Anti-Zika Virus ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2668-9601 G	Zika virus	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human antibodies of the immunoglobulin class IgG against Zika virus in serum or plasma for the diagnosis of Zika virus.

Application: The Anti-Zika Virus ELISA (IgG, IgM) is suitable for the serodiagnosis of acute and past Zika virus infections. Due to the use of virus-specific NS1 antigen, cross reactions can be virtually excluded. Thus, Zika virus infections can be discriminated from infections with other viruses such as dengue and chikungunya, which cause similar symptoms and are endemic in the same regions. The detection of virus-specific IgM antibodies or a significant increase in the IgG titer in a follow-up sample indicates an acute infection. Further, the determination of specific antibodies is relevant for epidemiological studies and for clarification of possible links between Zika virus infection and other diseases.

Principle of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with recombinant non-structural protein (NS1) of Zika virus. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgG (also IgA and IgM) antibodies will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (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 1 200 RU/ml (IgG, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2 20 RU/ml (IgG, human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 2 RU/ml, (IgG, human), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
8. Sample buffer ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
9. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
10. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
11. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
12. Protective foil	---	2 pieces	FOIL
13. Test instruction	---	1 booklet	
14. 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 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.

- **Calibrators 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.

- **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 calibrators 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 or 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.

Sample dilution: Patient samples are diluted **1:101** in sample buffer.
For example: dilute 10 µl sample in 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: The calibrators and controls are prediluted and ready for use, do not dilute them.

Incubation

For **semiquantitative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1, 2 and 3** along with the positive and negative controls and patient samples.

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 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 IgG) 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) (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.

Pipetting protocol

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

The pipetting protocol for microtiter strips 1 to 4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7 to 10 is an example for the **quantitative analysis** of 24 patient samples (P 1 to P 24).

The calibrators (C 1 to C 3), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test 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 should be assayed with each test run.

Calculation of results

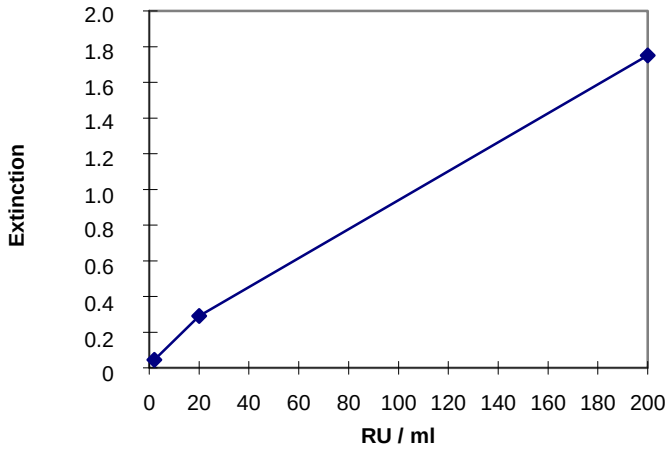
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2. Calculate the ratio according to the following formula:

$$\frac{\textbf{Extinction of the control or patient sample}}{\textbf{Extinction of calibrator 2}} = \textbf{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

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

Quantitative: The standard curve from which the concentration of antibodies in the patient samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 3 calibration sera against the corresponding units (linear/linear). Use “point-to-point” plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.



If the extinction for a patient sample lies above the value of calibrator 1 (200 RU/ml), the result should be reported as ">200 RU/ml". It is recommended that the sample be re-tested in a new test run at a dilution of e.g. 1:400. The result in RU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4.

The upper limit of the normal range of non-infected persons (**cut-off value**) recommended by EUROIMMUN is **20 relative units (RU)/ml**. EUROIMMUN recommends interpreting results as follows:

<16 RU/ml:	**negative**
≥16 to <22 RU/ml:	**borderline**
≥22 RU/ml:	**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 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 quantificated international reference serum exists for antibodies against Zika virus, the calibration is performed in relative units (RU).

For every group of tests performed, the extinction values of the calibrators and the relative units and/or 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.

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 calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The reagent wells are coated with recombinant non-structural protein (NS1) of Zika virus.

Linearity: The linearity of the Anti-Zika Virus ELISA (IgG) was determined by assaying 4 serial dilutions of different patient samples. The coefficient of determination R^2 for all sera was >0.95. The Anti-Zika Virus ELISA (IgG) is linear at least in the tested concentration range (2 RU/ml to 200 RU/ml).

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-Zika Virus ELISA (IgG) is 0.44 RU/ml.

Cross reactivity: Cross reactions can be almost entirely excluded due to the use of highly specific recombinant proteins.
Serum panels from clinically and serologically well characterised patients with high titers of antibodies of class IgG and/or IgM against flavivirus and chikungungya virus were analysed. Only a patient with acute JEV infection showed a positive result with the Anti-Zika Virus ELISA (IgG).
Double infection, particularly in endemic areas, and past infection with a different flavivirus should be taken into account. In this case, positive findings do not result from a cross reactivity of the respective antibodies.

