HISTOFLUOR® RODENT LKS FLUORESCENT ANTIBODY TEST SYSTEM

For in vitro Diagnostic Use
For Professional Use

INTENDED USE: This is an indirect fluorescent antibody test for the qualitative and semi-quantitative detection of IgG autoantibodies in human serum by manual fluorescent microscopy or with the Image Navigator® Fluorescence Semi-Automated Microscope. This test system is to be used as an aid in the detection of anti-mitochondrial (AMA), anti-parietal cell (APCA), and anti-smooth muscle (ASMA) autoantibodies associated with Type 1 Autoimmune Hepatitis, Primary Biliary Cholangitis, and Pernicious Anemia/Autoimmune Gastritis in conjunction with other laboratory and clinical findings. A trained operator must confirm results generated with the Image Navigator® semi-automated device and software.

SUMMARY AND EXPLANATION OF THE TEST

The autoantibodies found in humans are directed at a number of diverse antigens. Many of these antigens are highly conserved, so that virtually identical antigenic epitopes are found in the tissues of animals and humans. Thus, the use of animal tissues to detect these antibodies is a long-standing laboratory procedure. Many of the autoantibodies detected on animal tissue are closely associated with specific autoimmune diseases in humans. Detection and quantitation of specific autoantibodies allow for the diagnosis and monitoring of specific autoimmune diseases (1, 2).

PRINCIPLE OF THE TEST

The Immuno Concepts Histofluor® Rodent LKS Test System uses the indirect fluorescent antibody technique first described by Weller and Coons (3). Patient samples are incubated with antigen substrate (rodent liver/kidney/stomach) to allow specific binding of autoantibodies to cell components. If autoantibodies are present, a stable antigen-antibody complex is formed. After washing to remove non-specific and unbound antibodies, the substrate is incubated with an anti-human antibody conjugated to fluorescein. When results are positive, there is the formation of a stable three-part complex consisting of fluorescent antibody bound to human autoantibody, which is bound to cellular antigen. This complex can be visualized with the aid of a fluorescent microscope. In positive samples, the cells will show an apple-green fluorescence with a staining pattern characteristic of the particular antigen distribution within the cells. If the sample is negative for autoantibodies, the cells will not show a clearly discernible pattern of fluorescence.

SYSTEM COMPONENTS - MATERIALS PROVIDED

Use: All components come ready to use with no aliquoting or reconstitution required (except the PBS buffer which must be dissolved in deionized or distilled water before use).

Storage: All components can be stored under refrigeration at 2-10°C. After reconstitution, PBS buffer should be stored in screw cap containers at 2-25°C. Mounting medium can be stored at 2-25°C.

Stability: All components remain stable, both in the closed kit and after opening, at least 12 months from date of manufacture. Do not use any component beyond its expiration date.

REACTIVE REAGENTS

Substrate Slides SLIDE: Thin (approximately 4-5 micrometer) sections of rodent liver, kidney, stomach or kidney and stomach. The tissues have been fixed in a proprietary blend of acetone, alcohols, and other organic solvents to preserve the tissue proteins in their native configurations. Slides are available as 4-well slides (mouse, catalog number 12004-02; rat, catalog number 12004-03) and 8-well slides (mouse, catalog number 12008-02; rat, catalog number 12008-03). KS slides are available as 4-well slides (mouse, catalog number 12004-04; rat, catalog number 12004-05) and 8-well slides (mouse, catalog number 12008-04; rat, catalog number 12008-05).

Anti-Mitochondrial Antibody (AMA) Specific Positive Control CONTROL| + : Catalog No. 12021-02. Ready-to-use dropper vial containing 1.0 ml positive human control serum with IgG antibody specific to mitochondrial antigens. This serum demonstrates a positive anti-mitochondrial staining pattern on the rodent tissue substrate.

Negative Control Serum CONTROL| - : Catalog No. 12031. Ready-to-use dropper vial containing 1.0 ml negative human control serum. This control may demonstrate weak fluorescent staining, but it shows no discernable pattern.

Fluorescent Antibody Reagent CONJ|FITC: Catalog No. 12009-02 (9.0 ml), 12075-02 (23 ml). Goat anti-human IgG (gamma) conjugated to fluorescein isothiocyanate (FITC). Reagent comes ready-to-use in precision dropper bottles with 9.0 ml for every 10 slides in complete test kits.

