SARS-CoV-2 Antigens for Immunoassays

Background

Global spread of SARS-CoV-2

Since mid-December 2019, *Severe acute respiratory syndrome coronavirus Type 2* (SARS-CoV-2), the pathogen causing coronavirus disease 2019 (COVID-19), has been keeping the world on tenterhooks. Symptoms such as fever, dry cough, difficulties of breathing, headache, pneumonia or loss of smell and taste are observed to varying degrees in many patients. The course of the disease varies greatly in its symptoms and severity, ranging from asymptomatic progression to severe pneumonia with lung failure and death.

Although great efforts have been made in the development of antiviral drugs, only a few approvals are currently available. Therapy currently consists mainly of supportive measures. First vaccines were approved in December 2020. In order to control the spread of the virus, a majority of affected countries implemented - especially in the cold season - contact restriction measures and extensive laboratory diagnostics of sick people and people who have had contact with COVID-19 patients.

SARS-CoV-2 antigens

SARS-CoV-2 consists of 4 structural proteins and 16 non-structural proteins (Figure 1). The 4 structural proteins are spike (S), envelope (E), nucleocapsid (N) and membrane (M) protein. The N protein is associated with the RNA genome, the S, E and M proteins together form the virus envelope. The S protein is a glycoprotein consisting of two domains, the S1 domain, which contains the receptor binding domain (RBD) responsible for the virus being able to attach to and fuse with the ACE2 receptor of the host cell[1], and the S2 domain including transmembrane and endodomain. On the virus surface, the S protein forms trimeric structures.

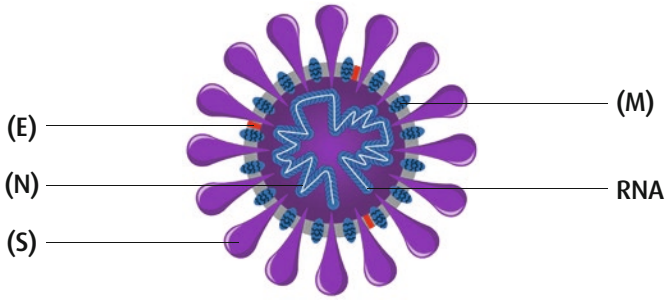

Figure 1: Schematic illustration of a coronavirus with the RNA genome in the nucleus, the RNA associated nucleocapsid protein (N) and the structural surface proteins envelope (E), membrane (M) and spike (S).

N and S protein are major immunogenic proteins of the virus family *Coronaviridae* and are very well suited for serological detection of anti-SARS-CoV-2 antibodies. Recombinant versions of both proteins are already widely used in different test systems[2, 3, 4, 5].

Antibody detection

Neutralizing antibodies during host immune responses are predominantly directed against S protein, which is supposedly suitable for very specific test settings as it is less conserved within the family of Coronaviridae than the other immunogenic antigens[2, 6].

However, recent studies have shown that antibodies against N protein are detectable earlier in mild infections than antibodies directed against S protein[3, 7]. As an acute marker in particular, it can be beneficial to use N protein for most sensitive early detection[7]. In the further course of the immune response, the N protein antibody level decreases, whereas antibodies against S protein are detectable for a longer time.

Various studies show that anti-SARS-CoV-2 antibodies of the IgA and IgM class are detectable in the median at the earliest from about six days after the onset of symptoms[3, 7, 9], increase in the further course of the disease and decrease again between 18 and 35 days[8, 9]. Antibodies of the IgG class are detectable in the median ten to eighteen days after onset of symptoms[4] and are detectable over several months[8].

Product Description

The new SARS-CoV-2 antigens complement SERION Immunologics growing raw material portfolio for IVD assay development. The antigens SARS-CoV-2 Spike Ectodomain (S1-S2) and SARS-CoV-2 Nucleoprotein are developed for the detection of IgA, IgG and IgM antibodies. Their superior performance has been shown in ELISA as well as in bead based immunoassays. The recombinant expression in insect cells in bioreactor systems within an ISO 13485 certified production environment guarantees highest lot-to-lot consistency and constant availability of bulk quantities up to several grams. The eukaryotic expression system was carefully chosen to provide best product performance for the development of highly specific detection assays.

