doi:10.2533/chimia.2021.446 Chimia 75 (2021) 446–452

FH HES

Universities of Applied Sciences

Fachhochschulen – Hautes Ecoles Spécialisées

Development of a Unique Rapid Test to Detect Antibodies Directed Against an Extended RBD of SARS-CoV-2 Spike Protein

Larissa Brosi[ab], Eric Kübler[a], Anna Weston[a], Patrick Romann[a], Sherin Panikulam[a], Lorin Dirscherl[b], Michael Gerspach[b], Cedric Giegelmann[b], Daniele Dolce[2], Marie-Eve Überschlag[b], Anna Melone[b], Frank I. Bantleon*[b], Thomas K. Villiger*[a], and Christian-B. Gerhold*[b]

*Correspondence: Dr. F. I. Bantleon[b], E-mail: fb@buhlmannlabs.ch ; Dr. T. K. Villiger[a], E-mail: thomas.villiger@fhnw.ch; Dr. C.-B. Gerhold[b], E-mail: cbg@buhlmannlabs.ch
[a]FHNW, School for Life Science, Hofackerstr. 30, CH-4132 Muttenz, Switzerland; [b]BÜHLMANN Laboratories AG, Baselstr. 55, CH-4124 Schönenbuch, Switzerland

Abstract: Serological testing for antibodies directed against SARS-CoV-2 in patients may serve as a diagnostic tool to verify a previous infection and as surrogate for an elicited humoral immune response, ideally conferring immunity after infection or vaccination. Here, we present the recombinant expression of an extended receptor binding domain (RBD) of the SARS-CoV-2 Spike protein used as capture antigen in a unique rapid immunoassay to detect the presence of RBD binding antibodies with high sensitivity and specificity. As currently available vaccines focus on the Spike RBD as target, the developed test can also be used to monitor a successful immune response after vaccination with an RBD based vaccine.

Keywords: Antibodies · Lateral Flow Assay · Point-of-Care · RBD · SARS-CoV-2

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the origin of the corona virus disease 2019 (COVID-19) pandemic that has a huge impact on the world's economy and people's health and has caused millions of deaths since its first report in China at the end of 2019.[1] Most SARS-CoV-2 infected individuals experience mild symptoms but it is still unknown whether these can induce a persistent immune memory for life-long immunity. The preferred methods to test for a SARS-CoV-2 infection comprise nucleic acid tests as well as viral antigen detection systems. While useful in terms of controlling the spread of the virus, the limitations of these tests lie in their positivity rate confined to the small time window during which the virus can be verified.[2] In contrast, serological tests detect immune system-induced antibodies directed against SARS-CoV-2 that may be detectable over a prolonged period of time. The finding that neutralizing anti-SARS-CoV-2 antibodies most often recognize the receptor binding domain (RBD) and within it the receptor binding motif (RBM) of the virus' spike protein led to the development of vaccines based on just this spike protein.[3] Thus, serological tests may also be useful in determining the immunological status of vaccinated people.

In support of the findings on the epitopes of the patient's serum anti-SARS-CoV-2 antibodies, several studies have shown that neutralizing mABs generated against a recombinant SARS-CoV-2 spike protein expressed in mammalian cells recognize the RBM and that some of these interfered with virus binding to the ACE2 receptor, thus with the virus entering the cell, by preventing the binding of the RBM to the ACE2 receptor.[4,5] The mABs recognizing epitopes outside the RBM but within the RBD are speculated to indirectly affect the conformation of the RBM and therefore its binding to the ACE2 receptor.[6] Taken together, the results show that the RBD alone could serve as an antigen to measure serum anti-SARS-CoV-2 antibodies but that retaining correct folding is of paramount importance as some of the antibodies show RBD-structure specific binding characteristics.

A new serological test system allowing a more precise determination of the antibody status in individual sera after SARS-CoV-2 infection or (RBD-based) vaccinations is presented. An extended RBD (RBD+) is the sole antigen used in this test that selectively probes for antibodies that hinder SARS-CoV-2 docking onto the ACE2 receptor of human host cells. Furthermore, this RBD+ exhibits a more compact structure than canonical RBD constructs due to an additional disulfide bond which also confers more thermal stability. Highly purified RBD+ is thus suggested to be a superior antigen for serological testing to assess potential immunity towards SARS-CoV-2.

2. Materials and Methods

2.1 *Cloning of the Avi-RBD / RBD+ Construct*

The RBD+ coding sequence of the spike protein (aa319 – 591) was amplified from a SARS-CoV-2 (2019-nCoV) Spike S1 Gene ORF template (Sino Biological, Wayne PA, USA). To create the Avi-RBD construct, the primers 5'-GGTGGTGAATTCGGCCTGAACGACATCTTCGAGGCCCAGAAGATCGAGTGGCACGAAAGGGTCCAACCAACAGAG-3' and 5'-GGTGGTGGATCCGAAGTTCACACACTTGTTCTTCACC-3' were used. For the RBD+ construct, the primers 5'-GGTGGTGAATTCAGGGTCCAACCAACAGAG-3' and 5'-GGTGGTGGATCCGGAACATGGTGTGATGTCCAG-3' were utilized. Amplified constructs were digested with EcoRI and BamHI and subsequently ligated into the plasmid pEXPR-IBA42 (IBA GmbH, Göttingen, Germany).

2.2 *Avi-RBD / RBD+ Plasmid Production and Purification*

The plasmids encoding either the Avi-RBD or RBD+ sequences were amplified in the *E. coli* DH5α strain in 25 g/L LB medium supplemented with 100 µg/mL ampicillin. The preculture was incubated for 16–17 h at 37 °C and 500 mL were used to inoculate a 20 L Techfors S bioreactor (Infors HT, Bottmingen, Switzerland). Plasmid extraction was performed following the manufacturer's instructions (Pure Yield Plasmid Maxiprep System, Promega, United Kingdom).

