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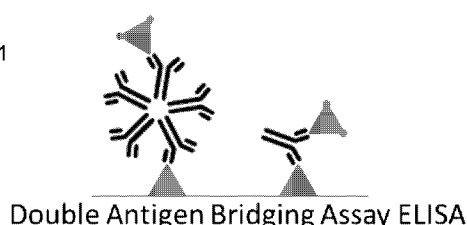
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(54) Title: HYBRID DOUBLE ANTIGEN SARS-COV-2 ANTIBODY DETECTION ASSAY

FIGURE 1



Double Antigen Bridging Assay ELISA

(57) Abstract: The present invention is directed to a method for detecting the presence or absence of SARS-Cov-2 antibodies in a sample, the method comprising: a. contacting the sample with a solid-phase support having a fusion protein immobilised thereto, said fusion protein comprising a first antigen and a scaffold polypeptide, i. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and ii. wherein the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support; b. allowing SARS-Cov-2 antibodies present in the sample to bind to the first antigen, thereby forming a complex of first antigen and SARS-Cov-2 antibody; c. contacting said complex with a labelled second antigen, wherein said labelled second antigen comprises a SARS-Cov-2 receptor binding domain polypeptide that binds SARS-Cov-2 antibody; d. allowing said labelled second antigen to bind to SARS-Cov-2 antibody present in the sample; e. removing labelled second antigen that is not bound to said complex; and f. detecting the presence of labelled second antigen bound to said complex; wherein the presence of labelled complex indicates the presence of SARS-Cov-2 antibody in the sample, and wherein the absence of labelled complex indicates the absence of SARS-Cov-2 antibody in the sample.

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HYBRID DOUBLE ANTIGEN SARS-COV-2 ANTIBODY DETECTION ASSAY

The present invention relates to methods for detecting antibody/ antibodies to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in a sample, and related kits for performing such methods.

SARS-CoV-2 is a strain of coronavirus that causes coronavirus disease 2019 (COVID-19), the respiratory illness characterised by the COVID-19 pandemic. The World Health Organization declared the pandemic a Public Health Emergency of International Concern on 30 January 2020, and a pandemic on 11 March 2020. As of 30 June 2020, more than 10.3 million cases were reported across 188 countries and territories, resulting in more than 505,000 deaths. A year later (as of 14 July 2021), the number of reported cases had risen to over 188 million, resulting in more than 4 million deaths.

Key to 'flattening' the coronavirus curve is (just like many other transmissible infections) the availability of reliable, rapid tests to detect infected individuals, who can then isolate from others to prevent transmission. Indeed, countries (such as South Korea) in which vigilant testing and tracing strategies were adopted early on during the pandemic succeeded in suppressing COVID-19 cases to numbers much lower than those seen in other countries, with such success being largely attributed to the widespread availability of testing. Testing is all the more important due to the current lack of a vaccine, and is an important resource for mitigating anxiety about spreading SARS-Cov-2 even where symptoms of COVID-19 are not being exhibited (although a number of vaccine options have subsequently become available, the need for vigilant testing has not waned).

Currently available tests include those for detecting the presence of the SARS-CoV-2 virus, and those for detecting the host response to the virus. These are molecular or antigen tests on the one hand (e.g. PCR-based or antigen assays) and antibody tests on the other hand (serological tests). Molecular tests (which detect viral genome) and antigen tests (which detect viral expression) can only diagnose the presence of infection as one finds in acute cases of COVID-19. They cannot tell whether someone has had the infection and since recovered. They also do not inform the immune status of an individual who has received a vaccine. Whilst these methods can provide high levels of sensitivity, they are only of value for investigation and identification of the infected patient from whom body fluids may be drawn that contain virus genome. Given the potentially short duration of viral replication and expression of viral antigens, coupled with the presence of mild clinical symptoms in some

patients, the question of past infection is often raised at a time that a diagnosis based on the detection of virus (e.g. viral DNA or antigen) is not possible. In addition, from a public health point of view of monitoring SARS-Cov-2 activity in a population of asymptomatic persons, direct tests for virus will not inform the prevalence of past infection.

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Advantageously, serological tests detecting anti-SARS-Cov2 antibodies have the potential to detect both past and present infection, even cases of infection with mild or no symptoms. Understanding if a patient has been previously infected with SARS-CoV-2 and defining whether antibody levels remain stable in the recovery patient will be an important step in understanding the durability and importance of post-infection susceptibility to reinfection. Such a serological test could also show the extent of viral spread in a community and provide useful public-health information. Furthermore, serological tests will prove indispensable to aid our understanding of the effectiveness (over time) of vaccines, allowing for the antibody levels in a vaccine -receiving individual to be probed and assessed as a readout of the immunisation response and potentially protection.

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Only a limited number serological tests (e.g. government approved tests) are available at the time of writing. These tests include the Abbott and Roche “SARS-CoV-2 IgG assay” (previously for the detection of IgG antibodies to the nucleocapsid protein of SARS-CoV-2, more recently for detection of the spike protein) and the Fortress/ Wantai “COVID-19 Total Ab” test (for the detection of antibodies to the receptor binding domain, RBD). A recent study published by Public Health England (PHE) entitled “Evaluation of the Abbott SARS-CoV-2 IgG for the detection of anti-SARS CoV-2 antibodies” ([https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/](https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/890566/Evaluation_of_Abbott_SARS_CoV_2_IgG_PHE.pdf) file/890566/Evaluation_of_Abbott_SARS_CoV_2_IgG_PHE.pdf) found this Abbot test to have a specificity of 100% and overall sensitivity of 92.7%. However, a subsequent study (Rosadas C, Randell P, Khan M, McClure MO, Tedder RS. Testing for responses to the wrong SARS-CoV-2 antigen? Lancet. 2020 Sep 5;396(10252):e23. doi: 10.1016/S0140-6736(20)31830-4. Epub 2020 Aug 28. PMID: 32866429; PMCID: PMC7455229, incorporated herein by reference) graphically displayed the serious loss of sensitivity of the Abbott anti-NP assay. Whilst the apparent sensitivity of the government approved assays may be adequate, a failure to detect antibody, particularly during the acute infection and when applied to population-based seroprevalence surveys, may allow current and past infections to go unrecognised. This will naturally under-ascertain the prevalence of this infection in any community. More importantly, false-reactivity has the potential to lead an individual to believe that having had the infection (which may have passed) and that they may be protected to

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some extent against reinfection. Though it cannot be guaranteed that the recovery confers resistance to reinfection, the double risk of falsely believing that one has had the infection when (when one has in fact not had the infection) is truly dangerous.

- 5 Furthermore, as the demand for antibody-testing increases, there will be a need for further tests from a variety of sources e.g. to prevent reliance on a limited number of suppliers.

The present invention solves one or more of the above-identified problems by providing a flexible, accurate, rapid, robust and sensitive method for detecting infection (e.g. past
10 infection) of a subject by SARS-Cov-2.

The method of the invention is performed *in vitro* on a sample (e.g. blood or plasma taken from a subject) and may be performed directly on said sample. The method is a serological assay, and therefore relies on identification of anti-SARS-Cov-2 antibody that is present in
15 the sample by capturing antibody with immobilised antigen bound to a solid-phase support and subsequently detecting immobilised antibody with a second appropriately labelled antigen. Of note, the invention is predicated on the surprising finding that presenting the immobilised antigen on a 'scaffold polypeptide', and away from the solid-phase support, leads to improved sensitivity and specificity *inter alia*. In other words, a scaffold having a
20 protein domain adjoining, but differing from, the desired epitope-holding antigen presents the immobilised antigen, yielding a sensitivity in a hybrid double antigen binding assay that surpasses that achievable with an otherwise equivalent assay (e.g. Fortress/ Wantai "COVID-19 Total Ab" test, an homologous double antigen binding assay) in which the antigen is bound directly to the solid-phase support e.g. the antigen is not presented/
25 displayed away from the solid-phase support by a scaffold polypeptide.

In more detail, the method of the present invention may typically comprise:

- 1) a capture step: anti-SARS-Cov2 antibody present in the patient sample that binds to a first antigen (e.g. RBD) is immobilised by scaffold-presented antigen;
- 30 2) a binding step to permit detection of antibody that binds to the first antigen: antibody immobilised by the scaffold-presented antigen is challenged with a labelled second antigen (e.g. having the same epitope as the first antigen, but a shorter sequence lacking the scaffold). In the presence of antibody that binds to the first antigen, labelled second antigen forms a labelled complex, with complex formation aided by
35 scaffold-presentation of the antigen. The antibody that binds to the first antigen

typically remains immobilised throughout, resulting in a labelled immobilised complex;
and

- 3) a detecting step that allows the identification of the presence (or absence) of labelled complex, and thus indicating the presence (or absence) of SARS-Cov-2.

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If desired, an optional quenching step may also be employed, in which the binding of the labelled second antigen may be challenged by the addition of a third unlabelled antigen from a non-SARS-Cov-2 virus, wherein the binding of said third antigen thereto suppresses (e.g. blocks) any inherent antigenic binding cross-reactivity of the immobilised antibody towards the non-SARS-Cov-2 virus. This quenching step effectively allows identification of immobilised antibody that specifically binds the first antigen from the SARS-Cov-2 virus.

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In other words, whilst the present assay may be a “double antigen bridging assay” (DABA) for the serological detection of SARS-CoV-2 (see Figure 1), it may better be described as a “hybrid DABA” whereby the solid-phase and the second labelled antigen components differ in their sourcing and are of different origin, sharing only the epitope against which the test is designed to detect antibody. The nature of the assay allows capture of all immunoglobulin classes, potentially conferring enhanced sensitivity. Furthermore, the assay is species-neutral and will detect specific antibody in hosts other than humans. Unlike conventional ELISA formats, double antigen bridging assays (DABA) use an antigen sandwich, with the second part of the sandwich being an antigen conjugated to label (e.g. an enzyme) to visualise specific antibody detection. This immunoassay design poses several advantages to conventional ELISA formats, especially when trying to reduce the effects of cross-reactive antibodies. A particular advantage conferred upon the DABA format comes from the potential to select (as in this case) a difference in the format of the solid-phase and the fluid phase and the additional option of quenching cross-reactive antibody from non-SARS-Cov-2 to prevent second label antigen signalling.

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In one aspect, the invention provides a method for detecting the presence or absence of SARS-Cov-2 antibodies in a sample, the method comprising:

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- a. contacting the sample with a solid-phase support having a fusion protein (aka protein e.g. multi-domain protein, aka a protein having two or more polypeptide domains immobilised thereto, said fusion protein comprising a first antigen and a scaffold polypeptide,

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- i. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and

- ii. the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support; and
- b. allowing SARS-Cov-2 antibodies present in the sample to bind to the first antigen, thereby forming a complex of first antigen and SARS-Cov-2 antibody;
- c. contacting said complex with a labelled second antigen, wherein said labelled second antigen comprises a SARS-Cov-2 receptor binding domain polypeptide that binds SARS-Cov-2 antibody;
- d. allowing said labelled second antigen to bind to SARS-Cov-2 antibody present in (e.g. captured from) the sample;
- e. removing labelled second antigen that is not bound to said complex; and
- f. detecting the presence of labelled second antigen bound to said complex;

wherein the presence of labelled complex indicates the presence SARS-Cov-2 antibody, and wherein the absence of labelled complex indicates the absence of SARS-Cov-2 antibody.

In one aspect, the invention provides a method for detecting the presence or absence of SARS-Cov-2 antibodies in a sample, the method comprising:

- a. contacting the sample with a solid-phase support having a protein (e.g. multi-domain protein, aka a protein having two or more polypeptide domains) immobilised thereto, said protein comprising a first antigen and a scaffold polypeptide,
 - i. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and
 - ii. the scaffold polypeptide immobilises the protein to the solid-phase support and presents the first antigen away from the solid-phase support; and
- b. allowing SARS-Cov-2 antibodies present in the sample to bind to the first antigen, thereby forming a complex of first antigen and SARS-Cov-2 antibody;
- c. contacting said complex with a labelled second antigen, wherein said labelled second antigen comprises a SARS-Cov-2 receptor binding domain polypeptide that binds SARS-Cov-2 antibody;
- d. allowing said labelled second antigen to bind to SARS-Cov-2 antibody present in (e.g. captured from) the sample;
- e. removing labelled second antigen that is not bound to said complex; and
- f. detecting the presence of labelled second antigen bound to said complex;

wherein the presence of labelled complex indicates the presence SARS-Cov-2 antibody, and wherein the absence of labelled complex indicates the absence of SARS-Cov-2 antibody.

5 One aspect of the invention provides a method for detecting the presence or absence of SARS-Cov-2 antibodies in a sample, the method comprising:

a. contacting the sample with a solid-phase support having a fusion protein (aka protein e.g. multi-domain protein, aka a protein having two or more polypeptide domains) immobilised thereto, said fusion protein comprising a first antigen and a
10 scaffold polypeptide,

i. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and

ii. the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase
15 support; and

b. allowing SARS-Cov-2 antibodies present in the sample to bind to the first antigen, thereby forming a complex of first antigen and SARS-Cov-2 antibody, such that SARS-Cov-2 antibody is captured from the sample;

c. contacting said complex with a labelled second antigen, wherein said labelled
20 second antigen comprises a SARS-Cov-2 receptor binding domain polypeptide that binds SARS-Cov-2 antibody;

d. allowing said labelled second antigen to bind to SARS-Cov-2 antibody captured from the sample;

e. removing labelled second antigen that is not bound to said complex; and

25 f. detecting the presence of labelled second antigen bound to said complex;

wherein the presence of labelled complex indicates the presence SARS-Cov-2 antibody, and wherein the absence of labelled complex indicates the absence of SARS-Cov-2 antibody.

30 One aspect of the invention provides a method for detecting SARS-Cov-2 antibody in a sample, the method comprising:

a. contacting a sample (e.g. isolated from a subject) with a solid-phase support having a first SARS-Cov-2 RBD antigen bound thereto by means of a scaffold (e.g. scaffold polypeptide), wherein the scaffold presents the first antigen (e.g.
35 away from the solid-phase support) for capturing and immobilising anti-SARS-Cov-2 antibody in the sample; and

- b. challenging the immobilised antibody with labelled second antigen, thereby forming a labelled antigen-antibody complex;

wherein the presence of labelled complex indicates the presence of SARS-Cov-2 antibody, and wherein the absence of labelled complex indicates the absence of SARS-Cov-2 antibody.

One aspect of the invention provides a method for detecting SARS-Cov-2 antibody in a sample, the method comprising:

- a. contacting a sample (e.g. isolated from a subject) with a solid-phase support having a first SARS-Cov-2 RBD antigen bound thereto by means of a scaffold (e.g. scaffold polypeptide), wherein the scaffold presents the first antigen (e.g. away from the solid-phase support) for capturing and immobilising anti-SARS-Cov-2 antibody in the sample; and
- b. allowing SARS-Cov-2 antibodies present in the sample to bind to the first antigen, thereby forming a complex of first antigen and SARS-Cov-2 antibody;
- c. contacting said complex with a labelled second antigen, wherein said labelled second antigen comprises a SARS-Cov-2 receptor binding domain polypeptide that binds SARS-Cov-2 antibody;
- d. allowing said labelled second antigen to bind to SARS-Cov-2 antibody from the sample;
- e. removing labelled second antigen that is not bound to said complex; and
- f. detecting the presence of labelled second antigen bound to said complex;

wherein the presence of labelled complex indicates the presence of SARS-Cov-2 antibody, and wherein the absence of labelled complex indicates the absence of SARS-Cov-2 antibody.

One aspect of the invention provides a method for detecting SARS-Cov-2 antibody in a sample, the method comprising:

- a. contacting a sample (e.g. isolated from a subject) with a solid-phase support having a first SARS-Cov-2 RBD antigen bound thereto by means of a scaffold (e.g. scaffold polypeptide), wherein the scaffold presents the first antigen (e.g. away from the solid-phase support) for capturing and immobilising anti-SARS-Cov-2 antibody in the sample; and
- b. allowing SARS-Cov-2 antibodies present in the sample to bind to the first antigen, thereby forming a complex of first antigen and SARS-Cov-2 antibody, such that SARS-Cov-2 antibody is captured from the sample;

- c. contacting said complex with a labelled second antigen, wherein said labelled second antigen comprises a SARS-Cov-2 receptor binding domain polypeptide that binds SARS-Cov-2 antibody;
- d. allowing said labelled second antigen to bind to SARS-Cov-2 antibody captured from the sample;
- e. removing labelled second antigen that is not bound to said complex; and
- f. detecting the presence of labelled second antigen bound to said complex;

wherein the presence of labelled complex indicates the presence of SARS-Cov-2 antibody, and wherein the absence of labelled complex indicates the absence of SARS-Cov-2 antibody.

A related aspect provides a kit for detecting the presence or absence of SARS-Cov-2 antibodies in a sample, the kit comprising:

- a. a solid-phase support having a fusion protein (aka protein e.g. multi-domain protein, aka a protein having two or more polypeptide domains) immobilised thereto, said fusion protein comprising a first antigen and a scaffold polypeptide,
 - i. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and
 - ii. the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support; and
- b. a labelled second antigen, wherein said labelled second antigen comprises a SARS-Cov-2 receptor binding domain polypeptide that binds SARS-Cov-2 antibody.

One aspect provides a kit for detecting the presence or absence of SARS-Cov-2 antibody in a sample (e.g. isolated from a subject), the kit comprising:

- a. a solid-phase support having a first SARS-Cov-2 RBD antigen bound thereto by means of a scaffold polypeptide, wherein the scaffold polypeptide presents the first antigen (e.g. away from the solid-phase support) for capturing and immobilising anti-SARS-Cov-2 antibody in a sample; and
- b. a labelled second antigen.

Another aspect of the invention provides an immunoassay solid-phase support for detecting the presence or absence of SARS-Cov-2 in a sample, the solid-phase support comprising a fusion protein (aka protein e.g. multi-domain protein, aka a protein having two or more

polypeptide domains) immobilised thereto, said fusion protein comprising a first antigen and a scaffold polypeptide,

a. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and

5 b. the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support.

One aspect provides an immunoassay solid-phase support for detecting SARS-Cov-2 antibody in a sample (e.g. isolated from a subject), the solid-phase support comprising a first
10 SARS-Cov-2 RBD antigen bound thereto by means of a scaffold (e.g. scaffold polypeptide), wherein the scaffold presents the first antigen (e.g. away from the solid-phase support) for capturing and immobilising anti-SARS-Cov-2 antibody in the sample.

Yet a further aspect of the invention relates to use of solid-phase support for detecting
15 SARS-Cov-2 in a sample, the solid-phase support comprising a fusion protein (aka protein e.g. multi-domain protein, aka a protein having two or more polypeptide domains) immobilised thereto, said fusion protein comprising a first antigen and a scaffold polypeptide,

a. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and

20 b. the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support.

In another aspect of the invention, there is provided a method for manufacturing a solid-phase support for detecting the presence or absence of SARS-Cov-2 in a sample, the
25 method comprising immobilising a fusion protein (aka protein e.g. multi-domain protein, aka a protein having two or more polypeptide domains) to a solid-phase support, said fusion protein comprising a first antigen and a scaffold polypeptide,

a. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and

30 b. the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support.

Reference to "SARS-Cov-2" means the strain of coronavirus that causes COVID-19 (e.g. having the sequence of NCBI reference no: NC_045512), and may be used interchangeably
35 with the terms "2019 novel coronavirus" (2019-nCoV), and "human coronavirus 2019" (HCoV-19 or hCoV-19).

The “scaffold polypeptide” (which may be referred to interchangeably with the term “carrier polypeptide”) provides a structural means for immobilising the first antigen (e.g. the fusion protein) to the solid-phase support, such that direct binding of a region of the RBD component of the fusion to the solid-phase support is not required. The first (RBD) antigen is
5 ‘presented’ (or ‘displayed’) on/ by the scaffold polypeptide away from the solid-phase support (e.g. is presented to the sample), thus the solid-phase includes first antigen (e.g. RBD antigen) that is not directly bound to the solid-phase support (i.e. the scaffold polypeptide is bound to the solid-phase support, allowing the first antigen to be free to interact with antibody). In other words, the scaffold polypeptide allows for orientating/ distancing the first
10 antigen away from the solid-phase support (and toward the sample). Without wishing to be bound by theory, it is believed that by employing a scaffold polypeptide to present the antigen, binding domains (e.g. epitopes) that would otherwise be ‘masked’ (upon direct solid-phase support binding of the RBD antigen) are left free to be recognised and bound by antibody in the sample.

15 An associated technical effect (e.g. of the scaffold) is improved sensitivity (see Table 2 in the Examples for further technical effects). The invention (e.g. method or immunoassay of the invention, use etc) preferably provides higher sensitivity when compared with an otherwise identical method lacking the scaffold of the fusion protein (e.g. in which the RBD is directly
20 bound to the solid phase support). Sensitivity is preferably defined as ability to correctly detect positive samples, for example where 98/100 positive samples (known to be positive for SARS-Cov-2 antibody) provide a positive result (e.g. detectable signal from the label), the sensitivity may be said to be 98%. Preferably, the invention provides a sensitivity (e.g. average sensitivity) of at least 95%; preferably at 96% (e.g. 96.95%). Further technical
25 effects are exemplified in the Examples, e.g. at Tables 3-6. For example, the invention (e.g. method, immunoassay, use of the invention, etc) preferably provides higher specificity when compared with an otherwise identical method lacking the scaffold of the fusion protein (e.g. in which the RBD is directly bound to the solid phase support). Specificity preferably refers to the specificity of providing a positive (detection) result for a SARS-Cov-2 antibody-positive
30 sample over a SARS-Cov-2 antibody-negative sample. For example, where 0/100 negative samples (known to be negative for SARS-Cov-2 antibody) do not provide a positive result (e.g. detectable signal from the label), the specificity may be said to be 100%. Preferably, the invention provides a specificity of at least 99.8 % (preferably 100%).

35 Where the term “is” is used to describe what an antigen is (e.g. the first antigen “is” a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody), it should

be understood that the (principle) antigenic component is as described, however the polypeptide as a whole may comprise an additional sequence, for example a 'tag' (such as a His-tag or Twin-Strep tag) fused to the antigenic component as a result of an expression/purification process.

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A "fusion protein" refers to a fusion formed from at least two domains of a protein (or from at least two proteins). For example, the RBD antigen (first domain), which is a region/domain of the spike protein of SARS-Cov-2, may be fused to another region/domain of the spike protein (second domain), such as an N-terminal region/domain. A "fusion protein" may be a
10 "chimeric protein", e.g. a protein created/ formed through the joining of two or more genes or gene domains that originally coded for separate proteins or protein domains. Translation of this "fusion gene" preferably results in a single or multiple polypeptides with functional properties derived from each of the original proteins. As used herein, the term "fusion protein" is also intended to encompass a fusion of two different domains of the same protein
15 from which said two different domains derive. For example, as will be explained in more detail below, an N-terminal domain of the SARS-Cov-2 spike protein can be fused to the RBD component of the SARS-Cov-2 spike protein to provide the "fusion protein" as described herein. The term "fusion protein" may be used synonymously with the term "protein" (e.g. where the latter comprise the first (RBD) antigen and scaffold polypeptide),
20 "multi-domain protein" or "protein comprising two or more polypeptide domains" (e.g. wherein the two or more domains comprise the first (RBD) antigen and scaffold polypeptide).

In one embodiment, the scaffold polypeptide is of 100-400 amino acids in length; preferably 150-350 amino acids in length; more preferably 175-325 amino acids in length.

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In one embodiment, the scaffold polypeptide is 10-50 kDa; preferably 15-45 kDa; more preferably 20-40 kDa.

For example, the scaffold polypeptide may present the first antigen for capturing and
30 immobilising anti-SARS-Cov-2 antibody in the sample. The term "immobilising" as used herein may refer to binding with an affinity (measured by the dissociation constant: K_d) of at least 10^{-4} M, e.g. at least 10^{-5} M, 10^{-6} M, 10^{-7} M, 10^{-8} M or 10^{-9} M. Alternatively, or additionally, "immobilising" as used herein may refer to binding with an affinity (measured by way of the association constant K_a) of 10^6 M, e.g. at least 10^7 M or at least 10^8 M.

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The scaffold polypeptide may be any polypeptide suitable for (i) fusion to an RBD antigen and (ii) binding to a solid-phase support. Examples of suitable scaffold polypeptides(s) include Keyhole Limpet Hemocyanin (KLH), Bovine Serum Albumin (BSA), and cationized BSA, or a fragment thereof (e.g. a fragment of 100-400 amino acids in length; preferably
5 150-350 amino acids in length; more preferably 175-325 amino acids in length).

In a preferable embodiment, the scaffold polypeptide comprises (or consists of) a polypeptide sequence corresponding to an N-terminal domain of the SARS-Cov-2 spike protein (SEQ ID NO. 1, NCBI Reference Sequence: YP_009724390.1), for example amino acid residues 1-
10 318, amino acid residues 2-318, amino acid residues 15-318, amino acid residues 50-250, or amino acid residues 100-200 of the SARS-Cov-2 spike protein. The scaffold may comprise (or consist of) amino acid residues 1-318 of the SARS-Cov-2 spike protein. The scaffold may comprise (or consist of) amino acid residues 2-318 of the SARS-Cov-2 spike protein. The scaffold may comprise (or consist of) amino acid residues 14-318 of the SARS-Cov-2 spike
15 protein. The scaffold may comprise (or consist of) amino acid residues 16-318 of the SARS-Cov-2 spike protein. Preferably, the scaffold comprises (or consists of) amino acid residues 15-318 of the SARS-Cov-2 spike protein (e.g. the scaffold preferably comprises or consists of SEQ ID NO.: 4). The invention also embraces variants of said sequences having less than 100% sequence identity thereto e.g. albeit with the proviso that said variant (scaffold
20 polypeptide) can immobilise the fusion protein to the solid-phase support and present the first antigen away from the solid-phase support. For example, the scaffold polypeptide may comprise or consist of a sequence having at least 75%, 80%, 85%, 90% or 95% (e.g. 90% or 95%) sequence identity with SEQ ID NO.: 4.

25 The first antigen may be covalently fused to the scaffold polypeptide, for example the scaffold polypeptide may be fused at the N-terminus or C-terminus of the antigen (preferably the N-terminus).

The "receptor-binding domain" (RBD) is a component (e.g. region or domain) of the S1 subunit of spike protein (S), a structural protein of the SARS-CoV-2 virus. The RBD component may be referred to as amino acid residues 319-541 of the SARS-Cov-2 spike protein (e.g. SEQ ID NO. 3). The "receptor-binding domain polypeptide" is a polypeptide having a sequence of the RBD component, and may, for example, comprise the whole sequence or a truncated sequence of the RBD, such as amino acid residues 319-530 (e.g.
30 with the proviso that the polypeptide retains an epitope for binding to a SARS-Cov-2 antibody). The invention also embraces variants of said sequences having less than 100%

sequence identity thereto e.g. albeit with the proviso that said variant can bind an anti-SARS-CoV-2 antibody. For example, the RBD antigen component of the first antigen may comprise or consist of a sequence having at least 75%, 80%, 85%, 90% or 95% (e.g. 90% or 95%) sequence identity with SEQ ID NO.: 3.

5

In more detail, SARS-CoV-2 contains four structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N), proteins. S protein plays a role in viral attachment, fusion and entry, and it serves as a target for development of antibodies. The S protein mediates viral entry into host cells by first binding to a host receptor, in this case ACE2,
10 through the receptor-binding domain (RBD) in the S1 subunit, and then fusing the viral and host membranes through the S2 subunit.

The first antigen may comprise (or consist of) amino acid residues 319-541, amino acid residues 319-530, or amino acid residues 350-500 of the SARS-Cov-2 spike protein
15 (preferably amino acid residues 319-530, or amino acid residues 350-500 of the SARS-Cov-2 spike protein). In a preferable embodiment, the first antigen comprises (or consists of) amino acid residues 319-530 of the SARS-Cov-2 spike protein (e.g. the first antigen preferably comprises or consists of SEQ ID NO.: 5). The invention also embraces variants of said sequences having less than 100% sequence identity thereto e.g. albeit with the proviso
20 that the variant (first antigen) can bind an anti-SARS-CoV-2 antibody. For example, the first antigen may comprise or consist of a sequence having at least 75%, 80%, 85%, 90% or 95% (e.g. 90% or 95%) sequence identity with SEQ ID NO.: 5.

The fusion protein may comprise (or consist of) amino acid residues 15-530, amino acid
25 residues 50-450, or amino acid residues 100-400 of the SARS-Cov-2 spike protein.

In a preferable embodiment, the fusion protein comprises (or consists of) amino acid residues 15-530 of the SARS-Cov-2 spike protein (e.g. the fusion protein preferably comprises or consists of SEQ ID NO.: 2). For example, in a preferable embodiment, the first
30 antigen is amino acid residues 319-530 of the SARS-Cov-2 spike protein, and the scaffold polypeptide is amino acid residues 15-318 of the SARS-Cov-2 spike protein, such that the fusion protein comprises (or consists of) amino acid residues 15-530 of the SARS-Cov-2 spike protein (SEQ ID NO.: 2). The invention also embraces variants of said sequences having less than 100% sequence identity thereto e.g. albeit with the proviso that the scaffold
35 protein of said variant (fusion protein) can immobilise the fusion protein to the solid-phase support and present the first antigen away from the solid-phase support, and the first (RBD)

antigen component of said variant (fusion protein) can bind an anti-SARS-CoV-2 antibody. For example, the first antigen may comprise or consist of a sequence having at least 75%, 80%, 85%, 90% or 95% (e.g. 90% or 95%) sequence identity with SEQ ID NO.: 2.

5 The first antigen may be amino acid residues 319-530 of the SARS-Cov-2 spike protein, and the scaffold polypeptide may be amino acid residues 1-318 of the of the SARS-Cov-2 spike protein, such that the fusion protein comprises (or consists of) amino acid residues 1-530 of the SARS-Cov-2 spike protein.

10 The fusion protein may comprise (or consist of) amino acid residues 1-530 of the SARS-Cov-2 spike protein. The fusion protein may comprise (or consist of) amino acid residues 2-530 of the SARS-Cov-2 spike protein.

The skilled would understand that the S1 polypeptide comprises an N-terminal cleavable
15 signal peptide, e.g. amino acids 1-14 of S1. Thus, any reference to a polypeptide herein (e.g. the fusion protein) that comprises the N-terminal signal peptide is intended to encompass said polypeptide in the absence of said signal peptide.

The fusion protein may comprise (or consist of) SEQ ID NO.: 6. The fusion protein may
20 preferably comprise (or consist of) SEQ ID NO.: 7.

The first antigen or fusion protein (preferably fusion protein) may comprise additional N-terminal or C-terminal residues as a result of the expression and purification methodology. The first antigen or fusion protein (preferably fusion protein) may comprise an N-terminal or
25 C-terminal (preferably C-terminal) fusion of SEQ ID NO.: 11 (e.g. TwinStrep tags). The first antigen may comprise (or consist of) SEQ ID NO.: 9. The first antigen as it is expressed may comprise (or consist of) SEQ ID NO.: 8 (e.g. said sequence including the N-terminal signal peptide).

30 The labelled second antigen preferably lacks the scaffold polypeptide (e.g. lacks the scaffold polypeptide of the fusion protein immobilised to the solid-phase support). A labelled second antigen that "lacks the scaffold polypeptide" may comprise $\leq 10\%$, $\leq 5\%$, or $\leq 2\%$ of the sequence of the scaffold polypeptide. Preferably, a labelled second antigen that "lacks the scaffold polypeptide" lacks any sequence of the scaffold polypeptide (e.g. comprises 0% of
35 the sequence of the scaffold polypeptide).

In a preferable embodiment, the labelled second antigen is present in a fluid phase.

The labelled second antigen may comprise (or consist of) amino acid residues 319-541, amino acid residues 319-530, or amino acid residues 350-500 of the SARS-Cov-2 spike protein (conjugated to a label). In a preferable embodiment, the second antigen comprises (or consists of) amino acid residues 319-541 of the SARS-Cov-2 spike protein (e.g. the second antigen preferably comprises or consists of SEQ ID NO.: 3), conjugated to a label. The invention also embraces variants of said sequences having less than 100% sequence identity thereto e.g. albeit with the proviso that said variant (second antigen) can bind an anti-SARS-CoV-2 antibody. For example, the second antigen component of the first antigen may comprise or consist of a sequence having at least 75%, 80%, 85%, 90% or 95% (e.g. 90% or 95%) sequence identity with SEQ ID NO.: 3.

The second antigen may comprise (or consist of) SEQ ID NO.: 9, conjugated to a label. The second antigen as it is expressed may comprise (or consist of) SEQ ID NO.: 8 (e.g. said sequence including the N-terminal signal peptide), conjugated to a label.

The labelled second antigen may comprise additional N-terminal or C-terminal residues as a result of the expression and purification methodology. The second antigen may comprise an N-terminal or C-terminal (preferably C-terminal) fusion of SEQ ID NO.: 11 (e.g. TwinStrep tags). The second (RBD) antigen may comprise (or consist of) SEQ ID NO.: 7. The second antigen as it is expressed may comprise (or consist of) SEQ ID NO.: 6 (e.g. said sequence including the N-terminal signal peptide).

The first antigen (e.g. as it is expressed) may be referred to as an S1 construct, and preferably takes amino acid residues 1-530 of the SARS-Cov-2 spike protein (e.g. the expression construction may comprise amino acid residues 1-530 of the SARS-Cov-2 spike protein). An advantage of this particular S1 construct is its high level of expression. The first antigen as secreted (from a cell) may comprise or consist of amino acid residues 15-530 of the SARS-Cov-2 spike protein, e.g. due to cleavage of amino acid residues 1-14 (the signal peptide).

Additionally, the use of hybrid double antigens (e.g. amino acid residues 1-530 or 15-530 of the SARS-Cov-2 spike protein for the first antigen, and amino acid residues 319-541 of the SARS-Cov-2 spike protein for the labelled second antigen) allows purification of the two different antigens separately, optionally providing excipients in the solid-phase protein which

differ from (e.g. potential) excipients in the fluid-phase protein (a feature which is not present in a normal/ homologous or non-hybrid double antigen assay with identical proteins). Thus, the first and second (e.g. recombinant) antigens may be derived from different sources. For example, the first antigen may be derived from a first expression and purification process, and the second antigen may be derived from a second (different) expression and purification process. In other words, the solid-phase and the second labelled antigen components differ in their sourcing and are of different origin, for example sharing only the epitope against which the test is designed to detect antibody.

10 In a preferable embodiment, the first and/or (preferably and) second antigen is a recombinant protein.

A step of "removing labelled second antigen that is not bound to said complex" may be referred to as a wash step (e.g. in which a wash buffer is used to wash away free labelled second antigen, thus ensuring visualisation of the label in subsequent steps allows visualisation of second antigen that is bound to immobilised SARS-Cov-2 antibody). Methods of the invention may comprise one or more additional wash steps. In one embodiment, a step of contacting said complex with a labelled second antigen (e.g. step c)) is preceded by a step of removing antibodies (and any other component present in the sample) that is not bound to the first antigen (e.g. that does not form a complex of first antigen and antibody).

The second antigen is labelled to allow visualisation of the detection of (immobilised) antibody. Any antigen label may in principle be employed. For example, the label may itself provide an observable/ detectable signal (e.g. visible dye), or it may require an activation partner (e.g. horseradish peroxidase (HRPO) plus substrate). Suitably, said label is conjugated directly to the antigen (e.g. by chemical conjugation or as a fusion protein).

Examples of suitable labels include detectable labels such as radiolabels or fluorescent or coloured molecules, enzymatic markers or chromogenic markers – e.g. dyes that provide a visible colour change upon binding of the detection antibody to an antigen. By way of example, the label may be fluorescein-isothiocyanate (FITC), R-phycoerythrin, Alexa 532, CY3 or digoxigenin. The label may be a reporter molecule, which is detected directly, such as by detecting its fluorescent signal, or by exposure of the label to photographic or X-ray film. Alternatively, the label is not directly detectable, but may be detected, for example, in a

two-phase system. An example of indirect label detection is binding of an antibody to the label.

In a preferable embodiment, the labelled (second) antigen is labelled with an HRPO.
5 Suitably, said HRPO labelled antigen is detected by means of an activation partner, e.g. a substrate that, when oxidized by HRP using hydrogen peroxide as the oxidizing agent, yields a characteristic colour change. Said activation partner may be one or more substrate selected from 3,3',5,5'-Tetramethylbenzidine (TMB), 3,3'-Diaminobenzidine (DAB), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), o-phenylenediamine
10 dihydrochloride (OPD), 3-amino-9-ethylcarbazole (AEC), AmplexRed, Homovanillic acid or Luminol. Preferably, said activation partner is the substrate TMB.

Thus, the presence or absence of labelled second antigen bound to said complex is detected via a signal emitted from a label. Preferably, the presence of labelled second antigen bound
15 to said complex is confirmed when the signal detected is greater than a signal detected in a control assay, wherein the control assay comprises contacting the solid-phase support with a control (e.g. seronegative) sample lacking SARS-Cov-2 antibody (e.g. lacking SARS-Cov-2 antibodies that bind to the first antigen).

20 The signal detected in the control assay may be determined either prior to carrying out a method of the invention or at the same time as carrying out a method of the invention (preferably at the same time).

The skilled person understands that where the methods of the invention comprise a
25 comparison step between two assays (e.g. between a "test assay" and a "control assay") that conditions (e.g. assay conditions during the method) should be kept consistent. For example, the amount of sample used should be the same, as should the time conditions, etc. Where a comparison is made between two samples herein, suitably the samples are equivalent. For example, the samples being compared may be the same sample types (e.g.
30 blood) and subjected to the same processing steps. Preferably, the samples are obtained from the same species (e.g. human).

Any solid-phase support may in principle be employed. For example, conventional multi-well plates and lateral flow devices. Preferably, the solid-phase support is a multi-well plate (such
35 as a 96 well plate).

In one embodiment, the sample is from a subject, typically an animal, most preferably a human. The terms “subject”, “individual” and “patient” are used interchangeably herein.

5 In such an embodiment, the sample is typically selected from blood (e.g. a dried blood spot), plasma, saliva, serum, sputum, urine, cerebral spinal fluid, semen, cells, a cellular extract, a tissue sample, a tissue biopsy, a stool sample, a swab from any body site and/or one or more organs; typically blood, serum, urine, saliva and/or organ(s).

10 In one embodiment, the sample is blood (e.g. plasma or serum). For example, the sample may be EDTA plasma (e.g. plasma treated with Ethylenediaminetetraacetic acid, for example to provide an EDTA concentration of about 1.5mg/mL plasma). In a preferable embodiment, the sample is serum.

15 In one embodiment, a sample may be processed to isolate an antibody from a sample.

Advantageously, the sample may be from a subject that was vaccinated against SARS-CoV-2. As such, the present assay may find utility in evaluating a person's level of immunity or protection from COVID-19 at any time post-vaccination.

20 In one embodiment, the method is for diagnosing the presence or absence of a viral infection with SARS-Cov-2 in a subject. In one embodiment, the presence of labelled complex indicates viral infection (or past infection) of a subject by SARS-Cov-2, and the absence of labelled complex indicates no viral infection (or no past infection) of a subject by SARS-Cov-2.

25 In one embodiment, upon identification of said infection in the subject, the subject is provided with an appropriate treatment or therapy. Said treatment or therapy may be an effective dosage of a medicament to relieve symptoms of infection (e.g. acetaminophen and/or ibuprofen).

30 The invention is highly suited to a number of important applications in the detection of antibody to SARS-Cov-2. For example, the invention can help support local surveillance of antibody to SARS-Cov-2, in the animal reservoir and in humans. This enables rapid targeting of vector control measures including the prediction of emerging SARS-Cov-2 outbreaks
35 before they enter (and/or spread throughout) the human population.

The invention may allow the screening of subject samples (such as blood/ serum), and can therefore be used to test (retrospectively) for past infection by SARS-Cov-2. The invention can also be used in screening travellers returning from an area affected (or an area suspected of being affected) with SARS-Cov-2. Not least, the invention may be used to assess the success of the vaccination programme by evaluating the presence (or absence) of anti-SARS-Cov-2 antibodies in the vaccinated population as time passes. Depending on whether this informs a decrease in antibodies or a stable level of antibodies over time will allow for the assessment of whether continued (e.g annual) vaccination programmes are necessary.

The invention is also suitable for confirming diagnosis of past infection of a subject with SARS-Cov-2 *e.g.* for confirming whether a subject suspected of being infected with a SARS-Cov-2 (e.g. due to the presence of symptoms or positive result in an alternative test for SARS-Cov-2) was indeed infected with SARS-Cov-2. In one embodiment, where the sample is isolated from the patient during a window (time-period) subsequent to acute infection and at a time that antibody is expected to be present, the presence of labelled complex confirms viral infection of a subject by SARS-Cov-2, and the absence of labelled complex confirms no viral infection of a subject by SARS-Cov-2.

Neutralising antibodies, IgM and IgG, for SARS-CoV-2 target nucleocapsid (N) and spike proteins (S). The RBD component of S1 in particular has been associated with a role in spike protein-induced viral attachment, fusion and entry, such that the RBD component is now known to represent a key target for neutralising antibodies. Thus, the present invention (directed to detecting antibody to RBD of the spike protein of SARS-Cov-2) may advantageously find utility in detecting the presence or absence of neutralising antibodies. In one embodiment, the detection of antibody to RBD is indicative of the presence of neutralising antibody and the absence of antibody to RBD is indicative of the absence of neutralising antibody. In one embodiment (where the sample is from a patient), the presence of labelled complex is indicative of patient immunity to SARS-Cov-2, and the absence of labelled complex is indicative of the absence of patient immunity to SARS-Cov-2.

The methods of the present invention find utility in detecting either recent (e.g. new) or historic viral infection of a subject by SARS-Cov-2. Discriminating between recent and historic infection may be achieved by determining the type of antibody detected by a method of the invention. Thus, in some embodiments a method of the invention may comprise a step of determining the type of antibody present in a labelled antigen-antibody complex.

In one embodiment the detected antibody is an IgM antibody. IgM is typically the first antibody to appear in response to exposure of a subject to an infection, therefore detection of an IgM antibody may be indicative of a recent (e.g. new, for example within 1 month) infection by a SARS-Cov-2. Detection of IgM indicated an individual may still be infected or has recently recovered. Others, IgA and IgE, are not found or only found in small amounts in the blood.

In another embodiment the detected antibody is an IgG antibody. IgG antibodies remain in the blood after an infection has passed (may take time to form). Detection of an IgG antibody may be indicative of a historic (e.g. more than 1 month or 1-2 months after infection) infection by a SARS-Cov-2. Where the sample is obtained from a vaccinated subject, detection of an IgG antibody may be indicative of vaccine protection (e.g. immunity) from SARS-Cov-2 in the subject.

In one embodiment, a method of the invention further comprises a step of contacting said complex with a third antigen from a non-SARS-Cov-2 species, wherein the binding of said third antigen thereto suppresses (e.g. blocks) any inherent antigenic binding cross-reactivity towards the non-SARS-Cov-2 species; wherein the second and third antigens are inter-species homologs of the same polypeptide/ protein (e.g. inter-species homologs of RBD). This step may be referred to as a 'quenching' step. More than one antigen from a non-SARS-Cov-2 species may be employed, in which case said antigen may be referred to as a third, fourth, fifth etc. antigen.

The term "suppresses" (or "suppressing") embraces both reduction of and/or complete blocking of any inherent antigenic binding cross-reactivity towards the non-SARS-Cov-2 species (e.g. reduction of and/or complete blocking of the availability of antibody binding domains (on the immobilised antibody) capable of binding to the third antigen).

In a preferable embodiment, said third antigen is an unlabelled antigen.

Advantageously, the capture/ contacting step (e.g. step a) leads to immobilisation of all antibodies capable of binding to SARS-Cov-2 RBD. As such, the quenching step can then be adapted based on user requirements by choosing any third antigen desired.

The second and third antigens are inter-species homologs of the same polypeptide/ protein, and typically demonstrate significant common antibody binding cross-reactivity. Said second

and third antigens typically demonstrate at least 60%, at least 70%, at least 80%, or at least 90% sequence identity to each other along their entire length (preferably at least 90% sequence identity to each other along their entire length). Reference to a polypeptide/ protein herein embraces fragments thereof having significant common antibody binding cross-reactivity.

The quenching step may comprise challenge of the immobilised antibody with third antigens from two or more non-SARS-Cov-2 species that are different from the SARS-Cov-2 to be detected. Thus, the quenching step may comprise challenge with two, three, four, or more third antigens from two, three, four, or more non-SARS-Cov-2 virus species. Should two or more second antigens from different non-SARS-Cov-2 species be employed, said two or more second antigens are inter-species homologs of the same polypeptide/ protein. Said two or more second antigens typically demonstrate at least 60%, at least 70%, at least 80%, or at least 90% sequence identity to each other along their entire length. Reference to polypeptide/ protein herein embraces fragments thereof having significant common antibody binding cross-reactivity.

The non-SARS-Cov-2 species may be selected from coronavirus selected from 229E (alpha coronavirus), NL63 (alpha coronavirus), OC43 (beta coronavirus), HKU1 (beta coronavirus) and SARS-Cov-1. For example, the non-SARS-Cov-2 species may be selected from coronavirus selected from 229E (alpha coronavirus), NL63 (alpha coronavirus), OC43 (beta coronavirus), and HKU1 (beta coronavirus).

In one embodiment, reference to said third antigen embraces two or more (e.g. 2, 3, 4 or 5) third antigens from two or more (e.g. 2, 3, 4 or 5) non-SARS-Cov-2 species. In such embodiments, said antigens may be referred to as third, fourth, fifth etc. antigens.

The quantity of said third antigen used in said contacting (e.g. quenching) step may be between about 0.25-2 µg, more preferably 0.5-1 µg. For example, the third antigen may be present at a concentration of 5-10 µg/mL, with 100 µl being used in the contacting step.

The contacting steps of a method of the invention may be carried out simultaneously or sequentially. In other words, the labelled second antigen and third (or further) antigen may be added simultaneously or sequentially.

Preferably the labelled second antigen and third (or further) antigens are added simultaneously. Advantageously, such simultaneous challenging allows for the direct competition between third antigen (e.g. cold, unlabelled third antigen) and labelled second antigen for antibody binding sites. This may allow for competition (e.g. fluid phase competition) for antibody binding sites, in which the third antigen out-competes the labelled second antigen for binding to (undesired) immobilised antibody having inherent antigenic binding cross-reactivity towards the non-SARS-Cov-2 species.

In one embodiment, a method of the invention may further comprise recording the output of said method on a data readable format.

S1 mutants having lower/ ablated binding affinity for said tetrapyrrole

Further embodiments of the invention provide an improved “antigen reagent” for use in serology tests, in which the Sars-Cov-2 spike protein (S1) sequence has been modified, thus significantly improving the performance of existing tests that otherwise use the “wild-type” S1 sequence. In summary, the modified S1 is better equipped to bind antibodies than a corresponding wild-type sequence. Thus, not only have the inventors identified a route to improvement of existing kits, but have provided the solution via modified S1.

In more detail, the present invention is predicated on the surprising finding that the SARS-CoV (more particularly SARS-CoV-2) spike protein binds biliverdin and bilirubin, tetrapyrrole products of haem metabolism, with nanomolar affinity. As explained in the Examples, the inventors have identified a tetrapyrrole interaction pocket within a deep cleft on the spike N-terminal domain (NTD) and have found the presence of biliverdin significantly dampened the reactivity of SARS-CoV-2 spike with immune sera and inhibited a subset of NTD-specific neutralising antibodies. The inventors’ work indicates that the virus co-opts the haem metabolite for the evasion of humoral immunity via allosteric shielding of a sensitive epitope. This is notable, as the spike protein, most particularly the S1 subunit (in which the tetrapyrrole interaction pocket has been identified) is regularly used as an antigen component in serology tests, as well as a key immunogenic antigen in vaccine compositions. With regard to the former, tetrapyrrole compounds (e.g. biliverdin) can be found in the blood, such that using blood/ plasma samples in serology tests will actually introduce tetrapyrrole compound(s) to the system, dampening interaction between S1 antigen (test reagent) and antibodies in the sample to be detected (thus inhibiting detection). Tetrapyrrole compounds (e.g. biliverdin) are also produced by cells in tissue culture conditions, such that

recombinantly manufactured S1 may (at least partially) have 'masked' epitopes due to tetrapyrrole binding.

As outlined in detail in the Examples (particularly at Example 4), the tetrapyrrole binding pocket has been mapped to the following amino acid residues of S1 (SEQ ID NO: 12): N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and L226. Closer interactions between biliverdin and S1 are seen with SARS CoV-2 S1 amino acid residues N99, W104, I119, N121, V126, F175, M177, R190, F192, H207, and L226.

The strongest interactions were seen with SARS-CoV-2 S1 residues N121, R190 and H207. Taking these residues thus as exemplary modification sites to disrupt the binding pocket, modified S1 'mutant' polypeptides were generated, having highly conservative substitutions at these positions (N121Q, R190K and H207A). The mutant S1 polypeptides demonstrated significantly reduced affinity for biliverdin. Noting that even conservative substitutions suppress the interaction between biliverdin and S1 (and lead to improved performance in serology tests as will be described below) the skilled person would appreciate that any other (e.g. less conservative) amino acid change also provides for suppressed biliverdin binding.

Furthermore, because tetrapyrrole binding pocket contains a histidine residue (H207), the Applicant rationalised that the interaction may be pH-dependent. They found that treating the S1-biliverdin complex with acids allowed purification of biliverdin-depleted spike antigens. However, because biological samples (e.g. human serum or plasma) contain variable amounts of biliverdin and bilirubin, the spike mutant may be preferable for the use in serological assays.

Unexpectedly, replacing the 'wild-type' S1 of existing immunoassays with such mutant S1 significantly improved the performance of these tests, particularly in terms of improving reactivity between the antigen (S1) and antibodies in human sera (see Example 4). These improved preparations of the spike protein antigen allow more sensitive detection of the antibodies. This is particularly important when antibody levels are low, for example, soon after exposure to the virus or vaccination.

It is remarkable that a small molecule with a footprint of 370 Å², corresponding to only ~0.9% of solvent-exposed surface (per spike monomer), competes with a considerable fraction of spike-specific serum antibody population. It is thus equally remarkable that suppressing the S1-tetrapyrrole interaction in immunoassays improves the performance of such assays.

The invention thus provides a modified S1 demonstrating lower affinity for a tetrapyrrole compound (e.g. lower epitope masking due to tetrapyrrole binding) compared to an unmodified equivalent.

5

Thus, the RBD/ S1 component of the assay described above may be represented a modified SARS coronavirus (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide (having a modified amino acid sequence relative to a wild-type S1 sequence, SEQ ID NO: 12) described herein. Thus, the “fusion protein” component of the above described assay may
10 be represented by said modified SARS-CoV-2 S1.

15

By employing the modified version, for example, epitopes of the modified S1 are not (or are less) suppressed due to binding of tetrapyrroles that may be present in the test sample (typically a blood sample). In a most surprising development, incorporating the modified S1 polypeptide of the present invention improves the performance of the serology tests significantly, and more particular promote signal such that the risk of a false-negative test is reduced.

20

With regard to the “DABA” (e.g. hybrid DABA) assay, it is believed that the antibodies to the N-terminal domain (NTD) of S1 are not detected. Instead, the NTD helps by “presenting” the RBD to antibodies (as described in more detail above). It is believed that, in the presence of biliverdin, the hydrophobic core of the NTD is hidden (by biliverdin itself and by loop structures of the S1). In the absence of biliverdin binding, it is believed the loops open up (like flower petals) exposing the hydrophobic residues inside. It is believed this allows the
25 NTD of the modified S1 polypeptide of the invention to “stick” to a solid-phase support (e.g. plastic of plate) and present the RBD for recognition of anti-SARS-CoV (e.g. SARS-CoV-2) antibodies.

30

Thus, in any aspect described herein, the fusion protein (e.g. protein) may be represented by a modified SARS-CoV (e.g. SARS-CoV-2) S1 as described herein.

35

In any aspect described herein, reference to the “fusion protein” may be replaced with reference to “a modified SARS-CoV (e.g. SARS-CoV-2) S1 as described herein”. In other words, step a. (which above reads “*contacting the sample with a solid-phase support having a fusion protein immobilised thereto, said fusion protein comprising a first antigen and a scaffold polypeptide, wherein the first antigen is a SARS-Cov-2 receptor binding domain*”

polypeptide that binds anti-SARS-Cov-2 antibody, and the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support") may instead read as follows:

- a. contacting the sample with a solid-phase support having a first antigen
5 immobilised thereto,
- i. wherein the first antigen comprises a modified SARS-CoV (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide as described herein.

All embodiments described above apply equally to this aspect employing the modified S1.

10

Thus, in one aspect, the invention provides a method for detecting the presence or absence of SARS-CoV (e.g. SARS-CoV-2) antibodies in a sample, the method comprising:

- a. contacting the sample with a solid-phase support having a first antigen
immobilised thereto,
- 15 i. wherein the first antigen comprises a modified SARS-CoV (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide as described herein;
- b. allowing SARS-CoV (e.g. SARS-CoV-2) antibodies present in the sample to bind to the modified S1 polypeptide, thereby forming a complex of modified S1 polypeptide and SARS-CoV (e.g. SARS-CoV-2) antibody;
- 20 c. contacting said complex with a labelled second antigen, wherein said labelled second antigen comprises a SARS-CoV (e.g. SARS-CoV-2) spike protein polypeptide (preferably wherein said labelled second antigen comprises or consists of a SARS-CoV receptor binding domain polypeptide, more preferably wherein said labelled second antigen comprises or consists of a sequence of
25 SEQ ID No.: 3) that binds SARS-CoV (e.g. SARS-CoV-2) antibody;
- d. allowing said labelled second antigen to bind to SARS-CoV (e.g. SARS-CoV-2) antibody present in the sample;
- e. removing labelled second antigen that is not bound to said complex; and
- f. detecting the presence of labelled second antigen bound to said complex;
- 30 wherein the presence of labelled complex indicates the presence SARS-CoV (e.g. SARS-CoV-2) antibody in the sample, and wherein the absence of labelled complex indicates the absence of SARS-CoV (e.g. SARS-CoV-2) antibody in the sample.

In other words, an aspect of the invention provides a method for detecting the presence or
35 absence of SARS-CoV-2 antibodies in a sample, the method comprising:

a. contacting the sample with a solid-phase support having a first antigen immobilised thereto, wherein the first antigen comprises a modified SARS-CoV-2 spike protein S1 subunit (S1) polypeptide having a modified amino acid sequence relative to a wild-type S1 sequence (SEQ ID NO: 12);

- 5 i. the modified S1 polypeptide comprising at least one amino acid residue change located within a tetrapyrrole binding pocket for binding to a tetrapyrrole compound;
- ii. wherein said at least one amino acid change is at a location on the modified S1 polypeptide sequence that corresponds to an amino acid location, of wild-type S1 (SEQ ID NO: 12), comprising: N99, I101, 10 W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and/or L226 (more particularly N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, and/or H207; even more particularly N99, N121, F175, M177, F194, and/or I203); and
- 15 iii. wherein the modified S1 polypeptide demonstrates lower binding affinity for said tetrapyrrole compound compared with wild-type S1;

b. allowing SARS-CoV-2 antibodies present in the sample to bind to the modified S1 polypeptide, thereby forming a complex of modified S1 polypeptide and SARS-CoV-2 antibody;

20 c. contacting said complex with a labelled second antigen, wherein said labelled second antigen comprises a SARS-CoV-2 receptor binding domain polypeptide that binds SARS-CoV-2 antibody;

d. allowing said labelled second antigen to bind to SARS-CoV-2 antibody present in the sample;

25 e. removing labelled second antigen that is not bound to said complex; and

f. detecting the presence of labelled second antigen bound to said complex;

wherein the presence of labelled complex indicates the presence SARS-CoV-2 antibody in the sample, and wherein the absence of labelled complex indicates the absence of SARS-CoV-2 antibody in the sample.

30

As per the earlier described aspects herein, the N-terminal domain (i.e. the sequence that is N-terminal to the RBD domain) of the modified S1 may help 'present' RBD for detection by antibodies. In other words, the modified S1 may comprise a first antigen and a scaffold polypeptide (e.g. the S1 sequence that is N-terminal to the first antigen), wherein the first 35 antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2

antibody, and the scaffold polypeptide immobilises the modified S1 to the solid-phase support and presents the first antigen away from the solid-phase support.

The structural features of the modified S1 will now be described in more detail.

5

In one aspect, the invention provides a modified SARS coronavirus (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide, having a modified amino acid sequence relative to a wild-type S1 sequence (SEQ ID NO: 12);

10

a. the modified S1 polypeptide comprising at least one amino acid residue change located within a tetrapyrrole binding pocket for binding to a tetrapyrrole compound;

b. wherein said at least one amino acid change is at a location on the modified S1 polypeptide sequence that corresponds to an amino acid location, of wild-type S1 (SEQ ID NO: 12), comprising:

15

i. N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and/or L226 (more particularly N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, and/or H207; even more particularly N99, N121, F175, M177, F194, and/or I203); and

20

c. wherein the modified S1 polypeptide demonstrates lower binding affinity for said tetrapyrrole compound compared with wild-type S1.

25

As the presence of a methionine residue at position 1 of a sequence having such methionine (e.g. as a start codon), such as SEQ ID NO: 12, is optional, the skilled person will take the presence/absence of the methionine residue into account when determining amino acid residue numbering. For example, where SEQ ID NO: 12 includes a methionine, the position numbering will be as defined above (e.g. N99 will be N99 of SEQ ID NO: 12). Alternatively, where the methionine is absent from SEQ ID NO: 12 the amino acid residue numbering should be modified by -1 (e.g. N99 will be N98 of SEQ ID NO: 52). Similar considerations apply when the methionine at position 1 of the other polypeptide sequences described herein is present/absent, and the skilled person will readily determine the correct amino acid residue numbering using techniques routine in the art.

30

In one aspect, the invention provides modified SARS coronavirus (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide, having a modified amino acid sequence relative to a wild-type S1 sequence (SEQ ID NO: 12);

35

- a. the modified S1 polypeptide comprising at least one amino acid residue change located within a tetrapyrrole binding pocket for binding to a tetrapyrrole compound;
- b. wherein said at least one amino acid change is at a location on the modified S1 polypeptide sequence that corresponds to an amino acid location, of wild-type S1 (SEQ ID NO: 12), comprising:
 - i. N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and L226 (more particularly N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, and H207; even more particularly N99, N121, F175, M177, F194, and I203); and
- c. wherein the modified S1 polypeptide demonstrates lower binding affinity for said tetrapyrrole compound compared with wild-type S1.

In one aspect, the invention provides modified SARS coronavirus (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide, having a modified amino acid sequence relative to a wild-type S1 sequence (SEQ ID NO: 12);

- a. the modified S1 polypeptide comprising at least one amino acid residue change located within a tetrapyrrole binding pocket for binding to a tetrapyrrole compound;
- b. wherein said at least one amino acid change is at a location on the modified S1 polypeptide sequence that corresponds to a polypeptide location, of wild-type S1 (SEQ ID NO: 12), comprising:
 - i. N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and/or L226 (more particularly N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, and/or H207; even more particularly N99, N121, F175, M177, F194, and/or I203); and
- c. wherein the modified S1 polypeptide demonstrates lower binding affinity for said tetrapyrrole compound compared with wild-type S1.

In one aspect, the invention provides modified SARS coronavirus (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide, having a modified amino acid sequence relative to a wild-type S1 sequence (SEQ ID NO: 12);

- a. the modified S1 polypeptide comprising at least one amino acid residue change located within a tetrapyrrole binding pocket for binding to a tetrapyrrole compound;

b. wherein said at least one amino acid change is at a location on the modified S1 polypeptide sequence that corresponds to a polypeptide location, of wild-type S1 (SEQ ID NO: 12), comprising:

i. N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and L226 (more particularly N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, and H207; even more particularly N99, N121, F175, M177, F194, and I203); and

c. wherein the modified S1 polypeptide demonstrates lower binding affinity for said tetrapyrrole compound compared with wild-type S1.

In one aspect, the invention provides modified SARS coronavirus (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide, having a modified amino acid sequence relative to a wild-type S1 sequence (SEQ ID NO: 12);

a. the modified S1 polypeptide comprising at least one amino acid residue change located within a tetrapyrrole binding pocket for binding to a tetrapyrrole compound;

b. wherein said at least one amino acid change is at a position on the modified S1 polypeptide sequence that corresponds to an amino acid position, of wild-type S1 (SEQ ID NO: 12), comprising:

i. N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and/or L226 (more particularly N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, and/or H207; even more particularly N99, N121, F175, M177, F194, and/or I203); and

c. wherein the modified S1 polypeptide demonstrates lower binding affinity for said tetrapyrrole compound compared with wild-type S1.

In one aspect, the invention provides modified SARS coronavirus (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide, having a modified amino acid sequence relative to a wild-type S1 sequence (SEQ ID NO: 12);

a. the modified S1 polypeptide comprising at least one amino acid residue change located within a tetrapyrrole binding pocket for binding to a tetrapyrrole compound;

b. wherein said at least one amino acid change is at a position on the modified S1 polypeptide sequence that corresponds to an amino acid position, of wild-type S1 (SEQ ID NO: 12), comprising:

- i. N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and L226 (more particularly N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, and H207; even more particularly N99, N121, F175, M177, F194, and I203); and
- 5 c. wherein the modified S1 polypeptide demonstrates lower binding affinity for said tetrapyrrole compound compared with wild-type S1.

