Anti-M2-3E ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EA 1622-9601 G	AMA-M2 (2-oxo-acid dehydrogenase complexes)	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human antibodies of the immunoglobulin class IgG against the mitochondrial antigens M2 in serum or plasma to support the diagnosis of primary biliary cirrhosis (PBC) and overlap syndrome with autoimmune hepatitis.

Application: Autoantibodies against the M2 antigen are highly specific diagnostic markers for PBC and are present in up to 96% of patients with PBC. High antibody titers are characteristic of PBC, whilst low titers have also been described in other chronic liver diseases. The combination of BPO fusion protein with native M2 protein further increases the diagnostic sensitivity compared to test systems which only use native M2.

Principles of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with M2 antigens (native M2 and recombinant BPO). In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgG antibodies (also IgA and IgM) 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 1 200 RU/ml (IgG, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2 20 RU/ml (IgG, human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 2 RU/ml (IgG, human), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
8. Sample buffer ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
9. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
10. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
11. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
12. Test instruction	---	1 booklet	
13. Quality control certificate	---	1 protocol	

LOT Lot description

IVD In vitro diagnostic medical device

CE

Storage temperature

Unopened usable until

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.

- **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.

- **Calibrators 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 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 calibrators 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 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 calibrators and controls are prediluted and ready for use, do not dilute them.

Incubation

For **qualitative/semiquantative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1, 2 and 3** along with the positive and negative controls and patient samples.

(Partly) manual test performance

Sample incubation: (1st step)
Transfer 100 µl of the calibrators, 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 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.
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 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 2	P 6	P 14	P 22			C 1	P 4	P 12	P 20		
B	pos.	P 7	P 15	P 23			C 2	P 5	P 13	P 21		
C	neg.	P 8	P 16	P 24			C 3	P 6	P 14	P 22		
D	P 1	P 9	P 17				pos.	P 7	P 15	P 23		
E	P 2	P 10	P 18				neg.	P 8	P 16	P 24		
F	P 3	P 11	P 19				P 1	P 9	P 17			
G	P 4	P 12	P 20				P 2	P 10	P 18			
H	P 5	P 13	P 21				P 3	P 11	P 19			

The pipetting protocol for microtiter strips 1 to 4 is an example for the **qualitative/semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7 to 10 is an example for the **quantitative analysis** of 24 patient samples (P 1 to P 24).

The calibrators (C 1 to C 3), 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

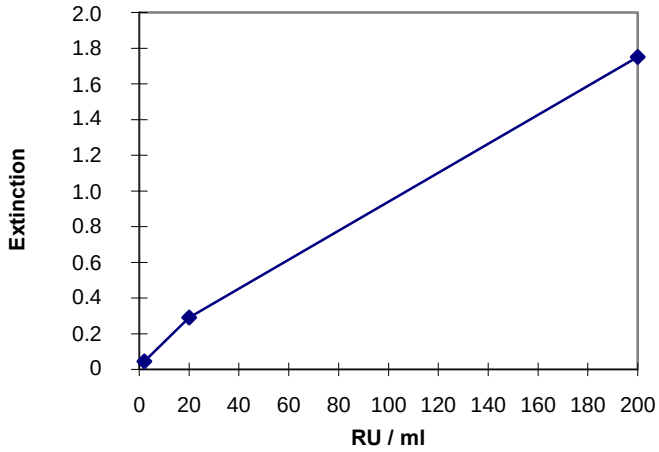
Qualitative/semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction of the control or patient sample over the extinction of calibrator 2. Calculate the ratio according to the following formula:

$$\frac{\textbf{Extinction of the control or patient sample}}{\textbf{Extinction of calibrator 2}} = \textbf{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <1.0: **negative**
Ratio ≥1.0: **positive**

Quantitative: The standard curve from which the concentration of antibodies in the serum samples can be taken is obtained by point-to-point plotting of the extinction readings measured for the 3 calibration sera against the corresponding units (linear/linear). Use “point-to-point” plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.



