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See external label



2°C-8°C



Σ= tests



cat.#290060-etho

## ANCA (ETHANOL)

Catalog No. 290060-etho

**PLEASE READ THIS MATERIAL BEFORE USING THE KIT**

### INTENDED USE

The DIAGNOSTIC AUTOMATION Corporation immunofluorescence assay (IFA) is for the in vitro diagnostic detection of anti-neutrophil cytoplasmic antibodies in human serum using ethanol fixed human neutrophils as substrate. The test is intended as an aid in the diagnosis of Wegener's granulomatosis, Idiopathic Crescentic Glomerulonephritis, Microscopic Polyarteritis, Pulmonary Renal Syndrome.

### CLINICAL SIGNIFICANCE

In 1939 Friedrich Wegener described a disease called "rhinogenic granulomatosis". Early in disease granulomas are found that, like the rheumatic nodules, develop independently from the vessels in the connective tissue. During the generalized stage, one will usually find the granuloma in close relation to the vessels in different regions of the body, sometimes in only one, sometimes in all organs. The systemic vasculitis occurs later. The connective tissue diseases are associated with autoantibodies to various non-organ-specific antigens, particularly nuclear antigens.<sup>1</sup> Autoantibodies to neutrophils have been known for a long time. As early as 1964 a granulocyte-specific antinuclear factor was reported.<sup>2</sup> The use of an ethanol fixation technique for granulocyte-specific antinuclear antibodies (GS-ANA) is the standard today. The cytoplasmic staining of granulocytes was first published in 1982 in a report of Australian patients with segmental necrotizing glomerulonephritis.<sup>3</sup> Four more patients were reported with vasculitis and glomerulonephritis.<sup>4</sup> In 1985 van der Woude<sup>5</sup> showed that the antineutrophil cytoplasmic autoantibodies (ANCA) occurred with Wegener's granulomatosis, as a result the interest for these antibodies significantly increased.<sup>6</sup> These antibodies, now termed ANCA, have proven useful for the diagnosis of disease in systemic vasculitis.

In the immunofluorescence assay for anti-neutrophil cytoplasmic antibodies, staining patterns that are different from the granular cytoplasmic pattern produced by the antibodies to the 29 kD serine protease have been recognized; in particular, a perinuclear pattern (P-ANCA) has been observed. This pattern is produced from antibodies against 2 other myeloid lysosomal enzymes - myeloperoxidase (MPO) and human leukocyte

elastase. P-ANCA antibodies have been identified by Falk and Jennette<sup>7,8</sup> in patients with either idiopathic or vasculitis-associated crescentic glomerulonephritis. The presence of antibodies to P-ANCA or C-ANCA have proven to be highly specific and sensitive for this group of disorders, and useful in the clinical analysis of patients suspected of having vasculitis.<sup>9-12</sup>

ANCA antibody detection by IFA has been a useful aid in the assessment of patient diagnosis, and to a certain extent their prognosis and response to therapy. Further identification and confirmation by enzyme immunoassay for both MPO and PR3 is suggested.<sup>13</sup> Simultaneous testing for MPO and PR3 antibodies are recommended for best patient assessment.<sup>6</sup>

## **PRINCIPLE OF THE TEST**

Diluted patient sera are incubated, in assigned slide wells, and antibodies against ANCA present in the specimen bind to the human granulocytes ethanol fixed to the slide. Unbound antibodies are washed off and a second incubation with anti-human IgG conjugated to fluorescein isothiocyanate (FITC) follows. Unbound conjugate is washed off and the bound conjugate pattern is read visually using a fluorescent microscope.

## **KIT COMPONENTS**

### **ITEMCONTENTS            QUANTITY**

1. ANCA (Human Granulocytes)
    - Substrate 6 Well Slide      10 X 1 ea.
  2. FITC Antihuman IgG Conjugate
    - w/Evans Blue                      1 X 2.5 mL
  3. C-ANCA Positive Control    1 x 1.0 mL
  4. ANCA Negative control      1 x 1.0 mL
  5. Mounting Medium            1 x 3.0 mL
  6. PBS Buffer (Powder)            2 X 1 L
  7. 6 Well Blotters                10 each
  8. Coverslips (22 X 70 mm) 1 X 12 each
- NOTE: Liquid reagents contain the preservative sodium azide (0.1%)

## **MATERIALS REQUIRED BUT NOT PROVIDED**

1. Test tube rack or microtiter system.
2. Micro-pipettor of 2 - 20  $\mu$ L range.
3. Graduated glass pipettes.
4. 1 L. graduated cylinder.
5. Staining dish.
6. Moist chamber.
7. Clean containers for diluted buffers.
8. Distilled or deionized water.
9. Fluorescent Microscope.
10. Lint free paper towel.
11. Timer (60 min. range).

