DRG® MPO (P-ANCA) (EIA-2579)

Revised 7 May 2007 **RUO in the USA**

Introduction

The **MPO Antibodies** Enzyme Immunoassay Kit provides materials for the quantitative or qualitative determination of human IgG autoantibodies specific for myeloperoxidase (MPO) in serum and plasma. This assay is intended for in vitro use only. In the United States, this kit is intended for Research Use Only.

The test should be employed as an aid to the diagnosis of conditions associated with raised anti-neutrophil cytoplasmic antibodies (ANCA) and is not definitive in isolation. MPO-ANCA is associated with Churg-Strauss syndrome, microscopic polyarteritis and necrotising glomerulonephritis. Autoantibody levels represent one parameter in a multi-criterion diagnostic process.

The systemic vasculitides are inflammatory diseases of blood vessels and comprise a heterogeneous group of disorders, the causes of which are generally unknown. The diseases have diverse presentations, and are often rapidly progressive, causing irreversible injury to the vessels of kidney and lungs. The presence of anti-neutrophil cytoplasmic antibodies (ANCA) in patients with vasculitis was first observed by Davies in 1982. ANCA are autoantibodies with specificities for proteins located in the primary and secondary granules of neutrophils and in the peroxidase-positive lysosomes of peripheral blood monocytes. They were originally detected by indirect immunofluorescence on ethanol-fixed neutrophils, producing characteristic staining patterns with accentuation of the fluorescent activity within the nuclear lobes. Two major patterns of immunofluorescent staining have been observed: a classical or cytoplasmic staining, designated cANCA, and a perinuclear pattern, designated pANCA. Other staining patterns have been described and are generally noted as atypical or snowdrift patterns. The nature of the antigens and clinical significance of the antibodies responsible for these atypical fluorescent patterns is currently unclear.

Antibodies producing a perinuclear staining pattern are directed against different cytoplasmic constituents of neutrophils and are not specific for a single disease entity, but tend to be associated with disease groups which show several common clinical and histological features. pANCA occurs in such conditions as vasculitis, glomerulonephritis, Churg-Strauss syndrome, polyarteritis nodosa, systemic lupus erythematosus, rheumatoid arthritis and associated disorders.

A major antigen of pANCA is myeloperoxidase, which, with co-factors, constitutes a potent microbiocidal system within neutrophil granulocytes. Additional target antigens, such as human leucocyte elastase, cathepsin-G and lactoferrin have been associated with the pANCA fluorescence pattern but in up to 50% of pANCA-positive sera, the target antigen(s) are unidentified. However, MPO antibodies are present in patients with pauci-immune glomerulonephritis who are negative for cANCA, in patients with Churg-Strauss syndrome and polyarteritis. MPO-ANCA can also be induced by drugs such as hydralazine, clozapine and L-tryptophan. Occupational exposure to environmental factors such as silica dust may provoke MPO-ANCA progressive glomerulonephritis.

Measurement of MPO-specific ANCA is an important adjunct to clinical findings in the evaluation of clinical sub-types within the systemic vasculitides spectrum.

Principle of the Test

This assay is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. The wells are coated with affinity-purified myeloperoxidase extracted from human neutrophils. During the first incubation, specific autoantibodies in diluted serum or plasma will bind to the antigen coating. The wells are then washed to remove unbound serum or plasma components. A monoclonal antibody to human IgG conjugated to alkaline phosphase binds to surface-bound antibodies in the second incubation. After a further washing step, specific antibodies are traced by incubation with substrate solution. Addition of the stop solution terminates the reaction and provides the appropriate pH for colour development.

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Precautions

- The assay calibrators and controls are of human origin and have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All standards, however, should be treated as potential biohazards in use and for disposal.
- The assay reagents contain sodium azide or thimerosal, which may be toxic if ingested. Sodium azide may react with copper and lead piping to form highly explosive salts. On disposal, flush with large quantities of water.
- The stop solution contains NaOH. If it comes into contact with skin, wash thoroughly with water and seek medical attention. Since the NaOH used to terminate the colour reaction is corrosive, the instrumentation employed to dispense it should be thoroughly cleaned after use.
- This kit is for in vitro use only. In the United States, this kit is intended for Research Use Only.
- Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes. If contact occurs, wash with germicidical soap and copious amounts of water.
- Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
- Wear disposable latex gloves when handling specimens and reagents, and wash hands thoroughly afterwards. Microbial contamination of reagents or specimens may give false results.

