

## Detection of SARS-CoV-2 antibodies in serum and dried blood spot samples of vaccinated individuals using a sensitive homogeneous proximity extension assay

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### ABSTRACT

A homogeneous PCR-based assay for sensitive and specific detection of antibodies in serum or dried blood spots (DBS) is presented and the method is used to monitor individuals infected with or vaccinated against SARS-CoV-2. Detection probes were prepared by conjugating the recombinant spike protein subunit 1 (S1), containing the receptor binding domain (RBD) of SARS-CoV-2, to each of a pair of specific oligonucleotides. The same was done for the nucleocapsid protein (NP). Upon incubation with serum or DBS samples, the bi- or multivalency of the antibodies (IgG, IgA or IgM) brings pairs of viral proteins with their conjugated oligonucleotides in proximity, allowing the antibodies to be detected by a modified proximity extension assay (PEA). Anti-S1 and anti-NP antibodies could be detected simultaneously from one incubation reaction. This Antibody PEA (AbPEA) test uses only 1  $\mu$ l of neat or up to 100,000-fold diluted serum or one  $\phi$ 1.2 mm disc cut from a DBS. All 100 investigated sera and 21 DBS collected prior to the COVID-19 outbreak were negative, demonstrating a 100% specificity. The area under the curve, as evaluated by Receiver Operating Characteristic (ROC) analysis reached 0.998 (95%CI: 0.993–1) for samples taken from 11 days after symptoms onset. The kinetics of antibody responses were monitored after a first and second vaccination using serially collected DBS from 14 individuals. AbPEA offers highly specific and sensitive solution-phase antibody detection without requirement for secondary antibodies, no elution step when using DBS sample in a simple procedure that lends itself to multiplex survey of antibody responses.

### Introduction

Specific antibodies in blood represent important biomarkers in infectious disease and for monitoring responses to vaccination. In

particular, assays for antibody responses to infections with and vaccination against SARS-CoV-2 virus provide useful information about the immune status of sampled individuals. Such analyses can also provide information about disease prevalence and population immunity levels

**Abbreviations:** SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; DBS, Dried blood spots; RBD, Receptor binding domain; NP, Nucleocapsid protein; PEA, Proximity extension assay; AbPEA, Antibody PEA; MSD, Meso scale discovery (MD, USA); ADAP, Agglutination-PCR; S, SARS-CoV-2 Spike protein; S1, SARS-CoV-2 subunit S1 protein; Ct, Cycle threshold;  $\Delta$ Ct, Ct value of negative control minus Ct value of positive samples; NTC, No template control.

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with rates of asymptomatic cases – information that is vital for developing policies to stem transmission [1]. Since the outbreak of the COVID-19 pandemic in 2019, there has been a rapid development of assays to assess immunity to this severe acute respiratory syndrome, caused by SARS-CoV-2. Most assays involve a solid-phase, such as in lateral flow assays (LFA), enzyme linked immunosorbent assay (ELISA), and chemiluminescence immunoassay (CLIA), a form of ELISA. ELISAs are the most commonly used methods for antibody detection in clinical laboratories and generally rely on dual-binding of patient antibodies, using virus antigen for capture and labeled secondary antibodies for detection. Among their advantages are the ability to detect both titers and isotypes of the antibodies. Frequently encountered problems in ELISA are weak signals and limited dynamic ranges. CLIA assays have excellent sensitivity with high signal-to-noise ratios. The Abbott (IL, USA) Architect SARS-CoV-2 IgG test, targeting antibodies directed against NP, is one of the most commonly used CLIAs, using magnetic beads coated with recombinant viral antigen and a luminescent substrate for automated analysis. The sensitivity for detection of virus-specific IgG reached 100% at 17 days after symptom onset or 14 days after PCR positivity for the virus [2]. Meso scale discovery (MD, USA; MSD) offers one of the most sensitive methods with a specificity of 97.4% and a sensitivity of 96.2% for samples collected 14 or more days after onset of symptoms [3]. Detection of antibodies directed against spike (S) or subunit 1 (S1) proteins is made after 50,000-fold dilution of serum or plasma.

Homogenous proximity extension assays (PEA) are protein detection reactions that differ in several respects from more standard immune assays for protein detection [4]. Like sandwich ELISAs, PEA depends on target recognition by pairs of affinity reagents, but no solid phases are needed for capture and no washes are required. Proteins are accurately quantified from levels below pg/ml and over wide dynamic ranges. Typically sample volumes of only 1  $\mu$ l or less are used for multiplex assays of a hundred or more target proteins [5]. The assays generally use pairs of oligonucleotide-conjugated antibodies that are brought into proximity by binding the same target protein molecules in a sample. This induced proximity allows oligonucleotides to produce amplifiable DNA strands via DNA polymerization. The amplicons then serve as surrogate markers for the targeted proteins in real-time PCR or DNA sequencing reactions. By conjugating barcoded oligonucleotides to pairs of antigens rather than to target-specific antibodies, the assay technique was adapted for detection of specific antibody responses against those antigens, as antibodies in the sample will have the effect of bringing pairs of antigen molecules into proximity in an approach termed AbPEA. A related solution phase assay, agglutination-PCR (ADAP), has been developed for detection of total levels of SARS-CoV-2 antibodies [6]. This assay consists of four reaction steps and a very good sensitivity of 98% and specificity of 99.55% was reported.

Serological testing for SARS-CoV-2 antibodies typically uses serum or plasma samples, collected by venipuncture. Collection of DBS by finger prick has proved to be a useful means of monitoring SARS-CoV-2 antibody levels, as samples can be collected at home and sent by post for analysis [8,9]. The Siemens (Munich, Germany) SARS-CoV-2 total antibody assay achieved a 100% and 94% agreement between results for plasma and DBS samples for negative and positive patient samples, respectively. The assay used 6 mm punches from the DBS [10]. A concordance of 98.4% was shown between DBS and venous plasma samples using DBS sample volumes of 150  $\mu$ l blood by the Architect SARS-CoV-2 IgG assay [11]. Similarly, there was 96.8% and 81.3% agreement for positive and negative samples, respectively, comparing serum and DBS with 3 mm punches by the Euroimmun (Lübeck, Germany) ELISA [12]. The rapid semi-quantitative Roche (Basel, Switzerland) Elecsys Immunochemistry assay can reveal SARS-CoV-2 antibodies from DBS samples in 18 min, but the sensitivity for detection of infected individuals was only 71.4% [2,13]. In contrast, the ADAP method achieved 100% sensitivity and specificity using DBS samples, but antibodies had to be eluted from the DBS for 90 min and

then concentrated [7]. The elution of antibodies from the DBS samples is a step that is in common for the published assays and takes more than 1 h.

The AbPEA approach, presented herein, consists of three simple reaction steps of incubation with liquid or dried sample, extension/prePCR and readout via real-time PCR without washing steps. The reaction uses 1  $\mu$ l of diluted or undiluted patient serum samples or one  $\phi$ 1.2 mm cut from a DBS to reveal the immune status of sampled individuals. The high specificity and sensitivity, as well as broad dynamic range, of this detection method are demonstrated using sets of pre-pandemic and patient serum samples, as well as DBS samples collected over time from vaccinated individuals.

## Materials and methods

### Antibodies and SARS-CoV-2 antigens

ChromPure Goat IgG (Catalog number: 005–000–003) was from Jackson ImmunoResearch, (Ely, UK). SARS-CoV-2 (2019-nCoV) recombinant nucleocapsid protein (Catalog number: 40588-T62) and rabbit anti-SARS-CoV-2 (2019-nCoV) spike-RBD antibody (Catalog number: 40592-T62) produced using a recombinant portion of the protein (Arg319-Phe541) were from Sino Biological, Frankfurt, Germany.

