INTENDED USE

The VIRGO® Antimitochondrial Antibody (AMA) IgG indirect fluorescent antibody (IFA) test is intended for the detection and titration of to antimitochondrial IgG antibodies in human sera.

SUMMARY AND EXPLANATION

Various autoantibodies have been held responsible for a number of human diseases. In "autoimmune" disorders, the immune response alone is responsible for the pathology of the disease (e.g., lesions), as opposed to an organism or external agent causing the pathology.

Autoimmune diseases of the liver are purely speculative at this time, because no liver-specific antibodies have yet been demonstrated, and immunization of animals with liver extracts has not given rise to a convincing form of hepatitis or to progressive cirrhosis.[1]

Increasing evidence, however, indicates that three chronic liver syndromes (primary biliary cirrhosis, chronic active hepatitis, and cryptogenic cirrhosis) represent expression of an underlying process closely associated with the autoimmune phenomena.[2] In many ways it can be compared with other, more easily accepted autoallergic disorders.[3]

Of the various autoantibodies detected in the serum in all three diseases, mitochondrial antibody is of particular interest because it has been found in most patients with primary biliary cirrhosis (approximately 90%[11]), in a significantly smaller proportion of those with chronic active hepatitis or cryptogenic cirrhosis, and only rarely in other disorders.[3-6] This antibody, which is non-organ and non-specific specific, is directed against a lipoprotein constituent of the mitochondrial inner membrane.[8,11]

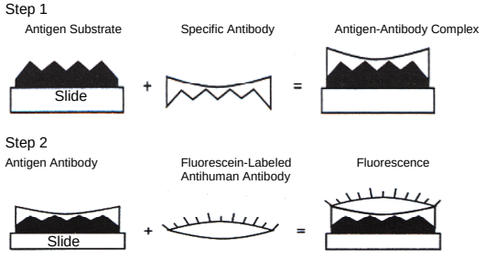
Tests for mitochondrial antibody has been recommended as a substitute for surgical exploration to provide confirmatory evidence when the diagnosis of primary biliary cirrhosis is suggested by the clinical laboratory or histological findings in the liver.[7]

The VIRGO AMA IFA kit manufactured by Hemagen Diagnostics, Inc., provides all the necessary reagents for the rapid determination of AMA in human sera. Antigenic substrate, control sera, FITC conjugate, buffer, coverslip mounting media and an instructional insert are included in the kit.

PRINCIPLE OF THE TEST

The VIRGO fluorescent antibody assays utilize the indirect method of fluorescent antibody staining, first described by Weller and Coons in 1954.[10] The procedure is carried out in two basic reaction steps. In step one, the human serum to be tested is brought into contact with the antigenic substrate. Antibody, if present in the test serum, will attach to the antigen, forming an antigen-antibody complex. If the serum being tested does not contain antibody for this particular antigen, no complex is formed and all the serum components are washed away in the rinse step. The second step involves adding a fluorescein labeled anti-human antibody to the test wells. If the specific antigen-antibody complex is formed in step one, the fluorescein labeled antibody will attach to the antibody moiety of the complex in step two. A positive reaction, bright apple-green fluorescence, can be seen with the aid of a fluorescence microscope.

Principle of Indirect Fluorescent Antibody Testing



CONTENTS OF THE KIT

902080	**Test Kit Product Number**
48 Tests	**Number of Tests per Kit**
12	**Slides (4-Well):** Noninfectious rabbit kidney cells, 50-70% confluent
1	**Vial Positive Control:** Lyophilized human serum
1	**Vial Negative Control:** Lyophilized human serum
1	**Vial FITC Conjugate:** Lyophilized, inactivated goat anti-human IgG (heavy and light chains) counterstain
3	***Packages Powdered Phosphate Buffer:** (PBS) pH 7.4 ± 0.2
1	***Vial (2 mL) Buffered Glycerol**
2	***Packages (5 each) Blotters**

*These components may be interchanged between different master lots. Additional supplies are available from Hemagen Diagnostics, Inc.

MATERIALS REQUIRED BUT NOT SUPPLIED

Test tubes and racks for making dilutions

Pipettes for preparing dilutions

Coverslips, 22 x 50 mm., No. 1 thickness

Humidified chamber

Magnetic stir plate (optional)

Staining dish and slide-holder rack

Fluorescence microscope. Refer to manufacturer's instruction manual for the filter system that gives optimum results for FITC (Maximum excitation wavelength = 490 nm. Mean emission wavelength = 520 nm.)

