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A cell-free high throughput assay for assessment of SARS-CoV-2 neutralizing antibodies

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ARTICLE INFO

Keywords:
Neutralization
Cell-free
SARS-CoV-2
Bead-based
Antibodies
Pseudoneutralization

ABSTRACT

Highly accurate serological tests are key to assessing the prevalence of SARS-CoV-2 antibodies and the level of immunity in the population. This is important to predict the current and future status of the pandemic. With the recent emergence of new and more infectious SARS-CoV-2 variants, assays allowing for high throughput analysis of antibodies able to neutralize SARS-CoV-2 become even more important. Here, we report the development and validation of a robust, high throughput method, which enables the assessment of antibodies inhibiting the binding between the SARS-CoV-2 spike protein and angiotensin converting enzyme 2 (ACE2). The assay uses recombinantly produced spike-f and ACE2 and is performed in a bead array format, which allows analysis of up to 384 samples in parallel per instrument over seven hours, demanding only one hour of manual handling. The method is compared to a microneutralization assay utilising live SARS-CoV-2 and is shown to deliver highly correlating data. Further, a comparison with a serological method that measures all antibodies recognizing the spike protein shows that this type of assessment provides important insights into the neutralizing efficiency of the antibodies, especially for individuals with low antibody levels. This method can be an important and valuable tool for large-scale assessment of antibody-based neutralization, including neutralization of new spike variants that might emerge.

Introduction

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) emerged during late 2019 in China and since then has spread around the world and caused a global crisis with more than 200 million detected infections and a death toll exceeding 4 million [1]. Due to the wide range of symptoms that overlap with other respiratory infections, confirmation of the disease largely depends on laboratory detection by reverse-transcriptase polymerase chain reaction (RT-PCR) to identify ongoing infection [2,3] or serological assays for post infection diagnosis [4,5]. Beside diagnosis, it is particularly important to monitor the prevalence of partial or full immunity in the population, as well as to evaluate vaccine-induced immune responses. There is also a large demand for knowledge regarding the longevity of antibody-based protection against the virus, for which studies to assess the duration of the induced antibodies upon vaccination or natural infection are needed.

The envelope of the coronavirus SARS-CoV-2 consists of a lipid bilayer with three different surface proteins; the spike, the membrane and the envelope proteins [6], all of which are exposed to the host upon entry. While the membrane and envelope proteins are mainly responsible for virion assembly inside host cells, the spike protein plays an important role in host cell entry. The first identified mechanism by

Abbreviations: ACE2, angiotensin converting enzyme 2; AU, arbitrary unit; CV, coefficient of variance; HCW, healthcare workers; HT, heat treatment; NC-C, C-terminal fragment of the nucleocapsid protein; PFA, paraformaldehyde; PNT, pseudoneutralization; RBD, receptor binding domain; RPE, R-phycoerythrin conjugated; SFR, surface plasmon resonance; WB, western blotting.

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https://doi.org/10.1016/j.nbt.2021.10.002
Received 20 May 2021; Received in revised form 4 October 2021; Accepted 5 October 2021
Available online 7 October 2021
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which the SARS-CoV-2 virus enters the host cell is via membrane-bound angiotensin converting enzyme 2 (ACE2) [7]. Through a high affinity interaction between the spike protein and ACE2, the viral and cellular membranes fuse allowing the viral genome to enter the cell [7–9].

The SARS-CoV-2 spike protein is a heavily glycosylated trimer constituted by two functional subunits, S1 and S2. The trimeric structure can be stabilized by exchanging the furin cleavage site for two prolines (spike-f) [10,11]. The S1 unit forms the outer part of the protein containing the receptor binding domain (RBD) which binds to ACE2, while the S2 unit is responsible for anchoring the protein in the bilipid layer of the virus envelope [6]. Serological tests that utilize various representations of the spike protein can assess the ability of antibodies to recognize different conformations and mutations of the spike protein. However, such tests do not evaluate the neutralizing capacity of the antibodies, which is important to assess the protection expected against an infection upon re-exposure to the virus. Hence, it is important not only to assess the amount of antibodies induced against the viral proteins, but also to understand whether they are able to neutralize the virus. This becomes even more necessary in the aftermath of a global vaccination campaign where it will be important to ensure that the vaccines give the expected protective effect.