Optional Positive Controls CONTROL| + :

Catalog No.12022-02 (0.5ml) – Anti-Parietal Cell Antibody (APCA). Ready-to-use dropper vial containing 0.5 ml positive human control serum with IgG antibody specific to parietal cell antigens. This serum demonstrates a positive anti-parietal cell pattern on the rodent tissue substrate.

Catalog No.12023-02 (0.5ml) – Anti-Smooth Muscle Antibody (ASMA). Ready-to-use dropper vial containing 0.5 ml positive human control serum with IgG antibody specific to smooth muscle antigens. This serum demonstrates a positive anti-smooth muscle staining pattern on the rodent tissue substrate.

Optional Titratable Control Sera TC:

Catalog No. 12261-02 (0.25ml) – AMA. Ready-to-use vial containing 0.25 ml anti-mitochondrial positive human control serum to be treated as an undiluted patient sample. See vial label for titer value.

Catalog No. 12262-02 (0.25ml) – APCA. Ready-to-use vial containing 0.25 ml anti-parietal cell positive human control serum to be treated as an undiluted patient sample. See vial label for titer value.

Catalog No. 12263-02 (0.25ml) – ASMA. Ready-to-use vial containing 0.25 ml anti-smooth muscle positive human control serum to be treated as an undiluted patient sample. See vial label for titer value.

NON-REACTIVE COMPONENTS

PBS Buffer Powder PWDR|PBS: Catalog No. 1011. Phosphate-buffered saline powder (0.01 M, pH 7.4 ± 0.2). Each pouch contains sufficient buffer powder to make 1 liter. (One pouch of buffer powder is supplied for every five slides in complete test kits.)

Preparation: Dissolve one pouch of buffer powder in 1 liter of deionized or distilled water, cover, and store between 2-25°C for up to four weeks or until signs of contamination or other visible changes occur.

Semi-permanent Mounting Medium SOLN|MM**:** Catalog No. 1111. Ready-to-use dropper vial containing 5.0 ml glycerol-based mounting medium.

Coverslips CVSLP**:** Catalog No. 1042. Each packet contains ten 24x64 mm No. 1 glass coverslips.

ADDITIONAL MATERIALS REQUIRED - BUT NOT PROVIDED

Volumetric pipettes to deliver 30-40 µl volumes
Coplin jars or staining dishes
Squeeze bottle or Pasteur pipettes
Serological pipettes
One-liter containers (for PBS buffer)
Deionized or distilled water
Test tubes to prepare serum dilutions
Bibulous paper or paper towels
Chamber for incubation
Disposable gloves
Lab timer
Fluorescent microscope equipped with 495 nm exciter filter and 515 nm barrier filter

PRECAUTIONS

1. All human source materials used for this product have been tested and found to be negative (not repeatedly reactive) for antibodies to Human Immunodeficiency Virus-1 (HIV-1), Human Immunodeficiency Virus-2 (HIV-2), hepatitis C virus (HCV), and for hepatitis B surface antigen (HBsAg) by FDA approved methods. However, no test method can offer complete assurance that HIV-1, HIV-2, hepatitis C, hepatitis B, or other infectious agents are absent. Thus, all kit materials should be handled in the same manner as potentially infectious materials.
2. All patient samples should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health Manual: *Biosafety in Microbiological and Biomedical Laboratories, 1999 Edition.*
3. Dilution of the components or substitution of components other than those provided in this system may yield inconsistent results.
4. Sodium azide (0.09%) is used as a preservative in some reagents. Sodium azide may react with lead or copper plumbing and form explosive metal azide salts. When disposing of reagents, flush with ample volumes of tap water to prevent potential residues in plumbing. Sodium azide is a poison and may be toxic if ingested.
5. This kit is for *in vitro* diagnostic use.
6. In the event hemolyzed or lipemic sera must be used, heat inactivate sera 30 minutes at 56°C for optimal results. Microbially contaminated sera should not be used.
7 Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
8. Avoid splashing or generation of aerosols at all times.
9. Incubation times and temperatures other than those specified may give erroneous results.
10. Cross contamination of reagents or samples may give false results.
11. Reusable glassware must be washed and thoroughly rinsed free of detergents prior to use. All glassware must be clean and dry before use.
12. Bring all reagents, slides, and specimens to room temperature (18-25°C) prior to use.
13. Wear disposable gloves when handling specimens and reagents, and wash hands thoroughly afterwards.
14. Microbial contamination of reagents or samples may give false results.
15. Never pipette by mouth and avoid contact of reagents and specimens with skin and mucous membranes. If contact occurs, wash with a germicidal soap and copious amounts of water.

