Triple IFA
- 96 determinations -

Indirect immunofluorescence assay for the determination of polyvalent antibodies to ANA/AMA/ASMA/APCA in human serum

Substrate: rat liver, stomach and kidney

**INTENDED USE**

Triple IFA is used for the qualitative and semi-quantitative determination of ANA/AMA/ASMA/APCA of IgA, IgG and IgM class in human serum on rat liver, stomach and kidney for the diagnosis of autoimmune diseases.

Auto-immune diseases are caused by a disorder of the cellular and/or humoral immunological reaction. These reactions which normally occur against external influences may under certain circumstances turn against the body itself and thereby cause various diseases.

The term anti-nuclear antibodies (ANA) comprises all antibodies which react with antigens in the cell nucleus. They are important markers in the diagnosis of the systemic lupus erythematosus (SLE), sclerodermas, Sjögren syndrome or various connective tissue diseases, commonly designated as MCTD. Although the ANA detection for these diseases proves highly important, the final diagnosis should be checked against the clinical results.

Antinuclear antibodies may also occur with non-auto-immune based diseases, especially in the case of infections, viral diseases, hepatitis, infectious mononucleosis, leukaemia, lymphomas, melanomas and others. Furthermore, ANA are often detected with chronic hepatitis, primary cirrhosis, thyroiditis and allergic encephalitis. Low ANA titres may occasionally be caused by medication or may be present in healthy persons.

Anti-mitochondrial antibodies (AMA) predominantly react with the inner membrane of the mitochondria (rich in phospholipids). AMA mostly appear with diseases such as primary biliary cirrhosis, pseudo-LE syndrome and various forms of chronic aggressive hepatitis. High titre AMA results are mainly found with non-suppurating gallbladder infections or primary biliary cirrhosis (positive results at about 90%). In these cases antibodies appear before the clinical symptoms and will hardly be influenced by therapy during the course of the disease. Low antibody titres are observed with scleroderma, Sjögren syndrome, rheumatoid arthritis and other autoimmune diseases.

ASMA: Antibodies against smooth, unstriated muscle occur in various liver diseases, for example acute and chronic hepatitis, primary biliary cirrhosis and other forms of liver cirrhosis. Furthermore, the detection of ASMA supports the diagnosis of SLE, infectious mononucleosis, breast and ovarian carcinoma and malignant melanomas.

APCA: Circulating antibodies against the structures of the parietal cell of the gastric mucosa are generally due to pernicious anemia. They may, however, also be detected with other diseases of the stomach (chronic atrophic gastritis, gastric ulcer), diseases of the thyroid (Hashimoto’s thyroiditis, myxedema), and more rarely with hypop ferric anemia, diabetes mellitus and in older patients.

**PRINCIPLE of the TEST**

Triple IFA is an indirect immunofluorescence assay for the qualitative and semi-quantitative determination of ANA/AMA/ASMA/APCA.

The antibodies of the diluted patient samples and controls react specifically with the antigens of the tissue sections immobilized on the slides. After an incubation period of 30 min at room temperature (RT), unbound serum components are removed by a wash step.

The bound antibodies react specifically with anti-human Ig (polyvalent) conjugated to Fluorescein-isothiocyanat (FITC). After an incubation period of 30 min at RT excessive conjugate is separated from the solid-phase immune complexes by an additional wash step.

Stained slides are read using a fluorescence microscope (excitation wavelength 490 nm, emission wavelength 520 nm). According to the histologic alignment of antigens in the tissue a specific fluorescent staining can be detected.

**PATIENT SAMPLES**

Specimen collection and storage

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. The samples may be kept at 2 - 8 °C for up to two days. Long-term storage requires - 20 °C. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at - 20 °C.
Lipaemic samples could bring about a film covering the cell substrate and should not be used. Contaminated samples should be avoided as they may contain proteolytic enzymes which might digest the cell substrate.

Preparation before use

Allow samples to reach room temperature prior to assay. Take care to agitate serum samples gently in order to ensure homogeneity.

**Screening**: Patient samples have to be diluted 1:20 (v/v) prior to the assay, e.g. 10 µl sample + 190 µl PBS buffer (made of C).

**Titration**: prepare a 4-fold serial dilution based on the 1:20 (v/v) dilution using PBS buffer solution (made of C), e.g. 100 µl sample dilution + 300 µl PBS (made of C), resulting the following dilutions: 1:20, 1:80, 1:320, 1:1280, etc.