### Production of recombinant SARS-CoV-2 spike receptor-binding domain (RBD)

To express recombinant S1-RBD, a codon-optimized cDNA, encoding amino acids 319–541 of the SARS-CoV-2 spike protein fused to the N-terminal signal peptide of immunoglobulin kappa, was synthesized by Eurofins Genomics (Ebersberg, Germany). C-terminal sortase and hexahistidine tags were inserted by PCR with nested primers (see [Supplementary Table S3](#) for full protein sequence). The cDNA was cloned downstream of the CAG promoter in an expression vector containing Epstein-Barr Virus-derived sequences for episomal replication using Gibson assembly [14] using the HiFi DNA Assembly kit (New England Biolabs, Ipswich, MA, USA).

Recombinant S1-RBD was produced in 293 F cells (Catalog number: R79007, Thermo Scientific, Waltham, MA, USA) cultured in Freestyle 293 medium and transfected at  $2 \times 10^6$  cells/ml, using FectoPro transfection reagent (Polyplus transfection, Illkirch, France) in a volume of 200 ml in a 1000 ml E-flask at 130 rpm in a Multitron 4 incubator (Infors, Velp, the Netherlands). Transfected cells were kept at 37°C and the supernatant was harvested after 72 h and filtered using Polydisc AS 0.45  $\mu$ m (Whatman; Cytiva, Marlborough, MA, USA) and loaded onto a 5 ml HisTrap Excel column (Cytiva). After sample loading, the column was washed with 10 column volumes of 500 mM sodium chloride, 30 mM sodium phosphate buffer with 30 mM imidazole before elution of the protein using the same phosphate buffer with a 15 column volumes linear gradient between 30 and 300 mM imidazole. Pooled fractions were concentrated using 10 kDa Vivaspinn concentrators (10 kDa MWCO; Sartorius, Göttingen, Germany), passed over a HiPrep 26/10 desalting column (Cytiva) in phosphate-buffered saline and finally concentrated again. Integrity and purity of the RBD preparation was checked by Coomassie-stained SDS-PAGE ([Supplementary Fig. 1](#)).

### Patient serum samples

Two sets of serum samples were collected. The first consisted of 20 positive and 20 negative sera, previously analyzed by the Abbott SARS-CoV-2 IgG test targeting antibodies directed against NP (Catalog number: G92798R02). This cohort was used to develop the assay. A second set of samples consisted of 100 negative samples collected in 2019 before the COVID-19 pandemic, and 94 serum samples collected between March 1 and November 30, 2020. The latter samples were from individuals with symptoms of COVID-19 and diagnosed with SARS-CoV-

2 infection by reverse transcription PCR (RT-PCR) or by characteristic symptoms. In addition, patient samples had been analyzed using the Abbott and ELISA tests for SARS-CoV-2 IgG antibodies at the Department of Clinical Microbiology, Laboratory medicine, Norrlands University hospital-region Västerbotten, Umeå University, Sweden. These patient serum samples were collected between 3 days and 8 months after diagnosed or suspected COVID-19.

#### *DBS from SARS-CoV2 vaccinated individuals*

DBS samples were collected from 101 individuals by unsupervised self-sampling. For 16 of these, DBS were obtained before vaccination and then weekly for 8 weeks, thereafter at 3, 4, 5 and 6 months after the first and second vaccinations. For this collection, one drop of blood of about 10–50 µl from a finger prick was spotted on Whatman™ paper (903 Protein Saver Card, Catalog number: 10531018, GE Healthcare Life Sciences, Sweden). DBS samples were dried for more than 60 min at room temperature (RT), and then stored at 4 °C. A circular piece of 1.2 mm in diameter was cut using a Uni-core™ puncher (Catalog number: WHAWB100074, GE Healthcare Life Sciences, Sweden) and used for AbPEA. DBS stored at either RT, 4 °C or 20 °C for 1 week yielded consistent results, as did repeated testing of the same samples, (data not shown). An earlier study measuring protein levels in DBS demonstrated that closely similar levels were detected in different samples cut from the same DBS [15].

Collections of all samples were approved by the Swedish Ethical Review Authority (Dnr 2020–06575 and 2021–03952). All DBS donors signed informed consent documents.

#### *Protein-oligonucleotide conjugation*

The sequences of all oligonucleotides in this study have been published previously [16], and those ones used here are described in [supplementary Table S1](#). Thirty micrograms of S1-RBD and NP was activated with the 2 mM crosslinker dibenzylcyclooctyne-NHS ester (DBCO-NHS ester) (Catalog number: 761524, Sigma Aldrich) at RT for 30 min. Unreacted crosslinker was removed using a 7 K MWCO Zeba spin desalting column (Thermo Scientific). Both activated proteins were then divided into two aliquots and each aliquot was incubated at 4 °C overnight with either one of a pair of azide-modified, barcoded oligonucleotides (Click-FWD1 and Click-Univ-REV for the S1-RBD protein, and Click-FWD2 and Click-Univ-REV for the NP). Oligonucleotides used for covalent coupling to antigen have an azide residue at the 5' ends. One of the oligonucleotides in each pair was assay-specific and 63 nt long, while the other protein aliquot was conjugated to a common 37 nt oligonucleotide called Click-Univ-REV. An assay-specific 89-mer oligonucleotide (hyb-REV), comprising 32- and 9-nt segments complementary to Click-Univ-REV and to Click-FWD, respectively, was then allowed to hybridize to the probe with the oligonucleotide Univ-REV. In addition, goat IgG was conjugated with both Click-FWD3 and Click-REV3, and used as an extension and PCR control. The extension primers included uracil residues in place of some T residues, as indicated in [supplementary Table S1](#). The molecular beacon included a fluorophore, FAM, and a quencher, DABSYL, at either end.

#### *Proximity extension assay*

One µl serum sample or dilutions thereof were combined with 3 µl incubation solution of PBS buffer containing 5 mM EDTA, 100 µg/ml salmon sperm DNA (Sigma Aldrich), 0.1% BSA, 1 mM biotin, 100 nM goat IgG, 0.05% tween 20 and 1.33 nM of 3-plex PEA reagents in the form of pairs of oligonucleotide-conjugated S1-RBD and NP, as well as a goat IgG conjugate, serving as extension control. The combined samples and reagents were incubated at 37 °C for 1 h or 4 °C overnight with similar results. After incubation, 96 µl extension solution containing 1X Hypernova buffer (BLIRT S.A. Gdańsk, Poland), 1.5 mM MgCl<sub>2</sub>, 0.2 mM

of each dNTP, 1 µM of universe forward and reverse primers, 2 U/ml Platinum™ Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 5 U/ml Hypernova DNA polymerase (Blirt Cat No. RP235) was added, thereby diluting the reactions 25-fold. The extension/pre-PCR reactions were incubated at 50 °C for 20 min, followed by 5 min heat-activation at 95 °C and then 17 cycles of pre-PCR of 95 °C for 30 s, 54 °C for 1 min, and 60 °C for 1 min using a primer pair common for all extension products. For the subsequent qPCR detection, 2.5 µl of the extension/pre-PCR products were transferred to a 96 or 384-well plate and combined with 7.5 µl qPCR mix to a final concentration of 1X PCR buffer (Invitrogen), 2.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 1.6 µM ROX reference Dye (Invitrogen), 0.3 µM molecular beacon (Biomers, Ulm, Germany), 0.9 µM of the appropriate amplicon-specific primer pair, 0.1 U uracil N-glycosylase and 0.5 U recombinant Taq polymerase (Invitrogen). Quantitative real-time PCR was run with an initial incubation at 25 °C for 30 min, followed by incubation at 95 °C for 5 min, and then 30 cycles of 15 s at 95 °C, and 1 min at 60 °C. To control for variation between experiments and microtiter plates, a standard serial dilution of rabbit anti-S1-RBD IgG (from 10 nM to 1 pM), extension/PCR control [15], as well as positive patient and negative patient controls, were also included in each 96 well microtiter plate.

