Avidity determination of antibodies against measles viruses (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2610-9601-1 G	Measles virus	IgG	Ag-coated microplate wells	96 x 01 (96)

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 identification 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 2610-9601-1 G:

Component	Colour	Format	Symbol
1. Test kit Anti-Measles Virus ELISA (IgG, order number EI 2610-9601 G)	---	---	---
2. Positive control HA High-avidity anti-measles (IgG, human), ready for use	red	1 x 1.3 ml	POS CONTROL HA
3. Positive control LA Low-avidity anti-measles (IgG, human), ready for use	blue	1 x 1.3 ml	POS CONTROL LA
4. Urea solution for Anti-Measles Virus 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		Storage temperature
IVD	In vitro diagnostic medical device	CE	Unopened usable until

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, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

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 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. The urea solution included in this test system may only be used for the avidity determination of antibodies against measles.

- **Phosphate buffer:** Ready for use.

Warning: The 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

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 must be incubated within one working day.

Sample dilution: Patient samples to be investigated are diluted **1:101** with sample buffer.
Example: Add 10 µl of sample 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: (1st step)
Transfer 100 µl of the 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 **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 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.
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: (2nd 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).

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

Note: Residual liquid (> 10 µl) 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.

Conjugate incubation: (3rd 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 (+18°C to +25°C).

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

Substrate incubation: (4th 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, carefully 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 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 minimises 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 is considerably reduced by urea treatment. For an objective interpretation the relative avidity index (RAI) is calculated and expressed in percent using the extinction with and without urea treatment.

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

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% RAI 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
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.

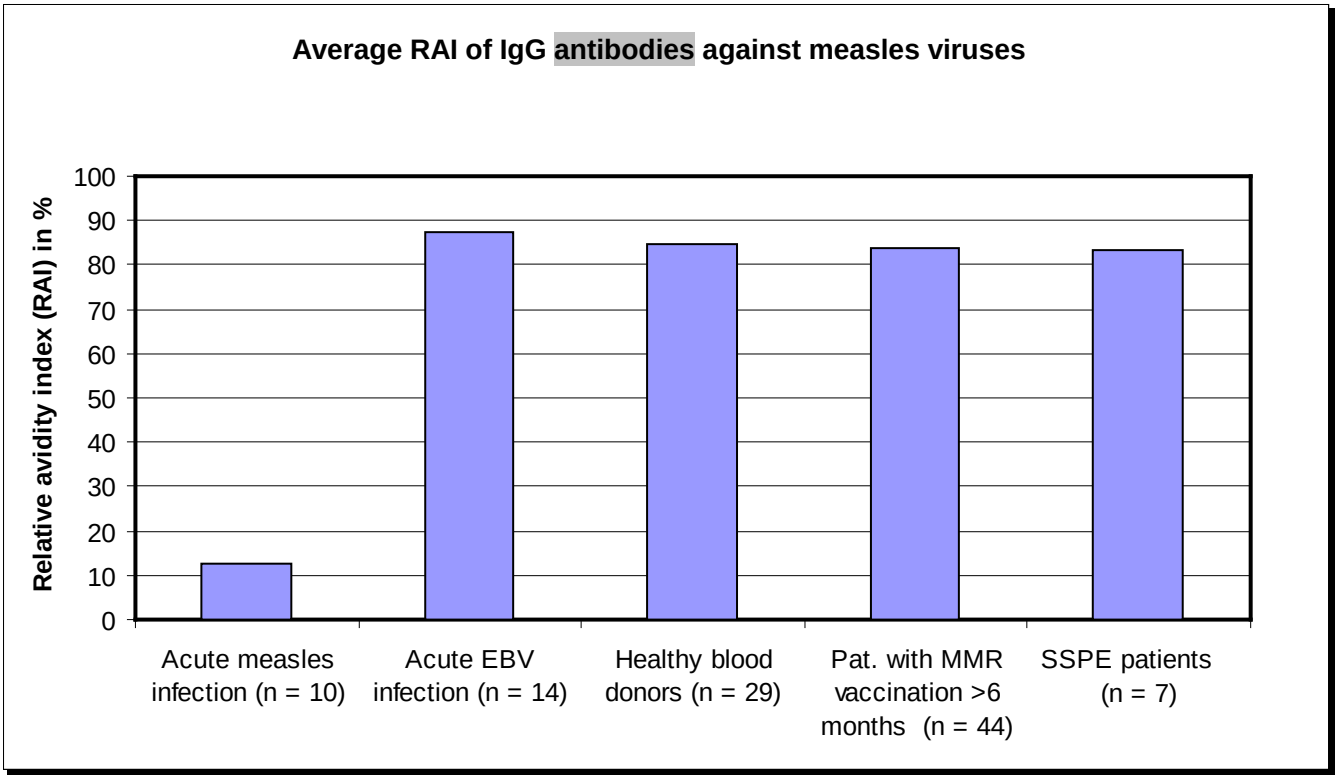
For diagnosis, the clinical symptoms of the patient should always be taken into account along with the serological results.

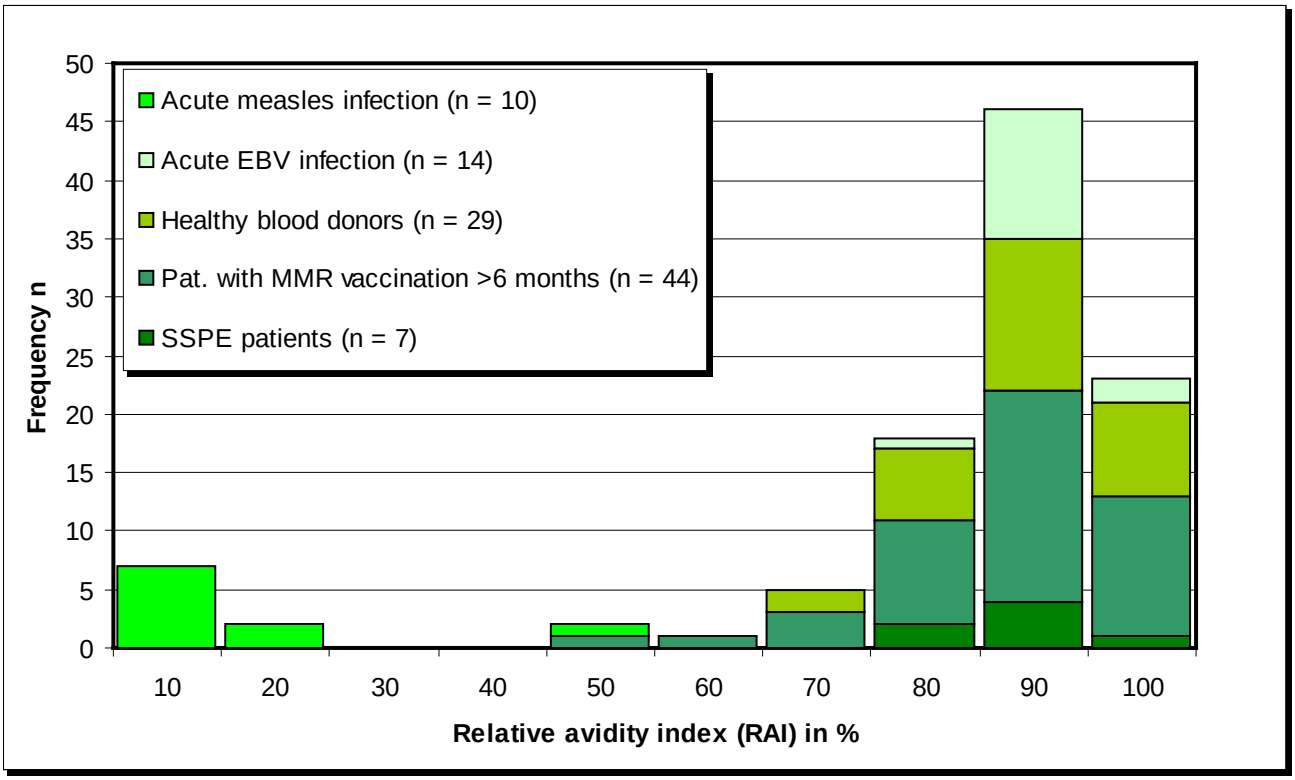
Attention:
In some patients with an acute infection, very high titers of IgG antibodies can be found. Even though the specific IgG antibody populations may be of both high and low avidity due to different maturation stages, the vacant antigen epitopes are predominantly occupied by high-avidity antibodies 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.

Test characteristics

A panel of 104 sera from patients with the following diseases was investigated:
10 patients with clinically and serologically diagnosed acute measles infection
14 patients with acute EBV infection and resulting polyclonal stimulation
29 healthy blood donors
44 patients with MMR vaccination more than 6 months ago
7 patients with SSPE after measles infection

In these panels patients with acute infection showed an average RAI of 13%, whereas the RAI determined for the remaining patients without acute measles infection was > 80%.





Clinical significance

The **measles virus (MV)** is the most instantly recognisable member of Morbilliviruses, a group of viruses belonging to the Paramyxoviridae family [1]. No animal reservoir is known. The measles virus causes an acute feverish illness which occurs mainly in childhood and is very infectious [2, 3]. In 1999, measles still caused worldwide 873,000 deaths per year [1, 4, 5]. Today they are less frequent because of vaccination, especially in the western hemisphere [6, 7]. However, measles epidemics are still observed in some countries [2, 3, 4, 5, 6, 8, 9]. Individuals acutely infected with the virus exhibit a wide range of clinical symptoms ranging from a characteristic mild self-limiting infection to death [1, 2, 8, 10].

MV infections are characterised by an incubation period of about 10 days, flue-like symptoms with fever, malaise, catarrh of the upper respiratory tract, cough, congestion and conjunctivitis. Soon afterwards the measles rash, a typical exanthema, appears first near the ears, then on the forehead, in the face and over the rest of the body [1, 5, 8, 11].

Complications arising from MV infections include secondary bacterial pneumonia, otitis media (approx. 1%), encephalitis (approx. 1%), myocarditis, miscarriage and a condition called subacute sclerosing panencephalitis (SSPE) [5, 12, 13]. Persistent MV infection of the otic capsule is an aetiologic factor in otosclerosis [9, 14]. Anti-measles IgG for the serological diagnosis of otoslcerotic hearing loss has a high specificity and sensitivity [15]. SSPE is a progressive, generally fatal brain disorder caused by chronic measles virus infection. It occurs about 7 to 10 years after the infection and generally kills within 3 years from the onset of the symptoms. The patients suffer from behavioural changes, cognitive deterioration, vision problems and eventually advanced neurological symptoms, such as severe spasms, and finally severe physical and mental impairment that leads to death [13]. Males are more commonly affected than females.

The risk of SSPE from measles was underestimated according to older data [12]. Actual papers put it at closer to 6.5 to 11 cases of SSPE per 100,000 measles infections; that means 7 to 13 times higher than the earlier estimates [1, 9, 12].

Women with acute measles infection during pregnancy and a negative result for measles-specific antibodies were observed e.g. in Japan, India, Thailand, Kenya and Brazil: 3 of 4 pregnancies ended in preterm delivery, spontaneous abortion or stillbirth; 2 of 4 neonates were found to have congenital measles with a positive result for IgM antibodies [5].

Antibodies against MV can be found in the serum of almost all patients during and after a measles infection. IgM antibodies develop soon after the onset of symptoms and can be measured using ELISA or indirect immunofluorescence tests (IIFT) [16, 20, 21, 22]. 50% of patients have IgM antibodies within three days, more than 90% within 10 days after occurrence of the rash [15, 17]. The Anti-Measles Virus IgM ELISA is more rapid and sensitive for the serological diagnosis of measles infections than other tests [15, 18, 19]. MV infections often cause an increase in heterologic antibodies. The statistically evaluated detection rate for antibodies is significantly higher for ELISA and IIFT in comparison with e.g. neutralisation tests [16, 20]. IgG and IgM antibodies against MV are reliable markers to confirm suspected measles infections.

Measles myelitis or encephalitis can be verified by detecting antibodies against measles in the cerebrospinal fluid (CSF) [23, 24, 25, 26]. These specific antibodies are synthesised in the brain [24]. The CSF-serum quotient (LSQ) allows to differenciate between a blood-derived and a pathological, brain-derived specific antibody fraction in CSF, taking into account individual changes in the blood/CSF barrier function [24, 25, 26, 27]. Therefore it is necessary to confirm the presence of antibodies against MV using ELISA both in CSF and in the serum. During measles myelitis or encephalitis an intrathecal synthesis of antibodies against MV in CFS takes place. Due to the fact that specific antibodies can pass the blood-cerebrospinal fluid barrier by diffusion from serum to CSF it is necessary to determine the relative CSF/serum quotient ($CSQ_{rel.}$, synonym: antibody specificity index) [24, 25, 26]. The quotient is calculated from the amount of specific anti-measles virus IgG antibodies in total CSF IgG in proportion to the amount of specific IgG antibodies in total serum IgG. During conversion the CSF/serum quotient of the pathogen-specific IgG-antibody concentrations $CSQ_{path.-spec.}$ (IgG) is put into relation to the CSF/serum quotient of the total IgG concentrations CSQ_{total} (IgG) [27]. A relative CSQ result above 1.5 indicates the production of specific antibodies in the central nervous system (CNS) and the involvement of the CNS in the disease [25, 26].

With respect to the severe complications known from measles infections, the Robert Koch Institute in Germany recommends vaccinating small children, with a first shot between the age of 11 to 14 months and a second between 15 and 23 months [2, 4, 10, 11]. Neutralisation activity and persistence of antibodies are induced in response to the immunisation [6, 15].

Life-long immunity is generally developed. However, antibody levels are 8 to 10 times lower in post-vaccination sera than in convalescent sera [6, 19, 28]. A passive immunisation with specific immunoglobulin concentrates is usually given to immunosuppressed seronegative individuals, such as tumour patients and recipients of transplants, as well as to seronegative pregnant women after exposure to the virus.

The European Regional Office of the WHO aims at eliminating measles from the region in the following years by area-wide vaccination campaigns [4, 9, 10]. This is expected to limit the number of apparent infections and especially of severe courses of the disease. For the diagnosis of the remaining cases of measles infection and of infections acquired outside Europe as well as for the clarification of atypical courses of the disease in partly immunised patients the antibody determination in serum and CSF will be of growing importance [3, 5, 8, 9].

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