研究用試薬

Updates with respect to the previous version are marked in grey.

Anti-SARS-CoV-2 NCP ELISA (IgM)

Instructions for use

For in vitro diagnostic use IVD

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2606-9601-2 M	SARS-CoV-2 NCP	IgM	Ag-coated microplate wells	96 x 01 (96)

CE

Intended use

The enzyme immunoassay (ELISA) provides semiquantitative in vitro determination of human antibodies of the immunoglobulin class IgM against SARS-CoV-2 NCP in serum, EDTA, or citrate plasma to support the diagnosis of SARS-CoV-2 infection and constitutes a supplement to the direct pathogen detection. The product is designed for use as IVD and can optionally be processed on fully automated equipment.

Clinical significance

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, previously called 2019-nCoV) belongs to the family of coronaviruses and, like SARS-CoV, is classified in the genus Betacoronavirus [1]. At the end of 2019, SARS-CoV-2 was identified as the causative pathogen of clustered cases of pneumonia of unclear origin. The virus caused an infection wave that has spread rapidly worldwide and was declared a pandemic by the WHO at the beginning of 2020 [2-5].

SARS-CoV-2 is predominantly transmitted by droplet infection via coughing or sneezing and through close contact with infected persons [3, 4, 6]. Health care personnel and family members are especially at risk [6]. The zoonotic reservoir of the virus appears to be bats [3, 4, 6].

The incubation time of SARS-CoV is three to seven, maximally 14 days [2]. The symptoms of SARS-CoV-2 infection are fever, coughing, breathing difficulties, fatigue and loss of the olfactory and taste sense [2-4, 6, 7]. In most patients the infection manifests with symptoms of a mild febrile illness with irregular lung infiltrates. Some patients, especially elderly or chronically ill patients, develop acute respiratory distress syndrome (ARDS) [2, 3, 5, 6]. In February 2020, the disease caused by SARS-CoV-2 was named COVID-19 by the WHO.

Suitable methods for the diagnosis of SARS-CoV-2 infections are the detection of viral RNA by reverse transcriptase polymerase chain reaction (RT-PCR) or of virus protein by means of ELISA or rapid test primarily in sample material from the upper (nasopharyngeal or oropharyngeal swab) or lower respiratory tract (bronchoalveolar lavage fluid, tracheal secretion, sputum, etc.) [4, 5]. Detection of viral antigens is less sensitive than RT-PCR testing.

The determination of antibodies enables confirmation of SARS-CoV-2 infections in patients with typical symptoms and in suspected cases. It also contributes to monitoring and outbreak control [4, 5]. The spike (S) and nucleocapsid (N) proteins of SARS-CoV-2 are highly immunogenic. More than 90% of the neutralising antibodies in COVID-19 patients are directed against the receptor-binding domain (RBD) of the spike protein. The spike protein is the target protein of almost all vaccines against COVID-19 [8].

Around 90% of SARS-CoV-2-infected persons develop specific antibodies until day 10 following symptom onset. IgG, IgA and IgM against the spike protein often occur simultaneously [8]. For significant serological results, two patient samples should be investigated, one from the acute phase (week 1 of the illness) and one from the convalescent phase (3 to 4 weeks later) [4, 6, 9]. SARS-CoV-2-specific T cells appear a few days after onset of symptoms. A specific T cell response is associated with a milder COVID-19 course [8].

Neutralising antibodies are associated with protective immunity against reinfection with SARS-CoV-2 or SARS-CoV. Neutralising antibodies against SARS-CoV could be detected 17 years after infection. SARS-CoV-2 reactive T cells are part of the T cell repertoire from persons who had a SARS-CoV infection in 2003. These cells proliferate following contact with SARS-CoV-2. Cross-reacting T cells were detected in some of the investigated persons without history of SARS-CoV-2 infection and are supposedly due to prior infections with coronaviruses causing common colds. This may indicate a long-lasting immunity following infection with betacoronaviruses [8, 10, 11].

With regard to COVID-19, the immunological memory is heterogeneous: virus-specific antibodies and memory B and T cells are present in different quantities and their levels change with different dynamics. Current findings indicate that the T and B cell memory and antibodies in most cases persist over years after SARS-CoV-2 [8].

Antigen

The reagent wells are coated with modified nucleocapsid protein (NCP) of SARS-CoV-2.