For DBS, disks 1.2 mm in diameter were cut from the filter papers used for collecting DBS samples and added directly to reactions or first eluted in a 100 µl volume from which 1 µl was incubated with the oligonucleotide-conjugated antigens. Elution of antibodies from DBS was performed by adding DBS paper disks into 100 µl elution buffer (PBS containing 0.5% tween20, 1% BSA and 1% Halt protease inhibitor cocktail (Thermo Scientific)) with shaking on a shaker set at 900 rpm for 60 min at RT [17]. The AbPEA assay for DBS samples was identical to that for serum samples as described above. Due to short supply of Hypernova DNA polymerase, Onetaq Hotstart DNA polymerase (Catalog number: M0481S, New England Biolabs) was used, which functions for both DNA extension and replication. The solution added for extension and pre-PCR contained 100 mM Tris-HCl pH 8.5, 50 mM KCl, 3 mM MgCl<sub>2</sub>, 0.75% TritonX-100, 0.2 mM dNTP, 5% DMSO, 5% glycerol, 1 µM forward and reverse primer and 1 unit Onetaq Hotstart DNA polymerase. The performance of Onetaq Hotstart DNA polymerase was equivalent to that of Hypernova DNA polymerase for both serum and DBS samples in corresponding buffer.

For DBS samples collected before the pandemic, pre-vaccination and after a first vaccination, AbPEA was performed directly without elution and dilution. For DBS samples collected after a second vaccination or after infection and first vaccination, AbPEA was carried out after elution and 100-fold dilution, and the Ct values were recalculated by subtracting 6.64 from detected Ct of 100-fold dilution to compensate for the sample dilution.

#### *Antibody detection by Abbott, Meso scale discovery and an in-house ELISA*

The Abbott SARS-CoV-2 IgG assay and MSD V-PLEX SARS-CoV2 panel 11 were analyzed according to instructions by the suppliers (Abbott 6R86–22 and MSD K15437U, respectively). An in-house ELISA was performed as previously described [18].

#### *Comparison between antibody detection using MSD and AbPEA*

Eight serum samples with different antibody levels were selected and subjected to an MSD assay using V-Plex IgG indirect serology. In order to compare the assay sensitive and dynamic ranges between MSD and AbPEA, serum samples were diluted 50-, 500-, 5000-, 50,000- or 500,000-fold. 50 µl of the diluted serum samples were used for MSD, while 1 µl samples were used for AbPEA. A serial dilution of rabbit anti-S1-RBD was also included in both assays.

Data and statistical analyses

The inter-assay coefficient of variation (CV) of the anti-S1-RBD assay was determined by analyzing a serial dilution of rabbit anti-S1-RBD IgG in duplicate on 10 different occasions. From the standard curve a high and a low concentration of rabbit antibodies (1 nM and 10 pM, respectively) were selected and used to calculate the overall mean, standard deviation (SD) and %CV using the formula: Overall %CV = (SD of plate Ct means / mean of plate means) x 100. The average %CV from the low and high concentration was used as the inter-assay %CV. The intra-plat%CV was investigated using goat IgG conjugated with both forward and reverse oligonucleotides, serving as an internal control. This reagent produces detectable extension products irrespective of the presence of SARS-CoV-2 antibodies in the sample and was included in all reactions. All graphs and figures were plotted in Rstudio.

Receiver operating characteristics (ROC) analysis was used to estimate the performance of anti-S1-RBD antibody detection by AbPEA. ROC analysis represents the rates of false negatives and positives detected by the assay across variable cutoffs and provides a means to determine the optimal cutoff point for the assay. ROC curve graphics was generated and the area under the curve (AUC) was calculated in Rstudio using the pROC package [19]. The optimal ΔCt cutoff values were determined as the ΔCt cutoff yielding the highest Youden statistics, using the Youden Index formula:  $J = Se + Sp - 1$ , where Se is sensitivity and Sp is specificity, both varying between 0 and 1.

Results

Assay sensitivity, dynamic range and optimal serum dilution

The PEA technique is commonly used for multiplex protein detection in, for instance, serum or plasma samples [16]. Here the technique was adapted for detection of antibodies against specific antigens. A schematic illustration of the AbPEA is shown in Fig. 1. The analytical sensitivity of the assay was first evaluated by investigating dilutions of a commercial purified rabbit anti-S1-RBD antibody preparation as shown in Fig. 2 A. Next, serial dilutions of convalescence serum samples were

investigated, as illustrated in Fig. 2B and C. At 1 pM of the rabbit anti-S1-RBD antibodies in the 1 µl samples (0.15 pg) the signal over background was greater than 2.2 Ct (this ΔCt is calculated as Ct value of negative control minus Ct value of positive samples), corresponding to 4.6-fold over background levels. Such serial dilution of rabbit anti-S1-RBD antibodies was included in each microtiter plate in the following experiments to control for variation between plates and experiments. The variation of the standard curves across 10 plates and experiments performed at different times was analyzed with very good reproducibility as demonstrated in Fig. 2A. The inter-assay coefficients of variations (CV) were 8.13%, 6.02%, 5.03%, 3.25%, and 5.5% at 10 nM, 1 nM, 100 pM, 10 pM and 1 pM of the rabbit anti-S1 antibody detection, respectively. An intra-assay CV variation of 1.3% was calculated using the internal control of goat IgG conjugated with both forward and reverse oligonucleotides.

A set of 40 sera was used to investigate the performance of the assay, 20 of the samples (P1-P20) were positive for antibodies against NP using the Abbott SARS-CoV-2 IgG kit and 20 were negative controls (H1-H20). Five randomly selected anti-S1-RBD positive sera (P1, P3, P5, P7, P10) and 3 negative controls (H1, H3, H5) were used in initial analyses. One µl of the undiluted samples as well as up to 10,000-fold diluted positive sera and up to 100-fold diluted negative sera were analyzed for both anti-S1-RBD and anti-NC antibodies. As shown in Fig. 2B, anti-S1-RBD antibody levels in 10,000-fold diluted patient samples P1 and P5 were quite high with ΔCt values of 3.9 and 5.6, respectively, whereas the limits of detection for P3 and P10 were reached already at a 1000-fold dilution, and for P7 at a 100-fold dilution. The signals for serum samples from control subjects with no known antibodies against the virus were similar to that for the no template controls (NTC). The detection signals for anti-NP antibodies were lower than those for anti-S1-RBD antibodies (Fig. 2C). The highest detectable dilutions of anti-NP antibodies of around 500-fold was seen in samples P1 and P5, and 100-fold for samples P3 and P10, while sample P7 could only be diluted 10-fold. Thus, the assay for anti-NP antibody detection was more than 20-fold less sensitive than that for anti-S1-RBD antibodies, and the dynamic range extended over only two orders of magnitude. It is not known whether the lower sensitivity reflects differences in antibody levels or in