SPECIMEN COLLECTION

Collection: Serum is the preferred specimen. Approximately 5 ml of whole blood should be collected aseptically by venipuncture using a sterile vacuum collection tube or other suitable collection system. Allow blood to clot at room temperature (18-25°C). Serum should be separated from the clot by centrifugation as soon as possible to minimize hemolysis.

Interfering Substances: Sera exhibiting a high degree of hemolysis, icterus, lipemia, or microbial growth should not be used because these conditions may cause aberrant results. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

Storage: Sera may be stored at 2-10°C up to one week. If testing is further delayed, sera should be stored frozen at –20°C or lower. Serum should not be stored in a self-defrosting refrigerator or freezer.

CAUTION: Repeated freeze/thawing of patient samples may yield false positive or false negative results.

INTERPRETATION OF RESULTS

QUALITY CONTROL

Positive, negative, and PBS controls should be tested one time per run. The positive control should show bright apple-green fluorescence in the appropriate tissue structures with a clearly discernible pattern characteristic of the control serum that was used. The negative control may show low intensity, nonspecific, dull green fluorescence in both the cytoplasm and nucleus, but with no discernible pattern of staining. The PBS control is used to observe non-specific staining by the antibody reagent and should not exhibit any green fluorescence. If the controls do not appear as described, the test is invalid and should be repeated.

It is important that the intensity of fluorescence not be confused with the presence or absence of antibodies. The key factor to consider in determining whether a given dilution of serum is positive is the appearance of a clearly discernible pattern, irrespective of the intensity of the fluorescent staining.

OPTIONAL TITRATABLE CONTROLS

The titratable control sera are intended for use in monitoring lot-to-lot and run-to-run reproducibility. They are not intended as a measurement of overall sensitivity or specificity of the assay.

When reading titers, many laboratories begin reading with the well that contains the most dilute sample and read “backwards” to the 1:20 dilution.

The mean titer and titer range (± one dilution on either side of the mean) determined for this lot number were established in our laboratory and are stated as a guide. This control is provided to allow each laboratory to assess the reproducibility (precision) of its testing. Since this control is not intended to be an indicator of titer accuracy, each laboratory should establish its own mean titer end-point for this sample and should use this information to assess run-to-run reproducibility (precision).

Through multiple testing of the titratable control, using the Immuno Concepts Histofluor® Rodent LKS Fluorescent Antibody Test System, a mean titer value has been established for each lot number. The lot number, mean titer and titer range (± one twofold dilution on either side of the mean) are stated on the vial label and should be used as a guide for the test system performance.

The values obtained in our laboratory may differ from your values.

Some of the many factors that can affect your results may include, but are not limited to:

1. The type of light source used. Mercury light sources will produce greater excitation energy at 495 nm than Quartz/Halogen. The 50-watt, 100-watt, and 200-watt Mercury light sources differ little in excitation energy at 495 nm. The 100-watt Quartz/Halogen light sources will produce greater excitation energy at 495 nm than 50-watt Quartz/Halogen.
2. The condition and age of the light source. This is particularly true for Mercury light sources, which generally exhibit a gradual reduction in excitation energy at 495 nm prior to burning out. This gradual reduction in excitation energy can result in a significant loss in sensitivity over several weeks. This problem can be avoided by keeping a time log. For best results, replace 50-watt mercury bulbs at 100 hours, and 100 or 200-watt mercury bulbs at 200 hours. Quartz/Halogen light sources generally do not exhibit a gradual reduction in excitation energy prior to burning out.
3. The type of exciter filter used. Interference exciter filters provide greater sensitivity over a much narrower wavelength than absorption exciter filters. Refer to your fluorescent microscope manual or sales representative for more information.
4. Proper alignment of the microscope light path. Refer to your fluorescent microscope manual for instructions.
5. The numerical aperture of the objective. With incident light fluorescence (Epi), fluorescence is increased exponentially as the numerical aperture (NA) of the objective is increased additively. This may cause a 40X objective with a NA of 0.65 to read one or more dilutions lower than a 40X objective with a NA of 0.85. The numerical aperture is printed on the side of the objective.
6. Suppression filters. Suppression filters reduce specific wavelengths of excitation and may be used in reducing

sensitivity. Refer to your fluorescent microscope manual or sales representative for more information.