- ✓ Structural antigens (S protein including S1, RBD and S2 domain & N protein)
- ✓ Eukaryotic expression system (Insect cells)
- ✓ Highest specificity – no cross reactivity
- ✓ ELISA and bead assay approved
- ✓ Bulk-quantities (several grams)
- ✓ ISO 13485 certified manufacturing conditions

Order Information and Related Products

Code	Description	Packaging
BA400R03	SARS-CoV-2 Spike Ectodomain (S1-S2) Recombinant spike glycoproteins comprised of S1 and S2 domains expressed in insect cells. Molecular weight: 135 kDa.	1 mg
BA400R04	SARS-CoV-2 Nucleoprotein Recombinant nucleocapsid protein expressed in insect cells. Molecular weight: 47 kDa.	1 mg

Experimental Data

Evaluation of antigens by WHO International Standard for anti-SARS-CoV-2 human Immunoglobulin

The proprietary antigens S protein ectodomain (Code BA400R03), S protein S1 domain, N protein produced in insect cells (Code BA400R04) and N protein produced in E. coli cells, as well as commercial antigens S protein trimer and S protein RBD, were coupled to proprietary paramagnetic particles of approximately 5 µm in size. The particle surfaces consisted of either carboxyl or amino groups. The antigens were irreversibly immobilized by a proprietary metal chelate coordination chemistry (SERION pro-Bind, Code CRC01) regardless of the surface type. The particles can be divided into different populations according to their intrinsic amount of fluorescent dye. Since each antigen is assigned to a specific particle population, antigen-specific antibodies can be detected in a multiplex bead assay format. Fluorescently labeled detection antibodies are used to detect the bound antibodies. Finally, a flow cytometer is used for readout to identify particle populations and quantify bound antibodies. The assay signal is expressed as median fluorescence intensity (MFI).

The antigens were evaluated using the First WHO International Standard for anti-SARS-CoV-2 Immunoglobulin (20/136, NIBSC). The WHO International Standard was serially diluted and analyzed by bead assay for the presence of antigen-specific antibodies of the immunoglobulin classes IgG, IgM and IgA (Figure 2).

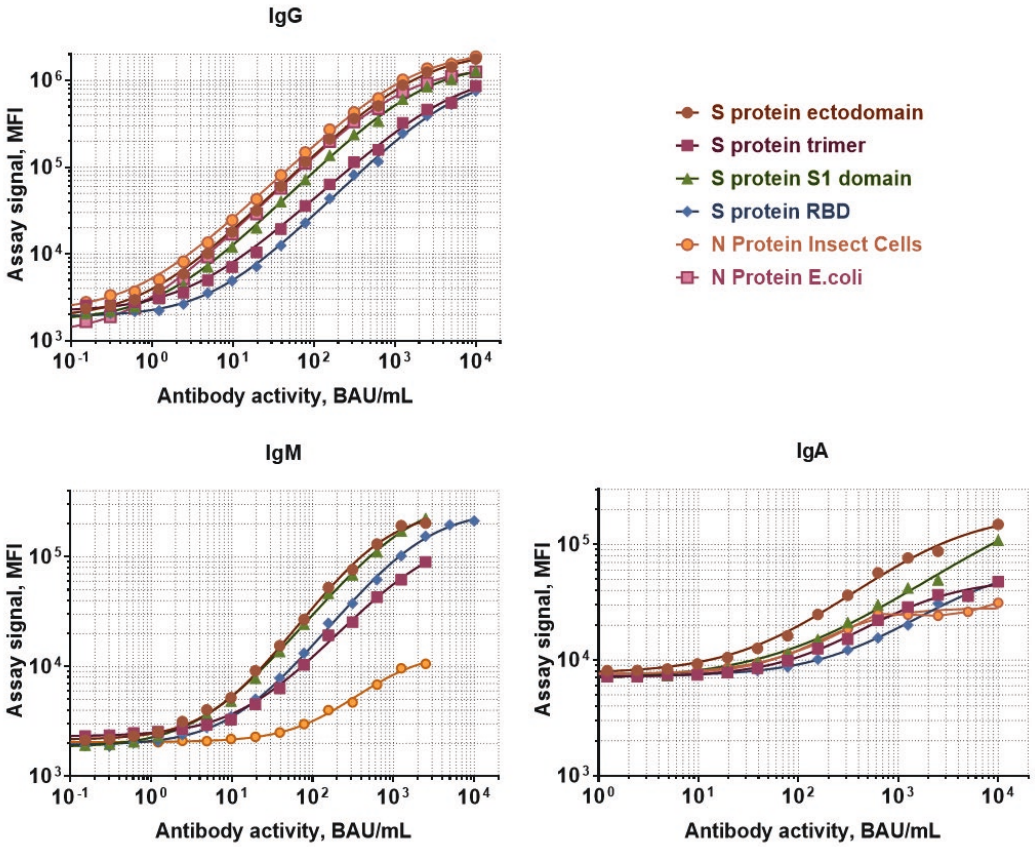

Figure 2: WHO International Standard for anti-SARS-CoV-2 human Immunoglobulin (NIBSC code: 20/136) was diluted and used in a multiplex bead assay with four proprietary antigens S protein ectodomain, S protein S1 domain, N protein produced in insect cells and N protein produced in E.coli cells and two commercial antigens S protein trimer and S protein RBD. Assay signal (MFI) is fitted with a four-parameter logistic-log model.

