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VERTEBRATE ANTIBODIES

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FOR RESEARCH USE

Technical Manual

EpitoGen® Differential

SARS-CoV-2 IgG

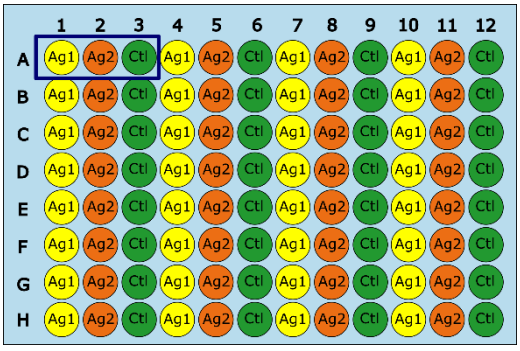
96 well ELISA plate

EpitoGen Technology

Detection of SARS-CoV-2 IgG Specific antibodies

To differentiate between vaccine-induced immunity and infection induced immunity

ELISA EpitoGen® Differential layout:



For 28 samples or 14 samples in duplicates

1. INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the causative viral agent of the disease COVID-19. The virus is transmitted *via* fomites and droplets during close unprotected contact between the infected and uninfected. The single-stranded 29.9 kb positive-sense RNA genome encodes four structural proteins, the nucleocapsid (N), spike (S), membrane (M), and envelope (E) protein, sixteen non-structural proteins (nsp1−16) and several accessary proteins. The N protein forms the capsid outside the genome and the genome is further packed by an envelope which is associated with three structural proteins S, M and E. The time between initial viral exposure and symptom onset is known as the incubation period. For COVID-19, the average incubation period has been reported to be between five and six days. However, there is a considerable variation in incubation time, with some studies suggesting symptoms can appear as soon as three days post-exposure or as late as thirteen days post-exposure. The major targets of humoral immune response are the S, N and M structural proteins. Antibody response has also reported against other proteins including ORF3 and ORF7a post SARS-CoV-2 infection.

2. INTENDED USE

EpitoGen Differential COVID-19 is an immunoassay intended for qualitative detection of IgG antibodies to SARS-CoV-2 in human serum and plasma. The test is intended for use to differentiate between vaccine-induced and infection induced antibody response to aid ongoing vaccine efficacy studies. The EpitoGen Differential assay is designed for Research Use Only.
Antibodies to SARS-CoV-2 are generally detectable in blood several days after initial infection/vaccination, although the duration of time antibodies are present post-infection/vaccination is not well characterized.

3. ABOUT EPITOGEN DIFFERENTIAL

To date, 102 candidate COVID-19 vaccines are in clinical development and 184 in preclinical development, by use of a range of vaccine platforms (WHO). An efficacious COVID-19 vaccine could reduce the likelihood of infection, severity of disease, or degree of transmission within a population. It is imperative that accurate assessment of vaccine performance is carried out, especially to evaluate the long-term protection. **COVID-19 vaccines are invariably based on the spike protein, therefore detection of antibodies against non-spike viral proteins is indicative of a natural infection with SARS-CoV-2.**
Currently, anti-N and anti-S assays are used with the deliberate intention of identifying natural infection as opposed to the effects of vaccine. However, both assays have limitations. It was observed that significant number of cases (5%) react only to the spike or nucleocapsid antigens, but not both. Therefore, traditional antibody assays using either the spike or nucleocapsid as antigens are limited in their detection. Also, accumulating mutations driven by antigenic drift, or by selection will further reduce the detection accuracy of existing serology tests. Mutations abolishes antibody binding sites and often create new epitopes to which antibody responses are generated. Our test can capture the new antibody responses by incorporating prevalent mutations into the test
A sensitive assay that can differentiate between the spike from the non-spike antibody response is needed to support vaccines' development and efficacy studies.
The Differential EpitoGen® COVID-19 test offers the ability to differentiate between humoral responses to vaccination and primary infection with SARS-CoV-2.

Differential EpitoGen® COVID-19 ELISA test adopts four principals designed for maximum performance.