7. Precision and accuracy of dilution technique, equipment, and performance of the test procedures.

INTERPRETATION OF PATIENT RESULTS

100X total magnification is recommended for screening positive/negative and titer end-point determination, while 200X total magnification is recommended for pattern recognition.

Negative: A serum is considered negative if it shows nonspecific, dull green fluorescence in both the cytoplasm and nucleus with no clearly discernible pattern. The tissue may demonstrate weak staining but with no clearly discernible pattern.

Positive: A serum is considered positive if the tissue shows a clearly discernible pattern of staining.

Titers: When reading titers, many laboratories begin reading with the well that contains the most dilute sample and read "backwards" to the 1:20 dilution. The first well in which a clearly discernible pattern is visible is the titer end-point. We recommend this technique for determining titer end-points. It is important that the intensity of staining not be confused with the presence or absence of anti-mitochondrial or anti-parietal cell antibodies. The key factor to consider in determining whether a given dilution of serum is positive is the appearance of a clearly discernible pattern, irrespective of the staining intensity.

REPORTING OF RESULTS

Screening: Results should be reported as positive or negative at the 1:20 dilution, and the staining pattern should be reported.

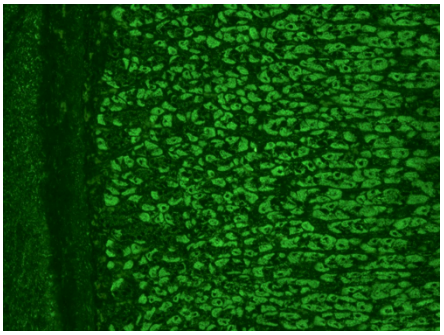
Titering: Results should be reported as the last serial dilution in which clearly discernible staining is seen. Results with a strong reaction at the 1:320 dilution should be reported as greater than 1:320.

PATTERN DETECTION

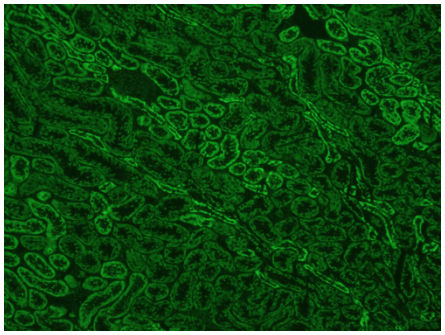
Anti-Mitochondrial Antibodies (**AMA):** Granular cytoplasmic staining of the Küpffer cells and hepatocytes in the liver section; granular cytoplasmic staining is also seen in the renal tubules, with the strongest staining in the distal tubules, which are more mitochondria-rich than the proximal tubules; the cytoplasm of gastric cells also shows granular staining, with stronger staining in the parietal cells than in the chief cells.

Antigen: The most commonly detected antigen in the mitochondria is M2, which is a complex of several antigenic peptides of the pyruvate dehydrogenase (PDH) complex.

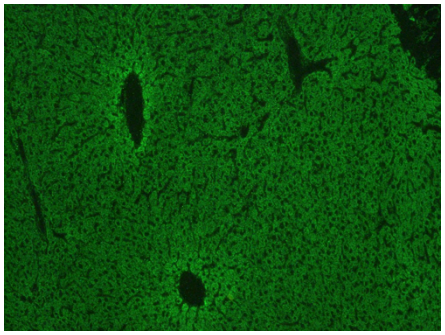
Disease Association: AMA are seen in 95% of patients with Primary Biliary Cholangitis (PBC) (4,5).



