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COVID-19 ELISA IgG DIATHEVA kit

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INTENDED USE

Immuno-enzymatic assay (ELISA) for the determination of IgG antibodies to the COVID-19 coronavirus Nucleocapsid (N) antigen from plasma and serum.

INTRODUCTION

Coronavirus are a large family of viruses that cause illness ranging from the common cold to more severe diseases such as Middle East Respiratory syndrome (MERS-CoV) and Severe Acute Respiratory syndrome (SARS-CoV).
The 2019 Novel Coronavirus, formerly known as 2019-nCoV and now known as SARS-COV-2, is a new strain of coronavirus that was first identified during an outbreak in Wuhan, China which started in December 2019.
Comparison of the genetic sequences of this virus have shown similarities to SARS-CoV and bat coronaviruses. Common signs of infection include respiratory symptoms, fever, cough, shortness of breath and breathing difficulties. In more cases, infection can cause pneumonia, severe acute respiratory syndrome, kidney failure and even death. 2019
Nucleocapsid protein is the most expressed protein of coronavirus. During virion assembly, N protein binds to viral RNA and leads to formation of the helical nucleocapsid. Nucleocapsid protein is a highly immunogenic phosphoprotein also implicated in viral genome replication and in modulating cell signaling pathways. Because of the conservation of N protein sequence and its strong immunogenicity, the N protein of coronavirus is chosen as an optimal diagnostic marker. Two types of antibodies against this virus (IgM and IgG) are produced and secreted into the circulation by the immune system; the presence of anti-SARS-CoV-2 IgM in the patient's serum indicates the acute phase of the infection and the presence of anti-SARS-CoV-2 IgG shows the long-lasting response against the infection and immunity against the virus

PRODUCT DESCRIPTION

The immune-enzymatic determination of specific antibodies is based on the ELISA (Enzyme Linked Immuno-Sorbent Assay) technique.
Microtiter plates are coated with specific antigens to bind corresponding antibodies of the sample.
Assays controls (Positive and Negative controls) are ready to use, the patient samples are diluted 1:100v/v and added to the plate.
After washing the wells to remove all unbound sample material a horseradish peroxidase (HRP) conjugated secondary Antibody is added into the well and incubated at 37°C. This conjugate binds to the captured IgG antibodies. In a second washing step unbound conjugated is removed. The immune complex formed by the bound conjugate is visualized by adding ABTS substrate which gives a green reaction product. Absorbance at 405nm is read using an ELISA microplate reader. The presence of specific anti SARS-CoV-2 IgG antibodies is directly proportional to the color intensity of the sample.
The kit is equipped with both positive and negative synthetic controls as well as a control on the blank value that define the quality control of the analysis session and allow the correct normalization of the data.

KIT CONTENTS **The kit provides all the reagents needed for the analysis** (Table 1)
***Note**: Kit contents are sufficient for an eventual use in automatic procedures*

Table 1: Kit Contents

Reagents	No vial x Volume	Description
Microtiter strips (12x8well strips)	1x96 wells	Coated plate with *COVID-19 Nucleocapsid recombinant protein* Preserved with sodium azide 0,02% w/v
Buffer A	2x50ml	Samples diluent; Ready to use With preservative
Buffer B	3x100ml	Wash buffer 10X concentrate To dilute to 1X with distilled water With preservative
Negative control (NC)	2x1ml	Ready to use
Positive Control (PC)	2x1ml	Ready to use
HRP-conjugated secondary antibody	1x18ml	Ready to use With preservative
ABTS solution	1x23ml	Ready to use With preservative

MATERIAL AND EQUIPMENT REQUIRED (NOT PROVIDED)

- Microplate reader equipped with 405nm filter
- Incubator at 37°C (avoid CO_2 that oxidizes the immune complexes) or thermo block
- Precision pipettes and pipette tips
- Glass or plastic pipettes
- Deionized or distilled water
- Semi-automated or automated microplate washer
- 1,000 mL graduated cylinder
- Vortex
- Tubes for diluting samples

LIMITATIONS OF THE ASSAY

- For laboratory use only
- No drugs have been investigated for assay interference
- Any variation in specimen diluent, operator, pipetting technique, washing technique, incubation time or temperature, kit age can cause variations in the formation of immune complexes.
- Infection novel coronavirus (COVID-19) patients the first week of the onset of which novel coronavirus IgG may be negative. In addition, patients with low autoimmunity or other diseases that affect autoimmune function, failure of important systemic organs, and use of drugs that suppress immune function can also lead to negative results of new coronavirus IgG. Previous infection of SARS or other coronavirus strain may cause a light IgG positive in view of similar of different strains
- The test cannot be used alone for a clinical diagnosis and the result must always be evaluated together with data from the patient's medical history and other diagnostic investigations.