The standard method to assess the neutralizing capacity of antibodies is by determining the infection rate, either of the virus itself or of a pseudovirus, in cultivated cells, where the level of antibodies needed to inhibit the viral infection of the cells is used as a measure of the neutralization capacity [12,13]. Although data from cell-based neutralization assays are considered the gold standard for the analysis of virus neutralization capacity, comparison of results from different analyses and different laboratories might be problematic due to varying cell types, cell numbers and virus inoculum. Also, cultivation of cells and viruses requires a high-level safety laboratory environment. Because of this, analysis of many samples in a high throughput setting demands expensive infrastructure and labour. Therefore, methods based on pseudoviruses measuring the cell infection rate [14], or binding assays (e.g. enzyme-linked immunosorbent assay, ELISA) measuring inhibited binding between recombinantly produced ACE2 and the spike protein, have been developed [15].

Presented here is the development and evaluation of a novel high throughput bead-based pseudoneutralization (PNT) assay for assessment of spike-specific antibodies with the ability to inhibit the interaction of spike-f with ACE2. The generated data is compared and benchmarked to a conventional neutralization assay, and to spike-targeting antibody levels detected in a serological assay.

Materials & methods

Protein production, purification, and labelling

Spike-f [11,12] and ACE2 [16] (Supplementary Fig. 1) were both produced by transient protein production in mammalian cells, but in different expression systems. Spike-f was produced in ExpiCHO-S cells (Thermo Fisher Scientific, Waltham, MA, USA) for the PNT assay and in Expi293-F cells (Thermo Fisher Scientific) for the serological assay. The ExpiCHO-S cells were cultivated and transiently transfected according to manufacturer’s user guide using the high titre protocol, and the Expi293-cells were cultivated in Expi293 Expression medium (Gibco, Waltham, MA, USA, A1435101). Transfection was performed with PEI MAX (Polysciences Inc., Warrington, PA, USA, 24765). ACE2 was produced using the QMCF Technology (Icosagen Cell Factory ÖÜ, San Francisco, CA, USA) according to [16]. Cells were harvested by centrifugation up to 13 d after transfection, depending on cell concentration and viability. The supernatant was filtered through a 0.2 μm filter, prior to purification. Spike-f protein was produced with a strep-tag [12], and therefore a biotin blocking solution of 18.1 μl/ml of supernatant (Bio-Lock, IBA Lifesciences, Göttingen, Germany, 2-0205-050) was added before filtration.

All purifications were performed using ÄKTAxpress chromatography systems (Cytiva, Uppsala, Sweden). ACE2 was purified on HP4 column according to [17] and spike-f was purified on StreptTrap (Cytiva) according to the manufacturer’s instructions. Protein concentration was determined using absorbance at 280 nm and quality control was performed by SDS-PAGE with Western blotting (WB) and mass spectrometry (MS/MS). After purification, spike-f protein was biotinylated using a 25x molar excess of EZ-link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific), according to the manufacturer’s protocol. In brief, EZ-link Sulfo-NHS-LC-Biotin was equilibrated to room temperature (RT) and dissolved in amine-free buffer to a 10 mM concentration immediately before use. A volume of biotin-reagent corresponding to a 25x molar excess was added to the spike-f of concentration 1 mg/ml, and the mixture was incubated at RT for 30 min. Unreacted reagent was removed from the sample through a NAP5 desalting column (Cytiva).

Serum samples

Positive control samples included blood samples donated from SARS-CoV-2 seropositive healthcare workers (HCW) after mild COVID19 infection (n = 76) and hospitalized qPCR-confirmed COVID-19 patients (n = 56) at Danderyd Hospital, Stockholm. The negative control samples were from SARS-CoV-2 seronegative HCW (n = 6) and healthy blood donors collected in 2019 (n = 163). In addition, 17 seropositive blood samples donated from HCW were utilized for comparison of plasma, serum, heated and non-heated samples. Informed consent was obtained by all participants, and the study was approved by the regional ethical committee (EPN: dnr 2020-01653, dnr 2020-01620).

