

As of 30 Dec. 2008 (Vers. 3.0)

NAME AND INTENDED USE

AMA-M2 is an indirect solid phase enzyme immunoassay (ELISA) for the quantitative measurement of IgG class autoantibodies against mitochondrial M2 subtype antigen in human serum or plasma.

The assay is intended for in vitro use only as an aid in the determination of primary biliary cirrhosis.

SUMMARY AND EXPLANATION OF THE TEST

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. 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 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 (E1a und E1b) 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 sub-type 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.

| AMA subtype | characterised autoantigens | localisation | clinical relevance |
|-------------|--|------------------------------|---|
| M1 | Cardiolipin | inner mitochondrial membrane | Lues-II |
| M2 | proteins of the α keto acid dehydrogenase complex | inner mitochondrial membrane | primary biliary cirrhosis (PBC) |
| M3 | | outer mitochondrial membrane | drug-induced LE (Pyrazolon) |
| M4 | associated with Sulfoxidase | outer mitochondrial membrane | PBC |
| M5 | | outer mitochondrial membrane | SLE and undifferentiated collagenosis, autoimmune haemolytic anemia |
| M6 | | outer mitochondrial membrane | drug-induced Hepatitis (Iproniazid) |

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| | | | |
|----|--|--|-----------------------------|
| 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 |

PRINCIPLE OF THE TEST

Highly purified mitochondrial M2 subtype antigen is bound to microwells. Antibodies against this antigen, if present in diluted serum or plasma, bind to the respective antigen. Washing of the microwells removes unspecific serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-human IgG immunologically detects the bound patient antibodies forming a conjugate/antibody/antigen complex. Washing of the microwells removes unbound conjugate. An enzyme substrate in the presence of bound conjugate hydrolyzes to form a blue color. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow color is measured photometrically at 450 nm. The amount of colour is directly proportional to the concentration of IgG antibodies present in the original sample.

WARNINGS AND PRECAUTIONS

1. All reagents of this kit are strictly intended for in vitro use only. In the United States, this kit is intended for Research Use Only.
2. Do not interchange kit components from different lots.
3. Components containing human serum were tested and found negative for HBsAg, HCV, HIV 1 and HIV2 by FDA approved methods. No test can guarantee the absence of HBsAg, HCV, HIV 1 and HIV2 and so all human serum based reagents in this kit must be handled as though capable of transmitting infection.
4. Avoid contact with the TMB (3,3',5,5'-Tetramethyl-benzidine). If TMB comes into contact with skin, wash thoroughly with water and soap.
5. Avoid contact with the Stop Solution which is acid. If it comes into contact with skin, wash thoroughly with water and seek medical attention.
6. Some kit components (i.e. Controls, Sample buffer and Buffered Wash Solution) contain Sodium Azide as preservative. Sodium Azide (NaN₃) is highly toxic and reactive in pure form. At the product concentrations (0.09%), though not hazardous. Despite the classification as non-hazardous, we strongly recommend using prudent laboratory practices (see 8., 9., 10.).
7. Some kit components contain Proclin 300 as preservative. When disposing reagents containing Proclin 300, flush drains with copious amounts of water to dilute the components below active levels.
8. Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
9. Do not pipette by mouth.
10. Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.
11. Avoid contact between the buffered Peroxide Solution and easily oxidized materials; extreme temperature may initiate spontaneous combustion.

Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

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CONTENTS OF THE KIT

Package size 96 determ.

| | |
|----------------------|---|
| Qty.1 | divisible microplate consisting of 12 modules of 8 wells coated with highly purified mitochondrial M2 subtype antigen. Ready to use. |
| 6 vials, 1.5 ml each | AMA-M2 Calibrators (A-F) in a serum/buffer matrix (PBS, BSA, NaN ₃ <0.1% (w/w)) containing: IgG: 0; 12.5; 25; 50; 100; and 200 IU/ml. Ready to use. |
| 2 vials, 1,5 ml each | AMA-M2 Controls in a serum/buffer matrix (PBS, BSA, NaN ₃ <0,1%(w/w)) positive (1) and negative (2), for the respective concentrations see the enclosed package insert. Ready to use. |
| 1 vial, 20 ml | Sample buffer (Tris, NaN ₃ <0.1% (w/w)), yellow, concentrate (5x). |
| 1 vial, 15 ml | Enzyme conjugate solution (PBS, Proclin 300 <0.5% (v/v)), (light red) containing polyclonal rabbit anti-human IgG; labelled with horseradish peroxidase. Ready to use. |
| 1 vial, 15 ml | TMB substrate solution. Ready to use. |
| 1 vial, 15 ml | Stop solution (contains acid). Ready to use. |
| 1 vial, 20 ml | Wash solution (PBS, NaN ₃ <0.1% (w/w)), concentrate (50x). |

STORAGE AND STABILITY

1. Store the kit at 2-8 °C.
2. Keep microplate wells sealed in a dry bag with desiccants.
3. The reagents are stable until expiration of the kit.
4. Do not expose test reagents to heat, sun or strong light during storage and usage.
5. Diluted sample buffer and wash buffer are stable for at least 30 days when stored at 2-8 °C.

