



ImmuGlo™ Anti-Neutrophil Cytoplasmic Antibody (ANCA) Test System

For *in vitro* Diagnostic Use

CDC Analyte Code: 0440

CDC Test System Code: 28549

CLIA Complexity: High

PRODUCT INSERT

Catalog No. 1116	ANCA Kit (ethanol fixed)	24 Determinations
Catalog No. 1140	ANCA Kit (ethanol fixed)	48 Determinations
Catalog No. 1141	ANCA Kit (formalin fixed)	48 Determinations
Catalog No. 1142	COMVI-ANCA (ethanol+formalin fixed)	48 Determinations

INTENDED USE

An indirect immunofluorescence antibody test for the detection and semi-quantitation of anti-neutrophil cytoplasmic antibodies (ANCA) in human serum. ANCA are found in the sera of patients with necrotizing vasculitides and hence, serve as an aid to the clinical and other laboratory findings in the diagnosis of these disorders.

SUMMARY AND EXPLANATION

ANCA occur in patients with *Wegener's granulomatosis*, *microscopic polyarteritis*, *necrotizing or crescentic glomerulonephritis*, other *vasculitides* and *inflammatory bowel disorders* (primarily *ulcerative colitis*)¹⁻¹¹. Indirect immunofluorescence staining of ethanol fixed neutrophils may exhibit different types of fluorescent staining patterns. These include:

1. Autoantibodies against cytoplasmic antigens of neutrophils giving a diffuse cytoplasmic staining (cANCA).
2. Autoantibodies against neutrophil antigens giving a perinuclear reaction pattern (pANCA).
3. Common antibodies against nuclear antigens (ANA).

The significance of these two types of ANCA differs¹². cANCA are found primarily in patients with *Wegener's granulomatosis* and *microscopic polyarteritis*, whereas pANCA occurs in various vasculitic disorders, *ulcerative colitis* (UC) and *primary sclerosing cholangitis* (PSC). Eighty percent of patients in whom pANCA are found have histologic evidence of vasculitis, UC or PSC. ANCA occur in more than 90% of patients with active generalized *Wegener's granulomatosis* and in 67% of patients with active limited disease. The incidence of ANCA varies in patients with clinical remission. Patients with active *Wegener's granulomatosis* may occasionally be ANCA negative. However, in such cases, repeat testing should yield positive ANCA reactions¹. The indirect immunofluorescence microscope method is considered to be the gold standard for detecting ANCA. Antigen fixation of PMN is usually performed in ethanol. When fixed in ethanol, two types of ANCA staining reaction patterns have been identified: cytoplasmic (cANCA) and perinuclear (pANCA). The pANCA reactions can be confirmed by repeat testing on formalin fixed slides where pANCA reactions convert to cANCA whereas ANA reactions either remain nuclear or become negative on formalin fixation.

For technical assistance please contact:



IMMCO Diagnostics, Inc.
60 Pineview Drive
Buffalo, NY 14228-2120
Telephone: (716) 691-0091
Fax: (716) 691-0466
Toll Free USA/Canada: 1-800-537-TEST
E-Mail: info@immcodiagnostics.com

or your local product distributor

PRINCIPLES OF PROCEDURE

In the indirect immunofluorescence method used in this kit, patients' sera are incubated on optimized preparations of human neutrophils to allow binding of antibodies to the substrate. Any antibodies not bound are removed by rinsing the slide. Bound antibodies of the IgG class are detected by incubation of the substrate with fluorescein-labeled, anti-human IgG conjugate. Reactions are observed under a fluorescence microscope equipped with appropriate filters. The presence of ANCA is demonstrated by an apple green fluorescence either of the cytoplasm with a diffuse granular cytoplasmic staining (cANCA) or a perinuclear staining (pANCA). The titer (the reciprocal of the highest dilution giving a positive reaction) is then determined by testing serial dilutions¹³.

REAGENTS

Storage and Preparation

Store all reagents at 2°-8°C. Reagents are ready for use after equilibration to room temperature.

Precautions

For *in vitro* Diagnostic Use. All human derived components used have been tested for HBsAg, HCV, HIV-1 and 2 and HTLV-I and found negative by FDA required tests. All human serum specimens and human derived products should be treated as potentially hazardous, regardless of their origin. Follow good laboratory practices in storing, dispensing and disposing of these materials¹⁴.

WARNING - Sodium azide (NaN₃) may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal of liquids, flush with large volumes of water to prevent azide buildup. Sodium azide may be toxic if ingested. If ingested, report incident immediately to laboratory director or poison control center.