Purification of antibodies

Two convalescent sera were purified using affinity chromatography. 1 ml HiTrap column coupled with Zeba-TetraCys ligand (Cytiva) was used to enable mild antibody elution at almost neutral pH, with less risk of damage to acid-sensitive antibodies compared with the standard Protein A matrices requiring low pH for product elution [20]. Prior to affinity chromatography, both sera were heat-inactivated at 56 °C for 30 min, filtered using 0.2 μm filters and spiked with 1 mM CaCl2 to promote interaction with Zeba-TetraCys. The purification was performed as described by [18] Affinity chromatography was conducted on an AKTA Start instrument (Cytiva) at 25 °C and a flow rate of 1 ml/min. The eluate was collected, and buffer exchanged to PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7.5) for subsequent SPR analysis.

Surface plasmon resonance (SPR) analysis

To confirm that the in vitro produced proteins (ACE2 and spike-f) interact with each other, an affinity measurement experiment was performed utilising SPR. Spike-f was immobilized via amine coupling on a CMS chip (Cytiva) at a flow rate of 10 μl/min to 750 RU. A multicycle kinetic analysis was performed on a T200 instrument (Cytiva) using PBS supplemented with 0.05 % (v/v) Tween20 (PBS-T) as running buffer. ACE2 was applied in three different concentrations (250 nM, 125 nM and 62.5 nM) and the purified antibodies were analysed in three concentrations (4 μM, 2 μM and 1 μM). ACE2 and the antibodies were allowed to associate with the spike-based proteins for 400 s before dissociation was monitored over 600 s at a flow rate of 30 μl/min. The kinetic parameters were determined using the Biacore T200 Evaluation Software (Cytiva) and a 1:1 binding model. All runs were followed by regeneration using 10 mM HCl at 30 μl/min for 30 s.

Pseudoneutralization (PNT) analysis

The PNT was performed as a high throughput bead-based assay. ACE2 was immobilized on the surface of colour-coded magnetic beads (MagPlex, Luminex corp., Austin, TX, USA) as described in [19]. In brief,
the protein was diluted in 100 mM 2-(N-morpholino) ethanesulfonic acid buffer, pH 4.5 (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 80 μg/mL. 1 × 10^6 colour-coded magnetic beads were activated by using 100 μL phosphate buffer complemented with 0.5 mg 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (ProteoChem, Inc., Hurricane, UT, USA) and 0.5 mg N-hydroxysulfosuccinimide (Thermo-Fisher Scientific). The ACE2 protein was conjugated with the beads in a 2 h incubation, followed by washing and overnight incubation in blocking buffer (Blocking Reagents for ELISA, Roche, Baslej, Switzerland, 11112589001), supplemented with 0.1 % (v/v) ProClin (Sigma-Aldrich, 48912-U).

Plasma samples were thawed at 4 °C and diluted in an assay buffer composed of PBS complemented with 3 % (w/v) bovine serum albumin (Saveen-Werner, Limhamn, Sweden), 5 % (w/v) non-fat milk (Sigma-Aldrich) and 0.05 % (v/v) Tween20 (Thermo-Fisher Scientific) to achieve dilutions of 1:5, 1:25 or 1:250. 15 μL of the diluted samples were mixed with 15 μL of biotinylated spike-f diluted to 2 μg/mL in assay buffer and incubated for 1 h at RT. 25 μl of the sample-antigen mix was then transferred to a microtitre plate containing the ACE2-conjugated beads (~150 beads/well) and the plate was incubated for a further 1 h at RT. The beads were then washed 3x with PBS-T using a plate washer (EL406, Biotek, Winooski, VT, USA) and incubated with 0.2 % paraformaldehyde (PFA) for 10 min at RT to crosslink the receptor-antigen complexes and prevent complex dissociation during the following steps. The 0.2 % PFA was freshly prepared from a 4 % PFA in PBS stored for up to one month. The beads were again washed 3x and incubated with 50 μL Streptavidin-R-phycocerythrin conjugate (Thermo-Fisher Scientific, SA10044) diluted to 0.2 μg/mL in PBS-T for 30 min at RT. After the final washing, the beads were resuspended in PBS-T and analysed on a FlexMap 3D instrument (Luminox corp.) equipped with the Luminox xPONENT software. The data was acquired as median fluorescent intensity and reported here as relative intensity [arbitrary unit, AU]. Only data based on at least 30 measured beads per sample were included in the subsequent analysis.

