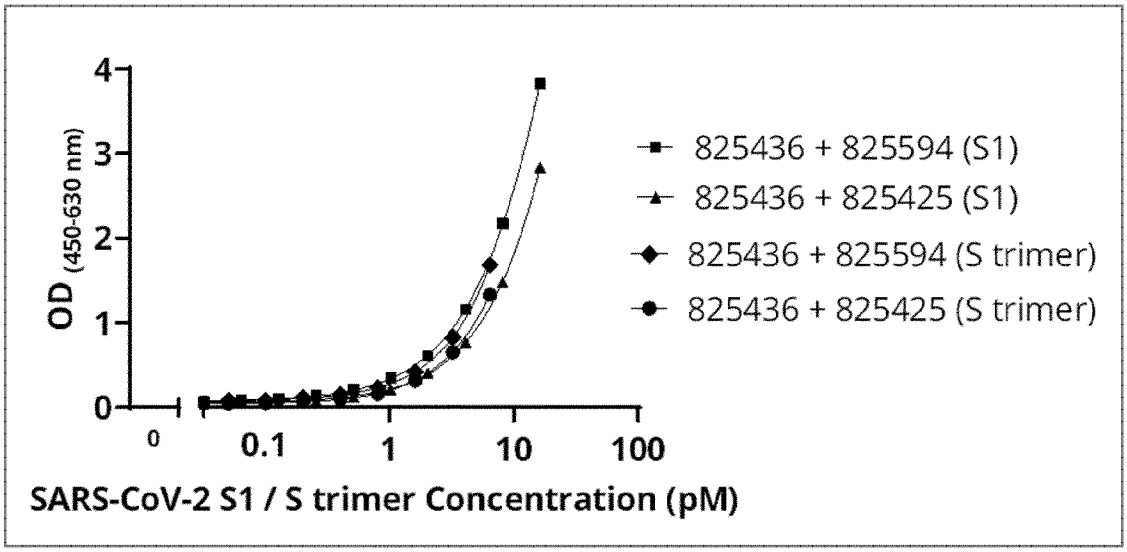


FIGURE 18A

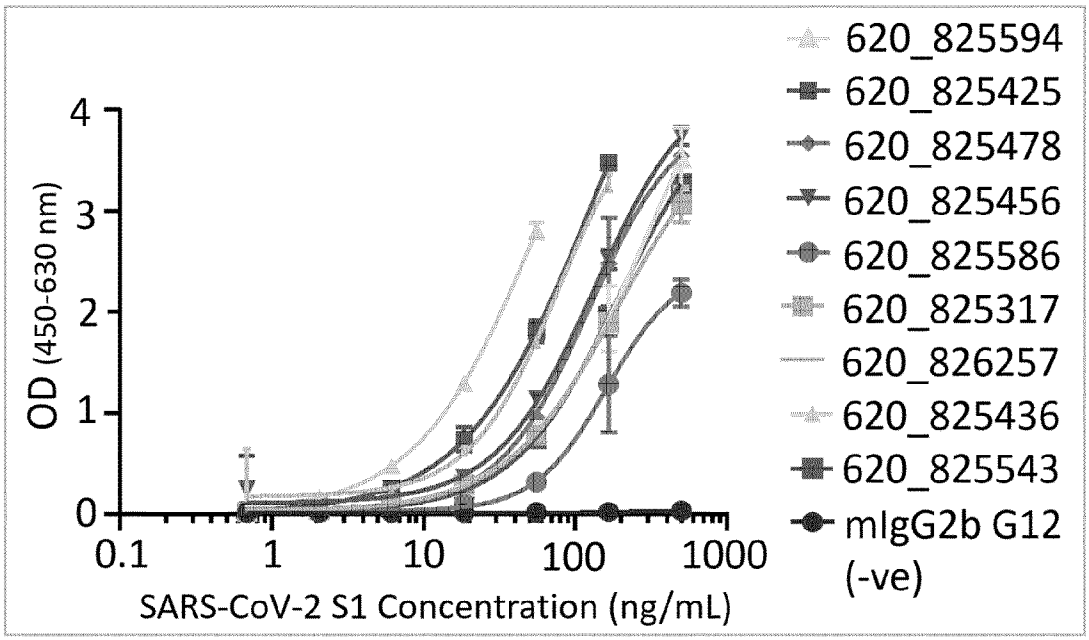


FIGURE 18B

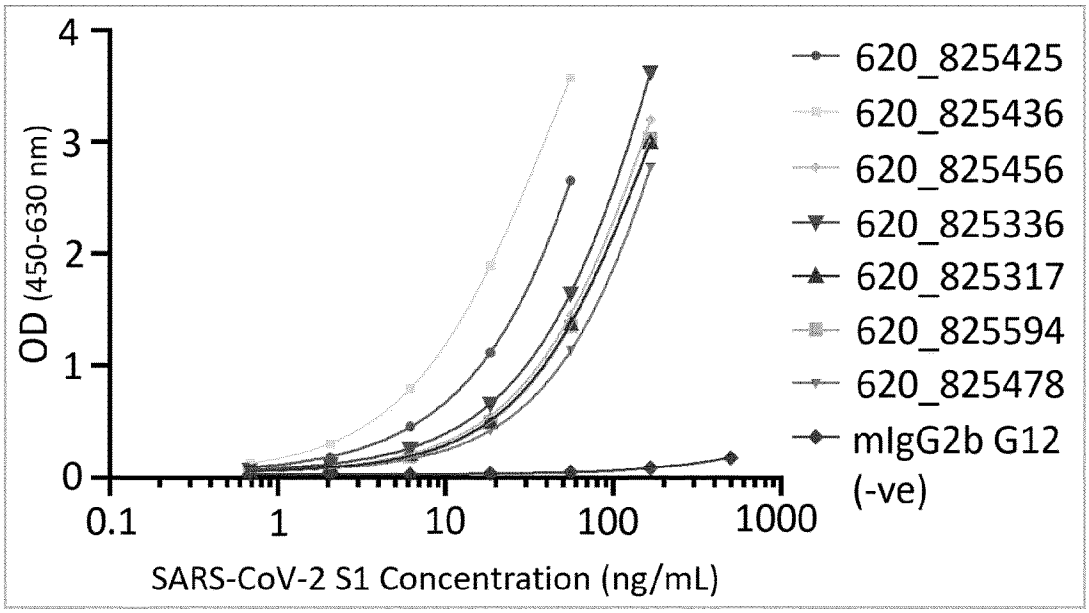

4
3
2
1
0
OD (450-630 nm)
0.1
1
10
100
1000
SARS-CoV-2 S1 Concentration (ng/mL)
620_825425
620_825436
620_825456
620_825336
620_825317
620_825594
620_825478
mIgG2b G12 (-ve)

