



Immunoassay Kits Beyond The Ordinary

Anti-PR3-ANCA (IgG) EIA

An enzyme-linked immunosorbent assay (ELISA) for detection and semi-quantitation of IgG antibodies to proteinase 3 (PR3) in human sera.

For In Vitro Diagnostic Use.

Catalog Number:	13-PR3-102X
Size:	96 Wells
Storage:	+2-8°C
Version:	031201 – ALPCO 12/13/05

ALPCO Diagnostics

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Intended use

The ALPCO PR3-ANCA test kit is an enzyme-linked immunosorbent assay (ELISA) for detection and semi-quantitation of IgG antibodies to proteinase 3 (PR3) in human sera. The assay is used to detect antibodies in a single serum specimen. The results of the assay are to be used as an aid in the diagnosis of Wegener's granulomatosis. The analysis should be performed by trained laboratory professionals.

FOR IN VITRO DIAGNOSTIC USE.

Summary and explanation

ANCAs (anti-neutrophil cytoplasmic antibodies) are a family of autoantibodies related to vasculitis and inflammatory disorders. Since 1985, when c-ANCA was shown to be related to Wegener's granulomatosis (1), interest in ANCAs has steadily increased, and today these antibodies are considered to be major diagnostic tools for the diagnosis and follow up of systemic vasculitis (13, 14).

The first method to detect ANCA was indirect immuno-fluorescence (IIF) performed on ethanol fixed granulocytes (2). This method yields two patterns, a cytoplasmic staining of the granulocyte denoting the presence of c-ANCAs, and a perinuclear staining denoting the presence of p-ANCAs. IIF was followed by ELISAs using the purified proteins (3,4).

The granulocyte is full of granules each with many different proteins. It was early shown that antibodies from systemic vasculitis patients bind to the alpha fraction containing the azurophil granules. The most important proteins were proteinase 3 (PR3)(5, 6) and myeloperoxidase (MPO)(7, 8). PR3 is a serine protease with a molecular weight of 29kD, and MPO is a dimer with a molecular weight of 140kD. Thus antibodies to proteinase 3 are termed PR3-ANCA, and antibodies to myeloperoxidase are termed MPO-ANCA.

Approximately 80-90% of WG patients manifest PR3-ANCA and 5-15% MPO-ANCA. One category of vasculitis is microscopic polyangiitis (MP). Most patients with active MP are characterised by positive ANCA test results, MPO-ANCA being more frequent than PR3-ANCA (9, 10, 11, 12).

Principle of the ALPCO PR3-ANCA

The wells of the microtitre strips are coated with purified proteinase 3. During the first incubation, specific antibodies in diluted serum, will bind to the antigen coating.

The wells are then washed to remove unbound antibodies and other components.

A conjugate of alkaline phosphatase-labelled antibodies to human IgG binds to the antibodies in the wells in this second incubation.

After a further washing step, detection of specific antibodies is obtained by incubation with substrate solution.

The amount of bound antibodies correlates to the colour intensity and is measured in terms of absorbance (optical density (OD)). The absorbance is then calculated against a calibrator curve and the results are given in arbitrary units.

Warnings and precautions

- For in vitro diagnostic use.

- The human serum components used in the preparation of the controls and calibrators in the kit have been tested for the presence of antibodies to human immunodeficiency virus 1 & 2 (HIV 1&2), hepatitis C (HCV) as well as hepatitis B surface antigen by FDA approved methods and found negative. Because no test methods can offer complete assurance that HIV, HCV, hepatitis B virus, or other infectious agents are absent, specimens and human-based reagents should be handled as if capable of transmitting infectious agents.
- The Centers for Disease Control and Prevention and National Institutes of Health recommended that potentially infectious agents be handled at the Biosafety Level 2.
- All solutions contain proclin 300 as a preservative. Never pipette by mouth or allow reagents or patient sample to come into contact with skin. Reagents containing proclin may be irritating. Avoid contact with skin and eyes. In case of contact, flush with plenty of water.
- The concentrations of anti-PR3 in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.

Specimen collection

The PR3-ANCA assay is for use with serum. Handle as if capable of transmitting infectious agents. Avoid using sera which are icteric, lipemic and hemolyzed.

Heat-inactivated sera can yield unspecific reactivities and should not be used.

Store serum between 2°-8°C if testing will take place within five days. If specimens are to be kept for longer periods, store at -20°C or colder. Do not use a frost-free freezer because it may allow the specimens to go through freeze-thaw cycles and degrade antibody. Samples that are improperly stored or are subjected to multiple freeze-thaw cycles may yield spurious results.

