







ANA HEp-2 plus

- 120 determinations -



IVD *In vitro* diagnostic device

Indirect immunofluorescence assay for the determination of antibodies to nuclear and cytoplasmic antigens in human serum

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



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INTENDED USE

ANA HEp-2 plus is used for the qualitative and semi-quantitative determination of antibodies to nuclear and cytoplasmic antigens in human serum.

Systemic autoimmune diseases such as systemic lupus erythematosus (SLE), progressive systemic sclerosis (PSS), rheumatoid arthritis (RA), Sjögren's syndrome, dermatomyositis, and mixed connective tissue disease (MCTD) are characterized by the appearance of a variety of autoantibodies directed against components of the cell nucleus. 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 diagnosis of systemic autoimmune diseases (1, 2).

Immunofluorescence test on HEp-2 cells is a sensitive screening test for the detection of antinuclear antibodies (ANA). The recognition of staining patterns may aid in the identification of the antigen targeted by ANA and the associated autoimmune disorders (3, 4).

PRINCIPLE of the TEST

ANA HEp-2 plus is an indirect immunofluorescence assay for the qualitative and semi-quantitative ANA determination.

The antibodies of the diluted patient samples and controls react specifically with the antigens of HEp-2 cells immobilized on 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-immunoglobulin (IgG and light-chain specific) conjugated to Fluorescein-isothiocyanat (FITC). After 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 HEp-2 cell specific fluorescence stainings 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 **1:80 (v/v)** prior to the assay, e.g. **10 µl sample + 790 µl PBS buffer (made of C)**.

Titration: prepare a 4-fold serial dilution based on the **1:80 (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: 1:80, 1:320, 1:1280, 1:5120 etc.

TEST COMPONENTS for 120 determinations

A (9483) Ag 12	Substrate slides 12 wells coated with HEp-2 cells	10 sealed in a foil pouch with desiccant
C (9018) BUF PBS	PBS Buffer for 2 x 1000 ml PBS solution	2 x 10 g dry substance
D (9510) CONJ	Conjugate anti-human-IgG (heavy- and light-chain specific), labeled to FITC	10.0 ml ready for use capped blue
E (8008) MOUNT	Mounting medium glycerol solution, PBS buffered, pH 7,4 ± 0,2	3.0 ml ready for use dropper bottle capped white
F (8046) TEMPL	Blotting templates	10
P (9102) CONTROL	Positive control specification on the label (diluted human serum)	2.0 ml ready for use dropper bottle capped red
N (9302) CONTROL	Negative control (diluted human serum)	2.0 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
- coverslips 24 x 60 mm
- fluorescence microscope (excitation wavelength 490 nm, emission wavelength 520 nm)

Size and storage

ANA HEp-2 plus (8101) has been designed for 120 (10 x 12) 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 ANA HEp-2 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 or de-ionized water to the mark (the use of distilled water is recommended). Dissolve dry substance by stirring or shaking. Reconstituted buffer solution should have a pH of 7.4 ± 0.2. Adjust with 1N NaOH or HCl if the pH value is outside the stated range.

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. Pipette 1 drop (25 – 30 µl) controls (P, N) 25 µl diluted patient samples onto the respective wells. 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 12-well slides run PBS stream from the midline of the slide successiv 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 (F). Apply 1 drop (25 – 30 µl) 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, shake of excess PBS, dry around the wells using the template (F), 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 continuous layer 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 paper.
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 ANA if the HEp-2 cells exhibit less than 1+ fluorescence and lack a clearly discernable pattern. Cells will appear reddish-orange due to Evans blue counterstain.

Positive result

A serum dilution is considered positive for ANA if the fluorescent staining is at an intensity of 1+ or greater with a clearly discernable pattern of fluorescence.

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:

1:80	=	3+	
1:320	=	2+	
1:1280	=	+/-	
1:5120	=	-	The extrapolated titer is 640.

REFERENCE VALUES

ANA HEp-2 plus	Titer
negative	< 80
positive	≥ 80

In general titers of 80 and 160 are considered low positive, 320 and 640 are considered medium titers and 1280 and greater are considered high positive.

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

INTERPRETATION of RESULTS

According to the staining of nucleus and cytoplasm of HEp-2 cells different patterns have been described:

1. Homogeneous

Diffuse staining of the entire nucleus, with or without apparent masking of the nucleoli. Especially as the antibody reaches its endpoint the pattern may appear granular. The chromosome region of mitotic cells exhibits a bright positive staining pattern.

Antigens: DNA, histones

Disease association: High titers are suggestive of SLE, while low titers may be found in SLE and RA; histone antibodies alone have a high association with drug-induced lupus (6).

2. Peripheral

Smooth staining primarily around the outer region of the nucleus with weaker staining in the center. Not all cells within a well may appear peripheral, some cells may appear homogeneous. The chromosome region of mitotic cells exhibits a bright positive staining pattern.

Note: A thin distinct line around the nucleus without staining of the chromosome region in the mitotic cells is not a peripheral ANA but a nuclear membrane pattern.

