Anti-Parvovirus B19 ELISA (IgM)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2580-9601 M	Parvovirus B19	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 parvoviruses B19 in serum or plasma to support the diagnosis of erythema infectiosum. Synonyms: megaloerythema, Sticker's disease, fifth disease.

Application: The determination of anti-parvovirus B19 antibodies of classes IgG and IgM, e.g. using ELISA, is after direct virus detection the most important method for diagnosis of a parvovirus B19 infection. Virus-specific IgM antibodies occur at the earliest around ten days after contact with the virus and often drop below the detection limit after a few weeks. A positive IgM result together with evidence of IgG seroconversion and/or viral DNA is proof of an acute infection.

Principles of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with parvovirus antigens. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgM antibodies (also IgA and IgG) 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. Test instruction	---	1 booklet	
11. Quality control certificate	---	1 protocol	
12. Protective foil	---	2 pieces	FOIL

LOT Lot description

IVD In vitro diagnostic medical device

CE

Storage temperature

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. After first use, the reagents are stable until the indicated expiry date if stored at +2°C and +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.

- **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 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 or EDTA, heparin or citrate 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 for **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) remaining 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 minimizes 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 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 the quantitative measurement of antibodies against parvovirus B19, the calibration is performed in ratio.

For every group of tests performed, the extinction readings of the calibrator and ratios 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 antigen source is a recombinant viral structural protein expressed in eukaryotic cells.

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-Parvovirus B19 ELISA (IgM) is ratio 0.1.

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 EUROIMMUN Anti-Parvovirus B19 ELISA (IgM).

Antibodies against	n	Anti-Parvovirus B19 ELISA (IgM) positive
Borrelia burgdoferi	9	0%
CMV	8	0%
EBV-CA	8	0%
HSV-1/2	2	0%
Measles virus	10	0%
Mumps virus	7	0%
Rubella virus	10	0%
Toxoplasma gondii	9	0%
VZV	3	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 samples. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed in 6 different test runs.

Intra-assay variation, n = 20		
Sample	**Mean value (Ratio)**	**CV (%)**
1	1.9	5.2
2	1.7	5.4
3	1.6	6.0

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (Ratio)**	**CV (%)**
1	2.3	7.7
2	1.9	5.9
3	1.4	5.6

Sensitivity and specificity:
Study I: 68 pre-characterised patient samples (origin: Europe; reference method: commercially available ELISA of another manufacturer) were investigated with the EUROIMMUN Anti-Parvovirus B19 ELISA (IgM). The sensitivity amounted to 100%, with a specificity of 97.9%.

n = 68		**ELISA of another manufacturer**	
		positive	negative
EUROIMMUN Anti-Parvovirus B19 ELISA (IgM)	positive	20	1
	negative	0	47

Study II: 225 clinically pre-characterised patient samples (INSTAND, NEQAS, RfB and IfQ) were investigated with the EUROIMMUN Anti-Parvovirus B19 ELISA (IgM). The sensitivity amounted to 98.0%, with a specificity of 100%. Borderline results were not included in the calculation.

n = 225		**INSTAND/NEQAS/RfB/IfQ**		
		positive	borderline	negative
EUROIMMUN Anti-Parvovirus B19 ELISA (IgM)	positive	48	0	0
	borderline	0	0	3
	negative	1	0	173

Reference range: The levels of anti-parvovirus B19 antibodies (IgM) were analysed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors. With a cut-off of 1.0 ratio 1.0% of the blood donors were anti-parvovirus B19 positive (IgM).

Clinical significance

Parvovirus B19 is the smallest (“parvo”) known virus, with a genome length of 5000 to 5500 base pairs. It is a single-stranded DNA virus from the family of Parvoviridae and has a diameter of 21 to 23 nm. The virus consists of two viral structural protein types (major and minor structural protein species), which form an icosaedric capsid. Until the discovery of human bocavirus in 2005, parvovirus B19 was the only known strictly human pathogenic virus from the genus Erythroviruses. Its replication takes place predominantly in haematopoietic cells. Parvovirus was discovered in blood donors in 1974 by the Australian virologist Yvonne Cossart. It obtained its name from sample B19 in which it was found by coincidence. The virus has a low sequence variability. Up until now three different genotypes (genotypes 1-3) have been identified. Parvovirus B19 is characterised by a very high stability with regards to environmental factors and detergents. The virus attacks a receptor on erythrocytes, the globoside blood group P antigen.

