

NOVA Lite™ HEp-2

708101

For *In Vitro* Diagnostic Use

CLIA Complexity: High

Intended Use

NOVA Lite™ HEp-2 is an indirect immunofluorescent assay for the screening and semi-quantitative determination of anti-nuclear antibodies (ANA) in human serum. The presence of anti-nuclear antibodies can be used in conjunction with other serological tests and clinical findings to aid in the diagnosis of systemic lupus erythematosus (SLE) or other connective tissue or rheumatic diseases.

Summary and Explanation of the test

The term "anti-nuclear antibodies" describes a variety of autoantibodies that react with constituents of cell nuclei including DNA, RNA and several proteins and ribonucleoproteins.¹ These autoantibodies occur with high frequency in patients with connective tissue or rheumatic diseases, especially systemic lupus erythematosus. Virtually all SLE patients are ANA positive. This diagnostic sensitivity has led to the incorporation of ANA testing into the 1982 Revised Criteria for the Classification of Systemic Lupus Erythematosus by an American College of Rheumatology subcommittee.² While ANA testing is an excellent screening test for SLE (a negative result virtually rules out active SLE³) it is by no means a specific test. Patients with other connective tissue diseases such as rheumatoid arthritis, scleroderma and dermatomyositis are frequently positive, and low ANA titers may be observed in other disease states and in the normal population. Positive ANA results can occur following severe burns or viral infection and have been reported in some normal, healthy people, especially in older populations. Because of this lack of specificity, it is recommended that all ANA positive samples be titered to endpoint and that more specific testing for autoantibodies to double stranded DNA (dsDNA) and extractable nuclear antigen (ENA) autoantibodies be performed.

Indirect immunofluorescence is the reference method for ANA testing. Common substrates are thin sections of rodent organs or various types of cell lines. It is generally agreed that cell line substrates are preferable to organ sections since these rapidly dividing cells have higher levels of certain clinically relevant antigens, including centromere, SS-A(Ro), Scl-70 and PCNA/Cyclin.

Besides the type of substrate, three other factors are critical to the performance of an ANA test: 1) the fixative used in preparing the slide, 2) the fluorescein to protein (F/P) ratio and 3) the immunoglobulin subclass specificity of the conjugate. Some fixatives or combinations thereof are known to destroy certain nuclear antigens and their use should be avoided. The sensitivity and non-specific background staining of a conjugate is determined by the F/P ratio while the disease specificity of a conjugate is determined by the immunoglobulin subclass reactivity. Virtually all clinically significant autoantibodies exhibit IgG subclass specificity even in the presence of IgM and IgA specific ANA.⁴ In contrast, ANA found in healthy blood donors are generally of the IgM and IgA subclass only.⁵ Because of this, conjugates specific for IgG are more disease specific. The substrate chosen for NOVA Lite™ HEp-2 is optimally-fixed human epithelial (HEp-2) cell line and the conjugate is affinity purified anti-human IgG possessing a carefully selected F/P ratio.

These reagent parameters allow the NOVA Lite™ HEp-2 test to detect clinically relevant autoantibodies (including SS-A and Scl-70) which can remain undetected by some other commercial ANA tests. In addition, the IgG conjugate specificity eliminates physiologic false positive results due to normally occurring low titer IgM autoantibodies, often found in older but otherwise healthy persons.

Principles of the Procedure

In the indirect immunofluorescence technique, samples are incubated with antigen substrate and unreacted antibodies are washed off. The substrate is incubated with specific fluorescein labeled conjugate and then unbound reagent is washed off. When viewed through a fluorescence microscope, autoantibody positive samples will exhibit an apple green fluorescence corresponding to areas of the cell or nuclei where autoantibody has bound.

