Anti-Chikungunya Virus ELISA (IgM)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 293a-9601 M	Chikungunya virus (CHIKV)	IgM	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides semiquantitative in vitro determination of human antibodies of the immunoglobulin class IgM against chikungunya virus (CHIKV) in serum or plasma to support the diagnosis of chikungunya virus infection.

Application: The Anti-Chikungunya Virus ELISA (IgG and IgM) is suitable for the serological diagnosis of an acute or past Chikungunya virus infection and supplements direct pathogen detection. Seroconversion or a significant increase in the IgG antibody titer indicates an acute infection. In addition, the determination of specific antibodies is important for epidemiological studies.

Principle of the test: The test kit contains microplate strips each with 8 break-off reagent wells coated with recombinant chikungunya virus antigens. 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	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 (goat), 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. Test instruction	---	1 booklet	
12. Quality control certificate	---	1 protocol	

LOT	Lot description	CE	Storage temperature
IVD	In vitro diagnostic medical device		Unopened usable until

Modifications to the former version are marked in grey.

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 reagents can be kept unopened until the specified expiration date if stored at +2°C to +8°C. After opening for the first time, they must still be stored at +2°C to +8°C and protected against contamination. The following table lists the stability of the reagents after first opening. These storage life limits only apply, if the indicated storage life is not exceeded:

Reagents	**Stability**
Coated wells	4 months
Calibators	12 months
Controls	12 months
Enzyme conjugate	12 months
Sample buffer	12 months
Diluted wash buffer	4 weeks
Chromogen/substrate solution	12 months
Stop solution	12 months

- **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 individual 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).

- **Calibrator and controls:** Ready for use. The reagents must be mixed thoroughly before use.

- **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed 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 diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionised or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.

- **Chromogen/substrate solution:** Ready for use. Close the bottle 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 for use.

Storage and stability: The test kit has to be stored at a temperature between +2°C and +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

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.

Warning: The calibrator and controls of human origin have tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain sodium azide in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the patient samples

Samples: Human serum, EDTA or heparin plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

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 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.

Incubation

(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.
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.
Incubate **60 minutes at +37°C ± 1°C**.

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 and 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: Residual liquid (>10 µl) in the reagent wells after washing can interfere with the substrate and lead to false low extinction readings.
Insufficient washing (e.g., less than 3 wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction readings.
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 for **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), protect 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 test instruction. However, these conditions were validated in respect of the combination of the EUROIMMUN Analyzer I, Analyzer I-2P or the DSX from Dynex and this EUROIMMUN ELISA. Validation documents are available on enquiry.
Automated test performance using other fully automated, open-system analysis devices is possible. However, the combination should 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 above pipetting protocol is an example of the **semiquantitative analysis** of antibodies in 24 patient samples (P 1 to P 24).

Calibrator (C), positive (pos.) and negative (neg.) control as well as the patient samples have been incubated in one well each. The reliability of the ELISA test can be improved by duplicate determinations of 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 should be assayed with each test run.

Calculation of results

The extinction value 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. Use the following formula to calculate the ratio:

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

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.

A negative serological result does not exclude an infection. Particularly in the early phase of an infection, antibodies may not yet be present or are only present in such small quantities that they are not detectable. In case of a borderline result, a secure evaluation is not possible. If there is a clinical suspicion and a negative test 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 affect the diagnostic relevance of positive findings. Significant IgG titer increases (exceeding factor 2) and/or seroconversion in a follow-up sample taken after at least 7 to 10 days can indicate an acute infection. To investigate titer changes, sample and follow-up sample should be incubated in adjacent wells of the ELISA microplate within the same test run. For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Calibration: As no international reference serum exists for IgM class antibodies against chikungunya virus, results are provided in the form of ratios which are a relative measurement of the antibody concentration in serum or plasma.

For every group of tests performed, the extinction readings of the calibrator and the ratio values of 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.

The binding activity of the antibodies and the activity of the enzyme used are temperature-dependent. It is therefore recommended using a thermostat in all three incubation steps. The higher the room temperature (+18°C to +25°C) during the incubation steps, the greater will be the extinction. Corresponding variations apply also to the incubation times. However, the calibrator is subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The wells were coated with a recombinant structural protein of the chikungunya virus.

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable antibody titer. The lower detection limit of the Anti-Chikungunya Virus ELISA (IgM) is ratio 0.05.

Cross-reactivity: The quality of the antigen used ensures a high specificity of the ELISA. Sera from patients with infections caused by various agents were investigated with the Anti-Chikungunya Virus ELISA (IgM). It needs to be taken into consideration that strong cross-reactions within the Alphavirus genus cannot be ruled out. However, it must also be taken into account that double infections, particularly in endemic areas, or infections with another alphavirus at an earlier time are possible. In this case, positive results are not caused by a cross reactivity of the corresponding antibodies.

Antibodies against	n	Anti-Chikungunya Virus ELISA (IgM) positive
Barmah Forest virus	46	4.3%
Mayaro virus	2	100%
Ross River virus	60	45%

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

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 3 samples. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 3 determinations performed in 10 different test runs.

