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# **AMA-M2**

**ORG 516**

**96 Tests**

**Immunometric Enzyme  
Immunoassay for the  
quantitative determination  
of anti-mitochondrial M2 -  
antibodies (AMA-M2)**

Instruction for use

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## WARNINGS AND PRECAUTIONS

All reagents of this test kit are strictly intended for in vitro use only.

Please adhere strictly to the sequence of pipetting steps provided in this protocol. Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.

All reagents should be stored refrigerated at 2 - 8 °C in their original container.

Do not interchange kit components from different lots. The expiration dates stated on the labels of the shipping container and all vials have to be observed. Do not use kit components beyond their expiration dates.

Allow all kit components and specimen to reach room temperature prior to use and mix well.

During handling of all kit reagents, controls and serum samples observe the existing legal regulations. The following precautions should be taken handling potentially infectious materials:

- do not eat, drink or smoke in areas where specimens or kit reagents are handled
- do not pipette by mouth
- wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.

The test kit contains components of human origin which, when tested by FDA-licensed methods, were found negative for hepatitis B surface antigen and for HIV antibody. No known test can guarantee, however, that products derived from human blood will not be infectious. Handle, therefore, all reagents and human blood derivatives, like plasma or serum samples, as if capable of transmitting infection.

Avoid contact with the TMB (3,3',5,5'-Tetramethyl-benzidine). If TMB comes into contact with skin wash thoroughly with water and soap.

The stop solution contains hydrochloric acid. If it comes into contact with skin, wash thoroughly with water and seek medical attention.

Avoid contact between the buffered Peroxide Solution and easily oxidized materials; extreme temperatures may initiate spontaneous combustion.

## MATERIALS SUPPLIED

<b>Package size</b>	<b>96 determ.</b>
divisible microplate consisting of 12 modules of 8 wells, ..... 1 coated with highly purified mitochondrial M2 subtype antigen	
AMA-M2 Calibrators in a serum/buffer matrix..... 6 vials, 1.5 ml each containing: 0; 12.5; 25; 50; 100 and 200 IU/ml	
AMA-M2 Controls in a serum/buffer matrix (positive and negative), .... 2 vials, 1.5 ml each for the respective concentrations see the enclosed package insert	
AMA-M2 sample buffer, yellow, Concentrate..... 1 vial, 20 ml	
enzyme conjugate solution (light red), containing polyclonal rabbit .... 1 vial, 15 ml anti-h-IgG-IgG, labelled with horseradish peroxidase	
TMB substrate solution ..... 1 vial, 15 ml	
stop solution (1 M hydrochloric acid) ..... 1 vial, 15 ml	
buffered wash solution, Concentrate ..... 1 vial, 20 ml	

## CONTROLS

A set of two controls is provided with the kit.

## TECHNICAL DATA

Sample material:	serum or plasma
Required sample volume:	10 µl of sample to be diluted 1:100 with sample buffer 100 µl prediluted sample per single determination
Total incubation time:	60 minutes at room temperature (20 - 28 °C)
Calibration range:	12.5 - 200 IU/ml
Sensitivity:	1 IU/ml
Storage:	refrigerated at 2 - 8 °C
Shelf life:	12 months after manufacturing or until the expiration date printed on the labels
Package size:	96 tests

## PRINCIPLE OF THE PROCEDURE

AMA-M2 is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for the quantitative measurement of IgG class autoantibodies directed against mitochondrial M2 subtype antigen. The assay is based on microplates coated with mitochondrial M2 antigen.

The microplates can be divided into 12 modules of 8 wells each or can be used completely for 96 determinations. Each well can be separated from the module ("break-away").

During this procedure the binding of present autoantibodies, as well as the formation of the sandwich complexes and enzymatic colour reaction take place during three different reaction phases:

### Phase 1:

Calibrators, controls and prediluted patient samples are pipetted into the wells of the microplate. Any present antibodies bind to the inner surface of the wells. After a 30 minutes incubation the microplate is washed with wash buffer for removing non-reactive serum components.