PRECAUTIONS

1. HANDLE ALL ASSAY SPECIMENS, SLIDES, POSITIVE AND NEGATIVE CONTROLS AS IF CAPABLE OF TRANSMITTING INFECTIOUS AGENTS.

   All human blood components of the kit have been tested by approved FDA methods and found to be negative for both hepatitis B surface antigen (HbsAg) and for antibodies to human immunodeficiency virus type 1. Because no test method can offer complete assurance that HIV, hepatitis B virus, or other infectious agents are absent, specimens and kit reagents should be handled at the Biosafety Level 2, as recommended for any potentially infectious human serum or blood specimen.[12,13]
2. The antigenic substrates are fixed in acetone.
3. Do not pipette by mouth.
4. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
5. All materials used in this assay, including reagents, samples and wiping materials should be disposed of in a manner that will inactivate infectious agents.

HANLDING PRECAUTIONS

1. **For *In Vitro* Diagnostic Use.**
2. Do not use the kit or individual reagents beyond their labeled expiration dates.
3. The components of this kit have been tested as a unit. **Do not** interchange components from other sources or from different master lots, except as noted.
4. Protect the conjugate from prolonged exposure to light.

REAGENT STORAGE AND STABILITY

1. Store kit at 2-8°C. Powdered PBS and Buffered Glycerol can be stored at 2-30°C if desired. The test kit can be used through the expiration date on the outer box label.
2. **After rehydration,** Positive Control, Negative Control and FITC Conjugate should be stored at 2-8°C or made up in aliquots and stored at -20°C or colder of not used within one week.

   NOTE: Precautions were taken in the manufacture of this product to protect the reagents from contamination. After reconstitution, care should be exercised to protect the reagents in this kit from contamination. If constant storage temperature is maintained, reagents and substrate will be stable for the dating period of the kit.

REAGENT PREPARATION

Allow reagents and slides to reach room temperature 15 to 30 minutes before use.

1. **Slides and Glycerol:** Ready to use.
2. **PBS:** Dissolve contents of one package in 1 liter of distilled or deionized water. Seal container to prevent contamination or evaporation.
3. **Controls:** Rehydrate with 1.0 mL of PBS. The controls are now at the appropriate working dilution.
4. **Conjugate:** Rehydrate with 2.0 mL of PBS. Aliquot for storage at -20°C.

SPECIMEN COLLECTION AND HANDLING

1. Serum samples may be stored at room temperature for up to 24 hours. For longer term storage, they may be stored at 2-8°C (for up to three days), or frozen at -20°C or colder. Place at 37°C only until the samples are thawed. Remove and mix thoroughly before use. Self-defrosting freezers are not recommended. Avoid multiple free-thaw cycles.[14]
2. Optimal performance of the VIRGO AMA IFA depends upon the use of fresh serum samples. Specimens should be collected aseptically. Early separation from the clot prevents hemolysis of serum.[15]
3. For best results, another sample should be drawn if bacteriological contamination or lipids are present. If another sample cannot be obtained, filtering (0.45µ) or centrifugation (approximately 3000 x G for 10 minutes) is required.
4. Excess lipids in the test serum may produce a "filming" reaction. The lipids "stick" nonspecifically to the glass and are extremely difficult to remove. Experience will enable the trained technician to differentiate this "film" reaction from the specific reaction.
5. Occasionally, the specimen may contain certain proteolytic enzymes which attack and digest the substrate. This is especially true of specimens contaminated with microorganisms. Such specimens may be heated to 56°C for 30 minutes. If this fails to reduce the enzymatic activity, another sample should be obtained from the patient.

TEST PROCEDURE

Specimens may contain infectious agents and should be handled accordingly.

For optimal results, DO NOT allow substrate wells to dry out while performing the test.

1. **Remove the slides and required reagents from the refrigerator** and allow them to reach room temperature (15 to 30 minutes).
2. **Remove the slides from the pouch** just before use and label.
3. **Rehydrate the controls to prepare the 1:10 screening dilutions.** These are now representative of typical positive and negative fluorescence patterns.
4. **Dilute the samples** with the PBS to the 1:10 screening dilution or prepare serial two-fold dilutions for quantitative determination, beginning with the 1:10 screening dilution.
5. **It is necessary to test 1 Positive Control, 1 Negative Control, and 1 PBS Buffer Control** for each batch of slides tested.
6. **Cover each well with diluted samples or controls** (~10-20 µL per well).
7. **Incubate in a humidified chamber at 23 ± 2ºC for 30 minutes.**
8. **Rinse the slides briefly** in a light stream of PBS. Do not direct the stream into the wells.
9. **Rinse the slides thoroughly for 7 minutes** in a staining dish of PBS. **Change the buffer and wash for an additional 8 minutes.** Handle slides gently. Gentle agitation of the buffer is necessary for efficient slide washing.
10. **Blot the painted mask of the slide** with the blotters provided. **Do not allow the wells to dry before conjugate addition.**
11. **Cover each well with one drop** (~10 µL) **of FITC Conjugate.**
12. **Incubate in a humidified chamber at 23 ± 2ºC for 30 minutes.** Protect from intense light.
13. **Repeat steps 7 and 8. Blot the painted mask of the slide** with the blotters provided. **Do not allow wells to dry before the addition of glycerol.**
14. **Place a small drop of Buffered Glycerol in each well** and cover with a coverslip.
15. For best results, **the slides should be read immediately at a magnification of 200-500X.** Alternatively, the slides may be read within 24 hours. However, they should be stored at 2-8°C in the dark, and sealed to prevent the mounting fluid from drying.

