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**User's Manual**

# Mitochondrial (AMA)-M2-Ab ELISA

REF **DEIA-NS2312-8**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.


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# PRODUCT INFORMATION

## Intended Use

Enzyme immunoassay for the quantitative and qualitative detection of antibodies against M2 in human serum.

## General Description

The Mitochondrial (AMA)-M2-Ab ELISA is a solid phase enzyme immunoassay employing native mitochondial M2 antigen for the quantitative and qualitative detection of IgG antibodies against M2 in human serum.

The assay is a tool in the research of the primary biliary cirrhosis (PBC).

Primary biliary cirrhosis (PBC) is a chronic inflammatory disorder of the small and medium bile ducts and serologically characterized by the presence of circulating M2 autoantibodies. Anti-M2 autoantibodies belong to the group of anti-mitochondrial antibodies (AMA). The heterogeneously reacting specific AMA of the M2 subtype are directed against three related proteins of the α-keto acid dehydrogenase complex, which is located at the inner mitochondrial membrane. The major epitope recognized is located on the E2 subunit and the protein X of the pyruvate dehydrogenase complex (PDC). Additionally, AMA-M2-G autoantibodies recognize the (E1α and E1β) subunits of the same complex and the E2 subunit of several other multienzyme complexes, such as the 2-oxo-glutarate dehydrogenase complex (OGDC) and the branched chain 2-oxo acid dehydrogenase complex (BCOADC). The determination of AMA-M2-G is a powerful tool in diagnosing PBC.

## Principles of Testing

Serum samples diluted 1:101 are incubated in the microplates coated with the specific antigen. Patient´s antibodies, if present in the specimen, bind to the antigen. The unbound fraction is washed off in the following step. Afterwards anti-human immunoglobulins conjugated to horseradish peroxidase (conjugate) are incubated and react with the antigen-antibody complex of the samples in the microplates. Unbound conjugate is washed off in the following step. Addition of TMB-substrate generates an enzymatic colorimetric (blue) reaction, which is stopped by diluted acid (color changes to yellow). The intensity of color formation from the chromogen is a function of the amount of conjugate bound to the antigen-antibody complex and this is proportional to the initial concentration of the respective antibodies in the patient sample.

## Reagents And Materials Provided

**1. Sample Buffer (5×):** 1 × 20ml, White Yellow, 5 × concentrated, Tris, sodium chloride (NaCl), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)

**2. Wash Buffer (50×):** 1 × 20ml White Green, 50 × concentrated, Tris, NaCl, Tween 20, sodium azide < 0.1%(preservative)

**3. Negative Control:** 1 × 1.5ml Green Colorless, Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)

**4. Positive Control:** 1 × 1.5ml Red Yellow, Human serum (diluted), bovine serum albumin (BSA), sodium

azide < 0.1% (preservative)

**5. Cut-off Calibrator:** 1 × 1.5ml Blue Yellow Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)

**6. Calibrators:** 6 × 1.5ml White Yellow * Concentration of each calibrator: 0, 3, 10, 30, 100, 300 U/ml. Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)

**7. Conjugate, IgG:** 1 × 15ml Blue Blue, Containing: Anti-human immunoglobulins conjugated to horseradish peroxidase, bovine serum albumin (BSA)

**8. TMB Substrate:** 1 × 15ml Black Colorless, Stabilized tetramethylbenzidine and hydrogen peroxide (TMB/$H_2O_2$)

**9. Stop Solution:** 1 × 15ml White Colorless, 1M Hydrochloric Acid

**10. Microtiter plate:** 12 × 8 well strips, With breakaway microwells. Refer to paragraph 1 for coating.

**Due to the lack of international reference calibration this assay is calibrated in arbitrary units (U/ml).**

## Materials Required But Not Supplied

Microtiter plate reader 450 nm reading filter and recommended 620 nm reference filter (600-690 nm). Glass ware (cylinder 100-1000ml), test tubes for dilutions. Vortex mixer, precision pipettes (10, 100, 200, 500, 1000 µl) or adjustable multipipette (100-1000µl). Microplate washing device (300 µl repeating or multichannel pipette or automated system), adsorbent paper. Our tests are designed to be used with purified water according to the definition of the United States Pharmacopeia (USP 26 - NF 21) and the European Pharmacopeia (Eur.Ph. 4th ed.).

## Storage

Store all reagents and the microplate at 2-8°C/35-46°F, in their original containers. Once prepared, reconstituted solutions are stable at 2-8°C/35-46°F for 1 month. Reagents and the microplate shall be used within the expiry date indicated on each component, only. Avoid intense exposure of TMB solution to light. Store microplates in designated foil, including the desiccant, and seal tightly.

## Specimen Collection And Preparation

Use preferentially freshly collected serum samples. Blood withdrawal must follow national requirements. Do not use icteric, lipemic, hemolysed or bacterially contaminated samples. Sera with particles should be cleared by low speed centrifugation (<1000 x g). Blood samples should be collected in clean, dry and empty tubes. After separation, the serum samples should be used during the first 8h, respectively stored tightly closed at 2-8°C/35-46°F up to 48h, or frozen at -20°C/-4°F for longer periods.