The amino acid change occurs within a “tetrapyrrole binding pocket” (of the modified S1 polypeptide) relative to the wild-type S1 sequence (SEQ ID NO: 12). By “binding pocket”, it is
10 meant herein a region of the S1 sequence which comprises one or more amino acids which are the contact points (e.g. via hydrogen-bond, van der Waals interactions, and/or π - π stacking between amino acid side chain and ligand) for binding to the corresponding binding site of a tetrapyrrole compound, and/or which provide the space to accommodate other substrate amino acid residue(s) (e.g. by modification, such as by substitution) capable to
15 bind a tetrapyrrole compound. The term “binding to” as used herein means “suitable for binding to” and forms part of Applicant’s rationale for the present invention – said rationale does not constitute an essential technical feature of the present invention. For example, the binding pocket which may be defined by N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and L226 of SEQ ID NO: 12, refers to a region of the S1
20 comprising amino acids N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and L226, and/or mutants thereof as described herein that Applicant believes cooperate to bind to a predicted binding site on a tetrapyrrole compound (particularly biliverdin).

25 The term “binding site” refers herein to a region of a tetrapyrrole compound comprising one or more groups that can be bound by the corresponding S1 binding pocket. For example, Pyrroles A and B of biliverdin represent a binding site, and are involved in a π - π stacking with an amino acid of the S1 binding pocket (e.g. a side chain of Arg190). The term “binding site” as used herein simply means “predicted binding site” (as predicted by Applicant) and forms
30 part of Applicant’s rationale for the present invention – said rationale does not constitute an essential technical feature of the present invention.

Without wishing to be bound by any theory, it is believed that the above-defined S1 tetrapyrrole binding pocket binding pocket effects an S1-ligand association within a deep cleft
35 on the spike N-terminal domain (NTD) e.g. the pocket providing a stabilising interaction between S1 and the ligand. Surprisingly, even modest (e.g. conservative substitutions) at

individual amino acid residues within the binding pocket may significantly reduce the S1 affinity for the ligand.

Thus, the present invention is predicated on the surprising finding (e.g. unexpected technical effect) that targeted amino acid substitutions as claimed allow for the generation of S1 constructs that remain free (e.g. substantially free) of bound tetrapyrrole compound, such as biliverdin. The present inventors have not only successfully identified suitable amino acid positions of S1 which can be altered (e.g. substituted) to decrease tetrapyrrole binding, but have also identified precise amino acid changes which provide this effect.

A key advantage of reducing/ preventing binding to tetrapyrrole binding is that the unbound S1 demonstrates improved epitope presentation, e.g. allowing for stronger binding of anti-SARS-CoV-2 antibodies (and thus improved detection thereof in immunoassays as will be described in more detail below). Again, not wishing to be bound by any theory, it is believed that S1 undergoes conformational rearrangements in the absence of tetrapyrrole (e.g. biliverdin) binding, allowing antibodies to bind to S1 epitope(s). It is believed that access to the epitope is gated by a solvent-exposed loop composed of predominantly hydrophilic residues ("gate", e.g. SARS-CoV-2 spike residues 174-188). To allow antibody binding, it is believed that the loop swings out of the way, with a backbone displacement in the middle of the loop of ~15 Å (see Fig. 11b). The gating mechanism is believed to be controlled by insertion of Phe175 and Met177, which are located in the beginning of the loop, into the hydrophobic pocket vacated by biliverdin (Fig. 11b).

As described in the Examples (e.g. Example 5), it has been identified that the pocket is lined by hydrophobic residues (Ile101, Trp104, Ile119, Val126, Met177, Phe192, Phe194, Ile203, and Leu226), which the inventors believe form van der Waals interactions with the tetrapyrrole ligand (e.g. biliverdin). As such, it is believed that effecting an amino acid change at any one of said positions disrupts van der Waals interactions between S1 and the tetrapyrrole ligand (e.g. biliverdin).

Biliverdin packs against His207, which projects its Nε2 atom towards pyrrolic amines, approaching three of them at ~3.6 Å (see Example 5). As such, it is believed that effecting an amino acid change at His207 disrupts projection of an S1 amino acid Nε2 atom toward the tetrapyrrole ligand (e.g. biliverdin).

Pyrroles A and B (e.g. of biliverdin) are involved in a π - π stacking with side chain of Arg190. As such, it is believed that effecting an amino acid change at Arg190 disrupts a π - π stacking interaction of an S1 amino acid with the tetrapyrrole ligand (e.g. biliverdin). Said π - π stacking (with side chain of Arg190) is stabilised by hydrogen bonding with Asn99 (see Example 5).
5 As such, it is believed that effecting an amino acid change at Asn99 disrupts stabilisation of said π - π stacking (with side chain of Arg190).

Ligand (e.g. biliverdin) binding largely buries the side chain of Asn121, which makes a hydrogen bond with the lactam group of pyrrole D (see Example 5). As such, it is believed
10 that effecting an amino acid change at Asn121 disrupts such hydrogen bond between an S1 amino acid and the tetrapyrrole ligand (e.g. biliverdin).

A modified S1 described herein may demonstrate a binding affinity for said tetrapyrrole compound that is 2-5 times lower (preferably 2-3 times lower) compared with wild-type S1 -
15 see the data described in Example 5.

When comparing a modified S1 of the invention with a wild-type S1, it will be appreciated that the polypeptide sequences of the modified S1 and the wild-type S1, other than the amino acid change, will otherwise be preferably identical. For example, if the modified S1
20 comprises or consists of amino acid residues 15-530 of S1 (in which the amino acid change is present), the wild-type S1 will likely preferably also comprises or consists of amino acid residues 15-530 of S1 (in which the amino acid change is not present).

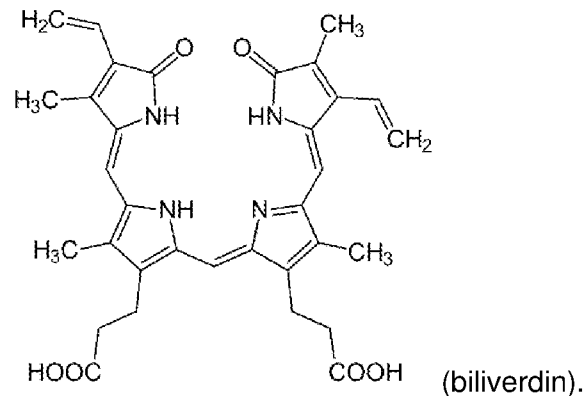
A "SARS coronavirus" (or SARS-CoV) is a virus that causes Severe acute respiratory syndrome (SARS), a viral respiratory disease. Examples include MERS-CoV (e.g. a beta coronavirus that causes Middle East Respiratory Syndrome, or MERS), SARS-CoV (e.g. a beta coronavirus that causes severe acute respiratory syndrome, or SARS) and SARS-CoV-2 (e.g. a coronavirus that causes coronavirus disease 2019, or COVID-19). Such coronaviruses each have highly homologous spike protein (including S1 subunit) sequences.
30

In all aspects and embodiments described throughout this specification, the SARS coronavirus is preferably SARS-CoV-2.

"Tetrapyrrole compounds" are a class of chemical compounds that contain four pyrrole or
35 pyrrole-like rings. The pyrrole/pyrrole derivatives are linked by (=CH)- or -CH₂- units), in

either a linear or a cyclic fashion. Pyrroles are a five-atom ring with four carbon atoms and one nitrogen atom. Tetrapyrroles are common cofactors in biochemistry and their biosynthesis and degradation feature prominently in the chemistry of life. Exemplary tetrapyrrole compounds include ham breakdown products, such as bilirubin and biliverdin. A

5 preferable tetrapyrrole compound is biliverdin:



The modified S1 as described herein may comprise an amino acid sequence having at least 60%, for example, at least 70% or at least 80% or at least 85% or at least 90% or at least 95% or at least 97% or at least 98% or at least 99%, sequence identity to the wild-type S1 (SEQ ID NO: 12), e.g. with the proviso that said modified S1 comprises said amino acid change within a tetrapyrrole binding pocket. The modified S1 amino acid sequence has less than 100% sequence identity to a wild-type S1 (e.g. SEQ ID NO: 12), e.g. because of said at least one amino acid change. Reference throughout this specification to a modified S1 embraces functional fragments thereof, that is fragments of said S1 that bind to anti-SARS-CoV (e.g. anti-SARS-CoV-2) antibodies at an equivalent or greater level than wild-type S1. For example, a modified S1 of the invention preferably comprises at least 400 (for example, at least 450 or at least 500) amino acids. By way of example, the N-terminal fourteen amino acids (e.g. cleavable signal peptide) and/ or the carboxyl-terminus (for example, amino acids 531-685) of S1 are not required for antibody binding, and indeed constructs corresponding the amino acid residues 15-530 of S1 have successfully been employed in serology tests for detecting anti-SARS-CoV-2 antibodies (see the Examples).

“Sequence identity” between amino acid or nucleic acid sequences can be determined by comparing a position in each of the sequences which may be aligned for the purposes of comparison. When a position in the compared sequences is occupied by the same nucleotide or amino acid, then the sequences are identical at that position. A degree of identity between amino acid sequences is a function of the number of identical amino acid sequences that are shared between these sequences. A degree of sequence identity

between nucleic acids is a function of the number of identical nucleotides at positions shared by these sequences.

To determine the “percentage of sequence identity” between two amino acid sequences or two nucleic acid sequences, the sequences are aligned for optimal comparison. For example, gaps can be introduced in the sequence of a first amino acid sequence or a first nucleic acid sequence for optimal alignment with the second amino acid sequence or second nucleic acid sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, the molecules are identical at that position.

The percentage (%) of identity between the two sequences is a function of the number of identical positions shared by the sequences. Hence, the percentage of identity can be calculated by multiplying the number of identical positions by 100 and dividing by the length of the aligned region (overlapping positions), including gaps (only internal gaps, not the gaps at the sequence ends).

In this comparison, the sequences can be of the same length, or may be of different lengths. Identity scoring only counts perfect matches, and does not consider the degree of similarity of amino acids to one another.

Optimal alignment of sequences may be herein preferably conducted by a global homology alignment algorithm should the alignment be performed using sequences of the same or similar length, such as by the algorithm described by Needleman and Wunsch (Journal of Molecular Biology; 1970, 48 (3): 443–53), by computerized implementations of this algorithm (e.g., using the DNASTAR® Lasergene software), or by visual inspection. Alternatively, should the alignment be performed using sequences of distinct length (e.g. the amino acid sequence of the light-chain according to the invention versus the entire amino acid sequence of a naturally-occurring botulinum neurotoxin), the optimal alignment of sequences can be herein preferably conducted by a local homology alignment algorithm, such as by the algorithm described by Smith and Waterson (Journal of Molecular Biology; 1981, 147: 195-197), by computerized implementations of this algorithm (e.g., using the DNASTAR® Lasergene software), or by visual inspection. The best alignment (i.e., resulting in the highest percentage of identity between the compared sequences) generated by the various methods is selected. Examples of global and local homology alignment algorithms are well-known to

the skilled practitioner, and include, without limitation, ClustalV (global alignment), ClustalW (local alignment) and BLAST (local alignment).

The skilled practitioner would further readily understand that the present invention embraces
5 modified S1 polypeptides that are substantially homologous, and which retain the capacity to
bind anti-SARS-CoV (e.g. anti-SARS-CoV-2) antibodies, i.e. functional variants or homologs.
These functional variants or homologs can be characterized as having one or more amino
acid mutations (such as an amino acid deletion, addition, and/or substitution) other than the
ones disclosed herein with regard to the tetrapyrrole binding pocket, and which do not
10 significantly affect the folding or epitope activity, in particular with regard to recognition by a
anti-SARS-CoV (e.g. anti-SARS-CoV-2) antibody. For example, such mutations include,
without limitation, conservative substitutions, small deletions (typically of 1 to about 30 amino
acids), small amino- or carboxyl-terminal extensions (such as an amino-terminal methionine
residue), addition of a small linker peptide of up to about 20-25 residues or of an affinity tag.

15 The modified SARS-CoV (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide may
comprise or consist of a sequence (otherwise) corresponding to the whole amino acid
sequence of wild-type S1 (e.g. amino acid residues 1-685 of S1 (SEQ ID NO: 12), in which
the at least one amino acid change is present. The N-terminal 13-15 amino acid residues
(signal peptide) are typically cleaved after expression. Thus, the modified SARS-CoV (e.g.
20 SARS-CoV-2) spike protein S1 subunit (S1) polypeptide may comprise or consist of a
sequence (otherwise) corresponding to amino acid residues 13-685 (preferably 15-685) of
S1 (SEQ ID NO: 12), in which the at least one amino acid change is present.

The modified S1 sequence may cease after the receptor binding domain, RBD, sequence
25 (which is at amino acid residues 319-541 of S1 (SEQ ID NO: 12)).

In one embodiment, the modified SARS-CoV (e.g. SARS-CoV-2) spike protein S1 subunit
(S1) polypeptide may comprise or consist of a sequence (otherwise) corresponding to amino
acid residues 1-541 of S1 (SEQ ID NO: 12), in which the at least one amino acid change is
30 present. The N-terminal 13-15 amino acid residues (signal peptide) are typically cleaved
after expression. Thus, the modified SARS-CoV (e.g. SARS-CoV-2) spike protein S1 subunit
(S1) polypeptide may comprise or consist of a sequence (otherwise) corresponding to amino
acid residues 13-541 (preferably 15-541) of S1 (SEQ ID NO: 12), in which the at least one
amino acid change is present.

The inventors have found that the S1 sequence can be truncated even further, having improved purification properties while retaining immunogenic activity/ epitopes (indeed, such truncated version was used in the immunoassays in the Examples).

5 In one embodiment, the modified SARS-CoV (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide may comprise or consist of a sequence (otherwise) corresponding to 1-530 of S1 (SEQ ID NO: 12), in which the at least one amino acid change is present. The N-terminal 13-15 amino acid residues (signal peptide) are typically cleaved after expression. Thus, the modified SARS-CoV (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide
10 may comprise or consist of a sequence (otherwise) corresponding to amino acid residues 13-530 (preferably 15-530) of S1 (SEQ ID NO: 12), in which the at least one amino acid change is present.

In one embodiment, the at least one amino acid change is at a location that corresponds to
15 an amino acid location, of wild-type S1 (SEQ ID NO: 12), comprising N99, W104, I119, N121, V126, F175, M177, R190, F192, H207 and/or L226.

The at least one amino acid change may be at a position that corresponds to an amino acid residue selected from N99, W104, I119, N121, V126, F175, M177, R190, F192, H207 and
20 L226, of wild-type S1 (SEQ ID NO: 12).

In a preferable embodiment, the at least one amino acid change is at a location that corresponds to an amino acid location, of wild-type S1 (SEQ ID NO: 12), comprising N121, R190, and/or H207.
25

Preferably, the at least one amino acid change may preferably be at a position that corresponds to an amino acid residue selected from N121, R190, and H207, of wild-type S1 (SEQ ID NO: 12).

30 A modified S1 polypeptide of the present invention having a binding pocket mutation (for binding a tetrapyrrole) may comprise one or more amino acid residue changes relative to the wild-type S1 sequence, as herein before defined. By way of illustration, a modified S1 of the present invention may have a single amino acid residue mutation (within the binding pocket, as defined above), for example a mutation corresponding to amino acid residue N121 of wild-
35 type S1 (SEQ ID NO: 12). Similarly, a modified S1 polypeptide of the present invention may comprise more than one amino acid residue mutation (within the binding pocket as defined

above), for example mutations corresponding to amino acid residues N121, R190K and H207 of wild-type S1 (SEQ ID NO: 12).

Thus, the modified S1 may comprise an amino acid change at 2 or more, 3 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, or all 14 position(s) on the modified S1 polypeptide sequence that corresponds to an amino acid residue, of wild-type S1 (SEQ ID NO: 12), selected from: N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and L226. The modified S1 may comprise an amino acid change at 1 or more, 2 or more, 3 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, or all 11 position(s) on the modified S1 polypeptide sequence that corresponds to an amino acid residue selected from N99, W104, I119, N121, V126, F175, M177, R190, F192, H207 and L226, of wild-type S1 (SEQ ID NO: 12). The modified S1 may comprise an amino acid change at 1 or more, 2 or more, or all 3 position(s) on the modified S1 polypeptide sequence that corresponds to an amino acid residue selected from N121, R190, and H207, of wild-type S1 (SEQ ID NO: 12).

For example, the modified S1 may comprise an amino acid change at positions on the modified S1 polypeptide sequence that corresponds to amino acid residues N121, R190, and H207, of wild-type S1 (SEQ ID NO: 12).

The modified S1 may comprise an amino acid change at a position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of wild-type S1 (SEQ ID NO: 12); and optionally the modified S1 may comprise at least one further amino acid change at a position on the modified S1 polypeptide sequence that corresponds to an amino acid residue selected from N99, I101, W104, I119, V126, F175, M177, R190, F192, F194, I203, H207 and L226 of wild-type S1 (SEQ ID NO: 12). The modified S1 may comprise an amino acid change at a position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of wild-type S1 (SEQ ID NO: 12); and the modified S1 may comprise at least one further amino acid change at a position on the modified S1 polypeptide sequence that corresponds to an amino acid residue selected from N99, W104, I119, V126, F175, M177, R190, F192, H207 and L226 of wild-type S1 (SEQ ID NO: 12). Preferably, the modified S1 may comprise an amino acid change at a position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of wild-type S1 (SEQ ID NO: 12); and the modified S1 may comprise at least one further amino acid change at a position on the modified S1 polypeptide sequence that corresponds to an amino acid residue selected from R190 and H207 of wild-type S1 (SEQ ID NO: 12).

The modified S1 may comprise an amino acid change at a position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of wild-type S1 (SEQ ID NO: 12); and optionally the modified S1 may comprise at least one further amino acid change at a position on the modified S1 polypeptide sequence that corresponds to an amino acid residue selected from N99, I101, W104, I119, N121, V126, F175, M177, F192, F194, I203, H207 and L226 of wild-type S1 (SEQ ID NO: 12). The modified S1 may comprise an amino acid change at a position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of wild-type S1 (SEQ ID NO: 12); and the modified S1 may comprise at least one further amino acid change at a position on the modified S1 polypeptide sequence that corresponds to an amino acid residue selected from N99, W104, I119, N121, V126, F175, M177, F192, H207 and L226 of wild-type S1 (SEQ ID NO: 12). Preferably, the modified S1 may comprise an amino acid change at a position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of wild-type S1 (SEQ ID NO: 12); and the modified S1 may comprise at least one further amino acid change at a position on the modified S1 polypeptide sequence that corresponds to an amino acid residue selected from N121 and H207 of wild-type S1 (SEQ ID NO: 12).

The modified S1 may comprise an amino acid change at a position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of wild-type S1 (SEQ ID NO: 12); and optionally the modified S1 may comprise at least one further amino acid change at a position on the modified S1 polypeptide sequence that corresponds to an amino acid residue selected from N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, and L226 of wild-type S1 (SEQ ID NO: 12). The modified S1 may comprise an amino acid change at a position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of wild-type S1 (SEQ ID NO: 12); and the modified S1 may comprise at least one further amino acid change at a position on the modified S1 polypeptide sequence that corresponds to an amino acid residue selected from N99, W104, I119, N121, V126, F175, M177, R190, F192, and L226 of wild-type S1 (SEQ ID NO: 12). Preferably, the modified S1 may comprise an amino acid change at a position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of wild-type S1 (SEQ ID NO: 12); and the modified S1 may comprise at least one further amino acid change at a position on the modified S1 polypeptide sequence that corresponds to an amino acid residue selected from N121 and R190 of wild-type S1 (SEQ ID NO: 12).

The term “modification”, “change” or “mutation” can be used herein interchangeably, and refer to the alteration in the amino acid sequence compared to that of a protein of reference,

i.e. herein relative to the wild-type S1 (SEQ ID NO: 12). The amino acid sequence illustrated herein as SEQ ID NO: 12 is 685 amino acid residues in length and ends with R685. It is understood that S686 is the first amino acid residue of the S1 subunit and most likely represents the C-terminal end of the S1 subunit. Thus, SEQ ID NO: 12 represents the subunit of the spike protein referred to as S1. In this regard, a wild-type S1 is typically 685 amino acid residues in length, which includes a short N-terminal signal peptide of about 13-15 amino acid residues.

As further explained below, the present invention reveals the identification of critical amino acid positions within a wild-type S1 that allow rational change to a different amino acid residue (or deletion of the amino acid) in order to render an S1 polypeptide incapable of (or having reduced capacity to) being bound by tetrapyrrole. In this regard, introduction of an amino acid change (i.e. a mutation), may be effected by means of an amino acid insertion, a deletion or a substitution, and preferably by means of an amino acid substitution. Methods allowing introduction of such mutation are known to the skilled person in the art. For example, it is possible to introduce a mutation by random or directed mutagenesis, by PCR using degenerate primers, e.g. in the nucleotide sequence coding for the protein of reference. Said techniques are notably described by Sambrook et al. in "Molecular Cloning: A laboratory Manual", 4th edition, Cold Spring Harbor Laboratory Press, (2012, and updates from 2014), and by Ausubel et al. in "Current Protocols in Molecular Biology", John Wiley & Sons (2012).

The amino acid change may be a deletion (e.g. designated by "del"). In such embodiments, the term "del" may be presented after recitation of the amino acid and its position e.g. as per the following: N99del, I101del, W104del, I119del, N121del, V126del, F175del, M177del, R190del, F192del, F194del, I203del, H207del, L226del. Other amino acid changes may include insertions, indels, duplications and frame shifts that lead to a lower level of tetrapyrrole binding compared to that for the wild-type S1.

In a preferable embodiment, the amino acid change is a substitution.

The amino acid change may comprise substitution of N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, or L226 (positions corresponding to that of wild-type S1, SEQ ID NO: 12) for any non-N, non-I, non-W, non-L, non-N, non-V1, non-F, non-M, non-R, non-F, non-F, non-I, or non-L amino acid, respectively. The amino acid change may comprise substitution of N99, W104, I119, N121, V126, F175, M177, F192, H207 or L226

(positions corresponding to that of wild-type S1, SEQ ID NO: 12) for any non-N, non-W, non-I, non-N, non-V, non-F, non-M, non-F, non-H or non-L amino acid, respectively. The amino acid change may comprise substitution of N121, R190, or H207 (positions corresponding to that of wild-type S1, SEQ ID NO: 12) for any non-N, non-R, or non-H amino acid, respectively.

Preferably, the amino acid change is a conservative substitution. For example, the amino acid change is preferably of a minor nature, that is a conservative amino acid substitutions (see below) and other substitutions that do not significantly affect the folding or activity of the polypeptide.

Basic:	arginine, lysine, histidine
Acidic:	glutamic acid, aspartic acid
Polar:	glutamine, asparagine
Hydrophobic:	leucine, isoleucine, valine
Aromatic:	phenylalanine, tryptophan, tyrosine
Small:	glycine, alanine, serine, threonine, methionine

In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-N-methyl lysine, 2-aminoisobutyric acid, isovaline and α -methyl serine) may be substituted for amino acid residues of the polypeptides, of the present invention. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for clostridial polypeptide amino acid residues. The polypeptides of the present invention may also comprise non-naturally occurring amino acid residues.

Non-naturally occurring amino acids include, without limitation, trans-3-methylproline, 2,4-methano-proline, cis-4-hydroxyproline, trans-4-hydroxy-proline, N-methylglycine, allo-threonine, methyl-threonine, hydroxy-ethylcysteine, hydroxyethylhomo-cysteine, nitro-glutamine, homoglutamine, pipecolic acid, tert-leucine, norvaline, 2-azaphenylalanine, 3-azaphenyl-alanine, 4-azaphenyl-alanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins.

The amino acid substitution may comprise the substitution of an amino acid comprising a certain physiochemical property (e.g. hydrophobicity) with an amino acid having a similar or alternative property. Examples of such substitutions are listed below:

Acidic amino acid substituted for a neutral, polar amino acid;

Polar amino acid substituted for a non-polar amino acid;
Non-polar amino acid substituted for a non-polar amino acid;
Non-polar amino acid substituted for a polar amino acid;
Polar amino acid substituted for a basic amino acid;
5 Non-polar amino acid substituted for an acidic amino acid;
Non-polar amino acid substituted for a polar amino acid.

10 An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N99 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of glutamine, aspartate and glutamate (preferably glutamine).

15 An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue I101 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of Glycine, Alanine, Valine, and Leucine.

20 An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue W104 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of Phenylalanine, and Tyrosine.

25 An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue I119 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of Glycine, Alanine, Valine, and Leucine.

30 An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of glutamine, aspartate and glutamate. Preferably, an amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12) may be amino acid residue glutamine (e.g. N121Q).

35 An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue V126 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of Glycine, Alanine, and Leucine.

An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F175 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of Tyrosine and Tryptophan.

5 An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue M177 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of Serine, Cysteine, Selenocysteine, and Threonine (preferably Serine, Cysteine, or Threonine).

10 An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of lysine and histidine. Preferably, an amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12) may be amino
15 acid residue lysine (e.g. R190K)

An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F192 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of Tyrosine and Tryptophan.

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An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F194 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of Tyrosine and Tryptophan.

25 An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue I203 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of Glycine, Alanine, Valine, and Leucine.

30 An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of alanine, lysine, arginine, glycine, valine, leucine and isoleucine. Preferably, an amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-
35 type S1 (SEQ ID NO: 12) may be amino acid residue alanine (e.g. H207A).

An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue L226 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of Glycine, Alanine, Valine, Isoleucine.

5 An amino acid change at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue L226 of the wild-type S1 (SEQ ID NO: 12) may be an amino acid residue selected from the group consisting of Glycine, Valine, Isoleucine.

In one embodiment, the at least one amino acid residue change comprises:

- 10 i. an amino acid residue selected from the group consisting of glutamine, aspartate and glutamate at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N99 of the wild-type S1 (SEQ ID NO: 12);
- ii. an amino acid residue selected from the group consisting of Glycine, Alanine, Valine, and Leucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue I101 of the wild-type S1 (SEQ ID NO: 12);
- 15 iii. an amino acid residue selected from the group consisting of Phenylalanine, and Tyrosine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue W104 of the wild-type S1 (SEQ ID NO: 12);
- iv. an amino acid residue selected from the group consisting of Glycine, Alanine, Valine, and Leucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue I119 of the wild-type S1 (SEQ ID NO: 12);
- 20 v. an amino acid residue selected from the group consisting of glutamine, aspartate and glutamate at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12);
- vi. an amino acid residue selected from the group consisting of Glycine, Alanine, and Leucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue V126 of the wild-type S1 (SEQ ID NO: 12);
- 25 vii. an amino acid residue selected from the group consisting of Tyrosine and Tryptophan at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F175 of the wild-type S1 (SEQ ID NO: 12);
- 30 viii. an amino acid residue selected from the group consisting of Serine, Cysteine, Selenocysteine, and Threonine, at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue M177 of the wild-type S1 (SEQ ID NO: 12);
- 35 ix. an amino acid residue selected from the group consisting of a lysine and histidine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12); and/or

- x. an amino acid residue selected from the group consisting of Tyrosine and Tryptophan at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F192 of the wild-type S1 (SEQ ID NO: 12);
- xi. an amino acid residue selected from the group consisting of Tyrosine and Tryptophan at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F194 of the wild-type S1 (SEQ ID NO: 12);
- xii. an amino acid residue selected from the group consisting of Glycine, Alanine, Valine, and Leucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue I203 of the wild-type S1 (SEQ ID NO: 12);
- xiii. an amino acid residue selected from the group consisting of an alanine, lysine, arginine, glycine, valine, leucine and isoleucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12); and/or
- xiv. an amino acid residue selected from the group consisting of an Glycine, Alanine, Valine, and Isoleucine (preferably selected from Glycine, Valine, Isoleucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue L226 of the wild-type S1 (SEQ ID NO: 12).

In one embodiment, the at least one amino acid residue change comprises:

- i. an amino acid residue selected from the group consisting of glutamine, aspartate and glutamate at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12);
- ii. an amino acid residue selected from the group consisting of a lysine and histidine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12); and/or
- iii. an amino acid residue selected from the group consisting of an alanine, lysine, arginine, glycine, valine, leucine and isoleucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12).

In a preferable embodiment, the at least one amino acid residue change comprises:

- i. a glutamine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12);
- ii. a lysine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12); and/or
- iii. an alanine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12).

Examples of modified S1 polypeptides (e.g. having a binding pocket change) include a modified S1 polypeptide comprising at least one of the following substitution(s):

- N121Q, N121D, N121E
- 5 - R190L, R190H
- H207A, H207K, H207R, H207G, H207V, H207L, H207I

In summary, most preferable examples of modified S1 polypeptides (e.g. having a binding pocket change) include a modified S1 polypeptide comprising the substitution:

- 10 N121Q;
- R190K; and/or (preferably or)
- H207A.

A particularly preferred modified S1 polypeptide comprises the substitution N121Q.

15

The labelled antigen (e.g. as it is expressed) may be referred to as an S1 construct, and preferably takes amino acid residues 1-530 of the SARS-CoV (e.g. SARS-CoV-2) spike protein, in which the amino acid change is present (e.g. the expression construction may comprise said amino acid residues 1-530). An advantage of this particular S1 construct is its

20 high level of expression. The labelled antigen as secreted (from a cell) may comprise or consist of amino acid residues 15-530 of the SARS-CoV (e.g. SARS-CoV-2) spike protein, in which the amino acid change is present e.g. due to cleavage of amino acid residues 1-14 (the signal peptide). The labelled antigen as secreted (from a cell) may comprise or consist of amino acid residues 13-530 of the SARS-CoV (e.g. SARS-CoV-2) spike protein in which

25 the amino acid change is present, e.g. due to cleavage of amino acid residues 1-12 (the signal peptide).

The labelled antigen may be a recombinant protein. In a preferable embodiment, the labelled antigen is a recombinant protein.

30

In DABA formats as described above, the use of hybrid double antigens (e.g. amino acid residues modified S1 polypeptide for a first antigen, and amino acid residues 319-541 of the SARS-CoV-2 spike protein for a (e.g. labelled) second antigen) allows purification of the two different antigens separately, optionally providing excipients in the solid-phase protein which

35 differ from (e.g. potential) excipients in the fluid-phase protein (a feature which is not present in a normal/ non-hybrid double antigen assay with identical proteins). Thus, the first and

second (e.g. recombinant) antigens may be derived from different sources. For example, the first antigen may be derived from a first expression and purification process, and the second antigen may be derived from a second (different) expression and purification process. In other words, the solid-phase and the second labelled antigen components differ in their sourcing and are of different origin, for example sharing only the epitope against which the test is designed to detect antibody.

In a preferable embodiment, the first and/or (preferably and) second antigen is a recombinant protein.

