Intended Use

NOVA Lite® ANCA is an indirect immunofluorescent assay for the screening and semi-quantitative determination of anti-neutrophil cytoplasmic antibodies (ANCA) in human serum. The presence of anti-neutrophil cytoplasmic antibodies can be used in conjunction with other serological tests and clinical findings aids in the assessment of various systemic vasculitides.

Summary and Explanation of the test

Anti-neutrophil cytoplasmic antibody (ANCA) testing has revolutionized the diagnosis and treatment of the various autoimmune mediated vasculitides.1,4 The perinuclear or pANCA and cytoplasmic or cANCA autoantibodies have been proven to be useful for the detection of diseases such as Wegener’s granulomatosis and crescentic glomerulonephritis.

There are at least six identified ANCA antigens and many are still unidentified.1,5 Most of these antigens appear to be enzymes residing in the neutrophil primary granules. These enzymes include myeloperoxidase (MPO), serine protease 3 (PR3), elastase, lactoferrin, cathepsin G and cationic protein 57 (CAP-57). ELISA tests have been developed for detection of many of the more important neutrophil antibodies yet most experts in the field of autoimmune vasculitis still recommend that the immunofluorescent assay (IFA) method be used for initial screening.

Two ANCA patterns are detectable using the standard ANCA IFA procedure. The classic ANCA pattern appears as a granular, cytoplasmic fluorescence. This pattern, suggestive of Wegener’s granulomatosis and to a lesser extent microscopic polyarteritis, has been designated cANCA.6 A second ANCA pattern has been described7,8 which stains the perinuclear region of alcohol-fixed neutrophils. This pattern has been designated pANCA. The pANCA pattern has been associated with a more organ-limited vasculitis, in particular, crescentic or rapidly progressive glomerulonephritis.9 The correlation of cANCA and Wegener’s granulomatosis has been well established.3,10 Wegener’s granulomatosis is characterized by a necrotizing inflammatory process with granuloma formation and vasculitis which primarily involves the upper and lower respiratory tracts and the kidneys. This triad of clinical involvement is considered the hallmark of Wegener’s granulomatosis. cANCA is present in up to 96% of Wegener’s granulomatosis patients with active generalized disease, but decreases to 65% in active limited disease and is present in 30-40% of patients in remission. The titer of cANCA will also follow the disease course with decreasing titers in disease remission and increasing titers in disease relapse.4,12

Unlike cANCA and its association with the predominantly systemic disease of Wegener’s granulomatosis, pANCA has frequently been associated with necrotizing glomerulonephritis. pANCA is typically not seen in systemic syndromes. The most common target antigen associated with pANCA is myeloperoxidase, but reactivity to elastase and lactoferrin is also detected. Nearly 90% of pANCA positive sera from patients with glomerulonephritis are reactive with myeloperoxidase; however, in patients with diseases other than glomerulonephritis that also have pANCA, a much lower percentage are reactive with myeloperoxidase.11,13 This would indicate that multiple antigens are detectable as perinuclear staining of ethanol-fixed neutrophils including sera which contain antinuclear antibodies. It is possible to confirm perinuclear patterns detected with ethanol-fixed neutrophils as pANCA by performing the IFA on formalin-fixed neutrophils. The primary target antigen of pANCA, myeloperoxidase, remains in the cytoplasm of formalin-fixed cells whereas in ethanol-fixed cells the antigen migrates to the nucleus causing the perinuclear pattern.

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. ANCA slides, ethanol-fixed human neutrophil substrate slides, 6 or 12 wells/slide, with desiccant

Kits Only

2. Anti-Human IgG Conjugate (Goat), fluorescein labeled in buffer containing Evans Blue and 0.09% sodium azide
3. cANCA Positive, 1 vial of buffer containing 0.09% sodium azide and human serum antibodies to cANCA antigens, prediluted
4. pANCA Positive, 1 vial of buffer containing 0.09% sodium azide and human serum antibodies to pANCA antigens, prediluted
5. IFA System Negative Control, 1 vial of buffer containing 0.09% sodium azide and no human serum antibodies to ANCA, prediluted

Product Codes: 708290, 708296, 708298, 708299
508290.10, 508296.20, 508298.20, 508299.10

CLIA Complexity: High
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 pANCA Positive, cANCA Positive and IFA System Negative Control should be handled in the same manner as potentially infectious material.14
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. 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 (formerly 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)
708290
10 6-well ANCA ethanol-fixed human neutrophil substrate slides
1 4mL FITC Anti-Human IgG Conjugate
1 0.5 mL cANCA Positive
1 0.5 mL pANCA Positive
1 0.5mL IFA System Negative Control
1 25mL PBS II Concentrate (40x)
1 7mL Mounting Medium
1 10 Coverslips

708296
20 12-well ANCA ethanol-fixed human neutrophil substrate slides
1 15mL FITC Anti-Human IgG Conjugate
1 0.5 mL cANCA Positive
1 0.5 mL pANCA Positive
1 0.5mL IFA System Negative Control
2 25mL PBS II Concentrate (40x)
1 7mL Mounting Medium
1 20 Coverslips
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 1:20 with diluted PBS II buffer (i.e., add 50µL of serum to 950µL 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. 1:40, 1:80,… 1:1280).