## Plate Preparation

We suggest pipetting calibrators, controls and samples as follows:

For *QUANTITATIVE* interpretation

| | 1 | 2 | 3 | 4... |
|---|---|---|---|---|
| A | Cal A | Cal E | P1 | |
| B | Cal A | Cal E | P1 | |
| C | Cal B | Cal F | P2 | |
| D | Cal B | Cal F | P2 | |
| E | Cal C | PC | P3 | |
| F | Cal C | PC | P3 | |
| G | Cal D | NC | ... | |
| H | Cal D | NC | ... | |

For *QUALITATIVE* interpretation

| | 1 | 2 | 3 | 4... |
|---|---|---|---|---|
| A | NC | P2 | | |
| B | NC | P2 | | |
| C | CC | P3 | | |
| D | CC | P3 | | |
| E | PC | ... | | |
| F | PC | ... | | |
| G | P1 | ... | | |
| H | P1 | ... | | |

CalA: calibrator A
CalB: calibrator B
CalC: calibrator C
CalD: calibrator D
CalE: calibrator E
CalF: calibrator F
PC: positive control
NC: negative control
CC: cut-off calibrator
P1: patient 1
P2: patient 2
P3: patient 3

## Reagent Preparation

**Dilute concentrated reagents:**

Dilute the concentrated sample buffer 1:5 with distilled water (e.g. 20 ml plus 80 ml).

Dilute the concentrated wash buffer 1:50 with distilled water (e.g. 20 ml plus 980 ml).

To avoid mistakes we suggest to mark the cap of the different calibrators.

**Samples:**

Dilute serum samples 1:101 with sample buffer (1×)

e.g. 1000 µl sample buffer (1×) + 10 µl serum. Mix well !

**Washing:**

Prepare 20 ml of diluted wash buffer (1×) per 8 wells or 200 ml for 96 wells e.g. 4 ml concentrate plus 196 ml distilled water.

**Automated washing:**

Consider excess volumes required for setting up the instrument and dead volume of robot pipette.

**Manual washing:**

Discard liquid from wells by inverting the plate. Knock the microwell frame with wells downside vigorously on clean adsorbent paper. Pipette 300 µl of diluted wash buffer into each well, wait for 20 seconds. Repeat the whole procedure twice again.

**Microplates:**

Calculate the number of wells required for the test. Remove unused wells from the frame, replace and store in the provided plastic bag, together with desiccant, seal tightly (2-8°C/35-46°F).

## Assay Procedure

1. Ensure preparations from **Reagent Preparation** above have been carried out prior to pipetting.
2. Use the following steps in accordance with quantitative/ qualitative interpretation results desired:

3. Pipette into the designated wells as described in **Plate Preparation** above, 100 µl of either:
   a. Calibrators (CAL.A to CAL.F) for QUANTITATIVE or
   b. Cut-off Calibrator (CC) for QUALITATIVE interp.and 100 µl of each of the following:
   c. Negative control (NC) and Positive control (PC), and Patients diluted serum (P1, P2...)
4. Incubate for 30 minutes at 20-32°C/68-89.6°F.
5. Wash 3× with 300 µl washing buffer (diluted 1:50).
6. Pipette 100 µl conjugate into each well.
7. Incubate for 30 minutes at 20-32°C/68-89.6°F.
8. Wash 3× with 300 µl washing buffer (diluted 1:50).
9. Pipette 100 µl TMB substrate into each well.
10. Incubate for 30 minutes at 20-32°C/68-89.6°F, protected from intense light.
11. Pipette 100 µl stop solution into each well, using the same order as pipetting the substrate.
12. Incubate 5 minutes minimum.
13. Agitate plate carefully for 5 sec.
14. Read absorbance at 450 nm (recommended 450/620 nm) within 30 minutes.

**General directions for use**

In case that the product information, including the labeling, is defective or incorrect please contact the manufacturer or the supplier of the test kit. Do not mix or substitute Controls, Calibrators, Conjugates or microplates from different lot numbers. This may lead to variations in the results.

Allow all components to reach room temperature (20-32°C/68-89.6°F) before use, mix well and follow the recommended incubation scheme for an optimum performance of the test.

Incubation: We recommend test performance at 30°C/86°F for automated systems. Never expose components to higher temperature than 37°C/ 98.6°F.

Always pipette substrate solution with brand new tips only. Protect this reagent from light.

Never pipette conjugate with tips used with other reagents prior.