Test principle

The test kit contains microplate strips each with 8 break-off reagent wells coated with modified nucleocapsid protein of SARS-CoV-2. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgM (also IgA and IgG) antibodies will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgM (enzyme conjugate) catalysing a colour reaction.

Contents of the test kit

Component	Colour	Format 96 x 01	Symbol
1. Microplate wells coated with antigens 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	-	12 x 8	STRIPS
2. Calibrator (IgM, human), ready for use	dark red	1 x 2.0 ml	CAL
3. Positive control (IgM, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
4. Negative control (IgM, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
5. Enzyme conjugate peroxidase-labelled anti-human IgM, ready for use	red	1 x 12 ml	CONJUGATE
6. Sample buffer containing IgG/RF-absorbent (anti-human IgG antibody preparation obtained from goat), ready for use	green	1 x 100 ml	SAMPLE BUFFER
7. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
8. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
9. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
10. Protective foil	-	2 pieces	FOIL
11. Quality control certificate	-	1 protocol	-
12. Instructions for use	-	1 booklet	-

Additional materials and equipment (not supplied in the test kit)

- Automatic microplate washer: recommended. Washing of the microplates can also be carried out manually.
- Microplate reader: wavelength of 450 nm, reference wavelength range from 620 nm to 650 nm
- Calibrated pipettes
- Pipette tips
- Stepper pipette: recommended for the pipetting of enzyme conjugate, substrate, and stop solution
- Distilled or deionised water
- Incubator: for incubation of the microplate at +37 °C
- Incubator or water bath: recommended to warm the wash buffer
- Stop watch

Storage and stability

The test kit has to be stored at +2 °C to +8 °C; do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

In-use stability

After initial opening, the reagents are stable until the indicated expiry date when stored at +2 °C to +8 °C and protected from contamination, unless stated otherwise below.

Warnings and precautions

- The product must only be used by healthcare professionals in an adequate laboratory environment.
- Do not use the test kit if the packaging of the reagents is damaged.
- Before using the product, read the instructions for use carefully. Only use the valid version provided with the product.
- EUROIMMUN reagents must not be mixed with or replaced by reagents from other manufacturers.
- Observe Good Laboratory Practice (GLP) and safety guidelines. Some of the reagents contain preservatives in non-declarable concentrations. Avoid eye and skin contact with samples and reagents. In case of eye or skin contact, rinse thoroughly with water. Remove and wash contaminated clothing. In case of ingestion, obtain medical advice.
- The calibrator and controls of human origin have tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all test kit components should be treated as potentially infectious and handled with care.

Preparation and stability of the samples

- **Samples:** Human serum or EDTA or citrate plasma.
- **Introduction:** Before the determination of specific antibodies of class IgM, antibodies of class IgG should be removed from the patient sample. This procedure must be carried out in order to prevent any rheumatoid factors of class IgM from reacting with specifically bound IgG, which would lead to false positive IgM test results, and to prevent specific IgG displacing IgM from the antigen, which would lead to false negative IgM test results.
- **Functional principle:** The sample buffer (green coloured!) contains an anti-human IgG antibody preparation from goat. IgG from a serum sample is bound with high specificity by these antibodies and precipitated. If the sample also contains rheumatoid factors, these will be absorbed by the IgG/anti-human IgG complex.
- **Separation properties:**
  - All IgG subclasses are bound and precipitated by the anti-human IgG antibodies.
  - Human serum IgG in concentrations of up to 15 mg per ml are removed (average serum IgG concentration in adults: 12 mg per ml).
  - Rheumatoid factors are also removed.
  - The recovery rate of the IgM fraction is almost 100%.
- **Performance:** The **patient samples** for analysis are diluted **1:101** with green-coloured sample buffer. For example, add 10 µl sample to 1.0 ml sample buffer and mix well by vortexing. Sample pipettes are not suitable for mixing. Incubate the mixture for at least **10 minutes** at room temperature (+18 °C to +25 °C). Subsequently, it can be pipetted into the microplate wells according to the pipetting protocol.
- **Notes:**
  - Antibodies of the class IgG should not be analysed with this mixture.
  - It is possible to check the efficacy of the IgG/RF absorbent for an individual patient sample by performing an IgG test in parallel to the IgM test using the mixture. If the IgG test is negative, the IgM result can be considered as reliable.
  - The calibrator and controls are ready for use, do not dilute them.
- **Stability of the patient samples:**
  - stored at +2 °C to +8 °C: up to 14 days

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incubate diluted samples within one working day

Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18 °C to +25 °C) approx. 30 minutes before use.

