Development of Genlisa™ SARS-CoV-2 ELISA (KBVH015-2 and KBVH015-8) for Estimation of Novel Coronavirus (nCoV/CoV-2) IgG Antibodies in serum and plasma

Krisha Jain, Atul Gadhave, Anitha Florance, and Dr Kalpesh Jain

Krishgen Biosystems, Mumbai 400018 India.

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Abstract- Researchers working on nCOV-2019 identified a unique protein, the Spike protein, on the surface of the coronavirus, which helped in understanding the method of transmission of the virus. Novel coronavirus uses this spike protein to infect a human cell by binding to the membrane. Detection of antibodies to SARS-COV-2 can help in diagnosis of disease, and also has implications in developing and monitoring patient therapeutics as well as in vaccine and therapy research. As many cases worldwide are asymptomatic (and particularly in India), detection of antibodies in serum and plasma can also identify infection in a patient. This study focuses in development of enzyme linked immunoassay for detection of IgG antibodies to Human SARS-COV-2 spike protein by using a recombinant SARS-COV-2 spike protein. In this assay, standards were calibrated over the concentration range 0 – 30 AU/ml. Intra-assay and inter-assay was determined to be <10% respectively. Spike recovery, assay comparison with a commercially available kit, and specificity of the kit were evaluated. Influences of anti-coagulants were studied using EDTA-plasma and Heparin-plasma. Overall, the results demonstrate that the SARS-CoV-2 Spike Protein ELISA kits have reliable diagnostic performance to support and perform accurate estimation of IgG antibody to SARS-CoV-2 levels in human serum and plasma.

Keywords: SARS-COV-2, Spike Protein, Covid-19, nCov, Coronavirus, Anti-Covid-19, Anti-SARS-CoV, Anti-SARS-CoV-2, Anti-Coronavirus, Coronavirus ELISA, Immunoassay, ELISA, Genlisa, Krishgen, Krishgen Biosystems

I. Introduction

SARS-CoV-2 (severe acute respiratory disease coronavirus 2) is a positive sense, single-stranded RNA virus, that is responsible for causing the respiratory illness, Coronavirus Disease (COVID-19).[1] Detected for the first time in late 2019 in Wuhan, China, the highly infectious SARS-CoV-2 has quickly lead to the current global pandemic with high mortality. Widespread availability of reliable and rapid testing methods will enable timely detection and diagnosis, effective treatment of patients, and the containment of the virus.

Nucleic acid tests and molecular tests to detect and diagnose COVID-19 are now abundantly available worldwide. However, one of the many challenges presented by COVID-19 is that a significant number of infected persons are asymptomatic, making it difficult to identify patients. Having a fast, convenient, and labour conservative test can help identify asymptomatic patients and reduce the spread caused by contact with these carriers.

SARS-CoV-2 has four structural proteins, known as the S (spike), E (envelope), M (membrane), and N (nucleocapsid) proteins [2]; the N protein holds the RNA genome, and the S, E, and M proteins together create the viral envelope. The N-protein is abundantly present on the surface of the virus, making it a popular target for using in ELISA tests [2]. Already there exists a large range of recombinant SARS-CoV nucleocapsid protein (N) ELISA-based antibody and antigen tests for diagnosis of SARS-CoV infections. However, SARS-CoV-2 Spike protein ELISA based tests are not easily commercially available.

The spike protein (S-protein) is the common target for neutralizing antibodies and vaccines. SARS-CoV-2 spike protein contains two subunits, S1 and S2. The S1 subunit contains a receptor binding domain (RBD), which is responsible for recognizing and binding with the cell surface receptor. S2 subunit contains other basic elements needed for the membrane fusion with ACE2 [3]. It is through the interaction of this protein with the human receptor angiotensin converting enzyme 2 (ACE2) that the virus infects the body.

ELISA based antibody tests for estimation of antibodies typically offer an easy method of detection. They are easy to use and standardize, offer high levels of reproducibility, and are priced competitively. SARS-CoV-2 Spike protein ELISA tests also allow us to study and monitor immune response in both a qualitative as well as quantitative manner. These determinations have implications in both patient therapy as well as vaccine development. Quantitatively estimating those with a strong antibody response in affected patients could even identify potential donors for convalescent plasma therapeutics.