AMA on Rodent Stomach



AMA on Rodent Kidney

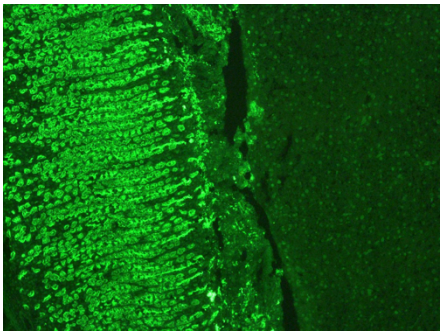


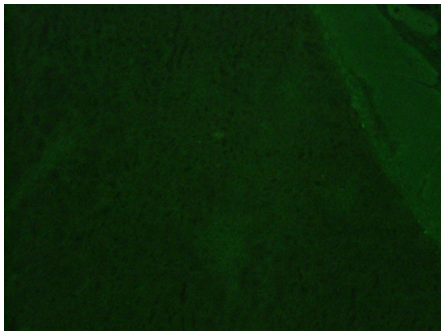
AMA on Rodent Liver

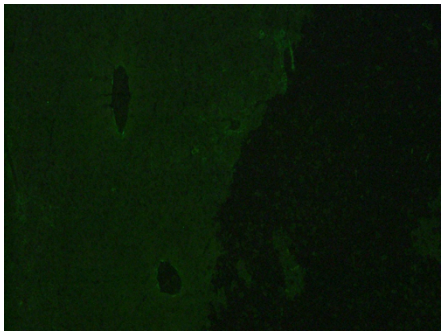
Anti-Parietal Cell Antibodies (**APCA):** In the stomach, the tissue shows bright granular cytoplasmic staining of the parietal cells in parallel linear pattern, with no staining of the chief cells. The antigen is not expressed in rodent kidney or liver, so these tissues are negative, with only background fluorescence.

Antigen: The antigen is the H+K+/ATPase proton pump.

Disease Association: APCA are present in more than 90% of patients with Pernicious Anemia. Atrophic gastritis is often a precursor to pernicious anemia, so antibodies can also be seen in patients with autoimmune atrophic gastritis (6,7).


APCA on Rodent Stomach

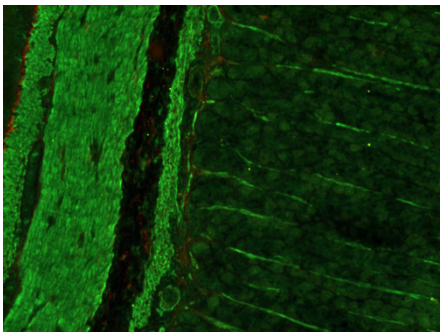

APCA on Rodent Kidney (Negative)

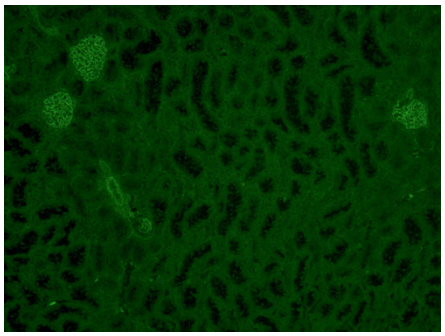

APCA on Rodent Liver (Negative)

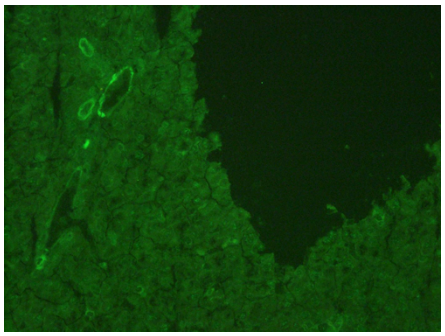
Anti-Smooth Muscle Antibody (ASMA): In the stomach, the tissue shows bright staining of smooth muscle fibers in the muscularis propria, the muscularis mucosa, the inter-gastric gland area, and the smooth muscle layers of arteries. In the liver, the tissue shows bright staining of smooth muscle fibers in the walls of the arteries. In the kidney, high titers of anti-actin antibodies may cross-react with tubulin fibers in the glomeruli.

Antigen: Actin is the major clinically important antigen, but antibodies to vimentin, tubulin, desmin, and other proteins in the smooth muscle fibers have also been described.

Disease association: Autoimmune chronic hepatitis (type 1) (8-10).


