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 a semiquantitative in vitro assay for human antibodies of the immunoglobulin class IgM against chikungunya virus (CHIKV) in serum or plasma for the diagnosis of chikungunya fever, and differential diagnosis of haemorrhagic fever.

Application: Suspected cases of chikungunya infections are confirmed with laboratory diagnostic means by a positive anti-chikungunya virus IgM result or by a 4-fold titer increase of specific IgG in 2 samples, alternatively by a positive PCR result. Since specific antibodies against chikungunya virus (IgG, IgM) are detectable at the earliest 6 to 8 days after onset of first symptoms, the PCR detection is essential for the diagnosis of acute infections. For the screening of blood reserves, the detection of viral antigens or virus-specific IgM antibodies is of great significance.

Principle of the test: The test kit contains microtiter 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		Storage temperature
IVD	In vitro diagnostic medical device	CE	Unopened usable until

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. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.
The thermostat adjusted ELISA incubator must be set at 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 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 bags).
  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. 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.
  The working strength wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.

- **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 to +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 the agent 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 IgM-negative 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 instrument manufacturer's recommendations with regard to microwell plate sealing.
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 values.
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 values.
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 value of the control or patient sample over the extinction value of 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 to retest 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 titer increases (exceeding factor 2) and/or seroconversion in a follow-up sample taken after 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 values 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 values. 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).

Antibodies against	n	Anti-Chikungunya Virus ELISA (IgM) positive
Borrelia	10	0%
CMV	7	0%
EBV-CA	16	0%
HSV-1/2	4	0%
Measles virus	7	0%
Mumps virus	6	0%
Parvovirus B19	10	0%
Rubella virus	10	0%
TBE virus	10	0%
Toxoplasma gondii	10	0%
VZV	5	0%

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 sera. 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		
Serum	**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		
Serum	**Mean value (Ratio)**	**CV (%)**
1	2.0	9.1
2	4.7	9.1
3	7.8	8.3

Sensitivity and specificity:
19 patient samples pre-characterised as Anti-Chikungunya virus IgM positive (origin: Europe) were investigated with the EUROIMMUN Anti-Chikungunya Virus ELISA (IgM). In comparison to the other ELISA, the sensitivity amounted to 100%, with respect to the positive pre-characterisation. Borderline results were not included in the calculation.

n = 19		**ELISA of another manufacturer**		
		positive	borderline	negative
Anti-Chikungunya Virus ELISA (IgM)	positive	17	1	1
	borderline	0	0	0
	negative	0	0	0

145 pre-characterised 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 98.9%. Borderline results were not included in the calculation.

n = 145		**Anti-Chikungunya Virus IIFT (IgM)**		
		positive	borderline	negative
Anti-Chikungunya Virus ELISA (IgM)	positive	51	1	1
	borderline	1	0	0
	negative	1	0	90

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

Clinical significance

The chikungunya virus is the pathogenic agent of chikungunya fever. This infectious tropical disease is characterised by fever and joint pain. It is transmitted by mosquitoes of the genus Aedes aegypti (Yellow fever mosquito) and Aedes albopictus (Asian tiger mosquito newly termed Stegomyia albopicta) that are active day and night. Congenital or transplacental transmission is also possible. The incubation time is 1 to 2 days, on average 2 to 3 days. Asymptomatic infections have also been described.

The chikungunya virus is a small enveloped single-stranded RNA virus belonging to the genus alphavirus from the togaviridae family and to the group of arboviruses. It is sensitive to heat (over 58°C), dehydration, soap and disinfectants.

Potential transmission cycles (human to human = urban cycle, or animal to human = sylvatic cycle) and clinical symptoms partially resemble those of dengue fever and yellow fever. The chikungunya virus is closely related to the O'nyong-nyong virus, the causative agent of O'nyong-nyong fever. So far, reservoir hosts have been monkeys and rodents.

Chikungunya fever was first reported in 1952/1953 during an epidemic in the Makonde plateau, which is the border region between Tanzania and Mozambique, east Africa. In the Makonde language the term chikungunya stands for "crookedly walking patient" due to its main symptom of severe joint and muscle pains accompanied by a high sensitivity to touch in the whole body (70 to 99% of cases). In addition to the generally rapidly rising high fever (38.5 to 40°C), chikungunya virus infections are characterised by lymph node swelling, mainly – rash with little or moderate itching interrupted by individual areas of normal skin (approx. 50%), punctual bleeding of the skin (petechia), milder forms of mucosa bleeding, e.g. of the nose or gums (approx. 25%), headache, fatigue and ophthalmitis (mainly in the form of conjunctiva infections).

Chikungunya fever subsides after around 10 days, generally without any lasting damage. Approx. 10% of patients experience joint pains which persist for more than 3 weeks or even months and years. In some cases, accompanying hepatitis, encephalopathy or meningo-encephalitis can occur. Chikungunya virus infections lead to life-long immunity.

According to its varying geographic distribution, the virus is divided into five subspecies which can be clearly genetically differentiated: the west African, central African, east and south African, Indian Ocean and Asian subtypes. To date, the chikungunya fever has spread dramatically worldwide and in several epidemics. It is suspected that the epidemic will spread further to Europe because today Aedes albopictus is already established in 12 European countries, which are Albania, Belgium, Bosnia-Herzegovina, Italy, France, Greece, Croatia, Montenegro, Netherlands, Switzerland, Slovenia and Spain. In summer 2007, Italy experienced an outbreak of chikungunya fever. In the same year, over 50 cases of chikungunya were diagnosed in Germany in travellers from endemic regions, with continuously increasing tendency. In 2007, eggs of the Asian tiger mosquito were first discovered in Baden-Württemberg. In 2011 and 2013, scientists caught living mosquitoes of this species in southern Germany.

The virus can only be detected during the viremic phase between the first and fourth day of the infection using RT-PCR or virus cultivation. The detection of chikungunya virus-specific IgM and IgG antibodies can be performed using HIT, NT, ELISA or IFA from the 8th to the 10th day of infection. Antibody investigation should be performed using serum samples from the acute and convalescent stage with a time period of at least 2 weeks between blood withdrawals.

There is no specific antiviral available for the therapy of chikungunya fever. Patients with neurological or liver diseases should be treated in intensive care. Other treatment options purely help to manage the symptoms. With no vaccine against chikungunya fever available, individual prevention of mosquito bites is of highest importance.

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