Anti-Rubella Virus Glycoprotein ELISA (IgM)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2590-9601-2 M	Rubella virus glycoprotein	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 class against Rubella virus glycoproteins in serum or plasma for the diagnosis of Rubella.

Application: Rubella infections can be diagnosed through the detection of specific antibodies of classes IgG and IgM. An increase of the IgG antibody titer within approximately ten days or the detection of antibodies of class IgM indicate an acute infection. A positive IgM test during pregnancy requires confirmation by means of other test methods (avidity determination of specific IgG antibodies, IgG immunoblot, if necessary, PCR or virus cultivation from chorion biopsy material or amniotic fluid, or investigation of foetal blood). By use of the anti-Rubella Virus Glycoprotein ELISA (IgM), the IgM diagnostic can be optimised, since unspecific reactions and cross reaction of antibodies against other infectious agents which sometimes occur in ELISAs based on virus lysate or in indirect immunofluorescence, can be minimised.

Principle of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with Rubella virus glycoproteins. 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, calibrator, 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 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** in 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. 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 and the Analyzer I-2P 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. Extinction values of patient samples above the extinction value of the calibrator are considered positive, extinction values 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 the calibrator. Use the following formula to calculate the ratio:

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

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 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 of IgM class antibodies against Rubella virus, results are provided in the form of ratio values 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 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 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: Highly purified glycoproteins from Rubella virus (strain HPV 77, cultivated in vero cells) is used as antigen.

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-Rubella Virus Glycoprotein ELISA (IgM) is ratio 0.04.

Cross reactivity: The quality of the antigen used ensures a high specificity of the ELISA. Sera from patients with acute infections caused by various agents were investigated with the Anti-Rubella Virus Glycoprotein ELISA (IgM).

Antibodies against	n	Anti-Rubella Virus Glycoprotein ELISA (IgM) positive
Toxoplasma	12	0%
CMV	16	12.5%
VZV	8	0%
HSV Pool	3	0%
Mumps virus	5	0%
Measles virus	11	0%
Borrelia	10	0%
EBV-CA	8	0%
Parvovirus B19	20	0%

Two of the 16 anti-CMV IgM positive samples were tested positive for IgM antibodies against Rubella glycoprotein. This reaction can presumably be attributed to a polyclonal B-cell stimulation, which has been described in detail in literature. Indications of cross-reactivity could not been determined.

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 on 6 different days.

Intra-assay variation, n = 20		
Sample	**Mean value (Ratio)**	**CV (%)**
1	2.2	5.9
2	2.8	6.8
3	10.2	3.6

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (Ratio)**	**CV (%)**
1	2.1	6.7
2	2.7	8.7
3	10.4	5.1

Specificity and sensitivity: 280 clinically characterised patient samples (INSTAND, NEQAS, Labquality, MQ and RfB) were examined with the EUROIMMUN Anti-Rubella Virus Glycoprotein ELISA (IgM). The test showed a specificity of 98.6% and sensitivity of 99.5%.

n = 280		**INSTAND/NEQAS/Labquality/MQ/RfB**		
		positive	borderline	negative
EUROIMMUN Anti-Rubella Virus-Glycoprotein ELISA (IgM)	positive	70	0	1
	borderline	0	0	0
	negative	1	0	208

To evaluate the specificity of the EUROIMMUN Anti-Rubella Virus Glycoprotein ELISA (IgM), a study was conducted with 125 patient sera which were seropositive for rheumatoid factor, different autoimmune diseases or hepatitis A or B. Of the 125 samples, 124 sera were tested negative with the EUROIMMUN Anti-Rubella Virus Glycoprotein ELISA (IgM). The specificity in this collective is therefore 99%.

Possible influencing factors	n	EUROIMMUN Anti-Rubella Virus Glycoprotein ELISA (IgM), positive
Various AAb	67	1.5%
HAMA	4	0%
Rheumatoid factor	39	0%
HAV + HBV	15	0%

Reference range: Levels of anti-Rubella virus glycoprotein antibodies were determined in a panel of healthy blood donors (n = 500, origin: Germany) using the EUROIMMUN ELISA. At a cut-off of 1.0, 1.6% of blood donors were anti-Rubella virus glycoprotein positive (IgM).

Clinical significance

The pathogenic agent of rubella is the Rubella virus, which is present worldwide. It is a positive single-stranded, enveloped RNA virus and the only species belonging to the genus Rubivirus of the Togaviridae family. The Rubella virus was first isolated in 1962 by Parkman, Weller and Neva. There are 2 genotypes, which are divided into further subgenotypes. The Rubella genotype I (RGI) occurs in the western hemisphere, whereas the Rubella genotype II (RGII) is mainly found in Asia.

A Rubella infection is transmitted by aerosols. It is considered contagious already during the incubation period of two to three weeks. Typical symptoms are headache, lymph node swellings, particularly in the neck area, and a blotchy exanthema, which generally persists for 3 days. This generalised, macular, not confluent, light red exanthema spreads from the face to the trunk and the extremities in a postauricular manner. A known complication is arthritis in the finger, hand, elbow and ankle joints, which may last for

up to three weeks in adults, especially in women. Further complications are myocarditis, neuritis, otitis, bronchitis and, very rarely, Rubella encephalitis with a good prognosis. The majority of infections occur between the ages of 5 to 14 years and lead to life-long immunity. In central Europe an infection spread of 80% to 90% is assumed, in the Near East, on the Arabian Peninsula and in the USA it amounts to approximately 91% to 93%. This means that 10% to 20% of women of child-bearing age are not immune to Rubella.

Rubella virus transmitted diaplacentally during the first trimester of pregnancy causes the highest rate of embryonic deformities. Severe forms of Rubella embryopathy are found in around 80% of cases. In the foreground are Gregg's Triad (first described in 1941 by the Australian eye specialist Gregg) consisting of heart deformations, eye defects and hearing damage such as congenital vitium cordis in around 48%, retinopathy in around 39%, cataract/myopia in around 29%, glaucoma in around 3% and deafness in around 67% of cases. Ideomuscular retardation (partly in combination with microcephalus) in around 45%, neonatal purpura with hepatosplenomegaly and diabetes mellitus in around 23% and death (incl. spontaneous abortion) in around 16% of patients are also known to occur. In many countries, an acute Rubella infection is considered to be a medical indication for termination of pregnancy.

In addition to the anamnesis and clinical analysis, laboratory diagnostic tests are of particular importance in the investigation of Rubella infections. They are indispensable with respect to the serological determination of the immune status in pregnant women in connection with a suspected Rubella embryopathy. The differentiation between acute and long-standing infections is one of the greatest challenges encountered in serology. Antibodies against Rubella virus structural proteins, mainly of the IgG class, can be found two to three days after the onset of the exanthema. Antibodies against the complete, intact Rubella virus only develop after 3 months to 1 year. Avidity determination of specific IgG antibodies contributes to the diagnosis of a fresh virus infection, particularly in IgM-negative individuals with fresh infections or patients showing persisting IgM. The ELISA avidity test is generally recommended due to its proven comparably high informative value and reliability.

A direct Rubella virus determination using PCR (polymerase chain reaction), such as in foetal blood, can only be performed in special laboratories.

The **HIT** (haemagglutination inhibition test, HAH test, HAI test) is used for the determination of the immunity status during early pregnancy. For the determination of a fresh infection two blood samples, one taken at the onset of the disease and the other 2 to 3 weeks later, are investigated. In case of a fresh infection the titer increases two to four times. If the result is borderline or negative, Rubella antibodies should be additionally determined using ELISA. It should be noted that in the HIT high titers without symptoms are evaluated as Rubella immunity, while low titers are considered to indicate insufficient immunity against Rubella reinfection following new contact with the virus. This interpretation is not reliable enough, since any titer in the HIT may indicate a fresh Rubella infection, as this test cannot distinguish IgG from IgM antibodies and a high HIT titer may result from IgM antibodies alone.

Alternatively, the Anti-Rubella Virus IgG ELISA and Anti-Rubella Virus IIFT are suitable for assessing immunity. An increase in antibody titer within 10 days or the detection of IgM antibodies indicates an acute infection. It must, however, be taken into consideration that anti-Rubella IgM antibodies may be present months after an infection.

Avidity determination of specific IgG antibodies provides reliable results for narrowing the period of infection, in particular the avidity test, the Anti-Rubella Virus **ELISA (IgG, avidity determination)** and the Anti-Rubella Virus **IIFT (IgG, avidity determination)**. High avidity excludes infections within the last 4 to 6 weeks. The Anti-Rubella Glycoprotein ELISA uses Rubella virus glycoproteins (rubella structure proteins) as antigens. In positive samples, the specific IgM (and IgA, IgG) antibodies will bind to the corresponding antigens and can already be determined 2 to 3 days after onset of the exanthema.

In encephalitis cases which are thought to be caused by Rubella virus, the presence of specific antibodies in the cerebrospinal fluid (CSF) should be investigated. For this therapy-relevant investigation the Anti-Rubella Virus **ELISA (IgG in CSF)**, which was developed especially for CSF diagnostics, can be used. In Rubella encephalitis agent-specific antibodies of class IgG are produced in CSF. The intrathecal agent-specific antibody production is defined by the relative CSF/serum quotient $CSQ_{rel.}$ (synonym: antibody specificity index). The quotient is calculated from the ratio of agent-specific antibodies to total IgG in CSF compared to the ratio of agent-specific antibodies to total IgG in serum.

The Anti-Rubella Virus **Westernblot** serves for the determination of IgG antibodies against Rubella virus. It should be used for the clarification of problematic Rubella IgM results. IgG conformation-specific

antibodies against the Rubella antigen E2 occur at the earliest 3 months after vaccination or recovery. If the E2 band is visible an infection within the last three months can be considered as unlikely.

Various inoculation strategies have been employed worldwide to prevent Rubella infections. Since active immunisation is well tolerated, vaccination programs aim to protect all young persons before puberty using a two-stage Rubella vaccination.

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