### Phase 2:

An anti-human-IgG horseradish peroxidase conjugate solution is pipetted into the wells of the microplate to recognise the autoantibodies bound to the immobilized antigens. After a 15 minutes incubation any excessive enzyme conjugate, which is not specifically bound is washed away with wash buffer.

### Phase 3:

A chromogenic substrate solution containing TMB (3,3',5,5'-Tetramethyl-benzidine) is dispensed into the wells. During 15 minutes of incubation the colour of the solutions change into blue. Colour development is stopped by adding 1 M hydrochloric acid as stop solution. The solutions colour change into yellow.

The amount of colour is directly proportional to the concentration of IgG present in the original sample. The optical density for each calibrator may be graphically plotted against the concentration of IgG and unknowns extrapolated from the curve.

To read the optical density a microplate reader with a 450 nm filter is required. Bi-chromatic measurement with a 600-650 nm reference is recommended.

## CLINICAL RELEVANCE

Anti-mitochondrial antibodies (AMA) are a heterogeneous group of autoantibodies directed against various proteins that are located in the outer and inner membrane of mitochondria. Development of their distinct target antigens take place in the tissues. Non-organ-specific subtypes of antibodies have been described besides those antibodies which exhibit a relatively high organ specificity.

Using fluorescence techniques with slices from different organs, i.e. liver, stomach, kidney, heart and pancreas, the mitochondrial autoantibodies of different specificity can be discriminated. For some of the AMA subtypes their specific target protein yet remains unclear.

AMA subtype	characterised autoantigens	localisation	clinical relevance
M1	Cardiolipin	inner mitochondrial membrane	Lues-II
M2	proteins of the $\alpha$ -keto acid dehydrogenase complex	inner mitochondrial membrane	primary biliary cirrhosis (PBC)
M3		outer mitochondrial membrane	drug-induced LE (Pyrazolon)
M4	associated with Sulfitoxidase	outer mitochondrial membrane	PBC
M5		outer mitochondrial membrane	SLE and undifferentiated collagenosis, autoimmune haemolytic anemia
M6		outer mitochondrial membrane	drug-induced Hepatitis (Iproniazid)
M7	sarcosin dehydrogenase	inner mitochondrial membrane	Cardiomyopathy, Myocarditis
M8		outer mitochondrial membrane	PBC
M9	associated with glycogen phosphorylase	outer mitochondrial membrane (probably also cytoplasmatic)	PBC

Specific anti-mitochondrial antibodies have been described for the primary biliary cirrhosis (PBC) as subtypes M2, M4, M8 and M9. Other AMA subtypes are related to other diseases, like collagenosis (AMA-M5) and drug induced LE and Hepatitis (AMA-M3 and AMA-M6).

The heterogeneously reacting specific anti-mitochondrial antibodies of the M2 subtype are directed against three related proteins of the  $\alpha$ -keto acid dehydrogenase complex which is located at the inside of the mitochondrial membrane. The recognized major epitope is located on the E2 subunit and the protein X of the pyruvate dehydrogenase complex (PDC). Additionally AMA-M2 autoantibodies recognise the (E1 $\alpha$  and E1 $\beta$ ) subunits of the same complex and the E2 subunit of several other multi enzyme complexes, such as the 2-oxo-glutarate dehydrogenase complex (OGDC) and the branched chain 2-oxo acid dehydrogenase complex (BCOADC).

Using HEp 2 Cell monolayers for indirect immune fluorescence AMA-M2 autoantibodies are characterised as a fine-speckled cytoplasmatic, perinuclear condensed fluorescence pattern.

For differential diagnosis of the primary biliary cirrhosis (PBC) determination of AMA-M2 by ELISA is recommended because of its high sensitivity and specificity.

In patients with other autoimmune diseases determination of AMA antibodies allows an early screening for the occurrence of subtype M2 and M9 antibodies which may be related with the development and / or association of PBC.