For the assays comparing heated and non-heated samples, the samples were heat-treated in a water bath for 30 min at 56 °C before dilution.

**Microneutralization analysis**

The microneutralization assay was performed as described earlier in [20]. Briefly, heat-inactivated serum (56 °C for 30 min) was first tested at a 1:10 dilution. Samples with neutralizing capacity were further incubated with biotinylated spike-f (green) (I) followed by incubation with ACE2-coupled magnetic beads (blue) (II). Non inhibited spike-f binds to the beads (III). Fluorescently labelled streptavidin is added (yellow) to enable read out of bead-bound spike-f (IV).

**Serological analysis**

Antibodies towards the SARS-CoV-2 virus were analysed as previously described in [10]. In brief, plasma and serum samples were diluted 1:50 and antibodies were detected by a bead-based assay using spike-f and a C-terminal fragment of the nucleocapsid protein (NC-C). The detection was through addition of an anti-human IgG-RPE (R-phycocerythrin conjugated) reagent (12-4888-82, eBiosciences, San Diego, CA, USA). Data are reported as relative intensity [arbitrary unit, AU]. The method was previously validated by using 442 negative samples collected before the pandemic and 243 samples from PCR-positive COVID19 subjects sampled at least 17 d after disease onset. The validation demonstrated a 99.2 % sensitivity and 99.8 % specificity [10].

**Data analysis and reproducibility**

Statistics and data visualizations were performed using the R Statistical Software (4.0.1) with RStudio (1.3.959) and additional packages tidyverse (1.3.0), ggplot2 (3.3.2) and ggbeeswarm (0.6.0). The figures were further modified for clarity using the vector graphic editor Affinity Designer (1.8.6) (Serif, West Bridgford, UK).

The mean coefficient of variance (CV) for the intra-assay technical variability of the PNT assay was calculated based on two triplicates present in each of the three sample dilutions (1:10, 1:50, 1:500; 6 values in total), and the range was defined by the lowest and highest number out of these values.

In the serology assay, the cut-off for seropositivity for spike-f was calculated as the mean + 6 x standard deviation (6 SD), rounded up, of 12 negative reference samples included in the analysis. The reference samples were carefully selected to represent a wide range of possible background signals as described in [16].

**Results and discussion**

Here we report the development and evaluation of a bead array-based PNT assay. In the assay, the diluted serum samples are first incubated with biotinylated spike-f, to allow for interaction with spike-f-specific antibodies in the serum sample (Fig. 1). When adding the ACE2-coated beads, remaining free biotinylated spike-f binds to the beads for detection by fluorescently labelled streptavidin. Hence, the more antibodies present in a sample binds the RBD region of spike-f and thereby
convalescent serum and across the surface (solid: 250 nM, dashed: 125 nM, dotted: 62.5 nM). The binding of antibodies purified from sera to spike-f, PNT assay and is defined as 3x the SD of the mean of the COVID19 (represents a cut-off between the samples classified as positive or negative in the assay). Colours indicate SARS-CoV-2 serology measurements at 1:50 dilution; grey: all individuals (n = 6) classified as seropositive in the microneutralization assay, HCW and Hospitalized: samples from HCW (n = 76) and hospitalized individuals (n = 56) classified as seropositive in the microneutralization assay (a sample is defined as positive if the antibody titre in the assay is 10 or above). Colours indicate SARS-CoV-2 serology measurements at 1:50 dilution; grey: all negative; orange: IgG-positive against the nucleocapsid NC-C; blue: IgG-positive against spike-f; red: IgG-positive against both spike-f and NC-C. The dashed line represents a cut-off between the samples classified as positive or negative in the PNT assay and is defined as 3x the SD of the mean of the COVID19 (+) samples.

