NOVA Lite® ANCA Kits/Substrate Slides  
**For In Vitro Diagnostic Use**

**Product Codes:** 708295, 708297  
508295, 508295.10, 508297, 508297.20

**Intended Use**

This product (kit or substrate slides) is intended for use in the screening and titration of circulating anti-neutrophil cytoplasmic antibodies (ANCA). These antibodies are markers for the diagnosis and treatment of Wegener’s granulomatosis and other systemic vasculitides.¹

**Summary and Explanation of the test**

The necrotising vasculitides, including Wegener’s granulomatosis, polyarteritis nodosa, Churg Strauss syndrome, idiopathic crescentic glomerulonephritis and ‘overlap’ systemic vasculitides are a group of related diseases which vary considerably in their clinical presentations and consequently cause diagnostic problems. Wegener’s granulomatosis is a severe vascular disease characterised by necrotising granulomas of the respiratory tract and focal glomerulonephritis. Early diagnosis is important as rapid immunosuppressive therapy has a major effect on renal outcome but initial symptoms at presentation and the histological examination by biopsies are frequently non-specific.²

In 1982 Davies et al described the presence of antineutrophil cytoplasmic antibodies (ANCA) in the serum of patients with necrotising glomerulonephritis.³ This was followed in 1985 with a publication by van de Woude et al who found that 25 out of 27 patients with active Wegener’s granulomatosis exhibited the classical cytoplasmic (c-ANCA) staining pattern in indirect immunofluorescence.³ The classical c-ANCA pattern on ethanol-fixed neutrophils shows characteristic granular cytoplasmic staining with minimal staining of the nuclear lobes. The major c-ANCA target antigen is proteinase 3, a serine protease.

A second ANCA staining pattern (perinuclear, p-ANCA) was described in 1988 by Falk et al in renal patients with systemic vasculitis.³ The major p-ANCA target antigen is thought to be myeloperoxidase, although several other antigens (e.g. lactoferrin, elastase, cathepsin G) are associated to a lesser degree. The p-ANCA pattern on ethanol-fixed neutrophils shows sharply delineated perinuclear staining.

Many samples submitted for ANCA testing will also be positive for anti dsDNA, anti histone and other antinuclear (ANA) autoantibodies which can mimic the p-ANCA staining pattern. Patient samples positive for ANA can also be positive for p-ANCA. It is obviously critical to distinguish true c-ANCA and p-ANCA staining patterns from other non-ANCA artifacts. This is achieved using a combination of ethanol-fixed and formalin-fixed neutrophil slides.

Ethanol fixation of neutrophils results in the positively charged cytoplasmic granule proteins migrating to the negatively charged DNA of the nucleus, resulting in perinuclear (p-ANCA) staining.⁴ If neutrophils are treated with a cross-linking fixative such as formalin, then this migration is prevented and the cytoplasmic granule proteins remain in the cytoplasm and true p-ANCA samples will show a granular cytoplasmic c-ANCA staining pattern on formalin-fixed slides. ANA positive samples, when tested on formalin-fixed neutrophils, will become negative or show greatly reduced staining intensity. Granulocyte specific ANA (GS-ANA) samples producing a nuclear or perinuclear reaction on ethanol-fixed neutrophils will become negative when tested on formalin-fixed neutrophils.

Indirect immunofluorescence on neutrophil slides is the method of choice for screening for ANCA. INOVA slides use purified human neutrophils which are prepared and fixed to achieve optimal staining patterns. Samples giving ANCA positive, ANA and atypical staining patterns should be titrated out.

**Principles of the Procedure**

This product uses an indirect immunofluorescence technique⁵. Patient samples and appropriate controls are incubated with the neutrophil substrate. The unreacted antibodies are washed off and then an appropriate fluorescein labelled conjugate is applied. Unbound conjugate is washed off as before. Slides are viewed with a fluorescence microscope and positive samples are seen as apple green fluorescence which corresponds to areas of the neutrophil where antibody has bound.