FIGURE 19A

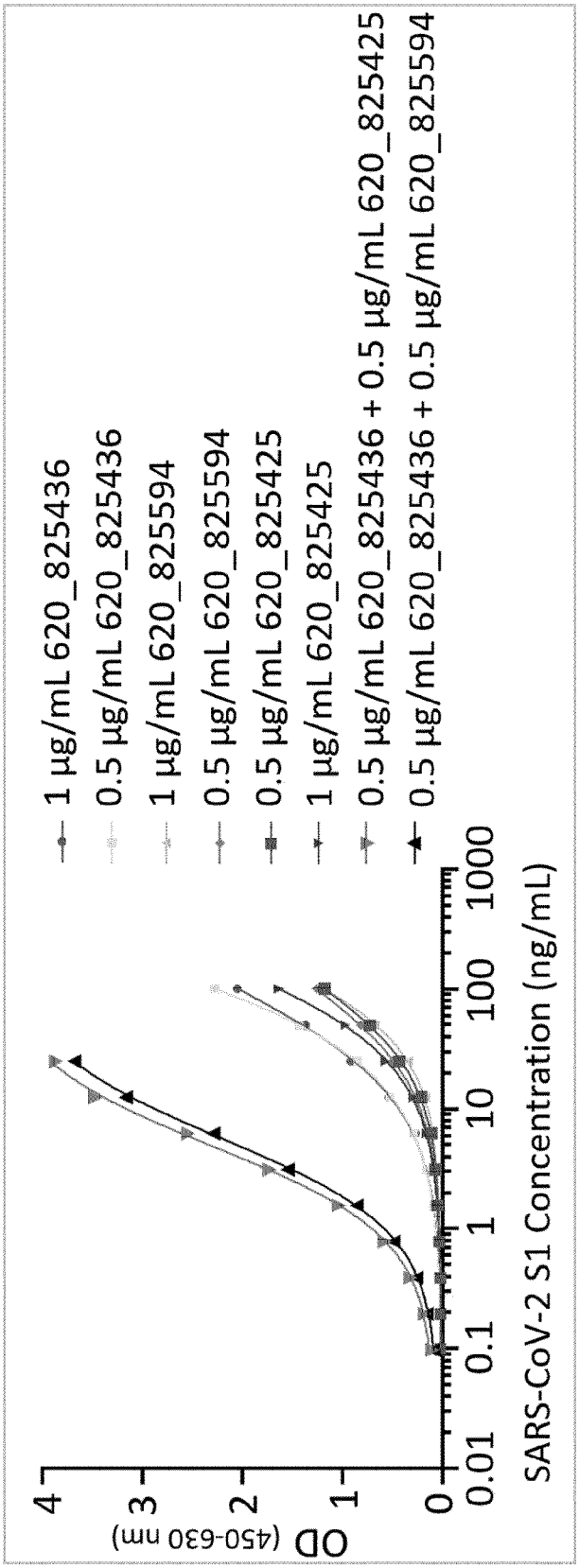

OD (450-630 nm)
0
1
2
3
4
0.01
0.1
1
10
100
1000
SARS-CoV-2 S1 Concentration (ng/mL)
1 µg/mL 620_825436
0.5 µg/mL 620_825436
1 µg/mL 620_825594
0.5 µg/mL 620_825594