If the extinction for a patient sample lies above the extinction of calibrator 1 (corresponding to 200 RU/ml), the result should be reported as “> 200 RU/ml”. It is recommended that the sample be re-tested at a dilution of e.g. 1:400. The result in RU/ml read from the calibration curve for this sample must then be multiplied by factor 4.

The upper limit of the normal range (**cut-off**) recommended by EUROIMMUN is 20 relative units (RU)/ml. EUROIMMUN recommends interpreting results as follows:

<20 RU/ml:	**negative**
≥20 RU/ml:	**positive**

The recommendation is based on data yielded in a ROC analysis (AUC: 0.966) using the results of 170 patients with primary biliary cirrhosis and 989 control samples. According to the analysis, the specificity was 98% at a cut-off of 19.6 RU/ml. The 99th percentile based on 400 healthy blood donors was 13.5 RU/ml (q.v. respective paragraphs under “Test characteristics”).

Please note that 16 of 22 positive results (72.7%) from the control panel (n = 989) but only 6 of 158 positive samples (3.8%) from patients with primary biliary cirrhosis ranged between 20 RU/ml and 50 RU/ml. Results from a weak positive range of 20 RU/ml to 50 RU/ml should be interpreted with prudence and possibly verified with a new patient sample taken several weeks later.

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.

For the medical diagnosis, the clinical symptoms of the patient and, if available, further findings should always be taken into account alongside the serological result. A negative serological result does not exclude the presence of a disease.

Test characteristics

Calibration: As no international reference serum exists for antibodies against M2 antigens, the calibration is performed in relative units (RU). The reactivity of the Anti- M2-3E ELISA (IgG) was verified using the human AMA reference serum AC-21 (Plasma Service Group, USA).

For every group of tests performed, the relative units or ratio values 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 calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The reagent wells were coated with a mixture of pyruvate dehydrogenase (isolated from bovine heart) and a recombinant fusion protein. The recombinant protein was produced in E.coli and comprises the immunogenic domains of the E2 subunits from branched-chain 2-oxo-acid dehydro-genase (**B**COADH), pyruvate dehydrogenase (**P**DH) and 2-oxoglutarate dehydrogenase (**O**GDH), together called **BPO**.

Linearity: The linearity of the Anti-M2-3E ELISA (IgG) was determined by assaying at least 4 serial dilutions of different patient samples. The Anti-M2-3E ELISA (IgG) is linear at least in the tested concentration range (3 RU/ml to 199 RU/ml).

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-M2-3E ELISA (IgG) is 1.1 RU/ml.

Cross-reactivity: This ELISA specifically detects autoantibodies of class IgG against M2-3E. Cross-reactions with other autoantibodies were not found in samples from patients with the following antibodies: anti-GBM (n = 10), anti-LKM (n = 9), anti-PCA (n = 10).

Interference: Haemolytic, lipaemic and icteric samples showed no influence on the result up to a concentration of 10.0 mg/ml for haemoglobin, 20.0 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 4 determinations performed in 6 different test runs.

Intra-assay variation, n = 20		
Sample	**Mean value (RU/ml)**	**CV (%)**
1	45	3.1
2	70	2.3
3	101	2.2
4	144	1.6

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (RU/ml)**	**CV (%)**
1	39	6.5
2	67	8.6
3	103	6.0
4	158	3.0

Clinical sensitivity and specificity: In total 1395 clinically characterised samples (251 from PBC patients, 15 from patients with PBC/AIH overlap syndrome and 1129 from control groups) were analysed using the EUROIMMUN Anti-M2-3E ELISA (IgG). The sensitivity of the ELISA for PBC was 93.2%, with a specificity of 97.9%. AMA can also be detected in other diseases overlapping with PBC such as autoimmune hepatitis, systemic sclerosis, systemic lupus erythematosus and Sjögren's syndrome.

