研究用試薬

Anti-Echinococcus ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2320-9601-1 G	Echinococcus	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides semiquantitative in vitro determination of human antibodies of the immunoglobulin class IgG against Echinococcus in serum or plasma to support the diagnosis of echinococcosis and hydatidosis**.**

Application: The detection of antibodies against Echincoccus spp. using the Anti-Echinococcus ELISA (IgG) is a useful supplement to clinical and imaging diagnostics of a cystic or alveolar echinococcosis, transmitted by Echinococcus granulosus or multilocularis. Owing to the use of purified Echinococcus multilocularis antigens, antibodies against E. granulosus, as well as antibodies against E. multilocularis can be determined semiquantitatively using the EUROIMMUN Anti-Echinococcus ELISA (IgG).

Principles of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with coated with antigens of Echinococcus preparations. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgG antibodies will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (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 (IgG, human), ready for use	dark red	1 x 2.0 ml	CAL
3. Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
4. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
5. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
6. Sample buffer ready for use	light blue	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. Protective foil	---	3 pieces	FOIL
11. Test instruction	---	1 booklet	
12. Quality control certificate	---	1 protocol	

LOT Lot description
IVD In vitro diagnostic medical device
CE
Storage temperature
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, 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°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.
The thermostat adjusted ELISA incubator must be set at **+37°C ± 1°C**.

- **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.

- **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 amount 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 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.

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 sodium azide in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the patient samples

Samples: Human serum or EDTA or heparin 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 are diluted **1:101** 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 calibrator and controls are prediluted and 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.
For manual test performance cover the reagent wells with the protective foil. When using an automated microplate processor for incubation follow the recommendations of the instrument manufacturer.
Incubate for **60 minutes** at **+37°C** ± **1°C**.

Washing:
Manual: Remove the protective foil, empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash.
Automatic: Remove the protective foil and 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 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.
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 IgG) into each of the microplate wells. For manual test performance cover the reagent wells with the protective foil.
Incubate for **30 minutes** at **+37°C** ± **1°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 **30 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.

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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 pipetting protocol for microtiter strips is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).

The calibrator (C), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for 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.

Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction of the control or patient sample over the extinction of calibrator. Use the following formula to calculate the ratio:

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

EUROIMMUN recommends interpreting results as follows:

Ratio <0.8: negative
Ratio ≥0.8 to <1.1: borderline
Ratio ≥1.1: positive

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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 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 quantificated international reference serum exists for antibodies against Echinococcus, the results are performed in ratio values.

For every group of tests performed, the extinction readings of the calibrator and the ratios determined for 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. 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 microplate wells were coated with native, highly purified antigen from Echinococcus multilocularis.

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-Echinococcus ELISA (IgG) is ratio 0.01.

Cross reactivity: Since cross reactions with other Helminth infections, especially Taenia spp., cannot be excluded, these pathogens should also be taken into account in differential diagnosis.

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 4 samples. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 3 determinations performed in 10 different test runs.

Intra-assay variation, n = 20		
Sample	**Mean value (ratio)**	**CV (%)**
1	0.6	7.6
2	1.4	3.6
3	2.0	2.2
4	2.9	3.5

Inter-assay variation, n = 3 x 10		
Sample	**Mean value (ratio)**	**CV (%)**
1	0.6	8.4
2	1.3	6.6
3	1.9	6.1
4	2.8	5.2

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Sensitivity and specificity:
Study I: Samples from 100 previously tested and evaluated patient samples (origin: National Center of Infectious and Parasitic Diseases (NCIPD) in Sofia, Bulgaria) were investigated using the EUROIMMUN Anti-Echinococcus ELISA (IgG). The analytical sensitivity was 95.5%, with a specificity of 95.7%. Borderline results were not included in the calculation.

n = 100		Previous findings		
		positive	borderline	negative
EUROIMMUN Anti-Echinococcus ELISA (IgG)	positive	42	0	2
	borderline	6	0	3
	negative	2	0	45

Study II: 58 clinically pre-characterised patient samples (INSTAND, NEQAS and RfB) were investigated with the EUROIMMUN Anti-Echinococcus ELISA (IgG). The sensitivity amounted to 100%, with a specificity of 100%. Borderline results were not included in the calculation.

n = 58		INSTAND/NEQAS/RfB		
		positive	borderline	negative
EUROIMMUN Anti-Echinococcus ELISA (IgG)	positive	20	0	0
	borderline	1	0	0
	negative	0	0	37

Study III: Serum panels of serologically characterised patients with high antibody titers mainly of class IgG against other helminth and protozoa species relevant for differential diagnosis due to the overlap of their endemic regions or their respective symptoms were investigated. Of the total of 127 samples, 4 samples were positive with the Anti-Echinococcus ELISA (IgG). The specificity in this panel amounted to 96.9%. The following table summarises the results:

Antibodies against	**n**	**Anti-Echinococcus ELISA (IgG) positive**
Presence in the endemic region		
Toxoplasma gondii	10	0%
Ascaris lumbricoides	10	10% (1 positive)
Toxocara canis	10	0%
Giardia lamblia	10	0%
Trichinella spiralis	10	10% (1 positive)
Opisthorchis viverrini	10	0%
Trichomonas vaginalis	10	10% (1 positive)
Similar symptoms		
Trypanosoma cruzi	10	0%
Plasmodium spp.	10	10% (1 positive)
Schistosoma mansoni	10	0%
Taenia spp.	27	0%

It must be taken into account that double infections may occur especially in endemic regions or that an infection with Echinococcus spp. may have been present at an earlier point in time. Positive results are then not due to a cross reactivity of the respective antibodies, but to a specific reaction.

Study IV: For evaluation of the specificity of the Anti-Echinococcus ELISA (IgG) a study was performed using 78 patient sera seropositive for rheumatoid factors, EBV and various autoantibodies. Of the total of 78 samples, 2 sera were positive with the Anti-Echinococcus ELISA (IgG). The specificity in this panel amounted to 97.4%. The following table summarises the results:

Possible influencing factors	n	Anti-Echinococcus ELISA (IgG) positive
Various ANA	34	0%
EBV-CA	7	0%
Rheumatoid factor	37	5.4% (2 positive)
Tumour patients	50	2% (1 positive)

Reference range: The levels of the anti-Echinococcus antibodies (IgG) were analysed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors. With a cut-off of ratio 1.0, 0.6% of the blood donors were anti-Echinococcus positive (IgG).

Clinical significance

Echinococcosis is an infectious disease caused by parasites of the genus Echinococcus. In Europe, the dog tapeworm (E. granulosus), causing cystic echinococcosis (CE), and the fox tapeworm (E. multilocularis), causing alveolar echinococcosis (AE), are most important from the medical point of view.
The development of all Echinococcus species includes an obligatory host change: the definitive hosts are carnivores, the intermediate hosts mostly herbivores.
The mature worms of the fox tapeworm, E. multilocularis, live in the intestine of their definitive hosts (in Europe, mainly red foxes, seldom dogs and cats) who release the mature cestode eggs with their droppings. These eggs are very robust and may be infectious for several months if the conditions are good.
The intermediate hosts (small mammals like field mice or European water voles) take the eggs in with their food. The larvae of the worms then develop in their inner organs (mostly in the liver).
Humans can be infected with E. granulosus by smear infection, through dealing with contaminated soil or consuming contaminated foods. Also here, the eggs are dispersed with the definitive hosts' droppings (mostly dogs, at times cats) and stay infectious for months. The larvae develop in liquid-filled blisters (hyatides) which are found in liver, lung, other organs and also in the skeletal system of the intermediate host (e.g. hoofed animals such as cows or sheep).
Humans are incidental hosts for fox and dog tapeworms. Human infection usually takes place by intake of cestodes after contact with infected hosts (e.g. smear infections or via the animal's fur).

The clinical image of both echinococcoses (CE and AE) differs in the different developmental behaviours of both parasites in the human body. The clinical appearance of AE corresponds to that of a malignoma. After haematogenous transport of the cestodes into the liver, infection of the liver takes place (usually unnoticed for a long time) and an alveolar tumour develops.

In infections with E. granulosus, larvae are released in the human intestine. They are transported haematogenously via the portal vein first into the liver and then into other organs, e.g. the lungs. The clinical course may vary significantly and is characterised by the slowly growing cysts and their different localisation.

In humans, both diseases remain asymptomatic for many years, until after 10 to 15 years they show symptoms like cholestatic icterus, epigastric pains, fatigue, weight loss and hepatomegaly. By invasion and destruction of the healthy liver tissue, an untreated echinococcosis may lead to the patient's death. Differential diagnosis from cysts, malignant and benign tumours, abscesses and the distinction between AE and CE are important for a diagnosis.

Imaging techniques, such as sonography, CT and MRT are used for diagnosis. The use of serological test systems for the detection of parasite-specific antibodies in serum or plasma helps to confirm results from imaging techniques. If whole antigens of *Echinococcus* are used, an echinococcosis can be detected with good sensitivity by ELISA or IFT tests. The use of species-specific antigens in Western-blot and ELISA tests enables in many cases the serological differentiation of *E. granulosus* and *E. multilocularis.* A negative result in this serological investigation does not exclude an infection. In liver CE, serum antibodies can be detected in 80% to 94% of cases. In lung echinococcosis, the prevalence amounts to only 65% to 70%.

In practice, molecular detection methods (PCR for the detection of *Echinococcus* DNA and RNA) have not proven to deliver.

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