1. Appreciation of the B-cell epitope immunodominance phenomenon. This enriches for the positive signal and improves sensitivity. Another advantage is that cross-reactive epitopes, especially those with homology with seasonal coronaviruses, can be eliminated, subsequently improving specificity.
2. Differential EpitoGen overcomes population heterogeneity (i.e. HLA genetic variation, co-morbidities, age, ethnicity, etc.) by selecting those epitopes prevalent in the population.
3. Differential EpitoGen is amenable to combining multiple SARS-CoV-2 proteins in one test, subsequently improving sensitivity of the assay.
4. Differential EpitoGen overcomes SARS-CoV-2 genetic variability (i.e. emerging mutations) by inclusion of prevalent mutations.

Using EpitoGen Technology, a set of 10 immunodominant non-spike reference immunodominant epitopes and 10 non-spike mutant epitopes corresponding to 29 prevalent mutations of SARS-CoV-2 viral proteins (N, M, ORF3a and ORF7a) were complexed to create a **non-spike antigen**. Whilst a set of reference spike immunodominant epitopes and spike mutant epitopes corresponding to 53 prevalent SARS-CoV-2 mutations were complexed to create **spike antigen**. The reason for the inclusion of spike mutations is to boost the detection accuracy of the test.

5. PRINCIPLE OF THE ASSAY

This ELISA kit uses Indirect-ELISA as the method to qualitatively detect IgG against SARS-CoV-2 proteins in human serum or plasma. The micro test plate provided in this kit is pre-coated with EpitoGens of immune dominant epitopes from 4 viral proteins (N, M, ORF7a, ORF3a and their corresponding mutants.
Prior to incubation, the serum/plasma samples or controls are diluted in the sample diluent buffer (provided). After incubation, specific anti-SARS-2-CoV antibodies are captured on the plate and detected by HRP conjugated anti-IgG secondary antibodies, while the circulating non-specific antibodies remain in the supernatant and are removed during washing. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of stop solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm.

6. MATERIALS

6.1. Reagents supplied

- **Micro ELISA Plate:** 96 wells plate coated with SARS-CoV-2 epitopes fused and displayed on a scaffold protein using EpitoGen Technology. All antigens are produced recombinantly in E. coli and purified using a His-tag purification method. Major circulating mutants (29 prevalent mutations), from four (N, M, ORF3a and ORF7a) SARS-CoV-2 proteins were complexed to create a universal mutant antigen.
- **Sample Diluent Buffer:** 1 vial containing 20 mL for sample dilutions, ready to use

6.2. Materials and equipment needed

- ELISA Microplate Reader with 450 nm wavelength filter or dual wavelength (450/630 nm)
- High-precision transfer pipettor
- EP tubes
- Disposable pipette tips
- Deionized or distilled water
- Absorbent paper
- Loading slot for Wash Buffer
- Goat anti-human IgG secondary antibody, (Fab')2 HRP conjugated
- Second antibody diluent
- Wash Buffer (1xPBS, pH7.4, 0.05% Tween)
- Colorimetric Stop Reagent (2M sulfuric acid)
- Colorimetric HRP Substrate (TMB)
- Plate Sealer
- Positive and negative control sera/plasma samples

For hazard and precautionary statements see section 16.

7. STABILITY AND STORAGE

Store the Micro ELISA Plate at 2 – 8 °C. Stable for 6 months.
Store the Sample Diluent Buffer at -20 °C. Stable for 6 months.

8. REAGENT PREPARATION

Bring all reagents and samples to room temperature 20 – 25 °C and mix them before starting the test.

9. SAMPLE COLLECTION AND PREPARATION

The human serum or plasma samples (heparin, citrate) should be kept at 2 – 8 °C if the assay is performed within 5 days. Otherwise samples should be stored at -70 to -20 °C. Mix samples well before testing. Avoid repeated freeze/thaw cycles.

9.1. Sample dilution

Antigens are diluted in the coating buffer at 100 ng/well/0.1 ml or as defined otherwise. Sera/plasma are diluted in diluent buffer. Recommended sera/plasma dilution is 1:50 to 1:100. The antigen distribution is described below (Plate Layout section 10)

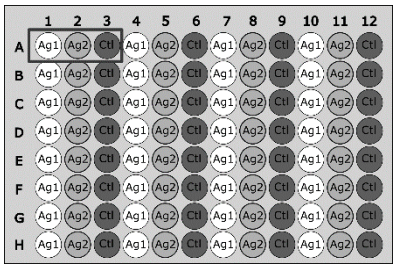
10. QUALITY CONTOL

Each assay must include both negative and positive controls. Negative control: Pool pre-COVID-19 serum samples. The acceptable signal/noise ratio of the negative control should be less than 2.5. Positive control: Pool serum of reactive samples then dilute by adding pooled negative serum to obtain a signal/noise ratio between 3 and 15.