II. Objective:

To develop an enzyme linked-immunosorbent assay (ELISA) for specific measurement of IgG antibodies to Human

SARS-COV-2 spike protein, as a tool for diagnosis of COVID-19, and provide a reproducible protocol for antibody detection using the same.

III. Materials and Method:

These kits employ a sandwich ELISA technique for higher specificity and increased sensitivity.

A DNA sequence encoding the SARS-CoV-2 (2019-nCoV) Spike Protein (RBD) (YP_009724390.1; Arg319-Phe541) [11] was expressed in baculovirus-insect cells with a polyhistidine tag at the C-terminus. Clear bands were visible when the expressed recombinant proteins were run on an SDS-PAGE (Figure 1). This SARS-CoV-2 RBD protein was pre-coated onto the microwells of a 96 well ELISA plate as capture antigen.

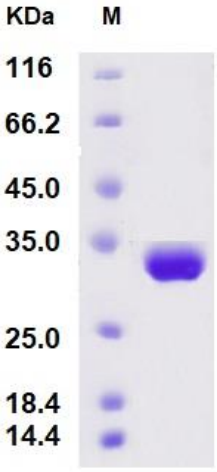
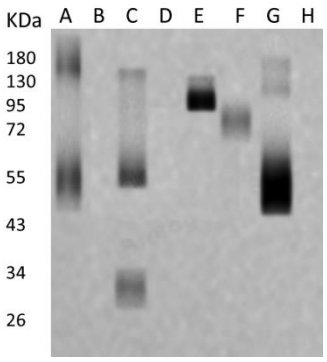


Figure 1

The kit uses recombinant SARS-CoV-2 (Covid-19) spike protein as standards/controls. The standard was produced in rabbits immunized with purified, recombinant SARS-CoV-2 / 2019-nCoV Spike/RBD Protein (NCBI: YP_009724390.1; Arg319-Phe541). The specific IgG was purified by SARS-CoV-2 / 2019-nCoV Spike/RBD affinity chromatography and were run on western blot (Figure 2).



Anti-SARS-S1 rabbit polyclonal antibody at 1:2000 dilution.
Lane A: SARS-CoV Spike S1 (His Tag) (30ng)
Lane B: SARS-CoV Spike S1 (His Tag) (5ng)
Lane C: SARS-CoV Spike RBD (His Tag) (30ng)
Lane D: SARS-CoV Spike RBD (His Tag) (5ng)
Lane E: SARS-CoV-2 (2019-nCoV) Spike S1 (His Tag) (30ng)
Lane F: SARS-CoV-2 (2019-nCoV) Spike S1 (His Tag) (5ng)
Lane G: SARS-CoV-2 (2019-nCoV) Spike RBD (mFc Tag) (30ng)
Lane H: SARS-CoV-2 (2019-nCoV) Spike RBD (mFc Tag) (5ng)
Secondary
Goat Anti-Rabbit IgG (H+L)/HRP at 1/10000 dilution.
Developed using the ECL technique.
Performed under reducing conditions.

Figure 2.

The SARS-CoV-2 antibody was quantified in terms of ng/ml by means of a Bradford assay but in absence of internationally recognized parameters for measurement of the antibodies to SARS-CoV-2, we assigned Arbitrary Units per ml (AU/ml) to ng per ml (ng/ml). Hence, this assay uses standards optimized for the linear range as AU/ml. Samples and standards are pipetted into microwells and IgG Antibodies to human SARS-CoV-2 (Covid-19) present in the sample are bound by the protein antigen.

After incubation, the wells are washed and followed by HRP-conjugated. Detection IgG Antibody is pipetted to the wells, and incubated to form a complex. After washing microwells in order to remove any non-specific binding, the substrate solution (TMB) was added to the microwells and color developed proportionally to the amount of IgG Anti-Human SARS-CoV-2 (Covid-19) in the sample. Color development was then stopped by addition of stop solution. The absorbance was measured at 450nm.

Each assay step was optimized for optimal noise-to-signal ratio, working range and for minimal %CV and relative error.

Antibody was then purified by affinity chromatography. Serum samples were collected to run intra & inter assays. Sera with known antibodies against confirmed past infections were used to check cross reactivity of non-specific antibodies.

Commercially available COVID-19 IgG / IgM Rapid Test was used to study comparison with ELISA where, 9 serum samples were measured using both assays. The influence of anti-coagulants like EDTA-plasma and Heparin-plasma were investigated in 6 serum samples.

IV. Results:

The GENLISA™ Human Anti-SARS-CoV-2 (Covid-19) IgG ELISA developed gives accurate, reproducible results. The assay was designed for 0 - 30 AU/ml as calibrator range and achieved a sensitivity of 2.5 AU/ml.

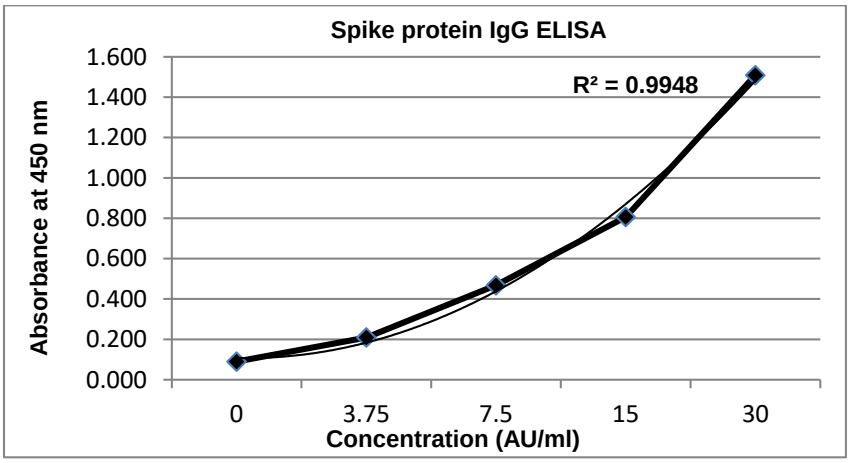
We have used a polyclonal antibody as standard and quantified for the total protein content of the antibodies raised as 1000 AU/ml. The kit shows a typical titration curve when used in dilutions as in the table below.

Standard Concentration	Standard Titre Dilution
30 AU /ml	1:33,333 titre
15 AU /ml	1:66,666 titre
7.5 AU /ml	1:133,332 titre
3.75 AU /ml	1:266,664 titre

Absorbance were measured at 450nm. Intra-assay and inter-assay precision was <10% and <10% respectively.

The coefficient of determination was 99% and absorbances were extrapolated using a polynomial regression 2nd order graph. Mean recoveries observed in serum and plasma on spiking was 96% - 104% for serum and plasma diluted at 1:5000 and obtained the best recoveries.

Standard Concentration (AU/ml)	Abs 1	Abs 2	Mean Abs	Interpolated Concentration	Interpolated Concentration % against Actual Concentration
0	0.091	0.088	0.090	0.3	
3.75	0.216	0.202	0.209	3.1	82.8
7.5	0.482	0.454	0.468	8.2	109.1
15	0.829	0.783	0.806	14.7	98.1
30	1.506	1.512	1.509	30.1	100.2



Spike-Recovery:

The ELISA kit was used to measure spike-recovery of Human Anti-SARS-CoV-2 (Covid-19) IgG spike protein in serum and plasma along with the kit assay diluent. Optimal recovery was obtained when the serum and plasma samples were diluted 1:5000. The standards in the kit were calibrated and >99% accuracy was maintained in their preparation for usage in the kit. The sensitivity was observed as 2.5 AU/ml.