Reagents

1. HEp-2 (human epithelial cell) substrate slides; 12 wells/slide, with desiccant
2. Anti-Human IgG Conjugate (Goat), fluorescein labeled in buffer containing Evans Blue and 0.09% sodium azide, 4mL
3. ANA Titratable Endpoint Pattern Control, 1 vial of buffer containing 0.09% sodium azide and human serum antibodies to HEp-2, prediluted, 0.5mL
4. IFA System Negative Control, 1 vial of buffer containing 0.09% sodium azide and no human serum antibodies to HEp-2, prediluted, 0.5mL
5. PBS Concentrate (40x), sufficient for 1000 mL
6. Mounting Medium, 0.09% sodium azide, 7mL
7. Coverslips

Warnings

1. All human source material used in the preparation of controls for this product has been tested and found negative for antibody to HIV, HbsAg, and HCV by FDA cleared methods. No test method, however, can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the ANA Titratable Endpoint Pattern Control and IFA System Negative Control should be

handled in the same manner as potentially infectious material.⁶

2. Sodium Azide is used as a preservative. Sodium Azide is a poison and may be toxic if ingested or absorbed through the skin or eyes. Sodium azide may react with lead or copper plumbing to form potentially explosive metal azides. Flush sinks, if used for reagent disposal, with large volumes of water to prevent azide build-up.
3. Use appropriate personal protective equipment while working with the reagents provided.
4. Spilled reagents should be cleaned up immediately. Observe all federal, state and local environmental regulations when disposing of wastes.

Precautions

1. This product is for *In Vitro* Diagnostic Use.
2. Substitution of components other than those provided in this system may lead to inconsistent results.
3. Incomplete or inefficient washing of IFA wells may cause high background.
4. Adaptation of this assay for use with automated sample processors and other liquid handling devices, in whole or in part, may yield differences in test results from those obtained using the manual procedure. It is the responsibility of each laboratory to validate that their automated procedure yields test results within acceptable limits.
5. A variety of factors influence the assay performance. These include the starting temperature of the reagents, the strength of the microscope bulb used, the accuracy and reproducibility of the pipetting technique, the thoroughness of washing and the length of the incubation times during the assay. Careful attention to consistency is required to obtain accurate and reproducible results.
6. Over time, the Anti-Human IgG Conjugate may change in color due to exposure to light. However, the color change does not affect the assay performance.
7. Strict adherence to the protocol is recommended.

Storage Conditions

1. Store all the kit reagents at 2-8°C. Do not freeze. Reagents are stable until the expiration date when stored and handled as directed.
2. Diluted PBS buffer is stable for 4 weeks at 2-8°C.

Specimen Collection

This procedure should be performed with a serum specimen. Addition of azide or other preservatives to the test samples may adversely affect the results. Microbially contaminated, heat-treated, or specimens containing visible particulates should not be used. Grossly hemolyzed or lipemic serum specimens should be avoided.

Following collection, the serum should be separated from the clot. NCCLS Document H18-A2 recommends the following storage conditions for samples: 1) Store samples at room temperature no longer than 8 hours. 2) If the assay will not be completed within 8 hours, refrigerate the sample at 2-8°C. 3) If the assay will not be completed within 48 hrs, or for shipment of the sample, freeze at -20°C or lower. Frozen specimens must be mixed well after thawing and prior to testing.

Procedure

Materials provided

- 5 12-well HEp-2 Substrate Slides
- 1 4mL FITC Anti-Human IgG Conjugate
- 1 0.5 mL ANA Titratable Endpoint Pattern Control
- 1 0.5mL IFA System Negative Control
- 1 25mL PBS Concentrate (40x)
- 1 7mL Mounting Medium
- 1 10 Coverslips

Additional Materials Required But Not Provided

Micropipets to deliver 15-1000µL volume
Distilled or deionized water
Squeeze bottles or Pasteur pipets
Moist chamber
1L container (for diluting PBS)
Coplin jar
Fluorescence microscope with 495nm exciter and 515nm barrier filter

Method

Before you start

1. Bring all reagents and samples to room temperature (20-26°C).
2. **Dilute PBS Concentrate:** IMPORTANT: Dilute the PBS Concentrate 1:40 by adding the contents of the PBS Concentrate bottle to 975mL of distilled or deionized water and mix thoroughly. The PBS buffer is used for diluting patient samples and as a wash buffer. The diluted buffer can be stored for up to 4 weeks at 2-8°C.
3. **Dilute Patient Samples:**
 - a. Initial Screening: Dilute patient samples 1:40 with diluted PBS buffer (i.e., add 50µL of serum to 1.95mL of PBS buffer).

