DiaMetra
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Anti MPO (p-ANCA)

for routine analysis

Quantitative determination of IgG class antibodies against myeloperoxidase (MPO) in human serum or plasma

IVD	☣	LOT See external label	2°C ↗8°C	Σ = 96 tests	REF DKO092

INTENDED USE

Eagle Biosciences Anti MPO (p-ANCA) ELISA Assay Kit is an indirect solid phase enzyme immunoassay (ELISA) for the quantitative measurement of IgG class autoantibodies against myeloperoxidase (MPO) in human serum or plasma. Anti MPO (p-ANCA) ELISA Assay Kit is intended for research use only and not intended for diagnostic procedures.

1. CLINICAL SIGNIFICANCE

Anti-neutrophilic-cytoplasm antibodies (ANCA) represent a group of autoantibodies directed towards the cytoplasmatic components of the neutrophilic granulocytes and monocytes. The classical methods for the determination of ANCA are the immunofluorescent methods.With these indirect immunofluorescence techniques two main patterns are recognized, a cytoplasmatic (c-ANCA) and a perinuclear (p-ANCA) type.

The main antigen for the c-ANCA is the proteinase 3 (PR3), which is a serine proteinase of the present in primary granules.Antibodies of p-ANCA positive sera are mainly directed to myeloperoxidase (MPO). Antibodies to other antigens e.g. lactoferrin, elastase, cathepsin-G and also lysozyme often result in a similar p-ANCA pattern. Beside different untypical variants of p- ANCA IF patterns granulocyte specific antinuclear antibodies (GS-ANA) is indistinguishable from p-ANCA. This makes a clear interpretation and classification of the IF patterns difficult.

Therefore every positive IF-ANCA findings esp. p-ANCA should be differenciated by ELISA techniques using purified antigens. A survey of documented clinical indications of specific ANCA is given in the table below. PR3- ANCA and MPO-ANCA are reliable serologic markers in the diagnostics of vasculitides.

PR3- ANCA is the classical autoantigen in Wegener´s granulomatosis with a clinical specificity of more than 95%. c-ANCA is documented to be present in different diseases. Anti-MPO antibodies are highly specific for idiopathic and vasculitis associated crescentic glomerulonephritis and also for classic polyarteritis nodosa, Churg-Strauss syndrome and the polyangitis overlap syndrome without renal involvement.7-10 With respect to sensitivity, either MPO or PR-3 antibodies were found in 77 to 100% of patients with idiopathic and vasculitis associated crescentic glomerulonephritis. In WG, anti-MPO antibodies were detected only occasionally and generally in patients negative for PR-3 antibodies.

The MPO and PR-3 specific ELISA methods can provide an important confirmatory result for two of the more important of the identified antigens. ELISA is also useful for interpreting "difficult" samples by IFA such as those which exhibit several antibodies simultaneously or those with high background fluorescence.

2. PRINCIPLE

Anti MPO (p-ANCA) ELISA Assay Kit is based on the binding of the antibodies in the sample to the human neutrophil myeloperoxidase coated on the microplates. In the first step the antibodies in calibrators, controls or prediluted samples bind to the inner surface of the wells. After a 60 minutes incubation the microplate is washed with a wash buffer to remove the non-reactive serum components. In the second step an anti-human-IgG horseradish peroxidase conjugated solution recognizes the IgG class antibodies bound to the immobilized antigens. After a 30 minutes incubation any excess of enzyme conjugate which is not specifically bound is washed away with the wash buffer. Then a chromogenic substrate solution containing TMB is dispensed into the wells. After 15 minutes of incubation the color development is stopped by adding the stop solution. The solutions color change into yellow. The amount of color is directly proportional to the concentration of IgG antibodies present in the original sample. The concentration of IgG antibodies in the sample is calculated through a calibration curve.

3. REAGENTS, MATERIALS AND INSTUMENTATION

3.1. Reagents and materials supplied in the kit

1. Anti MPO Calibrators (5 vials, 1.2 mL each)
Phosphate buffer 0,1M, NaN_3 < 0,1%, human serum

CAL0	REF DCE002/09206-0
CAL1	REF DCE002/09207-0
CAL2	REF DCE002/09208-0
CAL3	REF DCE002/09209-0
CAL4	REF DCE002/09210-0

2. Controls (2 vials, 1.2 mL each, ready for use)
Phosphate buffer 0,1M, NaN_3 < 0,1%, human serum

Negative control	REF DCE045/09201-0
Positive control	REF DCE045/09202-0

3. Sample Diluent (1 vial, 100 mL)
Phosphate buffer 0,1M, NaN_3 < 0,1%
REF DCE053-0

4. Conjugate (1 vial, 15 mL)
Anti h-IgG conjugated with horseradish peroxidase (HRP), BSA 0,1%, Proclin < 0,0015% REF DCE002/09202-0