### TEST COMPONENTS for 96 determinations

<table>
<thead>
<tr>
<th></th>
<th>Substrate slides</th>
<th>PBS Buffer</th>
<th>Conjugate</th>
<th>Mounting medium</th>
<th>Blotting templates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8 wells coated with rat liver, stomach and kidney</td>
<td>for 2 x 1000 ml PBS solution</td>
<td>anti-human IgG, heavy- and light-chain specific (sheep), labeled to FITC, containing Evans blue</td>
<td>glycerol solution, PBS buffered, pH 7,4 ± 0,2</td>
<td>covered in a sealed pouch</td>
</tr>
<tr>
<td>B</td>
<td>2 x 10 g</td>
<td>10 ml</td>
<td>3.0 ml</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>sealed in a foil pouch</td>
<td>ready for use</td>
<td>ready for use</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>dry substance</td>
<td>capped blue</td>
<td>capped white</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Materials required**

- micropipettes (10, 100, 1000 µl)
- disposable pipette tips
- disposable test tubes and rack
- graduated cylinders, volumetric flasks
- moist chambers
- plastic squeeze wash bottle
- coplin jars or staining dishes with slide racks
- distilled (or de-ionized) water
- fluorescence microscope (excitation wavelength 490 nm, emission wavelength 520 nm)

**Size and storage**

Triple IFA (85096) has been designed for 96 determinations.

The expiry date of each component is reported on its respective label and that of the complete kit on the box label.

After opening all kit components are stable for at least 2 months, provided proper storage.

Preparation before use

Allow all components to reach room temperature prior to use in the assay.

The substrate slides are individually covered in a sealed pouch. Allow the slides to reach room temperature before opening.

**PBS buffer preparation**: Plate content of a one-liter PBS packet into one-liter volumetric flask, add distilled water to the mark. Dissolve dry substance by stirring or shaking. Reconstituted buffer solution should have a pH of 7.4 ± 0.2.

Store the solution in a clean bottle at 25°C or lower. Stable for at least two months. Do not use if pH changes, or if a precipitate forms.

Avoid exposure of the conjugate to light.

### ASSAY PROCEDURE

1. Bring all reagents to room temperature (18…25°C) before use. Mix gently without causing foam. Remove slides from pouch immediately before use and identify slides using a permanent marking pen.
2. Apply 1 - 2 drops (30 - 50 µl) controls (P, N) 30 - 50 µl diluted patient samples onto the respective wells. Completely cover the immobilised tissue section. Do not touch antigen surface.
3. Incubate 30 min at RT (20…25°C) in a moist chamber.
4. Rinse gently with PBS solution (made of C) using a squeeze wash bottle. Do not focus the PBS stream directly onto the wells. To prevent cross contaminations avoid rinsing from one well across other wells. For multi row slides run PBS stream from the midline of the slide successively along both rows to the edge of the slide.
5. Wash 2 x 5 min in changing PBS solution in Coplin jars or staining dishes, agitate gently at entry and prior to removal.
6. Remove slides from the wash one at a time, shake off excess PBS tapping the edge of the slide onto absorbent towel, carefully dry around the wells using a blotting template (F). Apply one drop of conjugate (D) to each well of the slides, making sure each well is completely covered.
7. Incubate 30 min at RT (20-25°C) in a moist chamber, protected from direct light.
8. Rinse gently with PBS solution (made of C) using a squeeze wash bottle as described in 4.
9. Wash 2 x 5 min in changing PBS solution in Coplin jars or staining dishes, agitate gently at entry and prior to removal.
10. Remove slides from the wash one at a time, shake off excess PBS tapping the edge of the slide onto absorbent towel, carefully dry around the wells using a blotting template (F), apply 2-4 drops of mounting medium (E) across the slide. Rest the edge of a coverslip (G) against the bottom of the slide allowing the mounting medium to form a continous bead between coverslip and slide. Gently lower the coverslip from the bottom to the top of the slide, avoid air bubbles. Drain excess mounting medium from the edge of the slide with absorbent papera.
11. Read stained slides using a fluorescence microscope. Avoid longer exposition of one field of vision to minimize bleaching of FITC fluorescence.

**Preservation of slides**

It is recommended that slides are examined at the same day they are stained. If any delay is anticipated, store slides in a refrigerator (2…8°C) for some days. For long-term preservation, seal edges of slides using nail-varnish, store slides at −20°C.
**READING of the RESULTS**

**Fluorescence intensity**
Fluorescence intensity may be semi-quantititated following the guidelines established by the CDC, Atlanta, USA (3):
- $4^+ =$ maximal fluorescence, brilliant yellow-green
- $3^+ =$ less brilliant yellow-green fluorescence
- $2^+ =$ definite but dull yellow-green fluorescence
- $1^+ =$ very dim subdued fluorescence

The degree of intensity is not of clinically relevance and has only limited value as an indicator of titer. Differences in microscope optics, filters and light source may result in differences of +1 or more in intensity.

**Negative result**
A serum dilution is considered negative for ANA, AMA, ASMA or APCA if the fluorescence intensity is less than $1^+$ and the tissue lacks the specific fluorescence pattern. Tissue will appear reddish-orange due to Evans blue counterstain.

**Positive result**
A serum dilution is considered positive for presence of autoantibodies if the fluorescent staining is at an intensity of $1^+$ or greater with a clearly discernable pattern of fluorescence in the tissue sections.

The combined tissue section allows the differentiation of various antibodies within one test area and may thus be applied as a diagnostic test for the following autoimmune antibodies. (In case of diverse antibodies it is advisable to look for further diagnostic identification).

