INSTRUCTION MANUAL

REF 82050

March 04, 2008

nDNA IFA plus

- 50 determinations -

IVD *In vitro* diagnostic device

Indirect immunofluorescence assay for the sensitive determination of IgG antibodies to native DNA in human serum

Substrate: Crithidia lucilliae

REF	Catalogue number	LOT	Batch code
	Consult accompa-nying documents		Manufactured by
	Temperature limitation		Use by
	Consult operating instruction		Biological risk

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INTENDED USE

nDNA IFA plus is used for the sensitive qualitative and semi-quantitative determination of IgG antibodies to native (double-stranded) DNA in human serum using immunoflorescence using indirect immunoflorescence assay on Crithidia lucilliae for the diagnosis of systemic lupus erythematosus. A special sample diluent is used for increasing the test sensitivity compared with conventional test.

Systemic autoimmune diseases such as SLE are characterized by the appearance of a variety of autoantibodies directed against cell components of the nucleus or plasma. Although significance and pathological relevance of some autoantibodies are not completely revealed yet, the detection of autoantibodies is widely established and plays an important role in the diagnostics of systemic autoimmune diseases.

SLE has an unknown etiology and is characterized by multiorgan pathology. SLE has a female predominance. The onset of the disease occurs usually during childbearing age.

Antibodies to dsDNA are the hallmark for SLE diagnostics and are included in the diagnostic criteria of the American College of Rheumatology for SLE (1,2).

For the detection of antibodies to native DNA in immunofluorescence assay the haemoflaggelate Crithidia lucilliae can be used. These microorganism contains a special organ used as reservoir for native DNA, the kinetoplast. A fluorescent staining of the kinetoplast indicates the presence of antibodies to native DNA with a very high specificity.

PRINCIPLE of the TEST

nDNA IFA plus is an indirect immunfluorescence assay for the sensitive determination of IgG antibodies to native, double-stranded DNA.

The antibodies of the diluted patient samples and controls react specifically with the antigens of the cell culture 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 IgG 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 native DNA in the Crithidia a specific fluorescence stainings can be detected: a fluorescence of the kinetoplast situated between the nucleus and the basal body near the flagellum in the Crithidia cell.

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 with sample diluent (B) **1:10** (v/v) prior to the assay, e.g. **10 µl sample + 90 µl sample dikluent (B)**.

Titration: prepare a 4-fold serial dilution based on the **1:10** (v/v) dilution using sample diluent (B),
e.g. **100 µl sample dilution + 300 µl sample diluent (B),** resulting the following dilutions: 1:10, 1:40, 1:160, 1:640, etc.

TEST COMPONENTS for 50 determinations

A Ag 5	**Substrate slides** 5 wells coated with Crithidia lucilliae cell culture		10 sealed in a foil pouch containing drying template
B DIL	**Sample diluent**		100 ml ready for use capped black
C BUF PBS	**PBS Buffer** for 2 x 1000 ml PBS solution		2 x 10 g dry substance
D CONJ	**Conjugate** anti-human IgG (sheep), labeled to FITC,		3.0 ml ready for use dropper bottle capped white
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**		10
G COVER	**Coverslips** (22 x 70 mm)	12	1
P CONTROL	**Positive control** titer on the label (diluted human serum)	+	1.0 ml ready for use dropper bottle capped green
N CONTROL	**Negative control** (diluted human serum)	–	1.0 ml ready for use dropper bottle capped red

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
- coverslips 24 x 60 mm
- fluorescence microscope (excitation wavelength 490 nm, emission wavelength 520 nm)

Size and storage

nDNA IFA plus (82050) has been designed for 50 determinations.

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

Upon receipt, all components of the nDNA IFA plus 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) with sample diluent (B)**
- **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. Pipette
   25 µl controls (P, N)
   25 or 30 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 the template. 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, rinse Evans blue solution with PBS, shake of excess PBS, carefully dry around the wells using the template, apply **2-4 drops** of mounting medium (E) across the slide. Rest the edge of a coverslip 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 (5):
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 nDNA IgG if the fluorescence intensity is less than 1+ and the cells lack the specific fluorescence pattern of the kinetoplast. A fluorescent staining of the Crithidia cell, of the nucleus alone or of the basal body in the Crithidia cell is considered negative for antibodies to nDNA.

Positive result

A serum dilution is considered positive for nDNA IgG if a homogeneous fluorescent staining of the kinetoplast or the nucleus **and** the kinetoplast within the Crithidia cell is seen at an intensity of 1+ or greater.

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:

1:10 = 3+
1:40 = 2+
1:160 = +/-
1:640 = - The extrapolated titer is 80.

REFERENCE VALUES

nDNA IFA plus	Titer
negative	< 10
positive	≥ 10

It is recommended that each laboratory establishes its own normal and pathological nDNA IgG 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 with lack of fluorescent staining of the kinetoplast. The cells could appear reddish-orange due to the counterstain used.

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

A titered positive control allows to check 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.

Limitations of Method

Antibodies to native DNA are highly specific for systemic lupus erythematosus (SLE). Although low levels of nDNA antibody may be observed in other rheumatic diseases e.g. Sjögren syndrome, Mixed Connective Tissue Diseae (MCTD) and dermatomyositis, high titres of nDNA antibody are detected almost exclusively in SLE. For this, a specific determination of antibodies to native DNA should be a necessary tool in the diagnosis of SLE.

On the other hand the sensitivity of the traditional detection of nDNA antibodies on Crithidia is less compared with other diagnostic methods. This modified assay using a special sample diluent for high sensitivity detection of nDNA antibodies increases the sensitivity. Nevertheless, in case of suspected SLE and negative result of the Crithidia assay a test based on other methodology should be done in addition.

Antibodies to other parts of the tissue section could lead to a respective fluorescence pattern (e.g. cell nuclei, intercellular substance). These patterns are to be judged negative in relation to endomysial antobodies but can indicate other autoimmune diseases.

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 a 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 are unknown.

Sensitivity

In a group of 57 SLE patients with positive dsDNA antibody Elisa test the sensitivity was increased from 45.6% in the conventional Crithidia lucilliae immunofluorescence assay (CLIFT) to 78.9% in the nDNA IFA plus (6).

Specificity

In a group of 96 non-SLE patients with negative dsDNA antibody Elisa results the specificity was 98.9 % in the nDNA IFA plus compared with 100 % in the conventional CLIFT (6).

Precision and Reproducibility

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

INCUBATION SCHEME

nDNA IFA plus (82050)

Dilute patient sera: screening dilution / endpoint titration using sample diluent (B)

1	Bring all test reagents and slides to room temperature (20-25°C)			
			Controls	Patient samples
2	Dispense	Controls P, N	25 µl	
		Diluted patient samples		25 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 drop (25 µl)	1 drop (25 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 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.

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