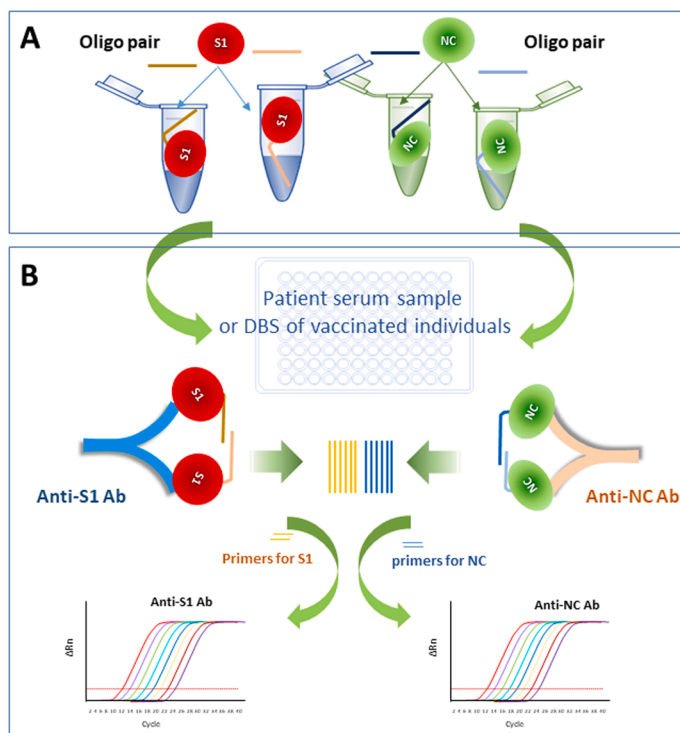
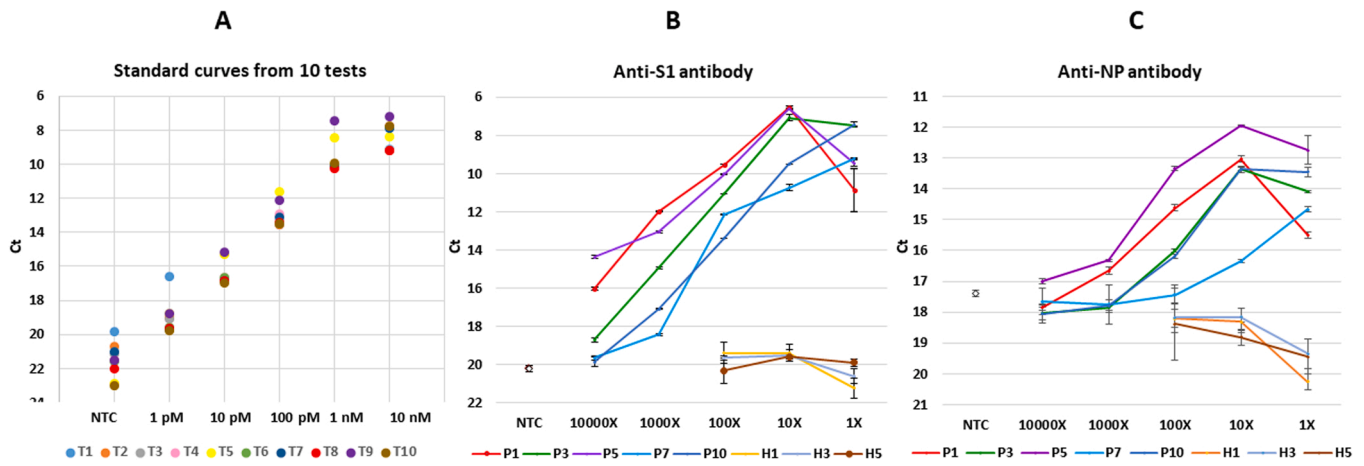


Fig. 1. Schematic illustration of AbPEA for antibody detection. A) Preparation of the probes. Two portions of the recombinant S1 or NP antigens were each conjugated to a pair of oligonucleotides. The pairs of oligonucleotides contain a mutually complementary region, and specific barcode each. One of them also has a target sequence for a molecular beacon. B) AbPEA tests for detecting antibodies to SARS-CoV-2. Upon incubation with serum samples or cuts of DBS from previously infected or vaccinated individuals, the di- of multimeric nature of antibodies in the sample bring pairs of oligonucleotide-conjugated protein probes in proximity. This allows their attached oligonucleotides to hybridize and be extended by a DNA polymerase. The extension products incorporate barcodes from both oligonucleotides conjugated to a particular antigen. The resulting DNA extension products are then amplified using universe primers to generate sufficient amount amplicons (pre-PCR). Finally, antigen-specific amplicons are quantified by real-time PCR using specific primers and molecular beacons to record at which PCR cycle (Ct) a signal exceeds a threshold value. ΔCt values are calculated as the Ct of the negative control minus that of the sample.

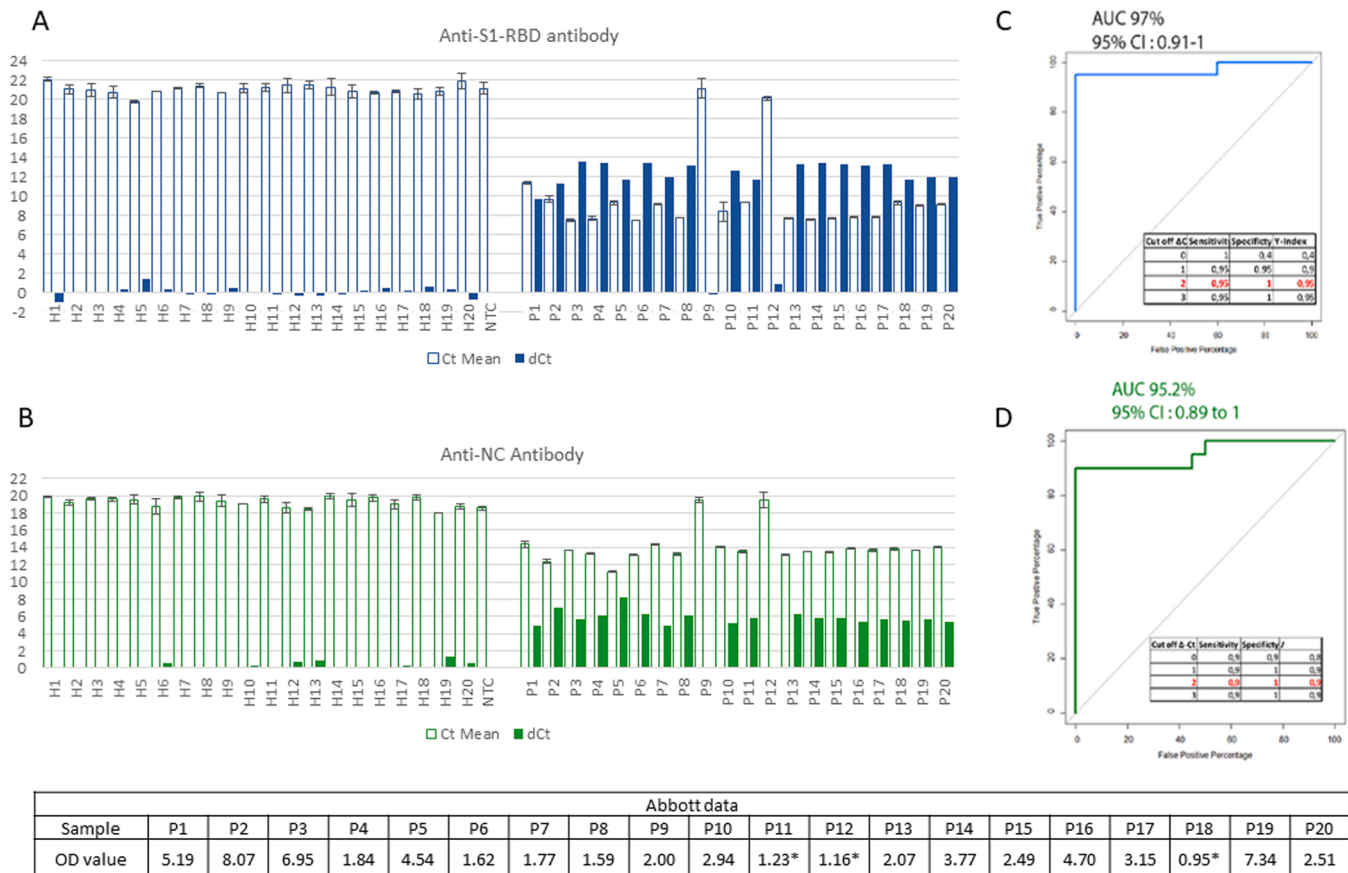


**Fig. 2.** Detection of antibodies via AbPEA in serial dilutions of rabbit antibodies or convalescent sera. A) Serial dilutions from 10 nM to 1 pM of a commercial rabbit anti-S1-RBD antibody preparation were analyzed on 10 different occasions with closely similar results. The Y axis indicate the Ct values for the corresponding antibody dilutions. B) Analysis of anti-S1-RBD antibody levels in serial dilutions of serum from 5 seropositive individuals (P1, 3, 5, 7 and 10), and from three seronegative individuals (H1, 3 and 5). C). The same patient and control serum samples were analyzed for reactivity to the NP antibody. NTC: No template control.

the performance of the AbPEA. The background in assays for NP antibodies was quite high. The levels of antibodies directed against S1-RBD and NP correlated among these samples (Fig. 2B and C). Titration tests showed that undiluted sera with high levels of antibodies against either the S1-RBD or NP proteins resulted in signals at plateau levels, while 10-fold dilutions of serum samples generated signals below plateau levels

for all samples. Therefore, 10-fold serum dilutions were used in all the following analyses unless otherwise stated.