Profiling the AMA subtypes allows an immunological and prognostic classification of the primary biliary cirrhosis. Beginning cases of symptomatic PBC often exhibit only AMA-M2 subtype antibodies (sometimes in combination with AMA-M9), whereas progressive cases and mixed syndromes with chronic acute hepatitis (CAH) are related with the occurrence of AMA-M2, -M4 and -M8 antibody subtypes.

## NORMAL VALUES

In a normal range study with serum samples from healthy blood donors the following ranges have been established with the AMA-M2 test:

	AMA-M2
normal:	< 10 IU/ml
elevated:	≥ 10 IU/ml

Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken individually.

It is recommended that each laboratory establishes its own normal and pathological ranges of serum AMA-M2.

## SPECIFICITY

The microplate is coated with highly purified mitochondrial M2 subtype antigen. The AMA-M2 test kit recognises only autoantibodies specific to the proteins of the  $\alpha$ -keto acid dehydrogenase complex. No crossreactivities to other mitochondrial autoantigens have been observed.

## CALIBRATION

The quantitative test system for AMA-M2 autoantibodies is calibrated against the WHO reference preparation 67/183 at 100 IU/ml.

## MATERIALS REQUIRED

### Equipment

- Microplate reader capable for endpoint measurements at 450 nm
- Vortex mixer
- Pipets for 10  $\mu$ l, 100  $\mu$ l and 1000  $\mu$ l

### Preparation of reagents

- distilled water
- graduated cylinder for 100 and 1000 ml
- plastic container for storage of the wash solution

### Optional

- Multi-Chanel Dispenser
- or repeatable pipet for 100  $\mu$ l
- data reduction software

## **SPECIMEN COLLECTION AND PREPARATION**

For determination of AMA-M2 antibodies serum or plasma are the preferred sample matrixes.

All serum and plasma samples are prediluted 1 : 100 with sample buffer. Therefore 10 µl of sample may be diluted with 1,000 µl of sample buffer.

The patients need not to be fasting, and no special preparations are necessary. Collect blood by venipuncture into vacutainers and separate serum or plasma from the cells by centrifugation after clot formation.

Samples may be stored refrigerated at 2 - 8 °C for at least 5 days. For longer storage of up to six months samples should be stored frozen at -20 °C. To avoid repeated thawing and freezing the samples should be aliquoted.

Neither Bilirubin nor Hemolysis have significant effect on the procedure.

## **PREPARATION AND STORAGE OF REAGENTS**

All components of this test kit are supplied in a liquid format and ready to use, except the sample buffer and wash buffer. When stored refrigerated at 2 - 8 °C the components are stable for at least 30 days after opening or until the expiration date printed on the labels.

Remaining modules of the microplate should be stored refrigerated at 2 - 8 °C protected from moisture; store together with desiccant and carefully sealed in the plastic bag.

### **Preparation of sample buffer**

Dilute the contents of each vial of the sample buffer concentrate (5x) with distilled water to a final volume of 100 ml prior to use. Store refrigerated: stable at 2 - 8 °C for at least 30 days after preparation or until the expiration date printed on the label.

### **Preparation of buffered wash solution**

Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled water to a final volume of 1000 ml prior to use. Store refrigerated: stable at 2 - 8 °C for at least 30 days after preparation or until the expiration date printed on the label.

## **NOTES ON TECHNIQUE**

Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay.

For all controls, the respective concentrations are provided on the labels of each vial. Using these concentrations a calibration curve may be calculated to read off the patient results semi-quantitatively.

### **Pipetting and Sample Handling**

Use a disposable-tip micropipette to dispense sera and plasma samples. Pipet directly to the bottom of the wells. To avoid carryover contamination change the tip between samples.

Patient samples expected to contain high concentrations should be additionally diluted with sample buffer before. Additional dilutions must be considered during calculation.

## IMMUNOASSAY PROCEDURE

Do not interchange components of different lots.

All components should be at room temperature before use.

Dilute all patient samples 1:100 with sample buffer before assay. Therefore combine 10 µl of sample with 1000 µl of sample buffer in a polystyrene tube. Mix well. Calibrators and controls are ready to use and need not to be diluted.

