Anti-ANCA (IgG) Rapid Screening EIA

An enzyme-linked immunosorbent assay (ELISA) for qualitative detection of IgG antibodies to proteinase 3 and myeloperoxidase (MPO) in human sera.

For In Vitro Diagnostic Use.

Catalog Number:	13-CP-101
Size:	96 Wells
Storage:	+2-8°C
Version:	031201 – ALPCO 12/05/05

ALPCO Diagnostics

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Intended use

The ALPCO ANCA screening test kit is an enzyme-linked immunosorbent assay (ELISA) for qualitative detection of IgG antibodies to proteinase 3 and myeloperoxidase (MPO) 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 to the diagnosis of systemic vasculitis, especially Wegener's granulomatosis (WG) and microscopic polyangiitis (MP). FOR IN VITRO DIAGNOSTIC USE.
A positive result should always be confirmed by a semi-quantitative assay.

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 ANCA screen kit

The wells of the microtiterstrip are coated with purified proteinase 3 and myeloperoxidase. 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 (goat) antibodies to human IgG binds to the antibodies in the wells in the 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 as absorbance (optical density (OD)).

Warnings and precautions

- 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.

Specimen collection

The ANCA screen assay is for use with serum. Handle as if capable of transmitting infections 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 doublestrips (6x (2x8) wells) coated with purified proteinase 3 (column 1) and myeloperoxidase (column 2), one lid sealed in a foil pack with a dry pack.
- 0.75 ml negative control (NC) containing human serum in diluent (green colour).
- 0.75 ml positive control (PC) containing human serum in diluent (red colour).
- 13 ml conjugate containing alkaline phosphatase-labelled (Goat) antibodies to human IgG in PBS with protein stabiliser (blue colour).
- 10 ml Diluent (Dil) containing PBS (reddish colour).
- 13 Substrate pNPP.
- 30 ml Wash solution 30x concentrated.

All reagents in the kit are ready for use except wash solution and and should be stored at + 2-8°C.
Remove only the number of wells 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.
- NaCl, Tween 20 and distilled water for preparing more washing solution.
- Washer for strips, absorbent tissue, tubes and a timer.

PROCEDURE

All solutions should be used at room temperature.

Remove only the number of wells needed for testing, resealing the aluminium package carefully.
If the assay is performed without a shaker, incubation times should be adjusted as follows: serum incubation 10 minutes, no change; conjugate incubation 20-30 minutes; substrate incubation 20-30 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

1. Pipet 75 µl diluent (Dil) into each well.
2. Pipet 25 µl negative control (NC) into wells A1 and A2.
3. Pipet 25 µl positive control (PC) into wells B1 and B2.
4. Pipet 25 µl of patient serum no.1 into wells C1 and C2, serum no.2 into wells D1 and D2, serum no.3 into wells E1 and E2 etc. according to the scheme below. Put on the lid and incubate for 10 minutes on a shaker.

	1	2
	Column 1 (PR3-ag)	**Column 2 (MPO-ag)**
A	75 µl Dil + 25 µl NC	75 µl Dil + 25 µl NC
B	75 µl Dil + 25 µl PC	75 µl Dil + 25 µl PC
C	75 µl Dil + 25 µl pat 1	75 µl Dil + 25 µl pat 1
D	75 µl Dil + 25 µl pat 2	75 µl Dil + 25 µl pat 2
E	75 µl Dil + 25 µl pat 3	75 µl Dil + 25 µl pat 3
F	75 µl Dil + 25 µl pat 4	75 µl Dil + 25 µl pat 4
G	75 µl Dil + 25 µl pat 5	75 µl Dil + 25 µl pat 5
H	75 µl Dil + 25 µl pat 6	75 µl Dil + 25 µl pat 6

After serum incubation

Wash 4 times with washing solution, 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 µl conjugate to each well. Put on the lid and incubate for 10 minutes on a shaker (without a shaker, incubate 20-30 minutes).

