

For Individual Laboratory to Complete:

**Anti-PR-3 (C-ANCA) IgG
Enzyme Immunoassay**

Laboratory Name		
Adopted		
Reviewed		
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Revised		
Supercedes		

Method: Diamedix Corp., Immunosimplicity®

Manual or in conjunction with one of the Diamedix Automated EIA Systems such as the MAGO Plus, the DSX, or the DS2. For *In Vitro* Diagnostic Use.

Clinical Significance

Anti-neutrophil cytoplasmic antibodies (ANCA) are autoantibodies directed at various lysosomal enzymes (1). Staining of alcohol-fixed neutrophils by indirect immunofluorescence (IFA) shows two main staining patterns: cytoplasmic (C-ANCA), and perinuclear (P-ANCA) (1,2). The C-ANCA pattern is caused by autoantibodies to proteinase-3 (PR-3) (3,4). These autoantibodies are found in 84-100% of patients with Wegener's granulomatosis (WG) (1,5). The P-ANCA pattern is caused by autoantibodies to myeloperoxidase (MPO) and is strongly associated with other types of vasculitides (3,4). Antibodies to MPO are found in 59% of patients with microscopic polyangiitis (4). Autoantibodies to PR-3 and MPO are highly specific for WG and microscopic polyangiitis respectively, and antibody titer often correlates with disease activity (1).

The Diamedix Is-anti-PR-3 IgG Test Kit is an EIA procedure intended for the qualitative detection and semi-quantitation of antibodies to proteinase-3 antigen. The results are reported in ELISA Units (EU) per ml and are determined by comparison to a standard curve.

Principle of the Procedure

Purified PR-3 antigen from human neutrophils is bound to microwells. Diluted patient sera, Standards and controls are placed in the microwells and incubated. Anti-PR-3 IgG antibodies, if present, will bind to the antigen forming antigen-antibody complexes. Residual sample is eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgG) is added and will bind to these complexes. Unbound conjugate is removed by aspiration and washing. Substrate is then added and incubated. In the presence of bound enzyme the substrate is converted to an end product. The absorbance of this end product can be read spectrophotometrically at 450 nm (reference 600-630 nm) and is directly

proportional to the concentration of IgG antibodies to PR-3 present in the sample.

Specimen Collection

Whole blood should be collected by accepted medical techniques. Separated serum should remain at 22°C for no longer than 8 hours. If assays are not completed within 8 hours, serum should be refrigerated (2-8°C). If assays are not completed within 48 hours, or the separated sample is to be stored beyond 48 hours, samples should be frozen at -20°C. Avoid multiple freeze-thaw cycles. Prior to testing, bring frozen sera to room temperature slowly and mix gently, avoiding foam formation. Specimens containing visible particulate matter should be clarified by centrifugation before testing. Grossly contaminated, hemolyzed, lipemic, or icteric specimens should not be used. The NCCLS provides recommendations for collecting and storing blood specimens, (Approved Standard - Procedures for the Handling and Processing of Blood Specimens, H18A).

CAUTION: Serum samples must not be heat-inactivated prior to use.

Reagents

Antigen Wells	Twelve, 8-well microwell breakapart strips, color-coded purple, coated with purified PR-3 antigen (from human neutrophils).
0 EU/ml Standard	One vial with yellow cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma, non-reactive for PR-3 IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned EU/ml value printed on label.
5 EU/ml Standard	One vial with green cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma, reactive for PR-3 IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned EU/ml value printed on label.
12.5 EU/ml Standard	One vial with brown cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma, reactive for PR-3 IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned EU/ml value printed on label.
25 EU/ml Standard	One vial with purple cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma, reactive for PR-3 IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned EU/ml value printed on label.
50 EU/ml Standard	One vial with white cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma, reactive for PR-3 IgG antibodies, 0.2% sodium