The thermostatically adjustable ELISA incubator must be set to +37 °C ± 1 °C.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag).
  Once the protective wrapping has been opened for the first time, the wells coated with antigens can be stored in a dry place and at a temperature between +2 °C and +8 °C for 4 months.
- **Calibrator and controls:** Ready for use. Mix reagents thoroughly before use.
- **Enzyme conjugate:** Ready for use. Mix the reagent thoroughly before use.
- **Sample buffer:** Ready for use. The green coloured sample buffer contains IgG/RF absorbent. Serum or plasma samples diluted with this sample buffer are only to be used for the determination of IgM antibodies.
- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallisation occurs in the concentrated buffer, warm it to +37 °C and mix well before dilution. Dilute the required volume 1:10 with deionised or distilled water (1 part reagent plus 9 parts water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working-strength wash buffer is stable for 4 weeks if stored at +2 °C to +8 °C and handled properly.
- **Chromogen/substrate solution:** Ready for use. Close the tube immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.
- **Stop solution:** Ready to use.

Waste disposal

Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Quality control

For every group of tests performed, the extinction readings of the calibrator and ratios determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

Reference material

As no quantified international reference serum exists for antibodies against SARS-CoV-2 NCP, the calibration is performed in ratios which are a relative measure for the concentration of antibodies in serum or plasma.

Assay procedure

(Partly) manual test performance

Sample incubation: (1st step)
Transfer **100 µl** of the **calibrator, positive and negative controls or diluted patient samples** into the individual microplate wells according to the pipetting protocol. Incubate for **60 minutes** at **+37 °C ± 1 °C**.
For manual processing of microplate wells, cover the finished test plate with the protective foil. When using an automated microplate processor for incubation, follow the recommendations of the instrument manufacturer.

Washing:
Manual: Remove the protective foil. Empty the wells and subsequently wash **3 times using 300 µl of working-strength wash buffer** for each wash.
Automatic: Remove the protective foil. Wash the reagent wells **3 times with 450 µl of working-strength wash buffer** (program setting: e.g. TECAN Columbus Washer "Overflow Mode").

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

Note:
Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

Conjugate incubation: (2nd step)
Pipette **100 µl of enzyme conjugate** (peroxidase-labelled anti-human IgM) into each of the microplate wells.
Incubate **30 minutes** at room temperature (+18 °C to +25 °C).

Washing:
Empty the wells. Wash as described above.

Substrate incubation: (3rd step)
Pipette **100 µl of chromogen/substrate solution** into each of the microplate wells. Incubate for **15 minutes** at room temperature (+18 °C to +25 °C) protected from direct sunlight.

Stopping:
Pipette **100 µl of stop solution** into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:
Photometric measurement of the colour intensity should be made at a **wavelength of 450 nm** and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution.** Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

Test performance using fully automated analysis devices

Sample dilution and test performance are carried out fully automatically using an analysis device. The incubation conditions programmed in the respective software authorised by EUROIMMUN may deviate slightly from the specifications given in the ELISA instructions for use, but have been validated in respect of the combination of the EUROIMMUN Analyzer I, the EUROIMMUN Analyzer I-2P, the Sprinter XL and this EUROIMMUN ELISA. Validation documents are available on enquiry.

Note: Processing on other fully automated systems is possible but must be validated by the user.

Pipetting protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	C	P 6	P 14	P 22								
B	pos.	P 7	P 15	P 23								
C	neg.	P 8	P 16	P 24								
D	P 1	P 9	P 17									
E	P 2	P 10	P 18									
F	P 3	P 11	P 19									
G	P 4	P 12	P 20									
H	P 5	P 13	P 21									

The pipetting protocol for microplate strips 1 to 4 is an example for the **semiquantitative analysis** of 24 patient sera (P 1 to P 24).

The calibrator (C), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA can be improved by duplicate determinations for each sample.

The wells can be broken off individually from the strips. This makes it possible to adjust the number of test substrates used to the number of samples to be examined and minimises reagent wastage.

Both positive and negative controls serve as internal controls for the reliability of the test procedure. They must be assayed with each test run.

Test evaluation

The extinction of the calibrator defines the upper limit of the reference range of non-infected persons (**cut-off**) recommended by EUROIMMUN. Values above the indicated cut-off are to be considered as positive, those below as negative.

Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction of the control or patient sample over the extinction of the calibrator. Calculate the ratio according to the following formula:

$$\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator}} = \text{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <0.8: negative
Ratio ≥ 0.8 to <1.1: borderline
Ratio ≥1.1: positive

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another, EUROIMMUN recommends retesting the samples.

Analytical performance

Measurement range:

Limit of blank (LoB): ratio 0.07
Limit of detection (LoD): ratio 0.09

LoB and LoD were defined according to the requirements defined in guideline EP17-A2 of the CLSI (Clinical and Laboratory Standards Institute, https://clsi.org/).

Precision: Studies on the intra-lab precision were carried out according to CLSI guideline EP05-A3. Six samples (reactivity distributed over the entire measurement range) were measured. The precision is given as standard deviation (SD) and coefficient of variation (CV).

Intra-lab precision

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
Mean	Ratio 0.13		Ratio 0.90		Ratio 1.17		Ratio 1.68		Ratio 2.28		Ratio 1.81	
	SD	% CV	SD	% CV	SD	% CV	SD	% CV	SD	% CV	SD	% CV
Repeatability	0.008	6.1%	0.038	4.3%	0.056	4.7%	0.063	3.7%	0.171	7.5%	0.053	2.9%
Between run	0.019	14.6%	0.061	6.8%	0.121	10.3%	0.145	8.6%	0.198	8.7%	0.186	10.3%
Within day	0.020	15.8%	0.072	8.0%	0.133	11.4%	0.158	9.4%	0.262	11.5%	0.194	10.7%
Between day	0.013	10.0%	0.056	6.2%	0.077	6.6%	0.151	9.0%	0.120	5.3%	0.112	6.2%
Within lab	0.024	18.7%	0.092	10.1%	0.154	13.1%	0.219	13.0%	0.288	12.6%	0.224	12.4%

Cross-reactivity (analytical specificity): Due to the use of a modified nucleocapsid protein, in which significant homologous regions were eliminated and the diagnostically relevant epitopes were combined, cross-reactions with most human pathogenic representatives of the coronavirus family are virtually excluded. 163 samples taken prior to the occurrence of SARS-CoV-2 (before January 2020) that were antibody-positive for at least one human pathogenic coronavirus (HCoV HKU1; HCoV OC43; HCoV NL63; HCoV 229-E) were investigated with the Anti-SARS-CoV-2 NCP ELISA IgG. Positive reactions were only observed in 0.6% of the analyses. Cross reactions with endemic HCoV are therefore unlikely. Cross-reactions between SARS-CoV(-1) and SARS-CoV-2, however, are likely to occur due to their close relationship.

Antibodies against	n (positive)/n (total)	Positive rate [%]
HCoV	1/163	0.6

Interference: Haemolytic, lipaemic and icteric samples showed no influence on the result up to concentrations of 10 mg/ml haemoglobin, 20 mg/ml triglycerides and 0.4 mg/ml bilirubin in this ELISA.

Clinical performance

Diagnostic sensitivity (Prevalence):

To determine the diagnostic sensitivity, samples from patients with confirmed SARS-CoV-2 infection were analysed. The following sensitivity therefore corresponds to the prevalence of antibodies against SARS-CoV-2 in COVID-19 patients.

The sensitivity was determined by investigating 102 samples from 79 European patients, using the Anti-SARS-CoV-2 NCP ELISA (IgM). In these patients, infections with SARS-CoV-2 had been confirmed by RT-PCR [10] based on a sample taken at the early phase of infection. In samples taken prior to day 10 (time point after onset of symptoms or positive RT-PCR), the Anti-SARS-CoV-2 NCP ELISA (IgM) showed a sensitivity of 88.2%. The sensitivity of the Anti-SARS-CoV-2-NCP ELISA (IgM) for samples collected in the period from day 11 to 15 is 70.6%. Further data on the sensitivity of the Anti-SARS-CoV-2-NCP ELISA (IgM) in samples collected after day 16 are presented in the following table. Borderline results (n = 6) were not considered in the calculation.

Days after symptom onset or positive RT-PCR	EUROIMMUN Anti-SARS-CoV-2 NCP ELISA (IgM)		
	positive	negative	Sensitivity
≤ 10	15	2	**88.2%**
11-15	12	5	**70.6%**
16-25	15	13	**53.6%**
26-35	5	6	**45.5%**
36-45	3	3	**50.0%**
≥ 46	2	15	**11.8%**

Specificity: The specificity of the Anti-SARS-CoV-2 NCP ELISA (IgM) was determined by analysing 199 patient samples that were positive for antibodies against other pathogens or for rheumatoid factors. Additionally, 622 samples from blood donors, children and pregnant women were analysed. The results in the borderline range (n = 7) were not included in the calculation, resulting in a specificity of the Anti-SARS-CoV-2 NCP ELISA (IgM) of 98.6%.