All 20 serum samples negative for NP antibodies according to the Abbott test had AbPEA values for anti-S1-RBD similar to those of the NTC, while the 20 positive samples had  $\Delta$ Ct values between 9.65 and 13.6, except for samples P9 and P12, for which the signals were similar



**Fig. 3.** Pilot tests to establish AbPEA assays for anti-S1-RBD and anti-NP specific antibodies. 20 serum samples were used that had tested positive by the Abbott SARS-CoV-2 IgG test (labeled as P) and 20 tested negative (labeled as H). A) AbPEA detection of anti-S1-RBD antibodies. B) AbPEA detection of anti-NP antibodies. The y axes display Ct (empty column) and  $\Delta$ Ct (solid column) values for the detection of anti-S1-RBD (blue) or anti-NP antibodies (green). C) and D) ROC curve analysis of the anti-S1-RBD and NP antibody tests. The table at the bottom displays the data of the Abbott SAR-CoV-2 IgG test. The asterisks mark OD values close to the cutoff of 1 of the Abbott test.

to those for the NTC (Fig. 3A). Detection of anti-NP antibodies showed results similar to those for anti-S1-RBD antibody reactivity, although signals were generally lower (Fig. 3B). Consistent with the AbPEA results for anti-S1-RBD antibodies, anti-NP antibodies were not detected in samples P9 and P12. A ROC analysis was performed for the above anti-S1-RBD antibody tests to identify an optimal cutoff between negative and positive samples (Fig. 3C and D). The ROC analysis shows the relationship between rates of false positive and negatives at different cutoff values for the recorded antibody levels using AbPEA. In general, the closer the AUC is to the maximum, the better the assay distinguishes among cases and controls. The inserted table in Fig. 3C shows the sensitivity and specificity of the anti-S1-RBD assay depending on the choice of  $\Delta\text{Ct}$  cutoff value. Samples P11 and P18 were clearly positive by AbPEA for both anti-S1-RBD and anti-NP, although they were close to the cutoff value according to the Abbott test for anti NP antibodies (See table at the bottom of Fig. 3). An optimal cutoff point was identified by finding the maximal Youden Index (J) [20]. Using a cutoff of  $\Delta\text{Ct}$  2, the S1-RBD antibody assays achieved 95% sensitivity with no false positives. This sensitivity was estimated on the assumption that all samples with positive Abbott test results were indeed true positives, but as mentioned the Abbott test measures antibodies directed against NP, while AbPEA is targeting anti-S1-RBD antibodies [21].

#### Test of AbPEA performance using a set of patient serum samples

The AbPEA assay for anti-S1-RBD protein antibody reactivity was applied to analyze sera collected before the COVID-19 pandemic from a cohort of 100 individuals and 94 serum samples collected from individuals previously scored as positive for COVID-19, either by RT-PCR for SARS-CoV-2 RNA (68 samples) or according to characteristic symptoms. In addition, data were also available from analyses by an in-house ELISA for all samples and by Abbott test for most of the samples. The sample donors had an age range spanning from 18 to 97-years, and they were collected from as early as 3 days after diagnosis to after more than 8 months. On the basis of the pilot test results, 1  $\mu\text{l}$  of 10-fold diluted sera was used for initial AbPEA analyses. However, for samples with a  $\Delta\text{Ct}$  of less than 4, as well as samples collected within one month of infection, undiluted sera were also used for the analysis. All 100 samples collected in 2019 before the pandemic had Ct values of 21 or more (23.7 on average), similar to those of the NTC, demonstrating that the assay had a specificity of 100% (Fig. 4). Among 50 samples

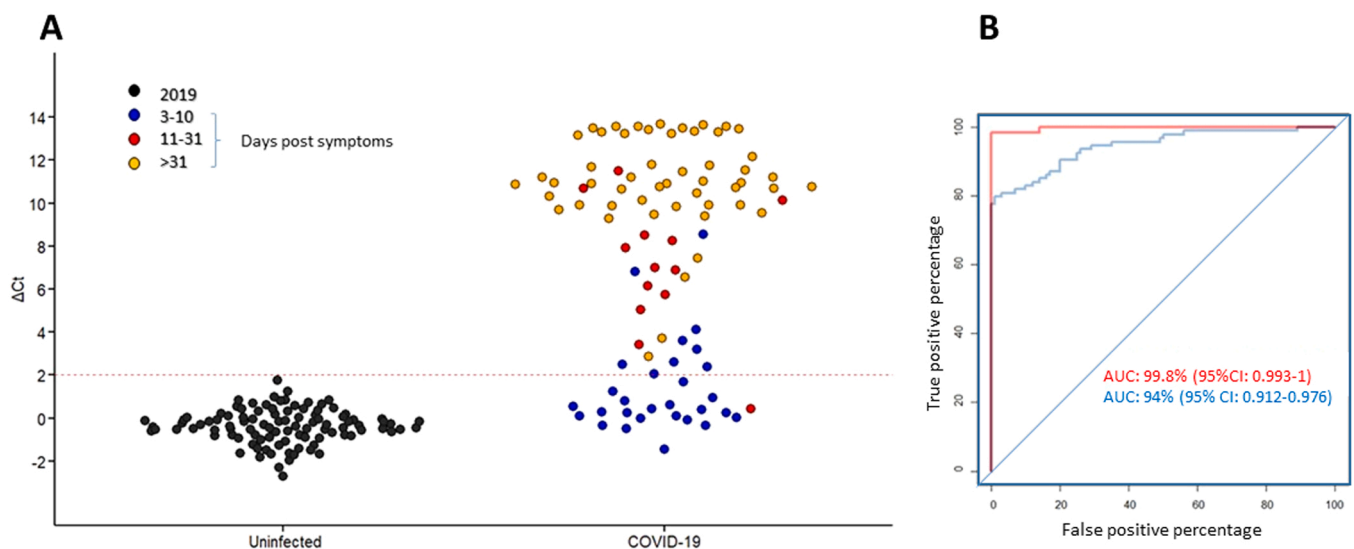
collected one month after onset of symptoms, 49 were positive with  $\Delta\text{Ct}$  over 6. One sample only yielded positive results when undiluted serum was used. All these samples were also positive by the Abbott and in-house ELISA tests. Among 15 samples collected between 11 and 31 days after symptom onset, only one was negative by both AbPEA and ELISA. Four out of 5 samples collected on the 10th day were positive by AbPEA, while 3 of them were positive by ELISA. Among 23 samples collected between days 4–9, 5 were positive by AbPEA, but none according to ELISA (results are included in supplementary Table S2). Furthermore, detection signals varied over a broad range, spanning from 2.4 to 13.6  $\Delta\text{Ct}$ , corresponding to amplicon levels ranging from 5.3- to 12,400-fold over background on a linear scale.

The assay performance in these cohorts was investigated by ROC analysis. A cutoff value of  $\Delta\text{Ct}$  2 maximized the diagnostic sensitivity and specificity and preserved a 100% specificity using the Youden Index. The ROC analyses revealed an AUCs of 0.94 (95%CI: 0.91–1) for all 94 collected patient samples from 3 days (Fig. 4), and 0.998 (95%CI: 0.993–1) for samples collected from 11 days.

