INSTRUCTION MANUAL

REF **85048**

December 28, 2007

Triple IFA

- 48 determinations -

CE

IVD *In vitro* diagnostic device

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

Substrate: rat liver, stomach and kidney

REF	Catalogue number	**LOT**	Batch code
	Consult accompanying documents		Manufactured by
	Temperature limitation		Use by
	Consult operating instruction		Biological risk

GA GENERIC ASSAYS GmbH

Ludwig-Erhard-Ring 3

15827 Dahlewitz, Germany

Telephone: **+49 (0) 33708-9286 - 0**
Fax: **+49 (0) 33708-9286 - 50**

www.genericassays.com

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, lymphoma, 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 phosholipids). 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, Sjörgen 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 hypoferric anemia, diabetes mellitus and in older patients.

PRINCIPLE of the TEST

Triple IFA is an indirect immunfluorescence 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 48 determinations

A Ag 4	**Substrate slides** 4 wells coated with rat liver, stomach and kidney		12 sealed in a foil pouch
C BUF PBS	**PBS Buffer** for 2 x 1000 ml PBS solution		2 x 10 g dry substance
D CONJ	**Conjugate** anti-human IgG, heavy- and light-chain specific (sheep), labeled to FITC, containing Evans blue		3.0 ml ready for use dropper bottle capped blue
E MOUNT	**Mounting medium** glycerol solution, PBS buffered, pH 7,4 ± 0,2		3.0 ml ready for use dropper bottle capped white
F TEMPL	**Blotting templates**		12
G COVER	**Coverslips** (22 x 70 mm)	12	1
P CONTROL	**Positive control** antigen specificity and titer on the label (diluted human serum)	+	1.0 ml ready for use dropper bottle capped red
N CONTROL	**Negative control** (diluted human serum)	–	1.0 ml ready for use dropper bottle capped green

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 (85048) has been designed for 48 determinations.

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

Upon receipt, all components of the Triple IFA have to be kept at 2…8 °C, preferably in the original kit box.

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:
Place 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, if the solution turns cloudy, or if a precipitate forms.

Avoid exposure of the conjugate to light.

ASSAY PROCEDURE

- **Dilute patient sera according to test demands (screening, titration)**
- **Do not allow the substrate slides to dry during the test 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 **1 - 2 drops** 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 papaer.
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-quantitated 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 interglandular 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:

1:20	=	3+	
1:80	=	2+	
1:320	=	-	The extrapolated titer is 160.

REFERENCE VALUES

Triple IFA	Titer
negative	**< 20**
positive	**≥ 20**

It is recommended that each laboratory establishes its own normal and pathological values Triple IFA reference ranges for serum levels as usually done for other diagnostic parameters, too.

Test validity

Both the positive and negative control provided in the test kit must be included in each test run. These controls must be examined prior to reading test samples and should demonstrate the following results:

Negative control: The cells should exhibit less than 1+ fluorescence and appear reddish-orange due to the counterstain.

Positive control: Fluorescence of the tissue section with an intensity of 3+ to 4+.

A titered positive control allows checking the test sensitivity as well as the reactivity of the reagents and microscope optical system. The endpoint titer stated on the label should be reproduced within one twofold difference in titer (+/-).

If the above mentioned quality criteria are not met, repeat the test and make sure that the test procedure is followed correctly (incubation times and temperatures, sample and wash buffer dilution, wash steps etc.). In case of repeated failure of the quality criteria contact your supplier. A troubleshoting guide is available to check laboratory procedure.

Diagnostic Relevance

ANA

1:20-1:80	A positive reaction is seen frequently in rheumatoid arthritis (RA) and MCTD. An increase in titre may indicate active SLE. Constant titres are observed in chronic or treated autoimmune diseases.
>1:160	An acute autoimmune disease is indicated, whereby in 80% of cases SLE is present.

AMA

1:20-1:80	A positive reaction is found in several liver diseases.
>1:160	Indicates biliary cirrhosis. AMA titres remain constant over a long period of time, and despite therapy so that the determination of titre as a measure of therapy control is not useful.

ASMA

1:20-1:80	A positive reaction is found in several liver diseases, viral hepatitis and primary biliary cirrhosis. However the titres here may fall below the determination border. Low titres may be observed in patients with gallbladder infections, alcoholic cirrhosis, SLE and in 2% of the normal, healthy population
>1:160	Chronic active hepatitis is indicated. In contrast to viral hepatitis the titres fall only slightly and may persist for several years. Patients with infectious mononucleosis may also show high ASMA titres.

APCA The APCA titer provides no information about the disease state of the patient. The antibody determination should be evaluated together with the measurement of Intrinsic factor and/or histopathology results.

Limitations of Method

The detection of antibodies largely depends on the tissue section used.

Faint fluorescence staining with titres of 1:20 - 1:40, or ambiguity concerning the clinical picture should be confirmed in diagnostic monitoring after 3-4 weaks.

Endpoint titer determination may vary depending on type and condition of the fluorescence microscope used and depending on subjective judgement of different observers.

Samples and wash solutions contaminated with bacteria or fungi could cause unspecific staining of the cell culture substrate.

Proteolytic enzymes in patient samples could result in damage or loss of the tissue sections fixed on the slide.

Any clinical diagnosis should not be based on the results of in vitro diagnostic methods alone. Physicians are supposed to consider all clinical and laboratory findings possible to state a diagnosis.

CHARACTERISTIC ASSAY DATA

Cross-reactivity

Cross-reactivity of other antibodies to the caracteristic 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 (85048)

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

1	Bring all test reagents and slides to room temperature (20…25°C)		
		Controls	Patient samples
2	Dispense Controls P, N	1 - 2 drops (30 - 50 µl)	
	Diluted patient samples		30 - 50 µl
3	Incubate	30 minutes, room temperature (20…25°C)	
4	Rinse with PBS solution (made of C)		
5	Wash	2 x 5 minutes in changing PBS solution (made of C)	
6	Dispense Conjugate (D)	1 - 2 drops (30 - 50 µl)	1 - 2 drops (30 - 50 µl)
7	Incubate	30 minutes, room temperature (20-25°C)	
8	Rinse with PBS solution (made of C)		
9	Wash	2 x 5 minutes in changing PBS solution (made of C)	
10	Place coverslip; 3-4 drops Mounting medium (E) per slide, lower the coverslip (G) gently		
11	Read using a fluorescence microscope		

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

1. Tan EM: Antibodies to nuclear antigens (ANA) and their immunobiology and medicine. Adv Immunol 1982 33:167-240
2. Tan EM, Cohen AS, Fries JF et al.: The 1982 revised criteria for the classification of systemic lupus erythematosus. Athritis Rheum 1982 25:1271-7
3. Humbel RL: Autoanticorps et Maladies Auto-Immunes, Elsevier 2e edition, 1997
4. Zachou K, Rigopoulou E, Dalekos GN: Autoantibodies and autoantigens in autoimmune hepatitis: important tools in clinical practice and to study pathogenesis of the disease. J Autoimmune Dis 2004, 1:2
5. Lyerla HC, Forrester FT: The Immunofluorescence (IF) test. In: Immunofluorescence methods in virology, USDHHS, Georgia, 1979, 71-81