1. Prepare a sufficient number of microplate modules to accommodate calibrators, controls and prediluted patient samples in duplicates.

	1	2	3	4	5	6
A	SA	SE	P1	P5		
B	SA	SE	P1	P5		
C	SB	SF	P2	P..		
D	SB	SF	P2	P..		
E	SC	C1	P3			
F	SC	C1	P3			
G	SD	C2	P4			
H	SD	C2	P4			

SA - SF: standards A to F

P1, P2... patient sample 1, 2 ...

C1: positive control

C2: negative control

2. Pipet **100 µl of calibrators, controls and prediluted patient samples** into the wells.
3. Incubate for **30 minutes** at room temperature (20 - 28 °C).
4. Discard the contents of the microwells and wash **3 times with 300 µl of wash solution**.
5. Dispense **100 µl of enzyme conjugate** solution into each well.
6. Incubate for **15 minutes** at room temperature.
7. Discard the contents of the microwells and wash **3 times with 300 µl of wash solution**.
8. Dispense **100 µl of TMB substrate solution** into each well.
9. Incubate for **15 minutes** at room temperature protected from light.
10. Add **100 µl of stop solution** to each well of the modules and leave untouched for 5 minutes.
11. Read the optical density at **450 nm** and calculate the results. Bi-chromatic measurement with reference at 600-650 nm is recommended.

**The developed color is stable for at least 30 minutes.  
Read optical densities during this time.**

## CALCULATION OF RESULTS

For the AMA-M2 tests a 4-Parameter-Fit with lin-log co-ordinates for optical density and concentration is recommended. Smoothed Spline approximation and log-log co-ordinates are also suitable.

### Recommended Lin-Log Plot

First calculate the averaged optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

## CALCULATION EXAMPLE

The figures below show typical results for AMA-M2. These data are intended for illustration only and should not be used to calculate results from another run.

No	Position	OD 1	OD 2	Mean	Conc. 1	Conc. 2	Mean	decl.Conc.	CV %
ST1	A 1/A 2	0.013	0.011	0.012	0.1	0.1	0.0	0.0	12
ST2	B 1/B 2	0.437	0.422	0.430	12.9	12.4	12.6	12.5	2
ST3	C 1/C 2	0.741	0.720	0.731	25	24	25	25	2
ST4	D 1/D 2	1.174	1.148	1.161	50	48	49	50	2
ST5	E 1/E 2	1.700	1.701	1.701	102	103	102	100	0
ST6	F 1/F 2	2.173	2.143	2.158	202	193	197	200	1

## ASSAY CHARACTERISTICS

### ***Sensitivity***

The lower detection limit for AMA-M2 has been determined at 1.0 IU/ml.

### ***Parallelism***

In dilution experiments sera with high antibody concentrations were diluted with sample buffer and assayed in the AMA-M2 kit. The assay shows linearity over the full measuring range.

### ***Precision***

Statistics were calculated for each of three samples from the results of 24 determinations in a single run for Intra-Assay precision. Run-to-run precision was calculated from the results of 5 different runs with 6 determinations each:

<b>Intra-Assay</b>		
Sample No	Mean (IU/ml)	CV (%)
1	39.8	7.0
2	81.3	3.8
3	177.3	3.6

<b>Inter-Assay</b>		
Sample No	Mean (IU/ml)	CV (%)
1	40.1	6.2
2	84.6	11.8
3	180.4	3.8

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## INCUBATION SCHEME

- ① Pipet **100 µl** calibrator, control or diluted patient sample  
→ Incubate for **30 minutes** at room temperature  
→ Discard the contents of the wells and wash 3 times with **300 µl** wash solution
- ② Pipet **100 µl** enzyme conjugate  
→ Incubate for **15 minutes** at room temperature  
→ Discard the contents of the wells and wash 3 times with **300 µl** wash solution
- ③ Pipet **100 µl** substrate solution  
→ Incubate for **15 minutes** at room temperature
- ④ Add **100 µl** stop solution  
→ Leave untouched for **5 minutes**  
→ Read at **450 nm**