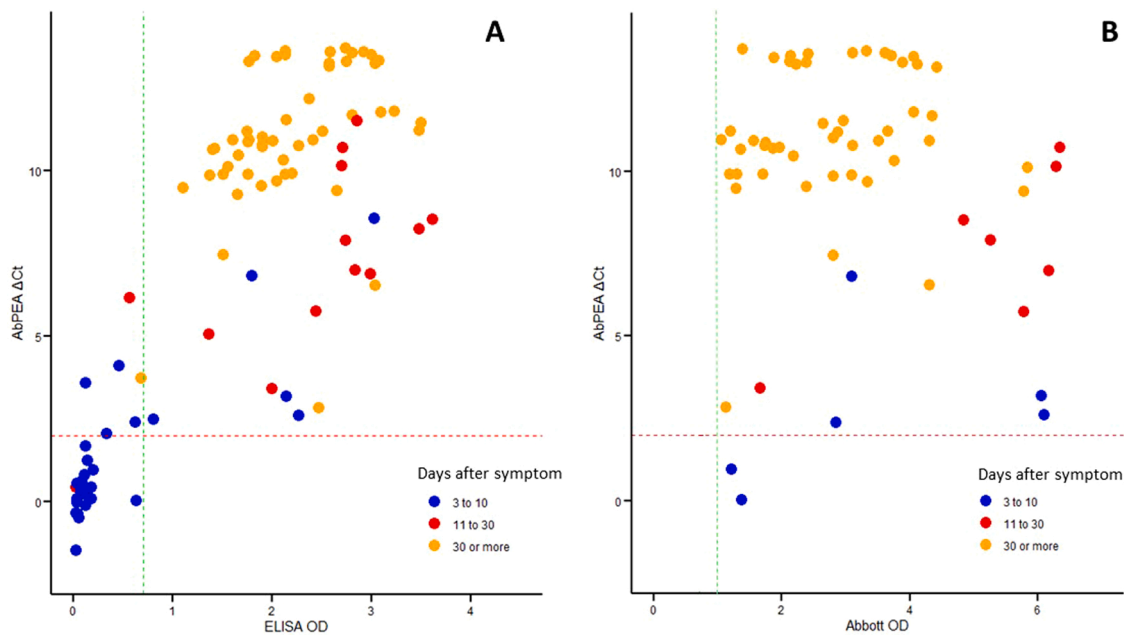
#### Comparison of AbPEA to other methods

As shown in Fig. 5A the results of AbPEA analyses of patient samples correlated slightly better with those of the in-house ELISA, both assays detecting S1-RBD antibodies. In contrast, the Abbott assay targets NP antibodies. The Abbott data presented in Fig. 5B were from 66 sera. All samples taken after 31 days of symptom were positive by all three methods. One of the significant differences between the three assay techniques was that  $\Delta\text{Ct}$  values from AbPEA tests correlated better with the time of sample collection, with higher values one month after symptom onset than within one month. Among 29 sera taken 3–10 days after symptom onset, 9 were positive by AbPEA and 5 positive by ELISA. Two samples that were positive by Abbott, but negative by both AbPEA and ELISA were collected 4 or 10 days after symptoms (data in Supplementary materials).

The assay sensitivity of AbPEA was further compared to one of the most sensitive assays, Meso Scale Discovery (MSD), by titration experiments. A selection of 8 serum samples with distinct antibody levels according to earlier AbPEA analyses were used for comparison. Sera were serially diluted from 50- to 500,000-fold for MSD and 10- to 100,000-fold for AbPEA. One  $\mu\text{l}$  and 50  $\mu\text{l}$  of each dilution, respectively, was used for AbPEA and MSD. In the case of AbPEA, SARS-CoV-2 S1-



**Fig. 4.** AbPEA analyses of sera from 94 COVID-19 positive patients and 100 negative control samples. A) The cutoff of  $\Delta\text{Ct}$  2 was used to distinguish samples positive or negative for S1 antibodies. Color codes represent the samples collection times after symptom onset. B) ROC analysis of the AbPEA analysis for S1-RBD-specific antibodies yielded an AUC of 94.4% for all samples collected from 3 days (blue) and 99.8% for samples collected from 11 days after symptom onset (red).

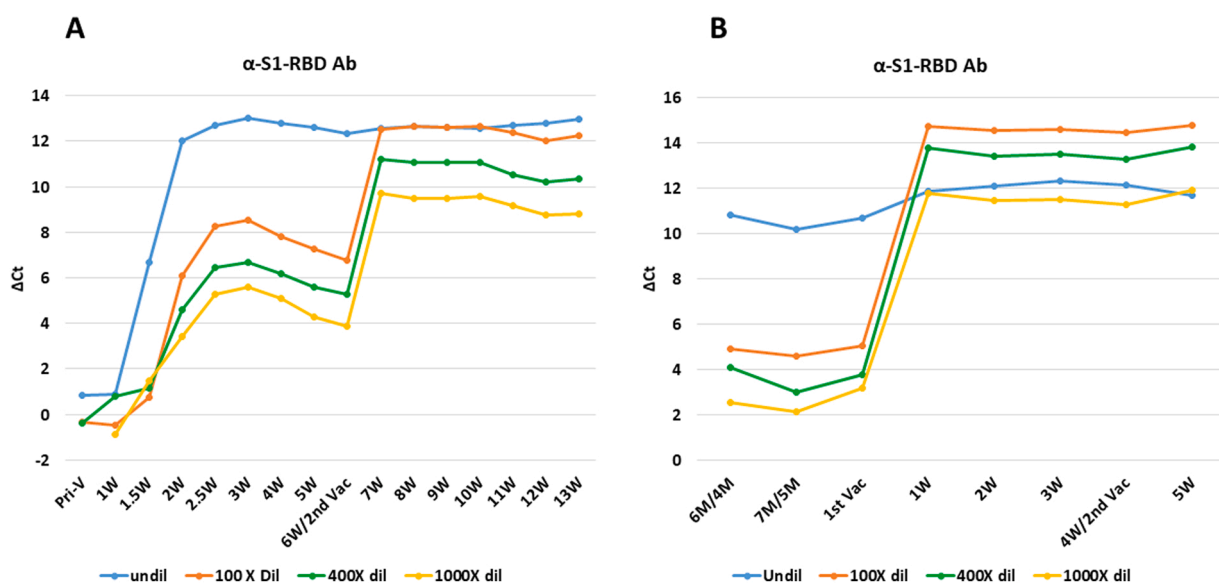


**Fig. 5.** Correlation between AbPEA results and those from ELISA (A) or from the Abbott assay (B). 94 serum samples were compared between AbPEA and ELISA. The  $\Delta Ct$  value for AbPEA vary over a log2 scale corresponding to a greater than 15,000-fold range. Data from the Abbott assay was only available for 66 out of the 94 samples for the comparison between AbPEA and the Abbott assay. Color codes represent the number of days the samples were collected after onset of symptoms. Dashed lines mark the cutoff value for positive and negative samples. The cutoff value for AbPEA is  $\Delta Ct$  of 2, ELISA is OD of 0.7 and Abbott is OD of 1.

RBD antibodies could be detected using as little as 0.1 nl of high-titer serum samples, such as samples 1 and 2, while a 1  $\mu$ l sample volume also gave positive results for samples with low antibody levels, taken 8 days after symptom debut (results are included in [supplementary Table S3](#)). Accordingly, samples of 1  $\mu$ l of undiluted serum should be used in AbPEA to maximize diagnostic sensitivity, although a 1:10 dilution is helpful to distinguish among samples with high antibody levels. In MSD, a 50,000-fold dilution or 0.1 nl of serum was optimal for most samples according to the manufacturer. However, at this dilution, positive samples with low antibody levels from 8 days after symptom debut were negative.