The NCCLS provides recommendations for storing blood specimens, (Approved Standard-Procedures for the Handling and Processing of Blood Specimens, H18A, 1990).

Kit components and storage of reagents

- One frame with strips (12x8 red coloured) coated with proteinase 3 one lid sealed in a foil pack with a dry pack.
- 1.5 ml negative control (NC) containing human serum in diluent.
- 1.5 ml positive control (PC) containing human serum in diluent.
- 13 ml conjugate containing alkaline phosphatase-labelled antibodies to human IgG (blue colour).
- 32 ml Diluent (Dil) containing PBS (red colour).
- 13 ml Substrate pNPP.
- 30 ml wash solution 30x concentrated.
- Five calibrators containing human serum in diluent. 1.5 ml Cal 1 = 320 units, 1.5 ml Cal 2 = 160 units, 1.5 ml Cal 3 = 80 units, 1.5 ml Cal 4 = 40 units, 1.5 ml Cal 5 = 10 units.

All reagents in the kit are ready for use except wash solution and should be stored at 2-8°C.

Remove only the number of strips needed for testing, resealing the aluminium package carefully.

Materials or equipment required but not provided

- Microplate reader with filter 405 nm.
- Precision pipettes with disposable tips.
- Washer for strips, absorbent tissue, tubes and a timer.

PROCEDURE

All solutions should be used at room temperature. Incubate in all steps at room temperature (20-25°C) with lid. Incubate serum for 30 minutes, conjugate for 30 minutes and substrate for 60 minutes (\pm 10 minutes).

Preparation of washing solution

Dilute 10 ml of the 30x concentrated wash solution in 290 ml distilled water. When stored at 2-8°C, the diluted wash solution is stable until the date of expiration of the kit.

Dilution of serum and incubation

Dilute the patient's serum 1/80 with diluent (395 μ l diluent +5 μ l serum).

Pipet 100 μ l/well in duplicate of diluent (as a blank), Calibrator 1, 2, 3, 4, 5, NC, PC and diluted patient's serum (P) according to the diagram below. Incubate for 30 minutes.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Dil	Cal 4	P1									
B	Dil	Cal 4	P1									
C	Cal 1	Cal 5	P2									
D	Cal 1	Cal 5	P2									
E	Cal 2	NC	etc									
F	Cal 2	NC										
G	Cal 3	PC										
H	Cal 3	PC										

After serum incubation

Wash 3 times with 300 μ l washing solution / well, filling and emptying the wells each time; after the last wash, empty the wells by tapping the strip on an absorbent tissue.

Adding conjugate

Add 100 μ l conjugate to each well. Incubate for 30 minutes.

After conjugate incubation

Wash as before.

Adding substrate solution

Add 100 μ l substrate pNPP to each well, incubate for 60 minutes (\pm 10 minutes).
Read the absorbance at 405 nm on a microplate reader.

Calculations

Subtract the OD value for the blank from the other OD values.

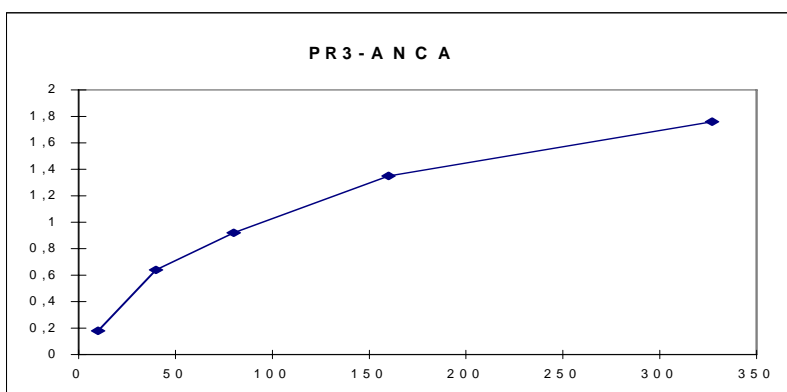
Construct a calibrator curve by plotting the OD against the unit values of the 5 calibrators. The five calibrators provided have been assigned arbitrary values of 320 units for calibrator 1,

160 units for calibrator 2.80 units for calibrator 3.40 units for calibrator 4 and 10 units for calibrator 5. Read the unit value of the patient from the constructed curve. Values greater than 320 should be reported as >320, or reassay them with a higher dilution.