ASMA on Rodent Stomach


ASMA on Rodent Kidney


ASMA on Rodent Liver

LIMITATIONS OF THE TEST

1. Diagnosis cannot be made on the basis of rodent tissue antibody detection alone. The physician must interpret these results in conjunction with the patient's history and symptoms, the physical findings, and other diagnostic procedures.
2. Treatment should not be initiated on the sole basis of a positive test for antibodies. Clinical indications, other laboratory findings, and the physician's clinical impression must be considered before any treatment is initiated.
3. Although a positive reaction may be highly suggestive of connective tissue disease, it should not be considered diagnostic but rather viewed as a part of the overall clinical history of a patient.
4. Staining patterns can change with progressive titration of sera. This phenomenon is generally due to the presence of more than one disease condition.
5. Because of the many options available in fluorescent microscopes, it is recommended that light sources, filters, and optics be standardized when comparing patient titers between laboratories.
6. Immuno Concepts recommends the use of cultured, transfected cells (HEp-2000®) to determine ANA and does not advocate the use of rodent tissue for ANA detection or identification.

Performance Characteristics

The Histofluor® Rodent LKS Test System was compared to another rodent tissue immunofluorescent antibody test kit that is in commercial distribution using 170 clinically characterized samples. The samples were tested at three sites and read by three operators at each site. Using conventional fluorescent microscopy, the data below were obtained. The agreement values represent consensus adjudication of best two out of three calls from the three sites.

Rat tissue
Positive agreement: 92.3% (79.0 – 98.1)
Pattern agreement: 92.3% (79.0 – 98.1)
Negative agreement: 97.8% (93.4 – 99.5)
Overall agreement: 98.2% (94.7 – 99.6)

Mouse tissue
Positive agreement: 94.4% (80.9 – 99.4)
Pattern agreement: 94.4% (80.9 – 99.4)
Negative agreement: 98.5% (94.5- 99.9)
Overall agreement: 98.8% (95.5 - 99.9)

USE WITH THE IMAGE NAVIGATOR®
The Image Navigator® is Immuno Concepts' semi-automated microscope system for reading the Histofluor® fluorescent slides. A comparison of the Image Navigator® monitor reading to conventional reading of Histofluor® Rodent LKS Test System fluorescent rodent tissue slides was performed using 170 clinically characterized samples tested at three sites and read by three operators at each site. The agreement values below represent consensus adjudication of best two out of three calls from the three sites.

Rat tissue
Positive agreement: 100% (87.9 – 100)
Pattern agreement: 100% (87.9 – 100)
Negative agreement: 100% (96.6 – 100)
Overall agreement: 100% (97.3 – 100)

Mouse tissue
Positive agreement: 100% (88.5 – 100)
Pattern agreement: 100% (88.5 – 100)
Negative agreement: 100% (96.7 – 100)
Overall agreement: 100% (97.3 – 100)

Clinical sensitivity for detection of autoantibodies in patients known to have Pernicious Anemia, Type 1 Autoimmune Hepatitis, or Primary Biliary Cholangitis is shown in the tables below. In these studies, a cutoff dilution of 1:20 was used to determine positive samples.

Clinical Sensitivity (95% CI)		Histofluor® Mouse Liver/Kidney/Stomach			
Diagnosis	N	Conventional	% Positive	Image Navigator®	% Positive
Pernicious Anemia	12	5	41.7% (19.3-68.1)	5	41.7% (19.3-68.1)
Type 1 Autoimmune Hepatitis	19	9	47.4% (27.3-68.3)	9	47.4% (27.3-68.3)
Primary Biliary Cholangitis	18	15	83.3% (60.0-95.0)	15	83.3% (60.0-95.0)

Clinical Sensitivity (95% CI)		Histofluor® Rat Liver/Kidney/Stomach			
Diagnosis	N	Conventional	% Positive	Image Navigator®	% Positive
Pernicious Anemia	12	6	50.0% (25.4-74.6)	6	50.0% (25.4-74.6)
Type 1 Autoimmune Hepatitis	19	9	47.4% (27.3-68.3)	9	47.4% (27.3-68.3)
Primary Biliary Cholangitis	18	15	83.3% (60.0-95.0)	15	83.3% (60.0-95.0)

Clinical specificity was determined in a group of 121 patients with autoimmune diseases other than Pernicious Anemia, Type 1 Autoimmune Hepatitis, or Primary Biliary Cholangitis. The data from these comparisons are shown in the table below. In these studies, a cutoff dilution of 1:20 was used to determine positive samples.