MATERIALS REQUIRED

Equipment

- Microplate reader capable of endpoint measurements at 450 nm
- Multi-Channel Dispenser or repeatable pipet for 100 µl
- Vortex mixer
- Pipets for 10 µl, 100 µl and 1000 µl
- Laboratory timing device
- Data reduction software

Preparation of reagents

- Distilled or deionized water
- Graduated cylinder for 100 and 1000 ml
- Plastic container for storage of the wash solution

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SPECIMEN COLLECTION, STORAGE AND HANDLING

1. Collect whole blood specimens using acceptable medical techniques to avoid hemolysis.
2. Allow blood to clot and separate the serum by centrifugation.
3. Test serum should be clear and non-hemolyzed. Contamination by hemolysis or lipemia is best avoided, but does not interfere with this assay.
4. Specimens may be refrigerated at 2-8 °C for up to five days or stored at -20 °C up to six months.
5. Avoid repetitive freezing and thawing of serum samples. This may result in variable loss of autoantibody activity.
6. Testing of heat-inactivated sera is not recommended.

PROCEDURAL NOTES

1. Do not use kit components beyond their expiration dates.
2. Do not interchange kit components from different lots.
3. All materials must be at room temperature (20-28 °C).
4. Have all reagents and samples ready before start of the assay. Once started, the test must be performed without interruption to get the most reliable and consistent results.
5. Perform the assay steps only in the order indicated.
6. Always use fresh sample dilutions.
7. Pipette all reagents and samples into the bottom of the wells.
8. To avoid carryover contaminations change the tip between samples and different kit controls.
9. It is important to wash microwells thoroughly and remove the last droplets of wash buffer to achieve best results.
10. All incubation steps must be accurately timed.
11. Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay.
12. Do not re-use microplate wells.

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.

PREPARATION OF REAGENTS

Preparation of sample buffer

Dilute the contents of each vial of the sample buffer concentrate (5x) with distilled or deionized 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 wash solution

Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled or deionized 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.

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Sample preparation

Dilute all patient samples **1:100** with sample buffer before assay.
Therefore combine 10 µl of sample with 990 µl of sample buffer in a polystyrene tube. Mix well.

Controls are ready to use and need not be diluted.

TEST PROCEDURE

1. Prepare a sufficient number of microplate modules to accommodate controls and prediluted patient samples.
2. Pipet 100 µl of calibrators, controls and prediluted patient samples in duplicate into the wells.

| | 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...C: patient sample 1, 2 ...
C1: positive control
C2: negative control

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 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.
10. Add 100 µl of stop solution to each well of the modules and incubate for 5 minutes at room temperature.
11. Read the optical density at 450 nm and calculate the results. Bi-chromatic measurement with a reference at 600-690 nm is recommended.
The developed colour is stable for at least 30 minutes. Read optical densities during this time.

Automation

The AMA-M2 ELISA is suitable for use on open automated ELISA processors. The test procedure detailed above is appropriate for use with or without automation.

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INTERPRETATION OF RESULTS

Quality Control

This test is only valid if the optical density at 450 nm for Positive Control (1) and Negative Control (2) as well as for the Calibrator A and F complies with the respective range indicated on the Quality Control Certificate enclosed to each test kit !

If any of these criteria is not fulfilled, the results are invalid and the test should be repeated.

Calculation of results

For AMA-M2 ELISA a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice.

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 ELISA. These data are intended for illustration only and should not be used to calculate results from another run.

| Calibrators | | | | | | | | | |
|-------------|----------|-------|-------|-------|---------|---------|------|-------------|------|
| 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 |

Interpretation of results

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

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

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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.

PERFORMANCE CHARACTERISTICS

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 (Reproducibility)

Statistics for coefficients of variation (CV) 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 of each sample:

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

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

Sensitivity

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

Specificity

The microplate is coated with highly purified mitochondrial M2 subtype antigen. The AMA-M2 test kit recognizes only autoantibodies specific to the proteins of the keto acid dehydrogenase complex. No cross reactivities 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.