Protein functionality

To confirm the functionality of the recombinantly produced spike-f and ACE2 proteins, their interaction was evaluated by SPR analysis. First, the affinity between spike-f, immobilized on the SPR-surface and ACE2 as analyte was measured. The affinity was shown to be 1.3 nM (koff = 2.4 x 10^3 M s^-1 and koff = 3.1 x 10^4 s^-1) (Fig. 2A) which is in agreement with prior data [21,22]. Next, the interaction between spike-f and two polyclonal antibody samples purified from two different sera, one COVID19 convalescent serum sample and one seronegative serum sample, was assessed. A distinct difference between the two samples was observed, indicating that antibodies from the convalescent serum sample effectively bind to the recombinantly produced spike-f while no interaction was observed in the seronegative serum sample (Fig. 2B and C).

Pseudoneutralization assay

To evaluate the performance of the PNT assay, a set of 301 serum samples from different cohorts was assembled. The set comprised samples from HCW following mild COVID19 disease (n = 76), hospitalized COVID19 patients following severe disease (n = 56) and seronegative samples from non-infected HCW (n = 6) as well as pre-pandemic control samples from 2019 (n = 163). For validation of the PNT method, all samples collected during 2020 were analysed using a cell-based micro-neutralization assay. Microneutralization data for the seropositive HCW samples revealed neutralization titres ranging from 10 to 320 with a median titre of 60 (Supplementary Fig. 2). However, there were two exceptions within this group displaying titres as high as 2560. Sera from hospitalized COVID19 patients overall displayed higher titres with a median of 200, albeit within a wide range from 60 to 3640. In the PNT assay, all samples were analysed in three different dilutions (1:10, 1:50 and 1:500) using the final concentration of spike-f, 1 μM Ab. Note the different scales on the Y-axis.

As can be seen in Fig. 3, the PNT assay performed at a 1:10 sample dilution distinguished well between positivity and negativity regarding neutralization capacity. By applying a cut-off based on 3x the SD of the mean of the COVID19 negative samples, no false positive classifications were made among the 169 negative samples. Among the 133 samples classified as positive in the microneutralization assay, all except one sample were shown to clearly inhibit the binding of spike-f and ACE2. One of the seropositive samples that were able to neutralize the virus in the microneutralization assay was on the border between the negative and positive samples in the PNT assay. This sample gave an antibody titre as low as 10 in the microneutralization assay. Higher dilutions are needed to discriminate between highly neutralizing samples (Fig. 4).

Further, the signals obtained from the PNT assay were compared to the microneutralization titres. As can be seen in Figs. 3 and 4A, a 1:10 dilution was sufficient to discriminate between negative and positive samples whereas further dilutions increased the signal for samples with low neutralization capacity resulting in an overlap with the truly negative samples. Samples with higher neutralization capacity, including the hospitalized individuals, showed dilution-dependent dynamics while the distribution among the negative controls were largely unaffected by sample dilution. The linearity within the different neutralization spans was highly dependent on the sample dilution, giving the possibility to clearly discriminate between all samples, regardless of neutralization capacity. When comparing signal intensities with neutralization titres over a larger interval, the 1:50 dilution gives a
good separation at microneutralization assay titres between 50 and ~200 and a dilution of 1:500 or more is required for neutralization titres of 200 and above. To control for potential signal variance caused by nonspecific binding of spike-f protein to the beads, beads without ACE2 were included in the PNT assay. As the measurements for all samples in all 3 dilutions were below 30 AU (Supplementary Fig. 3), it was concluded that the background signals did not influence the resulting data. Despite the variation in signal intensity between the dilutions, the technical intra-assay variation was similar, ranging from 2.4 % to 7.9 % with a mean of 4.8 %.