## **MATERIALS “AVAILABLE UPON REQUEST”**

1. ANCA (Human Granulocytes) Substrate Slides FORMALIN FIXED for confirmation of P-ANCA patterns.
2. P-ANCA Positive Control (Cat.# A003L) liquid ready-to-use.

Call Customer Service department for availability of these products.

## **PRECAUTIONS**

**Caution:** All blood products should be treated as potentially infectious. Human source materials from which this product was derived were found to be non-reactive for Hepatitis-B surface antigen (HBsAg) and Human Immuno- deficiency Virus (HIV) antibody when tested in accordance with current FDA required tests. No known test methods can offer total assurance products derived from human blood will not transmit HIV, Hepatitis or other potentially infectious agents. Therefore, these agents and all patients' specimens should be handled at Biosafety Level 2 as recommended for any potential infectious human serum or blood specimen in the CDC/NIH manual - "Biosafety in Microbiology and Biomedical Laboratories", 1984 or latest edition.

2. For in vitro diagnostic use only.
3. Do not use reagents past their expiration date.
4. Handle slides by the edges since direct pressure on the antigen wells may damage the antigen.
5. All reagents must be at room temperature (21-26 C) before running the assay. Temperature WILL affect the results of the assay.
6. Once the procedure has started, do not allow the antigen in the wells to dry out. This may result in false negative test results, or artifacts.
7. Use only distilled or deionized water and clean glassware.
8. Use separate pipette tips for each sample, control and reagent to avoid cross contamination.
9. ANCA Negative and Positive Controls must be run with each assay.
10. No assurance is given that these reagents are free of microbial or fungal contamination.
11. Incubation times and temperatures other than those specified may give erroneous results.

## **STABILITY AND STORAGE**

1. The kits and components should be stored at 2 - 8 C.
2. Rehydrated PBS buffer remains stable for 60 days at 2 - 8 C.

## **SERUM COLLECTION**

A whole blood sample should be collected by qualified personnel using approved aseptic venipuncture techniques. Obtain and/or clarify serum samples containing visible particulate matter by centrifugation. The samples may be stored at 2 - 8°C if testing is to be done within 5 days. If stored longer, they should be frozen at -20°C or lower. Do not use a frost-free freezer which may allow the specimens to go through freeze-thaw cycles that may denature the IgG antibody and cause spurious results. Do not use hyperlipemic, hemolytic, heat treated or contaminated samples.

## PREPARATION OF REAGENTS AND SAMPLES

1. Bring **all** reagents to room temperature before use. Remove the amount of slides to be used for the day's testing. Remove the slide from the foil pouch by tearing at notch. Carefully remove slide from the pouch and avoid touching the well areas. The slide is ready-to-use.
2. Prepare PBS buffer by pouring the contents of the package into a container and add deionized or distilled water to bring up to 1.0 L final volume. Mix thoroughly. Store 1X solution at 2 - 8°C.
3. Positive and Negative Controls: are ready-to-use, **Do not dilute further**. The Positive Control may be further diluted to the 1+ titer suggested on the vial label.
4. FITC Conjugate is ready-to-use liquid. The conjugate is stable at 2-8°C until the labeled expiration date.
5. Patient's Sera: **Screening:** Dilute each patient serum 1:20 (1+19) with PBS. Prepare dilutions (10  $\mu$ L + 0.20 ml) in test tubes. **Titration:** Set up doubling dilutions of serum starting at 1:20 (i.e. 1:20, 1:40, 1:80, 1:160, 1:320, etc.) Mix all dilutions by vortexing on low speed.
6. Assign each well of the slide the appropriate sample.