Panel	Anti-M2-3E ELISA (IgG)	
	n	positive (%)
Primary biliary liver cirrhosis	251	233 (92.8%)
PBC/AIH overlap syndrome	15	15 (100.0%)
Sensitivity for PBC	**266**	**248 (93.2%)**
Autoimmune hepatitis	131	6 (4.6%)
Viral hepatitis	239	0 (0.0%)
Primary sclerosing cholangitis	19	0 (0.0%)
Systemic lupus erythematosus	100	8 (8.0%)
Sjögren's syndrome	120	8 (6.7%)
Rheumatoid arthritis	120	2 (1.7%)
Asymptomatic blood donors	400	0 (0.0%)
Specificity for PBC	**1129**	**24 (97.9%)**

In a ROC analysis of the results (AUC: 0.966) of 170 PBC patient samples and 989 control samples the following characteristics were determined:

cut-off	specificity	sensitivity
13.7 RU/ml	95%	94%
19.6 RU/ml	98%	93%
26.5 RU/ml	99%	92%

Reference range: Levels of anti-M2 antibodies were analysed in 400 sera from healthy blood donors between 19 and 68 years of age (149 women, 251 men) using the EUROIMMUN ELISA. No differences with respect to age or gender were observed. The mean concentration of antibodies against M2 was 3.9 RU/ml (± 3.2 RU/ml of standard deviation) and the values ranged from 0.2 to 15.9 RU/ml. With a cut-off of 20 RU/ml no blood donors were anti-M2 positive.

cut-off	percentile
10.1 RU/ml	95.0%
12.6 RU/ml	98.0%
13.5 RU/ml	99.0%

Clinical significance

The Anti-M2-3E ELISA (IgG) provides highest sensitivity and specificity for the serological diagnosis of manifest primary biliary cirrhosis (PBC) and of early stage PBC (primary biliary cholangiitis) without significant liver function disorders or cholestatic symptoms. Co-reactions may occur in overlap syndrome (combination of PBC or autoimmune hepatitis with other autoimmune diseases that do not primarily affect the liver).

PBC is an autoimmune liver disease which initally causes destruction of the small biliary ducts due to chronic inflammatory processes occurring primarily in the biliary ducts (primary biliary cholangitis) and in later stages affects the entire liver tissue leading to cirrhosis (primary biliary cirrhosis). It is controversially discussed whether other pathogenic agents such as Chlamydia pneumoniae, Helicobacter pylori, Mycobacterium tuberculosis or retroviruses cause the disease. PBC occurs in over 90% of cases in women and is most frequently observed between the ages of 40 and 60. The disease has so far not been found in children. The prevalence of PBC has been quoted for an English patient collective as being 140 cases in 1 million inhabitants. The number of new disease cases worldwide is estimated to be 4 to 31 per 1 million per year.

The clinical picture of PBC begins with unspecific, very varying general symptoms, such as itching caused by micro-papular exanthemas, tiredness and pain in the upper right region of the abdomen. An obstructive jaundice develops after a varying period of time. Histologically, this stage is characterised by lymphocytic infiltration of the small intra-hepatic biliary ducts (canaliculi biliferi) and the build-up of bile (cholestasis). Accompanying symptoms may be arthritides, Sicca syndrome, xanthelasma, fatty stool, vitamin deficiency and, in approx. 20% of women, recurring urinary tract infections. The increase in serum lipids is an important indicator of PBC. The chronic, non-suppurative destructive cholangitis with granulating pericholangiitis and slowly progressing destruction of the small and medium-sized biliary ducts with subsequent fibrosis leads to complete cirrhosis in the final stage with ascites, oesophagus varices and hepatic encephalopathy. As well as the liver, other organs with exocrinous functions are frequently involved in the disease process, above all the lachrymal glands, salivary glands, the pancreas and often also the thyroid gland.

Although no cure for PBC is available, some of the symptoms can be reduced by treatment with e.g. ursodeoxycholic acid and cholestyramine. Ursodeoxycholic acid reduces cholestasis and improves the liver function. Cholestyramine absorbs bile acids in the gut and helps to relieve itching caused by bile acids in the blood stream. In Europe, advanced PBC is the most frequent indication for liver transplantation, which offers a favourable prognosis. Following transplantation 75% of PBC patients make a full recovery.