CRITERIA FOR GRADING FLUORESCENCE INTENSITY

4+	Brilliant apple-green fluorescence
3+	Bright apple-green fluorescence
2+	Clear distinguishable apple-green fluorescence
1+	Dull apple-green fluorescence, lacking in sharpness but readable
0	No fluorescence or barely visible fluorescence

GUIDELINES FOR CHARACTERIZING FLUORESCENCE

AMA Associated Fluorescence Pattern

Fine or granular-appearing staining of the mitochondria (within the cytoplasm) without fluorescent staining of the nucleus or nucleoli.

No Fluorescence

Absence of or less than 1+ specific fluorescence within the cytoplasm of the cells.

NOTES:

1. Samples that show a positive reaction at 1:10 should be tittered to determine the endpoint of reactivity.
2. In a quantitative determinations, the endpoint titer is the highest dilution showing a 1+ fluorescence in the inclusions.

QUALITY CONTROL

1. The control sera are representative of positive and negative reactions. At the 1:10 screening dilution, the Positive Control represents a strong (3-4+) reaction. If the fluorescence intensity of the Positive Control is less than the acceptable range, the test is invalid and should be repeated.
2. Each lot of Positive Control must be titrated to an endpoint dilution. The endpoint titer must be within ± one two-fold serial dilution of the Positive Control titer reported in the VIRGO AMA IFA 1+ Dilution Notice. If the results obtained are out of range, the test is invalid and should be repeated.
3. At the screening dilution, the Negative Control should not display apple-green fluorescence. If apple-green fluorescence is observed, the test is invalid and should be repeated.
4. Quality Control results were obtained on a Nikon® microscope equipped for epiilluimination with a 50W HBO mercury ARC lamp, B filter system for FITC and a 40X dry objective (NA 0.65). Differences in endpoint reactivity and fluorescence intensity may be affected by the type and condition of fluorescence equipment used (see Microscope Specifications at the end of the package insert).

INTERPRETATION OF SAMPLE RESULTS

RESULTS	SIGNIFICANCE
Screening:	
No fluorescence or fluorescence intensity < 1+ at the 1:10 screening dilution.	No detectable AMA antibodies by the IFA test.
≥1+ fluorescence at 1:10 or 1:40 on a single serum sample.	Positive by IFA for AMA antibodies. Titer for endpoint of reactivity.
Positive fluorescence at greater than 1:80 on a single serum sample.	Strongly suggestive of liver disease, particularly primary biliary cirrhosis.

The presence of antimitochondrial antibodies is in no way related to the duration of symptoms, suggesting that the antibody appears early in the course of the disease and persists.

LIMITATIONS OF THE PROCEDURE

1. An AMA titer is rarely helpful in distinguishing between various hepatic disorders. Patients may show wide variation in AMA titers depending upon clinical states of the disease.[11]
2. Routine screening of serum for alkaline phosphatase and cholesterol by automated methods and testing for antimitochondrial antibody in those patients with raised levels detect early stages of primary biliary cirrhosis.[9]
3. Results of this test should be interpreted in the light of other clinical findings and diagnostic procedures.

Results of Tests for Antimitochondrial Antibody[9]

Diagnosis	Positive Tests/Total # Pts.	% Positive
Primary Biliary Cirrhosis	158/188	84%
Chronic Active Hepatitis	8/77	11%
Cryptogenic Cirrhosis	2/33	6%
Other Forms of Cirrhosis	0/244	0%
Viral Hepatitis	0/332	0%
Drug Induced Hepatitis	3/73	4%
Other Forms of Hepatitis	0/87	0%
Extrahepatic Biliary Obstruction	4/180	2%
Nonobstructive BiliaryTract Disease	0/66	0%
Hepatic Malignancy	0/124	0%
Miscellaneous Hepatic Lesions	2/230	1%
Collagen Diseases	1/62	2%

MICROSCOPE SPECIFICATIONS

Comparable filter systems are shown below:

Transmitted Light Fluorescence:

Light Source	Exciter Filter	Barrier Filter
Mercury Vapor 200W 50W	**KP490 + BG38 (4 mm.) or BG12 (4 mm.) + BG38 (4 mm.)**	**KP510, K530**
Tungsten Halogen 50W 100W	KP490 + BG38 (4 mm.)	KP510, K515, K530

Incident Light Fluorescence:

Light Source	Exciter Filter	Dichroic Beam Splitting Mirror	Barrier Filter
Mercury Vapor 200W 100W 50W	**KP500 + BG38 (4 mm.) or BG23 (4 mm.) for stronger red suppression. Edgefilter 450 nm., 480 nm. For narrow band excitation, suppression of tissue auto-fluorescence**	**TK-510**	**K510 K520**
Tungsten Halogen 50W 100W	**KP500 + BG38 (4 mm.)**	**TK510**	**K510 K515 K530**

BIBLIOGRAPHY

1. **Parnetto, F and H Popper**. 1969. "Hetero, Iso, and Autoimmune Phenomena in the Liver," pp. 562-583. In Miescher, PA and HJ Muller-Eberhard, (eds.) *Textbook of Immunology,* Vol. 2 Grune Stratton, New York.
2. **Doniach, D, J Walker, I Roitt and P Berg.** 1970. Autoallergic hepatitis. N*ew England J. Med.,* **282:**86-88.
3. **Doniach, D and J Walker.** 1969. A unified concept of autoimmune hepatitis. *Lancet*. **1**:813-815.
4. **Walker, J, D Doniach and I Roitt, *et al.*** 1965. Serological tests in diagnosis of primary biliary cirrhosis. *Lancet,* **1**:827-831.
5. **Gouldie, R, R Macsween and D Goldbert.** 1966. Serological and histological diagnosis of primary biliary cirrhosis. *J. Clin. Path.* **19:**527-528.
6. **Kantor, F and G Klatskin.** 1967. Serological diagnosis of primary biliary cirrhosis: A potential clue to pathogenesis. *Trans. Assoc. Am. Phy.*, **80:**267-272.
7. **Sherlock, S.** 1968. Disease of the Liver and Biliary System, 4th edition, F.A. Davis Co., Philadelphia, pp. 310-313.
8. **Berg, PA, I Roitt and D Doniach.** 1969. Mitochondrial antibodies in primary biliary cirrhosis, IV. Significance of membrane structure for the complement fixing antigen. *Immunology,* **17**:281-293.
9. **Klatskin, G and F Kantor.** 1972. Mitochondrial antibody in primary biliary cirrhosis and other diseases. *Ann. Of Int. Med.* **77**:533-541.
10. **Weller, TH and AH Coons.** 1954. Fluorescent antibody studies with agents of varicella and herpes zoster propagated *in vitro*. *Proc. Soc. Exp. Biol. Med.* **86**:789-794.
11. **Bodenheimer, HC and F Schaffner.** 1979. State of the art. Primary biliary cirrhosis and the immune system. *Am. J. Gastroenterol.* **72**:285-296.
12. **NCCLS Doc M29-P.** 1988. *Protection of laboratory workers from infectious disease transmitted by blood and tissue, Proposed guideline.* National Committee for Clinical Laboratory Standards. Villanova, PA.
13. **CDC/NIH Manual.** 1988. Biosafety in Microbiological and Biomedical Laboratories, 2nd Ed. Pp 12-16.
14. **NCCLS Doc H18-T**. 1984. *Procedures for Handling and Processing of Blood Specimens.* National Committee for Clinical Laboratory Standards. Villanova, PA.
15. **NCCLS Doc H3-A2**. 1984. *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture, 2nd Edition: Approved Standard.* National Committee for Clinical Laboratory Standards. Villanova, PA.

SUMMARY OF VIRGO AMA IFA

IMPORTANT: It is recommended that one be familiar with the detailed procedure in the package insert before using this summary.

Cover wells with the 1:10 screening dilution of sample or control. For quantitative determination, prepare serial two-fold dilutions.

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Incubate slides in a humidified chamber at 23 ± 2ºC for 30 minutes.

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Rinse slides briefly with PBS. Wash for 15 minutes, changing buffer 1X. Blot slides.

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Cover each well with FITC Conjugate.

↓

Incubate slides in a humidified chamber at 23 ± 2ºC for 30 minutes.

↓

Repeat wash step described above.

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Place a small drop of Buffered Glycerol on each well and cover with a coverslip.

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Read slides immediately at 200-500X magnification on fluorescence microscope.

Antimitochondrial/ Antibody/ AMA IgG IFA

Immunofluorescence Test Kit for the Detection of Antimitochondrial IgG Antibodies

FOR *IN VITRO* DIAGNOSTIC USE

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