0.5 µg/mL 620_825425
1 µg/mL 620_825425
0.5 µg/mL 620_825436 + 0.5 µg/mL 620_825425
0.5 µg/mL 620_825436 + 0.5 µg/mL 620_825594

FIGURE 19B

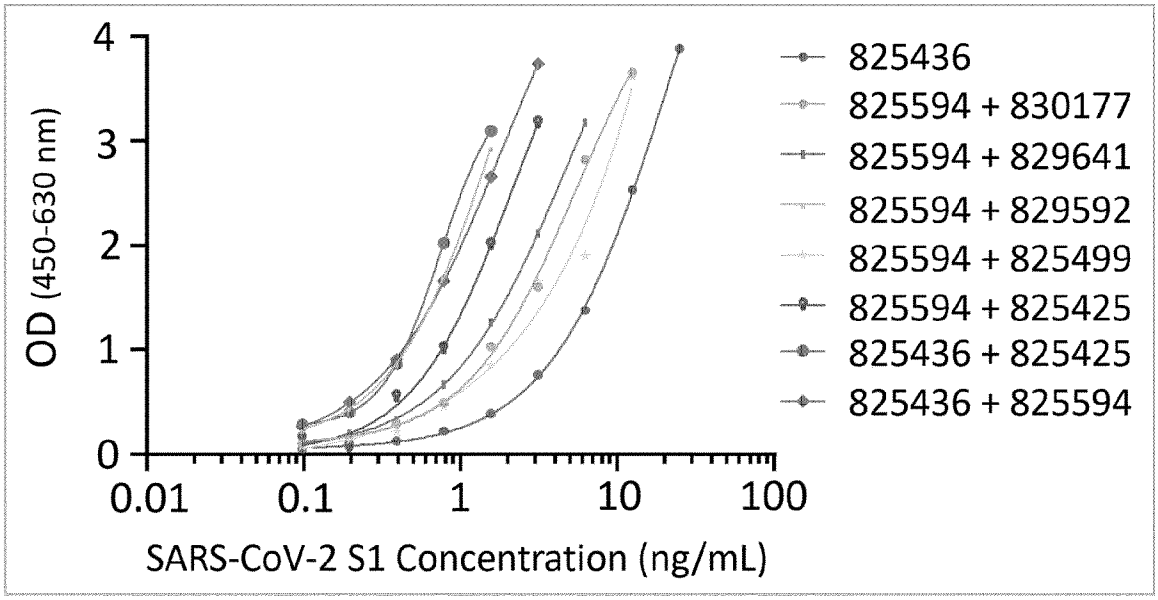
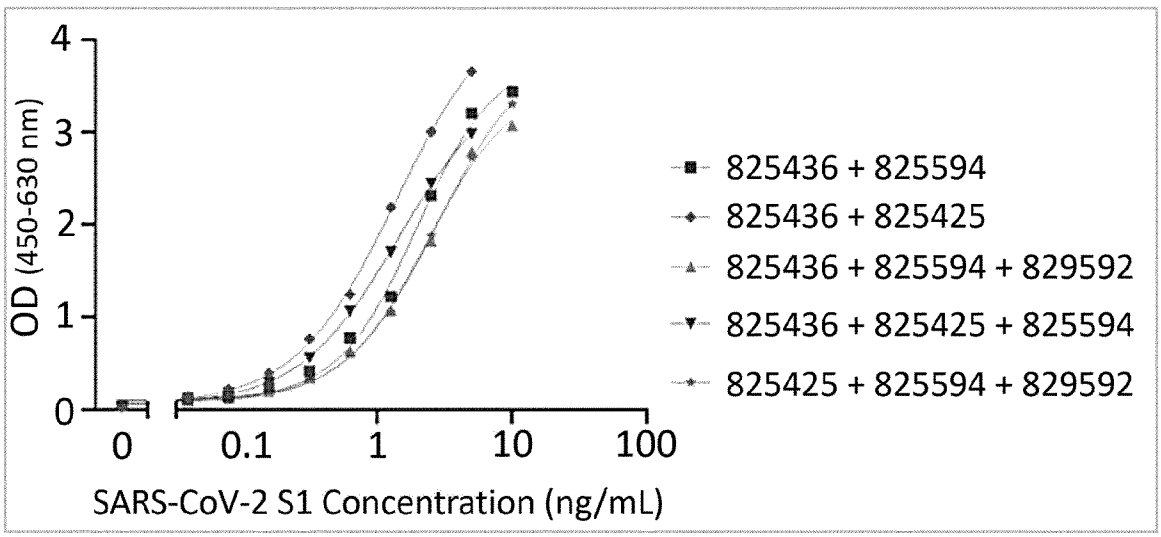


