Avidity determination of IgG antibodies against Rubella viruses
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EI 2590-9601-1 G	Rubella viruses	IgG	Ag-coated microplate wells	96 x 01 (96)

Intended Use: This test kit is intended for the avidity determination of IgG class antobodies against Rubella virus in human serum or plasma.

Background

The differentiation between fresh and long-standing infections is one of the greatest challenges in serology. Until now this was based mainly on determination of specific antibodies of the immunoglobulin class IgM, which generally only appear initially. However, the detection of these antibodies is often unreliable and problematic due to interfering factors such as persistence of the IgM response, too weak or delayed IgM production, and unspecific IgM production through polyclonal B-cell stimulation.

In recent years additional determination of the antibody avidity has become an established method for identifcation of primary infections. The immune system reacts to an infection by first forming low-avidity antibodies. With continued disease duration, IgG that are more precisely adapted to the antigens are produced – the avidity increases. If high-avidity IgG are detectable in the serum, it can be assumed that the infection is at a late stage.

Contents of the test system: EI 2590-9601-1 G

Component	Colour	Format	Symbol
1. Test kit Anti-Rubella ELISA (IgG, order number EI 2590-9601 G)	---		
2. Positive control HA High-avidity anti-Rubella (IgG, human), ready for use	red	1 x 1.3 ml	POS CONTROL HA
3. Positive control LA Low-avidity anti-Rubella (IgG, human), ready for use	blue	1 x 1.3 ml	POS CONTROL LA
4. Urea solution for Anti-Rubella ELISA, ready for use	yellow	1 x 12 ml	UREA
5. Phosphate buffer ready for use	light blue	1 x 12 ml	PBS BUFFER
6. Test instruction	---	1 booklet	
LOT Lot description IVD In vitro diagnostics	CE 0197	Storage temperature Unopened usable until	

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.

Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°Cto +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.

- **Controls:** Ready for use. The reagents must be mixed thoroughly before use.
- **Urea solution:** Ready for use.
- **Phosphate buffer:** Ready for use.

Warning: The calibrators and controls used have been tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2 using enzyme immunoassays and indirect immunofluorescence methods. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the toxic agent sodium azide. Avoid skin contact.

Preparation and stability of the patient samples

Sample material: 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.

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

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

Incubation

Sample incubation: (1. step)
Transfer 100 µl 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).

Wash:
Manual: Empty the wells and subsequently wash **1 time** using 300 µl of working strength wash buffer.
Automatic: Wash reagent wells **1 time** with 450 µl of working strength wash buffer (program setting: e.g. TECAN Columbus Washer “Overflow Modus”).

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. 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.

Urea incubation: (2. step)
Pipette 200 µl of urea solution into each of the microplate wells of the first microtiter strip and 200 µl of phosphate buffer into each of the microplate wells of the second microtiter strip. Incubate for **10 minutes** at room temperature (+18 °C to 25 °C).

Wash:
Empty the wells. Wash as described above, but wash **3 times** using working strength wash buffer for each wash.

Attention: 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 reaction times) can lead to false high extinction values.

Conjugate incubation: (3. step)
Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgG) into each of the microplate wells. Incubate for **30 minutes** at room temperature.

Wash:
Empty the wells. Wash as described above, but wash **3 times** using working strength wash buffer for each wash.

Substrate incubation: (4. 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 the reaction:
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	**pos HA**	**pos HA**	**P 7**	**P 7**	**P 15**	**P 15**						
B	**pos LA**	**pos LA**	**P 8**	**P 8**	**P 16**	**P 16**						
C	**P 1**	**P 1**	**P 9**	**P 9**	**P 17**	**P 17**						
D	**P 2**	**P 2**	**P 10**	**P 10**	**P 18**	**P 18**						
E	**P 3**	**P 3**	**P 11**	**P 11**								
F	**P 4**	**P 4**	**P 12**	**P 12**								
G	**P 5**	**P 5**	**P 13**	**P 13**								
H	**P 6**	**P 6**	**P 14**	**P 14**								

The above pipetting protocol is an example of the avidity determination of IgG antibodies in 18 patient samples (P 1 to P 18).