Clinical Specificity (95% CI)		Histofluor® Mouse Liver/Kidney/Stomach			
Group	N	Conventional	% Negative	Image Navigator®	% Negative
Autoimmune Patients	121	114	94.2% (88.3-97.4)	114	94.2% (88.3-97.4)

Clinical Specificity (95% CI)		Histofluor® Rat Liver/Kidney/Stomach			
Group	N	Conventional	% Negative	Image Navigator®	% Negative
Autoimmune Patients	121	113	93.4% (87.3-96.8)	113	93.4% (87.3-96.8)

Precision: According to CLSI EP05-A3 requirements, thirteen samples were tested in triplicate in ten test events at three independent sites. The sample set included four AMA positive samples, four APCA positive samples, four ASMA positive samples, and one negative sample. Each result was read conventionally and on the Image Navigator® by two readers. To demonstrate the data in a semi-quantitative format, titer endpoint agreement is shown below for within sites and between sites. All agreements were > 90.0%.

Multi-site, Multi-reader Reproducibility (% Agreements w/ 95% CI)								
Substrate	Measure	*n*	Conventional			Monitor (Semi-automated)		
			Site 1 v 2	Site 1 v 3	Site 2 v 3	Site 1 v 2	Site 1 v 3	Site 2 v 3
RAT	Positive	720	100.0% (99.4-100)	100.0% (99.4-100)	100.0% (99.4-100)	100.0% (99.4-100)	100.0% (99.4-100)	100.0% (99.4-100)
	Negative	60	100.0% (92.8-100)	100.0% (92.8-100)	100.0% (92.8-100)	100.0% (92.8-100)	100.0% (92.8-100)	100.0% (92.8-100)
	Pattern	720	100.0% (99.4-100)	100.0% (99.4-100)	100.0% (99.4-100)	100.0% (99.4-100)	100.0% (99.4-100)	100.0% (99.4-100)
	± 1 Titer	720	95.4% (93.6-96.7)	95.8% (94.1-97.1)	96.5% (94.9-97.7)	95.3% (93.5-96.6)	95.3% (93.5-96.6)	96.7% (95.1-97.8)
MOUSE	Positive	720	100.0% (99.1->99.9)	100.0% (99.4-100)	100.0% (99.1->99.9)	100.0% (99.1->99.9)	100.0% (99.4-100)	100.0% (99.1->99.9)
	Negative	60	100.0% (92.8-100)	100.0% (92.8-100)	100.0% (92.8-100)	100.0% (92.8-100)	100.0% (92.8-100)	100.0% (92.8-100)
	Pattern	720	100.0% (99.1->99.9)	100.0% (99.4-100)	100.0% (99.1->99.9)	100.0% (99.1->99.9)	100.0% (99.4-100)	100.0% (99.1->99.9)
	± 1 Titer	720	94.9% (93.0-96.3)	97.8% (96.4-98.7)	94.4% (92.5-95.9)	96.8% (95.2-97.9)	98.9% (97.8-99.5)	96.5% (94.9-97.7)

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In the event of damage to the protective packaging, please contact Immuno Concepts prior to use.


Manufacturer

Authorized Representative in the European Community


Temperature Limitation

Contains Sufficient for <n> tests


Consult Instructions for Use

In Vitro Diagnostic Medical Device

MDSS GmbH
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Rx Only – For Prescription Use Only

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HISTOFLUOR® RODENT TISSUE FLUORESCENT TEST PROCEDURE

NOTE: If the laboratory is using an automated sample processing system, the procedure and recommendations of the manufacturer of the processor should be followed. The slide processing system should be programmed for the appropriate sample dilutions, dispense volumes, and incubation times as outlined below.

1. **RECONSTITUTE BUFFER (PBS)**
   Dissolve contents of one buffer pouch in one liter of deionized or distilled water. The PBS buffer may be covered and stored at 2-25°C up to four weeks.

2. **DILUTE PATIENT SAMPLES**
   Screening: Dilute patient samples to 1:20 by adding 0.05 ml (50 µl) serum to 0.95 ml (950 µl) reconstituted PBS.
   Semi-Quantitative Titering: Make serial dilutions of screening sample(s), and the titratable control (e.g. 1:40, 1:80, 1:160, 1:320) using PBS.