5. Coated Microplate (1 breakable microplate coated with human neutrophil myeloperoxidase)
REF DCE002/09203-0

6. TMB Substrate (1 vial, 15 mL)
3,3',5,5'-tetramethylbenzidine 0.26 g/L, hydrogen peroxide 0.05% REF DCE004-0

7. Stop Solution (1 vial, 15 mL)
Sulphuric acid 0.15M REF DCE005-0

8. <u>10X Conc. Wash Solution</u> (1 vial, 50 mL)
Phosphate buffer 0,2M, proclin < 0,0015%

REF DCE054-0

3.2. Reagents necessary not supplied
Distilled water.
3.3. Auxiliary materials and instrumentation
Automatic dispenser.
Microplate reader (450 nm).

4. WARNINGS
- This Anti MPO (p-ANCA) ELISA Assay Kit is intended for research use by professional persons only. Not for internal or external use in Humans or Animals.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- All human source material used in the preparation of the Anti MPO (p-ANCA) ELISA Assay Kit reagents has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, Calibrators and Controls should be handled in the same manner as potentially infectious material.
- Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious.
- Some reagents of the Anti MPO (p-ANCA) ELISA Assay Kit contain small amounts of Sodium Azide (NaN_3) or Proclin 300^R as preservatives. Avoid the contact with skin or mucosa.
- Sodium Azide may be toxic if ingested or absorbed through the skin or eyes; moreover it may react with lead or copper plumbing to form potentially explosive metal azides. If you use a sink to remove the reagents, allow scroll through large amounts of water to prevent azide build-up.
- The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
- The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
- Avoid the exposure of reagent TMB/H_2O_2 to directed sunlight, metals or oxidants. Do not freeze the solution.

5. PRECAUTIONS
- Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
- All reagents of the Anti MPO (p-ANCA) ELISA Assay Kit should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
- Allow all Anti MPO (p-ANCA) ELISA Assay Kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange Anti MPO (p-ANCA) ELISA Assay Kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.

- **WARNING: the conjugate reagent is designed to ensure maximum dose sensitivity and may be contaminated by external agents if not used properly;** therefore, it is recommended to use disposable consumables (tips, bottles, trays, etc.). For divided doses, take the exact amount of conjugate needed and do not re-introduce any waste product into the original bottle. In addition, **for doses dispensed with the aid of automatic and semi-automatic devices,** before using the conjugate, it is advisable to clean the fluid handling system, ensuring that the procedures of washing, deproteinization and decontamination are effective in avoiding contamination of the conjugate; this procedure is highly recommended when the kit is processed using analyzers which are not equipped with disposable tips.
For this purpose, Diametra supplies a separate decontamination reagent for cleaning needles.
- If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.
- Maximum precision is required for reconstitution and dispensation of the reagents.
- Samples microbiologically contaminated, highly lipemeic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1. Preparation of the Calibrators (C_0…C_4)
Since no international reference preparation for Anti-myeloperoxidase antibodies is available, the assay system is calibrated in relative arbitrary units. The Calibrators are to use and have the following concentration:

	C_0	C_1	C_2	C_3	C_4
AU/mL	0	10	20	40	160

Once opened, the Calibrators are stable 6 months at 2-8°C.

6.2. Preparation of the Sample
Either human serum or plasma samples can be used for the test execution. Test samples should be clear. Contamination by lipemia is best avoided, but does not interfere with this assay.
Specimens may be refrigerated at 2-8°C for up to five days or stored at -20°C up to six months. Avoid repetitive freezing and thawing of serum samples. This may result in variable loss of autoantibody activity
Testing of heat-inactivated sample is not recommended.

All serum and plasma samples must be prediluted 1:100 with sample diluents; for example 10 µL of sample should be diluted with 990 µL of sample diluent.
The Controls are ready to use.

6.3. Preparation of the Wash Solution
Dilute the content of each vial of the "10X Conc. Wash Solution" with distilled water to a final volume of 500 mL prior to use. For smaller volumes respect the 1:10 dilution ratio. The diluted wash solution is stable for 30 days at 2-8°C.
In concentrated wash solution is possible to observe the presence of crystals; in this case mix at room temperature until the complete dissolution of crystals; for greater accuracy, dilute the whole bottle of concentrated wash solution to 500 mL, taking care to transfer completely the crystals, then mix until crystals are completely dissolved.