False negative results can result from:
• Incorrect collection or storage of the sample
• Secondary antibody degradation during transport / storage
• Failure to comply with the instructions for use

False positive results can result from:
• Contamination during sample collection, handling, or preparation
• Contamination when handling the product
• Contamination of the wells during the washing steps of the plate

TRANSPORT AND STORAGE CONDITIONS

After the arrival reagents must be stored at +2 / 8 °C. If stored at the recommended temperature all reagents are stable until the expiration date.

WARNING AND PRECAUTIONS

Carefully read all product information before using the kit
The operator must always pay attention to:

- Do not interchange components of different kit lots or with those of other kits
- Strict adherence to the test protocol will lead to achieving best results
- When using the kit, check the reagent solutions are clear
- Do not use the kit after expiration date
- Avoid cross-contamination between serum/plasma specimens

- Treat all specimens and kit serum-based reagents as potentially infectious
- Do not freeze any component of the kit
- Do not incubate in CO_2 incubators as immune complexes are oxidized
- Water deionized with polyester resins may inactive the horseradish peroxidase enzyme
- Use the chromogenic substrate at room temperature (22-27°C)

TEST PROCEDURE

1. Sample collection and storage

Serum Collect samples in serum-use pyrogen/endotoxin-free collecting tubes. After blood clotting, centrifuge the tubes at approximately 1,000 x *g* for 10 min and remove sera from the red cells.

Plasma Collect blood in serum-use pyrogen/endotoxin-free collecting tubes with anticoagulants. Centrifuge the tubes at 1,000 x *g* for 10 min. Remove plasma rapidly and carefully.
No interference was observed in sample preparation with citrate, EDTA or heparin

Storage Samples can be stored at 2–8°C for 24 hours for up to seven days or frozen up to six months. For longer periods, samples should be stored frozen in small aliquots. Avoid repetitive freezing and thawing cycles.

Recommendation *- Before use, thaw completely samples at room temperature. Do not thaw by heating at high temperature.*

2. Reagents preparation

Buffer B (Wash Buffer 10X Concentrate): Dilute 100ml of buffer B to 1,000ml with distilled water.
If crystals have formed in the concentrate, warm at room temperature and mix gently until the crystals are completely dissolved.

Important: *The performance of the kit may decrease if reagents are not properly prepared*

3. Sample preparation

Vortex sample 3-5 seconds and spin the specimen before taking the sample for dilution

Recommendation: *Avoid any foam or bubbles in the sample*.

4. Test execution

All reagents and specimens must be equilibrated to come to room temperature before use and must be GENTLY mixed without foaming. Once the procedure has started, all steps should be completed without interruption
Test execution time: 140 minutes

1. Extract the desired number of the strips from the aluminum bag which contains the plate and place them in the support (Frame)
   Note: *The leftover strips can be stored in the aluminum bag that houses the plate and placed at +4°C*

   Negative control, Positive control are ready to use.
   Dispense 100µl/well of negative control into duplicate wells.
   Dispense 100µl/well of positive control into a single well.
   For the reagent blank, dispense 100µl of sample diluent (Buffer A) into duplicate well (see Table 2)

2. Prepare 1:100v/v dilution of the samples by adding 1 µl of the sample to 99µl of Buffer A in the sterile tubes. Dispense 100µl/well of 1:100v/v diluted sera into the appropriate wells (see Table 2)

Table 2: Test configuration

ROW	Strip 1	Strip 2	Strip3
A	**Negative control (NC)**	Sample 4	
B	**Negative Control (NC)**	Sample 5	
C	**Blank**	Sample 6	
D	**Blank**	Sample 7	
E	**Positive Control (PC)**	Sample 8	
F	Sample 1	Sample 9	
G	Sample 2	Sample 10	
H	Sample 3	Sample 11	