3. **PREPARE SUBSTRATE SLIDES** (30-40 µl/well)
   Remove slide(s) from pouch(es) and place control sera on control wells as follows: Invert control dropper bottle and squeeze gently until drop is visible at the tip. Gently touch the drop to appropriate control well while avoiding direct contact of dropper tip with the tissue or slide surface. Add 1 drop (30-40 µl) patient sample to the numbered wells.
   NOTE: For general screening, the anti-mitochondrial positive control is recommended. For semi-quantitative titering, a Titratable Control, either catalog number 12261-02, 12262-02, or 12263-02 should be run with each batch of patient samples. All pattern controls should be run with each lot number of kits to demonstrate the expected appearance of the ANA patterns.
   CAUTION: DIRECT CONTACT OF DROPPER TIP WITH SLIDE SURFACE MAY RESULT IN DAMAGE TO THE TISSUE ANTIGEN SUBSTRATE.

4. **INCUBATE SLIDES** (30 ± 5 minutes at room temperature, i.e. 18-24°C)
   Place slide(s) into a moist covered chamber (a petri dish with moistened paper toweling will be adequate). Incubate, with lid in place, for 30 minutes (± 5 minutes) at room temperature (18-24°C).

5. **PBS RINSE**
   Remove slide(s) from incubator tray and rinse briefly with PBS using a squirt bottle, Pasteur, or serological pipette. Do not squirt buffer directly on the wells.
   NOTE: To avoid cross contamination on the slides, direct PBS stream along midline of slide, tilting first toward the upper row of wells followed by tilting toward the lower row of wells.

6. **PBS WASH** (10 minutes)
   Wash slide(s) 10 minutes with PBS in a slide staining dish or Coplin jar. This wash may be extended 10-30 minutes with no variability in final test results. Discard PBS wash solution after use.

7. **FLUORESCENT ANTIBODY REAGENT** (Cover the wells with 12-14 drops)
   Remove one slide at a time from PBS. Tap slide on its side against bibulous paper or paper toweling to remove excess buffer. Immediately return slide to the incubation chamber and cover the wells completely using fluorescent antibody reagent; begin by placing a drop over each well. Repeat for each slide. Fluorescent antibody reagent has been titered to compensate for residual buffer remaining on the slide after rinsing.
   NOTE: It is important that slide wells do not dry during this procedure or damage to the substrate may occur.
   DO NOT BLOT OR DRY THE SLIDE IN ANY MANNER OR ALLOW SLIDE TO SIT WITHOUT FLUORESCENT ANTIBODY REAGENT FOR LONGER THAN 15 SECONDS.

8. **INCUBATE SLIDES** (30 ± 5 minutes at room temperature, i.e. 18-24°C)
   Place lid on incubation chamber and cover with a paper towel to prevent exposure to light if the chamber is not opaque. Allow slide(s) to incubate 30 minutes (± 5 minutes) at room temperature (18-24°C).

9. **PBS RINSE**
   Remove slide(s) from incubator tray and rinse briefly with PBS. Do not squirt buffer directly on the wells.

10. **PBS WASH** (10 minutes)
    Wash slide(s) 10 minutes with PBS in a slide staining dish or Coplin jar. This wash may be extended 10-30 minutes with no variability in final test results when counterstain is not used.

11. **MOUNT COVERSLIP**
    Remove one slide at a time from PBS. Tap slide on its side against bibulous paper or paper toweling to remove excess buffer.
    DO NOT BLOT OR DRY THE SLIDE IN ANY MANNER OR ALLOW TO SIT WITHOUT COVERSLIP FOR LONGER THAN 15 SECONDS. Add 4-5 drops of semi-permanent mounting medium along midline of each slide. Carefully place coverslip in position, avoiding air pockets, by gently lowering coverslip from one end of the slide to the other.
    NOTE: Excess mounting medium on slide may result in high background fluorescence, due to light scattering, or lack of clear resolution of cells (blurred image). Excess mounting medium may be removed from slide by gently blotting coverslip with bibulous or lens paper while avoiding any direct movement of the coverslip.

12. **READ SLIDE**
    If the slide is to be read using conventional fluorescent microscopy, it can be read immediately, using the standard procedure of the laboratory. Slides must always be read by a trained user who is familiar with fluorescent tissue section interpretation and patterns. Consult the supplier of the fluorescent microscope for instructions for use of the microscope.

    If the slide is to be read using the Image Navigator®, Immuno Concepts' automated microscopy and image capture system, consult the Operator's Manual for the Image Navigator® system. The Image Navigator® captures digital images of each well and presents a panoramic view of the tissue section for review by the trained user. The Image Navigator® must only be operated by a trained user.

FOR TECHNICAL ASSISTANCE:
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