Avidity determination of IgG antibodies against CMV
Test instruction for the ELISA

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2570-9601-1 G	Cytomegalovirus (CMV)	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 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 2570-9601-1 G

Component	Colour	Format	Symbol
1. Test kit Anti-CMV ELISA (IgG, order number EI 2570-9601 G)	---		
2. Positive control HA High-avidity anti-CMV (IgG, human), ready for use	red	1 x 1.3 ml	POS CONTROL HA
3. Positive control LA Low-avidity anti-CMV (IgG, human), ready for use	blue	1 x 1.3 ml	POS CONTROL LA
4. Urea solution for Anti-CMV 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 — CE 0197 — Storage temperature

IVD In vitro diagnostics — 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 should be disposed of according to official regulations.

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.

- **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 votexing (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).

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 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).

Washing:
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.

Washing:
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 sera (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. In order to objectivate the results a relative avidity index (RAI) is calculated and expressed in percent using the extinction values with and without urea treatment.

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

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

The majority of cytomegalovirus (CMV) infections are subclinical or asymptomatic [1]. The disease can manifest itself in almost any organs. In the foreground, however, hepatitis and pneumonia, are most frequent and accompanied by a lasting fever [2]. Seropositive cytomegalovirus infections with an inflammatory response (interleukin-6 response) predict cardiac mortality in patients with coronary artery disease [3]. Antibodies against cytomegalovirus can be detected in the serum of nearly all patients after the disease has taken its course. Life-long immunity is normally developed [1]. The prevalence of CMV infection is heavily dependent on geographical location, social and economical status. The sero positive ratio (IgG) is listed in Western Europe and Canada as 30% to 50% in men and 20% to 40% in women who are in their fertile years. In the USA the prevalence of CMV infection is slightly higher [4, 5, 6, 7, 8, 9, 10]. The highest prevalence of CMV can be found in Asia and Africa in adults up to 95% [11, 12, 13]. Earlier infections can, however, be reactivated when the immunological defence mechanisms are weakened.

Cytomegalovirus is probably the most common agent of prenatal infection of the newborn, and one in 20 congenitally infected newborns shows serious symptoms [14, 15]. Connatal cytomegaly causes damage in particular to the liver, spleen and the central nervous system [16]. About 1% of all fatal cases are infected in utero and exhibit IgM class antibodies. CMV, the major viral cause of congenital disease, infects the uterine-placental interface with varied outcomes depending on the strength of maternal humoral immunity and gestational age [17]. CMV virions can disseminate to the placenta by co-opting the receptor-mediated transport pathway for IgG. These findings can explain the efficacy of hyper-immune IgG for treatment of primary CMV infection during gestation and support vaccination [18].

In the case of immunologically challenged seronegative patients, such as tumour patients and recipients of transplants, passive immunisation with specific immunoglobulin concentrates is frequently indicated [19, 20]. Such patients, however, and breast-feeding infants (particularly the newborn), should not be given blood products from CMV-infected (CMV antibody-positive) blood donors, because antigens are generally localised in the leucocytes [21].

Under certain circumstances, and especially in pregnant women, the diagnosis of CMV infections is essentially based on the detection of IgG and IgM antibodies; however, IgM antibodies are not an exclusive marker of acute infection. Complementary tests are needed to help in determinaing the stage of infection [17, 22, 23]. False-positive IgM antibody tests for CMV can be found in patients with acute Epstein-Barr virus infection [24]. Fetal damage is mostly linked to maternal primary infection. Therefore it is important to differentiate primary from recurrent or persistent CMV infection in pregnant females.

For this purpose, the measurement of the IgG avidity is currently the best method [25]. IgM tests and the IgG avidity determination can identify all women at risk of transmitting CMV. Furthermore, a high CMV DNA load in amniotic fluid could be an indicator of symptomatic congenital infection at a relatively early stage of pregnancy [25]. Detection of specific IgM antibodies in fetal blood is significantly correlated with severe outcome for the fetus or the newborn [26].

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