	azide and Proclin™ 300, 90 ppm active ingredient. Assigned EU/ml value printed on label.
100 EU/ml Standard	One vial with red cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma, reactive for PR-3 IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned EU/ml value printed on label.
Low Positive Control	One vial with blue cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma weakly reactive for PR-3 IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned EU/ml range printed on label. The positive control is used to control the low range of the assay.
Negative Control	One vial with black cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma non-reactive for PR-3 IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned EU/ml range printed on the label. The Negative Control is used to control the negative range of the assay.
Sample A Diluent	One bottle with blue cap containing 60 ml Phosphate buffer with protein stabilizers. Contains 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Color-coded blue.
Wash T Concentrate	Two bottles with clear caps containing 50 ml of Tris buffer with detergent and Proclin™ 300, 15 ppm active ingredient. Each bottle is sufficient to make 1 liter of wash solution.
Conjugate	One bottle with red cap containing 25 ml goat anti-human immunoglobulin G labeled with horseradish peroxidase. Also includes protein stabilizers and Proclin™ 300, 30 ppm active ingredient. Color-coded pink.
Substrate HRP	One amber bottle with brown cap containing 25 ml buffered TMB solution (3,3',5,5'-Tetramethylbenzidine).
Stop N Solution	One bottle with white cap containing 30 ml of 1 N Sulfuric Acid. CAUTION: Acids are corrosive. Avoid contact with skin or eyes. If contact is made, flush area with copious amounts of water. See Precautions section.

<p>Store these reagents at 2 to 8° C.</p>
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Other Materials Required

Manual Users:

1. Wash bottle or automated microplate washer
2. Pipettors capable of dispensing appropriate volumes
3. Timer
4. One liter graduated cylinder
5. One liter wash solution reservoir
6. Deionized or distilled water
7. Absorbent toweling
8. Tubes or microwell plate for serum dilution
9. Reader capable of reading absorbance at 450nm, reference at 600-630 nm.

Diamedix Automated EIA System Users:

1. One liter graduated container
2. Deionized or distilled water
3. Dilution containers as appropriate to system
4. Sample and Reagent tips required by system
5. Reagent containers required by system

Warnings:

1. Handle samples, standards, controls and the materials that contact them as potential biohazards. Each donor unit in the standards and controls has been found negative for Hepatitis B surface antigen and HIV-1 antibodies by FDA-approved third generation tests. However, because no method can offer complete assurance that HIV-1, Hepatitis B virus, or other infectious agents are absent, these materials should be handled at the Biosafety Level 2 as recommended for any potentially infectious serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual, "Biosafety in Microbiological and Biomedical Laboratories", 1993.
2. Never pipette by mouth.
3. Avoid contact with open skin and mucous membranes.
4. Certain of the test reagents contain Proclin™ 300 as a preservative. When disposing of reagents containing Proclin™ 300, flush drains with copious amounts of water to dilute the active components below active levels.
5. Serum components contain sodium azide as preservative. Azides are reported to react with lead and copper in plumbing to form compounds that may become explosive. When disposing of solutions containing sodium azide, flush with copious amounts of water to minimize the build up of metal azide compounds.
6. Sodium azide inhibits horseradish peroxidase activity. Care must be taken to ensure that azide is not carried over from other reagents into conjugate and substrate steps.
7. Avoid contamination of the TMB substrate solution with conjugate or other oxidants which will cause the solution to change color prematurely.
8. The substrate contains 3,3' 5,5' Tetramethylbenzidine (TMB) which has shown possible mutagenic effects in laboratory experiments.

9. Do not interchange reagents from different reagent lots except for Sample **A** Diluent, Wash **T** Concentrate, Substrate **HRP** and Stop **N** Solution.
10. Do not use reagents beyond their expiration date.
11. Store unused reagents at 2 to 8°C.
12. Incubations above or below the recommended temperatures or times may give erroneous results.
13. The EIA method is a very sensitive technique. Maintain consistent pipetting technique, incubation times, and temperature conditions throughout the test procedure. Cross contamination between reagents can invalidate the test.
14. Antigen coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use.
15. (*Manual Procedure Only*) The washing procedure is very important and requires special attention. Please refer to the Procedure section.)
NOTE: *Improperly washed wells may give erroneous results.*
16. The reported concentration of anti-PR-3 IgG in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.

Calibration

This test uses a 6-point calibration system (with qualitative reporting optional) based on in-house reference standards. These standards have been prepared from serum that is strongly positive for the antibody under investigation. The Standards have been assigned unitages in ELISA units (EU) per ml.

The positive cut-off has been assigned a value of 5 EU/ml and a Standard prepared at that level. The other Standards have been titrated from the positive serum accordingly. The test can be performed using all six Standards and reading the results from the point to point standard curve produced. If the user desires a qualitative result, i.e. negative or positive, then only the 5 EU/ml needs to be tested and patient absorbance values obtained compared to the 5 EU/ml Standard absorbance.

Patient samples which contain high levels of antibody may exceed the absorbance of the highest Standard. Such patient sample results should be reported as "Greater than 100 EU/ml". If numerical results are required for such samples, pre-dilute the sample using Sample Diluent and re-assay. The resulting EU/ml value should then be multiplied by the dilution factor to obtain estimated values.

Quality Control

- a) The Low Positive and Negative Controls must be included in each test run.
- b) The absorbance of the Blank or the 0 EU/ml Standard must be < 0.250.
- c) The absorbance of the Negative Control must be lower than that of the 5 EU/ml Standard.

- d) The absorbance of the Low Positive Control must be higher than that of the 5 EU/ml Standard.
- e) The absorbance of the 100 EU/ml Standard must be ≥ 3 times the absorbance of the 5 EU/ml Standard.
- f) When using the test semi-quantitatively the Low Positive Control and the Negative Control must be within their assigned ranges.
- g) When using the assay qualitatively the Low Positive Control Index Value must be ≥ 1.2 and the Negative Control Index Value must be < 0.8 .

If any of these criteria is not met, the run is invalid and must be repeated.

NOTE: Additional controls may be tested according to guidelines or requirements of local, state or federal regulations or accrediting organizations. For guidance on appropriate quality control practices, please refer to NCCLS document C24-A, Internal Quality Control Testing: Principles and Definitions.

Procedure

Allow all test components and patient samples to warm to room temperature before use. Invert reagent bottles gently several times before use. Return promptly to the refrigerator after use.

Prepare Wash Solution by adding 50 ml of Wash Concentrate (20X) to one liter deionized or distilled H₂O.

Manual Users:

1. **Standards and Controls are provided ready to use: DO NOT DILUTE FURTHER.**

For qualitative assays: The 5 EU/ml Standard is required and serves as the Cut-Off Standard for the assay. This Standard should be assayed in triplicate. In addition, a Blank (100 μ l Sample Diluent only, in the first well of the strip) is required. This will ultimately be used to "zero" the photometer before reading the test results.

For semi-quantitative assays: All six Standards are required. No Blank is required; the 0 EU/ml Standard will function as the "zero" and will be placed in the first well of the first strip. Standards (from 0-100 EU/ml) can be run singly or in duplicate.

* Low Positive and Negative Controls should be run for either assay option.

2. Prepare 1:21 dilutions of the patient samples in Sample Diluent. (e.g., by addition of 10 μ l sample to 200 μ l Sample Diluent).
3. Mix sample dilutions gently by withdrawing and expelling in a pipette tip 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100 μ l of Standards, controls and diluted patient samples, to the antigen wells. Avoid formation of bubbles when transferring diluted samples.
4. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 \pm 5 minutes.

5. Aspirate or discard the contents of the wells. Remove any excess moisture in the wells by tapping on paper toweling. Wash the wells by rinsing 3 times with at least 300 μ l of Wash Solution. Remove excess moisture from the wells after washing. When using an automated washer, follow the manufacturer's instructions.
6. Place 100 μ l of Conjugate into each well, avoiding bubble formation.
7. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 \pm 5 minutes.
8. Wash the wells as described in Step 4 above.
9. Place 100 μ l of Substrate into each well, avoiding bubble formation.
10. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 \pm 5 minutes.
11. Place 100 μ l of Stop Solution into each well, avoiding bubble formation.
12. Read the absorbance of each well at 450 nm using a reference wavelength of 600-630 nm. The plate should be read within 60 minutes of adding Stop Solution.