Dilution of Plasma at 1:1000 *using the kit assay diluent and spiked with known concentration of the antibodies to SARS-CoV-2.*

Standard Concentration (AU/ml)	Abs 1	Abs 2	Mean Abs	Interpolated Concentration	Interpolated Concentration % against Actual Concentration
0	1.092	1.060	1.076	---	---
3.75	1.189	1.222	1.205	4.28	114.1

7.5	1.354	1.386	1.370	7.14	95.2
15	1.943	1.619	1.781	15.33	102.2
30	1.935	2.168	2.051	29.55	98.5

Dilution of Plasma at 1:2000 *using the kit assay diluent and spiked with known concentration of the antibodies to SARS-CoV-2.*

Standard Concentration (AU/ml)	Abs 1	Abs 2	Mean Abs	Interpolated Concentration	Interpolated Concentration % against Actual Concentration
0	0.785	0.876	0.830	---	---
3.75	0.824	0.942	0.883	2.77	73.8
7.5	1.044	1.064	1.054	8.54	113.8
15	1.294	1.242	1.268	14.61	97.4
30	1.912	1.887	1.900	30.05	100.2

Dilution of Plasma at 1:4000 *using the kit assay diluent and spiked with known concentration of the antibodies to SARS-CoV-2.*

Standard Concentration (AU/ml)	Abs 1	Abs 2	Mean Abs	Interpolated Concentration	Interpolated Concentration % against Actual Concentration
0	0.611	0.611	0.611	---	---
3.75	0.814	0.812	0.813	3.50	93.2
7.5	1.126	1.135	1.130	7.85	104.6
15	1.521	1.563	1.542	14.73	98.2
30	2.010	2.015	2.013	30.15	100.5

Dilution of Plasma at 1:5000 *using the kit assay diluent and spiked with known concentration of the antibodies to SARS-CoV-2.*

Standard Concentration (AU/ml)	Abs 1	Abs 2	Mean Abs	Interpolated Concentration	Interpolated Concentration % against Actual Concentration
0	0.311	0.322	0.317	---	---
3.75	0.690	0.715	0.702	3.62	96.6
7.5	0.987	0.965	0.976	7.77	103.6
15	1.322	1.359	1.340	14.81	98.7
30	1.951	1.915	1.933	30.05	100.2

Dilution of Serum at 1:2000 *using the kit assay diluent and spiked with known concentration of the antibodies to SARS-CoV-2.*

Standard Concentration (AU/ml)	Abs 1	Abs 2	Mean Abs	Interpolated Concentration	Interpolated Concentration % against Actual Concentration
0	1.911	1.924	1.918	---	---

3.75	1.957	2.090	2.023	4.41	117.67
7.5	2.170	2.248	2.210	7.26	96.85
15	2.588	2.685	2.637	15.48	103.17
30	2.688	2.872	2.780	28.07	93.58

Dilution of Serum at 1:4000 *using the kit assay diluent and spiked with known concentration of the antibodies to SARS-CoV-2.*

Standard Concentration (AU/ml)	**Abs 1**	**Abs 2**	**Mean Abs**	**Interpolated Concentration**	**Interpolated Concentration % against Actual Concentration**
0	0.546	0.551	0.549	---	---
3.75	0.781	0.786	0.784	3.90	103.9
7.5	1.013	1.023	1.018	7.29	97.2
15	1.465	1.391	1.428	15.14	101.0
30	1.984	2.004	1.994	29.95	99.8

Dilution of Serum at 1:5000 *using the kit assay diluent and spiked with known concentration of the antibodies to SARS-CoV-2.*

Standard Concentration (AU/ml)	**Abs 1**	**Abs 2**	**Mean Abs**	**Interpolated Concentration**	**Interpolated Concentration % against Actual Concentration**
0	0.342	0.311	0.327	---	---
3.75	0.590	0.610	0.560	3.50	93.4
7.5	0.845	0.898	0.871	7.92	105.6
15	1.232	1.219	1.225	14.75	98.3
30	1.845	1.867	1.856	30.06	100.2

The data was interpreted using a polynomial regression curve to the 2nd order for net absorbances to remove any potential bias and get the best possible curve fit.

Analytical Specificity:

Until now, no serologically unique strains of SARS-CoV-2 have been described relative to the originally isolated virus.

Cross-reactivity of non-SARS-CoV-2 specific antibodies against SARS-CoV-2-S1 RBD protein in Anti-SARS-CoV-2 ELISA was examined using sera with known antibodies against confirmed past infections.