*Monitoring anti-S1-RBD antibody levels pre- and post-vaccination by AbPEA analysis of DBS*

Anti-S1-RBD antibody levels in self-sampled DBS collected prior or at different time points after a first and a second vaccination were examined. In total, 101 healthy individuals from the medical faculty of Uppsala University participated in this survey. Sixteen of the participants collected blood samples prior to or on the day of vaccination, as well as at weeks 1, 1.5, 2, 3, 4, 5 and 6 following their first and then also a second vaccination. 1.2 mm diameter discs (corresponding to approximately 0.5  $\mu$ l fresh blood) were added to an incubation solution



**Fig. 6.** Detection of anti-S1-RBD antibody levels in serially collected DBS samples pre- and post-vaccinations in an individual without previous infection (A), and one having undergone two infections before vaccination (B). Paper discs, 1.2 mm in diameter (corresponding to approximately 0.5  $\mu$ l fresh blood), were cut from a DBS and either added directly into the incubation solution (blue line) or eluted and used as 1  $\mu$ l of 100-fold (orange line), 400-fold (green line) and 1000-fold (yellow) dilutions.  $\Delta Ct$  was the Ct value of vaccinated individual samples subtracted from the average Ct values of 21 DBS collected in 2015 and 2019.

either directly or after elution and using 1  $\mu$ l of 100-, 400- and 1000-fold dilutions. DBS collected from 21 individuals in 2015 and 2019 were used as negative controls. In addition, repeated tests of DBS samples taken at the same time and stored either at RT, 4  $^{\circ}$ C or – 20  $^{\circ}$ C for 1 week, as well as collected 3–5 weeks after a second vaccination yielded consistent  $\Delta$ Ct value. Furthermore, the same results were obtained when analyzing anti-S1 antibodies from  $\phi$ 1.2 mm DBS and 0.5  $\mu$ l fresh blood from the same individual, indicating good efficiency of antibody detection from DBS (data not shown).

Anti-S1-RBD antibody levels were measured by AbPEA in a series of DBS collected from two individuals, one vaccinated individual without previous infection (Fig. 6A) and another having received one vaccination after two infections (7 and 5 months before the vaccination) (Fig. 6B). In the first case, antibody levels increased rapidly during the first two weeks after a first vaccination and reached the highest level in the 3rd week, remaining at similar levels even after a second vaccination when undiluted samples were analyzed (blue line). These results were at plateau levels for the assay, and therefore 100-fold, 400-fold and 1000-fold dilutions of the samples were also analyzed. A significant increase in antibody levels was observed at one week after a second vaccination, which was sustained for over three weeks and decreased slightly after one month. For the previously infected person, antibody levels were already high before vaccination. A significant increase of antibodies after a first vaccination was observed after sample dilution, but only a limited further increase was detected after the second vaccination. The results suggested that a 100-fold dilution was sufficient for accurate antibody detection after a second vaccination. However, at a 100-fold dilution, antibody levels were not detectable 1.5 week (11 days) after a first vaccination. To investigate antibody levels in DBS over a wide concentration range, both punched-out samples without elution, as well as eluted and 100-fold diluted samples were used in the following experiments.

Antibody levels were measured in consecutive samples from 14 vaccinated individuals using punched-out disc directly without elution and 100-fold diluted DBS samples after elution. Antibody levels varied among the individuals, but the changes across two vaccinations were similar, increasing to a maximum at two weeks after a first vaccination, and sustained before increasing further after a second vaccination (Fig. 7). Antibody levels increased already to a maximum at one week after the second vaccination, and remained at similar levels for about three months before slowly decreasing.

In addition, AbPEA analyses of S1-RBD antibody responses were performed on DBS collected from 21 individuals in 2015 or 2019 before

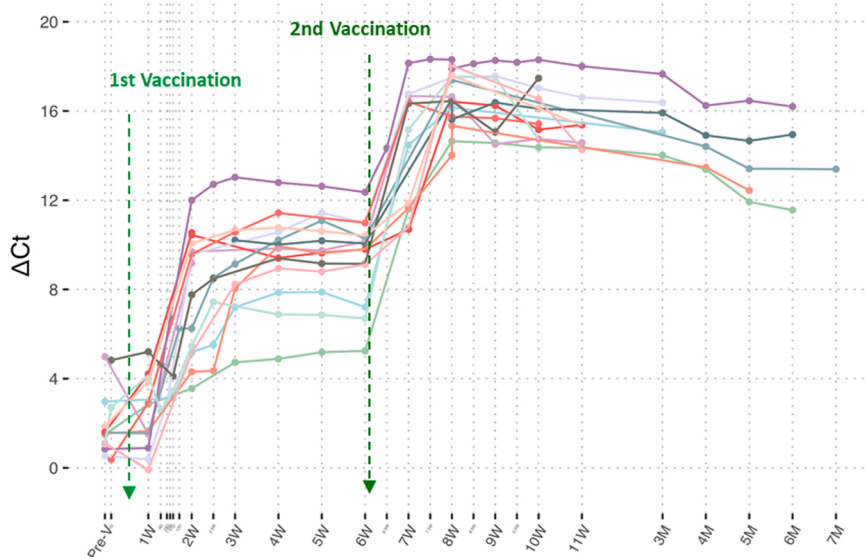
the pandemic and 141 DBS collected from 101 individuals during the pandemic, including 27 DBS collected before vaccination, 37 DBS collected after a first vaccination and 78 DBS obtained more than one month after a second vaccination (Fig. 8). The average AbPEA results increased by more than 8.76  $\Delta$ Ct values or 430-fold by 3 weeks after a first vaccination, compared to levels before vaccination. The second vaccination resulted on average in a further boost of 4.2  $\Delta$ Ct values, or another 20-fold. Antibody levels of individuals who had been infected before or after vaccination (red circles in Fig. 8) were higher than those of individuals who had not had the disease.

## Discussion

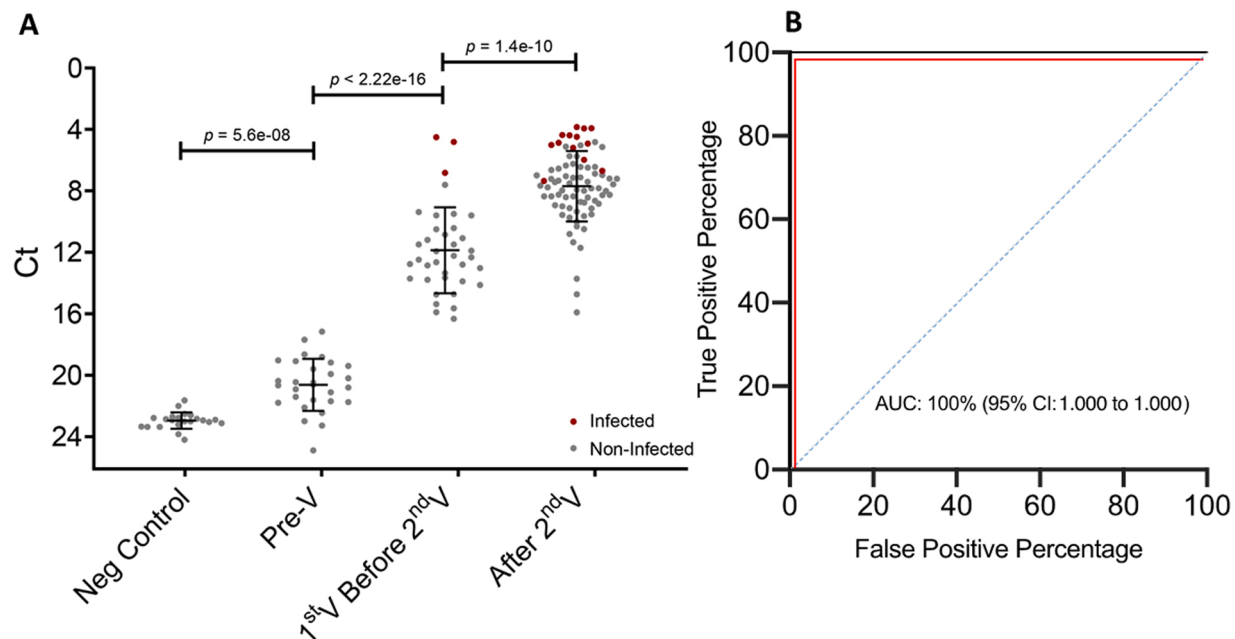
A homogenous PCR-based assay, AbPEA, is presented, serving to sensitively and conveniently detect antibody responses to the SARS-CoV-2 proteins RBD and NP after infection or vaccination. The assay is based on the proximity ligation assay technology for protein detection [4], which has been further developed and commercialized as proximity extension assays (Olink Proteomics, [www.olink.com](http://www.olink.com)). In AbPEA the dimeric or multimeric nature of antibodies serves to bring into proximity pairs of antigen proteins, conjugated with oligonucleotides. After incubation, the reactions are diluted with the addition of a DNA polymerase. This allows oligonucleotides on pairs of conjugates that remain in proximity by virtue of having been bound by the same antibodies to be enzymatically extended, generating amplifiable reporter DNA strands with no requirement for washes.