After conjugate incubation

Wash 4 times with washing solution, filling and emptying the wells each time; after the last wash, empty the wells by tapping the strip on an absorbent tissue.

Adding substrate

Add 100 µl substrate pNPP to each well, incubate for 10-20 minutes (without a shaker, incubate 20-30 minutes). Read the absorbance at 405 nm on a microplate reader at 10-20 minutes after substrate addition and 20-30 minutes after substrate addition without a shaker.

Calculations

An optical density (OD) ratio for each patient sample is calculated as follows:

$$\textbf{OD ratio} = \frac{\textbf{OD patient sample}}{\textbf{OD of negative control}}$$

The patient sample is negative if the OD ratio is < 3.0. Equivocal if the OD ratio is 3.0-4.0 and positive if the OD ratio is > 4.0.

Quality Control

The OD for the negative control should be < 0.3
The OD for the positive control should be > 0.9
If any of the values are not within their respective ranges, the test should be considered invalid and the test should be repeated.
The patient sample may contain unspecific reactivities if it is positive on both antigens and must be re-assayed in a quantitative assay. Sometimes unspecific reactivities will disappear with a higher dilution.

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 that an additional control be assayed that is close to the cut-off.
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

A sample with a OD ratio of:

< 3.0 = **Negative** 3.0 – 4.0 = **Equivocal;** Retest, if still equivocal retest by an alternative method
> 4.0 = **Positive**

Owing to the high sensitivity of the screening kit, in a few percent of cases samples with an OD ratio > 3 may be found to be negative in a quantitative assay.

An equivocal or a positive test result should always be confirmed by a quantitative assay.

Limitations

The individual patient's OD ratio 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 standardization of results.
The test should not be relied upon as the sole basis of decisions on clinical therapy, but should be used in combination with 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 auto antibodies. Some individuals may be positive, 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

MPO and PR3-ANCA are rarely found in normal healthy individuals. The ANCA screening kit was tested with 131 normal sera. 131 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. MPO-ANCA are found in approximately one half of the sera of MP (microscopic polyangiitis) patients. The MPO-ANCA was tested with 42 WG sera, 4 were found to be positive. The MPO-ANCA was tested with 43 MP sera, 20 were found to be positive. (Table 1)
The PR3-ANCA was tested with 42 WG sera, 39 were found to be positive.
PR3-ANCA are found in approximately one half of the sera of MP (microscopic polyangiitis) patients. The PR3-ANCA was tested with 43 MP sera, 21 were found to be positive. (Table 1)

Performance characteristics

Table 1. Clinical sensitivity and specificity. A total of 288 frozen retrospective sera with clinical characterisation were assayed. The following table summarises the results:

Control and Disease Groups	Total Number	Negative <3 units		Equivocal 3-4 units		Positive >4units	
		PR3	MPO	PR3	MPO	PR3	MPO
Blood Donors:(NS)	131	131	127	0	4	0	0
WG:	42	3	37	0	1	39	4
MP:	43	20	23	2	0	21	20
SLE:	31	31	24	0	2	0	*5
RA:	41	41	40	0	1	0	0

WG = Wegener's granulomatosis, MP = microscopic polyangiitis RA = rheumatoid arthritis
SLE = systemic lupus erythematosus GBM = glomerular basement membrane

*** All 5 sera was positive in quantitative MPO-ANCA test.**

Clinical sensitivity (Equivocal samples not included in the calculations)

PR3-ANCA: WG = 39/42 = 92.9 % 95% CI = 80.5-98.5% MP=21/41=51.2 % 95% CI = 35.1-67.1%
MPO-ANCA: WG = 4/41 = 9.7 % 95% CI = 2.7-23.1% MP=20/43=46.5 % 95% CI = 31.2-62.3%

Clinical specificity (Equivocal samples not included in the calculations)

