CYTOPLASMIC PATTERNS

Sera containing anti-mitochondrial and anti-smooth muscle antibodies as well as other non-defined cytoplasmic antibodies are evident on HEp-2 cells. The antibodies observed can be associated either with organs, molecular or cytoplasmic complexes, or the filaments of the cytoskeleton. The following fluorescence may also be observed:

Mitochondrial	Actin	Ribosomes	Trypomyosin	Lysosome
Vimentin	Peroxysomes	Cytokeratin	Golgi apparatus	

EXPECTED VALUES

1. A negative ANA test is expected in the normal population under the age of 50. A low titre positive ANA may be found in the normal population over the age of 50 (14).
2. The screening dilution is highly important for the evaluation of the positives and many authors recommend the use of a 1:80 dilution for the results to be significant.

PERFORMANCE CHARACTERISTICS

120 serum samples were tested on this ANA kit and on another commercial ANA kit. 71 were positive on both kits, one SSA serum gave a positive result on this kit but was missed by the other commercial kit and 48 were negative on both kits giving 100% relative sensitivity and specificity for this kit. All positive ANA patterns were the same on both kits and generally endpoint titres were similar.

TEST LIMITATIONS

1. No diagnosis should be based on a single serologic test since various host factors must be taken into consideration. Although the detection and identification of anti-nuclear or anti-cytoplasmic antibodies offers valuable assistance in the diagnosis of immunopathological disease but must be interpreted in the light of other biological data and the clinical context.
2. Substances such as drugs, viruses and bacteria, are likely to induce production of autoantibodies without there being any associated pathology or clinical condition (anti-histone antibodies, anti-nuclear matrix etc).
3. Certain drugs such as p-aminosalicylic acid, phenytoin, isoniazid, hydralazine, procainamide, etc. may induce autoantibody formation. (4, 12, 13) Positive results may also result in apparently healthy people and may be due to a host of other factors. A positive test suggests certain diseases but is not diagnostic and should be confirmed by clinical findings.
4. Anti-nuclear antibodies are rarely observed in the normal young population but the percentage of positive cases increases with age, reaching 12-18% over 60 years of age. The values are nonetheless low in this so-called "normal" population.

LITERATURE REFERENCES

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UK CA CE

INSTRUCTIONS FOR USE

ANA HEp-2 IFA Test System

Catalogue Number:

211140b - 140 Tests **211280b** - 280 Tests **211400b** - 1400 Tests

Also for:

14 well HEp-2 slides	**H149b**	12 well HEp-2 slides	**H129b**
HEp-2 FITC Conjugate (+EB)	**J501b/c**	HEp-2 Sample Diluent	**R008b/c**
HEp-2 Mounting Medium	**R007b**		

INTENDED PURPOSE

Bio-Diagnostics Ltd ANA HEp-2 IFA Test System is an indirect immunofluorescent assay (IFA) for the qualitative and/or semi-quantitative measurement of antinuclear antibodies in human serum. This test kit is for professional use only for the diagnosis of systemic autoimmune rheumatic diseases (SARD).

SUMMARY AND EXPLANATION

The presence or absence of antinuclear antibodies (ANA) are used in the diagnosis and classification criteria for several systemic autoimmune rheumatic diseases (1). These antibodies were first associated with systemic lupus erythematosus (SLE). Now ANA association includes Sjogrens Syndrome (SS), Systemic Sclerosis (SSc), Mixed Connective Tissue Disease (MCTD) and Polymyositis/Dermatomyositis. (2, 3, 4, 5, 6). Human epithelial type 2 (HEp-2) cells, originate from a human laryngeal carcinoma. As a substrate on IFA slides, they allow the recognition of over 30 different nuclear and cytoplasmic patterns that are given by more than 50 different autoantibodies and are associated with various autoimmune conditions (7,8). The Hep-2 IFA test is sensitive, screens for a wide variety of known and unknown antibodies and, through pattern recognition, enables experienced readers to the identity the antigen and associated autoimmune disorder. Hep-2 cells are cultured to maximise the number of mitotic figures that aid in pattern recognition (9, 10, 11). IFA ANA testing is currently considered the gold standard method for ANA screening and the first line screening tool for SARD (12).

PRINCIPLE OF THE TEST

Diluted human sera are incubated on the HEp-2 cell substrate. If antinuclear antibodies are present, they will bind to the cell nuclei forming antigen-antibody complexes. Fluorescein labelled anti-human immunoglobulin binds to these complexes. With a positive reaction, the nuclear pattern appears apple-green when viewed under a fluorescent microscope, whilst a negative reaction appears black or greenish-black.

Using Evans Blue counterstain, negative reactions lead to brownish-red patterns. The counterstaining intensity will vary according to the degree of counterstaining with the staining solution.