Antibodies against	**n**	**Anti-Zika Virus ELISA (IgG) positive**
Chikungunya virus	19	0%
Dengue virus	38	0%
TBE virus	15	0%
Yellow fever virus	12	0%
Japanese encephalitis virus (JEV)	25	4%
West Nile Virus	34	0%

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 6 samples. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 2 determinations performed in 10 different test runs.

Intra-assay variation, n = 20		
Sample	**Mean value (RU/ml)**	**CV (%)**
1	5.6	5.7
2	11.3	3.5
3	12.1	3.2
4	13.7	3.5
5	47.6	6.2
6	73.9	5.4

Inter-assay variation, n = 2 x 10		
Sample	**Mean value (RU/ml)**	**CV (%)**
1	6.3	11.6
2	12.3	5.6
3	13.7	4.7
4	15.8	5.5
5	51.5	10.6
6	74.1	9.7

Sensitivity and specificity:
115 samples were analysed using the Anti-Zika Virus ELISA (IgG). 15 samples were from patients infected with Zika virus according to tests performed by the BNI (Bernhard-Nocht-Institute, Hamburg). Serological and clinical data are available. The negative reference panel comprised sera from 100 healthy pregnant women who supposedly had never been in contact with Zika virus. Two of the patient samples showed a borderline result when tested for IgG antibodies. At the same time they were highly positive for IgM antibodies, which allowed a clear serological identification. The sensitivity amounted to 100%, with a specificity of 100%. Borderline results were not included in the calculation.

n = 115		Clinic		
		positive	borderline	negative
EUROIMMUN Anti-Zika Virus ELISA (IgG)	positive	13	0	0
	borderline	2	0	0
	negative	0	0	100

The specificity of the Anti-Zika Virus ELISA (IgG) was evaluated in a study performed on 72 patient sera which were seropositive for rheumatoid factors and diverse antibodies (ANA). 22 other samples originated from patients with acute EBV infection. None of the in total 94 samples tested positive using the Anti-Zika Virus ELISA (IgG). An overview of results can be found in the following table.

Possible influencing factors	n	Anti-Zika Virus ELISA (IgG) positive
Acute EBV infection	22	0%
Diverse antibodies (ANA)	35	0%
Rheumatoid factor	37	0%

Reference range: The levels of anti-Zika virus antibodies (IgG) were analysed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors. With a cut-off of 20 RU/ml, 0.2% of the blood donors were anti-Zika virus positive (IgG).

Clinical significance

Zika virus (ZIKV) is an arbovirus in the flaviviridae family. The virus is usually not transmitted between humans, although sexual transmission has been reported. Vectors are mosquitoes of the genus Aedes, which transfer the pathogen via a bite. Non-human primates are the virus reservoir.

First reported in African countries, major outbreaks in the last years occurred in tropical and subtropical regions in Asia and on Pacific islands. Most recently a rising number of infections was found in Brazil.

In most cases Zika virus infection is mild with symptoms almost identical to dengue or chikungunya fever. After an incubation time of 5 to 10 days a flu-like illness develops, with fever, rash, arthralgia, myalgia, headache and conjunctivitis.

There is no specific treatment for ZIKV infections. An option for prophylaxis is protection from mosquito bites and there is no vaccination available.

Infections with flaviviruses, including ZIKV, always lead to viraemia. Since direct detection of virus or virus components is only possible during viraemia (maximum 1 week), and the relatively mild course of disease may lead to a late presentation of patients to a physician, serology has a high diagnostic significance. Specific antibodies can be clearly detected a few days after the onset of symptoms. The detection of specific IgM antibodies or a significant titer increase of specific IgG is proof of an acute infection with ZIKV.

Possible cross reactions with antibodies against other flaviviruses, such as dengue, West Nile, yellow fever or TBE virus, following infection or vaccination should be taken into consideration when interpreting results. According to studies, these cross reactions are virtually excluded owing to the usage of the highly specific NS1 antigen.

Reference list

1. Weaver SC, Reisen WK. **Present and Future Arboviral Threats.** Antiviral Res 85 (2010) 1-36.
2. Dick GW, Kitchen SF, Haddow AJ. **Zika virus isolations and serological specificity.** Trans R Soc Trop Med Hyg 46 (1952) 509-20.
3. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, Stanfield SM, Duffy MR. **Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007.** Emerg Infect Dis. 14 (2008) 1232-9.
4. Tappe D, Rissland J, Gabriel M, Emmerich P, Günther S, Held G, Smola S, Schmidt-Chanasit J. **First case of laboratory-confirmed Zika virus infection imported into Europe, November 2013.** Euro Surveill. 19 (2014) pii=20685.
5. Zammarchi L, Tappe D, Fortuna C, Remoli ME, Günther S, Venturi G, Bartoloni A, Schmidt-Chanasit J. **Zika virus infection in a traveller returning to Europe from Brazil, March 2015.** Euro Surveill. 20 (2015) pii: 21153.