Arbitrary units have been adopted by ALPCO, as no generally recognised international standard exists for expressing PR3-ANCA titres.

Example

Calibrator	Units	Absorbance
1	320	2.3
2	160	1.7
3	80	1.1
4	40	0.7
5	10	0.2



A sample with an absorbance value of 1.36 will read on the X-axis as having 160 units of PR3-ANCA

Important: The curve is an example and should not be used for actual patient interpretation.

Quality Control

The OD for the negative control should be less than that of calibrator 5.

The OD for calibrator 1 should be > 1.0

The value for the positive and negative controls see lot certificate.

The negative and positive controls are intended to monitor for substantial reagent failure. The positive control will not ensure precision at the assay cut-off. It is recommended to assay an additional control at the assay cut-off.

If any of the values are not within their respective ranges, the test should be considered invalid and the test should be repeated. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organisations. Refer to NCCLS C24-A for guidance on appropriate QC practices.

Interpretation of results

< 10 units = **Negative**

10-20 units = **Equivocal**; Retest, if still equivocal retest by an alternative method or test a new sample

>20 units = **Positive**

Limitations

The individual patient's antibody titre can not be used as a measure of disease severity, as antibodies from different patients may differ from each other in affinity. Thus, it is difficult to obtain an absolute standardisation of results.

The test should not be relied upon as the sole basis of decisions on clinical therapy, but should be used as an adjunct to clinical symptoms and the results of other available tests.

Sera from patients with other autoimmune diseases and from normal individuals may contain potentially cross-reactive autoantibodies. Some individuals may be positive for PR3 antibodies with little or no evidence of clinical disease. On the other hand, some patients with active disease may have undetectable levels of these antibodies.

Immunosuppressive therapy should not be started on basis of a positive ANCA result. Initiation or changes in treatment should not be based on changes in ANCA concentration alone, but rather on careful clinical observation.

Expected results

PR3-ANCA are rarely found in normal healthy individuals. The PR3-ANCA was tested with 80 normal sera, of which all were found to be negative. One new patient with PR3-ANCA is expected per 100000 individuals per year. Around 10% of patients with WG are negative in both IIF and ELISA. The PR3-ANCA was tested with 39 WG sera, 33 (92%) were found to be positive.

PR3-ANCA is found in approximately one half of the sera of MP (microscopic polyangiitis) patients. The PR3-ANCA was tested with 28 MP sera, 11 (39%) were found to be positive.

Performance characteristics

Table 1. Clinical sensitivity and specificity. A total of 216 frozen retrospective sera with clinical characterisation were assayed. The following table summarises the results:

Control and Disease Groups	Total Number	Negative < 10 units	Equivocal 10-20 units	Positive > 20 units
Blood Donors:	80	80	0	0
WG:	39	3	3	33
MP:	28	17	0	11
SLE:	19	18	1	0
Others:	32	32	1	0
Anti-GBM:	18	17	0	1

WG = Wegener's granulomatosis, MP = microscopic polyangiitis

SLE = systemic lupus erythematosus Others = RA, UC, etc.

GBM = glomerular basement membrane

Clinical sensitivity (Equivocal samples excluded from calculations)

WG = $33/36 = 91.7\%$ 95% CI = 75.5-98.2 %

MP = $11/28 = 39.3\%$ 95% CI = 21.5-59.4 %

Clinical specificity (Equivocal samples excluded from calculations)

GBM = 17/18 = 94.4 % 95% CI = 72.7-99.9 %
 SLE = 18/18 = 100 % 95% CI = 81.5-100 %
 Others = 32/32 = 100 % 95% CI = 89.1-100 %
 Donors = 80/80 = 100 % 95% CI = 95.5-100 %

The 95% confidence interval (CI) was calculated using the exact method.

Table 2. Relative sensitivity and specificity of the PR3-ANCA kit compared to C-ANCA IIF.
 A total of 245 frozen retrospective sera were assayed. The following table summarises the results:

PR3-ANCA				
IIF C-ANCA	Positive	Equivocal	Negative	Total
Positive:	78	0	3	81
Negative:	13	3	148	164
Total:	91	3	151	245

Sera falling in the equivocal range were excluded from the following calculations.