Panel	n	EUROIMMUN Anti-SARS-CoV-2 NCP ELISA (IgM)
		Specificity
Blood donors	449	99.1%
Pregnant women	99	96.9%
Children	74	100.0%
Older people	97	100.0%
Influenza (freshly vaccinated including courses)	40	100.0%
Acute EBV infection & heterophilic antibodies	22	81.8%
Rheumatoid factors	40	100.0%
Total	**821**	**98.6%**

Limitations of the procedure

- The results should always be interpreted together with those of further laboratory diagnostic procedures and based on the clinical picture.
- A negative serological result does not exclude an infection. Particularly in the early phase of infection, antibodies may not yet be present or are only present in such small quantities that they are not detectable. In the case of a borderline result, a secure evaluation is not possible. If there is a clinical suspicion and a negative or borderline serological result, we recommend clarification by means of other diagnostic methods and/or the serological investigation of a follow-up sample. A positive result indicates that there has been contact with the pathogen. In the determination of pathogen-specific IgM antibodies, polyclonal stimulation of the immune system or antibody persistence may limit the diagnostic relevance of positive findings. A significant increase in the specific IgG antibody level (by more than factor 2) and/or seroconversion in a follow-up sample taken after at least 7 to 10 days may be interpreted as an indication of acute infection. The sample and the follow-up sample should be incubated in the same test run.
- The specifications in the instructions for use, e.g. pipetting volumes, incubation times, temperatures and preparation steps must be observed to avoid incorrect results.
- Correct sample collection and storage are crucial for the reliability of the test results.
- The test system is validated for the determination of anti-SARS-CoV-2 NCP IgM in human serum or plasma only.

- The binding activity of the antibodies and the activity of the enzyme used are temperature-dependent. It is therefore recommended to use a thermostatically controlled ELISA incubator in all incubation steps. The higher the room temperature during the incubation steps, the greater will be the extinction. The same variations also apply to the incubation times. However, the calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.
- Insufficient washing (e.g. less than 3 wash cycles, too small wash buffer volumes or too short residence times) can lead to false extinction readings.
- Residual liquid (>10 µl) in the reagent wells after washing can interfere with the substrate conversion and lead to false low extinction readings.
- Partial or complete adaptation of the test system for use with automated sample processors or other liquid handling devices may lead to differences between the results obtained with the automated and manual procedure. It is the responsibility of the user to validate the automated instruments used for the analysis to ensure that they yield test results within the permissible range.
- Since interferences with samples from patients with acute *Plasmodium* spp. infections cannot be excluded, malaria should always be considered in differential diagnostics.
- In the case of breakthrough infections of vaccinated persons or reinfections with SARS-CoV-2, the production of specific IgM antibodies may be very low, so that detection is not possible.

Literature

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Liability

The test kit, including original accessories, must only be used in accordance with the intended use. EUROIMMUN accepts no liability for any other use (e.g. non-compliance with the instructions for use and improper use) or for resulting damages.

Additional information

Regulatory information for customers in the European Union: Please observe the obligation to report any serious incidents occurring in connection with this product to the competent authorities and to EUROIMMUN.

Technical support

In case of technical problems you can obtain assistance via the EUROIMMUN website (https://www.euroimmun.de/en/contact/).

Meaning of the symbols

Symbol	Meaning	Symbol	Meaning
STRIPS	Microplate strips	LOT	Lot description
CAL	Calibrator		Protect from sunlight
POS CONTROL	Positive control		Storage temperature
NEG CONTROL	Negative control		Unopened usable until (YYYY-MM-DD)
CONJUGATE	Conjugate	CE	CE-labelled
SAMPLE BUFFER	Sample buffer		Manufacturing date (YYYY-MM-DD)
WASH BUFFER 10x	Wash buffer, 10x concentrate		Manufacturer
SUBSTRATE	Substrate		Observe instructions for use
STOP SOLUTION	Stop solution	REF	Order number
FOIL	Protective foil		Contents suffice for <n> analyses
IVD	In vitro diagnostic medical device		Biological risks