A step of "removing labelled second antigen that is not bound to said complex" may be referred to as a wash step (e.g. in which a wash buffer is used wash away free labelled antigen, thus ensuring visualisation of the label in subsequent steps allows visualisation of labelled antigen that is bound to captured/ immobilised SARS-CoV antibody). Methods of the invention may comprise one or more additional wash steps. In one embodiment, a step of contacting said complex with a labelled antigen (e.g. step c)) is preceded by a step of removing labelled antigen (and any other component present in the sample, such as free antibody) that is not bound to the anti-SARS-CoV antibody (e.g. that does not form a complex of first antigen and antibody).

A further aspect of the present invention provides a nucleic acid construct, comprising or consisting of a nucleic acid sequence that encodes a modified SARS-CoV (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide as herein described. A nucleic acid construct of the invention may include conventional regulatory elements such as a promoter and/or a terminator. The term "nucleic acid" may be used synonymously with the term "polynucleotide".

A nucleic acid construct described herein is preferably an isolated nucleic acid construct. The nucleic acid construct may be recombinant, synthetic, and/or purified.

In one embodiment, the nucleic acid construct is provided in the form of a bacterial plasmid or viral vector. Said nucleic acid construct can optionally be codon-biased for optimizing expression (e.g. recombinant expression) in a desired host cell (e.g. *E. coli*).

In one embodiment, a nucleic acid construct encoding a modified SARS-CoV (e.g. SARS-CoV-2) spike protein S1 subunit (S1) polypeptide as herein described can be employed for

administration to a patient, such as for therapeutic use. To this end, said nucleic acid construct can be typically optimised by way of conventional methodology for delivery into (followed by expression within) a target cell, preferably a human cell.

- 5 The nucleic acid construct may comprise DNA or RNA (preferably mRNA). An RNA nucleic acid construct may be particularly suited to nucleic acid immunisation.

The invention also provides a composition comprising (i) one or more nucleic acid(s) of the invention, or one or more nucleic acid(s) complementary thereto. Optionally, said
10 composition further comprises a pharmaceutically acceptable carrier or excipient. In one embodiment, said composition is for use in nucleic acid immunisation.

Another aspect of the invention provides a host cell comprising a nucleic acid construct described herein. Said host cell may be a mammalian cell, an insect cell, a yeast cell, a
15 bacterial cell (e.g. *E. coli*), or a plant cell. In a preferable embodiment, the host cell is a bacterial cell (preferably *E. coli*).

Another aspect of the invention provides a pseudotyped SARS-CoV (e.g. SARS-CoV-2) virus, wherein the pseudotyped virus comprises a modified SARS-CoV (e.g. SARS-CoV-2)
20 spike protein S1 subunit (S1) polypeptide as described herein. The pseudotyped SARS-CoV (e.g. SARS-CoV-2) virus may be provided in the form of a pseudotyped virus particle (e.g. a "pseudovirus"). Additionally or alternatively, the pseudotyped SARS-CoV (e.g. SARS-CoV-2) virus may be provided in the form of a viral vector. The modified SARS-CoV (e.g. SARS-CoV-2) spike protein S1 subunit (S1) preferably replaces the wild-type S1 in the pseudotyped
25 SARS-CoV-2 virus. However, the invention embraces embodiments in which the modified SARS-CoV (e.g. SARS-CoV-2) spike protein S1 subunit (S1) is present in addition to the wild-type S1.

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- 30 1. A modified SARS-CoV spike protein S1 subunit (S1) polypeptide, having a modified amino acid sequence relative to a wild-type S1 sequence (SEQ ID NO: 12);
a. the modified S1 polypeptide comprising at least one amino acid residue change located within a tetrapyrrole binding pocket for binding to a tetrapyrrole compound;

b. wherein said at least one amino acid change is at a location on the modified S1 polypeptide sequence that corresponds to an amino acid location, of wild-type S1 (SEQ ID NO: 12), comprising:

i. N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, H207 and/or L226 (more particularly N99, I101, W104, I119, N121, V126, F175, M177, R190, F192, F194, I203, and/or H207; even more particularly N99, N121, F175, M177, F194, and/or I203); and

c. wherein the modified S1 polypeptide demonstrates lower binding affinity for said tetrapyrrole compound compared with wild-type S1.

2. The modified S1 polypeptide according to clause 1, wherein the at least one amino acid change is at a location that corresponds to an amino acid location, of wild-type S1 (SEQ ID NO: 12), comprising N99, W104, I119, N121, V126, F175, M177, R190, F192, H207 and/or L226.

3. The modified S1 polypeptide according to clause 1 or clause 2, wherein the at least one amino acid change is at a location that corresponds to an amino acid location, of wild-type S1 (SEQ ID NO: 12), comprising N121, R190, and/or H207.

4. The modified S1 polypeptide according to clause 1, wherein the at least one amino acid residue change comprises:

i. an amino acid residue selected from the group consisting of glutamine, aspartate and glutamate at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N99 of the wild-type S1 (SEQ ID NO: 12);

ii. an amino acid residue selected from the group consisting of glycine, alanine, valine, and leucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue I101 of the wild-type S1 (SEQ ID NO: 12);

iii. an amino acid residue selected from the group consisting of phenylalanine, and tyrosine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue W104 of the wild-type S1 (SEQ ID NO: 12);

iv. an amino acid residue selected from the group consisting of glycine, alanine, valine, and leucine at the position on the modified S1 polypeptide

sequence that corresponds to amino acid residue I119 of the wild-type S1 (SEQ ID NO: 12);

v. an amino acid residue selected from the group consisting of glutamine, aspartate and glutamate at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12);

vi. an amino acid residue selected from the group consisting of glycine, alanine, and leucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue V126 of the wild-type S1 (SEQ ID NO: 12);

vii. an amino acid residue selected from the group consisting of tyrosine and tryptophan at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F175 of the wild-type S1 (SEQ ID NO: 12);

viii. an amino acid residue selected from the group consisting of serine, cysteine, selenocysteine, and threonine, at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue M177 of the wild-type S1 (SEQ ID NO: 12);

ix. an amino acid residue selected from the group consisting of a lysine and histidine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12); and/or

x. an amino acid residue selected from the group consisting of tyrosine and tryptophan at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F192 of the wild-type S1 (SEQ ID NO: 12);

xi. an amino acid residue selected from the group consisting of tyrosine and tryptophan at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F194 of the wild-type S1 (SEQ ID NO: 12);

xii. an amino acid residue selected from the group consisting of glycine, alanine, valine, and leucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue I203 of the wild-type S1 (SEQ ID NO: 12);

xiii. an amino acid residue selected from the group consisting of an alanine, lysine, arginine, glycine, valine, leucine and isoleucine at the position on

the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12); and/or

- xiv. an amino acid residue selected from the group consisting of glycine, alanine, valine, and isoleucine (preferably selected from glycine, valine, and isoleucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue L226 of the wild-type S1 (SEQ ID NO: 12)).

5. The modified S1 polypeptide according to any one of the preceding clause, wherein the at least one amino acid residue change comprises:

- i. an amino acid residue selected from the group consisting of glutamine, aspartate and glutamate at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12);
- ii. an amino acid residue selected from the group consisting of a lysine and histidine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12); and/or
- iii. an amino acid residue selected from the group consisting of an alanine, lysine, arginine, glycine, valine, leucine and isoleucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12).

6. The modified S1 polypeptide according to any one of the preceding clauses, wherein the at least one amino acid residue change comprises:

- i. a glutamine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12);
- ii. a lysine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12); and/or
- iii. an alanine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12).

7. The modified S1 polypeptide according to any one of clauses 1-6, wherein the at least one amino acid residue change comprises:
- 5 i. a glutamine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12);
8. The modified S1 polypeptide according to any one of clauses 1-6, wherein the at least one amino acid residue change comprises:
- 10 i. a lysine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12); and/or
9. The modified S1 polypeptide according to any one of clauses 1-6, wherein the at least one amino acid residue change comprises:
- 15 i. an alanine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12).
10. The modified S1 according to any one of the preceding clauses, wherein the modified S1 comprises or consists of a sequence corresponding to amino acid residues 15-530 of the SARS-CoV-2 spike protein S1 subunit (SEQ ID NO: 12), in which said at least one amino acid residue change is present.
- 20
11. A nucleic acid construct comprising or consisting of a nucleic acid sequence encoding the modified SARS-CoV-2 spike protein S1 subunit (S1) polypeptide as defined in any preceding clause.
- 25
12. A host cell comprising the nucleic acid construct of clause 11.
13. A pseudotyped SARS-CoV virus, wherein the pseudotyped virus comprises a modified SARS-CoV spike protein S1 subunit (S1) polypeptide as defined in any one of clauses 1-10.
- 30
14. A method for detecting the presence or absence of SARS-CoV antibodies in a sample, the method comprising:
- 35 a. contacting the sample with a solid-phase support having a first antigen immobilised thereto,

- i. wherein the first antigen comprises the modified SARS-CoV spike protein S1 subunit (S1) polypeptide as defined in any of clauses 1 to 10;
- b. allowing SARS-CoV-2 antibodies present in the sample to bind to the modified S1 polypeptide, thereby forming a complex of modified S1 polypeptide and SARS-CoV antibody;
- c. contacting said complex with a labelled second antigen, wherein said labelled second antigen comprises a SARS-CoV spike protein polypeptide (preferably wherein said labelled second antigen comprises or consists of a SARS-CoV receptor binding domain polypeptide, more preferably wherein said labelled second antigen comprises or consists of a sequence of SEQ ID No.: 3) that binds SARS-CoV antibody;
- d. allowing said labelled second antigen to bind to SARS-CoV antibody present in the sample;
- e. removing labelled second antigen that is not bound to said complex; and
- f. detecting the presence of labelled second antigen bound to said complex;

wherein the presence of labelled complex indicates the presence SARS-CoV antibody in the sample, and wherein the absence of labelled complex indicates the absence of SARS-CoV antibody in the sample.

15. The method according to any clause 14, wherein the labelled second antigen is amino acid residues 319-541 of the SARS-CoV-2 spike protein (SEQ ID NO.: 3) conjugated to a label.
16. The method according to any one of the preceding clauses, wherein the labelled antigen is present in a fluid phase.
17. The method according to any one of clauses 14-16, further comprising recording the output of said method on a data readable format.
18. An immunoassay solid-phase support for detecting the presence or absence of antibody to SARS-CoV (e.g. SARS-Cov-2) in a sample, the solid-phase support comprising a first antigen immobilised thereto,
 - a. wherein the first antigen comprises the modified SARS-CoV spike protein S1 subunit (S1) polypeptide as defined in any of clauses 1 to 10.

19. A kit for detecting the presence or absence of antibody to SARS-CoV (e.g. SARS-Cov-2) in a sample, the kit comprising:

- a. an immunoassay solid-phase support as defined in clause 18, and
- b. a labelled second antigen, wherein said labelled second antigen comprises a SARS-CoV spike protein polypeptide (preferably wherein said labelled second antigen comprises or consists of a SARS-CoV receptor binding domain polypeptide, more preferably wherein said labelled second antigen comprises or consists of a sequence of SEQ ID No.: 3) that binds SARS-CoV antibody; optionally wherein the labelled second antigen comprises or consists of amino acid residues 319-541 of the SARS-CoV-2 spike protein (SEQ ID NO.: 3) conjugated to a label.

20. The kit according to clause 19, further comprising instructions for use for detecting the presence or absence of antibody to SARS-CoV in a sample, preferably in a dried blood spot eluate sample.

21. Use of a solid-phase support as defined in clause 18, or a kit as defined in clauses 19 or 20, for detecting antibody to SARS-CoV (e.g. SARS-Cov-2) in a sample.

22. An immunogenic composition comprising:

- a. the modified SARS-CoV spike protein S1 subunit (S1) polypeptide as defined in any of clauses 1 to 10, or the nucleic acid construct of clause 11, or the pseudotyped SARS-CoV virus as defined in clause 13; and
- b. a pharmaceutically acceptable carrier or excipient.

23. An immunogenic composition according to clause 22 for use in preventing, treating or suppressing a SARS-CoV infection in a patient.

24. An immunogenic composition according to clause 22 comprising the nucleic acid construct of clause 11, for use in nucleic acid immunisation of a patient.

SEQUENCE HOMOLOGY

Any of a variety of sequence alignment methods can be used to determine percent identity, including, without limitation, global methods, local methods and hybrid methods, such as, e.g., segment approach methods. Protocols to determine percent identity are routine procedures within the scope of one skilled in the art. Global methods align sequences from

the beginning to the end of the molecule and determine the best alignment by adding up scores of individual residue pairs and by imposing gap penalties. Non-limiting methods include, e.g., CLUSTAL W, see, e.g., Julie D. Thompson et al., CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment Through Sequence Weighting, 5 Position- Specific Gap Penalties and Weight Matrix Choice, 22(22) Nucleic Acids Research 4673-4680 (1994); and iterative refinement, see, e.g., Osamu Gotoh, Significant Improvement in Accuracy of Multiple Protein. Sequence Alignments by Iterative Refinement as Assessed by Reference to Structural Alignments, 264(4) J. Mol. Biol. 823-838 (1996). Local methods align sequences by identifying one or more conserved motifs shared by all of 10 the input sequences. Non-limiting methods include, e.g., Match-box, see, e.g., Eric Depiereux and Ernest Feytmans, Match-Box: A Fundamentally New Algorithm for the Simultaneous Alignment of Several Protein Sequences, 8(5) CABIOS 501 -509 (1992); Gibbs sampling, see, e.g., C. E. Lawrence et al., Detecting Subtle Sequence Signals: A Gibbs Sampling Strategy for Multiple Alignment, 262(5131) Science 208-214 (1993); Align- 15 M, see, e.g., Ivo Van Walle et al., Align-M - A New Algorithm for Multiple Alignment of Highly Divergent Sequences, 20(9) Bioinformatics:1428-1435 (2004). Thus, percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48: 603-16, 1986 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-19, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using 20 a gap opening penalty of 10, a gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (ibid.) as shown below (amino acids are indicated by the standard one-letter codes). The "percent sequence identity" between two or more nucleic acid or amino acid sequences is a function of the number of identical positions shared by the sequences. Thus, % identity may be calculated as the number of identical nucleotides / 25 amino acids divided by the total number of nucleotides / amino acids, multiplied by 100. Calculations of % sequence identity may also take into account the number of gaps, and the length of each gap that needs to be introduced to optimize alignment of two or more sequences. Sequence comparisons and the determination of percent identity between two or more sequences can be carried out using specific mathematical algorithms, such as BLAST, 30 which will be familiar to a skilled person.

ALIGNMENT SCORES FOR DETERMINING SEQUENCE IDENTITY

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
5 N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
10 G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
15 M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
20 W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

The percent identity is then calculated as:

25

Total number of identical matches

_____ x 100

[length of the longer sequence plus the

30 number of gaps introduced into the longer

sequence in order to align the two sequences]

Substantially homologous polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see below) and other substitutions that do not significantly affect the folding or activity of the polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or an affinity tag.

40

CONSERVATIVE AMINO ACID SUBSTITUTIONS

Basic: arginine; lysine; histidine
Acidic: glutamic acid; aspartic acid
Polar: glutamine; asparagine
Hydrophobic: leucine; isoleucine; valine
5 Aromatic: phenylalanine; tryptophan; tyrosine
Small: glycine; alanine; serine; threonine; methionine

In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-N-methyl lysine, 2-aminoisobutyric acid, isovaline and α -methyl serine)
10 may be substituted for amino acid residues of the polypeptides of the present invention. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for polypeptide amino acid residues. The polypeptides of the present invention can also comprise non-naturally occurring amino acid residues.

15 Non-naturally occurring amino acids include, without limitation, trans-3-methylproline, 2,4-methano-proline, cis-4-hydroxyproline, trans-4-hydroxy-proline, N-methylglycine, allo-threonine, methyl-threonine, hydroxy-ethylcysteine, hydroxyethylhomo-cysteine, nitro-glutamine, homoglutamine, pipecolic acid, tert-leucine, norvaline, 2-azaphenylalanine, 3-azaphenyl-alanine, 4-azaphenyl-alanine, and 4-fluorophenylalanine. Several methods are
20 known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an in vitro system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of
25 plasmids containing nonsense mutations is carried out in a cell free system comprising an E. coli S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., J. Am. Chem. Soc. 113:2722, 1991; Ellman et al., Methods Enzymol. 202:301, 1991; Chung et al., Science 259:806-9, 1993; and Chung et al., Proc. Natl. Acad. Sci. USA 90:10145-9, 1993). In a
30 second method, translation is carried out in Xenopus oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., J. Biol. Chem. 271:19991-8, 1996). Within a third method, E. coli cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine,
35 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the polypeptide in place of its natural counterpart. See, Koide et al.,

Biochem. 33:7470-6, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by in vitro chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, Protein Sci. 2:395-403, 1993).

5

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

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

20

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

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR
35 BIOLOGY, 20 ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE

HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide the skilled person with a general dictionary of many of the terms used in this disclosure.

This disclosure is not limited by the exemplary methods and materials disclosed herein, and any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of this disclosure. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, any nucleic acid sequences are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

The headings provided herein are not limitations of the various aspects or embodiments of this disclosure.

Amino acids are referred to herein using the name of the amino acid, the three letter abbreviation or the single letter abbreviation. The term "protein", as used herein, includes proteins, polypeptides, and peptides. As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide". In some instances, the term "amino acid sequence" is synonymous with the term "enzyme". The terms "protein" and "polypeptide" are used interchangeably herein. In the present disclosure and claims, the conventional one-letter and three-letter codes for amino acid residues may be used. The 3-letter code for amino acids as defined in conformity with the IUPACIUB Joint Commission on Biochemical Nomenclature (JCBN). It is also understood that a polypeptide may be coded for by more than one nucleotide sequence due to the degeneracy of the genetic code.

Other definitions of terms may appear throughout the specification. Before the exemplary embodiments are described in more detail, it is to be understood that this disclosure is not limited to particular embodiments described, and as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be defined only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value

in that stated range is encompassed within this disclosure. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within this disclosure, subject to any specifically excluded limit in the stated
5 range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in this disclosure.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for
10 example, reference to "an antibody" includes a plurality of such antibodies and reference to "the antigen" includes reference to one or more antigens and equivalents thereof known to those skilled in the art, and so forth.

The publications discussed herein are provided solely for their disclosure prior to the filing
15 date of the present application. Nothing herein is to be construed as an admission that such publications constitute prior art to the claims appended hereto.

An "antibody" is a protein including at least one or two, heavy (H) chain variable regions (abbreviated herein as VHC), and at least one or two light (L) chain variable regions
20 (abbreviated herein as VLC). The VHC and VLC regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" (FR). The extent of the framework region and CDRs has been precisely defined (see, Kabat, E.A., et al. Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health
25 and Human Services, NIH Publication No. 91-3242, 1991, and Chothia, C. et al, J. Mol. Biol. 196:901-917, 1987). Preferably, each VHC and VLC is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FRI, CDRI, FR2, DR2, FR3, CDR3, FR4. The VHC or VLC chain of the antibody can further include all or part of a heavy or light chain constant region. In one embodiment, the antibody is a tetramer
30 of two heavy immunoglobulin chains and two light immunoglobulin chains, wherein the heavy and light immunoglobulin chains are interconnected by, e.g., disulfide bonds. The heavy chain constant region includes three domains, CH1, CH2 and CH3. The light chain constant region is comprised of one domain, CL. The variable region of the heavy and light chains contains a binding domain that interacts with an antigen. The term "antibody" includes intact
35 immunoglobulins of types IgA, IgG, IgE, IgD, IgM (as well as subtypes thereof), wherein the light chains of the immunoglobulin may be of types kappa or lambda. The term antibody, as

used herein, also refers to a portion of an antibody that binds to one of the above-mentioned markers, e.g., a molecule in which one or more immunoglobulin chains is not full length, but which binds to a marker. Examples of binding portions encompassed within the term antibody include (i) a Fab fragment, a monovalent fragment consisting of the VLC, VHC, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fc fragment consisting of the VHC and CH1 domains; (iv) a Fv fragment consisting of the VLC and VHC domains of a single arm of an antibody, (v) a dAb fragment (Ward et al, Nature 341:544-546, 1989), which consists of a VHC domain; and (vi) an isolated complementarity determining region (CDR) having sufficient framework to bind, e.g. an antigen binding portion of a variable region. An antigen binding portion of a light chain variable region and an antigen binding portion of a heavy chain variable region, e.g., the two domains of the Fv fragment, VLC and VHC, can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VLC and VHC regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 241:954-958; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also encompassed within the term antibody. These may be obtained using conventional techniques known to those skilled in the art, and the portions are screened for utility in the same manner as are intact antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the invention will now be described, by way of example only, with reference to the following Figures and Examples.

Figure 1 shows a schematic representation of a DABA assay. In this figure, both the solid phase and fluid phase antigens are represented as being identical. Embodiments of the present invention encompass a 'hybrid' DABA assay utilising two different proteins in each of the solid and fluid phases (hence the term hybrid). The hybrid DABA employs two different formulations where the only commonality between these two antigens, separately prepared so not sharing extraneous commonality, is the shared RBD epitope.

Figure 2 shows SDS-PAGE analysis of the purity of S1 and RBD proteins. A) SDS-PAGE analysis of three different batches of recombinant SARS-CoV-2 S1 (1-530) in a gel stained with Coomassie Blue. B) Size exclusion chromatography of S1; C) SDS PAGE analysis of three different batches of recombinant SARS-CoV-2 RBD; D) Size exclusion chromatography of RBD. Circle = A280; diamond = A260.

Figure 3 shows a scatter plot distribution of the S/CO of negative stored samples pre-dating SARS-CoV-2 epidemic evaluated by the SARS-CoV-2 Hybrid DABA.

Figure 4 shows a scatter plot distribution of the S/CO of seropositive samples evaluated by the SARS-CoV-2 Hybrid DABA.

Figure 5 shows ROC Curve analysis of SARS-CoV-2 Hybrid DABA.

Figure 6 shows a graphic representation of the comparison between SARS-CoV-2 Hybrid DABA and Abbot SARS-CoV-2 IgG.

Figure 7 shows correlation between the S/CO in Hybrid DABA and Abbot SARS-CoV-2 IgG.

Figure 8 shows a graphic representation of the comparison between SARS-CoV-2 Hybrid DABA, Abbot SARS-CoV-2 IgG and Fortress (e.g. Wantai) Total Antibody.

Figure 9 shows a Venn Diagram of positive samples in Hybrid DABA, SARS-CoV-2 Abbott IgG and Fortress total antibody. A) All seropositive samples; B) Samples obtained from RT PCR-confirmed individuals.

Figure 10 shows correlation between the S/CO in Hybrid DABA and A) Fortress total antibody; B) SARS-CoV-2 Abbott IgG.

Figure 11. Structures of SARS-CoV-2 spike-biliverdin (**a**, **b**) and spike-P008_056 Fab (**c**) complexes. (**a**) Cryo-EM 3D reconstructions of trimeric SARS-CoV-2 spike ectodomain in 3RBD-down (left) and 1RBD-up (right) conformations determined under saturation with biliverdin. Biliverdin is encircled, glycans are present at various positions with one being pointed to with an arrow. (**b**) Details of the biliverdin binding pocket in the crystal structure. SARS-CoV-2 NTD is shown as cartoons with selected amino acid residues and biliverdin in sticks. Carbon atoms of the protein chain, sugars (NAG), and biliverdin have different shadings, biliverdin is encircled. Dark grey dashes are hydrogen bonds.

Figure 12. UV-visible light absorption spectral properties of coronaviral spike antigen constructs. (**a**) WT SARS-CoV-2 S1 protein (corresponding to viral spike residues 1-530, produced by transient expression in suspension-adapted human embryonic kidney cells): analysis of three independent batches by SDS PAGE (*left*) and spectra of four independent S1 batches (250-800 nm, *right*). (**b-e**) Spectra of stabilised trimeric SARS-CoV-2 spike ectodomain (residues 1-1208), NTD (1-310), RBD (319-541), and biliverdin (**b**); SARS-CoV-1 S1 (residues 1-518), HCoV NL63 S1 (residues 1-664) and OC43 S1 (1-665) (**c**); SARS-CoV-2 S1 purified under acidic conditions in sodium acetate pH 5.2 or dialysed overnight against suspension of activated charcoal. Note the retention of biliverdin in dialysed sample, consistent with high affinity of the interaction at pH 8.0 (**d**); H207A, R190K and N121Q SARS-CoV-2 S1 (**e**), compared to a representative spectrum of WT SARS-CoV-2 S1 purified under standard conditions, shows as black lines. Spectra were acquired from proteins diluted to 1-1.2 mg/ml (in 150 mM NaCl, 1 mM EDTA, 20 mM Hepes-NaOH, pH 8.0) and normalised to absorption at 278 nm.

Figure 13. Representative SPR sensorgrams (**a-g**) and melting point analysis (**h**). The sensorgrams were recorded with WT (**a-e**), R190K (**f**), or N121Q (**g**) SARS-CoV-2 S1. The proteins were immobilised on a sensor chip and binding and dissociation of biliverdin IX alpha (**a, e-g**), bilirubin (**b**), hemin (**c**), and protoporphyrin IX (**d**) was measured. The analytes were injected at indicated concentrations at pH 8.0 (**a-d,f,g**) or pH 5.0. Estimated K_{ds} values are given in Table 6. Panel **h** shows melting behaviour of isolated SARS-CoV-2 NTD diluted to 30 mM in HBSE buffer (150 mM NaCl, 1 mM EDTA, 20 mM HEPES-NaOH, pH8.0) in the absence or presence of 100-1,000 mM biliverdin. The vertical axis corresponds to the first derivative of the ratios of fluorescence intensities measured at 350 and 330 nm wavelengths; the resulting melting points along with standard deviations ($n=4$) are given in the inset.

Figure 14. Pre- and post-vaccine anti-RBD levels as detected by the assay described herein, displayed in DABA binding ratios (BR) in 18 persons before and 23 persons following immunisation. Post vaccine samples were taken 14 or more days after the second dose of vaccine. Filled circles = 'Pfizer' vaccine, unfilled circles = 'AstraZeneca' vaccine. $P=0.00049$ (Wilcoxon matched pairs test) or $P<0.0001$ (Mann-whitney). Dotted line is the test cut off, lines are median value.

Figure 15. Titration of the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (NIBSC 20/136) in the DABA. Anti-RBD expressed as optical densities (OD). Dotted line is the assay cut off. Arrow marks the interpolated limit of detection 3 IU/ml. Error bars are SD.

Figure 16. Multiple samples from 10 COVIDITY patients over 16 weeks – All patients were seropositive initially but became seronegative for at least one subsequent sample during the follow up period, on WT S1 DABA. Panel A shows those samples which measured S/CO < 0.7 on WT S1 DABA. X axis is S/CO value for Hybrid DABA. Upper panel denotes individual samples, black is S1 blue is Mutant S1 (a.a. 1-530 of S1, but with N121Q), line denotes the cut-off. Lower panels combine all samples. Asterix displays significance compared to S1 calculated with Wilcoxon matched-pairs signed rank test.

Figure 17. Multiple samples from 10 COVIDITY patients over 16 weeks – All patients were seropositive initially but became seronegative for at least one subsequent sample during the follow up period, on WT S1 DABA. Panel shows those samples which measured S/CO 0.7-10 on WT S1 DABA. X axis is S/CO value for Hybrid DABA. Upper panels denote individual samples, black is S1 blue is Mutant S1, line denotes the cut-off. Lower panels combine all samples. Asterix displays significance compared to S1 calculated with Wilcoxon matched-pairs signed rank test.

Figure 18. Plots showing the follow up for 10 COVIDITY patients – All patients were seropositive initially but became seronegative for at least one subsequent sample during the

follow up period, on WT S1 DABA. S1 in black, Mutant in blue. Plots highlighted in box no longer become seronegative when tested with Mutant S1.

Figure 19. (A) Samples from patients. Panel A shows those samples which measured S/CO < 0.7 on WT S1 DABA. X axis is S/CO value for Hybrid DABA. Left panels denote individual samples, black is S1, blue is Mutant S1, line denotes the cut-off. Right panels combine all samples. Asterix displays significance compared to S1 calculated with Wilcoxon matched-pairs signed rank test. (B) Samples from patients. Panel B shows those samples which measured S/CO 0.7-5 on WT S1 DABA. X axis is S/CO value for Hybrid DABA. Left panels denote individual samples, black is S1, blue is Mutant S1, line denotes the cut-off. Right panels combine all samples. Asterix displays significance compared to S1 calculated with Wilcoxon matched-pairs signed rank test. (C) Samples from patients. Panel C shows those samples which measured S/CO >5 on WT S1 DABA. X axis is S/CO value for Hybrid DABA. Left panels denote individual samples, black is S1, blue is Mutant S1, line denotes the cut-off. Right panels combine all samples. Asterix displays significance compared to S1 calculated with Wilcoxon matched-pairs signed rank test.

MATERIALS AND METHODS

Pre-SARS-CoV-2 outbreak seronegative samples

To evaluate assay specificity, stored negative plasma and serum samples pre-dating the SARS-CoV-2 outbreak (n=825) were included in the study. These consisted of 1) plasma samples from blood donors kindly donated by NHSBT, Scotland (n=94); 2) stored serum samples from police officers from the REACT2 study (Imperial College London) (n=498); 3) plasma samples from antenatal screening donated by North West Pathology Service (n=100); 4) plasma collected from HTLV-1 infected patients from the National Centre for Human Retrovirology at Imperial College London (n=133). All samples were anonymously analysed.

SARS-CoV-2 seropositive samples

Assay sensitivity was tested on seropositive samples that included: 1) 103 serum samples from the REACT2 study (Imperial College London) – PCR confirmed infection, more than 21 days after symptoms onset and positive in an in-house tri-spike indirect immunoassay; 2) 51 serum samples from the Covidity study (Imperial College London), a longitudinal study of patients with suspected (later confirmed) COVID-19 infection. All patients had confirmed infection by PCR, were at least 14 days after symptoms onset and had antibodies detected by SARS-CoV-2 Abbot IgG by North West London Pathology; 3) 122 serum samples that

tested positive in the Fortress (Wantai) total antibody assay (samples taken from critical care staff).

SARS-CoV-2 antigens

5 SARS-CoV-2 Hybrid DABA of the present examples uses both S1 as the immobilising antigen and SARS-CoV-2 RBD as the second (labelled) antigen. The proteins were produced at the Francis Crick Institute. S1 antigen is used to coat the solid phase while RBD conjugated with horseradish peroxidase (HRP) is used as a revealing agent for captured antibodies.