NIBSC recommends an arbitrary unitage of 1,000 binding antibody units (BAU)/mL for the activity of the WHO International Standard. Considering the nominal assay dilution, the correct activity was assigned to each dilution of the WHO International Standard. In Figure 2, the assay signal (MFI) is plotted against antibody activity (BAU/mL). A logistic-log model with four parameters was used to mathematically describe the relationship between assay signal and activity:

$$y = A + \frac{D - A}{1 + e^{B(C - \ln x)}}$$

Equation 1: A logistic-log model with four parameters was used to describe the relationship between assay signal (y) and the activity of the antigen-specific antibodies (x) included in the WHO International Standard. Parameters A and D represent the lower and upper asymptote, C refers to the inflection point of the curve and B represents the slope of the curve at the inflection point.

The curve-fitting model used describes the binding behaviour of antigen-specific antibodies to their respective antigen very well. Table 1 shows the curve parameters obtained and the goodness of fit (R^2). For detection of IgG antibodies, a large dynamic range was observed, spanning almost four orders of magnitude from 1 to 10,000 BAU/mL. For IgM and IgA, the observed dynamic range is about one order of magnitude smaller, which could be due to the assay design or to the actual activity of the respective antigen-specific antibody and antibody class in the WHO International Standard. Although the shapes of the curves in each graph are very similar, the position of the curves in the graphs can be quite different. For example, the curve for RBD approaches the lower asymptote already at an activity of about 1 BAU/mL, while the curve for N protein from insect cells is still declining below activity of 1 BAU/mL.

Antibody class	Equation parameter	S protein ectodomain	S protein trimer	S protein S1 domain	S protein RBD	N protein insect cells	N protein E. coli
IgG	A	1,822	2,177	1,711	1,898	2,171	1,202
	D	2,116,000	1,298,000	1,575,000	1,188,000	2,189,000	1,420,000
	C	7.54	8.59	7.70	8.65	7.35	7.09
	B	0.915	0.858	0.917	0.935	0.893	0.923
	R^2	0.998	0.992	0.998	0.997	0.998	0.999
IgM	A	2,158	2,306	1,836	1,930	2,056	n.a.
	D	264,193	170,125	357,489	266,313	13,746	n.a.
	C	6.48	7.76	7.27	7.61	6.74	n.a.
	B	1.052	0.876	0.927	0.967	1.045	n.a.
	R^2	0.995	0.997	0.999	0.998	0.994	n.a.
IgA	A	7,638	7,132	6,770	7,229	7,597	n.a.
	D	189,558	50,297	346,401	72,238	28,212	n.a.
	C	7.83	7.18	10.50	8.67	5.61	n.a.
	B	0.818	0.944	0.671	0.855	1.243	n.a.
	R^2	0.993	0.990	0.993	0.995	0.984	n.a.

Table 1: Observed parameters of four-parameter logistic-log model curve-fitting and goodness of fit (R^2)

The actual parameters obtained from curve-fitting are a characteristic of the respective assay, i.e. combination of antigen and antibody class, and the actual activity of the respective antigen-specific antibody and antibody class in the WHO International Standard. Thus, for IgG assays, the fluorescence assay signal (MFI) spans almost three orders of magnitude, whereas for IgA assays the range is only slightly more than one order of magnitude. This is partly because a different fluorophore was used for IgA assays, and partly because there may be less IgA than IgG in the WHO International Standard.

In summary, we observed high antibody reactivity of the WHO International Standard against all SARS-CoV-2 antigens evaluated, including IgM and IgA. In each case, it was possible to set up a quantitative assay for a given antigen-specific antibody and antibody class. The IgG-type assays in particular promise a wide dynamic range and an excellent detection limit down to an activity below 1 BAU/mL

Diagnostic relevance of SARS-CoV-2 Spike Ectodomain (S1-S2) and SARS-CoV-2 Nucleoprotein

To analyze the diagnostic relevance of S protein ectodomain (Code BA400R03) and N protein produced in insect cells (Code BA400R04), these antigens as well as a commercial trimeric S protein antigen and another recombinant in-house S1 domain antigen were evaluated in a bead-based multiplex assay.