3. Cover the strips or plate with aluminum foil and incubate at 37±1°C for **60±5 minutes**

4. Wash the microtiter strips five times with 380µl of reconstituted Buffer B
   Before starting the washing phase, equilibrate the washer with Buffer B 1x
   (see paragraph "Reagents Preparation")
   Set the instrument as reported in Table 3

Table 3: Setting for automated washer

Strips washing with automated Washer	
Cycles number	5
Form	Plate/ (set the number of strips to wash)
Volume for well	350µL/well
Priming before Wash	yes

5. Dispense 100µl/well of HRP-conjugated secondary antibody
6. Cover the strips or plate with aluminum foil and incubate at 37°±1C for **60± 5 minutes**.
7. Wash the microtiter strips five times as point 4
8. Add 100 µl/well of ABTS solution
9. Cover the microtiter strips and incubate at room temperature (22-27°C) for **20±5 minutes**

Recommendation*: Avoid light exposure*

10. Read the absorbance at **405± 5 nm** using a microplate reader
    Note: *Shaker the plate for 3 seconds before reading*

5. Quality controls

The kit includes quality controls that allow the correct interpretation of the results
To be valid, the test must meet the VALIDITY CRITERIA OF THE TEST

To assure the validity of the results each assay must include both negative (double well) and positive controls (single well) and blank (double well).
Check that the values shown in the Table 4 are satisfied before proceeding with the interpretation of the results

Table 4: Validity criteria of the test

Blank (ABS at 405nm mean of the two replicates)	**≤ 0.160 OD**
Negative Control (NC) (ABS at 405nm mean of the two replicates)	**≤ 0.10 OD (after subtraction of the blank)**
Positive Control (PC) (ABS at 405nm)	**≥ 1.0 OD**

Recommendation*: If the above conditions are not found the assay is invalid. For invalid assays, technique may be suspect, and the assay should be repeated following a thorough review of the product information.*

6. Data analysis: Cutoff calculation

The results of the test are calculated through a Cut-Off value (Co) determined using the formula below which is based on the mean value of Negative Control (NC after subtraction of the blank).

Negative Control (abs a 405 nm mean) - Blank (abs a 405nm mean) = **NC**

Cut-Off (Co)= NC+ 0.480
The obtained value is used for the interpretation the results as described below

7. Data analysis: Results calculation

The results are interpreted by calculating the ratio between the Sample absorbance at 405nm (after subtracting the average blank value) and the Cut-Off value (Co) determined as described above (Cut-Off Calculation) with the following formula:

S/Co=Sample (ABS at 405nm) / Cut-Off (Co)

Note: *If the samples are tested in duplicate, average the two absorbances. The absorbance variation between the two samples replicate of the same sample is not acceptable if it is higher than 0.250 abs at 405nm. Re-test the sample.*

The results must be interpreted comparing the S/Co ratio with the ranges shown in the Table 5

Table 5: Results interpretation

NEGATIVE	EQUIVOCAL (*)	POSITIVE
S/Co ≤ 1.0	1.0 < S/Co < 1.5	S/Co ≥ 1.5

***Recommendation*:** (*) *In case of equivocal result repeat the test if the sample is still equivocal it is recommended to take a new serum/plasma sample from the patient after 4-5 days and repeat the Test for the presence/absence of IgG anti COVID-19*

PERFORMANCE CHARACTERISTIC

Analytical performance

The **Analytical Sensitivity** which defines the maximum dilution at which a sample is positive**,** was calculated utilizing the mean value of 10 positive and 10 negative samples and expressed as antibody titer.
The Analytical sensitivity determined for the COVID-19 ELISA IgG DIATHEVA kit is equal to 1,600 (or Log 1,600= 3.20).

Limit of Blank (LoB): 0.11 (Abs mean of blank replicates /Co)
Limit of Detection (LoD): 0.13 (Abs mean of samples positive /Co)
LoB and LoD were defined according to EP17-A2 CLSI (Clinical and Laboratory Standards Institute, https://clsi .org) guideline

Analytical Specificity (Cross reactivity): For the cross-reactivity evaluation against other disease, 18 samples derived from patients with the following infections: HIV1 and 2, HIV1 HAV, HBV, HCV, and Pneumocystis have been tested. No cross reactivity was observed in these samples, analytical specificity for the COVID-19 ELISA IgG DIATHEVA kit is 100%.