Antibody Positive Sera	**N**	**Anti-SARS-CoV-2 ELISA**
Beta Corona HKU1*	1	Negative
VCV	4	Negative
HCV	5	Negative
HAV	4	Negative
HBV	3	Negative
EBV	4	Negative
CMV	5	Negative
HSV	5	Negative

*The patient was tested PCR positive for Beta Corona HKU1 and PCR negative for SARS-CoV-2. Four weeks after PCR testing a serum sample was drawn from the patient and found to be negative in the Anti-SARS-CoV-2 ELISA.

Precision:

Intra Assay Variance

Serum samples were measured 10-fold within one assay. Mean variance was < 10%.

	Number of Determinations	Mean Value Abs	Standard Deviation Abs	CV (%)
Sample 1	10	0.346	0.025	7.3
Sample 2	10	0.939	0.038	4.1
Sample 3	10	2.257	0.066	2.9

Inter Assay Variance

6 Serum samples were measured in independent assays, in 3 different lots.

	Number of Determinations	Mean Value Abs	Standard Deviation Abs	CV (%)
Sample 1	11	1.040	0.039	3.8
Sample 2	12	0.320	0.035	10.9
Sample 3	12	0.750	0.039	5.2
Sample 4	10	0.220	0.025	11.4
Sample 5	12	0.210	0.024	11.0
Sample 5	7	0.120	0.120	11.7

Assay Comparison:

To demonstrate the identity of the Anti-SARS-CoV-2 ELISA and a commercially available COVID-19 IgG / IgM Rapid Test, 9 serum samples were measured using both the ELISA and the rapid test. As shown in the table below, the results of the IgG tests show an increased sensitivity with the anti-SARS-CoV-2 ELISA when compared to a Rapid test.

Sample No	**Anti-SARS-CoV-2 ELISA**	**COVID-19 IgG / IgM Rapid Test**
1	positive +	negative
2	positive +	positive
3	positive +	negative
4	negative -	negative
5	borderline +	slightly positive
6	positive +++	positive
7	negative -	negative
8	negative -	negative
9	positive ++	positive

*+ relative to the OD signal.

Samples:

The influences of anti-coagulants on measurements were investigated in 6 corresponding serum, EDTA and Heparin plasma samples. In comparison to the ODs of the serum samples, recovery was measured for Heparin and EDTA plasma samples on average 97.8% and 104.4 %, respectively.

	Serum OD	**%**	**EDTA-Plasma OD**	**%**	**Heparin- Plasma OD**	**%**
Sample 1	3.601	100	3.677	102	3.496	95.1
Sample 2	0.41	100	0.385	93.9	0.389	101
Sample 3	2.946	100	2.918	99	3.084	105.7
Sample 4	0.501	100	0.458	91.4	0.517	112.9
Sample 5	1.242	100	1.218	98.1	1.284	105.4
Sample 6	0.129	100	0.132	102.3	0.14	106.1

V. Conclusion:

Here, we describe a serological method to detect IgG antibodies to SARS-CoV-2 spike protein antibodies. The method used above is fast, easy and reproducible. Results from our data show that the ELISA test is both sensitive and specific. As per the results above, the GENLISA™ Human Anti-SARS-CoV-2 (Covid-19) IgG spike protein ELISA is an accurate assay, with sensitivity of 2.5 AU/ml. The assay allows the quantitative determination of samples of an unknown concentration titre (immunological titre) and the calibration of the kit standards. It is a faster, more useful assay for estimation of antibodies when working on convalescent plasma therapy as treatment of COVID-19 patients as it will accurately and quantitatively measure the antibody. Determination of antibody levels could also heavily support doctors customise their therapy as per each patient's responses. This ELISA kit could also help researchers working on vaccine and new drug development against Covid-19 to measure the antibody response quantitatively.

Conflict of Interest Statement

The authors declare that they are employees of Krishgen Biosystems.

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Authors

First Author – Krisha Jain, Krishgen Biosystems, Mumbai 400018 India.
Second Author – Atul Gadhave, Krishgen Biosystems, Mumbai 400018 India.
Third Author – Anitha Florance, Krishgen Biosystems, Mumbai 400018 India.
Fourth Author – Dr Kalpesh Jain, Krishgen Biosystems, Mumbai 400018 India.

Corresponding Author E-mail: krisha@krishgen.com