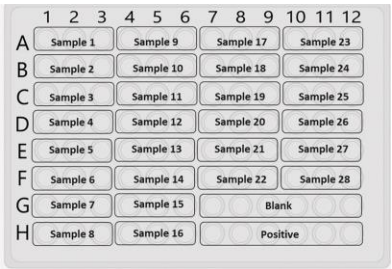
11. PLATE LAYOUT

Antigen 1 (Ag1) = A set of composites EpitoGens comprising 20 epitopes from four non-spike proteins and 29 mutations (N, M, ORF3a and ORF7a).
Antigen 2 (Ag2) = A set of composite EpitoGens comprising 15 immunodominant epitopes from the vaccine target spike protein and 53 spike mutations.
Control (Ctl) = The scaffold protein.

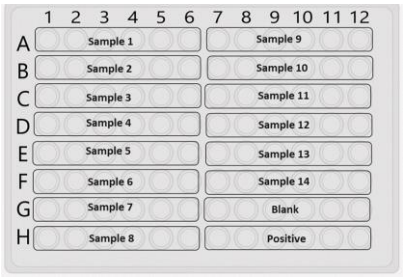
ELISA EpitoGen® Differnetial layout:



Sample distrubution (28 samples)



Sample distrubution (14 samples, in duplicates)



B – blank; *P* – positive control.

12. ASSAY PROTOCOL

Please read the instruction for use carefully before performing the assay. Result reliability depends on strict adherence to the instruction for use as described.

1. Remove plate from 4 °C, leave for 5 min to achieve room temperature (RT).
2. Wash plate once with Wash Buffer (PBST), 300 µl/well using a plate washer or manually.
3. Dilute plasma or serum samples in diluent buffer (recommended dilution 1:50 to 1:100)
4. Apply each sample to Ag1, Ag2 wells and the corresponding Ctl well, 100 µl/well. The same sample must be applied to both the Ag1, Ag2 wells and the Ctl well to accurately calculate the signal to noise ratio (see ELISA plate layout).
5. Incubate for 1 hour at RT.
6. Aspirate samples.
7. Wash plate three times with Wash Buffer (PBST), 300 µl/well using a plate washer or manually.
8. Prepare secondary antibody (HRP conjugated) solution and apply (100 µl/well) to wells for 30 mins at RT.
9. Wash plate three times with Wash Buffer (PBST), 300 µl/well using a plate washer or manually.
10. Apply colorimetric substrate (90 µl/well) substrate for 5 – 10 min.
11. Add stop solution (90 µl/well).
12. Read at 450nm.
13. The plate can be used for testing 28 serum samples singularly or 14 samples in duplicates.

13. RESULTS

13.1. Calculation of Results

The result of each sample is calculated as the signal to noise (S/N) **ratio** of the readout from the Ag1, Ag2 coated wells divided by the readout of Ctl well containing the same sera or plasma sample (ratio non-spike = Ag1 / Ctl); (ratio spike=Ag2 / Atl).
Example: The measured absorbance at 450 nm with the sera or plasma sample in the Ag1 coated well is 1.50 and the same sample in the Ctl coated well is 0.10, the Ag1/Ctl ratio is 1.5/0.10 = 15. Whilst the measured absorbance at 450 nm with the sera or plasma sample in the Ag2 coated well is 0.10 and the same sample in the Ctl coated well is 0.10, the Ag2 / Ctl ratio is 0.10/0.10 = 1

13.2. Interpretation of Results

Ag1/Ctl Ratio	Ag2/Ctl Ratio	Result	Interpretation
< 2.5	< 2.5	Negative	The sample contains no antibodies against the SARS-CoV-2 pathogen. A previous contact with the pathogen or antigen (pathogen respective vaccine) is unlikely.
2.5 to < 3.0	2.5 to < 3.0	Borderline	Antibodies against the SARS-CoV-2 pathogen cannot be detected clearly. It is recommended to repeat the test in 2 – 4 weeks. Alternatively, the assay could be repeated at lower sample dilution (1:50) or bellow.
≥3.0	<2.5	Spike Negative **Non-Spike Positive**	Antibodies against the SARS-CoV-2 non-spike viral antigens are present. There has been contact with a pathogen (i.e. infection).
<2.5	≥3.0	**Spike Positive** Non-Spike Negative	Antibodies against the SARS-CoV-2 spike viral antigens are present. No antibodies are detected to non-spike viral proteins. There has been contact with a spike-based respective vaccine and no previous exposure to SARS-CoV-2.