**Reagents**

1. Formalin-fixed neutrophil substrate slides, 6 or 12 wells/slide

**Kits Only**

2. Positive control sera (e.g. p-ANCA, c-ANCA, ANA) containing 0.09% sodium azide. Prediluted ready for use.
3. Negative control serum containing 0.09% sodium azide. Prediluted ready for use.
4. Affinity purified goat anti-human IgG fluorescein with Evans Blue containing 0.09% sodium azide. Prediluted ready for use.
5. 1% Evans Blue, as an optional counterstain.
6. Phosphate buffered saline (PBS II), a 40-fold concentrate in liquid form.
7. Mounting medium, containing an anti-fading agent
8. Covergrips

**Warnings/Precautions**

This product is for In Vitro Diagnostic Use. This product should only be used by suitably trained persons for the purposes stated. Adherence to the given procedure is recommended. All donors of human serum supplied in kits have been serum tested and found to be negative for Hepatitis B surface antigen and antibodies to Hepatitis C virus and Human Immunodeficiency Virus (HIV 1 & 2). However these tests cannot guarantee the absence of infective agents. Proper handling and disposal methods should be established as for all potentially infective material and only personnel adequately trained in such methods should be permitted to perform the procedures.
Some kit components contain 0.09% sodium azide as a preservative and must be handled with caution—do not ingest or allow contact with skin or mucous membranes. If contact does occur wash with a large volume of water and seek medical advice. Explosive metal azides may be formed with lead and copper plumbing; on disposal of reagent, flush with a large volume of water to prevent azide build up.

**Storage Conditions**

Unopened kits/slides should be stored at 2-8°C and can be used until the expiry date given on the box label. DO NOT FREEZE. Once slides are removed from a foil bag, they should be used immediately. Diluted PBS II buffer can be stored for up to one month at 2-8°C. The kit fluorescein conjugate should be kept out of sunlight, fluorescent or U.V. light whenever possible and stored at 2-8°C. All other reagents should be stored at 2-8°C.

**Specimen Collection**

Blood samples should be collected by venepuncture. Allow the blood to clot naturally and separate the serum as soon as possible to prevent haemolysis. The serum may be stored at 2-8°C for up to 7 days prior to assay, or for prolonged storage, aliquoted and stored at -20°C or below. Repeated freezing and thawing should be avoided. Avoid using lipaemic, haemolysed or microbially contaminated sera, as decreased titres or unclear staining patterns may occur.

**Procedure**

**Materials Provided (Kits)**

708295

10 6-well ANCA Neutrophil substrate slides (formalin-fixed)
1 1mL pANCA Positive Control (prediluted)
1 1mL ANA Positive Control (prediluted)
1 1mL IFA System Negative Control
1 7mL anti Human IgG AFF Fluorescein with Evans Blue
2 25mL PBS II Concentrate (40x)
1 3mL 1% Evans Blue Counterstain
1 3mL Mounting Medium
1 10 Coverslips
1 Instruction leaflet

708297

20 12-well ANCA Neutrophil substrate slides (formalin-fixed)
1 1mL pANCA Positive Control (prediluted)
1 1mL cANCA Positive Control (prediluted)
1 1mL ANA Positive Control (prediluted)
1 1mL IFA System Negative Control
1 15mL anti Human IgG AFF Fluorescein with Evans Blue
2 25mL PBS II Concentrate (40x)
1 3mL 1% Evans Blue
1 10mL Mounting Medium
1 20 Coverslips
1 Instruction leaflet

**Materials provided (slides)**

508295.10 10 x ANCA Neutrophil substrate slide-6 well (formalin-fixed)
508297.20 20 x ANCA Neutrophil substrate slide-12 well (formalin-fixed)

**Additional Materials Required But Not Provided**

1. Distilled water to dilute PBS II concentrate
2. Container for PBS II buffer
3. Micropipettes and disposable tips to apply patient samples
4. Humid chamber for incubation steps
5. Fluorescence microscope with 495nm exciter filter and 515nm barrier filter
6. Plastic squeeze bottle for initial wash in PBS II

If using substrate slides only, the following materials will also be required (INOVA part numbers given where applicable): positive control; e.g. c-ANCA (508191), p-ANCA (508291); negative control (508007), anti-human IgG AFF FITC conjugate (508113) or anti-human IgGAM AFF FITC (504031), PBS II (508998), Evans Blue (504049, optional), mounting medium (508001, 508005, 508006), coverslips.