WARNINGS AND PRECAUTIONS

1. All human components in the controls have been screened and found to be negative for STS, HBsAg and antibodies to HIV-1, HIV-2 and HCV. However, these tests cannot guarantee the absence of infectious agents. All human components should be handled with appropriate care.
2. The controls, conjugate and sample diluent included in the kit contain thiomersal as a preservative. Also, the conjugate contains sodium azide. Although these preservatives are at a low concentration, these reagents should be considered toxic. They should not be ingested or allowed to come into contact with the skin or the mucous membranes. Sodium azide may also cause the formation of potentially explosive lead or copper azides in sinks.
3. Do not use components beyond their expiration date.
4. Follow the procedural instructions exactly as they appear in this insert to ensure valid results.
5. For in vitro diagnostic use only.
6. Handle slides by the edges since direct pressure on the antigen wells may damage the antigen.
7. Once the procedure has started do not allow the antigen in the wells to dry out. This may result in false negative test results, or unnecessary artefacts.
8. The sample diluent is formulated to reduce non-specific fluorescence observed in some samples at the 1/40 screening dilution. It must NOT be used to prepare serial dilutions for endpoint titre or for any wash steps.

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ANA HEp-2 IFA Test System

KIT CONTENTS
All reagents except the PBS are READY-TO-USE.
Each ANA HEp-2 IFA Test System contains:

SLIDE	HEp-2 substrate antigen slides - 14 well (H149b); 12 well (H129b)
CONJ IgG	FITC Conjugate with Evans Blue Counterstain: J501b/J501c. This reagent contains antibodies that will react with the human IgG (H+L) Immunoglobulin class
CONTROL -	Universal Negative Control: C000N/C000N-0.5
CONTROL +	ANA Homogenous Positive Control: C001N/C001N-0.5
MM	HEp-2 Mounting Medium: R007b
SPE DIL	HEp-2 Sample Diluent: R008b/R008c (Do not use if solution turns cloudy or if a precipitate forms)
IFA/DFA PBS	Phosphate Buffered Saline (PBS) buffer pack: R002b = 5 litre or R003b = 1 litre
	Coverslips

Note: All kit components are available separately. Please see the Bio-Diagnostics Ltd catalogue for more details.

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

Test tubes and rack or microtitre system	Disposable pipettes	Staining Dish and Slide Forceps
Moisture Chamber	Volumetric Flask (500 ml)	Distilled Water
Fluorescence Microscope	Paper Towels – lint free	

REAGENT PREPARATION
1. Buffer Pack no: R002b/R003b. Rehydrate buffer with distilled water as per instructions on the label and leave for several hours to dissolve. Reconstituted PBS should have a pH of 7.4±0.2. Adjust with 1N NaOH or 1N HCl if pH is outside the stated range. Store in a clean screw capped bottle at 25°C or lower. Stable until labelled expiry date provided no gross contamination is seen. Do not use if pH changes, if solution turns cloudy or a precipitate forms.

KEY FOR OTHER SYMBOLS
Used in this instruction leaflet and on accompanying product packaging:

Manufacturer	Contains sufficient for <n> tests	RFU	Ready for use
Temperature limitation	IVD		In vitro diagnostic medical device

STORAGE AND STABILITY
The ANA HEp-2 IFA Test System kit is to be stored at a temperature of +2ºC to +8ºC. Do not freeze the test kit. The stability of the kit is as indicated by the expiry date on the packaging under the above storage conditions. This applies to unopened and opened reagents.

SPECIMEN COLLECTION
Serological specimens should be collected under aseptic conditions. Haemolysis is avoided through prompt separation of the serum from the clot. Serum should be stored at 2-8°C if it is to be analysed within 2 days. Serum may be held for 3 to 6 months by storage at -20°C or lower. Lipaemic and strongly haemolytic serum should be avoided.

TEST PROCEDURE
Screening: dilute test sera 1/40 (1 part patient sample to 39 parts diluent) in HEp2 Sample Diluent.
This diluent must be used for the initial screening dilution of patient samples only.
Titration: set up doubling dilutions of serum continuing from 1/40 using PBS, (i.e. 1/80, 1/160, 1/320, etc.).

NOTE: The initial screening dilution (1/40) is made in the Sample Diluent, subsequent dilutions are made in PBS. Do NOT use Sample Diluent for serial dilutions.

1. Once slides reach room temperature tear slide envelope at notch. Carefully remove the slide and avoid touching the antigen areas. The slide is now ready to use.
2. Apply a drop of diluted serum (≈20µl) and controls to assigned antigen wells.
3. Place slide in a moist chamber for 30 minutes at room temperature (approximately 20-30ºC). Once procedure has started, slides must not be allowed to dry. Process one at a time to avoid drying.