Storage and Stability

Store all reagents at 2 - 8 °C and use before expiry date. When stored at 2 - 8 °C unopened reagents will retain reactivity until expiration date. Do not use reagents beyond this date.

Once the foilbag of the coated microtiter strips has been broken, care should be taken to close it tightly again. The immunoreactivity of the coated microtiter strips is stable for approx. 6 weeks in the broken, but tightly closed bag.

Diluted wash buffer and diluted sample diluent are stable for up to 6 months at 2 - 8 °C provided that microbial contamination is avoided. Do not expose the substrate to light during storage. The substrate should be pale yellow in colour. Any pink colouring indicated that the reagent has been contaminated and must be discarded.

Allow all reagents and required number of strips to reach room temperature prior to use.

Contents of the Test Kit

Do components contain < 250 µl solution, please care that all the solution is on the bottom of the vial.

MPO-Coated Microtiter Strips 12 x 8 wells
break apart wells coated with MPO antigen in foilbag
with desiccant,
labelled component E and colour-coded bottle green.

anti-MPO Standards 1 - 5 5 vials
1 ml each, ready to use,
containing the below mentioned concentrations
of MPO antibodies in human plasma in buffer with
0.1 % (w/v) sodium azide as preservative:

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Standard	1	2	3	4	5
Concentration in U/ml	0	2	8	30	100

Conjugate 1 vial
15 ml, ready to use,
alkaline phosphatase-labelled murine monoclonal antibody
to human IgG in Tris buffer with protein stabiliser and preserved
with 0.1 % (w/v) sodium azide, labelled component A.

Substrate 1 vial
15 ml, ready to use,
containing phenolphthalein monophosphate (PMP)
and Mg^{2+} as cofactor in buffer solution, labelled component B.

Stop Solution 1 vial
15 ml, ready to use,
containg sodium hydroxide and EDTA as a chelating agent
in carbonate buffer pH > 10, labelled component C.
Caution: Caustic!

Wash Buffer, concentrate (16x) 2 vials
25 ml each, concentrate,
containing borate buffer and preserved
with 0.8 % (w/v) sodium azide, labelled component D.
Dilute contents of 1 vial with 375 ml distilled water prior to use.

Sample Diluent 2, concentrate (5x) 2 vials
25 ml each, concentrate,
containing phosphate buffer and BSA preserved
with 0.5 % (w/v) sodium azide, labelled component F.
Dilute contents of 1 vial with 100 ml distilled water prior to use.

anti-MPO Reference Control 1 vial
1.5 ml, ready to use,
containing human plasma in buffer preserved
with 0.1 % (w/v) sodium azide, labelled component G.

Autoantibody-Negative Control, concentrate (100x) 1 vial
50 µl, concentrate,
containing human plasma in buffer preserved with 0.1 % (w/v)
sodium azide, this control **requires pre-dilution (1:100)** with
diluted sample diluent 2 prior to use.

anti-MPO Positive Control, concentrate (100x) 1 vial
50 µl, concentrate,
containing human plasma in buffer preserved with 0.1 % (w/v)
sodium azide, this control **requires pre-dilution (1:100)** with

diluted sample diluent 2 prior to use.

Microtiter Strip Holder 1 piece

Material required but not provided

- Automatic pipettes to dispense 10 µl, 100 µl and 1 ml (a multichannel pipetting device such as Titertek is suitable for adding reagents to the wells)
- Distilled water
- Microtiter plate spectrophotometer (ELISA reader) with 550 nm filters. Filters in the range of 540 - 565 nm give acceptable results.
- Suitable tubes or containers (1 ml) for sample dilution

Specimen Collection and Storage

- Serum or EDTA plasma should be used, and the usual precautions for venipuncture should be observed. No special sample pretreatment **is necessary. Samples may be stored at 2 - 8 °C for up to 28 days, and should be frozen at -20 °C or** lower for longer periods. Diluted samples (1:100 in sample diluent 2) can be stored at 2 - 8 °C for up to 28 days.
- Repeated freeze - thawing should be avoided.
- Thawed samples should be inverted several times prior to testing.
- Do not use grossly hemolyzed or grossly lipemic specimens.
- Samples should not be heat-inactivated as this may yield false positive results.