2.3 *Avi-RBD / RBD+ Protein Expression and Purification*

HEK293 suspension cells were cultivated in Expi293 expression medium (Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured in vented 250 mL shake flasks (Corning, Inc., Corning, NY, USA) under controlled conditions (36.5 °C, 8% CO_2, >80% humidity, 280 rpm). Viable cell density and viability were determined with a Countess Automated Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA). Cells were further expanded in 1 L shake flasks (Corning, Inc., Corning, NY, USA), centrifuged and resuspended in fresh medium at a cell

density of 20×10^6 cells/mL. Avi-RBD or RBD+ plasmids and PEI Max (Polyscience Inc, IL, USA) were added in a 1:3 plasmid to PEI ratio at a concentration of 2.5 mg PEI per 10^9 cells. The transfection culture was incubated for 2 h and then seeded into fresh medium containing 3.5 mM valproic acid in a 5 L shake flask (Corning, Inc., Corning, NY, USA). The culture was incubated at 36.5 °C, 8% CO_2 and 95 rpm with a seeding density of 3×10^6 cells/mL for a maximum of 7 days. Cell culture fluids were harvested, centrifuged and filtered prior to Ni-Sepharose affinity chromatography using a HisTrap™ excel column. The eluted product stream was loaded on a HiLoad™ 16/600 Superdex™ 200 pg for further purification. All columns are from Cytiva Life Sciences, Marlborough, MA, USA. Loading, washing, and elution from the Ni-Sepharose and SEC columns were optimized according to the resin producers' recommendations.

2.4 *Western Blot Analysis*

Western blots were performed by initial SDS-PAGE with the Bolt™ system (Thermo Fisher Scientific, Waltham, USA) using MOPS buffer under reducing or non-reducing conditions. Proteins were transferred to a PVDF membrane with the Trans-Blot turbo™ system (Bio-rad Europe, Cressier, France) and the membrane was blocked for 1 h with PBS containing 4% skim milk powder followed by 1 h incubation with a serum pool from three Covid19 patients diluted 1:100 in PBS containing 2% skim milk powder. After washing with PBS-Tween20, the blot was incubated with anti-human IgG-HRP (Dako, Glostrup, Denmark) diluted 1:10'000 in PBS containing 2% skim milk powder for 1 h. After three washing steps with PBS-Tween20, the blot was developed using Western bright Quantum HRP substrate (Advansta, San Jose, USA) and recorded using the Fusion Solo Imager (Vilber, Collégien, France).

2.5 *Sybody Expression and Purification*

Sybody expressing *E. coli* MC1061 were obtained by the group of Prof. M. Seeger (University of Zurich).[7] The preculture was incubated for 16–17 h at 37 °C and 400 mL were used to inoculate a 20 L Techfors S bioreactor (Infors HT, Bottmingen, Switzerland). Sybodies were expressed overnight in TB medium supplemented with 25 µL/mL chloramphenicol. After centrifugation of the harvest, cells were disrupted by a high pressure homogenizer (Avestin EmulsiFlex C3, Ottawa, Canada) followed by Ni-Sepharose affinity chromatography using a HisTrap™ FF crude column (Cytiva Life Sciences, Marlborough, MA, USA). The eluted product stream was loaded on a HiLoad™ 16/600 Superdex™ 200 pg (Cytiva Life Sciences, Marlborough, MA, USA) for further purification.

2.6 *Evaluation of Thermal Stability*

To determine the thermal stability of the different RBD constructs, 70 µL of each RBD construct (1 mg/mL) in PBS was measured by static light scattering (SLS) using Zetasizer Ultra (Malvern Panalytical B.V., Netherlands) under reducing (1 mM dithiothreitol) and non-reducing conditions. The samples were centrifuged at 20'000 × g and the supernatants were transferred to a quartz cuvette (ZEN2112, Malvern Panalytical B.V., Netherlands). Three RBD constructs were analyzed in technical triplicates over a temperature range from 25 °C to 95 °C, increasing the temperature in 2.5 °C increments and incubating the samples for 30 s at each temperature point. Derived mean count rate was measured in kilo counts per second [kcps] and plotted versus temperature.

2.7 *Development and Validation of the Lateral Flow Assay (LFA)*

BÜHLMANN Laboratories developed the Quantum Blue® SARS-CoV-2 RBD+ assay under compliance to grant IVDD (In Vitro Diagnostic Directive) certification under self-conformity including a validation of the test according to CLSI (Clinical & Laboratory Standards Institute) standards. For development purposes, positively tested patient samples were provided by MediXtoujours (Basel, Switzerland) and the University Hospital Fribourg (Fribourg, Switzerland). The assay was validated and verified using samples purchased from TRINA Bioreactives AG (Naenikon, Switzerland), in.vent Diagnostica GmbH (Henningsdorf, Germany) and iSpecimen Inc. (Lexington, USA). The matrix equivalency test was performed in two replicates according to the instructions for use (IFU) and analyzed by visual inspection as well as with a Quantum Blue® Reader 2nd Generation (QB2) and a Quantum Blue® Reader 3rd Generation (QB3G). Finger-prick blood of donors, negatively tested for SARS-CoV-2 antibodies, were spiked with different amounts of SARS-CoV-2 antibody serum and 0.9% NaCl solution to obtain a positive, a low positive and a negative sample. Serum dilution in finger-prick blood did not exceed 5% (v/v). The spiked blood was transferred onto the sample application pad using a 20 µL PTS Capillary (PTS Diagnostics, Whitestown, USA). Three drops of Quantum Blue® SARS-CoV-2 RBD+ chase buffer was applied to the sample application pad and read-out was performed after 15 minutes.

2.8 *Vaccination Success Monitoring*

As part of the test development process, patients who received the SARS-CoV-2 vaccination were provided with the Quantum Blue® SARS-CoV-2 RBD+ kit for self-testing in order to assess usability of the assay. Patients performed the test at different timepoints following the two vaccination appointments according to the kit's instructions and recorded the lateral flow assay result photographically 15 minutes after blood sample application. Patients gave consent to anonymously publish the results of their tests.