- b. Titration: Make serial 2-fold dilutions from the initial screening dilution for all positive samples with diluted PBS buffer (i.e. 1:80, 1:160,... 1:2560).

Assay procedure

1. **Prepare Substrate Slides:** Allow the substrate slide to reach room temperature prior to removal from its pouch. Label it with pencil and place it in a suitable moist chamber. Add 1 drop (20-25 μ L) of the undiluted positive and the negative control to wells 1 and 2 respectively. Add 1 drop (20-25 μ L) of diluted patient sample to the remaining wells.
2. **Slide Incubation:** Incubate the slide for 30 \pm 5 minutes in a moist chamber (a dampened paper towel placed flat on the bottom of a closed plastic or glass container) will maintain the proper humidity conditions. **Do not allow the substrate to dry out during the assay procedure.**
3. **Wash Slides:** After incubation, use a plastic squeeze bottle or pipet to gently wash off the serum with diluted PBS buffer. Orient the slide and stream of PBS buffer so as to minimize wash-over of samples between wells. **Avoid directing the stream directly onto the wells to prevent substrate damage.** If desired, place the slides in a Coplin jar of diluted PBS buffer for up to 5 minutes.
4. **Addition of Fluorescent Conjugate:** Shake off the excess PBS buffer. Place the slide back in the moist chamber and **immediately** cover each well with a drop of fluorescent conjugate. Incubate the slides for an additional 30 \pm 5 minutes.
5. **Wash Slides:** Repeat Step 3.
6. **Coverslip:** Coverslip procedures vary from lab to lab; however, the following procedure is recommended:
 - a. Place a coverslip on a paper towel.
 - b. Apply mounting medium in a continuous line to the bottom edge of the coverslip.
 - c. Shake off the excess PBS buffer and touch the lower edge of the slide to the edge of the coverslip. Gently lower the slide onto the coverslip in such a way that the mounting medium flows to the top edge of the slide without air bubble formation or entrapment.

Quality Control

ANA Titratable Endpoint Pattern Control and IFA System Negative Control should be run on every slide to insure that all reagents and procedures perform properly. Additional suitable control sera may be prepared by aliquoting pooled human serum specimens and storing at $\leq -70^{\circ}\text{C}$. In order for the test results to be considered valid, all of the criteria listed below must be met. If any of these are not met, the test results should be considered invalid and the assay repeated.

1. The undiluted ANA Titratable Endpoint Pattern Control must be $\geq 3+$.
2. The IFA System Negative Control must be negative.

Interpretation of Results

Negative Reaction. A sample is considered negative if specific staining is equal to or less than the IFA System Negative Control. Samples can exhibit various degrees of background staining due to heterophile antibodies or low-level autoantibodies to cytoplasmic constituents such as contractile proteins.

Positive Reaction. A sample is considered positive if specific staining is observed to be greater than the IFA System Negative Control.

Determine the fluorescence grade or intensity using these criteria:

- | | |
|----|--|
| 4+ | Brilliant apple green fluorescence |
| 3+ | Bright apple green fluorescence |
| 2+ | Clearly distinguishable positive fluorescence |
| 1+ | Lowest specific fluorescence that enables the nuclear and/or cytoplasmic staining to be clearly differentiated from the background fluorescence. |

Pattern Interpretation. A variety of patterns of nuclear and/or cytoplasmic staining can be exhibited depending on the types and relative amounts of autoantibodies present in the sample. The following types of staining patterns can be observed:

Homogeneous: A solid staining of the nucleus with or without apparent masking of the nucleoli.

Nuclear antigens present: dsDNA, ssDNA, histones

Disease association: High titers are suggestive of SLE; lower titers are suggestive of SLE or other connective tissue diseases.

Peripheral: A solid staining, primarily around the outer region of the nucleus, with weaker staining toward the center of the nucleus.