Diagnostic performance

Diagnostic Sensitivity: For the DSe (Diagnostic sensitivity) evaluation 167 samples collected from individuals with or without clinical symptoms were tested, who have had clinical findings, positive for RT-PCR test. 50 positive samples were also evaluated with an ELISA assay anti COVID-19 IgG CE-IVD marked and 10 positive samples were also determined with CLIA IgG.
165 samples show positive results in accordance with both the molecular test and the immunoassay used as the reference standard.
The Diagnostic Sensitivity (DSe) determined for the COVID-19 ELISA IgG DIATHEVA kit is 98.80%. The positive predictive value is 97.06% (Table 6)

Specificity: For the DSp (Diagnostic specificity) the total number of 252 negative samples for novel coronavirus (COVID-19) infection previously collected and stored at -80°C were tested with our kit. Negative samples were represented by different cohorts as follow:
170 normal sera and plasma collected two years prior to January 2019.
29 pregnant woman samples collected two years prior to January 2019.
18 samples were from patients with various infections as HIV1 and 2, HAV, HBV, HCV, and pneumocystis collected two years prior to January 2019.
35 samples were from multi-ethnic individuals with high fragility and socio-sanitary margins
The Diagnostic Specificity (DSp) determined for the COVID-19 ELISA IgG DIATHEVA kit is 98.02%. The negative predictive value is 99.20% (Table 6 and Table 7)

Test Precision:
The **Accuracy** of the test calculated on the all cohort is 98.33%

Table 6: Diagnostic Sensitivity (Dse) And Diagnostic Specificity (Dsp)

	Percent (%)	Positive predictive value (%)	Negative predictive value (%)
Dse	98.80	97.06	-
Dsp	98.02	-	99.20
Accuracy	98.33	-	-

Table 7: Diagnostic Specificity for the different negative

	n.	Dsp (%)
Healthy Donors	170	99
Pregnant Women	29	97
Multi-ethnic individuals with high fragility and socio-sanitary margins	35	95
(*) Patients with various infections non COVID-19 correlated	18	100

(*) represented by HIV1;2, HAV, HBV, HCV, Pneumocystis patients

Repeatability: The repeatability is the precision intra-assay. Was evaluated on 20 sera and plasma sample (7 IgG COVID-19 positive, determined with CLIA and RT PCR and 13 negative sample collected two years prior to January 2019), positive controls, negative controls and background dispensed in ten twin wells, in the same experimental session with the same operator. Repeatability is determined with a CV (%) of OD values for each sample. The CV (%) values for the Repeatability are reported in Table 8

Table 8: CV (%) values for the Repeatability

REPEATABILITY	n.	Samples Replicates	CV (%) minimum	CV (%) maximum	CV (%)
COVID-19 Positive samples	**7**	**10**	**0**	**8**	**≤ 10**
COVID-19 Negative samples	**13**	**10**	**0**	**6**	**≤ 10**
Positive Control (PC)	**1**	**10**	**4**	**8**	**≤ 10**
Negative Control (NC)	**1**	**10**	**8**	**11**	**≤ 15**
Blank	**1**	**10**	**10**	**11**	**≤ 15**

Reproducibility: The Reproducibility is the precision inter-assay. Three lots of the kit were utilized for analyzing the same sample (27 sera and plasma: 7 IgG COVID-19 positive, determined with CLIA and RT PCR and 20 negative sample collected two years prior to January 2019), the same positive controls, negative controls and background, in three different experimental sessions by three different operator. Repeatability is determined with a CV (%) of OD values for each sample. The CV (%) values for the Reproducibility are reported in Table 9

Table 9: CV (%) values for the Reproducibility

REPRODUCIBILITY	n.	Samples Replicates	CV (%) minimum	CV (%) maximum	CV (%)
COVID-19 Positive samples	7	3 x 3 lots	2	7	≤ 10
COVID-19 Negative samples	20	3 x 3 lots	3	13	≤ 15
Positive Control (PC)	3	3 x 3 lots	6	8	≤ 10
Negative Control (NC)	3	3 x 3 lots	8	10	≤ 15
Blank	1	3 x 3 lots	10	16	≤ 20

Technical assistance:

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