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 ± 5minutes.

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

cANCA 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 undiluted cANCA 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 nuclear and cytoplasmic 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 nuclear or cytoplasmic staining as described below is observed to be greater than the negative control and the staining intensity is 1+ or greater. 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 cytoplasmic staining can be exhibited depending on the types and relative amounts of autoantibodies present in the sample. The following types of staining patterns may be observed:

**cANCA or cytoplasmic staining:** This pattern generally presents a coarse, speckled cytoplasmic fluorescence often with accentuated fluorescence in and around the nuclear lobes. This pattern is usually found to be produced by antibodies reacting with the primary granule enzyme Serine Protease 3 (PR3).

**pANCA or perinuclear staining:** This pattern generally appears as a homogeneous staining of the nuclear lobes, often with a perinuclear accentuation. Since some antinuclear antibodies (ANA) can also react with nuclei of ethanol-fixed human neutrophils, it is recommended that these samples also be tested for ANA. In addition, these samples can be tested on human neutrophils fixed in formalin instead of ethanol. Formalin, being a crosslinking fixative, destroys most nuclear antigens and shifts the staining pattern from perinuclear to cytoplasmic if the sample contains pANCA. Formalin-fixed slides for this purpose are available separately.

### Limitations of the Procedure

1. Heat inactivated, hemolyzed, microbially contaminated or incompletely defibrinated samples may cause high background staining and make interpretation difficult. Obtain a fresh sample and retest. Addition of 2% albumin or bovine serum to the PBS II buffer used to dilute samples may reduce the background staining of problematic samples.
2. ANA positive samples may react with ethanol-fixed neutrophils. Nuclear positive specimens should be tested for ANA and/or tested on formalin-fixed neutrophil slides.
3. Because ethanol-fixed human neutrophils are known to contain multiple primary granule antigens such as elastase, lactoferrin, cathepsin G, cationic protein 57 and possibly other as yet unidentified neutrophil antigens, samples appearing as p or cANCA positive may not always test positive for specific myeloperoxidase (MPO) or serine protease 3 (PR3) antibodies.
4. Besides ANA, certain other autoantibodies may also react with ethanol-fixed human neutrophils. These antibodies include anti-smooth muscle (actin) and certain alloantibodies such as Mart or NB1. Actin or smooth muscle antibodies react with neutrophil cytoplasm, but with a homogenous rather than a coarse speckled pattern, as with a typical cANCA. All alloantibodies also appear as a cytoplasmic pattern, but with a much finer speckling compared to cANCA and only a small percentage of cells (typically 40% or fewer) will fluoresce.
5. Occasionally samples can be seen with more than one autoantibody. For example, c and p ANCA or ANCA plus ANA.
6. Some patients with inflammatory bowel disease or ulcerative colitis have neutrophil reactive antibodies. These samples appear as a pANCA type pattern on ethanol-fixed neutrophils with very accentuated perinuclear staining. Typically these samples appear as negative or with a weak homogenous, cytoplasmic fluorescence on formalin-fixed neutrophil slides.
7. Mishandling of slides during the staining procedure, especially allowing slides to dry between steps, may result in a "washed out" pattern appearance and a high level of background staining.
8. Use of reagents from other types of fluorescent antibody kits (especially conjugate) may adversely affect the sensitivity and specificity of the ethanol-fixed neutrophil substrate slides.

9. 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.

10. If a band pass filter is used instead of a 515 barrier filter, increased artifactual staining may be observed.

11. Only pencil should be used to label the slides. Use of any other writing material may cause artifactual staining.

12. 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.

13. Results of this assay should be used in conjunction with clinical findings and other serological tests.

14. The assay performance characteristics have not been established for matrices other than serum.

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

**Expected Values**

One hundred and fifteen random normal samples were tested on ethanol-fixed NOVA Lite® ANCA slides. These samples were submitted as pre-employment or employee physical examinations and as such would not likely include elderly or pediatric individuals. The samples were a random mixture of males and females. All 115 samples were negative at the 1:20 screening dilution.