Comparison with serological data

The full set of 301 serum samples was additionally analysed in a serological assay using parallel detection of IgG antibodies to different virus proteins: spike-f and the NC-C. Classification of the serology data was performed according to [10] and the results are compared to the PNT and microneutralization data in Fig. 3. As can be seen, most of the samples classified as seropositive in the microneutralization assay four months post infection, were found to be seropositive also in the serological assay using a classification panel requiring antibodies against both the spike-f and NC-C [10]. Further, 12 of these samples were now shown to be negative against the NC-C, but positive against spike-f, which is in agreement with other studies showing that antibody signals against the nucleocapsid wane faster than those against spike-f [23]. Using the same classification panel, all negative controls but two were classified as seronegative, although, a few more samples were defined as reactive towards single antigens (Fig. 3). Interestingly, the neutralization capacity does not entirely follow the serological status, either when the more stringent read out demanding antibodies against two SARS-CoV-2 antigens [17] is used to avoid false positives, or when relying on the read out from one antigen, spike-f or NC-C. However, all sera with neutralizing capacity were shown to also have antibodies binding to spike-f (Fig. 3). To investigate this further, signal intensities from the pseudoneutralization and the serological assay against spike-f were compared. Here, dilutions of 1:10 and 1:50 were used in the PNT
as assay, and 1:50 and 1:500 for the serological assay (Fig. 5). As expected, a high concordance was found when comparing the relative antibody levels and their neutralizing capacity. However, as previously discussed, there were outliers in the COVID19 negative group showing low but positive anti-spike IgG levels according to the serological assay, but no neutralizing capacity according to the PNT assay. This indicates that the PNT assay could deliver more reliable results regarding previous infection and antibody protection of individuals compared to serological analysis.

**Dependence on sample preparation**

To assess if the assay read-out is dependent on the sample preparation, a set of serum and plasma samples from 17 seropositive HCW was used. To evaluate the effect of heat-treatment (HT), an aliquot of each sample was heated for 30 min at 56 °C. The different sample types and pre-treatment conditions were analysed for PNT capacity using a 1:50 sample dilution. Plasma and serum samples displayed comparable results, ensuring the assay utility for both sample types (Fig. 6A,B). A high correlation between the heated and non-heated samples could also be confirmed, suggesting that the assay can be used for both sample preparations (Fig. 6C,D).

**Conclusions**

During the SARS-CoV-2 pandemic, it is of particular importance to assess and map immunity by determining the presence of specific antibodies, both naturally acquired and obtained through vaccination. Such serological measurements are already efficiently performed by clinical laboratories around the world, but due to non-specific interactions within the antigens in the assays the analyses might report erroneously. The high throughput method presented here assesses the ability of the anti-spike antibodies to neutralize the spike protein’s interaction with ACE2. For this purpose, our assay would be superior to the less specific serological methods that measure all antibodies against the spike protein, regardless of their capacity to inhibit this specific interaction. In comparison with the gold standard microneutralization method, this novel bead-based method delivers comparable data regarding neutralization capacity, without the need to cultivate live viruses and cells. Due to the high throughput setting, the method could replace the less specific serological analyses mentioned previously and would not only report on earlier infection or vaccination but also provide information on the efficiency of the antibodies. Moreover, there are recent reports regarding new variants of the SARS-CoV-2 virus that show differences within the surface responsible for interacting with ACE2 and thereby affect virus entry into the cell [24]. This creates an emerging need for high throughput methods that can assess these small, but important variations. The method presented here can easily be amended to determine the neutralization capacity of the antibodies towards different novel virus variants.
Acknowledgements

This study was funded by Region Stockholm, Knut and Alice Wallenberg foundation, Science for Life Laboratory (SciLifeLab), Erling Persson family foundation, the Swedish Research Council and the European Union’s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 860197 (MIRIADE). The authors would like to express our gratitude to the Protein Factory at KTH for producing and purifying all proteins. Eni Andersson, Jennie Olofsson, Lovisa Skoglund and August Jernbom Falk is acknowledged for technical assistance and statistical analyses. The authors are grateful to Gordana Bogdanovic for sharing pre-pandemic samples as controls.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.nbt.2021.10.002.

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