Controls (pos HA and pos LA) as well as the patient samples have been incubated in duplicate in one well each of two different microtiter strips. The reagent wells of the microtiter strips 1, 3, 5 etc. are treated with urea solution after the incubation with patients samples, the reagent wells of the microtiter strips 2, 4, 6 etc. are treated with phosphate buffer.
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 controls with high-avidity and low-avidity antibodies serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

The presence of low avidity antibodies in a patient's serum has been proved if the ELISA extinction value is considerably reduced by urea treatment. For an objective interpretation the relative avidity index (RAI) is calculated and expressed in percent using the extinction values with and without urea treatment.

$$\frac{\textbf{Extinction of the sample with urea treatment x 100}}{\textbf{Extinction of the sample without urea treatment}} = \textbf{relative avidity index (RAI) in \%}$$

The upper limit of the range of low-avidity antibodies (**cut-off value**) recommended by EUROIMMUN is 40% RAI. Values below the indicated cut-off are to be considered as an indication of low-avidity antibodies, values between 40% and 60% RAI as equivocal, values above 60% as an indication of high-avidity antibodies. If a result is classified as equivocal, it is recommended to collect a second sample not less than 7 days later and to test it together with the first sample.

RAI < 40%:	Indication of low-avidity antibodies
RAI 40% - 60%:	Equivocal range
RAI > 60%:	Indication of high-avidity antibodies

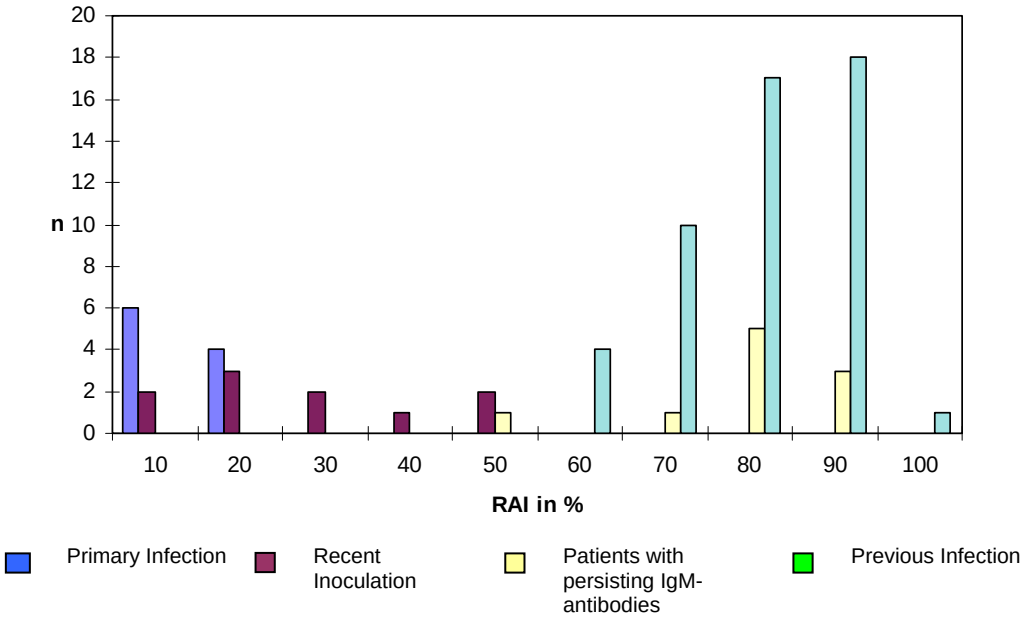
Reliable results in the the measurement of IgG antibody avidity can only be yielded if the patient sample contains a diagnostically significant concentration of specific antibodies. Generally, the determination of the relative avidity index is not helpful in samples which have an O.D. of <0.140 after incubation without urea treatment.

For diagnosis, the clinical symptoms of the patient should always be taken into account alongside the serological results.

Attention:
In some patients with an acute infection, very high titers of IgG antibodies can be found. Even though the specific IgG antibody population is in different maturation stages, both high-avidity and low-avidity, the vacant antigen epitopes are predominantly occupied by high-avidity antibodies in high titer samples. The determination of the avidity of the whole specific IgG antibody population can lead to false high RAI values in results.
False high RAI values were found in some cases of acute infections when the extinction value of the IgG measurement without urea treatment was >1.200.
It is recommended for samples with extinction values of >1.200 to repeat the avidity determination with a higher sample dilution (e. g. 1:401). If low avidity of IgG antibodies is already found at extinction values of >1.200, no further testing is necessary.

Clinical significance

1. In a study performed at EUROIMMUN 10 sera of patients with a recent infection (RAI 5% to 18%, mean 9.0%) as well as 10 blood donors with a recent inoculation (RAI 6% to 43%, mean 22.9%) showed a relative avidity index below 50%. In each of the 10 serum samples of patients with persisiting IgM antibodies (48% to 83%, mean 73.6%) as well as in 50 serum samples of patients with previous infection (55% to 95%, mean 75.4%) the RAI was significantly higher.



2. In an external study carried out by the Department of Medical Microbiology and Infectious Diseases at the University of Manitoba, Winnipeg and by the National Microbiology Laboratory at the Public Health Agency of Canada, the EUROIMMUN ELISA for the determination of the avidity of antibodies against Rubella virus was compared together with four other commercial tests with a well-established in-house reference test. A panel of n = 66 characterised serum samples was investigated. In this study, the present test showed an excellent correlation with the reference test and proved to be the best ELISA in comparison with the commercial tests of the other manufacturers.

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 [1]. 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 [2].
A rubella infection is transmitted by aerosols. It is considered contagious already during the incubation period of two to three weeks [1]. 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 [1, 3]. The majority of infections occur between the ages of 5 to 14 years and lead to life-long immunity [1, 4]. 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% [5, 6, 7, 8, 9]. This means that 10% to 20% of women of child-bearing age are not immune to rubella [9].

Rubella virus transmitted diaplacentally during the first trimester of pregnancy causes the highest rate of embryonic deformities [10]. 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 [11]. 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 [10, 12]. 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 [10, 12]. The differentiation between acute and long-standing infections is one of the greatest challenges encountered in serology [13, 14]. Antibodies against rubella virus structural proteins, mainly of the IgG class, can be found two to three days after the onset of the exanthema [15, 16, 17]. Antibodies against the complete, intact rubella virus only develop after 3 months to 1 year [1]. 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 [14, 15, 17, 18, 19, 20, 21, 22, 23]. The ELISA avidity test is generally recommended due to its proven comparably high informative value and reliability [24].

A direct rubella virus determination using PCR (polymerase chain reaction), such as in foetal blood, can only be performed in special laboratories [10, 12].

The **HIT** (haemagglutination inhibition test, HAH test, HAI test,) is used for the determination of the immunity status during early pregnancy [24, 26]. 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 [18]. 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 [25].

Alternatively the Anti-Rubella Virus IgG ELISA and Anti-Rubella Virus IIFT are suitable for assessing immunity [21, 30, 31]. An increase in antibody titer within 10 days or the detection of IgM antibodies indicates an acute infection [21, 23]. It must, however, be taken into consideration that anti-rubella IgM antibodies may be present months after an infection [21].

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)** [15, 17, 18, 19, 22, 23, 25]. 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 [16]. 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 [15, 22, 23].

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 [3]. For this therapy-relevant investigation the Anti-Rubella Virus **ELISA (IgG in CSF)**, which was developed especially for CSF diagnostics, can be used [4, 27, 28]. In rubella encephalitis agent-specific antibodies of class IgG are produced in CSF [29]. 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 [1, 17, 18, 19, 21, 25, 27, 28].

The Anti-Rubella Virus **Westernblot** serves for the determination of IgG antibodies against rubella virus [36]. It should be used for the clarification of problematic rubella IgM results [36]. For the confirmation of the test result, avidity can be determined in addition [25, 34, 35]. 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 [16, 17, 36].

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. In the case of a negative result, unknown status, or detection of specific IgM, passive immunisation in the early stages of pregnancy is possible, although only within 7 days of exposure or 2 to 3 days after onset of the exanthema [1, 10, 35].

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