≥3.0	≥3.0	**Spike Positive** **Non-Spike Positive**	Antibodies against the SARS-CoV-2 spike and non-spike antigens are present. There has been a contact with the pathogen or pathogen-attenuated respective vaccine.

13.3. Antibody isotypes and state of infection

Antibody isotype	Significance
IgM	Primary antibody response. High IgM titer suggests of a current or very recent infection/immunization.
IgG	Secondary antibody response. Follows IgM production. Persists from few months to several years. High IgG titer with low IgM titer suggests past infection.
IgA	Produced in mucosal linings throughout the body. Usually produced early in the course of infection.

14. SPECIFIC PERFORMANCE CHARACTERISTICS

The results refer to the groups of samples investigated.

14.1. Precision

Inter-assay and intra-assay % CVs of less than 10%.

14.2. Diagnostic Specificity

The diagnostic specificity is defined as the probability of the assay of scoring negative in the absence of the specific analyte. The cross-reactivity of the EpitoGen Universal assay was evaluated by testing SARS-CoV-2 seronegative specimens from patients with antibodies to other coronaviruses.

Analyte	Total Number Tested	Number Reactive
anti-229E (alpha coronavirus)	16	0
anti-NL63 (alpha coronavirus)	19	0
anti-OC43 (beta coronavirus)	20	0
anti-HKU1 (beta coronavirus)	18	0

Cross-reactivity of EpitoGen Universal assay was examined using sera from 100 healthy blood donors collected prior to the outbreak of the COVID-19 pandemic were tested. The specificity of serum was 99% (99/100).

14.3. Diagnostic Sensitivity

The diagnostic sensitivity is defined as the probability of the assay of scoring positive in the presence of the specific analyte.

Differential test	Patients No: >8 days post PCR+ test	Positives	Diagnostic sensitivity
Spike-based antigen	123	120	97.6%
Non-Spike-based antigen	110	108	98.1%

15. LIMITATIONS

Bacterial contamination or repeated freeze-thaw cycles of the sample may affect the absorbance values.

16. PRECAUTIONS AND WARNINGS

- For Research use only.
- All materials of human or animal origin should be handled as potentially infectious.
- Do not use reagents after expiry date (6 months after production date).
- Use only clean pipette tips, dispensers, and lab ware.
- Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.

- After first opening and subsequent storage check the diluent buffer microbial contamination prior to further use.
- To avoid cross-contamination and falsely elevated results pipette patient samples and dispense reagents without splashing inaccurately into the wells.
- The ELISA is only designed for qualified personnel who are familiar with good laboratory practice.

16.1. Safety note for reagents containing hazardous substances

The following components are defined as non-hazardous and do not require SDS. The products do not contain hazardous materials above the concentration thresholds defined as hazardous by the Globally harmonized system of classification and labelling of chemicals (GHS). The products do not contain any hazardous components above 1% or any carcinogens above 0.1% as defined in 29 CFR 1910. 1200, the OSHA Hazard Communication Standard, nor are they controlled under the classifications defined by the Workplace Hazardous Materials Information System (WHMIS 2015).

- Diluent buffer (contains 0.01% Proclin300)
- Microwell ELISA plate (wells coated with EpitoGen® epitope)

17. SCHEME OF THE ASSAY

EpitoGen® Universal SARS-CoV-2 IgG ELISA Assay

17.1. Test Preparation

Prepare reagents and samples as described.

17.2. Assay Procedure

	Substrate Blank (G7-G12)	Positive Control (H7-H12)	Sample (diluted 1:100)
Positive Control	-	100 µl	-
Sample (diluted 1:100)	-	-	100 µl
Incubate for 1 h at room temperature Wash each well three times with 300 µl Wash Buffer			
Conjugate	-	100 µl	100 µl
Incubate for 30 min at room temperature Wash each well three times with 300 µl Wash Buffer			
TMB Substrate solution	90 µl	90 µl	90 µl
Incubate for 10 – 15 min at room temperature in dark			
Stop Solution	90 µl	90 µl	90 µl
Photometric measurement at 450 nm			

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