10

The SARS-CoV-2 RBD and S1 constructs (the former used as the labelled antigen, the latter as the immobilising antigen), spanning SARS-CoV-2 S (NCBI reference NC_045512) residues 319-541 and 1-530, respectively, are produced with C-terminal twin Strep tags (Figure 1). The SARS-CoV-2 RBD construct has the sequence of SEQ ID NO.: 8 as
15 expressed, SEQ ID NO.: 9 as secreted and used in the challenging/ detecting step. The S1 construct has the sequence of SEQ ID NO.: 6 as expressed, and SEQ ID NO.: 7 as secreted and coated on the plate.

To prepare the construct(s), the corresponding codon-optimised DNA fragments were cloned
20 into mammalian expression vector pQ-3C-2xStrep (PubMed ID 31907454). A signal peptide from immunoglobulin kappa gene product directs secretion of the RBD construct. Expi293F cells (Thermo Fisher Scientific, Catalog number: A14528) growing at 37 °C in 5% CO₂ atmosphere in shaking flasks in FreeStyle 293 medium (Thermo Fisher Scientific, Catalog number: 12338001) were transfected with the corresponding plasmids using ExpiFectamine reagent (Thermo Fisher Scientific, ExpiFectamine™ 293 Transfection Kit, Catalog number:
25 A14525). Conditioned medium containing secreted proteins is harvested twice (3-4 and 6-8 days post-transfection). Twin Strep- and His6-tagged proteins are captured onto Streptactin XT (IBA LifeSciences) affinity resin, respectively, and purified to homogeneity by size-exclusion chromatography through Superdex 200 (GE Healthcare) in 150 mM NaCl, 1 mM
30 EDTA, 20 mM Tris-HCl, pH 8.0.

SARS-CoV-2 RBD conjugation was performed using LYNX Rapid HRP Conjugation kit (Bio-Rad Laboratories Ltd, Watford, UK, e.g. product code LNK001P) according to the manufacturer's instructions. Briefly, 10µL of modifier reagent was added to 100µL of antigen
35 (in an optimal concentration range of 0.5-5.0mg/ml). The mix was transferred to the lyophilized HRPO and incubated overnight. The quencher reagent was added to the antigen

mix (10µL). The conjugate was diluted 1:10 in HRP Stabilising Buffer (ClinTech, Guildford, UK) and stored at -20°C. Prior to use, RBD-HRP was diluted to a final working concentration of 1:5K in conjugate diluent (ClinTech, Guildford, UK).

5 Hybrid DABA Immunoassay

Solid phase 96 microwells plates (NUNC Immunomodule, U8 Maxisorp wells) were coated with 100µl of S1 antigen (SEQ ID NO.: 7) diluted in MicroImmune Coating Buffer at a concentration of 5µg/ml. The plates were incubated overnight at 2-8°C, followed by 3 hours at 35-37°C (under moist condition) and 1 hour at room temperature. Wells were washed
10 once with PBS Tween 20 (0.05%) and blocked with MicroImmune Blocking Solution (3-4 hours at 37°C in a moist box). Wells were aspirated and the plates were dried overnight at 37°C. They were stored dry at 4°C in sealed pouches with desiccant. The assay was performed by adding 50µl of sample diluent (MicroImmune Sample Diluent; ClinTech, Guildford, UK) to each well, followed by the addition of 50µl of controls and test sera to their
15 respective wells. Plates were incubated for 1 hour at 37°C followed by washing five times with ClinTech wash buffer (ClinTech, Guildford, UK). RBD-HRP conjugate was added (100µl) to the wells. After further incubation for 1 hour at 37°C the solid phase was washed five times and 100µl of TMB substrate added (ClinTech, Guildford, UK), incubated for 30 minutes at 37°C, when the reaction was stopped and measured at 450nm. The cut-off was established by
20 adding 0.1 to the average of optical density (OD) obtained for the negative controls, assayed in triplicate in each run. The signal/cut-off value for each sample was determined by dividing the sample's OD by the cut-off. A sample is considered positive if S/CO ≥ 1.

Buffers and reagents as typical in the field are employed additionally or alternatively, for
25 example as described in Cox KL, Devanarayan V, Kriauciunas A, et al. (Immunoassay Methods. 2012 May 1 [Updated 2019 Jul 8]. In: Sittampalam GS, Grossman A, Brimacombe K, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK92434/>), which describes coating buffer (e.g. 50 mM sodium bicarbonate, pH 9.6), blocking buffer (e.g. 1% BSA or 10% host serum in TBS, or
30 TBS with 0.05% Tween-20), wash buffer (e.g. PBST, 0.05% Tween-20), sample/antibody/antigen diluent (e.g. PBS with 0.05% Tween-20), HRP substrate (3, 3', 5,5'-tetramethyl benzidine), stop solution (e.g. 2M H₂SO₄).

35 Buffers are also available from ClinTech, Guildford, UK, e.g. sample diluent (Clin-tech cat. no. MI2040), wash buffer (Clin-tech cat. no. MI20024), conjugate diluent (Clin-tech cat. no.

100171), TMB substrate (Clin-tech cat. no. MI2030b), stop solution (Clin-tech cat. no. MI20031).

Positive control: serum positive for SARS-CoV-2 antibody and an antimicrobial agent (0.1% Bronidox). The positive control is a pool of seropositive samples. The positive pool is serial diluted in negative plasma until it reaches an optimal OD of approximately 1.5. The optimal dilution should be confirmed by a second independent run.

Negative control: serum negative for SARS-CoV-2 antibody and an antimicrobial agent (0.1% Bronidox).

Neutralization assay

Samples obtained from PCR confirmed patients, at least 21 days after symptoms onset (n=50) were tested in the Hybrid DABA and by pseudotype neutralization assay.

Any serum that inhibits the PV signal by more than 98% is deemed positive.

A setting of 25 samples were titrated and IC50 was calculated. Correlation between the S/CO obtained in the Hybrid DABA and the IC50 was calculated.

Assay comparative performance

Hybrid DABA performance was compared to the performance of SARS-CoV-2 Abbot IgG Assay and to the Fortress Total antibody. Both assays are commercial assays and were recently approved by Public Health England (PHE). Abbot SARS-CoV-2 IgG is an automated CE approved chemi-luminescence immunoassay (CMIA) that detects IgG to the N protein of SARS-CoV-2 in serum and plasma. Fortress total antibody is a double binding antigen assay that also targets RBD.

In an initial stage, Hybrid DABA was compared to SARS-CoV-2 Abbot IgG Assay using 100 samples from Covidity study, comprising patients with suspected (later confirmed) COVID-19. Later, to compare the performance between Hybrid DABA with the Fortress (total antibody) and SARS-CoV-2 Abbot IgG samples obtained from critical care staff were assayed in all three assays (n=596). The Hybrid DABA was performed at the Molecular Diagnostic Unit (MDU, Imperial College London), while SARS-CoV-2 Abbott IgG was performed by North West London Pathology and elsewhere and Fortress Total antibody was tested elsewhere. Both commercial assays were tested according manufacturers'

instructions. A sample is considered positive in SARS-CoV-2 Abbot IgG if it has a signal > 1.4, which is in effect a binding ratio. In Fortress, a sample with a S/CO < 0.9 is negative, while 0.9 - 1.1 is equivocal and those with S/CO > 1.1 are considered positive. The operators were blind for the serostatus.

5

The interpretation of discordant results was done as follows: 1) Equivocal samples in Fortress and negative in both other assays were considered negative; 2) the sample was considered positive if: had SARS-CoV-2 infection confirmed by RT-PCR or if the sample was positive in two assays; 3) Indeterminate: a sample reactive in only one out of three assays and donor was known to have been swab RT-PCR negative or data not available. Indeterminate samples were tested in two other in-house DABA assays, one targeting NP and the other one targeting Tri-Spike. If the indeterminate sample was positive in at least one further assay it was considered positive, if the sample was negative in both confirmatory assays it was deemed as negative.

15

The concordance between tests were analysed and described. Kappa index was calculated. The assay sensitivity, specificity, positive and negative predictive value, and accuracy was determined for each assay with this setting of samples.

20 **Protein expression and purification**

DNA fragments encoding SARS-CoV-2 S1 (Uniprot ID: P0DTC2; residues 1-530), NTD (1-310), RBD (319-541), SARS-CoV-1 S1 (Uniprot ID: P59594; residues 1-518), HCoV NL63 (Uniprot ID: Q6Q1S2; residues 1-618), HCoV OC43 (isolate LRTI_238, NCBI accession code KX344031; residues 1-619) were codon-optimised for expression in human cells and cloned into pQ-3C2xStrep vector under control of the cytomegalovirus (CMV) promoter for production of the recombinant proteins carrying a C-terminal extension containing human rhinovirus 14 3C protease recognition site followed by a TwinStrep tag. The signal peptide from immunoglobulin kappa gene product (METDTLLLWVLLLWVPGSTGD – SEQ ID NO.:10) was used to direct secretion of the RBD construct. The vector for production of the His₆-tagged stabilised trimeric SARS-CoV-2 has been described. Expression constructs encoding heavy and light chains of P008_056 Fab were made by inserting the respective coding sequences into pHLsec, including a sequence encoding a hexa-histidine (His₆) tag on the heavy chain fragment C-terminus.

35 With exception of trimeric stabilized SARS-CoV-2 spike ectodomain, the proteins were produced by transient transfection of Expi293 (Thermo Fisher Scientific) cells with endotoxin-

free preparations of the corresponding DNA constructs using ExpiFectamine293 (Thermo Fisher Scientific). The cells were maintained in shake flasks in FreeStyle293 (Thermo Fisher Scientific) medium at 37°C in humidified 5% CO₂ atmosphere. To produce SARS-CoV-2 S1 NTD fragment for crystallography, cell culture medium was supplemented with 5 µM kifunensine (Sigma-Aldrich) to suppress complex glycosylation. Conditioned medium containing recombinant products was harvested twice, 4- and 8-days post-transfection, or once, for production of the NTD and P008_056 Fab, 5 days post-transfection. For production of the trimeric SARS-CoV-2 spike ectodomain, Expi293 transfected with the pcDNA3-based expression construct were selected with 250 µg/ml geneticin. Stably transfected cells, grown to a density of 3.5 million per ml at 37°C, were shifted to 32°C for 3 days prior to harvesting conditioned medium to enhance secretion of the viral glycoprotein.

TwinStrep-tagged proteins were captured on Strep-Tactin XT (IBA LifeSciences) affinity resin. Following extensive washes in TBSE (150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 25 mM Tris-HCl, pH 8.0), the proteins were eluted in 1xBXT buffer (IBA LifeSciences). His₆-tagged proteins were captured on HisTrap Excel (Sigma-Aldrich) resin and eluted with 300 mM imidazole in phosphate buffered saline. For the use in crystallography, SARS-CoV-2 S1 NTD was digested with Endo Hf (New England Biolabs) and rhinoviral 3C protease to trim glycans and to remove the C-terminal twin Strep tag; Endo Hf was depleted by absorption to amylose resin (New England Biolabs). The proteins were further purified by size exclusion chromatography through a Superdex 200 16/600 column (GE Healthcare) in HBSE (150 mM NaCl, 1 mM EDTA, 20 mM Hepes-NaOH, pH 8.0) and concentrated by ultrafiltration using a Vivaspinn-20 with 10-kDa cut-off (Sartorius). To deplete biliverdin from SARS-CoV-2 S1, recombinant protein eluted from Strep-Tactin XT resin was supplemented with 0.5 M sodium acetate, pH 5.2 and subjected to size exclusion chromatography through a Superdex 200 16/600 column in 200 mM sodium acetate, pH 5.2; fractions containing S1 were pooled and dialyzed overnight against HBSE buffer.

Surface Plasmon Resonance (SPR)

Experiments were performed on a Biacore S200 (GE Healthcare); S1 protein, diluted to 50 µg/ml in 10 mM sodium acetate, pH 5.0, was injected into a CM5 sensor chip (Cytiva product code BR100530) to achieve immobilisation to a level of 4,000 response units. Analyte binding was studied in running buffer comprising 150 mM NaCl, 50 mM HEPES-NaOH, pH 8.0 or 50 mM BisTris-HCl, pH 5.0, 0.05% Tween-20, and 1% dimethyl sulfoxide (DMSO). Biliverdin, bilirubin, haem, and protoporphyrin were obtained from Sigma-Aldrich (product codes 3089, 14370, 51280, and P8293, respectively). Generally, analyte stock

solutions were prepared in DMSO prior to dilution in running buffer, maintaining the final DMSO concentration of 1%. The final analyte concentration was verified by spectrophotometry, using the following molar extinction coefficients: biliverdin 39,900 (at a wavelength of 388 nm), bilirubin 53,846 (460 nm), haem 58,440 (385 nm), and protoporphyrin IX 107,000 (407 nm). Alternatively, biliverdin, which is highly soluble at pH>7, was dissolved directly in running buffer, allowing to omit the solvent from the experiment. The presence of DMSO did not affect the observed K_d of S1-biliverdin interaction (Table 6). All experiments were conducted using a CM5-kinetics-multicycle template at 25°C. Flow rate was 30 µl/min with a contact time of 180 s, followed by a dissociation time of 10 min; three start-ups were performed at the beginning of each experiment. Solvent correction was deemed unnecessary for the assays that contained DMSO. Biliverdin displayed very fast association. Data were analysed using the affinity software tool to calculate estimated K_d values in equilibrium regime.

Protein thermostability assay

Biliverdin-depleted SARS-CoV-2 NTD (corresponding to spike residues 1-310) was diluted to 1 mg/ml in 150 mM NaCl, 20 mM HEPES-NaOH, pH 8.0 and supplemented with biliverdin from a 5-mM stock prepared in 100 mM Tris-HCl, pH8.0 where appropriate. Melting curves were recorded using 20–95 °C 1.5 °C/min temperature ramps on a Promethius NT.48 instrument (Nanotemper). Melting points were determined from inflection points of fluorescence intensity ratios (350 and 330 nm) using first derivative analysis.

Cryo-electron microscopy

Four µl stabilised trimeric SARS-CoV-2 spike ectodomain (0.6 mg/ml final concentration in TBSE supplemented with 0.1% n-octylglucoside) with 25 µM biliverdin or 0.2 mg/ml P008_056 Fab, was applied onto glow-discharged 200-mesh copper holey carbon R2/2 grids (Quantifoil) for 1 min, under 100% humidity at 20 °C, before blotting for 3-4 sec and plunge-freezing in liquid ethane using Vitrobot Mark IV (Thermo Fisher Scientific). The data were collected on Titan Krios microscopes operating at 300 keV (Thermo Fisher Scientific). Single particles of spike-biliverdin were imaged using a Falcon III direct electron detector (Thermo Fisher Scientific). A total of 15,962 movies were recorded with a calibrated pixel size of 1.09 Å and a total electron exposure of 33 e⁻/Å², spread over 30 frames in single electron counting mode. The spike-Fab complex was imaged on a GIF Quantum K2 detector with a post-column energy filter (Gatan), selecting a 20-eV window, in single electron counting mode. A total of 17,010 movies were collected with a pixel size of 1.38 Å and total electron exposure

of 51 e⁻/Å² spread over 40 frames. Both datasets were acquired with a defocus range of -1.6 to -4 μm .

Crystal Structure of the NTD in complex with biliverdin

5 Protein construct (spanning SARS-CoV-2 S1 residues 1-310) at 10 mg/ml was supplemented with 90 μM biliverdin before mixing with crystallization mother liquor in a 1:1 ratio. Plate-like crystals grew to 80-120 μm in two dimensions and ~10-20 μm in the third dimension in conditions containing 24% PEG 3350 (w/v) and 0.25 M NaSCN by hanging drop vapour diffusion over 1-2 weeks at 18°C. Crystals were cryoprotected by the addition of PEG 400 to
10 a final concentration of 30% (v/v) to the drop solution before flash freezing in liquid nitrogen.

X-ray diffraction data were collected at the PX1 beamline, Swiss Light Source, using wavelength 1 Å, 100% transmission, a 40-μm beam, 0.1-sec exposure and 0.5° rotation per image. Data were indexed, scaled and merged using XDS and Aimless via Xia2. SARS-CoV-
15 2 spike NTD (residues 14-290; PDB ID 6ZGE) was used as a model for molecular replacement and yielded a solution containing one NTD per asymmetric unit, with a log likelihood gain of 490 and translation function Z-score of 22.7, in space group C222₁ using Phaser within the Phenix package. The initial molecular replacement solution was subjected to morph model in Phenix before commencing with rounds of manual fitting in Coot and
20 refinement using phenix.refine, version 1.19rc4-4035. First, the protein chain was fitted and extended where possible, and refined, then glycosylation moieties were added where visualized in the positive *F_o-F_c* density, followed by conceivable PEG and water molecules. The electron density around the disulphide bonds suggested that they were labile and as such were modelled as alternative conformations between oxidized and reduced where
25 appropriate and the occupancy refined between these states. The stability of the disulphide bonds could have been affected by trace amounts of DTT introduced during the treatment of the protein with 3C protease and EndoH. The *R*_{free} and *R*_{work} were 21.5 and 18.5%, respectively, before a biliverdin molecule was fitted into the prominent positive difference density. The final refinement included four TLS groups (residues 14-67, 68-202, 203-278,
30 279-319) that had been segmented by the TLSMD server. All ligand geometry definition files were generated by Grade (Global Phasing) and model quality was assessed using Molprobit. The final model consists of spike residues 14-319, one biliverdin molecule, seven N-linked glycans (attached to asparagine residues at positions 17, 61, 122, 149, 165, 234, and 282), 10 PEG moieties, and 351 water molecules and has reasonable geometry and fit to the
35 electron density. The model and the associated X-ray diffraction data were deposited with the Protein Data Bank under accession code 7B62.

EXAMPLES

The preferred SARS-CoV-2 Double Antigen Bridging Assay (SCov2 DABA) has been designed to detect antibodies to the SARS-CoV-2 receptor binding domain (RBD) within the corona virus spike glycoprotein (NCBI reference NC_045512). The SARS-CoV-2 DABA is designed to be sensitive and specific using recombinant protein S1 antigen (The Francis Crick Institute) coated on the solid phase and recombinant RBD antigen (The Francis Crick Institute) conjugated to horse radish peroxidase (HRP).

In other words, the invention is directed to a SARS-CoV-2 Hybrid DABA designed to be sensitive and specific using recombinant protein S1 antigen coated onto the solid phase and recombinant RBD antigen conjugated to Horse Radish Peroxidase (HRP) in the fluid phase. The SARS CoV2 RBD and S1 constructs, spanning SARS CoV2 S (NCBI reference NC_045512) residues 319-541 and 1-530, respectively, are produced by The Francis Crick Institute with C-terminal twin Strep tags using the mammalian expression vector, pQ-3C-2xStrep (PubMed ID 31907454).

Test principle

The SARS-CoV-2 DABA is a three-step enzyme linked immunoassay that utilises a recombinant antigen pre-coated onto the polystyrene microwell solid phase, a second antigen of the same type, conjugated to the enzyme horseradish peroxidase (HRP), and a final enzyme reaction. In the first incubation, SARS-CoV-2 antibodies - if present - in the sample will be captured. Unspecific antibody is removed by the first wash step. Antibodies have more than one binding site, therefore the enzyme-conjugated antigen is able to bind to the captured antibody during the second incubation. Excess, unbound enzyme-conjugated antigen is removed in the second wash step. The presence of the antigen-antibody-antigen-enzyme immune complex is detected in the final step, an enzyme reaction. TMB Substrate is added to initiate this reaction. In the presence of peroxidase, TMB breaks down to form blue coloured products which change to yellow on adding the acid Stop Solution.

The yellow-coloured solution is measured using a photometric plate reader at 450 nm with background correction set between 620 and 650 nm. The presence of SARS-CoV-2 specific antibody is inferred by optical density values above the cut-off. The optical density is proportional to the amount of antibody present. Wells containing samples negative for RBD antibody remain colourless.

Immunoassay procedure

Instructions for the kit are as follows:

1. Remove and assemble the required number of microwell strips to perform the test. A minimum of 4 wells is needed for the controls which must be included in each test run.
- 5 Return unused microwell strips and the desiccant to the foil pouch and reseal.
2. Pipette 50 μ L of Sample Diluent into each well required.
3. Pipette 50 μ L of the Positive Control (Reagent 4) and Negative Control (Reagent 5) to each assigned well, one well for the Positive Control and three (3) wells for the Negative Control.
- 10 Pipette 50 μ L of each serum specimen to the assigned wells. Use a separate disposal pipette tip for each Sample, Negative Control and Positive Control to avoid cross contamination.
4. Place the microwell plate in a plastic bag (or else cover with lid or sealing tape) and mix gently by tapping the side of the plate strip holder.
5. Incubate at $37 \pm 2^\circ\text{C}$ in a moist chamber for 60 ± 2 minutes.
- 15 6. Wash wells five times with working strength Wash Buffer (see reagent preparation). The wash cycle is carried out as follows: aspirate the contents of the well and dispense 350 μ L/well of diluted wash buffer, leave to soak for approximately 30 seconds and aspirate. Repeat the wash cycle four further times. It is recommended to use an automatic plate washer for this procedure. Tap the wells dry face down onto absorbent paper.
- 20 7. Dilute the stock conjugate (e.g. stock conjugate/ labelled antigen) into the volume of conjugate diluent required for the number of wells to be tested, to provide Working Strength Conjugate. Pipette 100 μ L of Working Strength conjugate to each well, cover plate and incubate at $37 \pm 2^\circ\text{C}$ in a moist chamber for 60 ± 2 minutes.
8. Wash the wells five times with working strength Wash Buffer as in step 6.
- 25 9. Pipette 100 μ L of TMB Substrate (Reagent 8) to each well. Incubate for 30 ± 2 minutes, protected from strong light at room temperature ($18\text{-}25^\circ\text{C}$).
10. Pipette 100 μ L of Stop Solution (Reagent 9) to each well.
11. Within 10 minutes, read the optical densities (OD) at 450 nm in an ELISA plate reader. If the feature is available, set the reference wavelength between 620 and 650 nm.

30

Quality control

The optical density $\text{OD}_{450-620 \text{ nm}}$ of the Positive Control (PC) should be greater than 0.8 (preferably >1). The $\text{OD}_{450-620 \text{ nm}}$ of each of the three Negative Control (NC) wells should less than 0.1.

35

Interpretation of the results

Calculate the mean OD of the three Negative Control wells (NC_{mean}). If one of the three OD values differs by more than 30% from the NC_{mean} , omit it and re-calculate the mean value.

- 5 The Cut-Off value for the assay is $(NC_{mean} + 0.10)$.

The following criteria are used for a specimen to be identified as SARS-COV-2 Antibody Reactive, Non-Reactive or Equivocal.

10 SARS-COV-2 Antibody Reactive

Specimen OD $\geq (NC_{mean} + 0.10) \times 1.1$

SARS-COV-2 Antibody Non-Reactive

Specimen OD $\leq (NC_{mean} + 0.10) \times 0.9$

15

Equivocal for SARS-COV-2 Antibody

$(NC_{mean} + 0.10) \times 0.9 < \text{Specimen OD} < (NC_{mean} + 0.10) \times 1.1$

- 20 A sample giving an equivocal result is re-tested. If the equivocal status cannot be resolved on re-testing, follow up samples taken between 7 and 21 days after the initial sample are tested in parallel with a further re-test of the first sample. If an equivocal result is obtained on re-testing a follow up sample, it is reported as SARS-COV-2 Antibody Non-Reactive.

- 25 To interpret results across plates, optical densities should be normalised using the Cut-Off value as below,

Binding Ratio = Specimen OD / Cut-Off

In other words: each sample absorbance test OD result is valid if the Quality Control criteria are verified as below:

- 30 • The OD of each Negative Control must be less than 0.100
 • The OD of blank must be less than 0.100
 • The OD of each Positive Control should be greater than 0.8 (preferably >1.000)

- 35 Negative results ($S/CO \leq 1$): Samples giving an OD less or equal to the cut-off value are considered negative, that is, no anti-SARS-CoV-2 RBD antibodies have been detected using this kit.

Positive results ($S/CO > 1$): Samples giving OD greater than the cut-off value are positive for this assay, that is, antibodies to SARS-CoV-2 RBD have been detected.

Results are considered to be equivocal in the OD range 0.86 to 1.0. In these cases, samples are retested either by the same or by another assay and an explanation for the assay selected is provided.

EXAMPLE 1 – Demonstrating anti-RBD SARS-Cov-2 antibody detection

Antigens Purity and quality control

10 The proteins were more than 99% pure when evaluated by SDS PAGE and Coomassie Blue stain (Figure 2) and had a single peak upon separation of 100µg protein by size exclusion chromatography through an analytical Superdex-200 increase column in HBSE buffer (10 mM HEPES, 150 mM NaCl, 2 mM EDTA pH 7.4).

Assay specificity

15 All 825 stored samples were negative ($S/CO < 1$) (Figure 3). Therefore, the assay specificity was 100%. With a 95% confidence interval, the assay sensitivity is 99.8% (98.8-100%).

Assay sensitivity

20 Three out of 276 (seropositive) samples were negative in the Hybrid DABA, being 2/3 with a S/CO of 0.9 and one with a S/CO of 0.7, giving a sensitivity of 98.91% (96.8-99.8%) (Figure 4).

The above-mentioned samples were included in a ROC analysis using the MedCalc program
25 ($n=276$ seropositives; $n=825$ seronegatives). The ROC curve analysis showed that with a S/CO of 0.86 the assay has 100% of specificity and 99.6% of sensitivity with this setting of samples (Figure 5).

Neutralization activity

30 Forty-eight out of 50 PCR-confirmed samples were positive in the Hybrid DABA and had neutralizing activity. The Hybrid DABA S/CO and the respective neutralization percentual for these two samples were: 0.9 and 49.7%; 0.5 and 0%.

A titration was performed for an additional setting of nine samples. There was a strong
35 correlation between S/CO obtained in Hybrid DABA and the half maximal inhibitory concentration (IC_{50}) ($r = 0.885$, $p = 0.003$) (Table 1).

Table 1. SARS-CoV-2 Hybrid DABA S/CO and Pseudotype Neutralization assay.

Sample ID	Pseudotype Neutralization Assay (IC50)	SARS-CoV-2 Hybrid DABA S/CO
Sample 1	3819.8	17.5
Sample 2	1763.9	17.2
Sample 3	119.2	6.2
<u>Sample 4</u>	9.3	0.4
Sample 5	74.9	4.5
Sample 6	353.5	11.8
<u>Sample 7</u>	10	0.4
<u>Sample 8</u>	10	0.4
Sample 9	392.7	3.4

* Negatives samples are underlined in blue

EXAMPLE 2 – Comparison of anti-RBD SARs-Cov-2 antibody detection hybrid DABA vs Abbot and Fortress assays

Hybrid DABA versus SARS-CoV-2 Abbot IgG: Initial comparison

At the initial comparative analysis 23/100 samples were concordantly negative by both assays and 63 concordantly positive. Fourteen samples gave discordant results. Hybrid DABA did not detect three samples that was detected by Abbot. Two of the three had S/CO=0.9 in the Hybrid DABA. Abbott anti-NP did not detect 11 samples that were detected in the Hybrid DABA, or whom 10/11 had PCR confirmed infection. The remaining serum donor had not been tested, despite being symptomatic.

There was a significant correlation between S/CO in Hybrid DABA and Abbot SARS-CoV-2 IgG (Figure 7). There was a strong agreement between these two assays.

Large scale performance comparison Hybrid DABA versus Wantai (total antibody) Assay and SARS-CoV-2 Abbot IgG

Among 596 samples (samples from critical care staff) analysed in all three assays, 76.5% (456) were concordantly negative, 18.3% (109) were concordantly positive and 4.9 % (29) had discordant results. Two samples (0.4%) were equivocal in Fortress and negative in Hybrid DABA and SARS-CoV-2 Abbott IgG, so they were considered negative and excluded from further analysis. Figure 9 shows a diagram regarding positive samples in these three different assays. Of 29 discordant samples, 22 were considered positive, seven were considered negative. Based on this classification, sensitivity, specificity, positive and negative predictive values, assay accuracy were calculated for each assay (Table 2). The

performance of Hybrid DABA was better, being similar to that of the Fortress total antibody. The sensitivity of SARS-CoV-2 Abbot IgG was lower (86%). This assay did not detect anti-SARS-CoV-2 NP IgG antibodies in 20 samples. From these, only 3 had detectable NP antibodies in the in-house NP DABA assay. On the other hand, all 20 samples had detectable S antibodies in at least two different assays.

Table 2.

	Hybrid DABA	Fortress Total Antibody	SARS-CoV-2 Abbott IgG
Sensitivity % (95%CI)	96.95 (92.4-99.2)	96.18 (91.3-98.7)	86.3 (79.2-91.6)
Specificity % (95%CI)	99.8 (98.8-100)	99.6 (98.4-99.9)	99.1 (97.8-99.8)
Positive Likelihood Ratio	448.9 (63-3180)	222.7 (56-888)	99.8 (37-265)
Negative Likelihood Ratio	0.03 (0.01-0.08)	0.04 (0.02-0.09)	0.14 (0.09-0.21)
Positive Predictive Value* % (95%CI)	99.1 (94.1-99.9)	98.2 (93.3-99.5)	96.1 (90.4-98.5)
Negative Predictive Value* % (95%CI)	99.2 (98-99.7)	99 (97.8-99.6)	96.6 (94.9-97.8)
Accuracy* % (95%CI)	99.2 (98.1-99.8)	98.9 (97.7-99.6)	96.6 (94.8-97.9)

* Considering a prevalence of 20%

Hybrid DABA had an almost perfect agreement with Fortress total antibody (Kappa Index = 0.96; 95%CI = 0.93-0.99) and also a very strong agreement with SARS-CoV-2 Abbott IgG (Kappa Index= 0.861; 95%CI = 0.81-0.91). The S/CO obtained in Hybrid DABA correlated with the S/CO of Fortress ($r=0.9654$) and with Abbott ($r=0.7835$) ($p<0.0001$) (Figure 10).

Considering only PCR-confirmed patients ($n=37$), Hybrid DABA (e.g. as per claim 1) detected 97.3% (36/37), followed by Fortress 94.6% (35/37) and Abbot IgG 91.9% (34/37). The RT-PCR confirmed sample that Hybrid DABA missed had a S/CO of 0.9 and was detected by SARS-CoV-2 Abbot IgG only (Figure 9). This is a clear demonstration that, as the tests are deployed at scale, the present invention will correctly detect much higher numbers of patients with Sars-Cov-2 antibody.

As can be seen from Table 2, not only does the hybrid DABA of the present invention show improved sensitivity vs the commercial assays, but also improved positive/negative predictive values and accuracy.

Take, for instance, the "sensitivity %". Table 3 below proposes a forecast of the number of cases detectable by each of these three serological tests, based on sensitivity %. As can be seen in the forecasting table below, as these kits are deployed at scale, the improved sensitivity of the assay of the present invention is expected to provide higher sensitivity compared to existing assays.

Table 3.