Also tested were 45 Wegener’s, 39 microscopic polyarteritis and 20 crescentic glomerulonephritis patients as well as patients with a variety of connective tissue diseases. The results are summarized in the following table:

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Number of Patients</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random Normals</td>
<td>115</td>
<td>0</td>
</tr>
<tr>
<td>Wegener’s</td>
<td>45</td>
<td>89 (all cANCA)</td>
</tr>
<tr>
<td>Microscopic polyarteritis</td>
<td>39</td>
<td>100 (all pANCA)</td>
</tr>
<tr>
<td>Crescentic glomerulonephritis</td>
<td>20</td>
<td>100 (all pANCA)</td>
</tr>
<tr>
<td>SLE</td>
<td>27</td>
<td>7 (both pANCA and cANCA)</td>
</tr>
<tr>
<td>Sjogrens Syndrome</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>Scleroderma</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Polymyositis</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

NOVA Lite® ANCA slides were compared side by side with cytocentrifuged neutrophil preparations used routinely in a university-based ANCA reference center. Fifty-five positive sera (23 cANCA, 28 pANCA, 4 Atypical) and 14 ANCA negative samples were tested. Of the 55 positive samples, 54 produced an identical staining pattern on the NOVA Lite® ANCA slides. One low titer cANCA sample produced a negative result on the INOVA slide. All 14 ANCA negative samples were similarly negative on the NOVA Lite® ANCA slides. Titrations of the 55 positive samples on both substrates produced the same result or a one doubling dilution difference with 53 of the samples tested. The other 2 samples differed by 2 doubling dilutions.

Forty-eight random serum samples submitted to a major U.S. reference laboratory for ANCA testing were tested simultaneously for ANCA, using NOVA Lite® ANCA slides and also for specific MPO and PR-3 antibodies using INOVA QUANTA Lite® ELISA kits. The results are summarized in the following table.

<table>
<thead>
<tr>
<th>IFA Result</th>
<th>No. Samples</th>
<th>% ELISA Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative</td>
<td>8</td>
<td>0 MPO 0 PR3</td>
</tr>
<tr>
<td>pANCA</td>
<td>13</td>
<td>84 MPO 0 PR3</td>
</tr>
<tr>
<td>cANCA</td>
<td>21</td>
<td>0 MPO 95 PR3</td>
</tr>
<tr>
<td>c and pANCA</td>
<td>5</td>
<td>80 MPO 0 PR3</td>
</tr>
<tr>
<td>Atypical</td>
<td>1</td>
<td>0 MPO 0 PR3</td>
</tr>
</tbody>
</table>

Eight samples were ANCA negative by immunofluorescence. All eight samples were negative for both MPO and PR-3 by ELISA. MPO and PR-3 results ranged from 1-5 units, well below the 20 unit positive cutoff. Thirteen samples were scored as pANCA positive by IFA. Eleven of these were MPO positive and none were PR-3 positive. Twenty-one samples were cANCA positive by immunofluorescence. None of these samples were MPO positive, while 20 of the 21 samples were PR-3 positive. Five samples appeared to have both p and c ANCA type patterns by IFA. Four of these were MPO positive and none PR3 positive. One sample was scored as an atypical pANCA. This atypical pANCA sample was negative on both the MPO and PR3 kits.

**Accordance of ANCA results - ELISA vs. IFA**

While MPO and PR3 are major antigens comprising what can be identified as p and c ANCA, the user should be aware that antibodies to several other neutrophil primary granule enzymes can also produce c and p ANCA patterns by immunofluorescence on ethanol-fixed neutrophils. This is especially true in the case of pANCA antibodies where at least 3 other autoantibodies to antigens other than MPO can produce a pANCA type IFA pattern. To illustrate this point, samples from the above mentioned Wegener’s, microscopic polyarteritis and crescentic glomerulonephritis groups as well as the 48 samples submitted for routine serologic testing for vasculitis were grouped. Both the ethanol-fixed human neutrophil immunofluorescence and the ELISA results appear below.
<table>
<thead>
<tr>
<th></th>
<th>QUANTA Lite® PR-3 IgG</th>
<th>QUANTA Lite® MPO IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+ 60 Relative sensitivity 90.9%</td>
<td>+ 47 Relative sensitivity 61.0%</td>
</tr>
<tr>
<td></td>
<td>6 Relative specificity 98.8%</td>
<td>30 Relative specificity 100%</td>
</tr>
<tr>
<td>-</td>
<td>- 1 Relative specificity 98.8%</td>
<td>- 0 Agreement 80.3%</td>
</tr>
<tr>
<td></td>
<td>85 Agreement 95.4%</td>
<td>75 Agreement 80.3%</td>
</tr>
</tbody>
</table>

Of 66 cANCA IFA positive samples, 6 were negative for PR3 and 1 sample was ELISA positive yet negative by IFA. Of 77 pANCA IFA positive samples, 30 were negative by specific MPO ELISA. No pANCA, IFA negative samples were found positive by the MPO ELISA.
References


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