**ANA**: positive samples cause a fluorescence of the cell nuclei of the liver tissue. Highly positive samples should be titrated to determine hidden fluorescence patterns or mixed reactions. For the differentiation of various patterns (e.g. homogenous, nucleolar, speckled, centromere) the HEp-2 cell should be used as the substrate (REF 8000, 60 determinations; REF 8100, 120 determinations).

**AMA**:
- The presence of anti-mitochondrial antibodies displays a fine granular cytoplasmatic fluorescence of the renal tubules. The distal tubules are richer in mitochondria and therefore display a more intense fluorescence in contrast to the proximal tubules.

**ASMA**: The presence of ASMA is indicated by a fluorescence of the smooth muscle fibres of the blood vessels of kidney and stomach, of muscularis mucosa, tunica muscularis ventriculi, as well as the inter glandular contractile fibrillae of the stomach mucosa.

**APCA**: Finely granular fluorescence of the parietal cells in the gastric mucous membrane indicates APCA. Since AMA also reacts with parietal cells, anti-mitochondrial antibodies (renal tubules) should be excluded in the APCA assessment.

Beside these, several other autoantibodies can be identified on triple section combi substrate, e.g. antibodies to LKM (microsomes of liver and kidney), brush border antibodies (kidney tubules), antibodies to liver cell cytoplasm antigens.

**Titration**
If semi-quantitative titration is performed, the result should be reported as the reciprocal of the last dilution in which $1^+$ apple-green fluorescent intensity with a clearly discernable staining pattern is detected.

Using the recommended fourfold serial dilution the endpoint titer can be extrapolated:

<table>
<thead>
<tr>
<th>Titer</th>
<th>1:20</th>
<th>1:80</th>
<th>1:320</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative</td>
<td>$3^+$</td>
<td>$2^+$</td>
<td>-</td>
</tr>
<tr>
<td>positive</td>
<td>&lt; 20</td>
<td>≥ 20</td>
<td></td>
</tr>
</tbody>
</table>

**REFERENCE VALUES**

**CHARACTERISTIC ASSAY DATA**

**Cross-reactivity**
Cross-reactivity of other antibodies to the characteristic antigen structure is unknown.

**Precision and Reproducibility**
With this immunofluorescence assay, no difference in the interassay and Interlot variability by using the controls could be detected.
INCUBATION SCHEME

Triple IFA (85096)

Dilute patient sera: screening dilution / endpoint titration using PBS solution (made of C)

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bring all test reagents and slides to room temperature (20…25°C)</td>
</tr>
<tr>
<td>2</td>
<td>Dispense Controls P, N</td>
</tr>
<tr>
<td></td>
<td>Diluted patient samples</td>
</tr>
<tr>
<td>3</td>
<td>Incubate 30 minutes, room temperature (20…25°C)</td>
</tr>
<tr>
<td>4</td>
<td>Rinse with PBS solution (made of C)</td>
</tr>
<tr>
<td>5</td>
<td>Wash 2 x 5 minutes in changing PBS solution (made of C)</td>
</tr>
<tr>
<td>6</td>
<td>Dispense Conjugate (D)</td>
</tr>
<tr>
<td>7</td>
<td>Incubate 30 minutes, room temperature (20-25°C)</td>
</tr>
<tr>
<td>8</td>
<td>Rinse with PBS solution (made of C)</td>
</tr>
<tr>
<td>9</td>
<td>Wash 2 x 5 minutes in changing PBS solution (made of C)</td>
</tr>
<tr>
<td>10</td>
<td>Place coverslip; 3-4 drops Mounting medium (E) per slide, lower the coverslip (G) gently</td>
</tr>
<tr>
<td>11</td>
<td>Read using a fluorescence microscope</td>
</tr>
</tbody>
</table>

SAFETY PRECAUTIONS

- **This kit is for in vitro use only.** Follow the working instructions carefully. GA GENERIC ASSAYS GmbH and its authorized distributors shall not be liable for damages indirectly or consequentially brought about by changing or modifying the procedure indicated. The kit should be performed by trained technical staff only.
- The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for reconstituted reagents.
- The substrate slides are individually covered in a sealed pouch. Do not use if pouch has been punctured.
- Mixing of reagents from different kit lots and from other manufacturers could lead to differences in assay results.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept at 2 - 8 °C before use in the original shipping container.
- Some of the reagents contain small amounts of Sodium azide (< 0.1 %) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa. Sodium azide may react with lead and copper plumbing building highly explosive metal azides. Flush with sufficient water when disposing of reagents to prevent potential residues in plumbing.
- Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed:
  - Do not smoke, eat or drink while handling kit material,
  - Always use protective gloves,
  - Never pipette material by mouth,
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.

REFERENCES

5. Lyerla HC, Forrester FT: The Immunofluorescence (IF) test. In: Immunofluorescence methods in virology, USDHHS, Georgia, 1979, 71-81