Ten serum samples from patients with confirmed COVID-19 infection and another ten pre-pandemic serum samples from blood donors from 2015 were used for this evaluation. The same bead assay setup was used as described above. Figure 3A shows the IgG antigen-specific antibody profile. High titers of antibodies against each antigen were found in the serum of all patients, while only minimal reactivity was detectable in blood donors.

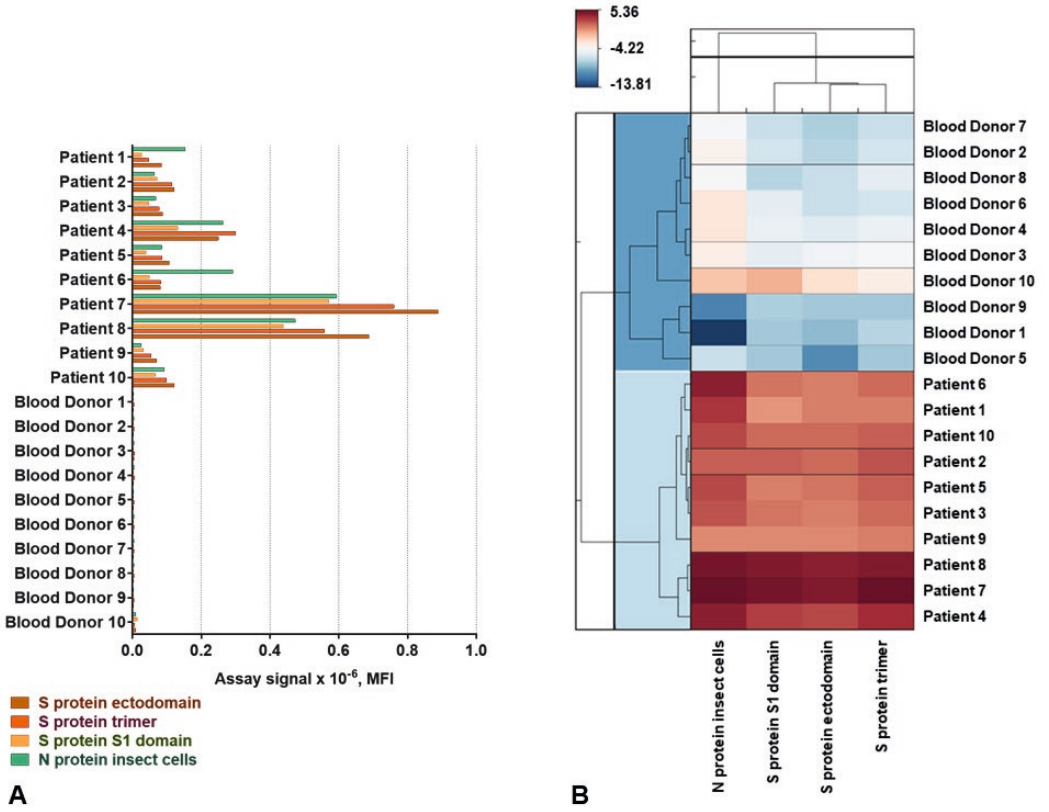

Figure 3: Sera from individuals with confirmed COVID-19 infection and from blood donors were tested in a multiplex bead assay using three proprietary antigens produced in insect cells, S protein ectodomain, S protein S1 domain and N protein, and a commercial S protein trimer antigen. Here, assay signals indicate the presence of antigen-specific IgG antibodies (A). A deeper statistical analysis was performed by hierarchical clustering (B).

The data were used to perform a deeper analysis by hierarchical clustering. Therefore, the obtained assay signals (MFI) were first normalised for each antigen in a range from 0% to 100%. Then, median centring and log2 transformation were performed for each data set before hierarchical cluster analysis was performed. The data are illustrated by a colour code, with red representing high assay signals and blue representing low assay signals. As can be seen in Figure 3B, patients and blood donors are grouped into two clearly separated clusters, demonstrating that this multiplex bead assay constitutes a valuable diagnostic tool. When looking at antigen reactivity, the dendrogram on the top of the graph shows two clusters. The first cluster includes only the N protein, while the second cluster includes all three S protein variants. The length of the vertical branches in the dendrogram represents the diversity of the antigens or vice versa: the shorter the vertical branches, the more similar the antigens are.

Overall, all three S protein variants included in this evaluation show the same diagnostic significance. Thus, although N protein shows different reactivity in patient samples than the S protein antigens, it also has a high diagnostic relevance and is an ideal complementary partner for the S protein antigen in a multiplex assay setup.

High specificity of SARS-CoV-2 Spike Ectodomain (S1-S2) and SARS-CoV-2 Nucleoprotein

The S protein ectodomain (Code BA400R03) and N protein produced in insect cells (Code BA400R04) antigens were also tested for specificity and potential cross-reactivity, respectively, by testing sera containing high titers of antibodies to coronaviruses and common pathogens relevant to the differential diagnosis that could potentially cause false positive results. This includes samples from other coronaviruses (n = 9), adenovirus (n = 26), *Chlamydia pneumoniae* (n = 20), cytomegalovirus (CMV, n = 10) and Epstein–Barr virus (EBV, n = 10). In addition, samples from patients with PCR-confirmed COVID-19 (n = 32) as a positive collective and samples from blood donors (n = 138) with blood collections from December 2019 and April 2020 collected by the German Red Cross as a negative collective were added. Antigen-specific antibodies of the immunoglobulin classes IgG, IgM and IgA were detected by bead assay as described above.

The results presented in Figure 4 show that no relevant cross-reactivity with the S protein ectodomain and N protein is observed. Only one *C. pneumoniae*-positive serum shows a weak S protein ectodomain-specific IgM signal. Remarkably, this serum also shows significant reactivity in the S protein ectodomain IgM ELISA, which is based on the same antigen (data not shown). Two sera obtained from patients with other coronavirus infections show a weak IgG-specific binding to the N protein. It should be noted that the corresponding titers of anti-S protein antibodies are not elevated in these two sera. The reason for these elevated titers remains unclear, but might be due to a high homology of the N protein within the coronavirus family. Overall, the analysis of 213 putative negative samples revealed for the S protein ectodomain antigen an outstanding specificity of more than 99.5% and for the N protein assay an excellent specificity of more than 99%.

