Anti-CMV ELISA (IgM)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2570-9601 M	Cytomegalovirus (CMV)	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 CMV in serum or plasma for the diagnosis of the infection with cytomegalovirus.

Application: Infections with cytomegaloviruses (CMV) can be diagnosed by the detection of specific antibodies of classes IgG and IgM. Antibodies of class IgM quite reliably indicate an acute infection. However, their detection in CMV cannot be used for differentiation from a primary infection of a reactivation as they may occur in both conditions. In cases of positive IgM findings, the avidity determination of pathogen-specific IgG antibodies is therefore a suitable method to differentiate between primary infection and reactivation.

Principle of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with CMV 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	

LOT Lot description
IVD In vitro diagnostic medical device
CE 0197
Storage temperature
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.

- **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 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 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 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. Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Manual: Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash.
Automatic: 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.

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 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 international reference serum exists for antibodies against CMV, results are provided in the form of ratios which are a relative measure for the concentration of antibodies in serum or plasma. The calibration is performed with internal reference sera, which were used for evaluation of the test system.

For every group of tests performed, the extinction values of the calibrator and the 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 control sera 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 provided by inactivated cell lysates of MRC-5 cells infected with the “AD169” strain of cytomegalovirus.

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-CMV 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-CMV ELISA (IgM). Cross reactivities to other herpes viruses cannot be excluded.

Antibodies against	n	Anti-CMV ELISA (IgM) positive
Borrelia	10	0%
EBV-CA	17	35.3%
Measles virus	7	0%
Mumps virus	6	0%
Parvovirus B19	10	0%
Rubella virus	10	0%
HSV Pool	4	0%
VZV	5	0%
Toxoplasma gondii	10	0%
TBE virus	10	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	2.4	6.1
2	2.9	11.3
3	5.2	7.0

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (Ratio)**	**CV (%)**
1	2.4	6.2
2	2.9	8.6
3	5.8	9.3

Sensitivity and specificity:
328 clinically pre-characterised patient samples (INSTAND, NEQAS, Labquality, MQ and RfB) were investigated with the EUROIMMUN Anti-CMV ELISA (IgM). The sensitivity amounted to 100%, with a specificity of 99.6%. Borderline results were not included in the calculation.

n = 328		**INSTAND/NEQAS/Labquality/MQ/RfB**		
		positive	borderline	negative
EUROIMMUN Anti-CMV ELISA (IgM)	positive	99	0	1
	borderline	1	0	3
	negative	0	0	224

Reference range: Levels of anti-CMV antibodies were investigated in a panel of healthy blood donors (n = 500) using the EUROIMMUN ELISA. With a cut-off ratio of 1, 0.6% of the blood donors were anti-CMV positive (IgM).

Clinical significance

Cytomegalovirus (CMV) is a human pathogenic DNA virus from the herpes virus family. It is also known as human cytomegalovirus (HCMV) or human-pathogenic herpes virus 5 (HHV-5). CMV is the causative agent of cytomegaly.

The virus is transmitted by saliva, lachrymal fluid, urine, blood and blood components, genital secretion and breast milk. In one third of pregnant women with primary cytomegaly the foetus is also infected. The time between infection and the onset of first symptoms is two to six weeks. Like other herpes viruses, CMV persists in the body lifelong. The virus is secreted via the saliva and urine during acute infection and up to several weeks afterwards.

The majority of CMV infections in immunocompetent persons proceed inapparently or with only a few symptoms. Main signs are lymph node swelling and fever. Occasionally, headache and joint pains additionally occur. In severe cases almost any organ can be affected, in particular the liver (hepatitis) and lungs (pneumonia), accompanied by long lasting fever. Accompanying diseases of the coronary blood vessels may be the cause of heart failure at a later period. Ulceration of the mucosa to haemorrhagic necrosis can occasionally be found. Gastrointestinal symptoms of CMV infection include difficulties in swallowing, a burning sensation behind the breastbone or symptoms similar to those known from stomach and duodenal ulcers. CMV retinitis, an inflammation of the retina, is most feared.

Cytomegaly proceeds asymptomatically in 90% of children and infants. CMV is the most common agent of prenatal infection in the newborn. It is transmitted transplacentally by blood contact with the infected mother, with varied outcomes depending on the strength of maternal humoral immunity and the gestational stage. Miscarriages and deformities are found. Around 5% of congenitally infected newborns show severe symptoms, particularly damage to the liver, spleen and the central nervous system. The most frequent consequences are hearing loss (approx. 10%), loss of vision, mental retardation and growth disorders. CMV infection is fatal in around 30% of infected newborns and infants. Approximately 1% of fatal cases were infected in utero. Postnatal transmission of CMV via breast milk is possible. CMV-positive mothers of premature infants should therefore have their breast milk tested and, in case of a positive CMV result, pasteurise the milk before feeding. CMV infection in pregnancy is notifiable in many countries.

It is also possible that CMV is transmitted by a transplanted infected organ or that a latent infection is reactivated during immunosuppressive therapy after transplantation. The new organ is often rejected in both cases.

Due to the uncharacteristic symptoms of CMV infection, which can sometimes be found in a similar form in other viral, bacterial or fungal infections, clinical diagnosis is very difficult. The seroprevalence (CMV IgG) depends on such factors as the country, the environment and the age. It is reported to be around 60% in industrialised countries and over 90% both in developing countries and in elderly people of over 80 years old. In Germany, approximately 40% of women of child-bearing age are seropositive for CMV.

Antibodies against CMV can be detected in the serum of nearly all patients with past infections. Latent CMV can be reactivated when the immunological defence mechanisms of the body are weakened.

In suspected active CMV infection in patients under immunosuppression (after organ or bone marrow transplantation and in HIV-positive persons) analysis of the pp65 antigen and/or determination of the viral load in blood using PCR are recommended.

Blood test results in CMV infections often show unspecific changes, such as increased transaminase levels, hyperbilirubinaemia or thrombocytopenia, which may indicate hepatitis. Tissue samples, on the other hand, show typical, so-called "owl's eye cells".

The determination of antibodies against CMV in serum, plasma and CSF is of particular diagnostic imortance. Various test systems (ELISA, IIFT, immunoblot) are available for the detection of specific antibodies. The main problem in CMV diagnostics is the differentiation between acute primary infection and past or recurrent infection, This is most relevant in prenatal care since primary CMV infection in the mother often results in foetal damage.

CMV-specific antibodies of class IgM are considered as a marker of acute infection. They can be detected in primary infection, but also in polyclonal stimulation of the immune system, e.g. in patients with acute Epstein-Barr virus infection, as well as in CMV reactivation. The antibodies may persist for a long time. For reliable differentiation between primary infection and past infection or reactivation, positive IgM results should be followed up by further serological tests. The determination of the avidity of pathogen-specific IgG antibodies using ELISA or IIFT has proved to be particularly useful. High-avidity IgG antibodies against CMV can be detected only after 20 weeks, which practically excludes acute primary infection. Moreover, stage-specific antibodies against certain CMV antigens, which are used as substrates in ELISA or immunoblot, can provide information on the presence of an early or late infection. IgM antibodies against p52 (ppUL44), for instance, are considered to be a marker of an early infection stage, whereas IgG antibodies against gB (glycoprotein B) indicate a late or past infection. In case of an isolated positive IgM result, seroconversion in a follow-up sample taken after a suitable time span is proof of a primary CMV infection. After successful seroconversion CMV-specific IgG antibodies are detectable lifelong. Furthermore, a high CMV DNA load in amniotic fluid (determined by PCR) and the detection of CMV IgM in foetal blood (using ELISA) could be an indicator of symptomatic congenital infection at a relatively early stage of pregnancy. This procedure is especially indicated in suspicious ultrasonic results, since the presence of specific IgM antibodies in foetal blood is significantly correlated with a severe outcome for the foetus or the newborn.

So far, there is no vaccine against CMV infection. In organ transplantation the situation has improved significantly due to effective medication (virostatics) for the prevention and treatment of CMV infection. These drugs, however, are unsuitable for pregnant women. For seronegative pregnant women, exposure prophylaxes by hygienic measures are, for the present, the only way of protection. Preventive measures include avoidance of regular occupational contact with children below the age of three for seronegative pregnant women. Pregnant women from risk occupational groups, who have close contact to known virus carriers, may be given CMV-specific hyperimmunoglobulin (CMVIG). Passive immunisation is also indicated for seronegative individuals under immunosuppressive therapy, such as tumour patients or transplant recipients. Such patients, but also infants and particularly newborns, should not receive blood products from CMV-antibody positive blood donors.

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