Instructions should be followed exactly as they appear in this insert to ensure valid results. Do not interchange kit components with those from sources other than the same catalog number from IMMCO DIAGNOSTICS. Do not use beyond expiration date.

Materials Provided

ImmuGlo™ Anti-Neutrophil Cytoplasmic Antibody (ANCA) Test Systems. Kits contain sufficient reagents to perform 24 or 48 determinations

4 x	6-well Human Neutrophil Substrate Slides (<i>ethanol fixed</i>) or	<i>Catalog No. 1116</i>
8 x	6-well Human Neutrophil Substrate Slides (<i>ethanol fixed</i>) or	<i>Catalog No. 1140</i>
8 x	6-well Human Neutrophil Substrate Slides (<i>formalin fixed</i>) or	<i>Catalog No. 1141</i>
8 x	COMVI 6+6 well slide, human neutrophil substrate for ANCA screen and pANCA confirmation (<i>ethanol and formalin fixed slides</i>)	<i>Catalog No. 1142</i>
1 x 0.5 ml	cANCA Positive Control*	
1 x 0.5 ml	pANCA Positive Control*	
1 x 0.5 ml	Negative Control*	

Reproducibility:

Studies were performed to demonstrate intra and inter-assay variability. Four ANCA positive (two each of c and p ANCA and one ANCA negative sera were tested starting at 1:20 dilution to endpoint. They were tested on 3 different lots each of ethanol, formalin and COMVI slides for four days to determine intra as well as inter-reproducibility. Negative samples remained negative and positive samples provided the expected titer.

REFERENCES

1. Nolle B, Specks U, Lüderman J, Rohrbach M, DeRemee RA and Gross WL. Anticytoplasmic antibodies: Their immunodiagnostic value in Wegener's granulomatosis. *Ann Int Med* 111: 28-40, 1989.
2. Venning MC, Quinn A, Broomhead V and Bird AG. Antibodies directed against neutrophils (cANCA and pANCA) are of distinct diagnostic value in systemic vasculitis. *Quart J Med* 77: 1287-1296, 1990.
3. Van der Woude FJ, Daha MR and Van Es LA. The current status of neutrophil cytoplasmic antibodies. *Clin Exp Immunol* 78: 143-148, 1989.
4. Specks U, Wheatley CL, McDonald TJ, Rohrbach MS and DeRemee RA. Anticytoplasmic autoantibodies in the diagnosis and follow up of Wegener's granulomatosis. *Mayo Clin Proc* 64: 28-36, 1989.
5. Tervent JWC, van der Woude FJ, Fauci AS and Ambrus JL. Association between active Wegener's granulomatosis and anticytoplasmic antibodies. *Arch Int Med* 149: 2461-2465, 1989.
6. Cross CE and Lillington GA. Serodiagnosis of Wegener's granulomatosis: Pathobiologic and clinical implications. *Mayo Clin Proc* 64: 119-122, 1989.
7. Seibold F, Slametschka D, Gregor X and Weber P. Neutrophil Autoantibodies: A genetic marker in primary sclerosing cholangitis and ulcerative colitis. *Gastroenterol* 107:532-536, 1994.
8. Claise C, Johanet C, Bouhnik Y et al. Antineutrophil cytoplasmic autoantibodies in autoimmune liver and inflammatory bowel diseases. *Liver* 16:28-34, 1996.
9. Gigase P, DeClerck LS, Van Cotthem KA et al. Anti-Neutrophil cytoplasmic antibodies in inflammatory bowel disease with special attention for IgA-class antibodies. *Dig Dis and Sci* 42:2171-2174, 1997.
10. Shanahan F. Neutrophil autoantibodies in inflammatory bowel disease: Are they important? *Gastroenterol* 107:586-589, 1994.
11. Shanahan F and Bernstein CN. ANCA's aweigh in colitis. *Gastroenterol* 105:946-947, 1993.
12. Lüdemann J, Utecht B and Gross WL. Laboratory methods for detection of antineutrophil cytoplasm antibodies. *Clin Immunol Newsletter* 10:159-166, 1990.
13. Beutner EH, Kumar V, Krasny SA and Chorzelski TP. Defined immunofluorescence in immunodermatology. In "Immunopathology of the Skin", Beutner EH, Chorzelski TP and Kumar V, Eds, John Wiley and Sons, New York, 3rd Ed, 3-40, 1987.
14. Biosafety in Microbiological and Biomedical Laboratories. Center for Disease Control, National Institute for Health, HHS Pub. No {CDC} 93-8395) 1993.
15. Streicher J, Fabian B, Herkner K et al. Anti-cytokeratins are a potential source of false positive indirect immunofluorescence assays for cANCA. *J Clin Lab Analysis*. 12:54-59, 1998.
16. Hagen CF, Daha MR, Hermand J et al. Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis. *Kidney Int* 53:743-753, 1998.
17. Jennette JC, Wilkman AS, Falk RJ. Diagnostic predictive value of ANCA serology. *Kidney Int* 53:796-798, 1998.