Preparation of Samples and Reagents

Sample Diluent 2

Dilute the sample diluent 2 concentrate by pouring the contents of the vial into a clean vessel and adding 100 ml distilled or deionized water. Mix thoroughly.

Samples and Controls

Dilute patient samples as well as positive and negative controls by dispensing

1 ml of **diluted** sample diluent 2 into a suitable container and adding 10 µl of each sample or control. Mix thoroughly.

Do not dilute the reference control!

Wash Buffer

Dilute the wash buffer concentrate by pouring the contents of 1 vial into a clean vessel and filling up to 400 ml (i.e. adding 375 ml) with distilled or deionized water. Mix thoroughly.

Microtiter strips

Calculate the total number of wells to enable the running of standards, controls, and samples:

Qualitative test - run reference control, positive and negative control + samples

Quantitative test - run standards, positive and negative control + samples

If individual, or less than 8 wells are required, the exact number of wells can be broken off by removing the plastic strip from the plate holder. Remove the well strip from the plastic strip holder by pushing from the underside of the wells. The required number of wells can then be snapped off and repositioned in the plastic strip. Replace the plastic strip in the microtiter plate holder by first hinging the rectangular hole into the bottom edge (row H) of the holder groove, and ensuring that the square hole, with nick on the left-hand side, is firmly held along the top edge (row A). Colour-coding BOTTLE GREEN identifies the anti-MPO coated strips.

Assay Procedure
GENERAL REMARKS:

It is recommended to use control samples according to state and federal regulations. The use of control sera or plasma is advised to assure the day-to-day validity of results. Use controls at both normal and pathological levels.

All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming. Once the test has been started, all steps should be completed without interruption.

Use new disposable plastic pipette tips for each reagent, standard or specimen in order to avoid cross contamination.

Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents be ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.

Qualitative Protocol

Reference the wells of the microtiter strips to allow identification of the kit reference control, positive and negative control and patient samples.

Pipet **100 µl of reference control** into the first and second wells.

Pipet **100 µl of prediluted controls and samples** into the appropriate wells of the strips.

Note: Controls and samples should be added in a continuous operation, remembering to change the pipette tips between samples. The additions from start to finish should not exceed 15 minutes.

Incubate for 60 ± 10 minutes at room temperature (18 - 24 °C).

Washing: discard the incubation solution, rinse the wells 3 x with 200 µl of wash buffer and remove any residual by firmly blotting the inverted strips with absorbent paper towels.

Add **100 µl of enzyme conjugate** to each well in sequence.

Incubate for 30 ± 5 minutes at room temperature (18 - 24 °C).

Washing: discard the incubation solution, rinse the wells 3 x with 200 µl of wash buffer and remove any residual by firmly blotting the inverted strips with absorbent paper towels.

Promptly pipet **100 µl of substrate** into the rinsed wells.

Incubate for 30 ± 5 minutes at room temperature (18 - 24 °C).

Stop the reaction by adding **100 µl stop solution** to each well.

Shake gently the microtiter strips being careful not to let the content come from the wells and **read at 550 nm** (540 - 565 nm) within 24 hours.

Quantitative Protocol

Reference the wells of the microtiter strips to allow identification of the kit standards, positive and negative control and patient samples.

Pipet **100 µl of standards 1 to 5** into the appropriate wells.

Pipet **100 µl of prediluted controls and samples** into the remaining wells.

Note: Controls and samples should be added in a continuous operation, remembering to change the pipette tips between samples. The additions from start to finish should not exceed 15 minutes.

Incubate for 60 ± 10 minutes at room temperature (18 - 24 °C).

Washing: discard the incubation solution, rinse the wells 3 x with 200 µl of wash buffer and remove any residual by firmly blotting the inverted strips with absorbent paper towels.

Add **100 µl of enzyme conjugate** to each well in sequence.

Incubate for 30 ± 5 minutes at room temperature (18 - 24 °C).