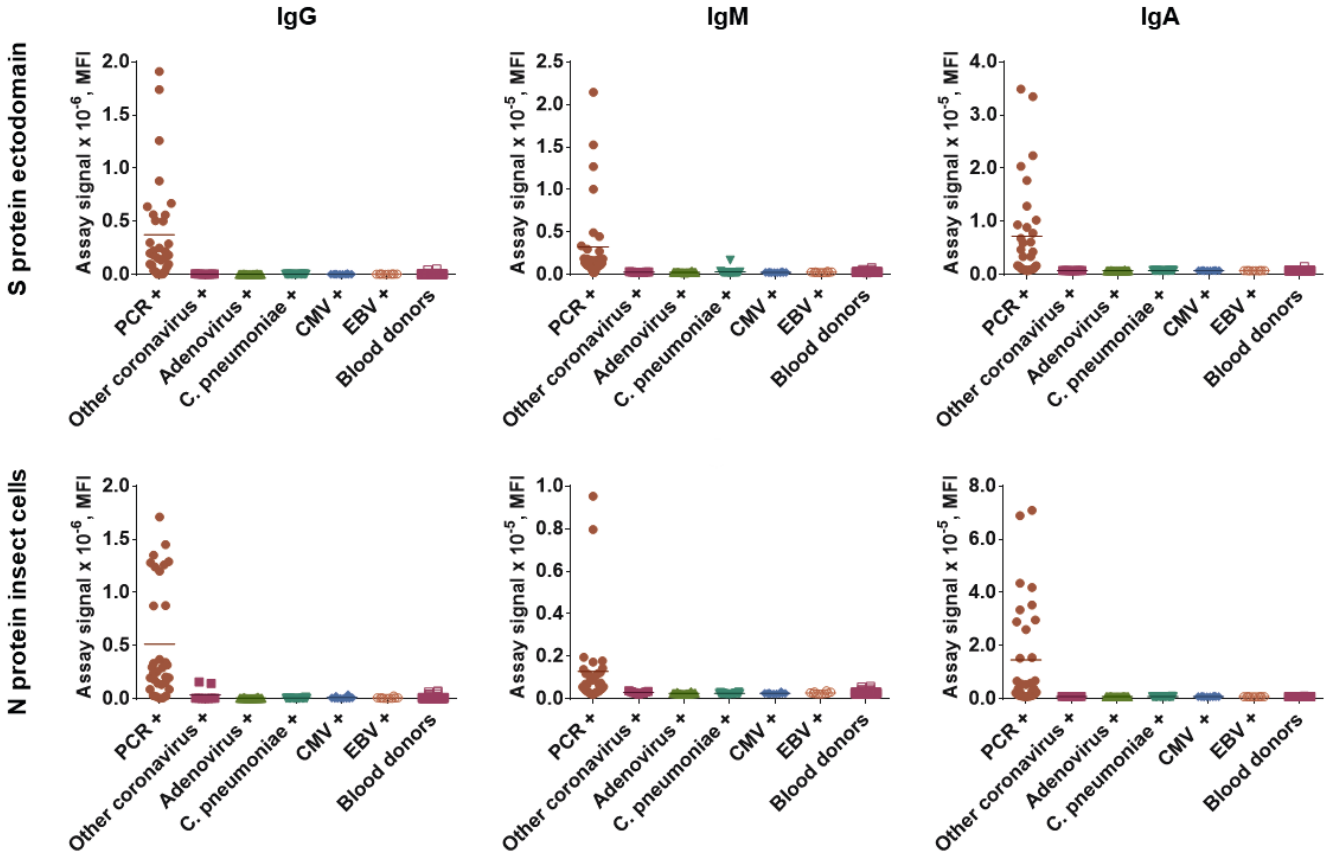

Figure 4: Sera of patients infected with SARS-CoV-2 were tested in a bead assay for the presence of IgG, IgM and IgA type antibodies against the S protein ectodomain antigen and N protein antigen. In addition, sera from other corona virus-positive individuals as well as sera from individuals with confirmed infections by other viruses and blood donors (blood collection Dec 2019 and Apr 2020) were tested

SARS-CoV-2 Spike Ectodomain (S1-S2) and SARS-CoV-2 Nucleoprotein antigens perform well on different platforms

The sample panel described above (n = 245, Figure 4) was also analyzed for anti-SARS-CoV-2 antibodies of different immunoglobulin classes using in-house ELISAs, which are based on the S protein ectodomain (Code BA400R03) and N protein produced in insect cells (Code BA400R04), respectively. Using this data set, a method comparison between bead assay and ELISA was performed to demonstrate the suitability of the antigens on different platforms. Figure 5 shows the reaction profile for the bead assay for S protein ectodomain IgG. Samples are classified as positive (n = 26) or negative (n = 218) according to their ELISA test result. Equivocal results were excluded from this analysis. At first glance, the S protein ectodomain antigen has the same discriminatory power regardless of the test arrangement.

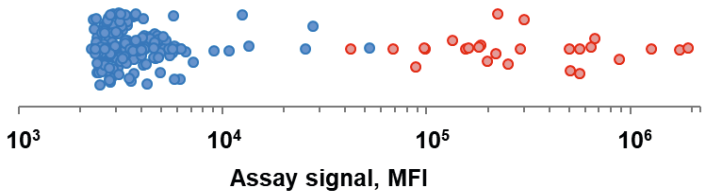

Figure 5: A total of 245 sera (already described in the previous chapter), which had been interpreted positive (red) and negative (blue) in the S protein ectodomain IgG ELISA were also tested in the bead assay using the same antigen.

In addition, ROC analysis was performed and the area under the curve (AUC) was calculated as a quantitative measure of diagnostic similarity between the bead assay and ELISA. Figure 6 shows the ROC analysis for comparison of bead assay and ELISA based on the S protein ectodomain antigen for detection of IgG class antibodies.

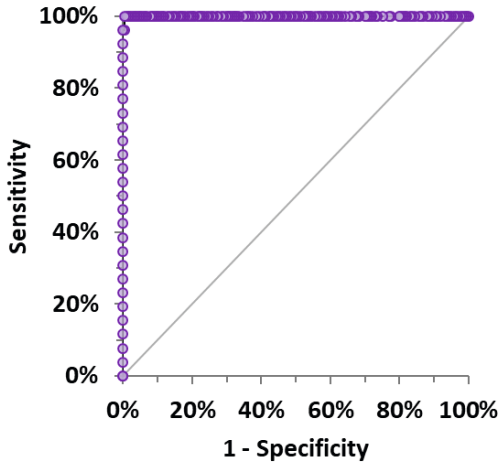

Figure 6: ROC analysis was performed for S protein ectodomain IgG bead assay based on the ELISA results. The same analysis was performed for other parameters and area under the curve was determined (Table 1).