Table 2: Sensitivity and Specificity of the Indirect Immunofluorescence Test in Patients with Systemic Vasculitides¹⁶

	N	cANCA	Sensitivity %	
			pANCA	c- or pANCA
Patients				
Wegener's granulomatosis	97	64	21	85
Microscopic polyangiitis	44	23	58	81
Idiopathic RPGN	12	36	45	81
Classical polyarteritis nodosa	10	10	30	40
Churg-Strauss syndrome	6	33	33	66
Specificity %				
Controls				
Disease Controls	184	95	81	76
Healthy controls	740	98	96	94

Adapted from reference 16

PERFORMANCE CHARACTERISTICS

ImmuGlo™ Anti-Neutrophil Cytoplasmic Antibody (ANCA) Test was compared with another indirect immunofluorescence ANCA test. The comparison included a total of 129 serum samples obtained from a diagnostic reference laboratory specializing in the detection of autoimmune diseases. These samples were tested according to the procedures recommended by the manufacturers. The results are as follows:

		ImmuGlo™		
		Positive	Negative	Total
Other	Positive	49	0	49
	Negative	7	73	80
	Total	56	73	129

Relative Specificity: 91%
Relative Sensitivity: 100%
Relative Agreement: 95%

Seven discrepant samples observed by indirect immunofluorescent method above were tested by ELISA for antibodies to myeloperoxidase (MPO) or proteinase 3 (PR3) antigens, the two major antigens associated with ANCA. Of the seven positives on ImmuGlo and negative on the other test kit, all but one tested positive by ELISA, suggesting thereby, the true positivity of these samples.

Cross Reactivity:

Antinuclear antibodies (ANA) may exhibit positive reactions on PMN. To determine reactivities on PMN of ANA, which may be confused for ANCA reactivity, we tested a total of 25 ANA positive samples of varying antibody specificities on ethanol, formalin and COMVI slides. All ANA positive sera with the exception of SS-A (Ro) and SS-B (La) antibody specificities, showed nuclear reactions. These ANA reactions either remain nuclear or become negative on formalin fixed slides.

- 1 x 5.0 ml** Ready to use goat anti-human IgG FITC Conjugate*. **Protect from light.**
- 1 x 60 ml** Buffered Diluent. Ready to use*
- 2 vials** Phosphate Buffered Saline. **Dissolve each vial to 1 liter.**
- 1 x 5.0 ml** Mounting Medium. **Do not freeze*.**
- 1 x 1.0 ml** Evans Blue Counterstain
- 1 x 12** Cover Slips

Other Available Components:

- 1 x** 6-well Human Neutrophil Substrate Slides *Ethanol fixed* Catalog No. 2162
- 1 x** 6-well Human Neutrophil Substrate Slides *Formalin fixed* Catalog No. 2186
- 1 x** COMVI 6 + 6 well slide, human neutrophil substrate ANCA screen and pANCA confirmation slide *ethanol and formalin fixed* Catalog No. 2189
- 1 x 0.5 ml** cANCA Positive Control* Catalog No. 2252
- 1 x 0.5 ml** pANCA Positive Control* Catalog No. 2240

*CAUTION - Contains <0.1% NaN₃

Materials Required but not Provided

- Fluorescence microscope
- Micropipette or Pasteur pipette
- Serological pipettes
- Staining dish (e.g. Coplin jar)
- Small test tubes (e.g. 13 x 75 mm) and test tube rack
- Distilled or deionized water
- 1 liter container
- Wash bottle
- Paper towels
- Incubation chamber

SPECIMEN COLLECTION AND PREPARATION

Only serum specimens should be used for this procedure. Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of this test and should not be used. Store specimens at 2-8°C for no longer than one week. For longer storage, serum should be frozen at -20°C. Avoid repeated freezing and thawing of samples.

PROCEDURE

Test Method

The indirect immunofluorescence staining procedure is illustrated in the following figures:

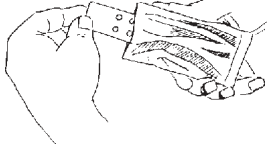
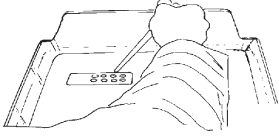
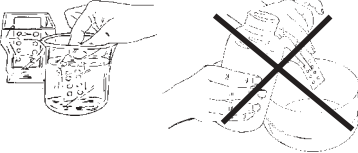
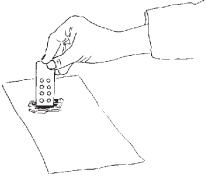
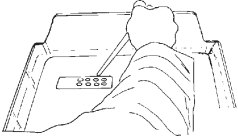
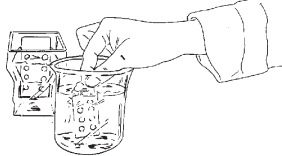
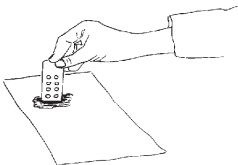
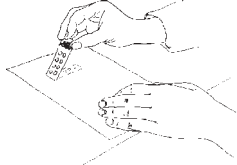
1. Let pouch equilibrate to room temperature, then remove slide(s) from pouch. 
2. Place slide(s) into moisture chamber and add samples and controls. Cover and incubate 30 minutes. 
3. Rinse slide(s) by dipping into beaker with PBS. Transfer slide(s) into Coplin jar and wash 10 minutes. 
4. Blot edge of slide(s) on absorbent paper. Proceed immediately with next step. 
5. Apply Conjugate to each well. Cover and incubate 30 minutes. 
6. Rinse slide(s) by dipping into beaker with PBS. Transfer slide(s) into Coplin jar and wash 10 minutes. 
7. Blot edge of slide(s) on absorbent paper. Proceed immediately with next step. 
8. Mount cover slip and read under fluorescent microscope. 

Table 1

Incidence of anti-Neutrophil Cytoplasmic Antibodies¹

Clinical Condition	% Positive
Wegener's granulomatosis	
Generalized	
Active	96
Partial remission	71
Full remission	41
Local recurrence	80
Localized	
Active	67
Partial remission	54
Full remission	32
Inflammatory Bowel Disorders	
Ulcerative colitis	70
Primary sclerosing cholangitis	82
Crohn's disease*	27
Disease Controls	
Blood Donors	0
Connective tissue autoimmune disorders**	5
Misc. medical conditions	0
Granulomatosis disease	0
Primary renal disease	1

* ANCA titers are usually low

** ANCA Reactivity is pANCA

In this study, ANCA was found to be present in 85% of patients with *Wegener's granulomatosis*, of these, 64% were positive for cANCA and 21% for pANCA. The pANCA was more prevalent than cANCA in microscoping polyangitis (58% vs 23%) with sensitivity of 81% for microscoping polyangitis and 82% for *idiopathic rapidly progressive glomerulonephritis*. Of the disease and healthy controls, ANCA was present in 19% and 6% respectively, thus providing a specificity of 76% in disease controls and 94% in healthy individuals (Table 2).

However, the positive predictive value of the ANCA test is much better and more significant if evaluated in conjunction with clinical signs and symptoms. In an editorial, Jennette, Wilmant and Falk¹⁷ reported a positive predictive value of 92% in a patient with serum creatinine >3 mg/dl. Similarly, in patients in which the initial positive predictive value is low, a positive ANCA result increases the likelihood of a disease to a level that may warrant further evaluation. The authors conclude that ANCA testing in patients with strong clinical evidence for pauci-immune crescentic glomerulonephritis is most useful for substantiating the diagnosis whereas ANCA testing in patients with weak clinical evidence is most useful for ruling out the diagnosis.

In some patients with *Wegener's granulomatosis*, ANCA tests may be negative. In such cases repeat testing may yield positive results. Patients with *Wegener's granulomatosis* on treatment invariably are negative for ANCA.

ANA reactions may sometimes be confused with or mimic pANCA staining. To confirm pANCA reactivity, sera providing pANCA reactions should be retested either on formalin fixed slides (*Cat. Nos. 2189, 2186*) or for ANA on HEp-2 or tissue sections. pANCA specimens on formalin fixed ANCA substrate should give cANCA reactivity, whereas reactions to ANA should be either negative or remain nuclear. Anti-cytokeratin antibodies may result in false positive cANCA reaction¹⁵. In such cases, indirect IF tests on HEp-2 cells may help in distinguishing "pseudo-ANCA" from true cANCA reaction (*Cat. Nos 1102, 1103*).

The antibody titers do not necessarily associate with disease activity. The results of the ANCA test should be evaluated in light of clinical findings as the presence or absence of ANCA may not be directly associated with a vasculitis disorder.

The positive ANCA results obtained by immunofluorescence should be confirmed by ELISA. ANCA of certain antigen specificities are more indicative of a specific vasculitic disorder. Also, ANCA have been associated with immunological disturbances other than vasculitis disorders such as ulcerative colitis^{7,8}.

EXPECTED VALUES

The ANCA reactions should be negative on normal sera.

Sixty-four (64) normal samples were tested for ANCA. All samples were negative for ANCA at 1:20 dilution.

A positive ANCA in the appropriate clinical setting is useful in the diagnosis of systemic vasculitides and inflammatory bowel disorders⁷⁻¹¹. cANCA staining occurs most commonly in *Wegener's granulomatosis* and pANCA staining in microscopic polyangitis, pauci-immune crescentic glomerulonephritis and ulcerative colitis⁷⁻¹¹. In other vasculitides (*polyarteritis nodosa, takayasu disease, giant cell arteritis, Behcet's disease*) ANCA are rare or absent. The incidence of ANCA in *Wegener's granulomatosis* and other vasculitides as abstracted from the literature is summarized in Table 1.

In the European ANCA collaborative assay standardization project, Hagen and his collaborators¹⁶ evaluated the usefulness of the indirect IF test towards the diagnosis of idiopathic systemic vasculitides.

A. Screening:

- Step 1.** Dilute each patient serum **1:20** with the Buffered Diluent provided (50 µl serum + 1.0 ml Diluent). **Do not dilute Positive or Negative Controls.** Save the undiluted sera to determine antibody titers if screening tests are found to be positive.
- Step 2** Allow pouches containing substrate slides to equilibrate to room temperature for **10-15 minutes**. Carefully remove the slides without touching the substrate.
- Step 3** Label the slides and place them in an incubation chamber lined with paper towels moistened with water to prevent drying.
- Step 4** Invert dropper vial and gently squeeze to apply **1 drop** (approximately 50 µl) of the **Negative Control** to well #1. Similarly apply **1 drop of Positive Control** to well #2. Avoid overfilling the wells.
- Step 5** Using a micropipette or Pasteur pipette, apply **1 drop** of patient's diluted serum (approximately 50 µl) to the other wells. Avoid overfilling the wells.
- Step 6** Place the lid on the incubation chamber and incubate slides **30 minutes** at room temperature.
- Step 7** Remove a slide from the incubation chamber. Hold slide at tab end and rinse gently with approximately **10 ml** of PBS using a pipette, or rinse slide in a beaker filled with PBS. Do not use wash bottle. Transfer slide immediately into Coplin jar and wash **10 minutes**. Repeat process with all remaining slides.
- Step 8** Remove slide(s) from Coplin jar. Blot the edge of the slide on a paper towel to remove excess PBS. Place the slide in the incubation chamber. Immediately invert the **Conjugate** dropper vial and gently squeeze to apply **1 drop** (approximately 50 µl) to each well.
- Step 9.** Repeat **Steps 7 and 8** for each slide.
- Step 10** Replace the lid on the incubation chamber. Incubate **30 minutes** at room temperature.
- Step 11** Remove a slide from incubator. Hold the slide at the tab end and dip the slide in a beaker containing PBS to remove excess conjugate. Place slide(s) in a staining dish filled with PBS for **10 minutes**. If desired, 2-3 drops of Evans blue counterstain may be added to the final wash. Repeat for the remaining slides.
NOTE: Improper washing may impact the morphology of the PMN cells and may lead to increased background fluorescence.
- Step 12** Remove a slide from the staining dish. Blot the edge of the slide on a paper towel to remove excess PBS. **To prevent slide from drying, proceed immediately with Step 13 while the slide is still wet.**
- Step 13** Mount the coverslip by applying **1 drop** of Mounting Medium gently onto each well and place cover slip over slide. **Avoid applying undue pressure and prevent lateral movement of the coverslip.**
- Step 14** Repeat **Steps 12 and 13** for each slide.
- Step 15** Examine for specific fluorescence under a fluorescence microscope at a magnification of **200x** or greater.

Slides may be read as soon as prepared. However, because of the presence of antifading agent in the mounting medium, no significant loss of staining intensity occurs if reading is delayed for up to 48 hours. Slides should be stored in the dark at 2°- 8°C.

B: End Point Determination (Titration)

A serum positive in the screening test may be further tested following **Steps 5 through 13** to determine the titer. Each test run should include the Positive and Negative Controls. Make serial two-fold dilutions starting at 1:20. Using one slide, a serum may be tested at dilutions ranging from 1:20 to 1:160. If positive at a 1:160 dilution, the results may be reported as greater or equal to 160. Additional slides may be used to obtain endpoints for those sera still positive at a 1:160 dilution. The reciprocal of the highest dilution producing a positive reaction is the titer.

Preparation of Serial Dilutions

Number four tubes 1 through 4. Add 1.0 ml of Buffered Diluent to tube 1 and 0.2 ml to tubes 2 through 4. Pipette 0.05 ml of undiluted serum to tube 1 and mix thoroughly. Transfer 0.2 ml from tube 1 to tube 2 and mix thoroughly. Continue transferring 0.2 ml from one tube to the next after mixing to yield the dilutions depicted in the following table:

Tubes	1	2	3	4
Serum	0.05 ml			
	+			
Buffered Diluent	1.0 ml	0.2 ml	0.2 ml	0.2 ml
		↪	↪	↪
Transfer	0.2 ml	0.2 ml	0.2 ml	
Final dilution	1:20	1:40	1:80	1:160 etc.

Quality Control

Both a Positive and Negative Control should be included with each test run. The Negative Control should show no specific fluorescence of the neutrophil, whereas the Positive Control should have 2+ or greater staining intensity of the cytoplasm of the neutrophil with cANCA positive control and perinuclear with pANCA positive control on ethanol fixed slides. On formalin fixed slides, the c-ANCA Positive Control remains cytoplasmic whereas pANCA reaction will become cytoplasmic and not perinuclear as observed on ethanol fixed slides.

If expected results are not obtained, the run should be repeated. If inadequate results continue to occur with the controls, these may be due to:

- Turbidity. Discard and use another control.
- Problems with the optical system of the fluorescence microscope. These may include: improper alignment, bulb beyond useful life expectancy, etc.
- Allowing the slide to dry during the procedure.

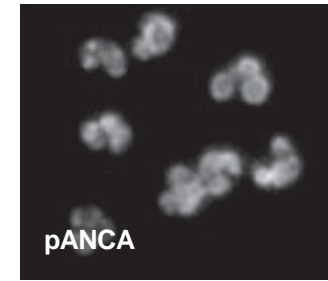
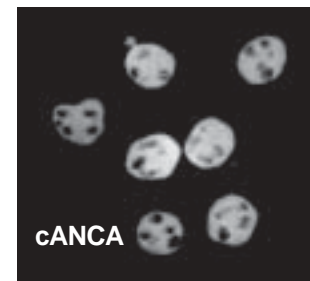
RESULTS

The results of the tests for ANCA should be reported as negative (<20), positive greater or equal to 160, or preferably, positive with titer and pattern.

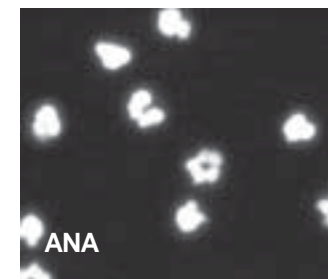
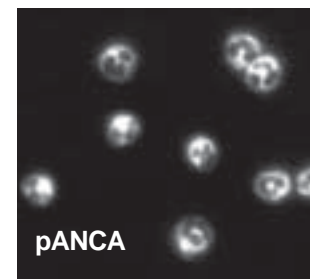
Read for specific diffuse, granular cytoplasmic staining (cANCA) or perinuclear staining (pANCA).

Other detectable antibodies include antinuclear antibodies (ANA), which sometimes may mimic pANCA reactions.

ANCA Reactions on Ethanol Fixed Slides



ANCA Reactions on Formalin Fixed Slides



LIMITATIONS OF THE PROCEDURE

In some cases, sera positive for ANCA may either be very weak or negative at the initial screening dilution (prozone phenomenon). In such doubtful cases the sera should be screened at higher dilutions and, if positive, antibody titers determined.

In some cases the presence of two or more antibodies in a serum which are reactive with the same substrate may cause an interference in their detection by immunofluorescence. This interference may cause either a failure to detect ANCA or a suppression of its titer if the interfering antibody has a higher titer than ANCA. The most common cause of the interference phenomenon in ANCA tests is the coexistence of ANA.