3. Results and Discussion

3.1 *Comparison of RBD Antigens*

The receptor binding domain (RBD) of SARS-CoV-2 binds to the human ACE2 receptor as an initial step for cell invasion.[8] Hence, antibodies targeting the RBD have the potential to block the interaction of the virus with the receptor and thereby hampering viral docking to human cells.[9] In the development of the Quantum Blue® SARS-CoV-2 RBD+ rapid test, the RBD was chosen as the sole antigen in order to specifically probe for antibodies demonstrating the highest probability for neutralizing viral potency. For this purpose, three different RBD constructs (RBD commercial, Avi-RBD and RBD+) were evaluated regarding their ability to bind antibodies of COVID-19 patients and their thermal stability. The canonical RBD without and with Avi-Tag differ from RBD+ in their length and the number of cysteines (Spike S AA 319-541 vs. Spike S AA 319-591).

A western blot analysis comparing three different RBD constructs revealed the importance of disulfide bonds in RBD constructs for antibody recognition (Fig. 1A, B). Transferred RBD constructs (319-541) under reducing conditions and thus without disulfide bridges were not recognized by the polyclonal antibodies of these patients, highlighting the importance of disulfide bond formation in the RBD antigen. The RBD+ showed a slight signal also under reducing conditions suggesting that a linear epitope between AA542 and AA591 can elicit an immune response.

Moreover, the SDS-PAGE analysis and size exclusion chromatography (SEC) of RBD and RBD+ constructs emphasized the significant difference in the monomer to dimer ratio and the overall purity (Fig. 1B, D). In the SEC chromatogram, RBD+ showed a monodisperse distribution in contrast to the RBD construct exhibiting a higher molecular weight 'shoulder' that cor-

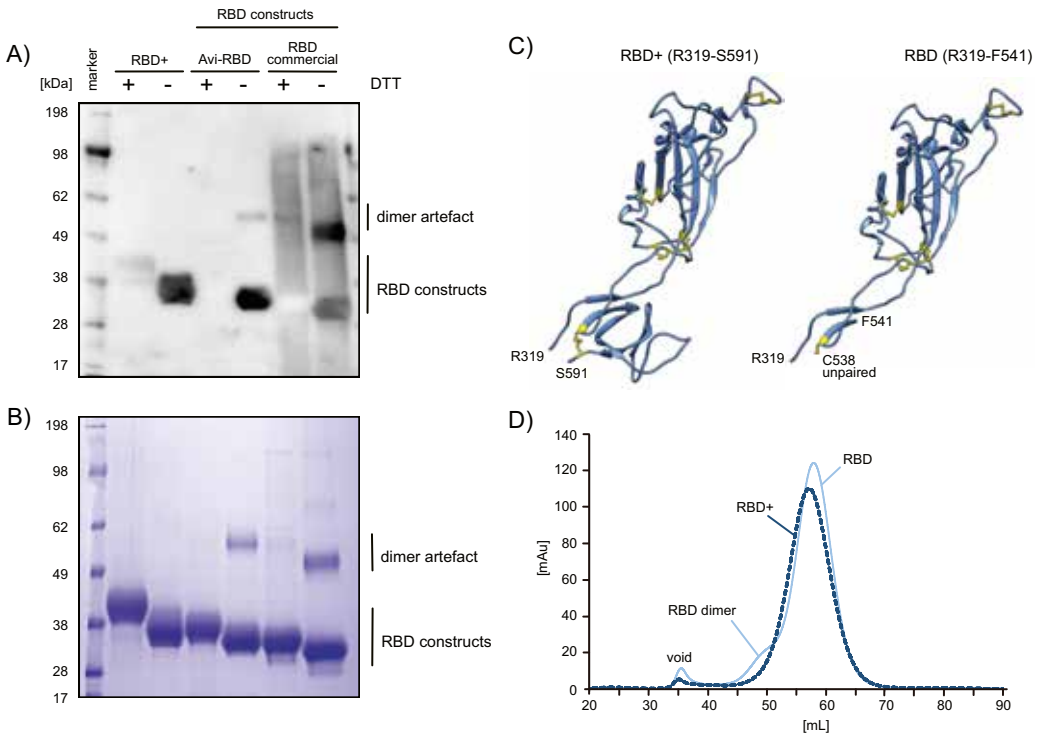

Fig. 1. Comparison of RBD constructs. A) Western blot of different RBD constructs with pooled sera of three Covid19 patients and anti-human IgG-HRP with B) corresponding SDS-PAGE. C) Structural depiction of the RBD+ and the RBD constructs illustrating the unpaired Cysteine 538, which binds Cys 590 in the RBD+ construct.[10] D) Overlay of size exclusion chromatograms of RBD and RBD+.

responds to the dimer artefact originating from intermolecular disulfide bonds between Cysteine 538 of two RBD molecules. Such an artefact may pose several reproducibility problems if used as raw material in an antibody test. Structural evaluation of spike protein confirmed that an RBD antigen construct with an even number of cysteines (RBD+) reduces the probability of intermolecular disulfide bridges (Fig. 1C).[11]

The additional disulfide bond of the RBD+ impairs dimer artefacts arising during recombinant expression and purification and is hypothesized to have a more compact structure compared to the RBD. This was corroborated by the finding that the RBD+ remains more confined upon heat exposure as evidenced in static light scattering (Fig. 2).

Static light scattering-derived mean count rate revealed different thermostability of the three RBD constructs. The RBD+ construct exhibited a high thermal stability and could not be fully denatured even at temperatures above 90 °C. The Avi-RBD construct with its unstructured N- and C-terminal parts appeared to be more susceptible to unfolding compared to RBD+. The commercial RBD with lower purity showed denaturing effects already at significantly lower temperatures, which may be explained by initial aggregation of impurity proteins thereby accelerating RBD denaturing (Fig. 2A). Since long-term stability of *in vitro* diagnostic (IVD) tests plays an important role for production, storage and logistics of these assays, it seems that highly pure and structurally confined RBD+ antigen may be superior to other RBD constructs for *in vitro* diagnostics. In presence of 1 mM DTT, the overall stability of the RBD constructs strongly decreased and did not cause significant differences between the constructs, highlighting once more that disulfide bridges in the RBD region of the SARS-CoV-2 Spike protein are essential for proper folding of this domain (Fig. 2B). Due to the monodisperse distribution during chromatographic purification and higher thermal stability, RBD+ was chosen as the sole antigen for the lateral flow assay.