FIGURE 19C



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/064289

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/81 A61K38/16 G01N33/53
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K G01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AVACTA: "Affimer-based rapid test for COVID-19", , 29 April 2020 (2020-04-29), XP002804035, Retrieved from the Internet: URL:https://www.avactaanimalhealth.com/2020/04/29/affimer-based-rapid-test-for-covid-19/ [retrieved on 2021-08-26] the whole document	1-60
A	WO 2019/008335 A1 (AVACTA LIFE SCIENCES LTD [GB]) 10 January 2019 (2019-01-10) cited in the application stefin-based scaffold 3r2 (page 49); adhiron-based scaffold T2 (example 12); page 49	1-60

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 September 2021	10/09/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Galli, Ivo

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TIEDE CHRISTIAN ET AL: "Affimer proteins are versatile and renewable affinity reagents", ELIFE, ELIFE SCIENCES PUBLICATIONS LTD, GB, vol. 6, 27 June 2017 (2017-06-27), XP009527450, ISSN: 2050-084X, DOI: 10.7554/ELIFE.24903 the whole document	1-60
A	D'CRUZ ROSHAN J. ET AL: "Laboratory Testing Methods for Novel Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2)", FRONTIERS IN CELL AND DEVELOPMENTAL BIOLOGY, vol. 8, 4 June 2020 (2020-06-04), XP055835615, CH ISSN: 2296-634X, DOI: 10.3389/fcell.2020.00468 Retrieved from the Internet: URL:http://dx.doi.org/10.3389/fcell.2020.00468> the whole document	1-60
X,P	AVACTA: "AffiRX product specification", , October 2020 (2020-10), XP002804036, Retrieved from the Internet: URL:https://bioservuk.com/wp-content/uploads/2021/06/SARS-CoV-2-S1-Affimer-Pair-ELISA-IFU-1.pdf [retrieved on 2021-08-26] the whole document	1-60

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2021/064289

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2021/064289

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

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

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

2. ☒ Claims Nos.: 1-60(partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

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

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

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

see additional sheet

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-60

Affimer against SARS-CoV2, characterized by loop2+loop4=SEQ5+6

1.1. claims: 1-60(partially)

Inventions 2-57:
Affimer against SARS-CoV2, characterized by loop2+loop4=8+9, 11+12....173+174.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1-60(partially)

The claims could be searched only partially: The "loop2+loop4" pairs defined by sequences 65+66, 92+93, 116+117, 134+135 could not be searched, because sequences 66, 63, 117, 135 are missing from the listing.
The invention is about affimers that bind the spike protein of SARS-COV2. The more general claims, however, concern unspecified polypeptides where the sequences supposed to bind the virus protein can be inserted anywheres in the scaffold, and the polypeptides are not even functionally limited to such that actually turn out to be functional affimers. Generic polypeptides that do not act as bona fide affimers are devoid of inventive step as they do not solve any technical problem: a search covering such useless compounds would be meaningless. Support and disclosure are provided only for affimers where the "loop2" and "loop4" are inserted between position corresponding to amino acids 48-50 and 73-78 of the stefine-derived scaffold 3r2, or between positions corresponding to amino acids 46-51 and 75-79 of the adhiron-derived scaffold T2. The search is so limited.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) PCT declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2021/064289

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2019008335 A1	10-01-2019	AU 2018295447 A1	16-01-2020
		CA 3067682 A1	10-01-2019
		CN 111132999 A	08-05-2020
		EP 3649151 A1	13-05-2020
		JP 2020532949 A	19-11-2020
		KR 20200024869 A	09-03-2020
		SG 11201912298Q A	30-01-2020
		TW 201920256 A	01-06-2019
		US 2020181239 A1	11-06-2020
		WO 2019008335 A1	10-01-2019