**Test Procedure**

**Quality control**

Positive and negative controls should be used every time a batch of samples is tested.

1. Dilute PBS II concentrate: Dilute kit PBS II concentrate with distilled water 1 part PBS II concentrate + 39 parts distilled water and mix. The PBS II is used for diluting patient samples and as a wash buffer. NB: Only make up the total amount of kit PBS II if the entire kit is to be used within one month.
2. Dilute patient samples

**Screening:** Dilute patient samples 1/20 by adding 10µL of serum to 190µL of PBS II buffer.

**Titration:** Make serial dilutions of positive screened samples with PBS II buffer (e.g. 1/40, 1/80, 1/160, 1/320 and 1/640 etc). For example: Take 100µL of the 1/20 dilution, mix with 100µL of PBS II to give a 1/40 dilution. Repeat for further dilutions.
3. Substrate slides: Allow substrate slide(s) to reach room temperature (18-28°C) for approximately 30 minutes prior to removal from pouch(es). Label slides appropriately, place in the humid chamber and add one drop of each kit’s positive and negative controls to the appropriate wells. Add 25μL of diluted patient samples to the remaining wells.

4. Slide incubation: Incubate slides for 30 minutes in a humid chamber at room temperature (18-28°C).

5. PBS II wash: Remove slides from humid chamber and rinse briefly with PBS II squeeze bottle. Do not squirt directly on to the wells. Place slides in a rack and immerse in PBS II and agitate or stir for 10 minutes.

6. Addition of fluorescent conjugate: Shake off excess PBS II. Return slides to humid chamber and immediately cover each well with a droplet of fluorescent conjugate. DO NOT LEAVE WELLS UNCOVERED FOR LONGER THAN 15 SECONDS. Drying out of the substrate seriously affects the results.

7. Slide incubation: Incubate slides for 30 minutes in humid chamber at room temperature (18-28°C) in the dark.

8. PBS II wash: Wash again as described in step 5. Optional counterstain: Add 2-3 drops of 1% Evans Blue per 100mL of PBS II prior to slide immersion.

9. Mounting with coverslip: Remove one slide at a time from PBS II wash. Add a drop of mounting medium to each well. Carefully lower the slide onto the coverslip, avoiding air bubbles, but if present do not attempt to remove.

10. View slides under fluorescence microscope: Finished slides should be stored at 2-8°C and viewed as soon as possible.

Quality Control

On formalin-fixed neutrophils c-ANCA and p-ANCA samples show granular cytoplasmic staining. On formalin-fixed neutrophils no ANA positivity should be seen. The negative control should show no discernible fluorescence. If the controls do not appear as described, the test is invalid and should be repeated.

Interpretation of Results

Negative
A sample is considered negative if specific neutrophil staining is equivalent to or less than the negative control well.

Positive
A sample is considered positive if cytoplasmic staining is observed.

N.B: Each laboratory should establish at which point a positive result is considered clinically significant.

Pattern interpretation

c-ANCA (Cytoplasmic staining)
Formalin-fixed slides, c-ANCA positive samples will show generalised granular staining of the cytoplasm.

p-ANCA pattern (Perinuclear pattern)
On formalin-fixed slides, pANCA samples will show generalised granular staining of the cytoplasm, indistinguishable from that obtained with c-ANCA positive samples.

ANA pattern
On formalin-fixed slides, most nuclear antigens have been destroyed, therefore the ANA positive samples will show negative or greatly reduced fluorescence compared to the p-ANCA positive control.

Limitations of the Procedure

1. Heat inactivated haemolysed or incompletely defibrinated samples may cause high background staining and make interpretation difficult. Fresh samples should be obtained and the test repeated. Problematic samples may be diluted with PBS II buffer containing 2% albumin or bovine serum, in order to reduce background staining.

2. The light source, filters and optics of different fluorescence microscopes will influence the sensitivity of the kit. The performance of the microscope is significantly influenced by correct maintenance especially centring of the vapour lamp and changing of the lamp after the recommended period of time.

3. Positive IFA results should be confirmed by myeloperoxidase (MPO) and proteinase 3 (PR3) enzyme immunoassays (EIA).\textsuperscript{11,12} Formalin fixed ANCA slides may also contribute to the determination of autoantibody specificity, especially for moderate and high titre samples.