3.2 *Set-up of the Quantum Blue® SARS-CoV-2 RBD+ Assay*

The purified RBD+ is used as the antibody-detecting antigen in the Quantum Blue® SARS-CoV-2 RBD+ assay. For this purpose, RBD+ is immobilized on the membrane as well as conjugated to cellulose nanobeads. With this set-up, bi- or multivalent antibodies recognizing RBD+ antigen are able to cross-link the

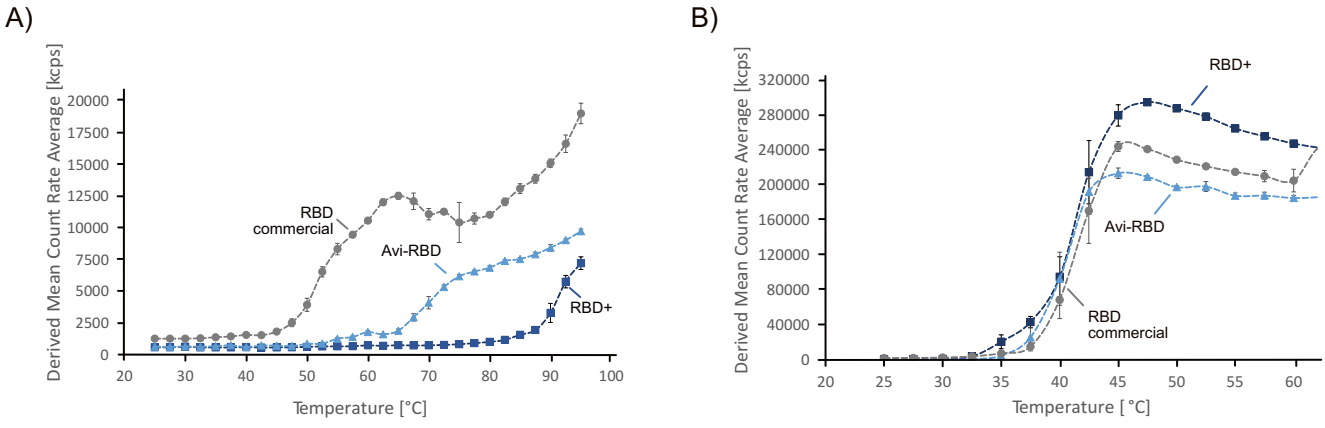

Fig. 2. Thermal stability evaluation of RBD constructs using SLS under A) non-reducing and B) reducing conditions (1 mM DTT).

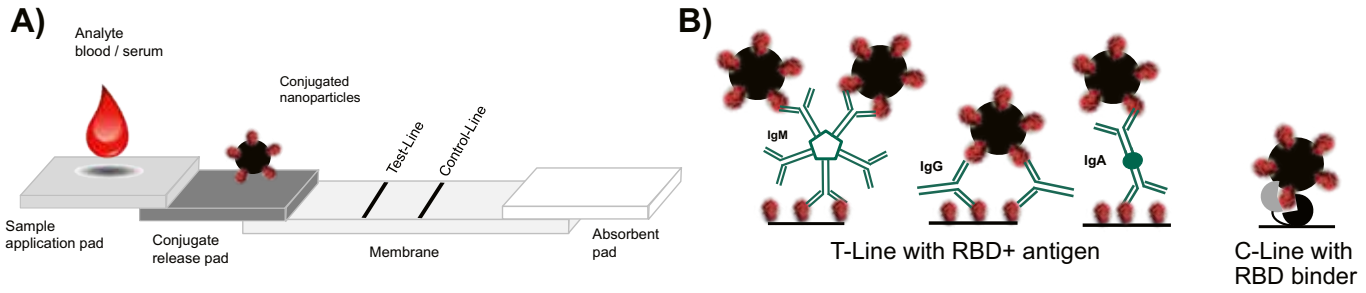

Fig. 3. A) Schematic representation of the Quantum Blue® SARS-CoV-2 RBD+ Lateral Flow test. RBD+ conjugated nanoparticles reside on the conjugate release pad. B) RBD+ specific antibodies of various isotypes cross-link the RBD+ antigen on the test line with RBD+ nanoparticles, while a synthetic binder against two RBD epitopes captures remaining RBD+ conjugated nanoparticles on the C-line. For more detailed information: Quantum Blue SARS CoV-2 RBD+ Antibody Rapid Test - YouTube: https://www.youtube.com/watch?v=5VH_hQB21RY

antigens on the test line (T-line) and on the cellulose nanoparticles. Hence, RBD+ detecting antibodies of different isoforms (*i.e.* IgG, IgM and IgA) will be visualized by immobilizing RBD+ conjugated cellulose nanoparticles on the test line.

The Quantum Blue® SARS-CoV-2 RBD+ assay therefore probes for potentially neutralizing antibodies irrespective of their isoform, without the ability to discriminate between them. Most SARS-CoV-2 antibody tests of various providers measure the presence of SARS-CoV-2 IgM and IgG antibodies in a patient sample separately. Interestingly, IgA antibodies are usually neglected in competitor assays despite their important role as first line defense in the mucosa, which might be particularly important for the defense against a respiratory virus, such as SARS-CoV-2.[12,13]

Every rapid test based on lateral flow also comprises a control line (C-line) to verify correct test handling. For this purpose, the Quantum Blue® SARS-CoV-2 RBD+ assay uses specific RBD binders originating from an *in vitro* selection from sybody libraries.[7] Sybodies are synthetic binders based on the VHH scaffold of camelid antibodies or nanobodies. Instead of camelid immunization, a fully *in vitro* selection process based on ribosome and phage display with large libraries of sybody sequences generated specific RBD binders. A fusion of two of these RBD sybodies recognizing different epitopes on the RBD antigen is immobilized on the C-line of the Quantum Blue® SARS-CoV-2 RBD+ assay. This fusion protein immobilizes remaining RBD+ conjugated nanoparticles on the C-line to ensure proper test handling. Additionally, this set-up makes it obsolete to use additional nanoparticles in this LFA system (Fig. 3).