Nuclear antigens present: dsDNA, ssDNA, DNP, histone

Disease association: High titers are suggestive of SLE; lower titers are suggestive of SLE or other connective tissue diseases.

Speckled: A fine or grainy appearing staining of the nucleus, generally without fluorescent staining of the nucleoli.

Nuclear antigens present: Sm, RNP, Scl-70, SS-A, SS-B, and other antigen/ antibody systems not yet characterized.

Disease association: High titers suggestive of SLE (Sm antibody), mixed connective tissue disease (RNP antibody), scleroderma (Scl-70 antibody), or Sjogren's syndrome-sicca complex (SS-B antibody); lower titers may be suggestive of other connective tissue diseases.

Nucleolar: Large coarse speckled staining within the nucleus, generally less than 6 in number per cell, with or without occasional fine speckles.

Nuclear antigens present: 4-6S RNA and other unknown nuclear antigens.

Disease association: High titers are prevalent in scleroderma and Sjogren's syndrome.

Centromere: A discrete, speckled staining pattern. The nuclear speckles are very discrete and usually in

some multiple of 46.

Nuclear antigens present: Chromosomal centromere (kinetochore).

Disease association: Highly suggestive of the CREST syndrome, a variant of progressive systemic sclerosis (PSS). CREST is a form of PSS with prominent calcinosis, as well as Raynaud's phenomenon, esophageal dysmotility and limited involvement of the skin (often confined to the fingers or face), telangiectasia.

Mitochondrial: A discrete speckling of the cytoplasm with relative sparing of the nuclear area.

Antigen present: Various types of mitochondrial antigens.

Disease association: High titers indicate primary biliary cirrhosis.

It is important to caution the user about relying on patterns to determine autoantibody specificity, except for the nucleolar and centromere patterns in which each of the antigens is very well defined and their patterns are characteristic. Since many autoantibodies or combinations thereof can induce a homogenous or speckled pattern, it is recommended that specific, follow-up autoantibody testing (such as for dsDNA and ENA) be performed on all speckled or homogeneous samples.

Limitations of the Procedure

1. High-titered ANA is suggestive of connective tissue disease but should not be considered diagnostic. The ANA result should be considered in combination with other serological results as well as the overall clinical history of the patient.
2. ANA patterns often change as the sample is titered out to endpoint. This phenomenon is due to lower titer antibodies dropping below the sensitivity of the system as more dilute sample is tested.
3. A variety of external factors influence the test sensitivity including the type of fluorescence microscope used, the bulb strength and age, the magnification used, the filter system and the observer.
4. If a band pass filter is used instead of a 515 barrier filter, increased artifactual staining may be observed.
5. Only pencil should be used to label the slides. Use of any other writing material may cause artifactual staining.
6. All coplin jars used for slide washing should be free from all dye residues. Use of coplin jars containing dye residue may cause artifactual staining.
7. Results of this assay should be used in conjunction with clinical findings and other serological tests.
8. The assay performance characteristics have not been established for matrices other than serum.

Expected Values

Using the NOVA Lite™ HEp-2 test, a variety of connective tissue disease patients as well as 200 random blood donors were tested. The results appear below:

Patient Group	Number	NOVA Lite™ HEp-2 Number Positive
SLE	105	101
Drug Induced Lupus	24	24
Rheumatoid Arthritis	40	28
Scleroderma	24	18
Dermatomyositis	14	10
Sjogren's Syndrome	14	12
Normals	200	5

References

1. Tan EM: Autoantibodies to nuclear antigens (ANA): Their immunobiology and medicine. *Advances in Immunology* **33**: 167-239, 1982.
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4. Gonzalez E and Rothfield N: Immunoglobulin class and pattern of nuclear fluorescence in systemic lupus erythematosus. *The New England Journal of Medicine* **274**: 1333-1338, 1966.
5. Wiik A: Antinuclear factors in sera from healthy blood donors. *Acta Path Microbiol Scand.* **84**: 215-220, 1976.
6. Biosafety in Microbiological and Biomedical Laboratories. Centers for Disease Control/National Institute of Health, Fourth Edition, 1999 (HHS Pub. #(CDC) 93-8395).

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