ROC analysis was also performed for IgM and IgA assays. For N protein, data were only obtained for IgM and IgA assays. Table 2 shows the calculated AUCs for all comparisons. The remarkably high AUCs of more than 98% or even more indicate that both antigens are very well suited for use in the bead assay and ELISA set-up.

Assay	AUC	95% CI	n (ELISA +)	n (ELISA +)
IgG S protein ectodomain	1.000	0.999 to 1.000	26	218
IgM S protein ectodomain	0.995	0.990 to 1.000	40	284
IgM N protein insect cells	0.982	0.952 to 1.000	22	302
IgA S protein ectodomain	0.980	0.956 to 1.000	39	290
IgA N protein insect cells	0.999	0.998 to 1.000	29	301

Table 2: ROC analysis was performed for various parameters for comparison of bead assay based on ELISA results. The area under the curve (AUC) and 95% confidence intervals (CI) were calculated. In addition, the number of positive and negative ELISA results is shown.

Wide dynamic range: enabling accurate tracking of SARS-CoV-2 antibody levels

In this study, the SARS-CoV-2-specific antibody titers of COVID-19 patients were followed over a period of up to seven months. For each patient, blood was drawn on four or five different days. The starting point, day zero, is the date of the first blood collection and is between 3 weeks and 6 weeks after infection or a positive PCR result. Using the multiplex bead assay presented above, the activities of specific antibodies of the immunoglobulin classes IgG, IgM and IgA against the proprietary antigens S protein ectodomain (Code BA400R03), S protein S1 domain and N protein from insect cells (Code BA400R04) as well as N protein from E.coli cells were detected. In addition, the commercial antigens S protein trimer and S protein RBD were used as reference.

Figure 7 shows the changes in antibody levels over time for all five patients, immunoglobulin classes and antigens. The large dynamic range allows proper tracking of individual antibody levels. Apart from therapeutic insights, this may help to monitor the development of antibody levels after vaccination.