Intra-assay variation, n = 20		
Sample	**Mean value (Ratio)**	**CV (%)**
1	2.0	3.8
2	4.7	2.3
3	8.0	2.0

Inter-assay variation, n = 3 x 10		
Sample	**Mean value (Ratio)**	**CV (%)**
1	2.0	9.1
2	4.7	9.1
3	7.8	8.3

Sensitivity and specificity:
Study I: 351 precharacterised patient samples of different origins were investigated with the EUROIMMUN Anti-Chikungunya Virus ELISA (IgM). The sensitivity amounted to 99.2%, with a specificity of 98.2%. Borderline results were not included in the calculation.

n = 351		**Precharacterised**		
		positive	borderline	negative
EUROIMMUN Anti-Chikungunya Virus ELISA (IgM)	positive	234	0	2
	borderline	5	0	0
	negative	2	0	108

Study II: 219 precharacterised patient samples (origin: Europe; reference method: EUROIMMUN Anti-Chikungunya Virus IIFT (IgM)) were investigated with the EUROIMMUN Anti-Chikungunya Virus ELISA (IgM). The sensitivity amounted to 98.1%, with a specificity of 98.2%. Borderline results were not included in the calculation.

n = 219		**EUROIMMUN Anti-Chikungunya Virus IIFT (IgM)**		
		positive	borderline	negative
EUROIMMUN Anti-Chikungunya Virus ELISA (IgM)	positive	105	0	2
	borderline	2	0	0
	negative	2	0	108

The specificity of the Anti-Chikungunya Virus ELISA (IgM) was evaluated in a study performed on 391 patient sera which were seropositive for different pathogens, for rheumatoid factors or for diverse autoantibodies. Of the total of 391 samples, 3 samples were positive tested with the Anti-Chikungunya Virus ELISA (IgM). An overview of results can be found in the following table.
Since interference with samples from acute Plasmodium spp. infections cannot be excluded, malaria should be taken into consideration in differential diagnosis.

Possible influencing factors	n	Anti-Chikungunya Virus ELISA (IgM) positive
Borrelia	20	5.0% (1 positive)
CCP (rheumatoid arthritis)	16	0%
CMV	7	0%
Dengue virus	19	5.3% (1 positive)
Diverse autoantibodies	24	0%
EBV	22	0%
Hepatitis B virus	6	0%
Hepatitis C virus	6	0%
HSV-1/2	4	0%
Influenza virus	40	0%
Leptospira	17	0%
Measles virus	7	0%
Mumps virus	6	0%
Parvovirus B19	10	0%
Polio virus	12	0%
Rheumatoid facktor	37	2.7% (1 positive)
Rubella virus	10	0%
TBE virus	15	0%
Toxoplasma gondii	10	0%
Treponema	50	0%
VZV	5	0%
Yellow fever virus	12	0%
Zika virus	30	0%

Reference range: Levels of anti-chikungunya virus antibodies (IgM) were analysed in a panel of 498 healthy blood donors using the EUROIMMUN ELISA. With a cut-off ratio of 1.0, 0.8% of the blood donors were anti-chikungunya virus positive (IgM).

Clinical significance

Chikungunya virus (CHIKV) is an arbovirus of the genus *Alphavirus* belonging to the *Togaviridae* family [1]. According to its geographic distribution there are 4 subtypes: West African (WA), central, east and south African (ESCA), Indian Ocean (IOL) and Asian [2, 3, 4]. CHIKV is transmitted, for instance to humans, by mosquitoes of the genus *Aedes* [1, 3, 4, 5, 6].

Two transmission cycles have been described for CHIKV. The “sylvatic” cycle encompasses several mosquito species as vectors (e.g. *A. furcifer, A. vittatus, A. fulgens*) and non-human primates as reservoirs. The “urban” cycle is based on anthropophilic vectors such as *A. aegypti* and *A. albopictus*, which infect humans with CHIKV [1]. Humans are the link between both transmission cycles. CHIKV can be transmitted via blood transfusions [5] and vertically [7]. Human-to-human transmission has not yet been observed.

The first cases of chikungunya fever caused by CHIKV occurred in 1952/3 in Tanzania [1, 3, 8]. Shortly after, CHIKV outbreaks were reported in Asia. CHIKV has spread rapidly with a series of epidemics and can be found today on all five continents [1, 3, 4, 8].

Chikungunya fever cannot be distinguished clinically from, for instance, dengue, Mayaro and Zika fever [1, 3]. The disease lasts for approximately 10 days and generally takes a mild course [1]. Beside rapidly rising high fever, the symptoms include headache, muscle pains, skin rash, retinitis and arthralgia. The joint pains can persist for several months or years [1, 2, 3, 5, 6, 8]. Complications or comorbidities can occur in some cases, for example, hepatitis, nephritis, myocarditis, arthritis and meningitis/encephalitis [1, 8]. Less than 15% of infections are asymptomatic [8].

Diagnosis of CHIKV infection is based on clinical and epidemiological criteria as well as laboratory findings. Direct detection of the virus is generally possible for one week after onset of symptoms. CHIKV-specific IgM antibodies can be detected a few days after onset of symptoms; anti-CHIKV IgG is detectable around 2 days later. Seroconversion or a significant titer increase in a follow-up sample taken at least 7 to 10 days later also indicates an acute infection. Anti-CHIKV IgG can persist for many years [1, 6, 8].

Possible cross reactions with antibodies against other alphaviruses, such as Mayaro, Ross River, Barmah Forest and equine encephalitis viruses should be taken into account when interpreting findings [9].

There is no specific antiviral medication for the treatment of chikungunya fever [1, 3, 8]. Protection from mosquito bites serves as a preventative measure. Vaccination is not yet available.

Literature

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