3.3 *Performance of the Quantum Blue® SARS-CoV-2 RBD+ Assay*

In addition to the qualitative visual read-out the Quantum Blue® SARS-CoV-2 RBD+ can be analyzed by Quantum Blue® readers of the second and third generation. In order to establish a cut-off that discriminates between positive and negative samples, more than 100 serum samples collected before December 2019 and thus negative for SARS-CoV-2 antibodies were measured in the QB2 and QB3G to assess the cut-off of the Quantum Blue® SARS-CoV-2 RBD+. The cut-off for a positive SARS-CoV-2 antibody result with the Quantum Blue® SARS-CoV-2 RBD+ was chosen to be the mean of blank plus four times the standard deviation of the measurements.

Based on the data set of 104 negative samples, the cut-off for the readers was determined to be 37 mV (millivolts) for the QB2 and 1.5 GV (grey values) for the QB3G. These values correspond to a faint, but visual discrete band on the T-line of the test. Overall, 39 positive SARS-CoV-2 antibody serum samples according to RT-PCR tests were used for test development. A first benchmark test with the readily developed Quantum Blue® SARS-CoV-2 RBD+ test led to 85% sensitivity and 100% specificity in the QB2 (Fig. 4). One of the negative serum samples (N79) was just below QB2 cut-off, nevertheless a faint yet discrete band was visible, resulting in a 99% specificity by visual read-out.

After the design freeze of the Quantum Blue® SARS-CoV-2 RBD+ a validation and verification according to CLSI guidelines was performed to assess the overall performance of the assay. This included various aspects, such as test stability, robustness of the test, cross-reactivities etc. that are evaluated to assess test limitations listed in the IFU. A total of 57 positive control patients with a previous positive RT-PCR test on SARS-CoV-2 infection and 86 negative control patients with blood sample collection before December 2019 and tested positive for IgG antibodies against at least one other respiratory pathogen were included in the study. Positive and negative percent agreements between Quantum Blue® SARS-CoV-2 RBD+ and reference diagnosis (negative and positive controls) were estimated based on these cohorts.

The clinical agreement study of the validation phase was comparable to results obtained during the development of the as-

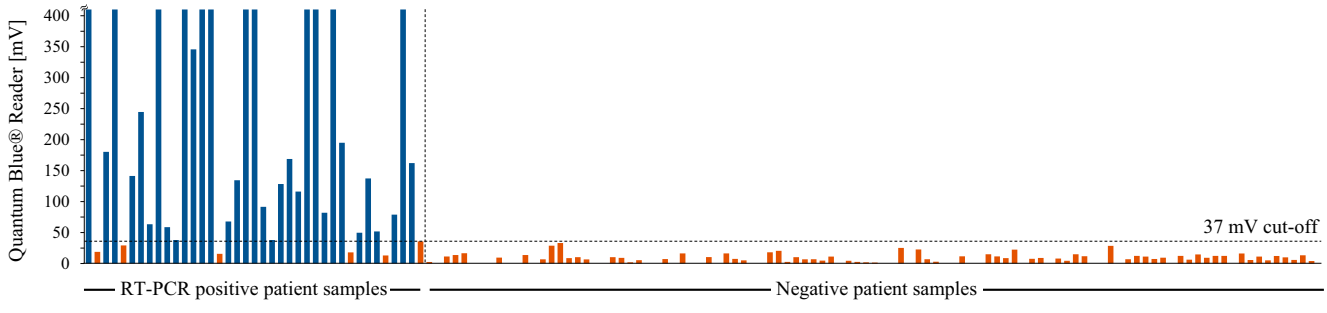

Fig. 4. Read-out of the Quantum Blue® SARS-CoV-2 RBD+ test with a Quantum Blue® Reader 2nd generation. In a cohort of 39 RT-PCR positive and 104 negative patient serum samples this cut-off resulted in a sensitivity of 85% and a specificity of 100% in the QB2 reader.

say with a different sample set. Depending on the read-out, specificity of the test was determined to be between 97.7% and 100%, while a sensitivity of 86% to 87.7% was measured (Table 1).

Table 1. Comparison of visual read-out and Quantum Blue® Reader. Positive percent agreement (PPA), negative percent agreement (NPA) and their respective 95% confidence interval (CI) were determined with 143 subjects (57 positive and 86 negative control samples).

Metrics	Visual read-out	Quantum Blue® Reader (2nd and 3rd generation)
PPA (95% CI) NPA (95% CI)	86.0% (74.2%, 93.7%) 100.0% (95.8%, 100.0%)	87.7% (76.3%, 94.9%) 97.7% (91.9%, 99.7%)

Serum applications may not be applicable for Point-of-Care or self-testing settings. Therefore, a matrix equivalency study using spiked finger-prick whole blood was conducted to show that the Quantum Blue® SARS-CoV-2 RBD+ Lateral Flow assay is capable to qualitatively assess the presence of antibodies in small blood samples. Plotting QB reader results of duplicate measurements of serum and whole blood antibody dilutions reveal similar measuring ranges for the accordant dilutions. Hence, antibodies from whole blood measured with the Quantum Blue® SARS-CoV-2 RBD+ Lateral Flow assay provide comparable results to serum samples (Fig. 5).

In a qualitative assessment, 100% positive percent agreement (PPA) and 100% negative percent agreement (NPA) were estimated between capillary blood and the corresponding serum matrix (Table 2). Therefore, the acceptance criteria for a whole blood application was fulfilled and the test is technically feasible for Point-of-Care or even self-testing use.

Table 2. Estimates of percent agreement between capillary blood results and corresponding serum results.

Metric	Read-out System	Estimate	95% CI[a]
PPA	Visual read-out, Quantum Blue Reader	20/20=100%	83% to 100%
NPA	2nd and 3rd Generation	10/10=100%	69% to 100%

[a]The exact binomial confidence interval (CI) was calculated with Analyse-it using the Clopper Pearson method.