ANA HEp-2 IFA Test System

4. Remove slide from moisture chamber and tap the slide on its side to allow the serum to run off onto a piece of paper towel. Using a wash bottle, use PBS to gently rinse remaining sera from slide being careful not to aim the rinse stream directly onto the well. **Never rinse with distilled or deionised water**.
5. Wash in PBS for 5 minutes. Repeat using fresh PBS.
6. Remove slide from PBS and absorb excess buffer. Avoid touching the wells. **Do not allow tissue to dry.**
7. Apply 1 drop (20-30µl) of conjugate to each antigen well. Repeat steps 3-6.
8. Place 4-5 drops of mounting medium on slide.
9. Apply a coverslip. Examine the slide under a fluorescent microscope. A 400x magnification pattern gives optimal patterns and allows easy identification. Note: To maintain fluorescence, store mounted slide in a moisture chamber placed in a dark refrigerator.

QUALITY CONTROL
1. Positive and negative serum controls must be included in each day's testing to confirm reproducibility, sensitivity and specificity of the test procedure.
2. The negative serum control should result in little or no fluorescence. If this control shows bright fluorescence, either the control, antigen, conjugate or technique may be at fault.
3. The positive serum control should show a homogeneous high-intensity (3+ to 4+) pattern. If this control shows little or no fluorescence, either the control, antigen, conjugate or technique may be at fault.
4. In addition to positive and negative serum controls, a PBS control should be run to establish that the conjugate is free from non-specific staining of the antigen substrate. If the antigen shows bright fluorescence in the PBS control repeat using fresh conjugate. If the antigen still fluoresces, either the conjugate or antigen may be at fault.

RESULTS
NEGATIVE:
A serum is considered negative when the fluorescent intensity of the nuclei approximates that of the negative control and there is no discernible pattern in the nucleus.

POSITIVE:
A serum is considered positive when the fluorescent intensity of the nuclei is greater than the negative control and there is a clearly discernible pattern in the nucleus. Fluorescent intensity may be determined by the guidelines established by the CDC, Atlanta, Georgia:
4+: Maximal brilliant green fluorescence; clear-cut cell with sharply defined cell centre.
3+: Less brilliant green fluorescence; clear-cut cell with sharply defined cell centre.
2+: Definite cell pattern but dim fluorescence; cell outline less well defined.
1+: Subdued fluorescence; cell almost indistinguishable from cell centre in most instances.
Positive results are reported with titre and pattern. Titres of 1/40 and 1/80 are considered low; 1/160 and 1/320, significant, and 1/640 or greater, highly significant.

NUCLEAR PATTERNS – Using ICAP (https://www.anapatterns.org**)**
Homogeneous (AC-1)
Smooth, even staining of the nucleus. Mitotic figures are solidly stained with more intense outer edges.
High titres suggest SLE. Also detected in patients with chronic autoimmune hepatitis or juvenile idiopathic-arthritis. Antigens detected: dsDNA, nucleosomes and histones.
Speckled (AC- 2,4,5 and 29)
Grainy staining throughout the nucleus, sometimes affecting the nucleoli. Mitotic figures are either negative or give a fine grainy appearance.
High titres suggest SLE, MCTD, dermatomyositis, SSc, & SjS. Antigens detected: Sm, hnRNP, U1RNP, RNA polymerase III, SS-B/La, SS-A/Ro, DFS70/LEDGF, Mi-2, TIF1γ, TIF1β and Ku.
Nucleolar (AC- 8,9 and 10)
Solid staining of the nucleoli, usually 3 or 4 in number, large and multi-lobed. Mitotic figures are negative. Associated with SSc, SSc/PM overlap and SjS. Antigens: Pm/Scl-75, PM/Scl-100, Th/To, B23/nucleophosmin, nucleolin, No55/SC65, U3-snoRNP/fibrillarin, RNA Polyermase I, hUBF/NOR-90.
Centromere (AC-3)
Discrete speckling, usually 23 or 46 grains per nucleus. Mitotic figures have discrete speckles over the chromosome region. Associated with limited cutaneous SSc and PBC. The mitotic figures differentiate centromere pattern from discrete speckled pattern; i.e. the chromosomes appear positive in a centromere pattern and negative in a discrete speckled pattern.
Discrete nuclear dots (AC-6, 7)
Countable few (1-6) or multiple (6-20) discrete nuclear speckles. Antigens detected: p80-coilin, SMN, Sp-100, PML proteins, MJ/NXP-2. Associated with SLE, SjS, SSc, Pm, SARD, PBC, dermatomyositis and asymptomatic individuals.