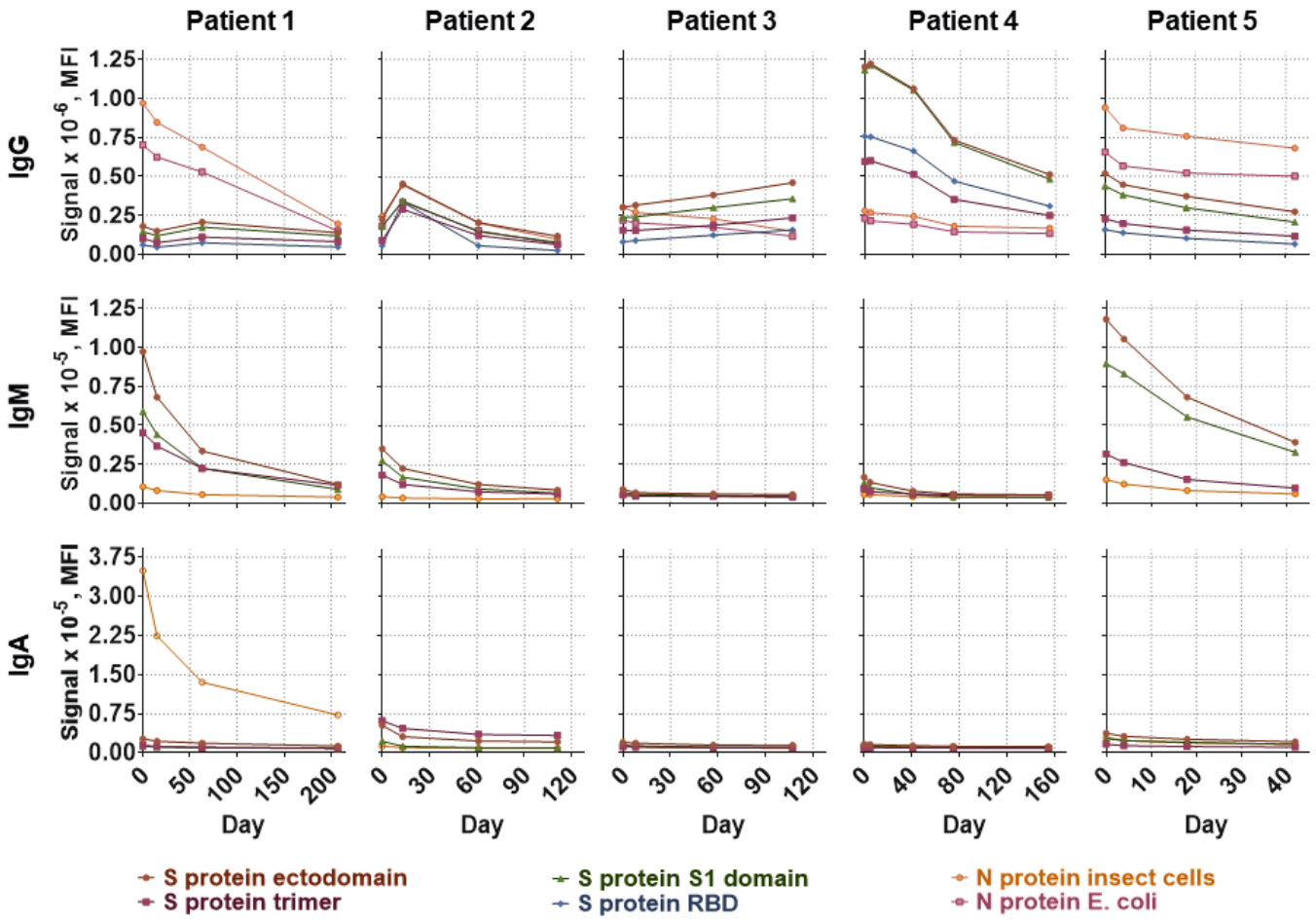

Figure 7: Sera of patients infected with SARS-CoV-2 were tested in a bead assay for presence of IgG, IgM and IgA type antibodies against up to four proprietary antigens S protein ectodomain, S protein S1 domain, N protein produced in insect cells and N protein produced in E.coli cells and two commercial antigens S protein trimer and RBD.

Summary

The new antigens *SARS-CoV-2 Spike Ectodomain (S1-S2) (Code BA400R03) and SARS-CoV-2 Nucleoprotein (Code BA400R04)* have been developed for the detection of IgA, IgG and IgM antibodies. Recombinant expression in insect cells in bioreactor systems within an ISO 13485 certified production environment guarantees highest lot-to-lot consistency and constant availability of bulk quantities up to several grams.

Analysis of the *First WHO International Standard for anti-SARS-CoV-2 Immunoglobulin* revealed high reactivity for the immunoglobulin classes IgA, IgG and IgM. In addition, great dynamic range was observed when using these antigens in a bead-based assay format, rendering them perfect for sample comparison or tracking of antibody levels over time.

Results with trimeric Spike protein showed that the *SARS-CoV-2 Spike Ectodomain (S1-S2)* displays no reduced sensitivity. In addition, the antigens also enable high specificity: no relevant cross-reactivity was observed with samples from patients suffering from other infections. Finally, the antigens were successfully tested in different test set-ups as ELISA and bead-based assay format.

References

[1] Walls et al, „Structure, Function, and Antigenicity of the SARSCoV-2 Spike Glycoprotein" Cell 180, 281–292 April 16, 2020 ª 2020 Elsevier Inc.

[2] Meyer et. al., "Serological Assays for Emerging Coronaviruses: Challenges and Pitfalls", Virus Research 194 (December 2014)

[3] Nisreen M.A. Okba et al., "SARS-CoV-2 Specific Antibody Responses in COVID-19 Patients", preprint (Infectious Diseases (except HIV/AIDS), March 20th 2020)

[4] Liu W. et al., "Evaluation of Nucleocapsid and Spike Protein-Based ELISAs for Detecting Antibodies against SARS-CoV-2", Journal of Clinical Microbiology, 30. März 2020, JCM.00461-20, jcm;JCM.00461-20v1.

[5] Lassauniere et al, "Evaluation of nine commercial SARS-CoV-2 immunoassays", medRxiv, April 2020

[6] Suresh et. al Molecular Targets for Diagnostics and Therapeutics of Severe Acute Respiratory Syndrome (SARS-CoV)", J Pharm Pharm Sci. Author manuscript; available in PMC 2009 May 7

[7] Guo L. et al., "Profiling Early Humoral Response to Diagnose Novel Coronavirus Disease (COVID-19)", Clinical Infectious Diseases, March 21st 2020

[8] Liu W. et al., "Evaluation of Nucleocapsid and Spike Protein-Based ELISAs for Detecting Antibodies against SARS-CoV-2", Journal of Clinical Microbiology, 30. März 2020, JCM.00461-20, jcm;JCM.00461-20v1

[9] Padoan A. et al., "IgA-Ab Response to Spike Glycoprotein of SARS-CoV-2 in Patients with COVID-19: A Longitudinal Study", Clinica Chimica Acta 507 (August 2020): 164–66

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