3.4 *Cross-reactivity Assessment of the Quantum Blue® SARS-CoV-2 RBD+ Assay*

Potential cross-reactivities negatively affect the specificity of the test. For this reason, serum samples with antibodies directed against various viruses were tested. In a sample set with a mix of antibodies directed against common coronaviruses (HCoV-229E, -OC43 and -NL63) one sample out of nine was determined to be positive by visual inspection and by both reader generations. A systematic cross-reactivity may therefore be excluded for HCoV-229/-OC43/-NL63. The cause of positivity of this single positive sample is unknown. In total, 130 samples that are positive for various anti-virus antibodies, but negative for antibodies directed against SARS-CoV-2 did not show more than three positive results irrespective of the read-out method. The specimens include viral antibodies with specificity for hepatitis, influenza, respiratory syncytial virus (RSV), HIV, Epstein-Barr virus, cytomegalovirus, measles, herpes simplex, amongst others (detailed list included in Quantum Blue® SARS-CoV-2 RBD+ IFU). The specificity on this viral antibody sample set was determined to be 97.7% for visual read-out and QB3G read-out, while the QB2 cut-off was exceeded by two samples, resulting in a specificity of 98.5%. Therefore, the Quantum Blue® SARS-CoV-2 RBD+ assay shows no systematic cross-reactivity with antibodies directed against various other common viruses. Nevertheless, it is recommended to perform additional testing if individual patients with a known clinical history of hepatitis and other corona viruses exhibit a positive result.

3.5 *Quantum Blue® SARS-CoV-2 RBD+ Assay Vaccination Monitoring*

In addition to an elicited immune response as a consequence of infection, specific vaccinations also trigger the immune system to produce antibodies that confer immunity or some degree of resistance towards subsequent infections. Due to the neutralizing capacity of RBD binding antibodies, the RBD is also the preferred antigen for current vaccines that prove to be highly effective. Therefore, the Quantum Blue® SARS-CoV-2 RBD+ Lateral Flow assay should be able to monitor an elicited immune response based on RBD vaccines. As part of the assay development, a first self-testing set-up with finger-prick blood from patients vaccinated with the BioNTech/Pfizer or Moderna/Lonza vaccines illustrated that the test format has a usability for lay users and it showed a gradual increase of the T-line signal over time, which was significantly boosted after the second vaccination. It cannot be fully ruled out that a concomitant asymptomatic infection leads to an increase of RBD antibodies and hence to a

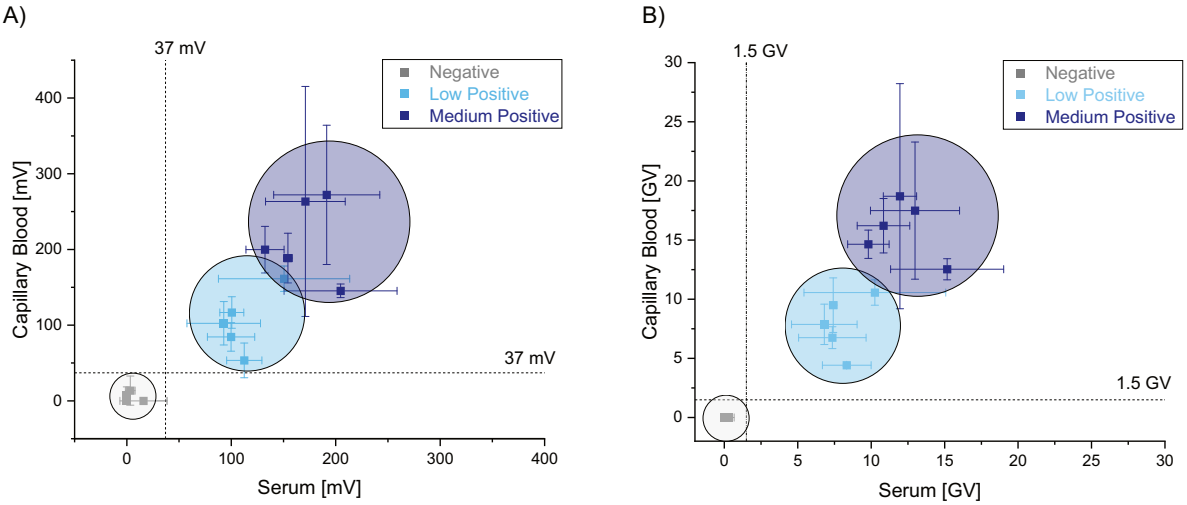

Fig. 5. Plots of comparable dilutions of SARS-CoV-2 antibodies in whole blood versus serum reveal similar categories based on read-outs with the QB2 (A) or the QB3G (B).

stronger signal in the Quantum Blue® SARS-CoV-2 RBD+ in this case, but the slight increase in signal over time and the significant leap in signal after the second vaccination suggest that the immune system was indeed triggered by the vaccine (Fig. 6A, B). Although these test results were not recorded with a QB3G reader, visual inspection clearly shows the increase in signal on the T-line. After the second vaccination, the T-Line intensity suggests a high titer of RBD binding antibodies, indicative of neutralizing potency.

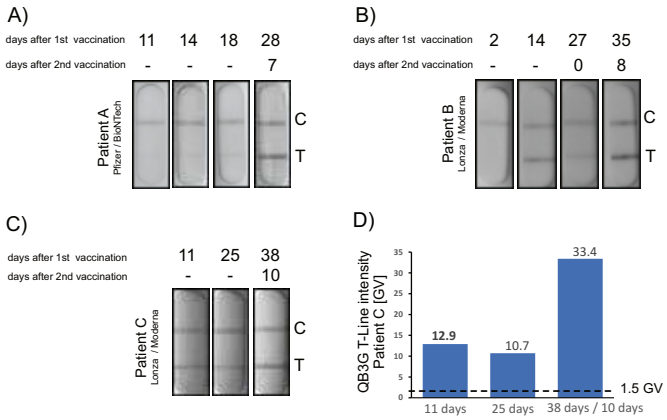

Fig. 6. Visualization of Quantum Blue® SARS-CoV-2 RBD+ results during self-testing of patients receiving vaccines from A) BioNTech or B, C) Moderna. D) Quantum Blue® SARS-CoV-2 RBD+ read-out by QB3G of patient C.

A third person (patient C) was also vaccinated with the Lonza/Moderna vaccine and the accordant immune response was monitored using the Quantum Blue® SARS-CoV-2 RBD+ with a concomitant QB3G read-out (Fig. 6C, D). The first data point was recorded 11 days after the first vaccination and exhibited a signal of 12.9 GV. Due to missing earlier data points, it remains unclear if the positive result can be solely attributed to the vaccination effect. A second test was conducted prior to the second vaccination leading to 10.7 GV which corroborates the first test result. Again, the second vaccination proved to significantly raise the antibody titers, as a third test 10 days after the second administration of the Lonza/Moderna vaccine resulted in 33.4 GV, which is indicative of an elevated antibody titer directed against the SARS-CoV-2 RBD domain. Patient C also receives an immunocompromising antibody therapy directed against TNFα.

Initial Quantum Blue® SARS-CoV-2 RBD+ tests with other immunocompromised patients 19 days after their second vaccination with the Lonza/Moderna vaccine suggest that treatment with anti-TNFα medication does not strongly interfere with vaccination success. However, receiving a cocktail of immunocompromising reagents, such as Tacrolimus after organ transplant may hamper the antibody response towards the RBD vaccine (data not shown). It has to be noted that the latter observation is preliminary and based on a single patient, but we suggest to closely monitor vaccination outcomes of immunocompromised patients after organ transplantation.

4. Conclusions

The COVID-19 pandemic triggered an unprecedented amount of research. Structural analysis of viral proteins, fast development of vaccines and a plethora of IVD tests to probe for viral antigens or specific antibodies against the virus. All of these insights and developments may help to manage and eventually overcome this pandemic. The Quantum Blue® SARS-CoV-2 RBD+ test was developed with the aim to assess potential immunity to the virus from 20 µL of finger-prick capillary blood. Due to its high specificity and the choice of the extended RBD+ antigen, it is highly probable that a positive signal in the Quantum Blue® SARS-CoV-2 RBD+ relates to the presence of neutralizing antibodies. Therefore, this test can serve as an inexpensive and rapid alternative to sophisticated SARS-CoV-2 neutralizing antibody assays. Moreover, the test allows to monitor vaccination success of RBD-based vaccines and can help to improve current and future vaccination strategies.

The use of RBD+ for the production of diagnostic assays is clearly advantageous due to the higher stability of this novel antigen in comparison to the commercially available variants. Furthermore, the RBD+ construct (319-591) as opposed to the widely used RBD constructs (319-541) bears a linear epitope within its structure while the canonical RBD sequence appears to have conformational epitopes only. This finding is corroborated by reports that a neutralizing linear epitope was identified at the position of SARS-CoV-2 Spike 556-570,[14,15] which is included in the sequence of the RBD+, but not in the RBD. The choice of the RBD+ as an antigen for the Quantum Blue® SARS-CoV-2 RBD+ thus has several advantages. The RBD+ domain structure is expected to have a higher structural similarity to the natural form of the domain embodied in the spike protein of SARS-CoV-2. The increased stability of the RBD+ over the RBD likely stems from the structural integrity of the RBD+ and the additional disulfide bridge in this domain. The unpaired cysteine 538 of the RBD construct on the other hand fosters intermolecular dimerization artefacts that do not form with the RBD+. Additionally, the RBD+ harbors an important linear epitope for neutralizing antibodies that is missing in the RBD sequence. Therefore, highly purified RBD+ is a well-suited antigen to generate a robust lateral flow assay that specifically probes for neutralizing antibodies.

At this point it is not possible to foresee if a certain cut-off value in this test is required for immunity. The current strictly qualitative cut-off indicates the mere presence of RBD+ directed antibodies, but the increasing intensity of the test band after a second vaccination may correlate with the degree of immunity to SARS-CoV-2.

Finally, novel, mutated SARS-CoV-2 strains that are better adapted to the human host are currently replacing the initial strain. Randomly occurring mutations in a virus' genome are selected for by the virus' ability to multiply more efficiently than the non-mutated version. This does not necessarily lead to a more harmful virus but a large cohort study suggests that different strains lead to altered mortality rates.[16] The so-called British (B.1.1.7), South African (B.1.351) and Brazilian (B.1.1.28) virus strain lineages each contain an individual set of mutations that seem to ameliorate viral spread. Some of the mutations lie within the RBD of the spike protein and are therefore thought to be the primary cause for the significant dominance of these strains over the non-mutated strain because they could facilitate binding to the ACE2 receptor for cellular entry. In this respect, it is interesting to note that the N501Y mutation appears in all the three mutant viral lineages but probably evolved independently of each other. Also, the mutation E484K and K417N or K417T lie within the RBD of the spike protein and have evolved independently in more than one viral lineage. While these examples are just a few in the variety of new mutant strains, they highlight the dynamics of genomic changes within SARS-CoV-2. The important question whether or not these mutated viral strains are still sensitive towards the human immune response generated by an earlier infection or by vaccination using the original version of the spike protein remains to be answered by ongoing investigations. As of now it seems that the current vaccines confer potent immunity also against these new variants and it is therefore highly likely that the Quantum Blue® SARS-CoV-2 RBD+ will also be able to

detect antibodies directed against the mutated variants. This, however, is subject of ongoing research.

Conflict of interest

The authors Larissa Brosi, Michael Gerspach, Lorin Dirscherl, Cedric Giegelmann, Daniele Dolce, Frank I. Bantleon and Christian-Benedikt Gerhold are employed by BÜHLMANN Laboratories AG.

Acknowledgements

We would like to thank PD Dr. Alexander Eggel, University of Bern and Fabian Rudolf, D-BSSE, ETH Zürich for valuable support during the development of the Quantum Blue® SARS-CoV-2 RBD+. Moreover, we would like to thank Prof. Markus Seeger and Dr. Cedric Hutter, University of Zurich for the provision of RBD binding sybody sequences and plasmids. The expression and production of RBD+ and sybody was assisted by Simon Zoss, Francesco Lipari and Daniela Tobler. Validation of the test assay was supported by Terry Müller, Sabine Kräuchi, Daniela Trapani, Sadieskumar Velayutham, Joana Afonso and Thomas Schuster (all BÜHLMANN Laboratories AG). Valuable support during assay development was given by Jakob Weber, Michele Romano, Michael Schneider, Andreas Abel, Joëlle Jourdan and Chris Moore (all BÜHLMANN Laboratories AG).