Antigens: DNA, histones

Disease association: High titers are suggestive of active SLE; lower titers are suggestive of SLE and other connective tissue diseases (7).

3. Speckled

Fluorescent aggregates throughout the nucleus which can be very fine to very coarse depending on the type of antibody present. More than one type of speckle may be seen in one specimen. The chromosome region of mitotic cells is usually negative.

a.) Sm and nRNP antibodies usually present as coarse speckles, nucleoli often left unstained, chromosome region of mitotic cells negative.

Disease association: Sm antibodies are highly specific for SLE; appear to be a marker of this disease; high levels of nRNP antibodies alone are characteristic of MCTD, along with other types of ANA anti-nRNP are found in SLE, RA, PSS (8).

b.) SS-A and SS-B antibodies present as small uniform speckles in a uniform distribution with the chromosome region of mitotic cells negative.

Disease association: SS-A and SS-B antibodies are frequently present in patients with Sjögren's syndrome without associated RA, less frequently in patients with SLE, anti-SS-A are found in a high percentage of congenital heart block and neonatal lupus (9).

c.) Scl-70 antibodies are indicated as fine dense speckles with positive staining of the nucleoli and chromosome region of mitotic cells.

Disease association: Anti-Scl-70 appear to be a marker of PSS (8).

d.) PCNA antibodies present as varying fluorescence of fine to coarse speckles in approximately 30-60% of cells.

Disease association: Anti-PCNA has been found in a small percentage of patients with SLE (10).

4. Centromere

Discrete uniform speckles throughout the nucleus, the number corresponds to a multiple of the normal chromosome number. The staining pattern of the mitotic cells will follow that of the chromosomes, with pairs of dots arranging themselves in an equatorial plane during metaphase and then moving towards their respective centrosomes during anaphase.

Note: A similar staining (multiple nuclear dots) is caused by NSP-1 (SP100) antibodies. This antibodies can be differentiated from centromere antibodies in that the chromosome region of mitotic cells does not stain.

Antigens: centromere proteins of chromosomes

Disease association: Centromere antibodies are considered as marker of CREST syndrome, infrequently found in diffuse scleroderma and Raynaud's disease (11, 12).

5. Nucleolar

Fluorescent staining of the nucleoli within the nucleus, sharply separated from the unstained nucleoplasm. The nucleolar fluorescence may be homogeneous, speckled or clumpy. Frequently accompanied by a speckled pattern.

Antigens: PMScl, RNA polymerase I, fibrillarlin

Disease association: High titers are highly specific for PSS and overlap with polymyositis, with lower titers found in SLE, Sjögren's syndrome, Raynaud's disease (13).

6. Anti-spindle antibodies

A network of threads connecting the centrosomes in mitotic cells.

Antigens: spindle apparatus in cells undergoing mitosis

Disease association: Less frequent in some autoimmune and non-autoimmune diseases (RA, SLE, PBC, Carpal Tunnel Syndrome) (14).

7. Cytoplasmic fluorescence

Granular or fibrous fluorescence in the cytoplasm.

a.) Ribosomal RNP

Diffuse granular fluorescence throughout the cytoplasm (confirmation on appropriate tissue sections is recommended).

Disease association: Characteristic in a small percentage of cases of SLE (15).

b.) Jo-1 (PL-7, PL-12)

Fine speckles generally with low fluorescent intensity concentrated in the perinuclear region.

Disease association: polymyositis, dermatomyositis (16)

c.) Mitochondrial

Discrete speckles throughout the cytoplasm in a fibrous network (confirmation on appropriate tissue sections is recommended).

Disease association: Marker for Primary biliary cirrhosis (PBC).

d.) Cytoskeleton

Fluorescent strands in the cytoplasm in a spidery network, with fibrils extending from the cell membrane, caused by antibodies to actin (smooth muscle antibodies) and other components of the cytoskeleton (e.g. vimentin, tubulin), confirmation on appropriate tissue sections is recommended.

Disease association: Different, anti-Actin frequently in autoimmune hepatitis and infectious diseases.

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: The cells should exhibit a staining pattern as stated on the label with a fluorescent intensity of 3+ to 4+.

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 troubleshooting guide is available to check laboratory procedure.

Limitations of Method

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.

Up to 10% of the normal adult population show antinuclear antibodies. ANA are known to be age and sex related. With increasing age there is an increased ANA incidence. Therefore a low positive titer result maybe normal for certain individuals in the absence of other clinical signs. However, in normal young individuals ANA should not be found. SLE patients undergoing steroid therapy or in remission may have a negative ANA. Some positive ANA have been reported in relatives of patients suffering from a connective tissue disease who may develop such a disease at a later time.

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.

INCUBATION SCHEME

ANA HEp-2 plus (8101)

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 drop (25 - 30 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 – 30 µl)	1 drop (25 - 30 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 under protective gas. Do not use if pouch has been punctured, as indicated by a flat pouch.
- 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 Thimerosal (< 0.005 %) and Sodium azide (< 0.1 %) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- 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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