B19 infections (fifth disease, erythema infectiosum, megaloerythema, Sticker's disease) occur world-wide, mainly in spring. They occur in local epidemics, especially in child day care centres, schools, families and hospitals. In central Europe they can be described as endemic.

Parvovirus B19 is transmitted by droplets, skin contact, via blood or blood products or diaplacentally. The incubation time is 4 to 14 days, occasionally 3 to 17 days. The virus can be detected in the serum of the infected person between the 3rd and 16th day after infection. When the exanthema appears, the patient is no longer infectious.

Typically headaches, itching, myalgia and fever occur in the prodrome phase. Fresh B19 infections (anti-B19 IgM) can occur in all age groups. Acute infections are found most frequently in 6 to 15-year-olds. The prevalence of antibodies against parvovirus B19 (anti-B19 IgG) increases with age. In Germany, this amounts to around 35% for 4 to 6-year-olds, 58% for 10 to 15-year-olds, 70% for 25 to 29-year-olds and 79% for 65 to 69 year olds.

In children parvovirus B19 causes fifth disease. The exanthema generally begins with an intense redness and swelling on the cheeks (butterfly form; "slapped cheek"). Individual large areas of bright red colour are found on the forehead and around the ears. The exanthema extends to the extensor side of the arms, as well as the buttocks and legs. The extremities are most severely affected; surfaces of the hands and feet can also be afflicted. The trunk is not greatly affected, and mucous membranes remain free from exanthema. The exanthema is characteristically garland-shaped or net-like. It lasts for 6 to 21 days and subsides with an undulating form. As well as exanthema, lymph node swelling and flu-like symptoms are frequently observed. Accompanying symptoms are occasionally pruritus, subfebrile temperature and arthralgia. Symmetrical arthritis of the small joints can occur as a complication in children. An acute B19 infection can also proceed with purpura Schoenlein-Henoch or trigger various diseases, such as pseudoappendicitis, coxitis, enteritis, myocarditis, neuropathy of the brachial plexus, and erythema nodosum.

In adults, the infection can trigger acral erythema and arthritis (acute symmetrical polyarthropathy), which is difficult to differentiate clinically from chronic polyarthritis. 17 to 33% of all heart muscle inflammation cases can be attributed to parvovirus B19.

Parvovirus B19 multiplies in erythroblastocytes, causing temporary anaemia. The infection can lead to complications and even death in immunocompromised patients. The condition "pure red cell aplasia" described in AIDS patients is caused by chronic B19 infection.

Diaplacental B19 infections during pregnancy can lead, via inhibition of foetal erythropoiesis, to anaemia, hypoxia and in extreme cases to hydrops fetalis (in around 12% of cases) and foetal death. Further symptoms are caused by hypoproteinemia: oedema, pericardial and pleural effusion, ascites.

Clinically, fifth disease is often difficult to distinguish from rubella. Therefore, clinicians often rely on serology (anti-B19 IgM/IgG). Particular in adults, fifth disease often proceeds with atypical exanthema.

Banked blood is currently not tested for B19 virus. Since B19-infected persons are mostly still asymptomatic in the viraemic stage, B19 virus infections via transfusion can occur. Tests in Germany and France showed a prevalence of B19 virus in banked blood of 0.01 to 0.03%. Since the detection of B19 antigen is time-consuming, high-risk patients should only be given blood that has tested positive for anti-B19 IgG. Anti-B19 IgG-positive blood no longer contains B19 virus.

Due to the differing manifestations of a B19 infection, it is necessary to confirm or exclude an acute B19 infection. The detection of B19 antigen or DNA (PCR) plays a secondary role in diagnosis, since patients in the viraemic stage of a B19 infection are mostly asymptomatic. Thus, the detection of B19-specific antibodies (anti-B19 IgG and IgM) is of particular significance. Diagnostics for a B19 infection is performed using ELISA or immunoblot, which selectively detect anti-B19 IgG or anti-B19 IgM using a viral structural protein as antigen.

The detection of anti-B19 IgM indicates a fresh B19 infection. Anti-B19 IgM can be detected from around 10 days up to 3 to 5 months after infection. Anti-B19 IgG appears at the end of the 3rd week after infection at the earliest and is assumed to persist lifelong. To narrow down the time of infection, the avidity of specific IgG antibodies (anti-B19 IgG avidity) is determined using microtiter ELISA. This method provides reliable results, in particular when anti-B19 IgM is absent. High avidity excludes infections within the last 4 to 6 weeks.

Therapeutic measures are limited to treating the symptoms. With hydrops fetalis an intrauterine exchange transfusion can substantially improve prognosis. A vaccine is being developed.

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