6.4. Procedure
- **Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes.**
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C_0-C_4), two for each Control, two for each sample, one for Blank.

Reagent	Calibrator	Sample/ Controls	Blank
Calibrator C_0-C_4	100 µL		
Controls		100 µL	
Diluted Sample		100 µL	
Incubate 60 minutes at room temperature (22-28°C). Remove the contents from each well, wash the wells 3 times with 300 µL of diluted wash solution.			
Conjugate	100 µL	100 µL	
Incubate 30 minutes at room temperature (22-28°C). Remove the contents from each well, wash the wells 3 times with 300 µL of diluted wash solution.			
TMB Substrate	100 µL	100 µL	100 µL
Incubate 15 minutes in the dark at room temperature (22-28°C).			
Stop Solution	100 µL	100 µL	100 µL
Shake the microplate gently. Read the absorbance (E) at 450 nm against Blank within 5 minutes.			

7. QUALITY CONTROL
- The MPO IgG Positive and the Negative Control should be run with every batch of samples to ensure that all reagents and procedures perform properly.
- Because Positive and the Negative Control are prediluted, they do not control for procedural methods associated with dilution of specimens.

- Additional suitable control sera may be prepared by aliquoting pooled human serum specimens and storing at < -20°C.
- In order for the test results to be considered valid, all of the criteria listed below must be met. If any of these are not met, the test should be considered invalid and the assay repeated:
- The absorbance of the prediluted MPO IgG Positive must be greater than the absorbance of the prediluted Negative Control.
- The Negative and Positive Control are intended to monitor for substantial reagent failure and they will not ensure precision at the assay cutoff.
- This test is only valid if the optical density at 450 nm for Positive Control (1) and Negative Control (2) as well as for the Calibrator S0-S5 complies with the respective range indicated on the Quality Control Certificate enclosed to each test kit: If any of these criteria is not met, the results are invalid and the test should be repeated.

8. RESULTS
For Anti MPO (p-ANCA) test a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice. Smoothed-Spline Approximation and log-log coordinates are also suitable. Recommended Lin-Log Plot
First calculate the averaged optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

9. REFERENCE VALUES
In a normal range study with serum samples from healthy blood donors the following ranges have been established with the Anti MPO (p-ANCA) tests:

	Anti MPO (p-ANCA)
Cut-off	20 AU/mL

Please pay attention to the fact that the determination of a range of expected values for a "normal" population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacurer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.
Positive results should be verified concerning the entire status of the samples. It is recommended that each laboratory establishes its own normal and pathological ranges of serum Anti-MPO.

10. LIMITATIONS OF PROCEDURE
The presence of immune complexes or other immunoglobulin aggregates in the sample may cause an increased level of non-specific binding and produce false positives in this assay.

11. PERFORMANCE AND CHARACTERISTICS

11.1. Specificity:
Comparison test against a commercial reference kit, performed on 32 sera (8 of them positive sera and 24 negative sera) showed a 100% specificity.

11.2. Sensitivity:
Comparison test against a commercial reference kit, performed on 32 sera (8 of them positive sera and 24 negative sera) showed a 100% sensitivity.

11.3. Detection Limit:
The lowest concentration of anti MPO antibodies that can be distinguished from the Calibrator 0 is about 0.73 AU/mL with a confidence limit of 98%.

11.4. Precision and reproducibility
11.4.1. Intra-Assay
Within run variation was determined by replicate the measurements of three different sera with values in the range of the calibration curve. The within assay variability is ≤ 6.1%.
11.4.2. Inter-Assay
Between run variation was determined by replicate the measurements of two different control sera with different lots of kits and/or different mix of lots of reagents. The between assay variability is ≤ 12.3%.

12. WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

BIBLIOGRAPHY
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5. Hagen, E.C. et al. Antineutrophil cytoplasmic autoantibodies: a review of the antigens involved, the assays, and the clinical and possible pathogenic consequences.Blood 1993,Vol.81: 1996 - 2000.
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ERROR POSSIBLE CAUSES / SUGGESTIONS
No colorimetric reaction
- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)
Too low reaction (too low ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low
 Too high reaction (too high ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)
Unexplainable outliers
- contamination of pipettes, tips or containers
insufficient washing (conjugates not properly removed) too high within-run
- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)
too high between-run - incubation conditions not constant (time, CV % temperature)
- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation