NOVA Lite® HEp-2 ANA Kits/Substrate Slides
For In Vitro Diagnostic Use

Product Code: 708100, 708100.100
708101, 508100.20
508100.80, 508100.100
508105.10

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 ANA 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 ANA 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 substrate slides on 6 or 12 well slides

Kits only:
2. HEp-2 (human epithelial cell) substrate slides; 12 wells/slide or 6 wells/slide, with desiccant
3. Anti-Human IgG Conjugate (Goat), fluorescein labeled in buffer containing Evans Blue and 0.09% sodium azide, 15mL
4. ANA Titratable Endpoint Pattern Control, 1 vial of buffer containing 0.09% sodium azide and human serum antibodies to HEp-2, prediluted, 0.5mL
5. IFA System Negative Control, 1 vial of buffer containing 0.09% sodium azide and no human serum antibodies to HEp-2, prediluted, 0.5mL
6. PBS II Concentrate (40x), sufficient for 2000 mL
7. Mounting Medium, 0.09% sodium azide, 7mL
8. Coverslips

Warnings
1. All human source material used in the preparation of kit 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.
2. Sodium Azide is used as a preservative in some kit components. 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 (Kit) 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 slides and 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 II 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. CLSI (NCCLS) Document H18-A3 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 hours, 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 (kits)
708100
20 12-well HEp-2 Substrate Slides
  15mL FITC Anti-Human IgG Conjugate
  1 0.5 mL ANA Titratable Endpoint Pattern Control
  1 0.5mL IFA System Negative Control
  25mL PBS II Concentrate (40x)
  1 7mL Mounting Medium
  1 20 Coverslips
708100.100
100 12-well HEp-2 Substrate Slides
10 15mL FITC Anti-Human IgG Conjugate
10 0.5 mL ANA Titratable Endpoint Pattern Control
10 0.5mL IFA System Negative Control
20 25mL PBS II Concentrate (40x)
10 7mL Mounting Medium
10 20 Coverslips

708101
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 II Concentrate (40x)
1 7mL Mounting Medium
1 20 Coverslips

Materials provided (slides)
508100.20 20 x HEp-2 slides (12 well)
508100.80 80 x HEp-2 slides (12 well)
508100.100 100 x HEp-2 slides (12 well)
508105.10 10 x HEp-2 slides (6 well)

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 II)
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) and mix well.
2. **Dilute PBS II Concentrate:** IMPORTANT: Dilute the PBS II Concentrate 1:40 by adding the contents of the PBS II Concentrate bottle to 975mL of distilled or deionized water and mix thoroughly. The PBS II 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 II buffer (i.e., add 50μL of serum to 1.95mL of PBS II buffer).
   b. Titration: Make serial 2-fold dilutions from the initial screening dilution for all positive samples with diluted PBS II 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 (20-25μL) of the undiluted positive and the negative control to wells 1 and 2 respectively. Add 1 drop (20-25μL) of diluted patient sample to the remaining wells.
2. **Slide Incubation:** Incubate the slide for 30 ± 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 II buffer. Orient the slide and stream of PBS II 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 II buffer for up to 5 minutes.
4. **Addition of Fluorescent Conjugate:** Shake off the excess PBS II 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 ± 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 II 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 < -70°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 ≥ 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), telangectasia.

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.

Slides sold separately are classified as “Analyte specific reagents”.
Except as a component of NOVA Lite® HEp-2 ANA Kit, analytical and performance characteristics are not established.

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

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Number</th>
<th>NOVA Lite® HEp-2 ANA Number Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>105</td>
<td>101</td>
</tr>
<tr>
<td>Drug Induced Lupus</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Rheumatoid Arthritis</td>
<td>40</td>
<td>28</td>
</tr>
<tr>
<td>Scleroderma</td>
<td>24</td>
<td>18</td>
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<tr>
<td>Dermatomyositis</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Sjogren's Syndrome</td>
<td>14</td>
<td>12</td>
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<tr>
<td>Normals</td>
<td>200</td>
<td>5</td>
</tr>
</tbody>
</table>

References

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