## Interpretation Of Results

For quantitative interpretation establish the standard curve by plotting the optical density (OD) of each calibrator (y-axis) with respect to the corresponding concentration values in U/ml (x-axis). For best results we recommend log/lin coordinates and 4-Parameter Fit. From the OD of each sample, read the corresponding antibody concentrations expressed in U/ml.

| Normal Range | Equivocal Range | Positive Results |
|---|---|---|
| < 12 U/ml | 12 - 18 U/ml | >18 U/ml |

**Example of a standard curve**

Do NOT use this example for interpreting sample´s result

| Calibrators IgG | OD 450/620 nm | CV % (Variation) |
|---|---|---|
| 0 U/ml | 0.032 | 2.8 |
| 3 U/ml | 0.152 | 2.6 |
| 10 U/ml | 0.281 | 1.2 |
| 30 U/ml | 0.646 | 2.4 |
| 100 U/ml | 1.214 | 1.7 |
| 300 U/ml | 2.104 | 1.6 |

**Example of calculation**

| Patient | Replicate (OD) | Mean (OD) | Result (U/ml) |
|---|---|---|---|
| P 01 | 0.794/0.792 | 0.793 | 45.4 |
| P 02 | 1.453/1.477 | 1.465 | 135.8 |

Samples above the highest calibrator range should be reported as >Max. They should be diluted as appropriate and re-assayed. Samples below calibrator range should be reported as < Min.

For lot specific data, see enclosed quality control leaflet. Medical laboratories might perform an in-house quality control by using own controls and/or internal pooled sera, as foreseen by national regulations.

Each laboratory should establish its own normal range based upon its own techniques, controls, equipment and patient population according to their own established procedures.

In case that the values of the controls do not meet the criteria the test is invalid and has to be repeated.

The following technical issues should be verified: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, photometer, incubation conditions and washing methods.

If the items tested show aberrant values or any kind of deviation or that the validation criteria are not met without explicable cause please contact the manufacturer or the supplier of the test kit.

For qualitative interpretation read the optical density of the cut-off calibrator and the patient samples. Compare patient´s OD with the OD of the cut-off calibrator. For qualitative interpretation we recommend to consider sera within a range of 20% around the cut-off value as equivocal. All samples with higher ODs are considered positive, samples with lower ODs are considered negative.

**Negative:** OD patient < 0.8 × OD cut-off

**Equivocal:** 0.8 × OD cut-off ≤ OD patient ≤ 1.2 × OD cut-off

**Positive:** OD patient > 1.2 × OD cut-off

## Precision

To determine the precision of the assay, the variability (intra and inter-assay) was assessed by examining its reproducibility on three serum samples selected to represent a range over the standard curve.

| Intra-assay | | |
|---|---|---|
| **Sample No.** | **Mean (U/ml)** | **CV (%)** |
| 1 | 153.3 | 3.4 |
| 2 | 63.7 | 2.8 |
| 3 | 24.8 | 1.4 |

| Inter-assay | | |
|---|---|---|
| **Sample No.** | **Mean (U/ml)** | **CV (%)** |
| 1 | 144.8 | 4.2 |
| 2 | 59.7 | 2.1 |
| 3 | 21.4 | 1.5 |

## Detection Range

0-300 U/ml

## Sensitivity

1. 0 U/ml

Testing sample buffer 30 times on Mitochondrial (AMA)-M2-Ab ELISA gave an analytical sensitivity of 1.0 U/ml.

## Specificity

The microplate is coated with highly purified mitochondrial M2 antigen. No crossreactivities to other autoantigens have been found. Anti-M2 antibodies have been shown to be specific for the diagnosis of PBC and were found in 96% of PBC patients.

## Linearity

Chosen sera have been tested with this kit and found to dilute linearly. However, due to the heterogeneous nature of human autoantibodies there might be samples that do not follow this rule.

| Sample No. | Dilution Factor | Measured (U/ml) | Expected (U/ml) | Recovery (%) |
|---|---|---|---|---|
| 1 | 1 / 100 | 154.0 | 157.0 | 98.1 |
| | 1 / 200 | 77.0 | 78.5 | 98.1 |
| | 1 / 400 | 40.8 | 39.3 | 103.8 |
| | 1 / 800 | 20.4 | 19.6 | 104.1 |
| 2 | 1 / 100 | 65.0 | 63.0 | 103.2 |
| | 1 / 200 | 32.0 | 31.5 | 101.6 |
| | 1 / 400 | 16.4 | 15.8 | 103.8 |
| | 1 / 800 | 8.2 | 7.9 | 103.8 |

## Precautions

Only staff trained and specially advised in methods of in vitro diagnostics may perform the kit. Although this product is not considered particularly toxic or dangerous in conditions of the intended use, refer to the following for maximum safety:

### Recommendations and precautions

This kit contains potentially hazardous components. Though kit reagents are not classified being irritant to eyes and skin we recommend to avoid contact with eyes and skin and wear disposable gloves. WARNING ! Calibrators, Controls and Buffers contain sodium azide ($NaN_3$) as a preservative. $NaN_3$ may be toxic if ingested or adsorbed by skin or eyes. $NaN_3$ may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up. Please refer to decontamination procedures as outlined by CDC or other local/national guidelines. **Do not smoke, eat or drink when manipulating the kit. Do not pipette by mouth.** All human source material used for some reagents of this kit (controls, standards e.g.) has been tested by approved methods and found negative for HbsAg, Hepatitis C and HIV 1. However, no test can guarantee the absence of viral agents in such

material completely. Thus handle kit controls, standards and patient samples as if capable of transmitting infectious diseases and according to national requirements. The kit contains material of animal origin as stated in the table of contents, handle according to national requirements.

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