Number of Tests	Number cases detected				Additional cases detected by Hybrid DABA vs. Fortress
	Abbot (86.3%)	IgG	Fortress (96.18%)	Hybrid DABA (96.95%)	
1,000	863		961.8	969.5	7.7
10,000	8630		9618	9695	77
25,000	21575		24045	24237.5	192.5
50,000	43150		48090	48475	385
100,000	86300		96180	96950	770
500,000	431500		480900	484750	3,850
1,000,000	863000		961800	969500	7,700

Turning now to the “Positive Predictive Value %” (the probability that subjects with a positive screening test truly have the disease) the forecasting table below (Table 4) provides yet further demonstration of the significant advantages of the Hybrid DABA as these kits are deployed at scale.

Table 4.

Number of Tests	Positive Predictive Value				Difference: Hybrid DABA vs. Fortress
	Abbot (96.1%)	IgG	Fortress (98.2%)	Hybrid DABA (99.1%)	
1,000	961		982	991	9
10,000	9,610		9820	9910	90
25,000	24,025		24550	24775	225
50,000	48,050		49100	49550	450
100,000	96,100		98200	99100	900
500,000	480,500		491000	495500	4,500
1,000,000	961,000		982000	991000	9,000

Table 5 below proposes a forecast of the number of false-positive assay results (in the three middle columns) that are expected from each of these three serological tests, based on specificity %. As can be seen in the forecasting table below, as these kits are deployed at scale, the improved specificity of the assay of the present invention is expected to provide less false-positive results compared to existing assays, mitigating risk of providing an individual with erroneous belief that they have had the infection (which by extension may lead to erroneous belief that they potentially have immunity), the least desirable outcome.

Table 5.

Number of Tests	Assay specificity				Difference: Hybrid DABA vs. Fortress
	Abbot (99.1%)	IgG	Fortress (99.6%)	Hybrid DABA (99.8%)	
1,000	9		4	2	2
10,000	90		40	20	20
25,000	225		100	50	50
50,000	450		200	100	100
100,000	900		400	200	200
500,000	4,500		2,000	1,000	1,000
1,000,000	9,000		4,000	2,000	2,000

EXAMPLE 3 – Vaccine monitoring and WHO standard titration

The assay described herein was used to successfully detect antibodies to RBD of the spike protein in vaccinated individuals. This is demonstrated in Figure 14, which compares data from samples isolated from unvaccinated individuals with data from individuals that were vaccinated (with either the 'Pfizer' or 'AstraZeneca' vaccine). In more detail, pre- and post-vaccine anti-RBD levels (as detected by the assay described herein) are displayed in DABA binding ratios (BR) in 18 persons before and 23 persons following immunisation. Post vaccine samples were taken 14 or more days after the second dose of vaccine. Filled circles = 'Pfizer' vaccine, unfilled circles = 'AstraZeneca' vaccine. $P = 0.00049$ (Wilcoxon matched pairs test) or $P < 0.0001$ (Mann-whitney). Dotted line is the test cut off, lines are median value.

The assay described herein was also used successfully to titrate the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (NIBSC 20/136) (see Figure 15, where anti-RBD levels are expressed as optical densities (OD); dotted line is the assay cut off; and an arrow marks the interpolated limit of detection 3 IU/ml. Error bars are SD). Anti-RBD levels detected by the present assay can thus be expressed in the 'WHO' units.

EXAMPLE 4 - Mutation of amino acids in S1 biliverdin binding pocket leads to improved performance of S1 mutant in DABA assay

It will now be demonstrated biliverdin binding masks the antigenic properties of the viral spike, which masking can be suppressed by conservative substitution of amino acids in biliverdin binding pocket of S1.

It is remarkable that a small molecule with a footprint of 370 Å², corresponding to only ~0.9% of solvent-exposed surface (per spike monomer), competes with a considerable fraction of spike-specific serum antibody population

- 5 In these experiments, the S1 construct used was amino acids 1-530 of the spike protein, with N121Q in the case of “mutant” S1. WT means this construct without substitution.

The S1 antigen was immobilised to the plate, and used to capture anti-SARS-CoV-2 serum antibodies. A horseradish peroxidase labelled RBD (amino acids 319-541 of the spike
10 protein) was used to detect antibodies. The (immobilised) S1 construct used was amino acids 1-530 of the spike protein, with N121Q in the case of “mutant” S1. WT means this construct without substitution.

The mutant S1 was used as an immobilised capture antigen in a DABA assay (explained
15 under materials and methods above, heading “Double antigen bridging assay (DABA) assay”).

Two sets of patient samples were used, including COVIDITY samples. The COVIDITY samples are 56 samples taken from 10 different patients across a 19 week period. All
20 patients were seropositive initially, but all subsequently became seronegative on the standard S1 DABA (e.g. otherwise equivalent assay, but using non-mutated S1).

As can be seen from Figures 16-19, assay performance was improved with the mutant S1.

25 **EXAMPLE 5 – Demonstrating biliverdin binding masks the antigenic properties of the viral spike**

The coronaviral spike glycoprotein is the dominant viral antigen and the target of neutralising antibodies. It is here shown that SARS-CoV-2 spike binds biliverdin, a tetrapyrrole product of haem metabolism, with nanomolar affinity in a pH-sensitive manner. Using cryo-electron
30 microscopy and X-ray crystallography we mapped the tetrapyrrole interaction pocket to a deep cleft on the spike N-terminal domain (NTD). At physiological concentrations, biliverdin significantly dampened the reactivity of SARS-CoV-2 spike with immune sera and inhibited a subset of NTD-specific neutralising antibodies. We show that access to the biliverdin-sensitive epitope is gated by a flexible loop on the distal face of the NTD. Accompanied by
35 profound conformational changes in the NTD, antibody binding requires relocation of the gating loop, which folds into the cleft vacated by biliverdin. Our results indicate that the virus

co-opts the haem metabolite for the evasion of humoral immunity via allosteric shielding of a sensitive epitope.

Trimeric coronaviral spike glycoproteins form prominent features on viral particles that are responsible for the attachment to a receptor on the host cell and, ultimately, fusion of the viral and cellular membranes. Encoded by a single viral gene, the mature spike glycoprotein comprises two subunits, S1 and S2, which mediate binding to the receptor and facilitate fusion, respectively. The recognition of the betacoronavirus SARS-CoV-2 host receptor, the cellular membrane protein angiotensin-converting enzyme 2, maps to the S1 C-terminal domain (referred to as the receptor binding domain, RBD), while the function of the N-terminal domain (NTD) remains enigmatic. Both S1 domains can be targeted by potent neutralising antibodies that arise in infected individuals. The majority of characterized neutralizing antibodies bind the RBD, while minimal structural information exists about neutralizing epitopes on the NTD.

Example 5.1 – S1 interacts with biliverdin

The immune properties of the spike glycoprotein underpin ongoing SARS-CoV-2 vaccine development efforts. Spike-derived antigens allow for specific detection of antibodies to SARS-CoV-2 on the background of recurrent infections with seasonal coronaviruses. In the course of our activities to support SARS-CoV-2 serology, we produced a range of recombinant coronaviral spike antigens by expression in human cell lines. Surprisingly, preparations of SARS-CoV-2 trimeric spike and S1 carried a distinct green hue, with prominent peaks at ~390 and 670 nm in their light absorbance spectra (Fig. 12a). These unusual features were also evident in spectra of isolated SARS-CoV-2 S1 NTD, but not RBD (Fig. 12b). In contrast, recombinant S1 constructs derived from the seasonal alphacoronavirus NL63 or the betacoronavirus OC43 did not absorb visible light (Fig. 12c). The spectra of SARS-CoV-2 spike constructs were consistent with biliverdin (Fig. 12b), a product of haem metabolism responsible for coloration of bruises and green jaundice. Biliverdin is produced at the first step of haem detoxification by oxygenases and is then reduced to bilirubin, the final product of tetrapyrrole catabolism in humans. We followed tetrapyrrole binding to immobilised SARS-CoV-2 S1 using surface plasmon resonance (SPR) and estimated the dissociation constant (K_d) for the interaction (biliverdin) at 9.8 ± 1.3 nM (Fig. 13; Table 6). Bilirubin and haem bound S1 considerably more weakly, with apparent K_d s of 720 ± 240 nM and 6.9 ± 1.2 μ M, respectively, while no interaction was observed with protoporphyrin IX (Fig. 13, Table 6).

Table 6. Affinity of SARS CoV2 S1 interaction with tetrapyrroles measured using surface plasmon resonance.

S1 protein	Ligand	Buffer ¹	Kd (nM) ²	Mean Kd (nM) ³
WT	Biliverdin	Hepes pH 8.0	8.0; 9.2	9.8 ±1.3
WT	Biliverdin	1% DMSO, Hepes pH 8.0	10.6; 11.5	
WT	Biliverdin	1% DMSO, Hepes pH 7.0	13.5; 14.5	
WT	Biliverdin	1% DMSO, BTP pH 6.0	13.6; 14.2	
WT	Biliverdin	1% DMSO, BTP pH 5.5	34.5; 29.9	
WT	Biliverdin	1% DMSO, BT pH 5.0	186.8; 194.1; 358.5	250 ±100
WT	Bilirubin	1% DMSO, Hepes pH 8.0	540; 620; 1,000	720 ±250
WT	Hemin	1% DMSO, Hepes pH 8.0	5,800; 6,640; 8,200	7,000 ±1,200
WT	Protoporphyrin	1% DMSO, Hepes pH 8.0	>10,000	
R190K	Biliverdin	Hepes pH 8.0	1,500; 2,230	
N121Q	Biliverdin	Hepes pH 8.0	16,800; 19,200	

¹Running buffer variables (DMSO and pH) are shown; full composition is given in Methods section. BTP, BisTris Propane; BT, BisTris.

²Results of individual experiments performed in equilibrium mode.

³Mean and standard deviation shown for $N > 2$ measurements.

Example 5.2 – revealing structural basis for S1-biliverdin interaction

Next, we imaged single particles of the trimeric SARS-CoV-2 spike ectodomain in the presence of excess biliverdin using cryo-electron microscopy. Image processing resulted in 3D reconstruction of closed (3RBDs-down) and partially open (1RBD-up conformation) states of the spike at 3.35 and 3.50 Å resolution, respectively (Fig. 11a). Close inspection of the cryo-EM maps revealed features interpretable as a biliverdin molecule buried within a deep cleft on one side of each of the NTD domains (Fig. 11a). Unidentified entities at this position can be observed in prior cryo-EM reconstructions, presumably obtained with partial occupancy by the metabolite. To define the structural basis for the interaction more precisely, we co-crystallised the isolated NTD with biliverdin and determined the structure at 1.8 Å resolution (Fig. 11b). The metabolite fits snugly into the cleft with the pyrrole rings B and C buried inside and propionate groups appended to rings A and D projecting toward the outside. The pocket is lined by hydrophobic residues (Ile101, Trp104, Ile119, Val126, Met177, Phe192, Phe194, Ile203, and Leu226), which form van der Waals interactions with the ligand. Biliverdin packs against His207, which projects its Nε2 atom towards pyrrolic amines, approaching three of them at ~3.6 Å. Pyrroles A and B are involved in a π - π stacking with side chain of Arg190, which is stabilised by hydrogen bonding with Asn99. Ligand binding largely buries the side chain of Asn121, which makes a hydrogen bond with the lactam group of pyrrole D.

Example 5.3 – conservative substitution of amino acids in biliverdin binding pocket of S1 suppresses the interaction between S1 and biliverdin

The presence of a histidine residue in the biliverdin binding pocket suggested that the interaction may be pH-dependent. In agreement with this hypothesis, the K_d of the S1-biliverdin interaction increased to $250 \pm 100 \mu\text{M}$ at pH 5.0 (Fig. 13; Table 6), and purification under acidic conditions greatly diminished the biliverdin content of recombinant SARS-CoV-2 S1 (Fig. 12d). Substitutions of spike residues closely involved in ligand binding (H207A, R190K and N121Q) diminished pigmentation of purified recombinant protein (Fig. 12e). The biliverdin binding affinity of SARS-CoV-2 S1 was reduced by two and three orders of magnitude by the R190K and N121Q amino acid substitutions, respectively (Fig. 13, Table 6).

Example 5.4 - the structural basis for SARS-CoV-2 neutralisation by a biliverdin-sensitive antibody

Access to the epitope is gated by a solvent-exposed loop composed of predominantly hydrophilic residues ("gate", SARS-CoV-2 spike residues 174-188; Fig. 11). To allow antibody binding, it is believed the loop swings out of the way. The gating mechanism is controlled by insertion of Phe175 and Met177, which are located in the beginning of the loop, into the hydrophobic pocket vacated by biliverdin (Fig. 11). Antibody binding is additionally accompanied by an upward movement of a β -hairpin ("lip", SARS-CoV-2 residues 143-155), which overlays a cluster of aromatic residues (Fig. 11).

Summary/ Discussion of Example 5

It is well-established that viruses employ extensive glycosylation of their envelopes to shield antibody epitopes from recognition by humoral immunity, and ~40% of SARS-CoV-2 spike surface is covered by glycans. Here, we identified and structurally characterised a novel class of a neutralizing epitope, present on SARS-CoV-2 S1, which is differentially exposed through recruitment of a metabolite. In contrast to glycosylation, co-opting a metabolite may allow conditional unmasking, for example under acidic conditions within the endosomal compartment. Biliverdin is the product of haem metabolism, and its concentration in the lung can be expected to drastically increase as Covid-19 progresses to erythrocyte damage. Biliverdin levels in plasma of healthy individuals ($0.9\text{-}6.9 \mu\text{M}$) and more so under pathological conditions ($>50 \mu\text{M}$) greatly exceed the K_d of its interaction with the spike ($\sim 10 \text{ nM}$) and are therefore sufficient to affect SARS-CoV-2 antigenic properties and neutralisation. It would be of great interest to determine the levels of biliverdin and the related tetrapyrroles in lung

tissue and how these may change during the course of mild versus severe disease. This is particularly important given the role of biliverdin in upregulating IL-10, one of the major markers of severe COVID-19. Finally, our results suggest that controlling biliverdin levels in vaccines and serological assay reagents may substantially alter outcomes.

5

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

15

SEQUENCES

Where an initial Met amino acid residue or a corresponding initial codon is indicated in any of the following SEQ ID NOs, said residue/codon is optional.

20

SEQ ID NO. 1 (SARS-Cov-2 spike protein, NCBI Reference Sequence: YP_009724390.1)

25

MFVFLVLLPLVSSQCVNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFSNV
TWFHAIHVSGTNGTKRFDNPVLPFNDGVYFASTEKSNIIRGWIFGTTLDSTQSLIVNNATN
VVIKVCEFQFCNDPFLGVYYHKNNKSWMESEFRVYSSANNCTFEYVSQPFLLMDLEGKQGN
FKNLREFVFKNIDGYFKIYSKHTPINLVRDLPPQGSALPLVDLPIGINITRFQTLALHRSYLT
PGDSSSGWTAGAAAYYVGYLQPRFTLLKYNENGTITDAVDCALDPLSETKCTLKSTVEKGI
YQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSASF
STFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAW
NSNNLDSKVGGNYNYLYRLFRKSNLKPFRDISTEIYQAGSTPCNGVEGFNCYFPLQSYGF
QPTNGVGYQPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTGVLTESNKK
FLPFQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITPGTNTSNQVAVLYQDVNCTEVP
VAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEHVNNSECDIPGAGICASYQTQTNPPRR
ARSVASQSIAYTMSLGAENSVAYSNNISAIPTNFTISVTTEILPVSMTKTSVDCTMYICGDSTE
CSNLLLQYGSFCTQLNRALTGIAVEQDKNTQEVFAQVKQIYKTPPIKDFGGFNFSQILPDPSK
PSKRSEFIEDLLFNKVTADAGFIKQYGDCLGDIAARDLCAQKFNGLTVLPPLLTDEMIAQYTS
ALLAGTITSGWTFGAGAAALQIPFAMQMAYRFNGIGVTQNVLYENQKLIANQFNSAIGKIQDSL
SSTASALGKLQDVVNQNAQALNTLVKQLSSNFGAISSVLNDILSRDLKVEAEVQIDRLITGRL
QSLQTYVTQQLIRAAEIRASANLAATKMSECVLGQSKRVDFCGKGYHLMSFPQSAPHGVVF
LHVTYVPAQEKNFTTAPAICHGDKAHFPREGVVFVSNGTHWFTQRNFYEPQIITDNTFVSG
NCDVVIGIVNNTVYDPLQPELDSFKEELDKYFKNHTSPDVDLGDIGINASVUNIQKEIDRLNE
VAKNLNESLIDLQELGKYEQYIKWPWYIWLGFIAGLIAIVMVTIMLCCMTSCCSCLKGCCSCG
SCCKFDEDDSEPVLKGVKLHYT

40

SEQ ID NO. 2 (amino acid residues 15-530 of the SARS-Cov-2 spike protein)

45

CVNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFSNVTWFHAIHVSGTNGT
KRFDNPVLPFNDGVYFASTEKSNIIRGWIFGTTLDSTQSLIVNNATNVVIKVCEFQFCNDP

FLGVYYHKNNKSWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQGNFKNLREFVFKNIDG
YFKIYSKHTPINLVRDLPGGFSALEPLVDLPIGINITRFQTLALHRSYLT PGDSSSGWTAGAA
AYYVGYLQPRTFLLKYNENGTITDAVDCALDPLSETKCTLKSTVEKGIYQTSNFRVQPTESI
VRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLVNSASFSTFKCYGVSPTKLN
5 DLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNY
NYLYRLFRKSNLKPFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRV
VVLSFELLHAPATVCGPKKS

SEQ ID NO. 3 (amino acid residues 319-541 of the SARS-Cov-2 spike protein)

RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLVNSASFSTFKCY
GVSPTKLNLDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLD
SKVGGNYYLYRLFRKSNLKPFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQPTNGV
15 GYQPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNF

SEQ ID NO. 4 (scaffold sequence, amino acid residues 15-318 of the SARS-Cov-2 spike protein)

CVNLTTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFSNVTWFHAIHVSGTNGT
KRFDNPVLPFNDGVYFASTEKSNIIRGWIFGTTLDSTQSLIVNNATNVVIKVFCEFCNDP
20 FLGVYYHKNNKSWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQGNFKNLREFVFKNIDG
YFKIYSKHTPINLVRDLPGGFSALEPLVDLPIGINITRFQTLALHRSYLT PGDSSSGWTAGAA
AYYVGYLQPRTFLLKYNENGTITDAVDCALDPLSETKCTLKSTVEKGIYQTSNF

SEQ ID NO. 5 (binding RBD antigen, amino acid residues 319-530 of the SARS-Cov-2 spike protein)

RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLVNSASFSTFKCY
GVSPTKLNLDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLD
SKVGGNYYLYRLFRKSNLKPFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQPTNGV
25 GYQPYRVVLSFELLHAPATVCGPKKS

SEQ ID NO. 6 (S1 Construct sequence, including N-term signal peptide and C-term TwinStrep tags)

MFVFLVLLPLVSSQCVNLTTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFSNV
TWFHAIHVSGTNGTKRFDNPVLPFNDGVYFASTEKSNIIRGWIFGTTLDSTQSLIVNNATN
35 VVIKVFCEFCNDPFLGVYYHKNNKSWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQGN
FKNLREFVFKNIDGYFKIYSKHTPINLVRDLPGGFSALEPLVDLPIGINITRFQTLALHRSYLT
PGDSSSGWTAGAAAYYVGYLQPRTFLLKYNENGTITDAVDCALDPLSETKCTLKSTVEKGI
YQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLVNSASF
STFKCYGVSPTKLNLDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAW
40 NSNNLDSKVGGNYYLYRLFRKSNLKPFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGF
QPTNGVGYQPYRVVLSFELLHAPATVCGPKKSgssglevlfqpgpgssggsawshpqfekggsgsgsgg
sawshpqfek

SEQ ID NO. 7 (S1 Construct sequence, without N-term signal peptide and with C-term TwinStrep tags)

CVNLTTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFSNVTWFHAIHVSGTNGT
KRFDNPVLPFNDGVYFASTEKSNIIRGWIFGTTLDSTQSLIVNNATNVVIKVFCEFCNDP
FLGVYYHKNNKSWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQGNFKNLREFVFKNIDG
YFKIYSKHTPINLVRDLPGGFSALEPLVDLPIGINITRFQTLALHRSYLT PGDSSSGWTAGAA
45 AYYVGYLQPRTFLLKYNENGTITDAVDCALDPLSETKCTLKSTVEKGIYQTSNFRVQPTESI
VRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLVNSASFSTFKCYGVSPTKLN
DLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNY
NYLYRLFRKSNLKPFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRV
VVLSFELLHAPATVCGPKKSgssglevlfqpgpgssggsawshpqfekggsgsgsggsawshpqfek

SEQ ID NO. 8 (RBD second antigen construct, including N-term signal peptide and C-term TwinStrep tags)

METDTLLLWVLLLWVPGSTGdaaqpattgRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAW
 NRKRISNCVADYSVLYNSASFSTFKCYGVSPSTKLNDLCFTNVYADSFVIRGDEVQRQIAPGQT
 GKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSNLKPFRDISTEIQAGS
 TPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVLSFELLHAPATVCGPKKSTNLVKNK
 CVNFGssglevlfqgpgsggsawshpqfekgggsgggsggsawshpqfek

SEQ ID NO. 9 (RBD second antigen construct, without N-term signal peptide and with C-term TwinStrep tags)

aaqpattgRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRRKRISNCVADYSVLYNSASF
 TFKCYGVSPSTKLNDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAW
 NSNNLDSKVGGNYNYLYRLFRKSNLKPFRDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQ
 PTNGVGYQPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFGssglevlfqgpgsggsawshpqf
 ekgggsgggsggsawshpqfek

SEQ ID NO. 10 (signal peptide)

METDTLLLWVLLLWVPGSTGD

SEQ ID NO. 11 (TwinStrep tags)

gssglevlfqgpgsggsawshpqfekgggsgggsggsawshpqfek

SEQ ID NO. 12 (the wild-type S1 subunit sequence of SARS-CoV-2 spike protein, NCBI Reference Sequence: YP_009724390.1) – the N-terminal methionine is optional

MFVFLVLLPLVSSQCVNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFSNV
 TWFHAIHVSGTNGTKRFDNPVLPFNDGVYFASTESNIIRGWIFGTTLDSTQSLIVNNATN
 VVIKVCEFCNDPFLGVYYHKNNKSWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQGN
 FKNLREFVFKNIDGYFKIYSKHTPINLVRDLPQGFSALEPLVDLPIGINITRFQTLALHRSYLT
 PGDSSSGWTAGAAAYVGYLQPRTFLLKYNENGITDAVDCALDPLSETKCTLKSFTVEKGI
 YQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRRKRISNCVADYSVLYNSASF
 STFKCYGVSPSTKLNDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAW
 NSNNLDSKVGGNYNYLYRLFRKSNLKPFRDISTEIQAGSTPCNGVEGFNCYFPLQSYGF
 QPTNGVGYQPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTGVLTESNKK
 FLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFSGGVSVITPGTNTSNQVAVLYQDVNCTEVP
 VAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEHVNNSECDIPIGAGICASYQTQTNSPRR
 AR

CLAIMS

1. A method for detecting the presence or absence of SARS-Cov-2 antibodies in a sample, the method comprising:

5 a. contacting the sample with a solid-phase support having a fusion protein immobilised thereto, said fusion protein comprising a first antigen and a scaffold polypeptide,

i. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and

10 ii. wherein the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support;

b. allowing SARS-Cov-2 antibodies present in the sample to bind to the first antigen, thereby forming a complex of first antigen and SARS-Cov-2 antibody;

15 c. contacting said complex with a labelled second antigen, wherein said labelled second antigen comprises a SARS-Cov-2 receptor binding domain polypeptide that binds SARS-Cov-2 antibody;

d. allowing said labelled second antigen to bind to SARS-Cov-2 antibody present in the sample;

20 e. removing labelled second antigen that is not bound to said complex; and

f. detecting the presence of labelled second antigen bound to said complex;

wherein the presence of labelled complex indicates the presence of SARS-Cov-2 antibody in the sample, and wherein the absence of labelled complex indicates the absence of SARS-Cov-2 antibody in the sample.

25 2. The method according to claim 1, wherein the fusion protein is provided by a modified SARS-CoV-2 spike protein S1 subunit (S1) polypeptide having a modified amino acid sequence relative to a wild-type S1 sequence (SEQ ID NO: 12);

30 the modified S1 polypeptide comprising at least one amino acid residue change located within a tetrapyrrole binding pocket for binding to a tetrapyrrole compound;

wherein said at least one amino acid change is at a location on the modified S1 polypeptide sequence that corresponds to an amino acid location, of wild-type S1 (SEQ ID NO: 12), comprising: N99, I101, W104, I119, N121, V126, F175, M177, 35 R190, F192, F194, I203, H207 and/or L226; and

wherein the modified S1 polypeptide demonstrates lower binding affinity for said tetrapyrrole compound compared with wild-type S1.

3. The method according to claim 1 or claim 2, wherein the first antigen is covalently
5 fused to the scaffold polypeptide.
4. The method according to any one of the preceding claims, wherein the second antigen is lacking the scaffold polypeptide.
- 10 5. The method according to any one of the preceding claims, wherein the scaffold polypeptide is of 100-400 amino acids in length, preferably 150-350 amino acids in length, more preferably 175-325 amino acids in length.
6. The method according to any one of the preceding claims, wherein the scaffold
15 polypeptide is 10-50 kDa, or 15-45 kDa, or 20-40 kDa.
7. The method according to any one of the preceding claims, wherein the scaffold polypeptide comprises (or consists of) amino acid residues 15-318 of the SARS-Cov-2 spike protein (SEQ ID NO.: 1).
20
8. The method according to claim 7, wherein the first antigen comprises (or consists of) amino acid residues 319-530 of the SARS-Cov-2 spike protein, such that the fusion protein comprises (or consists of) amino acid residues 15-530 of the SARS-Cov-2 spike protein (SEQ ID NO.: 2).
25
9. The method according to any one of the preceding claims, wherein the labelled second antigen comprises (or consists of) amino acid residues 319-541 of the SARS-Cov-2 spike protein (SEQ ID NO.: 3) conjugated to a label.
- 30 10. The method according to any one of the preceding claims, wherein the labelled second antigen is present in a fluid phase.
11. The method according to any one of the preceding claims, further comprising a step of contacting said complex with a third antigen from a non-SARS-Cov-2 species, wherein
35 the binding of said third antigen thereto suppresses (e.g. blocks) any inherent antigenic binding cross-reactivity towards the non-SARS-Cov-2 species;

wherein the second and third antigens are inter-species homologs of the same polypeptide/ protein.

12. The method according to claim 11, wherein the contacting steps are carried out simultaneously or sequentially, preferably wherein the contacting steps are carried out simultaneously.

13. The method according to claim 11 or 12, wherein the third antigen is unlabelled.

14. The method according to any one of claims 11-13, wherein the third antigen is from a coronavirus selected from 229E (alpha coronavirus), NL63 (alpha coronavirus), OC43 (beta coronavirus), and HKU1 (beta coronavirus).

15. The method according to any one of the preceding claims, further comprising recording the output of said method on a data readable format.

16. A kit for detecting the presence or absence of antibody to SARS-Cov-2 in a sample, the kit comprising:

a. a solid-phase support having a fusion protein immobilised thereto, said fusion protein comprising a first antigen and a scaffold polypeptide,

i. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and

ii. wherein the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support; and

b. a labelled second antigen, wherein said labelled second antigen comprises a SARS-Cov-2 receptor binding domain polypeptide that binds SARS-Cov-2 antibody.

17. An immunoassay solid-phase support for detecting the presence or absence of antibody to SARS-Cov-2 in a sample, the solid-phase support comprising a fusion protein immobilised thereto, said fusion protein comprising a first antigen and a scaffold polypeptide,

a. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and

- b. the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support.

18. The kit or solid-phase support according to any one of claims 16-17, wherein the scaffold polypeptide comprises (or consists of) amino acid residues 15-318 of the SARS-Cov-2 spike protein (SEQ ID NO.: 1).

19. The kit or solid-phase support according to claim 18, wherein the first antigen comprises (or consists of) amino acid residues 319-530 of the SARS-Cov-2 spike protein, such that the fusion protein comprises (or consists of) amino acid residues 15-530 of the SARS-Cov-2 spike protein (SEQ ID NO.: 2).

20. The kit or solid-phase support according to any one of claims 16-19, wherein the labelled second antigen comprises (or consists of) amino acid residues 319-541 of the SARS-Cov-2 spike protein (SEQ ID NO.: 3) conjugated to a label.

21. The kit according to any one of claims 16 or 18-20, further comprising instructions for use.

22. A method for manufacturing a solid-phase support for detecting the presence or absence of antibody to SARS-Cov-2 in a sample, the method comprising immobilising a fusion protein to a solid-phase support, said fusion protein comprising a first antigen and a scaffold polypeptide,

- a. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and
- b. the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support.

23. Use of solid-phase support for detecting antibody to SARS-Cov-2 in a sample, the solid-phase support comprising a fusion protein immobilised thereto, said fusion protein comprising a first antigen and a scaffold polypeptide,

- a. wherein the first antigen is a SARS-Cov-2 receptor binding domain polypeptide that binds anti-SARS-Cov-2 antibody, and
- b. the scaffold polypeptide immobilises the fusion protein to the solid-phase support and presents the first antigen away from the solid-phase support.

24. The method according to any one of claims 2-15, wherein the at least one amino acid change is at a location that corresponds to an amino acid location, of wild-type S1 (SEQ ID NO: 12), comprising N99, W104, I119, N121, V126, F175, M177, R190, F192, H207 and/or L226.

5

25. The method according to any one of claims 2-15 or claim 24, wherein the at least one amino acid change is at a location that corresponds to an amino acid location, of wild-type S1 (SEQ ID NO: 12), comprising N121, R190, and/or H207.

