NOVA Lite® Skin Antibody
Primate Esophagus Autoantibody Screen Assay
For In Vitro Diagnostic Use
CLIA Complexity: High

Intended Use
NOVA Lite® SA is an indirect immunofluorescent assay for the screening and semi-quantitative determination of skin autoantibodies 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 autoimmune skin diseases, especially pemphigus and pemphigoid.

Summary and Explanation of the test
The term "skin autoantibodies" describes a variety of autoantibodies that react with constituents of skin and mucosal surfaces. These autoantibodies occur with high frequency in patients with autoimmune skin diseases, especially pemphigus and pemphigoid. Autoantibodies which react with an intercellular antigen of skin and mucosa are found in virtually all active cases of the bullous skin disease pemphigus. Titers of these antibodies are found to parallel the disease activity and decrease with successful therapy.

Bullous pemphigoid, another important autoimmune skin disease, is characterized serologically by the presence of antibodies to the basement membrane zone of skin and mucosa. Circulating antibodies to basement membrane zone are found in 70-80% of pemphigoid patients. Although there is not such a striking correlation between the disease severity and titer in pemphigoid as there is in pemphigus, there is some relationship. During remission, either spontaneous or after treatment, there is a decrease in the basement membrane zone antibodies, usually to undetectable levels. In most cases, relapse is accompanied by the reappearance of basement membrane zone autoantibody.

Indirect immunofluorescence is the most commonly used method for skin autoantibody testing. Common substrates used are either thin sections of rodent or primate skin and mucosa with primate esophagus section serving as the substrate of choice. Besides the substrate type, other factors of vital importance to the performance of the skin autoantibody test include: 1) the fixative used in preparing the substrate slide and 2) the specificity and fluorescein to protein (F/P) ratio of the FITC labeled conjugate used. Some fixatives or combinations thereof are known to destroy certain skin antigens and their use should be avoided. The F/P ratio of a conjugate determines the sensitivity and the degree of background staining. The specificity of a conjugate is vitally important. The vast majority of autoantibodies are of the IgG subclass. It is known that when autoantibodies are found in healthy blood donors, they are generally of the IgM and IgA subclass and rarely IgG. Because of this, conjugates specific for IgG are more disease specific. The substrate chosen for NOVA Lite® Skin Antibody is optimally fixed primate esophagus section. The conjugate is affinity purified anti-human IgG.

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. Skin Antibody Slides (Primate Esophagus), 8 wells/slide, with desiccant
2. Anti-Human IgG Conjugate (Goat), fluorescein labeled in buffer containing Evans Blue and 0.09% sodium azide, 7mL
3. Pemphigoid Positive, 1 vial of buffer containing 0.09% sodium azide and human serum antibodies to pemphigoid, prediluted, 0.5mL
4. IFA Systems Negative Control, 1 vial of buffer containing 0.09% sodium azide and no human serum antibodies to pemphigoid, prediluted, 0.5mL
5. PBS II 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 Pemphigoid Positive and IFA Systems Negative Control should be handled in the same manner as potentially infectious material.12
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 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. Microbiologically 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-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
10 8-well Skin Antibody Substrate Slides
1 7mL FITC Anti-Human IgG Conjugate
1 0.5 mL Pemphigoid Positive
1 0.5mL IFA Systems Negative Control
1 25mL PBS II 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 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 1:10 with diluted PBS II buffer (i.e., add 100µL of serum to 900µL 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. 1:20, 1:40,... 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 (70-90µL) of the undiluted positive and the negative control to wells 1 and 2 respectively. Add 1 drop (70-90µL) 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**

Pemphigoid Positive 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 Pemphigoid Positive 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 of the intercellular areas, basement membrane zone or other structures is equal to or less than the negative control. Samples can exhibit various degrees of background staining due to heterophile antibodies, or other specific reactions due to reticulin antibodies, anti-nuclear, anti-endoendomysial or anti-smooth muscle antibodies.

**Positive reaction for intercellular and basement membrane zone antibodies.** A sample may be considered positive if specific staining of intercellular or basement membrane zone areas of the esophageal mucosa are observed.

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 intercellular or basement membrane zone areas staining to be clearly differentiated from the background fluorescence

**Limitations of the Procedure**

1. The presence of skin autoantibodies is suggestive of certain autoimmune bullous skin diseases but should not be considered diagnostic. The autoantibody result should be considered in combination with other serological or biopsy results as well as the overall clinical history of the patient.
2. In addition to skin autoantibodies, other autoantibodies can react with the esophagus substrate. Some of these include anti-nuclear (ANA), anti-mitochondrial (AMA), anti-smooth and anti-skeletal muscle autoantibodies. Presence of these antibodies should be noted and their presence should be confirmed and titrated on other approved substrates.
3. It should be noted that as a sequela to tissue damage, some burn patients produce weakly reactive antibodies that also bind to intercellular areas. These so-called "pemphigus-like" antibodies give a weak and uneven staining of the mucosa, are transient in nature, and do not react with patient skin in vivo.\textsuperscript{13,14,15}
4. Because esophageal mucosa can contain certain blood group substances, anti-A and anti-B blood group isoa ntibodies can react with esophagus.\textsuperscript{16} The pattern of reactivity can, in some patients, mimic the pattern observed with pemphigus intercellular autoantibodies (although blood group antibody will also stain blood capillaries in the musculature of the esophagus section). Reactivity due to blood group antibody can be absorbed out with blood group antigen.\textsuperscript{17}
5. 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.
6. If a band pass filter is used instead of a 515 barrier filter, increased artifactual staining may be observed.
7. Only pencil should be used to label the slides. Use of any other writing material may cause artifactual staining.
8. 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.
9. Results of this assay should be used in conjunction with clinical findings and other serological tests.
10. The assay performance characteristics have not been established for matrices other than serum.

## Expected Values

NOVA Lite® Skin Antibody substrate was used to test pemphigus and pemphigoid skin disease patients as well as 100 random blood donors. The results appear below:

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Number</th>
<th>Intercellular</th>
<th>Basement Membrane Zone</th>
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<tbody>
<tr>
<td>Pemphigus</td>
<td>24</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Pemphigoid</td>
<td>18</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Normals</td>
<td>100</td>
<td>0</td>
<td>0</td>
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## References