Washing: discard the incubation solution, rinse the wells 3 x with 200 µl of wash buffer and remove any residual by firmly blotting the inverted strips with absorbent paper towels.

Promptly pipet **100 µl of substrate** into the rinsed wells.

Incubate for 30 ± 5 minutes at room temperature (18 - 24 °C).

Stop the reaction by adding **100 µl stop solution** to each well.

Shake gently the microtiter strips being careful not to let the content come from the wells and **read at 550 nm** (540 - 565 nm) within 24 hours.

CALCULATION OF RESULTS

Qualitative Protocol

An absorbance value (optical density) ratio for each sample and positive and negative control should be calculated as follows:

$$\text{Absorbance Ratio} = \frac{\text{Sample or Control Absorbance Value}}{\text{Reference Control Absorbance Value (mean)}}$$

It is recommended that users of the MPO antibodies kit determine a cut-off ratio for their patient population which gives optimal discrimination between negative and positive samples. Results from the patient populations used in an in-house clinical trial suggest:

Absorbance Ratio	Result Interpretation
< 0.95	Negative
0.95 to 1.0	Borderline result – repeat testing recommended
> 1.0	Positive

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It is recommended that positive samples are re-assayed using the quantitative protocol.

The absorbance ratio of the negative control should be less than 1.0. The absorbance ratio of the positive control is quoted on the label.

Quantitative Protocol

Any ELISA reader capable of determining the absorbance at 550 nm (540 - 565 nm) may be used. The antigen concentration of each sample is obtained as follows:

Using semi log graph paper, construct a standard curve by plotting the mean absorbance (Y) of each reference standard against its corresponding concentration (X) in U/ml.

Use the average absorbance of each sample to determine the corresponding antigen value by simple interpolation from this standard curve.

Alternatively the use of electronic device is possible. The results can also be calculated with normal programs for automatic data processing, i.e. 4 or 5 parameter, spline, logit-log.

Any sample reading greater than the highest standard should be diluted appropriately with the diluent buffer and reassayed. In this case, the sample concentration obtained must be corrected by this **further** dilution factor.

Typical Example:

Standards (U/ml)	OD 550 nm
0	0.050
2	0.085
8	0.420
30	1.360
100	2.140

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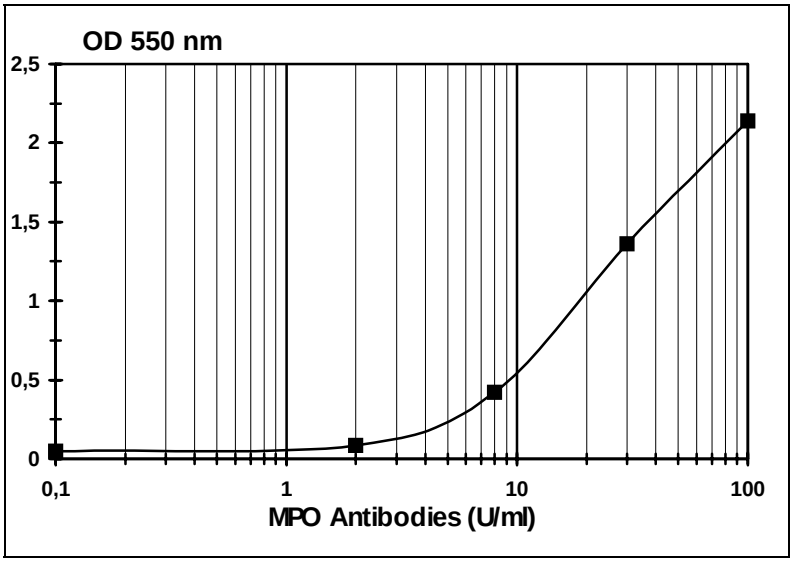
Typical Standard Curve
MPO Antibodies ELISA



Do not use this calibration curve. In the laboratory the standard curve should be established in each assay run.

The concentration of the negative control should be less than 6 U/ml. The acceptable expected range (U/ml) of the positive control is given on its label.

Assay Characteristics
Expected values
The following values can be used as preliminary guidelines until each laboratory establishes its own normal ranges.