10 26. The method according to any one of claims 2-15, 24 or 25, wherein the at least one amino acid residue change comprises:

15

i. an amino acid residue selected from the group consisting of glutamine, aspartate and glutamate at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N99 of the wild-type S1 (SEQ ID NO: 12);

ii. an amino acid residue selected from the group consisting of glycine, alanine, valine, and leucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue I101 of the wild-type S1 (SEQ ID NO: 12);

20

iii. an amino acid residue selected from the group consisting of phenylalanine, and tyrosine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue W104 of the wild-type S1 (SEQ ID NO: 12);

25

iv. an amino acid residue selected from the group consisting of glycine, alanine, valine, and leucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue I119 of the wild-type S1 (SEQ ID NO: 12);

30

v. an amino acid residue selected from the group consisting of glutamine, aspartate and glutamate at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12);

35

vi. an amino acid residue selected from the group consisting of glycine, alanine, and leucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue V126 of the wild-type S1 (SEQ ID NO: 12);

- vii. an amino acid residue selected from the group consisting of tyrosine and tryptophan at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F175 of the wild-type S1 (SEQ ID NO: 12);
- 5 viii. an amino acid residue selected from the group consisting of serine, cysteine, selenocysteine, and threonine, at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue M177 of the wild-type S1 (SEQ ID NO: 12);
- 10 ix. an amino acid residue selected from the group consisting of a lysine and histidine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12); and/or
- 15 x. an amino acid residue selected from the group consisting of tyrosine and tryptophan at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F192 of the wild-type S1 (SEQ ID NO: 12);
- 20 xi. an amino acid residue selected from the group consisting of tyrosine and tryptophan at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue F194 of the wild-type S1 (SEQ ID NO: 12);
- 25 xii. an amino acid residue selected from the group consisting of glycine, alanine, valine, and leucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue I203 of the wild-type S1 (SEQ ID NO: 12);
- 30 xiii. an amino acid residue selected from the group consisting of an alanine, lysine, arginine, glycine, valine, leucine and isoleucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12); and/or
- 35 xiv. an amino acid residue selected from the group consisting of glycine, alanine, valine, isoleucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue L226 of the wild-type S1 (SEQ ID NO: 12).
27. The method according to any one of claims 2-15 or 24-26, wherein the at least one amino acid residue change comprises:

- 5 i. an amino acid residue selected from the group consisting of glutamine, aspartate and glutamate at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12);
- ii. an amino acid residue selected from the group consisting of a lysine and histidine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12); and/or
- 10 iii. an amino acid residue selected from the group consisting of an alanine, lysine, arginine, glycine, valine, leucine and isoleucine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12).
- 15 28. The method according to any one of claims 2-15 or 24-27, wherein the at least one amino acid residue change comprises:
- i. a glutamine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12);
- 20 ii. a lysine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12); and/or
- iii. an alanine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12).
- 25 29. The method according to any one of claims 2-15 or 24-28, wherein the at least one amino acid residue change comprises:
- i. a glutamine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue N121 of the wild-type S1 (SEQ ID NO: 12).
- 30 30. The method according to any one of claims 2-15 or 24-29, wherein the at least one amino acid residue change comprises:
- i. a lysine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue R190 of the wild-type S1 (SEQ ID NO: 12); and/or
- 35

31. The method according to any one of claims 2-15 or 24-30, wherein the at least one amino acid residue change comprises:

- i. an alanine at the position on the modified S1 polypeptide sequence that corresponds to amino acid residue H207 of the wild-type S1 (SEQ ID NO: 12).

32. The method according to any one of claims 2-15 or 24-31, wherein the modified S1 comprises or consists of a sequence corresponding to amino acid residues 15-530 of the SARS-CoV-2 spike protein S1 subunit (SEQ ID NO: 12), in which said at least one amino acid residue change is present.

FIGURE 1

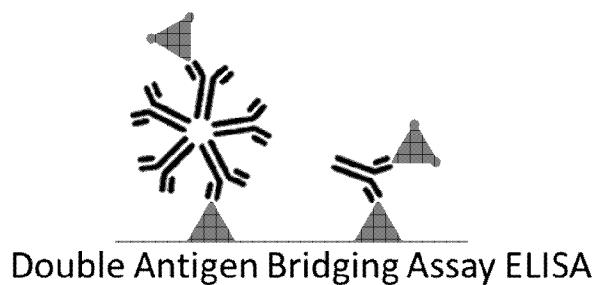


FIGURE 2

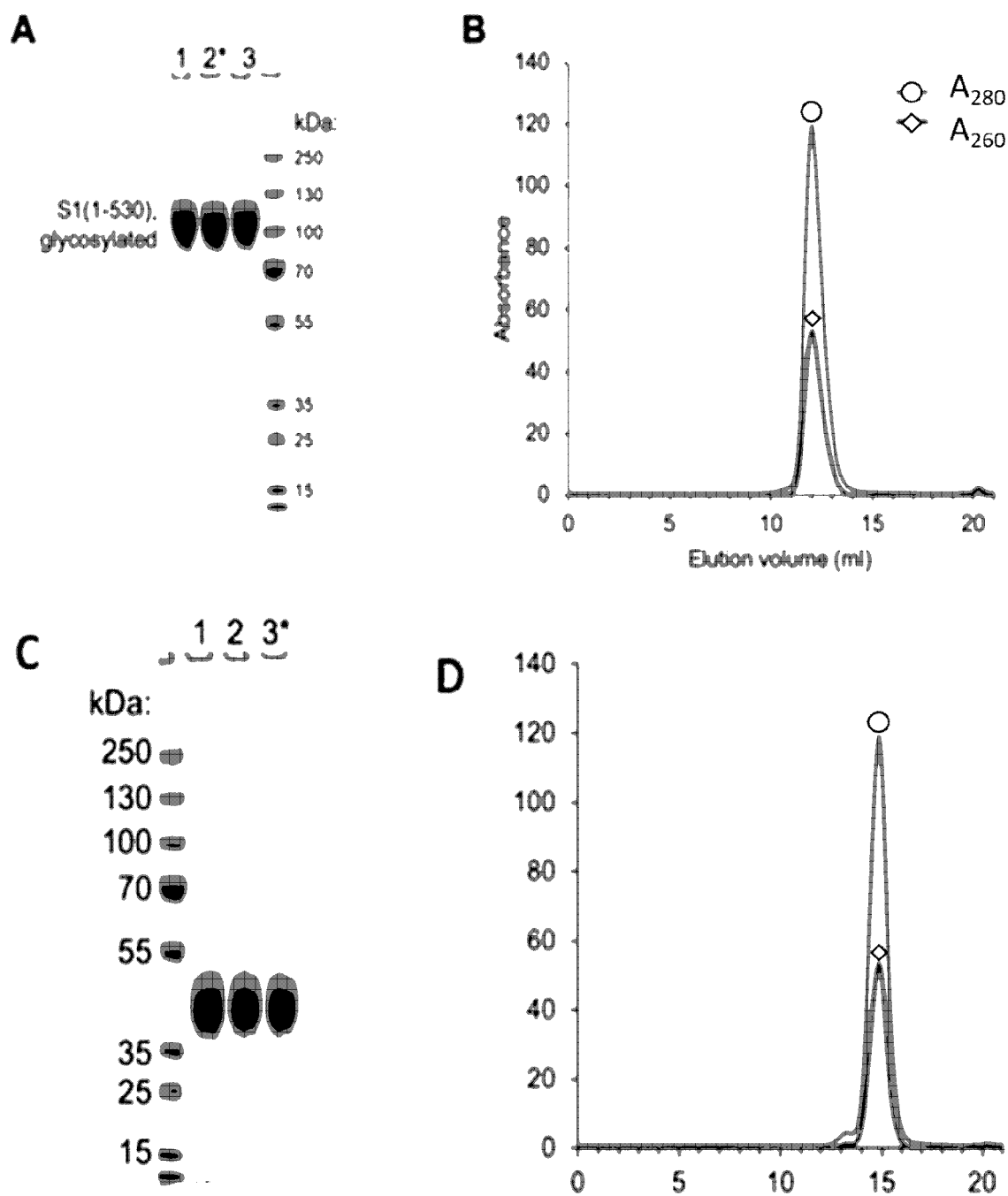


FIGURE 3

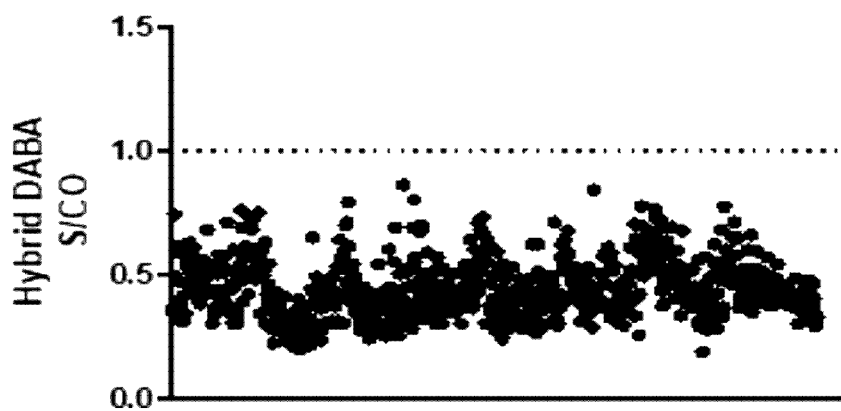


FIGURE 4

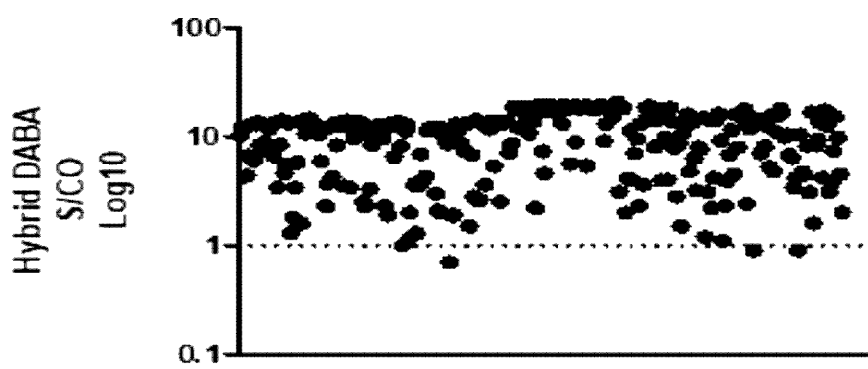


FIGURE 5

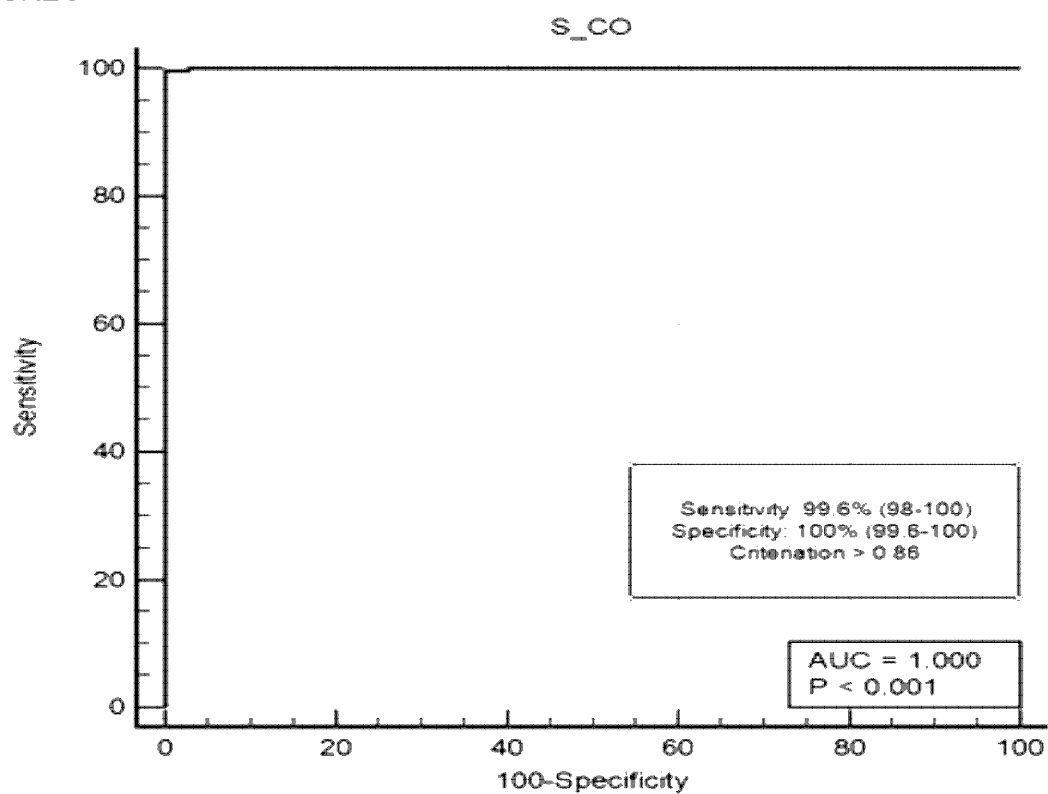


FIGURE 6

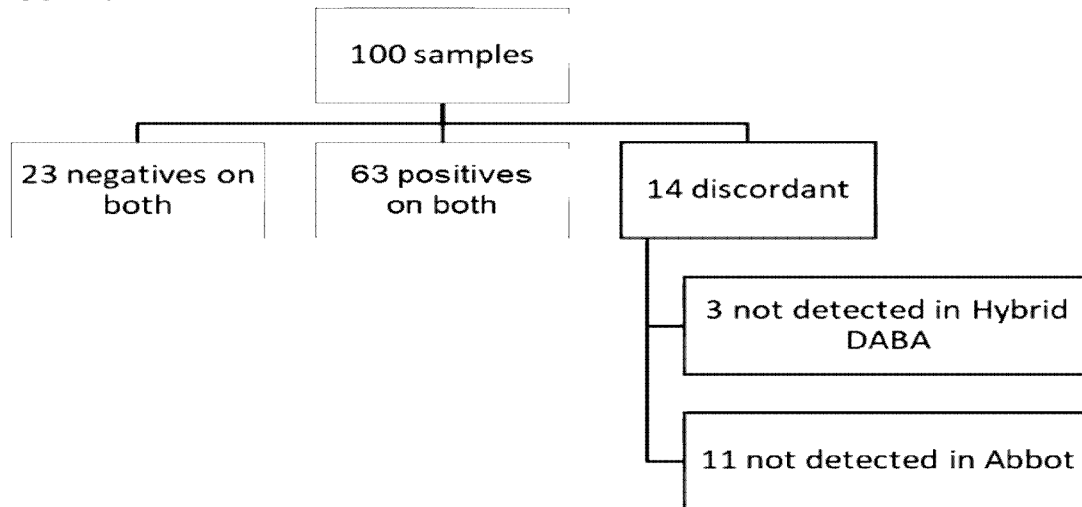


FIGURE 7

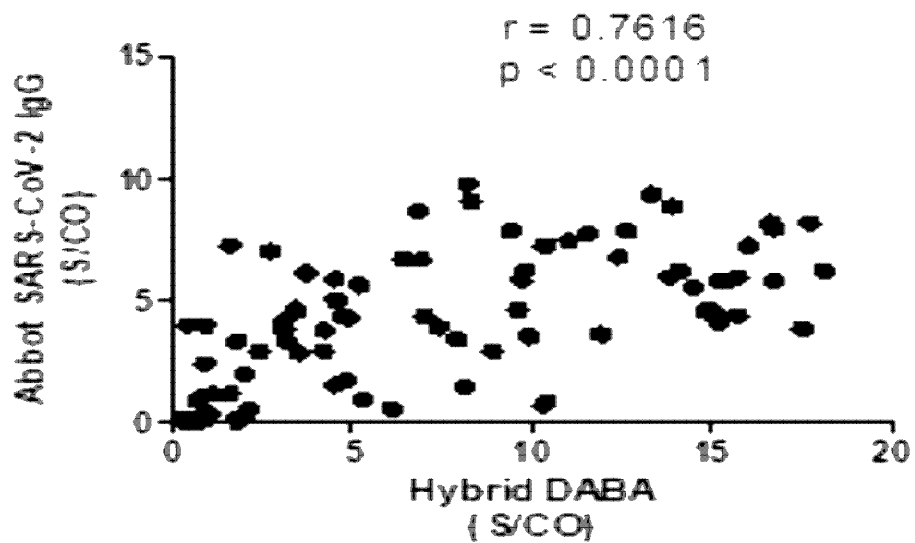


FIGURE 8

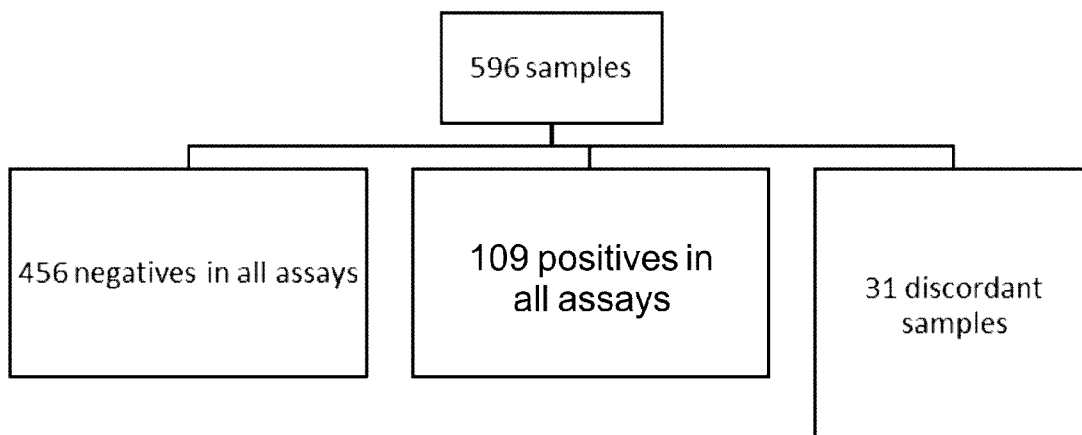


FIGURE 9

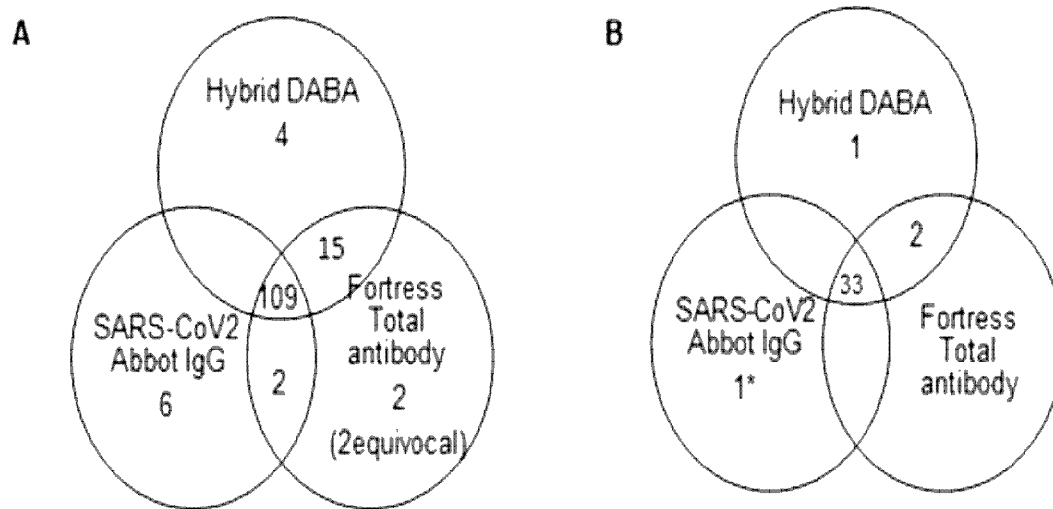


FIGURE 10

A

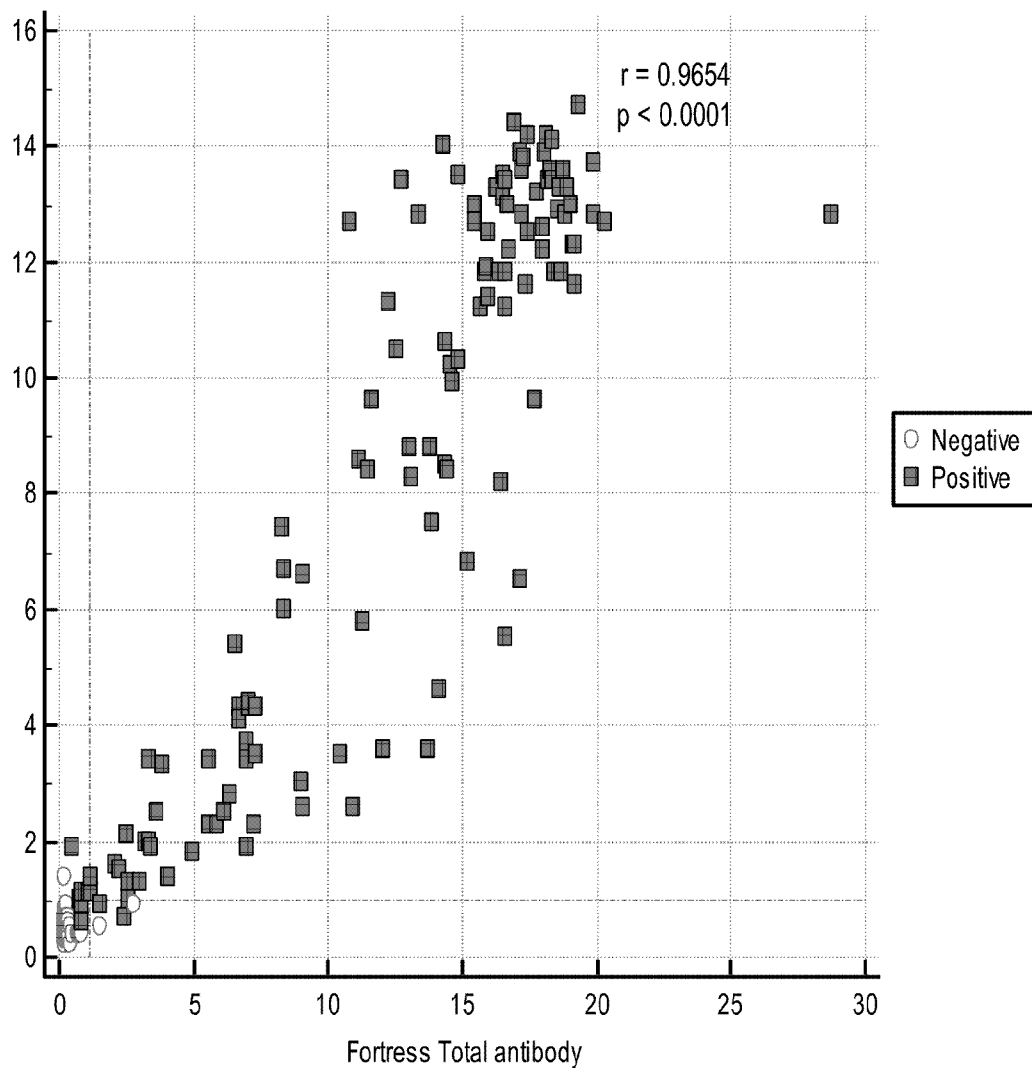


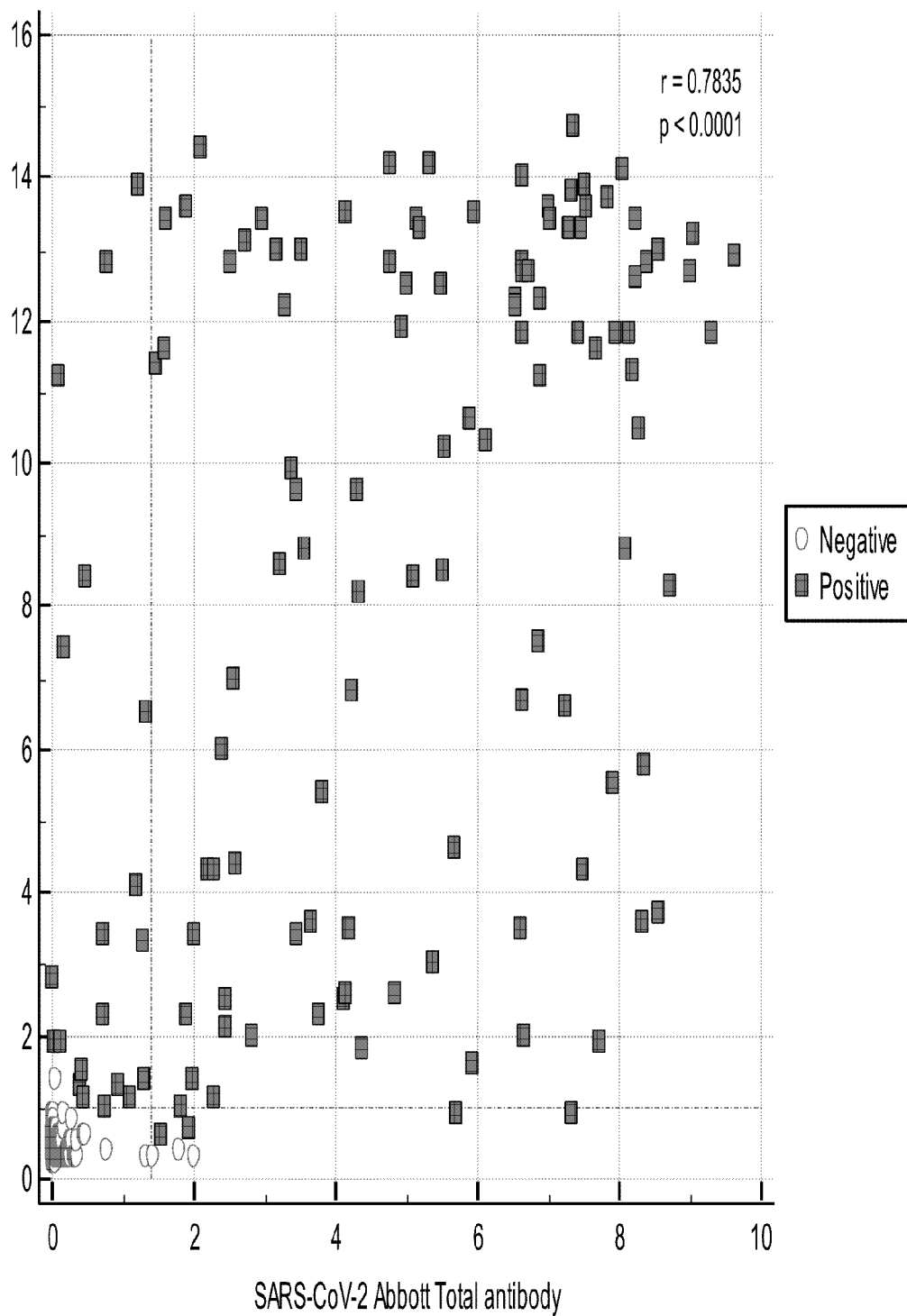
FIGURE 10 (continued)
B

FIGURE 11

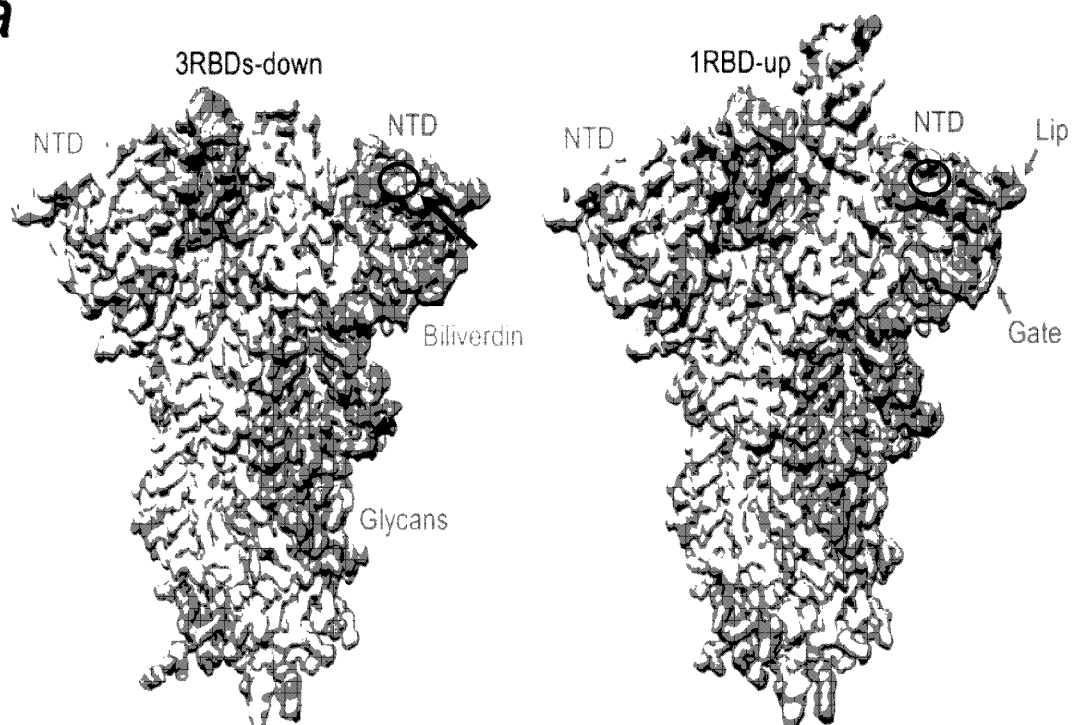
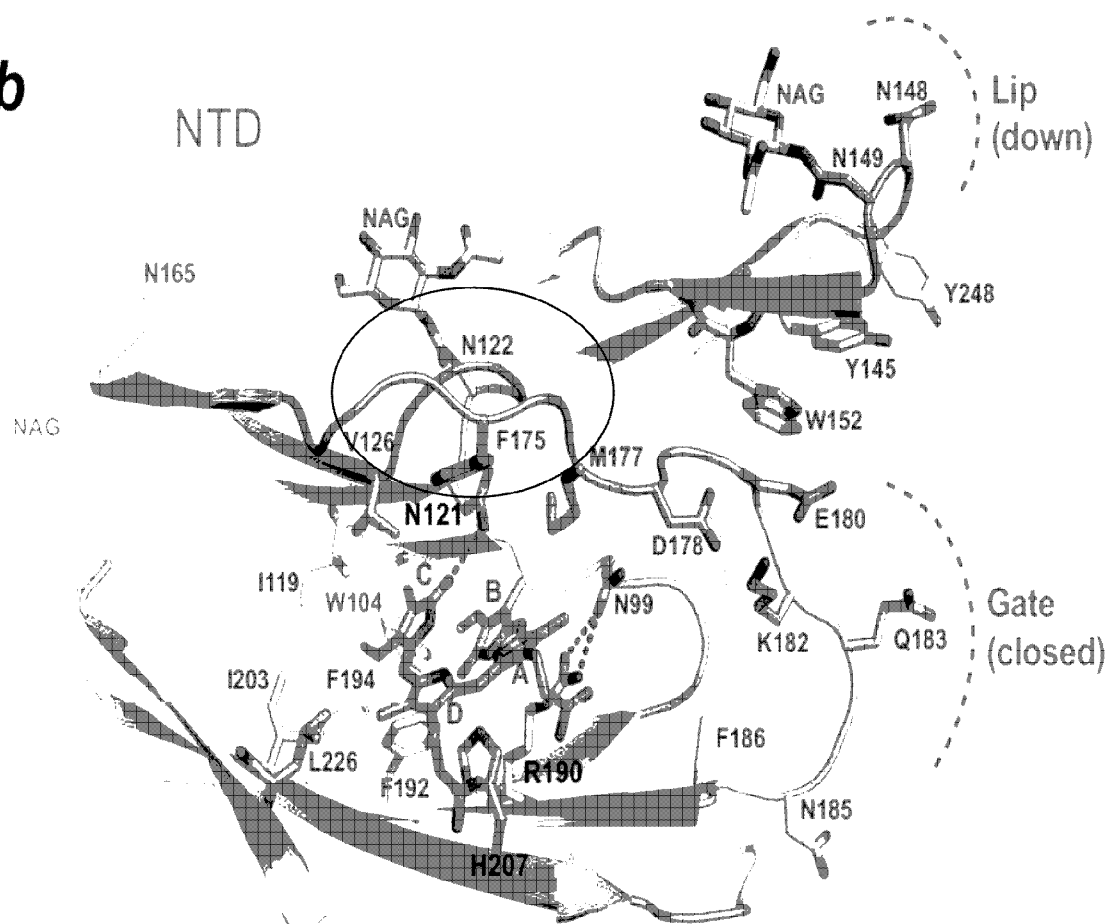
a**b**