A closely related DNA-assisted antibody detection technique, agglutination-PCR (ADAP), has previously been applied for detection of anti-SARS-CoV-2 antibodies both in serum/plasma and DBS samples from patients [6,7]. The assay is similar to AbPEA in that pairs of oligonucleotides are conjugated to S1-RBD protein molecules, which are brought into proximity upon binding to antibody, although a proximity ligation rather than extension mechanism is used to create the amplicon that is then measured via PCR. Their assay consists of four steps and uses 4  $\mu$ l sample, while AbPEA generates results in three reaction steps using 1  $\mu$ l sample. For analysis of DBS by ADAP, antibodies are first eluted in 1000  $\mu$ l buffer and then concentrated.

In this report, the assay sensitivity and dynamic range of AbPEA were investigated, and the assay was benchmarked to other methods for measuring specific antibody responses. Furthermore, the changes in antibody levels were monitored during the course of two consecutive vaccinations using serially self-collected DBS. The performance of AbPEA was evaluated using both liquid serum samples and DBS from



**Fig. 7.** Time-course of antibody production by 14 vaccinated individuals using DBS. The individuals were followed from before a first vaccination at time 0 and a boost at 6 weeks. The times of vaccinations are indicated by vertical lines.  $\Delta$ Ct was the Ct value of vaccinated individual samples subtracted from the average Ct value of 21 DBS collected in 2015 and 2019. The antibody levels after second vaccination were measured after 100-fold dilution, but their  $\Delta$ Ct values were calculated by adding 6.64 [ $\log_2$  100] to compensate for the 100-fold dilution.



**Fig. 8.** Comparison of antibody levels for negative control samples and those collected at different stages of vaccination using DBS samples. A) Samples were collected in 2015 and 2019 before the pandemic (Neg control), and among vaccinated individuals sampled were obtained pre-vaccination (PreV), 4–6 weeks after a 1st vaccination (1stV before 2ndV) and 1–8 month after 2nd vaccination (After 2nd) as reflected in bee swarm plots. Red circles indicate individuals who also had been infected by the virus. For DBS samples collected before the pandemic, pre-vaccination and after 1st vaccination, AbPEA were performed directly on paper punches without elution and dilution. For DBS samples collected after a 2nd vaccination or after infection and 1st vaccination, AbPEA was performed after elution and 100-fold dilution, but the Ct values were calculated by subtracting 6.64 from detected Ct of 100-fold dilution. P-values were determined by a Wilcoxon test. B) ROC values were calculated using data after the first vaccination.

individuals having been infected by or vaccinated against SARS-CoV-2. The specificity for both samples reached 100%, as all serum samples collected during 2019 from patients infected with other virus as well as DBS collected in 2015 or 2019 were negative. The ROC analyses revealed an AUCs of 0.998 (95%CI: 0.993–1) for samples collected from 11 days after COVID-19 symptom debut. Anti-S1-RBD antibodies could be also detected from DBS collected from 11 days after vaccination. Assay sensitivity of AbPEA was similar to that of commercial assays from Abbott and MSD using the same serially diluted samples. AbPEA could detect anti-S1-RBD antibody levels in high-titer serum samples even after 10,000-fold dilution corresponding to 0.1 nl aliquots of serum. To achieve sensitive detection early in immune responses undiluted samples were used, while distinction among further elevated antibody levels after boosting required that samples were diluted.

By using both undiluted and 10-fold diluted serum samples, antibody concentrations could be monitored over more than 3 logs. Since AbPEA measures how antibodies can bring oligonucleotide-conjugated antigen molecules into proximity, the assay can detect all classes of immunoglobulins (Ig). Since no anti-Ig reagents are required, the same assay can be applied to assess antibody responses to a given protein in any species, for example for veterinary purposes (data not shown).

Most data presented here concern detection of anti-S1-RBD antibodies. However, one of the defining features of the PEA assay mechanism is that it lends itself to multiplexing. The assays developed by Olink Proteomics measure 96 or even more proteins and controls in individual samples. The reason for this far greater potential for multiplexing PEA reactions compared to sandwich ELISA reactions is that the assays are designed so that only the correct pairs of reagents can give rise to detectable amplicons. The AbPEA therefore has excellent potential to allow measurements of antibody responses to large numbers of antigens in the same assay. Such measurement of antibody repertoires can prove of diagnostic value in many contexts. Besides immune responses to infectious agents or vaccines, as demonstrated here, it is also of interest to survey responses to autoantigens, and antibodies to interferon have for

example been linked to poor outcome in COVID-19 [22]. In preliminary experiments a four-plex panel was used to simultaneously assess antibody levels directed against S1-RBD, NP, IFN $\alpha$ 1 and IFN $\omega$ 1 (data not shown). Consistent results were obtained when measuring antibody responses to S1-RBD and NP in individuals who had been infected with the virus. Furthermore, significant levels of anti-NP antibodies could be detected from DBS collected from individuals that had been infected by SARS-CoV2 after 2 doses of vaccinations against the S1-RBD protein (data not shown). The anti-NP assay showed a higher background and a relatively low signal compared to that for S1-RBD, however, and further optimization is warranted. Applying AbPEA to self-sampled DBS, antibody responses were detected by 1.5 weeks after a first vaccination. In order to demonstrate the further increased antibody levels after a second vaccination, DBS portions were eluted and diluted 100-fold.

In summary, AbPEA allows convenient detection of antibody levels across wide dynamic ranges in a convenient format with no requirement for washes and with a potential for extensive multiplexing.

#### CRediT authorship contribution statement

HZ: designed, developed and performed experiments, collected and analyzed samples, interpreted data, wrote first draft. MW: performed experiments, analyzed and interpreted data, performed statistical analyses, revised manuscript. PM: analyzed and interpreted data, performed statistical analyses. LL: undertook experiments, revised manuscript. FS: contributed reagents, wrote manuscript. RG: performed experiments, revised manuscript. N-VK: contributed reagents, performed experiments. TM: collected samples, revised manuscript. KN: provided samples, proof read manuscript. MÅ: contributed reagents, guidance on requirements for SARS-CoV-2 antibody testing, revised and edited manuscript. MK-M: secured ethical permissions, revised and edited manuscript. Y-FM: collected samples, performed assay, revised and edited manuscript. UL: obtained funding, designed assay, supervised work, edited manuscript. All authors have approved the final

manuscript.

### Declaration of Competing Interest

UL is a founder and shareholder of Olink Proteomics, having rights to the PEA technology. All other authors declare that they have no competing financial interests or personal relationships that could influence the work reported in this paper.

### Data availability

Data will be made available on request.

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.nbt.2022.11.004](https://doi.org/10.1016/j.nbt.2022.11.004).

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