179 serum samples from asymptomatic, healthy white caucasians aged between 17 and 68 years, with no history of autoimmune or rheumatic disease, were assayed for the presence of MPO IgG antibodies. From this population, a cut-off of 6 U/ml was established, which approximates to the mean + 4 SDs.

Reference Range	
< 6 U/ml	negative
≥ 6 U/ml	positive

Using a value of equal to, or greater than 6 U/ml as representing a positive result, the distribution of anti-MPO antibodies in specific disease groups was calculated.

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	anti-MPO Distribution				
anti-MPO Range U/ml	**Asymptom. Healthy**	**pANCA IFA Positive**	**cANCA IFA Positive**	**SLE**	**Viral Infections**
0 – 5.99	177	37	143	89	13
6 – 29.99	2*	17	0	3	0
30 – 99.9	0	14	1	1	0
>100	0	3	0	0	0
Total	179	71	144	93	13
% Positive	1.12%	47.9%	0.69%	4.3%	0%

*Values – 6.2, 12.5U/ml respectively

IFA = Immunofluorescence Assay
SLE = Systemic lupus erythematosus

Accordance with Immunofluorescence (IFA)
108 serum samples were evaluated externally using the MPO antibodies ELISA and a commercially available pANCA IFA assay, which uses whole human neutrophils as substrate. pANCA IFA positivity was determined using ethanol-fixed slides, and confirmed by formalin-fixed slide preparations. The evaluation was carried out by an established reference centre for systemic rheumatic diseases.

The objective of the trial was to assess the pANCA IFA status of samples which had been identified as MPO positive or negative using the MPO antibodies ELISA.

31 MPO positive samples and 77 MPO negative samples, the latter comprising samples from asymptomatic healthy patients and patients with non-vasculitic auto-immune diseases were re-assayed by the external evaluator, using the MPO antibodies ELISA.

The following results were obtained:

		MPO ab ELISA	
		+	–
pANCA IFA	+	31	17
	–	0	60

100% (31/31) of MPO positive samples were positive by pANCA IFA.

Of the 77 MPO negative samples:

77.9% (60/77) were pANCA IFA negative;

22.1% (17/77) were pANCA IFA positive.

or in this selected population of 48 pANCA IFA positive samples, 64.6% (31/48) were MPO positive, and, of 60 pANCA IFA negative samples, 100% (60/60) were MPO negative.

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Precision

Intra assay precision

Intra assay precision was determined by testing three controls in a total of six assays. Results were obtained by three different operators using three different kit batches.

Control	Mean Value (U/ml)	Root Mean Square %CV
1	9.01	11.0
2	20.5	3.9
3	48.2	8.8

Inter assay precision

Inter assay precision was determined by testing three controls in at total of 20 assays. Results were obtained by three different operators using 3 different kit batches.

Control	Mean Value (U/ml)	Root Mean Square %CV
1	9.11	5.5
2	20.4	7.6
3	49.1	10.7

Linearity

4 dilutions of 3 patient samples were assayed using 2 separate kits. The mean values and calculated percentage recoveries are given.

Sample	Dilution	Mean Value (U/ml)	Recovery (%)
1	A	50.6	100
	A/2	24.4	96.4
	A/4	14.2	112
	A/8	7.60	120
2	A	21.8	100
	A/2	13.0	119
	A/4	7.32	134
	A/8	4.36	160
3	A	64.4	100
	A/2	33.2	103
	A/4	18.3	114
	A/8	10.3	128

Limitations of use

- Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instructions and with adherence to good laboratory practice.
- The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbances.

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- The presence of myeloperoxidase antibody is associated with some forms of vasculitis, but is in itself not diagnostic. The data must be considered in the light of other clinical and laboratory information.
- Some individuals may have a positive anti-myeloperoxidase antibody titre, with little or no evidence of clinical disease. By contrast, some individuals with active disease may have undetectable levels of these antibodies.
- Serum or plasma samples which contain very high levels of antibody may give absorbances greater than the top standard. These samples should be diluted further with sample diluent 2 and re-assayed.
- When the MPO antibodies ELISA is used for monitoring antibody levels, it is advisable to use the same type of sample (serum or plasma) throughout the study period.

Warranty

Any modification of this test as well as exchange or mixture of any components from different lots might influence the results. In such cases there is no claim for a replacement.

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