Received: March 15, 2021

[1] P. Mehta, D. F. McAuley, M. Brown, E. Sanchez, R. S. Tattersall, J. J. Manson, *Lancet* **2020**, *395*, 1033, https://doi.org/10.1016/S0140-6736(20)30628-0.

[2] B. Meyer, G. Torriani, S. Yerly, L. Mazza, A. Calame, I. Arm-Vernez, G. Zimmer, T. Agoritsas, J. Stirnemann, H. Spechbach, I. Guessous, S. Stringhini, J. Pugin, P. Roux-Lombard, L. Fontao, C. A. Siegrist, I. Eckerle, N. Vuilleumier, L. Kaiser, *Clin. Microbiol. Infect.* **2020**, *26*, 1386, https://doi.org/10.1016/j.cmi.2020.06.024.

[3] G. A. Poland, I. G. Ovsyannikova, S. N. Crooke, R. B. Kennedy, *Mayo Clin. Proc.* **2020**, *95*, 2172, https://doi.org/10.1016/j.mayocp.2020.07.021.

[4] W. H. Chen, P. J. Hotez, M. E. Bottazzi, *Hum. Vaccines Immunother.* **2020**, *16*, 1239, https://doi.org/10.1080/21645515.2020.1740560.

[5] Y. He, J. Li, S. Heck, S. Lustigman, S. Jiang, *J. Virol.* **2006**, *80*, 5757, https://doi.org/10.1128/jvi.00083-06.

[6] Y. He, H. Lu, P. Siddiqui, Y. Zhou, S. Jiang, *J. Immunol.* **2005**, *174*, 4908, https://doi.org/10.4049/jimmunol.174.8.4908.

[7] J. D. Walter, C. A. J. Hutter, A. A. Garaeva, M. Scherer, I. Zimmermann, M. Wyss, J. Rheinberger, Y. Ruedin, J. C. Earp, P. Egloff, M. Sorgenfrei, L. M. Hürlimann, I. Gonda, G. Meier, S. Remm, S. Thavarasah, G. Zimmer, D. J. Slotboom, C. Paulino, P. Plattet, M. A. Seeger, *bioRxiv* **2020**, https://doi.org/10.1101/2020.11.10.376822.

[8] M. Hoffmann, H. Kleine-Weber, S. Schroeder, N. Krüger, T. Herrler, S. Erichsen, T. S. Schiergens, G. Herrler, N. H. Wu, A. Nitsche, M. A. Müller, C. Drosten, S. Pöhlmann, *Cell* **2020**, *181*, 271, https://doi.org/10.1016/j.cell.2020.02.052.

[9] J. Huo, A. Le Bas, R. R. Ruza, H. M. E. Duyvesteyn, H. Mikolajek, T. Malinauskas, T. K. Tan, P. Rijal, M. Dumoux, P. N. Ward, J. Ren, D. Zhou, P. J. Harrison, M. Weckener, D. K. Clare, V. K. Vogirala, J. Radecke, L. Moynié, Y. Zhao, J. Gilbert-Jaramillo, M. L. Knight, J. A. Tree, K. R. Buttigieg, N. Coombes, M. J. Elmore, M. W. Carroll, L. Carrique, P. N. M. Shah, W. James, A. R. Townsend, D. I. Stuart, R. J. Owens, J. H. Naismith, *Nat. Struct. Mol. Biol.* **2020**, *27*, 846, https://doi.org/10.1038/s41594-020-0469-6.

[10] D. J. Benton, A. G. Wrobel, C. Roustan, A. Borg, P. Xu, S. R. Martin, P. B. Rosenthal, J. J. Skehel, S. J. Gamblin, *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2022586118, https://doi.org/10.1073/pnas.2022586118.

[11] A. C. Walls, Y. J. Park, M. A. Tortorici, A. Wall, A. T. McGuire, D. Veesler, *Cell* **2020**, *181*, 281, https://doi.org/10.1016/j.cell.2020.02.058.

[12] Z. Wang, J. C. C. Lorenzi, F. Muecksch, S. Finkin, C. Viant, C. Gaebler, M. Cipolla, H.-H. Hoffmann, T. Y. Oliveira, D. A. Oren, V. Ramos, L. Nogueira, E. Michailidis, D. F. Robbiani, A. Gazumyan, C. M. Rice, T. Hatziioannou, P. D. Bieniasz, M. Caskey, M. C. Nussenzweig, *Sci. Transl. Med.* **2021**, *13*, eabf1555, https://doi.org/10.1126/scitranslmed.abf1555.

[13] A. Padoan, L. Sciacovelli, D. Basso, D. Negrini, S. Zuin, C. Cosma, D. Faggian, P. Matricardi, M. Plebani, *Clin. Chim. Acta* **2020**, *507*, 164, https://doi.org/10.1016/j.cca.2020.04.026.

[14] C. M. Poh, G. Carissimo, B. Wang, S. N. Amrun, C. Y. P. Lee, R. S. L. Chee, S. W. Fong, N. K. W. Yeo, W. H. Lee, A. Torres-Ruesta, Y. S. Leo, M. I. C. Chen, S. Y. Tan, L. Y. A. Chai, S. Kalimuddin, S. S. G. Kheng, S. Y. Thien, B. E. Young, D. C. Lye, B. J. Hanson, C. I. Wang, L. Renia, L. F. P. Ng, *Nat. Commun.* **2020**, *11*, https://doi.org/10.1038/s41467-020-16638-2.

[15] Y. Li, D. yun Lai, H. nan Zhang, H. wei Jiang, X. Tian, M. liang Ma, H. Qi, Q. feng Meng, S. juan Guo, Y. Wu, W. Wang, X. Yang, D. wei Shi, J. biao Dai, T. Ying, J. Zhou, S. ce Tao, *Cell. Mol. Immunol.* **2020**, *17*, 1095, https://doi.org/10.1038/s41423-020-00523-5.

[16] R. Challen, E. Brooks-Pollock, J. M. Read, L. Dyson, K. Tsaneva-Atanasova, L. Danon, *BMJ* **2021**, *372*, n579, https://doi.org/10.1136/bmj.n579.