FIGURE 12

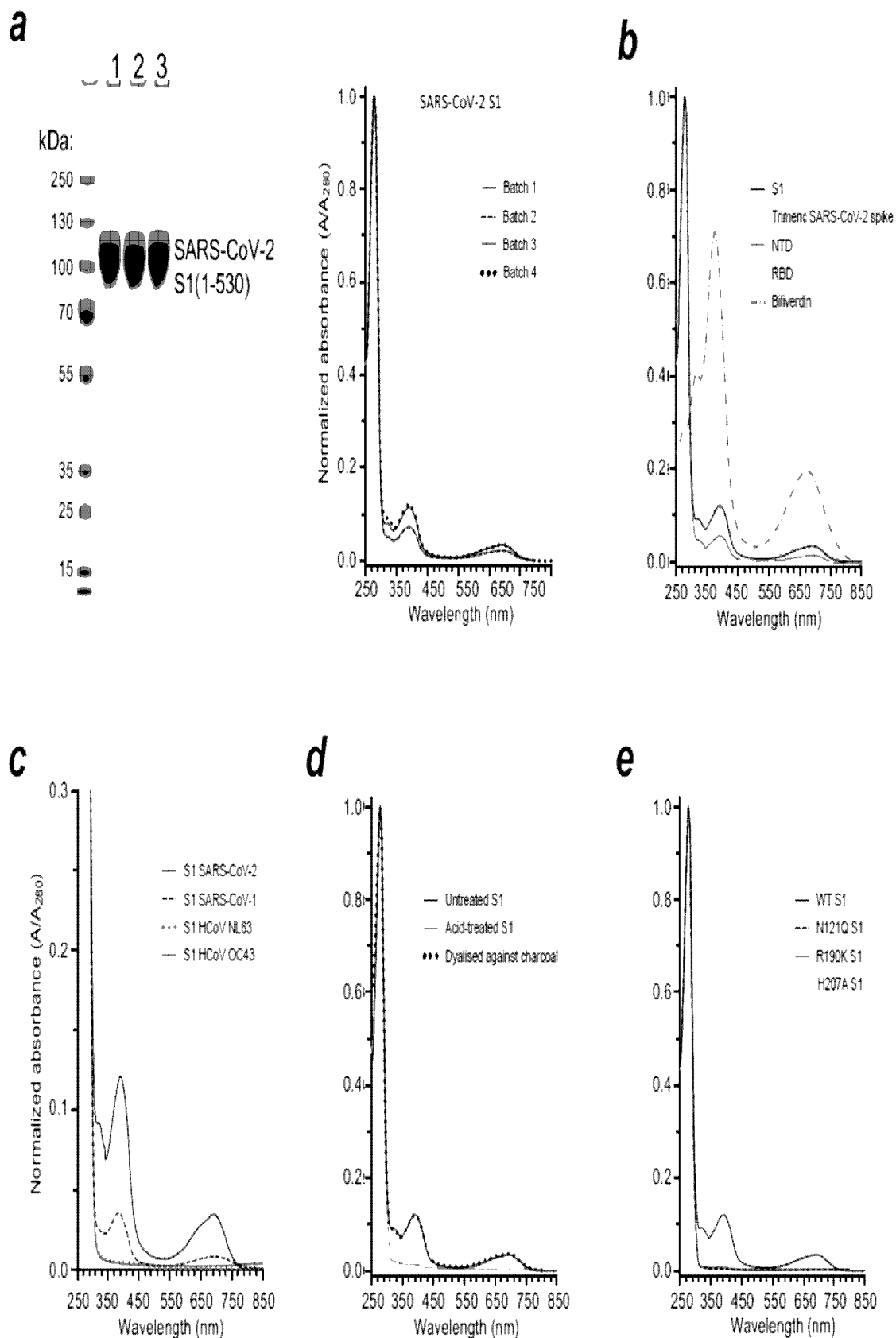


FIGURE 13

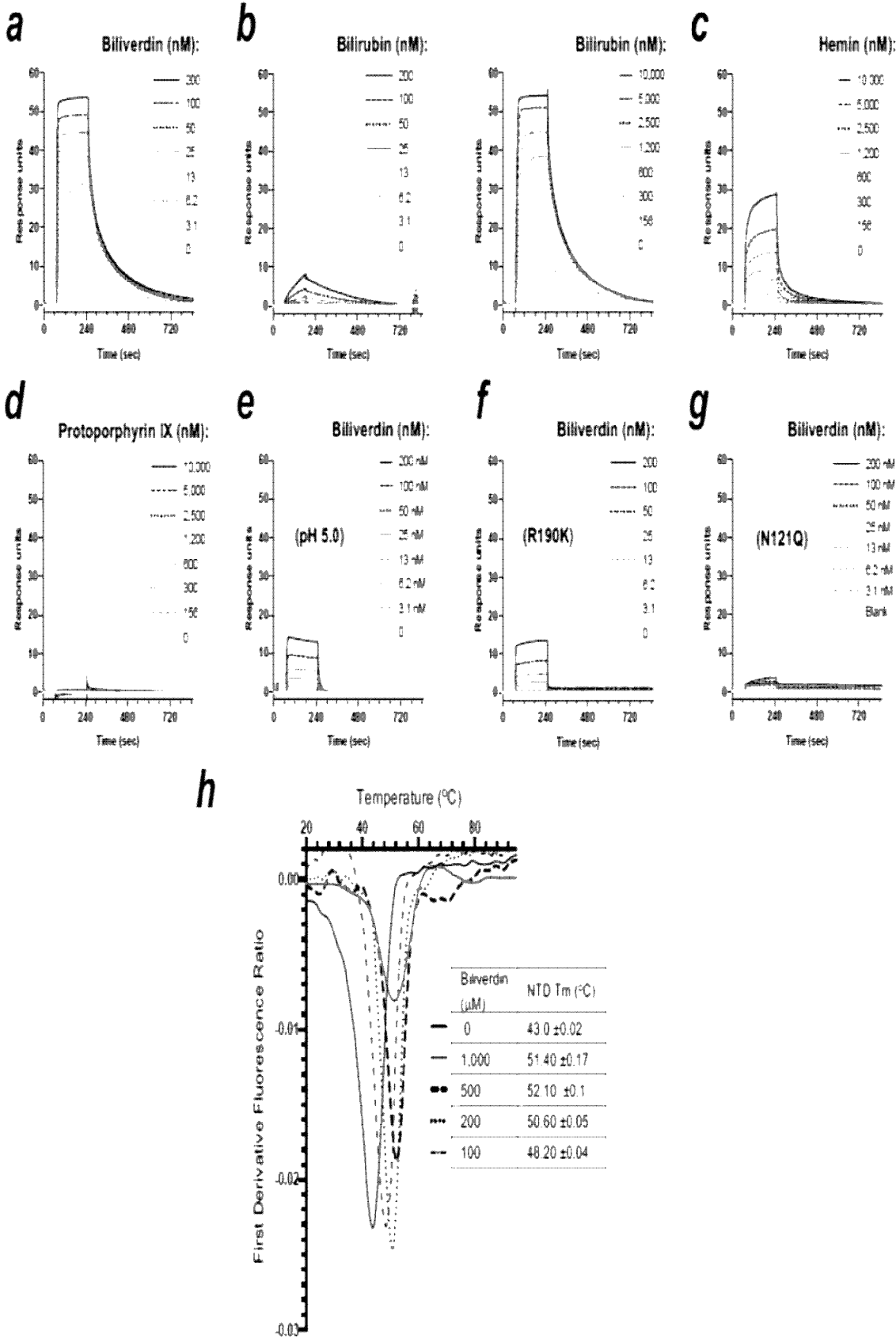


FIGURE 14

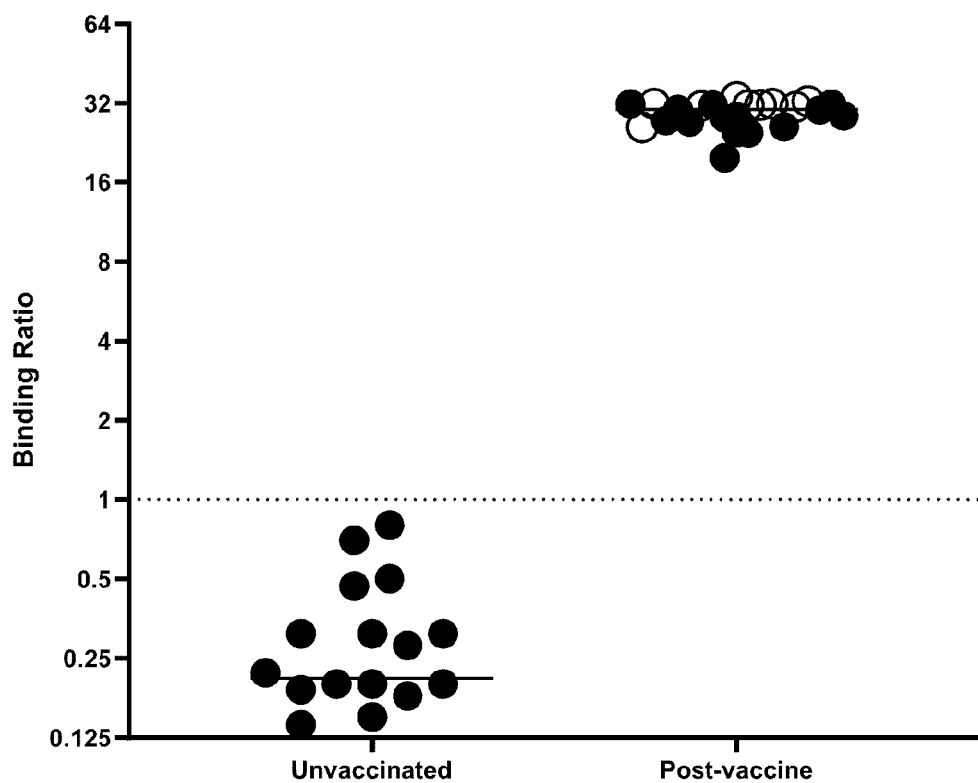
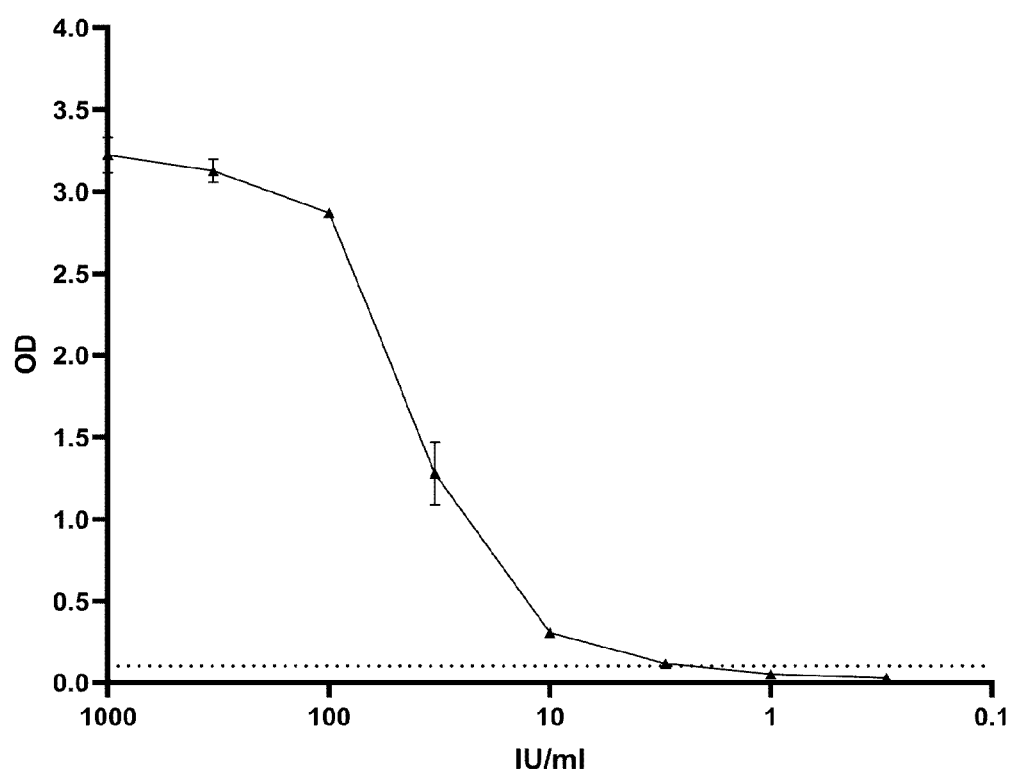


FIGURE 15



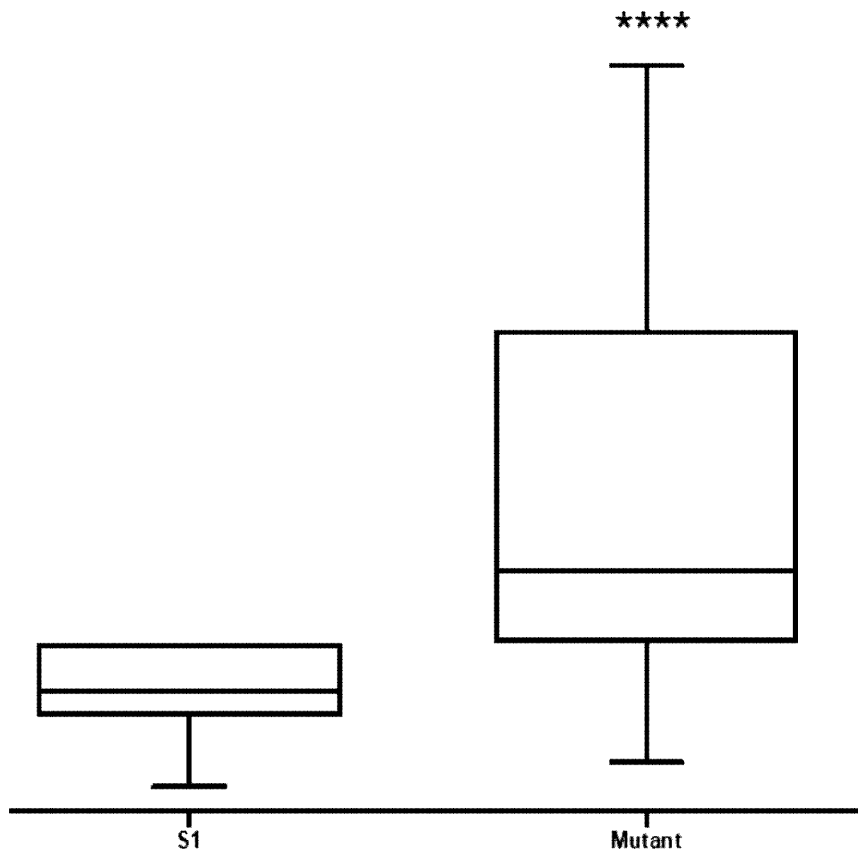


FIGURE 17

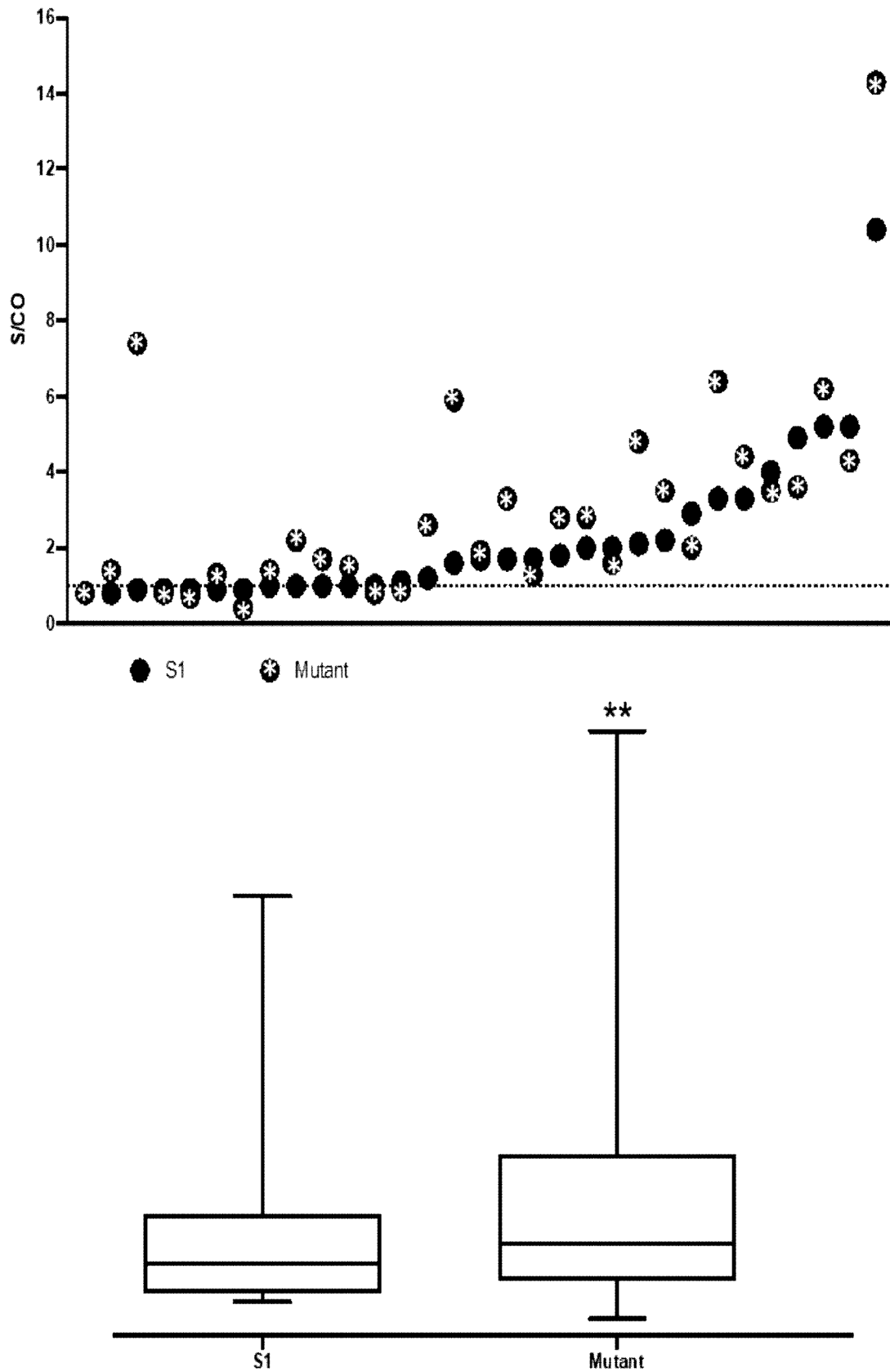


FIGURE 18

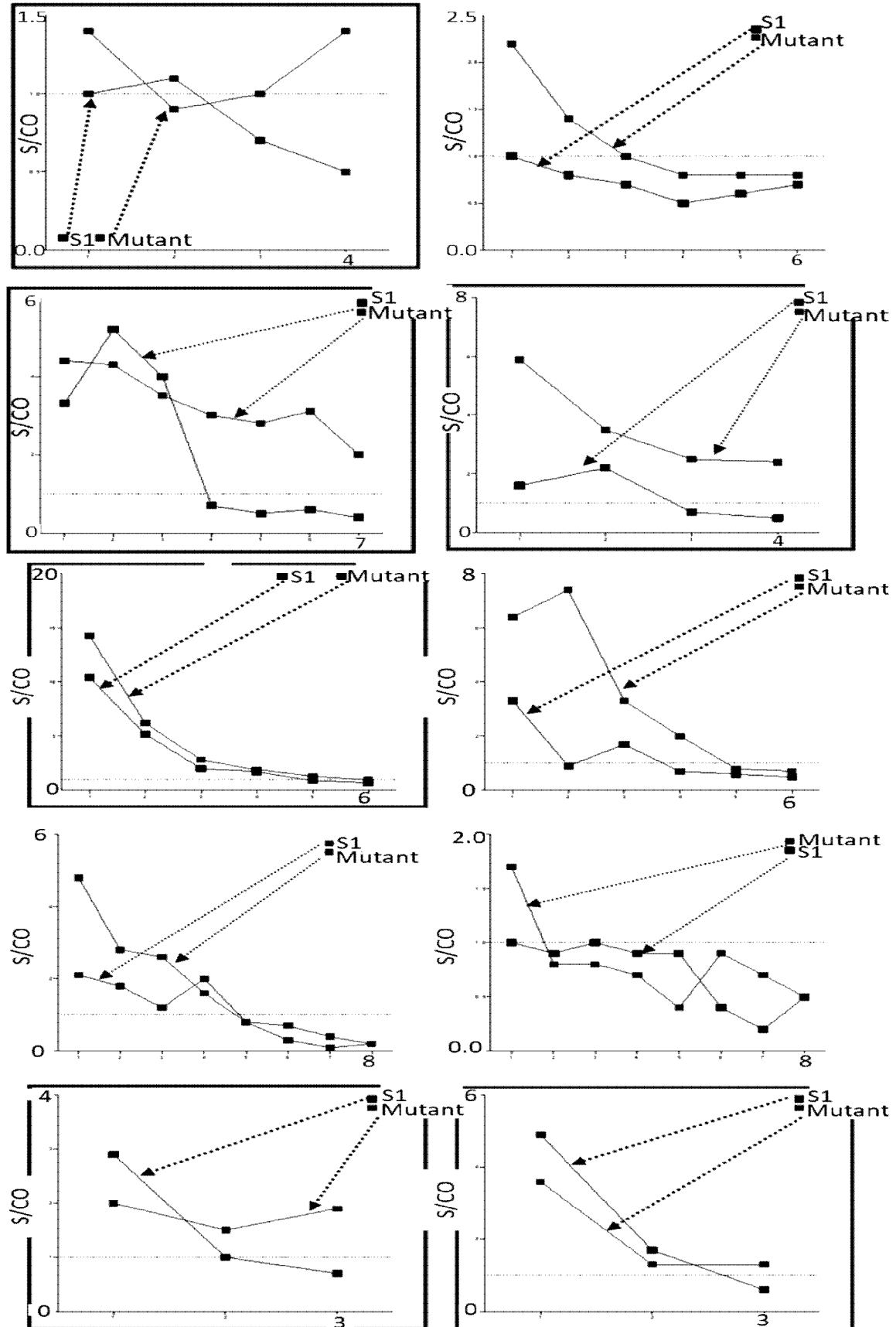
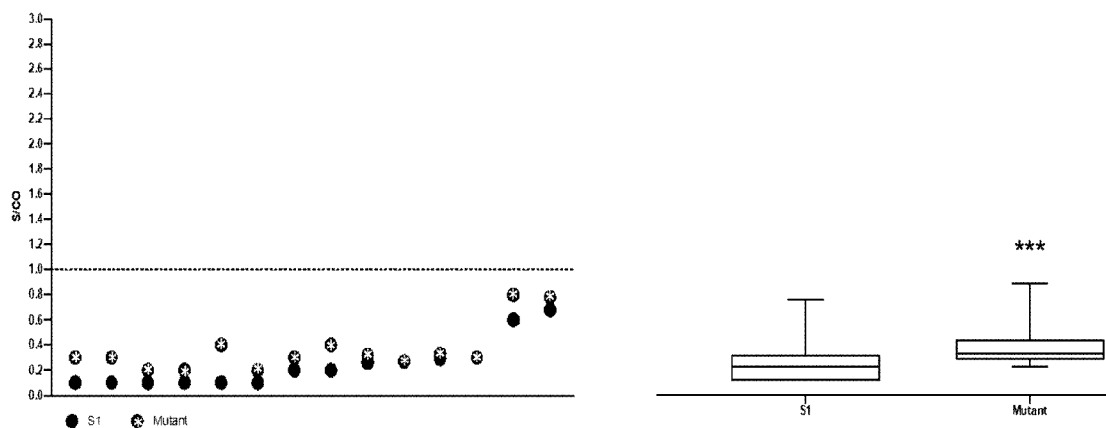


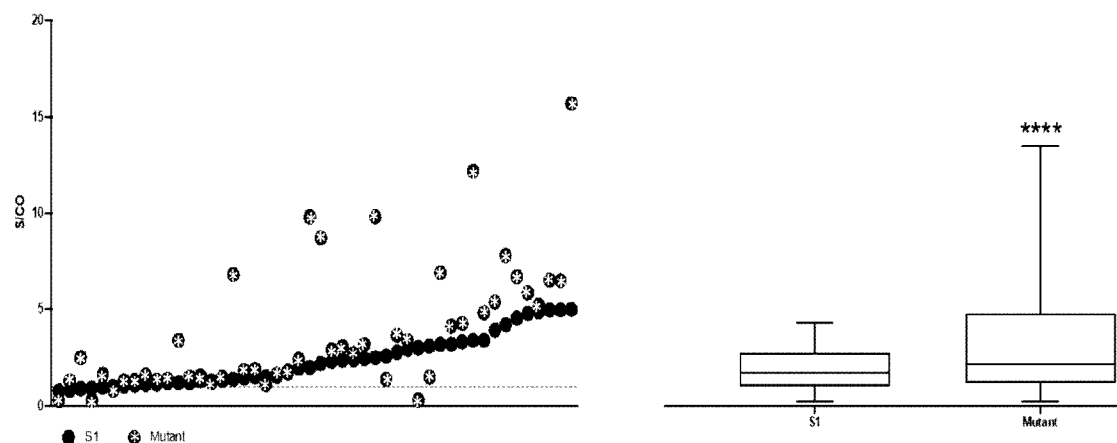
FIGURE 19

A

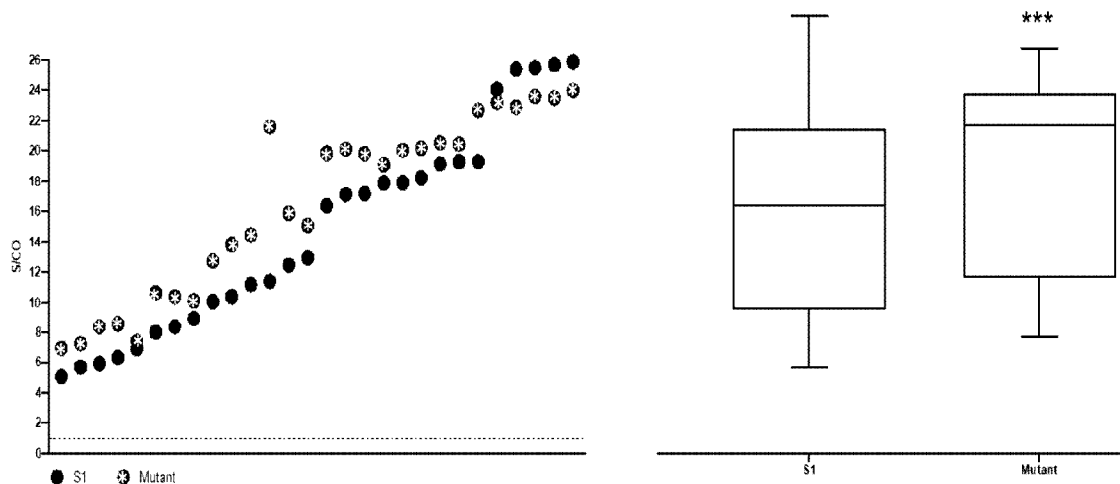
Negatives (S/CO < 0.7)



Borderline and Low positives (S/CO 0.7 - 5)

B

High positives (S/CO > 5)

C

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2021/051837

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
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)
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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XIAO KANGPENG ET AL: "Isolation of SARS-CoV-2-related coronavirus from Malayan pangolins", NATURE, MACMILLAN JOURNALS LTD., ETC, LONDON, vol. 583, no. 7815, 7 May 2020 (2020-05-07), pages 286-289, XP037289886, ISSN: 0028-0836, DOI: 10.1038/S41586-020-2313-X [retrieved on 2020-05-07] the whole document ----- -/--	1-32

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

☐ 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

28 September 2021

Date of mailing of the international search report

07/10/2021

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

C.F. Angioni

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2021/051837

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEN JIANJUN ET AL: "Severe Acute Respiratory Syndrome Coronavirus 2-Specific Antibodies in Pets in Wuhan, China", JOURNAL OF INFECTION, ACADEMIC PRESS, LONDON, GB, vol. 81, no. 3, 21 June 2020 (2020-06-21), XP086240706, ISSN: 0163-4453, DOI: 10.1016/J.JINF.2020.06.045 [retrieved on 2020-06-21] the whole document	1-32
X	----- DENG JUNHUA ET AL: "Serological survey of SARS-CoV-2 for experimental, domestic, companion and wild animals excludes intermediate hosts of 35 different species of animals", VETERINARY MEDICINE, vol. 67, no. 4, 7 May 2020 (2020-05-07), pages 1745-1749, XP055844227, ISSN: 1865-1674, DOI: 10.1111/tbed.13577 Retrieved from the Internet: URL:https://onlinelibrary.wiley.com/doi/full-xml/10.1111/tbed.13577> the whole document	1-32
X	----- Lassaunière Ria ET AL: "Evaluation of nine commercial SARS-Cov-2 immunoassays", medRxiv, 10 April 2020 (2020-04-10), XP055779188, DOI: 10.1101/2020.04.09.20056325 Retrieved from the Internet: URL:https://www.medrxiv.org/content/10.1101/2020.04.09.20056325v1.full.pdf [retrieved on 2021-02-24] the whole document In particular: Title; Abstract; Materials and methods, ELISA assays.	1-32
X,P	----- KIM SEUNGHYEON ET AL: "Vertical Flow Cellulose-Based Assays for SARS-CoV-2 Antibody Detection in Human Serum", ACS SENSORS, vol. 6, no. 5, 6 April 2021 (2021-04-06), pages 1891-1898, XP055839862, ISSN: 2379-3694, DOI: 10.1021/acssensors.1c00235 Retrieved from the Internet: URL:https://pubs.acs.org/doi/pdf/10.1021/acssensors.1c00235> the whole document In particular: Title; Abstract; Scheme 1; Figures 1-6; Materials and methods.	1-32

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB2021/051837

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. ☒ forming part of the international application as filed:
 - ☒ 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: