Anti-Adenovirus ELISA (IgM)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2680-9601 M	Adenovirus	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 adenovirus in serum or plasma for the diagnosis of respiratory tract infections, conjunctivitis, keratitis, meningitis and gastroenteritis.

Application: Diagnosis of acute adenovirus infections is usually achieved by direct detection of the pathogenic agent (e.g. using PCR). The determination of specific antibodies using Anti-Adenovirus ELISA can supplement the direct detection of the virus and secure the diagnosis. Detection of IgA and/or IgM antibodies against adenovirus and a significant titer increase of specific IgG in a follow-up sample taken 7 to 14 days later indicate an acute infection. Moreover, serological investigations can clarify questions about the epidemiology of adenovirus infections.

Principle of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with adenovirus 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
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: (2rd 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 minimizes 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. Values above the indicated cut-off are to be considered as positive, those below 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 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 IgM class antibodies against adenovirus, results are provided in the form of ratios which are a relative measurement of the antibody concentration in serum or plasma.

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 (+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 cell lysates of MRC-5 cells infected with the “Adenoid 6“ strain of adenovirus.

Detection limit: The lower detection limit is defined as 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-Adenovirus 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 infections caused by various agents were investigated with the Anti-Adenovirus ELISA (IgM). Only one of 8 samples from patients with an acute EBV infection was positive. It is known, that infections with EBV viruses can lead to a polyclonal stimulation of B cells, resulting in the production of IgM antibodies against various infectious pathogens that the patient has had contact with. Thus, false positive IgM results can occur.

Antibodies against	n	Anti-Adenovirus ELISA (IgM) positive
CMV	17	0%
Mumps virus	13	0%
Measles virus	10	0%
Toxoplasma gondii	14	0%
VZV	15	0%
Rubella virus	10	0%
HSV Pool	4	0%
Borrelia	10	0%
EBV-CA	8	12.5%

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	1.9	3.5
2	3.1	3.6
3	5.3	4.1

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (Ratio)**	**CV (%)**
1	1.8	8.2
2	2.9	7.4
3	5.5	9.0

Reference range: The levels of the anti-adenovirus antibodies (IgM) were analyzed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors. With a cut-off ratio of 1.0, 0.2% of the blood donors are were anti-Adenovirus positive (IgM).

Clinical significance

Human pathogenic adenoviruses, which belong to the Adenoviridae family, are non-enveloped viruses with a double-stranded, linear DNA as their genome. They were first isolated from human tonsils and other adenoid tissue and propagated in a cell culture in 1953 by a group of scientists under the direction of W. P. Rowe. Adenoviruses have a high stability against chemical and physical influences and a high tolerance for extreme pH values and alcoholic disinfectants. The viruses can be fully inactivated by heating to 56°C for 10 minutes. Today, six human-pathogenic adenovirus species are known (human adenovirus A to F), which are again divided into a total of 53 serologically distinguishable subtypes. The way that the genetic information is transmitted within the host cell is largely understood.

Adenoviruses are transmitted by direct contact, faecal-orally and, occasionally, through water. They mainly cause respiratory diseases. Spreading of the virus in the patient's body can take several months or years. The symptoms of respiratory disease range from common colds to bronchitis and pneumonia. Patients with a weakened immune system are particularly prone to developing severe complications from adenovirus infections, e.g. ARDS (acute respiratory distress syndrome).

Typical manifestations caused by human pathogenic adenoviruses include keratoconjunctivitis epidemica (types 8, 19, 37), acute respiratory syndromes (types 1-3, 4, 6, 7, 14, 21), pharyngoconjunctival fever (types 3, 7, 14), follicular conjunctivitis (types 3, 4, 7), gastroenteritides (types 40, 41, 31), gastroenteritides with mesenteric lymphadenopathy (types 1, 2, 5, 6), pneumonia (types 1-4, 7) and acute, febrile pharyngitis (types 1-3, 5-7). Serotypes 1, 2, 5 and 6 are endemic in some regions throughout the world and infections here generally occur during childhood. Other species, in particular serotypes 8, 19 and 37, lead to epidemic keratoconjunctivitis, with increasing tendency (by approx. 300% in the past two years). Adenovirus types 1 to 39 are involved in about 6% of infections of the respiratory tract worldwide. Types 40 and 41 can cause gastroenteritis, and in this respect take second place after rotaviruses for virally causes illness in small children. Various conditions such as persisting bronchiolitis, dilatative cardiomyopathy, type 1 diabetes or hearing loss are discussed as late manifestations of the infection. A connection between virus type Ad-36 and obesity in humans is also suspected.

Most adenovirus infections have a mild course and do not require therapy but merely treatment of the symptoms. Due to a lack of virus-specific therapy, even in severe cases treatment is largely aimed at reducing the symptoms and complications of the infection.

Vaccines have been developed for serotypes 4 and 7, but were only available until 1998 for the prevention of severe respiratory infections in US Army recruits. There are no vaccines available for the general public. Therefore, the spread of adenovirus-associated infections can only be effectively prevented by careful infection control.

For the serological diagnosis of acute and chronic adenovirus infections, in particular epidemic keratoconjunctivitis and adenovirus pneumonia, there are several methods available that allow the detection and typing of adenoviruses. Viral DNA and the relevant serotypes 3, 7, 8, 19, 37, 53 and 4, 6, 14 can be identified rapidly, sensitively and reliablly in a variety of clinical materials (including environmental samples) using qualitative PCR (generic), thus allowing diagnosis of an adenovirus infection. The viral load assay (quantitative PCR, generic) helps not only to detect adenovirus DNA, but also to determine the virus load in body fluids (mainly blood). This is very useful for reliable diagnosis of disseminated adenovirus infection, e.g. in immunosuppressed individuals. Viral culture/isolation (e.g. isolation of A549 or HKK 293 cells) is the reference method for the diagnosis of adenovirus infections. However, this method is time-consuming, hence PCR is often preferred. For molecular typing (by sequencing), primarily a conserved region of the hexon gene is sequenced to determine the species. This generally suffices for the few types of species A, E, F and G. A second sequencing must be carried out for species B, C and D due to their large variety of types. The neutralisation test (type-specific antibody determination) is useful for ascertaining the serological status of a patient with respect to a specific adenovirus type. Electron microscopy, which is indicated in exceptional cases, is only available in a few laboratories and is an extremely laborious procedure.

Serological methods for the diagnosis of acute or chronic adenovirus infections are IIFT (indirect immunofluorescence test) and ELISA (enzyme linked immunosorbent assay), which were introduced in 1976 and 1983, respectively. They provide rapid and exact diagnostics through the determination of specific antibodies of class IgA, IgG, and IgM. It should be noted, however, that serological diagnosis can only be established on the basis of at least two serum samples taken and investigated at an interval of two to three weeks. Furthermore, clinical symptoms must be taken into account for diagnosis. A single sample does not yield sufficient information. Sometimes specific IgM is not produced in the early stage of the infection. In other cases antibodies of class IgM persist for a long time. For this reason, IgM detection is not sufficiently significant with respect to acute adenovirus infection. Therefore, investigation of follow-up samples is indispensable. Cross-reactivity exists between the various adenovirus serotypes. In the IIFT using infected EU 38 cells as the standard substrate and the ELISA based on inactivated cell lysates of MRC-5 cells infected with adenovirus of the "adenoid 6" strain, cross reactivity is reduced to a minimum.

Serological adenovirus detection methods do not only play a decisive role in the diagnosis of acute or chronic adenovirus infection, but also in the determination of the immunity status, e.g. with respect to pandemics.

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