LIMITATIONS OF PROCEDURE

The AMA-M2 ELISA is a diagnostic aid. A definite clinical diagnosis should not be based on the results of a single test, but should be made by the physician after all clinical and laboratory findings have been evaluated.

Not all primary biliary cirrhosis patients are positive for AMA-M2.

DRG



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INTERFERING SUBSTANCES

No interference has been observed with haemolytic (up to 1000 mg/dL), lipemic (up to 3 g/dL triglycerides) or bilirubin (up to 40 mg/dL) containing sera.

Nor have any interfering effects been observed with the use of anticoagulants.

However for practical reasons it is recommended that grossly hemolyzed or lipemic samples should be avoided.

REFERENCES

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5. Fussey, S.P.M., Guest, J.R., James, O.F.W. et al. Identification and analysis of the major M2 autoantigens in primary biliary cirrhosis. PNAS, USA 1988; 85: 8654 - 8658.

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Symbols used with DRG Assays

| Symbol | English | Deutsch | Français | Español | Italiano |
|---|--------------------------------------|--|--|---------------------------------------|-------------------------------------|
|  | Consult instructions for use | Gebrauchsanweisung beachten | Consulter les instructions d'utilisation | Consulte las instrucciones de uso | Consultare le istruzioni per l'uso |
|  | European Conformity | CE-Konformitätskennzeichnung | Conformité aux normes européennes | Conformidad europea | Conformità europea |
|  | In vitro diagnostic device | In-vitro-Diagnostikum | Usage Diagnostic in vitro | Para uso Diagnóstico in vitro | Per uso Diagnostica in vitro |
|  | For research use only | Nur für Forschungszwecke | Seulement dans le cadre de recherches | Sólo para uso en investigación | Solo a scopo di ricerca |
|  | Catalogue number | Katalog-Nr. | Numéro de catalogue | Número de catálogo | Numero di Catalogo |
|  | Lot. No. / Batch code | Chargen-Nr. | Numéro de lot | Número de lote | Numero di lotto |
|  | Contains sufficient for <n> tests/ | Ausreichend für "n" Ansätze | Contenu suffisant pour "n" tests | Contenido suficiente para <n> ensayos | Contenuto sufficiente per "n" saggi |
|  | Storage Temperature | Lagerungstemperatur | Température de conservation | Temperatura de conservación | Temperatura di conservazione |
|  | Expiration Date | Mindesthaltbarkeitsdatum | Date limite d'utilisation | Fecha de caducidad | Data di scadenza |
|  | Legal Manufacturer | Hersteller | Fabricant | Fabricante | Fabbricante |
| Distributed by | Distributor | Vertreiber | Distributeur | Distribuidor | Distributore |
| Content | Content | Inhalt | Conditionnement | Contenido | Contenuto |
| Volume/No. | Volume / No. | Volumen/Anzahl | Volume/Quantité | Volumen/Número | Volume/Quantità |
| Symbol | Portugues | Dansk | Svenska | Ελληνικά | |
|  | Consulte as instruções de utilização | Se brugsanvisning | Se bruksanvisningen | Εγχειρίδιο χρήστη | |
|  | Conformidade com as normas europeias | Europeaisk overensstemmelse | Europeisk överensstämmelse | Ευρωπαϊκή Συμμόρφωση | |
|  | Diagnóstico in vitro | In vitro diagnostik | Diagnostik in vitro | in vitro διαγνωστικό | |
|  | | | | | |
|  | Catálogo n.º | Katalognummer | Katalog nummer | Αριθμός καταλόγου | |
|  | No do lote | Lot nummer | Batch-nummer | Αριθμός Παρτίδος | |
|  | | Indeholder tilstrækkeligt til "n" test | Innehåller tillräckligt till "n" tester | Περιεχόμενο επαρκές για «n» εξετάσεις | |
|  | Temperatura de conservação | Opbevarings-temperatur | Förvaringstemperatur | Θερμοκρασία αποθήκευσης | |
|  | Prazo de validade | Udløbsdato | Bäst före datum | Ημερομηνία λήξης | |
|  | Fabricante | Producent | Tillverkare | Κατασκευαστής | |
| Distributed by | | | | | |
| Content | Conteúdo | Indhold | Innehåll | Περιεχόμενο | |
| Volume/No. | Volume/Número | Volumen/antal | Volym/antal | Όγκος/αριθ.. | |