## ASSAY PROCEDURE

1. Dispense one drop (20-30  $\mu$ L) of each diluted patient serum or Control over the appropriate slide well. Do not touch the surface of the slide.
2. Place the slide into a moist chamber for 20 minutes at room temperature (22-26°C).
3. Remove the slide(s) from the moist chamber and tap the slide on its edge to allow the serum to run off onto a lint-free absorbent paper towel. Using a wash bottle, gently rinse the remaining sera from the slide using an indirect stream of PBS buffer above the wells, while the slide is tipped, to avoid cross-reactivity between wells. Care should be taken to avoid aiming the stream directly on to the wells.
4. Place the slide(s) into a staining dish containing PBS buffer and incubate for 5 min. Incubate for a second 5 minutes in fresh PBS.  
**Note:** Caution should be taken to not extend incubation of the wash times. The substrate will be affected and poor morphology will result.
5. Place a blotter on the lab table with absorbent side up. Remove the slide(s) from the PBS and invert so that substrate side faces absorbent side of the blotter. Line up wells to blotter holes. Place the slide on top of the blotter. Do not allow the substrate to dry.
6. Wipe the back of the slide with lint-free paper towel. Apply sufficient pressure while wiping to absorb buffer.
7. Remove the slide from the blotter and place substrate side up on the bench.
8. Deliver 1 drop (20-30  $\mu$ L) of Conjugate per well, and incubate in a moist chamber for 20 minutes at room temperature.
9. Wash slide as described in steps 3-7.
10. Place 4-5 drops of Mounting Medium randomly on the slide.
11. Apply a 22 X 70 mm coverslip to the slide.
12. Examine the reactions on the slide under a fluorescent microscope.  
**Note:** To maintain fluorescence, store the mounted slide in a moist chamber placed in a dark refrigerator. Read and record reactions.

## INTERPRETATION OF RESULTS

**Positive** = C-ANCA is identified as a positive result when there is intense positive granular staining of the **cytoplasm** that extends to the border of the human granulocyte substrate displaying a 1+ or greater fluorescence and there is absence of nuclear staining.

P-ANCA exhibits intense positive **perinuclear** staining of the multi-lobed nucleus with a poorly defined cell border. A 1+ or greater fluorescence is considered a positive result.

Differentiation between C-ANCA, P-ANCA and possible cross-reactivity with GS-ANA (Granular Specific ANA) should be made on HEp2 cells and formalin fixed ANCA slides.

**Negative** = A serum is considered negative for ANCA if there is an absence or minimal staining of the cytoplasm, or a low level of uniform (not granular) staining of the cytoplasm. The fluorescence is less than 1+ at 1:20 dilution. The multi-lobed nucleus should also be negative.

## QUALITY CONTROL

A positive and negative control serum is supplied with the kit. The controls verify test performance, test integrity and operator reliability. Good laboratory practice dictates running the positive and negative control each time the kit is used.

The Negative Control should result in little or no fluorescence. If this Control shows bright fluorescence, either the Control, antigen, conjugate or technique may be at fault.

The Positive Control should result in bright 3+ to 4+ fluorescence. If this Control shows little or no fluorescence, either the Control, antigen, conjugate or technique may be at fault. Note: This control may be used at the 1+ titer suggested on the label.

In addition to positive and negative controls, a PBS control well should be run to establish that the conjugate is free from non-specific staining of the substrate. If the antigen shows bright fluorescence in the PBS control, repeat assay using fresh conjugate. If the antigen still fluoresces, neither the conjugate or antigen may be at fault.

A titratable positive control should be run when patient samples are being titered. Either an in-house control of known titer or the Positive Control included in the kit may be run. The 1+ titer of the Positive Control (C-ANCA) is included on the vial label. The Control should titer to +/- 1 two-fold serial dilution in the assay. A P-ANCA control (Cat.# A003L or in-house) of known titer should also be run for quality control of the P-ANCA pattern.

## LIMITATIONS

1. The antibody titer obtained from individual samples do not necessarily correlate with disease severity and should not be reported as such. Antibodies from different patients may have different avidities. Paired sera run at the same time under the same conditions will give a better indication of the disease process. However, the result of an assay is not diagnostic proof of the presence or absence of vasculitic disease. Immunosuppressive therapy or treatment should not be started based on a single positive result. ELISA should be used for confirmation.
2. The results obtained from this assay are intended to be an aid to diagnosis only. Each physician must interpret the results in light of the patient's history, physical findings and other diagnostic procedures. Low positive samples should be evaluated in light of clinical symptoms and results of testing for other autoimmune antibodies, such as SLE, GBM, etc. Confirmation of results is advised by redrawing and retesting the patient.
3. Positive results obtained from this assay should be confirmed by EIA since ANCA antigens contain additional specificities such as cathepsin, lactoferrin and elastase. Due to sequence similarities some cross-reactivity is possible. In addition, a small number of SLE patients, especially SLE with neurologic disease,

will have antibodies to myeloid elastase, cathepsin and lactoferrin.<sup>14,15</sup> In certain diseases (UC and/or PSC), a similar ANCA pattern has been identified but does not react with PR3 or MPO in ELISA.<sup>16-18</sup>

4. Only if test instructions are rigidly followed will optimum results be achieved.
5. Use fresh serum or samples frozen and thawed only once. Samples that are improperly stored or are subjected to multiple freeze-thaw cycles may yield spurious results.
6. Patients should be screened on ANA HEp2 substrate to avoid confusion. Granulocyte-specific antinuclear antibodies (GS-ANA) also called neutrophil nuclear antibodies (ANNA), are a class of specific antinuclear antibodies that react with neutrophil nuclei in a homogenous pattern, but not with the other substrates used for ANA detection (e.g., rat liver or HEp2 cells). The exact non-histone antigen(s) of GS-ANA have not yet been identified.

This class of antibodies is said to be associated with active rheumatoid arthritis (RA), especially when associated with vasculitis and/or neutropenia in frequencies up to 75%. No reactivity to myeloperoxidase or proteinase -3 is detected in these patients. An atypical P-ANCA pattern associated with inflammatory bowel disease (IBD) is not completely defined. It is detected in 59-84% of ulcerative colitis, only 10-20% of Crohn disease, and also in 65-84% of primary sclerosing cholangitis with and without IBD.<sup>23,24</sup> These IBD-related P-ANCA are not associated with antibodies to PR3 or to MPO. Atypical ANCA patterns are considered "suspect" and further testing by EIA is recommended. Testing serum samples for their reactivity with HEp2 cells and formalin fixed slides helps to differentiate atypical pattern antibody specificities such as GS-ANA, elastase or lactoferrin from P-ANCA for confirmation of a positive result.

## EXPECTED RESULTS

Published clinical studies have demonstrated that < 1% of patients who had a suspected diagnosis of vasculitis and/or glomerulonephritis exhibited a positive C- or P-ANCA pattern. Anti-PR3 and anti-MPO antibodies are highly sensitive and specific, therefore the combination of IFA and EIA testing is suggested. Combined testing by IFA and EIA for ANCA antibodies will result in the best specificity and sensitivity.

The presence of C-ANCA antibodies is associated with many different vasculitic disease states with classic Wegener's resulting in a sensitivity range of >90%. Classic or extended Wegener's has been characterized by granulomatous inflammation of the respiratory tract, necrotizing crescentic glomerulonephritis and systemic vasculitis. Patients with more benign disease such as pauci-immune necrotizing glomerulonephritis are detected approximately 40- 50% of the time. In patients with limited Wegener's without renal involvement the rate of detection is 67-86%.<sup>19-22</sup>

It should be noted that not all ANCA positive sera will react with PR3 or MPO antigens. Depending on the methods and antigens used in the test, the sensitivity range is approximately 70-100%. Positive ANCA sera may contain additional antibodies that are not present in the PR3 antigen or MPO antigen.<sup>19-22</sup> Published studies have demonstrated a high percentage of P-ANCA antibodies in various systemic vasculitic and glomerulonephritis disease states. Positive ANCA results obtained in patients with other types of vasculitides (sometimes in the form of microscopic polyarteritis, Churg-Strauss syndrome, and polyangitis) typically give a P-ANCA pattern and are usually due to antibodies to MPO. Some studies have shown up to 92% positive incidence of anti-MPO in Systemic Small Vessel Vasculitis, polyarteritis, primary Glomerulonephritis and Rapidly Progressive Glomerulonephritis confirmed patients. In a group with systemic necrotizing vasculitis of the polyarteritis group, 50% of the patients had MPO antibodies. In another study, 50 of 424 sera with a perinuclear staining pattern were positive for MPO antibodies as tested by ELISA.<sup>10,11,17,18</sup>

An agreement at the 1st International Workshop on ANCA in 1988 recommended that sera should be screened for ANCA antibodies at a 1:20 dilution for immunofluorescent assays. Based on this recommendation, this IFA was established using a 1:20 serum dilution. As confirmation, a series of 100 normal blood donors (male and

female, ages 20-65) from the East coast were tested and found negative in this assay using the 1:20 serum dilution.

The following table shows the incidence of ANCA antibodies found in the various known disease state and normal sera obtained from the clinical studies performed in the evaluation of this ANCA Antibody IFA kit:

<b>Disease State</b>	<b>Total Tested</b>	<b>No. C-ANCA Negative</b>	<b>No. C-ANCA Positive</b>	<b>No. P-ANCA Negative</b>	<b>No. P-ANCA Positive</b>
Wegener's	44	6	38	39	5
ICGN	41	31	10	13	28
PAN	4	1	3	4	0
PRS	8	6	2	3	5
No DX	36	20	16	23	13
MPA	21	19	2	2	19
CGN	7	4	3	3	4
UC	24	20	4	14	10
SLE	17	16	1	14	3
All other Autoimmune	30	28	2	25	5
All other Vasculitic	12	8	4	8	4
Normals	198	195	3	196	2

ICGN = Idiopathic Crescentic Glomerulonephritis; PAN = Polyarteritis Nodosa; PRS = Pulmonary Renal Syndrome; No DX = No Diagnosis; MPA = Microscopic Polyarteritis; CGN = Crescentic Glomerulonephritis; UC = Ulcerative Colitis; SLE = Systemic Lupus Erythematosus

The overall clinical sensitivity/specificity/agreement between the DIAGNOSTIC AUTOMATION ANCA IFA results and **clinical diagnosis** of Idiopathic Crescentic Glomerulonephritis, Pulmonary Renal Syndrome, Microscopic Polyarteritis in this study is as follows.

		ICGN, MPA and PRS diagnosed	
		+	-
DIAGNOSTIC AUTOMATION P-ANCA	+	<b>50</b>	<b>51</b>
	-	<b>16</b>	<b>325</b>

Sensitivity = 75.7%

Specificity = 86.4%

Agreement = 84.8%

The overall clinical sensitivity/specificity/agreement between the DIAGNOSTIC AUTOMATION ANCA IFA results and **clinical diagnosis** of Wegener's Granulomatosis in this study is as follows.

		Wegener's diagnosed	
		+	-
DIAGNOSTIC AUTOMATIO N C-ANCA	+	38	52
	-	6	346

Sensitivity = 86.3% Specificity = 86.9%

Agreement = 86.9%

## PERFORMANCE CHARACTERISTICS

### Comparison

A study was performed using 244 patient samples obtained from a laboratory specializing in autoimmune diseases and 198 normal blood donor samples. These samples were assayed in the DIAGNOSTIC AUTOMATION ANCA IFA kit using the C-ANCA pattern results compared to an ELISA anti-PR3 kit. The results yielded an 86.0% sensitivity, 97.1% specificity, and 94.8% agreement to the ELISA procedure. The results are summarized as follows:

		reference EIA (PR3)	
		+	-
DIAGNOSTIC AUTOMATION ANCA IFA	+	80	10
	-	13	339

Relative Sensitivity = 86.0%

Relative Specificity = 97.1%

Relative Agreement = 94.8%

These same samples were assayed with the DIAGNOSTIC AUTOMATION ANCA IFA kit using the P-ANCA pattern interpreted results compared to an ELISA anti-MPO kit. The results yielded a 98.7% sensitivity, 93.1% specificity, and 94.1% agreement to the ELISA procedure. The results are summarized as follows:

		Reference EIA (MPO)	
		+	-
DIAGNOSTIC AUTOMATION P-ANCA	+	76	25
	-	1	340

Relative Sensitivity = 98.7%

Relative Specificity = 93.1%

Relative Agreement = 94.1%

## Cross Reactivity

A study was performed using 50 autoimmune disease characterized samples obtained from an outside laboratory. The samples were run on the DIAGNOSTIC AUTOMATION ANCA IFA. Only one patient exhibited a positive C-ANCA pattern on IFA, for which the diagnosis indicated SLE autoimmune disease for this patient. The other SLE known patients did not demonstrate a positive reaction on the C-ANCA IFA. The nature of the SLE related disease can cause a number of anomalous antibody responses, which are unknown, and may demonstrate a slightly positive result. Eight samples exhibited a positive P-ANCA pattern in IFA. Three of the samples were SLE, but the other diagnoses were mixed.

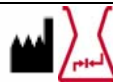
## Reproducibility

Studies were performed to demonstrate the Intra-Run, Inter Day and Inter-Lot reproducibility of the DIAGNOSTIC AUTOMATION ANCA IFA Test kit. The reproducibility studies were performed using the screening dilution of 1:20 for five sera; 3 positive sera samples (one high, one mid-range and one low) and two negative sera samples. The fluorescence intensities were interpreted on a graded scale of 1+ to 4+. The Intra-Run study used the sera in replicates of 6 wells each. The same 5 samples were run on three lots of kits over 5 days for Inter-Day and Inter-Lot Reproducibility. The negative samples remained negative in all wells throughout the testing period. All of the readings for the three positive sera demonstrated a consistent fluorescence intensity of 1+2, 2+3, and 4+ respectively.

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