Overlap syndrome is characterised by a combination of two or more autoimmune diseases, most frequently in connection with autoimmune hepatitis and PBC, but it may also occur together with other autoimmune diseases, e.g. systemic sclerosis (in approx. 6% of overlap cases) or Sjögren's syndrome.

In PBC the serological detection of autoantibodies against mitochondria (**AMA**) is diagnostically very relevant. But AMA can also be found in other diseases (see table). Sometimes they also occur in patients with chronic hepatitis C or rheumatic diseases, such as systemic lupus erythematosus, although the autoantibody titers in the serum are generally low.

Antibodies against	Associated disease	Prevalence
M1	Lues (indication of activity) Systemic lupus erythematosus Progressive systemic sclerosis, Sjögren’s syndrome, Sharp syndrome, rheumatoid arthritis	100% 50% 5-15%
M2	Primary biliary liver cirrhosis (high titers) Other chronic liver diseases Progressive systemic sclerosis	up to 96% 30% 7-25%
M3	Pseudolupus syndrome	100%
M4	Primary biliary liver cirrhosis	up to 55%
M5	Non-specific collagenoses	rare
M6	Hepatitis (Iproniazide-induced)	100%
M7	Acute myocarditis Cardiomyopathies	60% 30%
M8	Primary biliary liver cirrhosis	up to 55%
M9	Primary biliary liver cirrhosis Other forms of hepatitis	37-82% 3-10%

So far, four different AMA types have been detected in the sera of PBC patients: antibodies against the antigens M2, M4, M8 and M9. Antibodies against M2 antigen (2-oxoacid dehydrogenase complex) are highly specific (approx. 98%) and highly sensitive (approx. 93%) diagnostic markers for PBC. They are detectable in up to 96% of PBC patients. The prevalence of antibodies against M4, M8 and M9 antigens is significantly lower. Antibodies against M4 and M8 only occur together with antibodies against M2. Data obtained so far indicate that antibodies against M4 are a sign of an unfavourable progress of the disease. Antibodies against M9 occur mainly in patients with PBC in its early stage (82%) in which antibodies against M2 have in some cases not (yet) been produced. In such cases, the M9 antibodies are of the IgM class (greater than 90%). If antibodies against M2 can already be detected, the prevalence of M9 antibodies is only 37%, of which 50% are exclusively of the IgM class. The presence of M9 antibodies appears to be an indication of a favourable progress of the disease.

High titers of antibodies against **M2** are characteristic of PBC, whereby the E2 enzyme and protein X of the pyruvate dehydrogenase complex are the preferred antigens. Low anti-M2 antibody titers can also be detected in other chronic liver diseases and in progressive systemic sclerosis, as shown in the table. Patients with progressive systemic sclerosis exhibiting antibodies against M2 may suffer from overlap syndrome with PBC.

The M2 antigens used in the Anti-M2-3E ELISA (IgG), native M2 and recombinant BPO, ensure that the assay provides a high diagnostic relevance. The artifical BPO protein, which contains all relevant epitopes, was designed by fusing the lipoyl-binding areas of BCOADH-E2 (E2 subunit of branched-chain 2-oxoacid dehydrogenase), PDH-E2 (E2 subunit of pyruvate dehydrogenase) and OGDH-E2 (E2 subunit of 2-oxoglutarate dehydrogenase) using recombinant techniques. This fusion protein (produced in E. coli) mixed with native M2 (purified protein from porcine pyruvate dehydrogenase complex) in this monospecific test system for the determination of antibodies against M2 increases the sensitivity compared to test systems based on native M2 alone.

Note: With a negative AMA result and continued suspicion of PBC, the additional determination of antibodies against nuclear granules (nuclear dots, SP100) is indicated, since these are also pathognomonically relevant.

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