4. ANCA-positive samples may not always test positive for myeloperoxidase (MPO) or serine proteinase 3 (PR3) using EIA, as other multiple primary granule antigens may be responsible for classical p- or c-ANCA positive staining pattern. These include elastase, lactoferrin, cathepsin G, cationic protein 57 and other as yet unidentified neutrophil antigens.

5. Anti-smooth muscle (actin) antibodies may react with ethanol-fixed human neutrophils as well as alloantibodies such as mart or NB1\textsuperscript{5}. Actin or smooth muscle antibodies react with neutrophil cytoplasm giving a homogeneous rather than the typical coarse speckled c-ANCA staining pattern. Alloantibodies also react with the neutrophil cytoplasm giving a fine speckled staining pattern. Typically, only 40% of cells or less will fluoresce.

6. Samples may contain more than one antibody, e.g. c-ANCA and p-ANCA or c-ANCA and ANA.

7. Neutrophil reactive antibodies may also be found in the serum of patients with inflammatory bowel disease or ulcerative colitis\textsuperscript{6}. On ethanol-fixed neutrophil slides, these samples appear as a p-ANCA pattern with much accentuated perinuclear staining. On formalin-fixed neutrophil slides these samples appear negative or give very greatly reduced fluorescence.

8. This test alone should not be considered diagnostic. All other factors including the clinical history of the patients and other serological or biopsy results must also be taken into account.

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

Expected Values

<table>
<thead>
<tr>
<th>Patient group</th>
<th>% positive No.</th>
<th>p-ANCA</th>
<th>c-ANCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy persons</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>80</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>46</td>
<td>46</td>
<td>0</td>
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<tr>
<td>Primary sclerosing cholangitis with inflammatory bowel disease</td>
<td>17</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>Primary biliary cirrhosis</td>
<td>25</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>15</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>Wegener’s granulomatosis</td>
<td>30</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Polyarteritis nodosum *</td>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
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</table>

All of the above information has been derived from Seibold et al², except that marked *, which was obtained by in-house studies at The Binding Site Ltd.

Precision

Ten patient sera (three p-ANCA-positive, three c-ANCA-positive, two ANA-positive and two negative) were assayed nine times (in triplicate on three separate kit lots). The staining pattern for each sample differed by no more than one staining unit (based on a scale of 1+ (weak staining) to 3+ (strong staining)).

Comparison study

100 clinical serum samples and 40 normal adult donor sera (20 males, 20 females) were tested on Binding Site’s ANCA combi kit and a competitor’s slide kit. Results were as follows:

<table>
<thead>
<tr>
<th>Competitor</th>
<th>The Binding Site</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>c-ANCA</td>
<td>p-ANCA</td>
<td>ANA</td>
</tr>
<tr>
<td>c-ANCA</td>
<td>60</td>
<td>3</td>
</tr>
<tr>
<td>p-ANCA</td>
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<tr>
<td>p/c-ANCA</td>
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<td>0</td>
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<tr>
<td>ANA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Neg.</td>
<td>0</td>
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</tr>
</tbody>
</table>

134 out of 140 samples tested gave identical results by the two methods. For the c-ANCA pattern, the relative sensitivity was 95%, the relative specificity was 96%, and the relative agreement was 96%. For the p-ANCA pattern, the relative sensitivity was 90%; the relative specificity was 97% and the relative agreement was 96%. For the six samples giving different patterns, all demonstrated very strong or very weak staining on one or both of the kits, which can make interpretation difficult.

Summary of the Procedure

1. Dilute PBS II with distilled water.
2. Dilute patient sera 1/20 with PBS II.
3. Remove slides from refrigerator and equilibrate to room temperature (18-28°C).
4. Remove slide from foil bag and place in a humid chamber. Add 25µL of controls and patient sera.
5. Incubate for 30 minutes at room temperature (18-28°C).
6. Rinse slides with a stream of PBS II.
7. Wash slides for 10 minutes in a rack.
8. Return slide to humid chamber and immediately add a drop of fluorescent conjugate.
9. Incubate slides for 30 minutes.
10. Wash again as described in step 7.
11. Add mounting media to each well and coverslip.
12. View slides under a fluorescence microscope.

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References