PR3-ANCA: SLE = 31/31 = 100 % 95% CI = 88.8-100%
RA=41/41=100 % 95% CI = 91.4-100% NS = 131/131 = 100 % 95% CI = 97.2-100%

MPO -ANCA: SLE =24/29 =82.8 % 95% CI = 64.2-94.2%
RA=40/40=100 % 95% CI = 91.2-100% NS = 127/127 = 100 % 95% CI = 97.1-100%

Table 2. Relative sensitivity and specificity of the PR3-ANCA screen kit compared to a semi-quantitative ELISA. A total of 216 frozen retrospective sera were assayed. The table summarises the results:

Semi-quantitative ELISA PR3-ANCA MPO-ANCA	ANCA Screen					
	Negative <3 units		Equivocal 3-4 units		Positive >4units	
	PR3	MPO	PR3	MPO	PR3	MPO
Postive	1	1	0	0	59	23
Negative	152	182	2	4	0	0
Equivocal	1	4	0	1	1	1
Total	154	187	2	5	60	24

Sera falling in the equivocal range were are not included in the calculations:

Relative sensitivity PR3-ANCA = 59/60 = 98.3 % 95% CI = 91.1-100%
Relative sensitivity MPO-ANCA = 23/24 = 95.8 % 95% CI = 78.9-99.9%

Relative specificity PR3-ANCA = 152/152 = 100 % 95% CI = 97.6-100%
Relative specificity MPO-ANCA = 182/182 = 100 % 95% CI = 98-100%

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

Table 3. Batch to batch variation was determined by testing three different samples in 4 different batches:

Sample		OD ratio		SD		CV %	
PR3	MPO	PR3	MPO	PR3	MPO	PR3	MPO
2	3	34.5	16.3	1.0	3.9	3	24
5	6	19.8	27.8	2.2	2.1	11	7
8	9	33.8	23.0	1.5	2.9	4	13

Table 4. Inter-assay precision was determined by testing one sample. Results were obtained for six different runs:

Sample		OD ratio		SD		CV %	
PR3	MPO	PR3	MPO	PR3	MPO	PR3	MPO
PK	PK	27.3	17.3	2.9	3.8	11	21
5	6	12.1	16.5	1.2	0.8	10	5

Table 5. Intra-assay precision was determined by testing one sample in 22 wells.

Sample		OD ratio		SD		CV %	
PR3	MPO	PR3	MPO	PR3	MPO	PR3	MPO
PK	PK	1.3	1.9	0.07	0.06	6	3
5	6	1.3	1.5	0.06	0.07	5	5

Troubleshooting

Problem:	Possible causes:	Solution:
Control values out of range. test	**1.** Incorrect temperature, timing or pipetting; reagents not mixed.	**1.**Check that the time and temperature was correct. See ”Poor precision” below. Repeat
	2. Cross contamination of controls.	**2.** Pipette carefully.
	3. Improper dilution.	**3.** Repeat test.
	4. Optical pathway not clean.	**4.** Check for dirt or air bubbles in the wells. Wipe bottom and reread.
All test results negative	**1.**One or more reagents not added, or added in wrong sequence.	**1.** Recheck procedure. Check for unused Reagent. Repeat test.
	2. Antigen coated plate inactive.	**2.** Check for obvious moisture in unused wells. Wipe bottom and reread.
All test results yellow.	**1.** Contaminated buffers or reagents.	**1.** Check all solutions for turbidity.
	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.

References:

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Explanation of symbols

Symbol	Explanation
	Expiration date.
	Biological material.
	Store at.
	Manufacturer.
LOT	Lot Number.
IVD	In vitro diagnostic medical device
REF	Product number.
	See instruction for use.
Ag	Antigene.
DIL	Diluent.
CONJ	Conjugate.
WASHBUF 30X	Wash solution 30x conc.
SUBSBUF	Substrate buffer.
SUBS pNPP	Substrate pNPP.
CAL X	Calibrator.
CONTROL X	Control.