Refer to the BP-96 Plate Reader Operation Manual for complete instructions on set-up and operating procedures.

Diamedix Automated EIA System Users:

If using one of Diamedix's Automated EIA Systems, please refer to the corresponding Operating Manual for the test setup, procedure, and accessories/consumables needed.

Calculation of Results

Qualitative Assay: Qualitative results may be obtained using the 5 EU/ml Standard as a Cut-Off Standard, run in triplicate, following a single Blank well (100 μ l Sample Diluent only). If performing the qualitative assay option, set the reader for absorbance mode and calculate the mean absorbance of the three 5 EU/ml Standard wells. Alternatively, a reader with a Cut-Off Control test option may be used.

Note: When calculating the mean absorbance value for the Cut-Off Standard exclude any absorbance value that deviates by more than 15% from the mean of the three absorbance values. Use the mean of the remaining two replicates in calculations. Exclusion of more than one of the three absorbance values invalidates the run.

*Example: Absorbance values obtained for Cut-Off Standard:
0.276, 0.288, 0.258 (after subtraction of the blank)
Mean Absorbance of Cut-Off Standard = 0.274
Sample Absorbance = 1.150
Index Value = 1.150 / 0.274 = 4.2*

When using one of the Diamedix Automated EIA Systems, results are automatically calculated.

Semi-quantitative Assay: Semi-quantitative results may be obtained from the point-to-point curve fit using all six Standards. For the BP-96 or Stat-Fax readers the point-to-point option should be selected and Standard values entered accordingly. The Diamedix Automated EIA Systems will calculate results automatically.

Reference Ranges

The following is only a guide to interpretation. **Each laboratory can establish its own "normal" ranges based on populations encountered.**

Is anti-PR-3 Value	Index Value*	Interpretation
< 4.0 EU/ml	< 0.80	Negative for antibodies to PR-3.
≥ 6.0 EU/ml	≥ 1.20	PR-3 IgG antibody detected.
4.0-5.9 EU/ml	0.80 - 1.19	Equivocal for antibodies to PR-3. Sample should be retested. If retest results are equivocal, the sample should be reported as equivocal, tested by another method, or a new sample should be tested.**

*For Qualitative Results only.

**Equivocal samples that give positive results on retest should be reported as positive. Equivocal results that give negative results on retest should be reported as negative.

Samples which yield absorbances greater than that of the 100 EU/ml Standard may be reported as greater than 100 EU/ml. Alternatively, such samples may be pre-diluted in Sample Diluent and retested. The resulting EU/ml or Index Value must be multiplied by the dilution factor for reporting.

Example: If the specimen was pre-diluted 1:5 before testing, the resulting EU/ml value should be multiplied by 5.

Limitations

1. The results obtained with the Is-anti-PR-3 IgG Test Kit serve only as an aid to diagnosis and should not be interpreted as diagnostic in themselves.
2. Assay performance characteristics have not been established for visual result determination.
3. The test should be performed on serum. The use of whole blood or plasma has not been established.
4. The analysis of a single serum sample should not be used as the sole criteria for diagnosis of an autoimmune disease.

5. Screening of the general population should not be performed. The positive predictive value depends on the likelihood of Wegener's Granulomatosis being present. Testing should only be performed when clinical symptoms are present or disease suspected.
6. Results from immunosuppressed patients should be interpreted with caution.
7. The performance characteristics of the Is-anti-PR-3 IgG Test Kit with automated equipment other than the Diamedix Automated EIA Systems have not been established.
8. Icteric, lipemic, hemolyzed, or heat inactivated sera may cause erroneous results and should be avoided.

References

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4. Hagen, E.C., et al. 1992. Anti-neutrophil cytoplasmic antibodies: a review of the antigens involved, the assays, and the clinical and possible pathogenetic consequences. *Blood*. 91:1996-2002.
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