Relative sensitivity = 78/81 = 96.3 % 95% CI = 89.6-99.2 %
 Relative specificity = 148/161 = 91.9 % 95% CI = 86.6-95.6 %
 Relative accuracy = 226/242 = 93.4% 95% CI = 89.5-96.2 %

Table 3. Relative sensitivity and specificity of the PR3-ANCA kit compared to an alternative ELISA. (60x60x60)
 A total of 159 frozen retrospective sera were assayed. The following table summarises the results:

PR3-ANCA					
		Positive	Equivocal	Negative	Total
Alternative ELISA	Positive	34	0	0	34
	Equivocal	0	3	0	3
	Negavitive	0	0	122	122
	Total	34	3	122	159

Sera falling in the equivocal range were excluded from the following calculations.

Relative sensitivity = 34/34 = 100 % 95% CI = 89.7-100 %
 Relative specificity = 122/122 = 100 % 95% CI = 97.0-100 %
 Relative accuracy = 156/156 = 100% 95% CI = 97.7-100 %

The 95% confidence interval (CI) was calculated using the exact method.

Table 4. Batch to batch variation was determined by testing four different samples in duplicate. Results were obtained for six different batches:

Sample	Mean value	SD	CV %
1	56 units	2	4
2	24 units	4	15
3	27 units	1	4
PK	80 units	7	9

Table 5. Inter-assay precision was determined by testing two different samples in duplicate. Results were obtained for four different runs:

Sample	Mean value	SD	CV %
1	68 units	5.4	8
2	27 units	2.5	9

Table 6. Intra-assay precision was determined by testing one sample in 30 wells:

Sample	Mean value	SD	CV %
1	143 units	8.9	6

Table 7. Linearity. The values were determined for serial two-fold dilutions of four positive sera. The values were compared to log 2 of dilution by standard linear regression. The data indicates that the assay has a linear relationship with serum dilution:

Serum	Neat	1:2	1:4	1:8	1:16	r
1	140	79	39	20	11	0.998
2	340	86	45	22	10	0.971
3	135	81	48	28	12	0.993
4	56	30	14	6		0.998

Troubleshooting

Problem:	Possible causes:	Solution:
Control values out of range. test	<ol style="list-style-type: none"> 1. Incorrect temperature, timing or pipetting; reagents not mixed. 2. Cross contamination of controls. 3. Improper dilution. 4. Optical pathway not clean. 	<ol style="list-style-type: none"> 1. Check that the time and temperature was correct. See "Poor precision" below. Repeat 2. Pipette carefully. 3. Repeat test. 4. Check for dirt or air bubbles in the wells. Wipe bottom and reread.
All test results negative	<ol style="list-style-type: none"> 1. One or more reagents not added, or added in wrong sequence. 2. Antigen coated plate inactive. 	<ol style="list-style-type: none"> 1. Recheck procedure. Check for unused Reagent. Repeat test. 2. Check for obvious moisture in unused wells. Wipe bottom and reread.
All test results	<ol style="list-style-type: none"> 1. Contaminated buffers or reagents. 	<ol style="list-style-type: none"> 1. Check all solutions for turbidity.

yellow.	2. Washing solution contaminated	2. Use clean container. Check quality of water solution used to prepare
	3. Improper dilution of serum.	3. Repeat test.
Poor precision.	1. Pipette delivery CV greater than 5%.	1. Check calibration of pipette. Use reproducible technique.
	2. Serum or reagents not mixed sufficiently or not equilibrated to room temperature.	2. Mix all reagents gently but thoroughly and equilibrate to room temperature.
	3. Reagent addition taking too long; inconsistency in timing intervals.	3. Develop consistent uniform technique and use multi-tip device or auto dispenser to decrease time.
	4. Optical pathway not clean.	4. Check for air bubbles in the wells. Wipe bottom and reread.
	5. Washing not consistent; trapped bubbles; washing solution left in the wells	5. Check that all wells are filled and aspirated uniformly. Dispense liquid above level of reagent in well. After the last wash, empty the wells by tapping the strip on an absorbent tissue.
	6. Improper pipetting.	6. Avoid air bubbles in pipette tips.













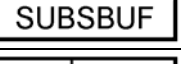
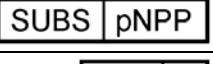


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Explanation of symbols

	Expiration date.
	Biological material.
	Store at.
	Manufacturer.
	Lot Number.
	In vitro diagnostic medical device
	Product number.
	See instruction for use.
	Antigene.
	Diluent.
	Conjugate.
	Wash solution 30x conc.
	Substrate buffer.
	Substrate pNPP.
	Calibrator.
	Control.