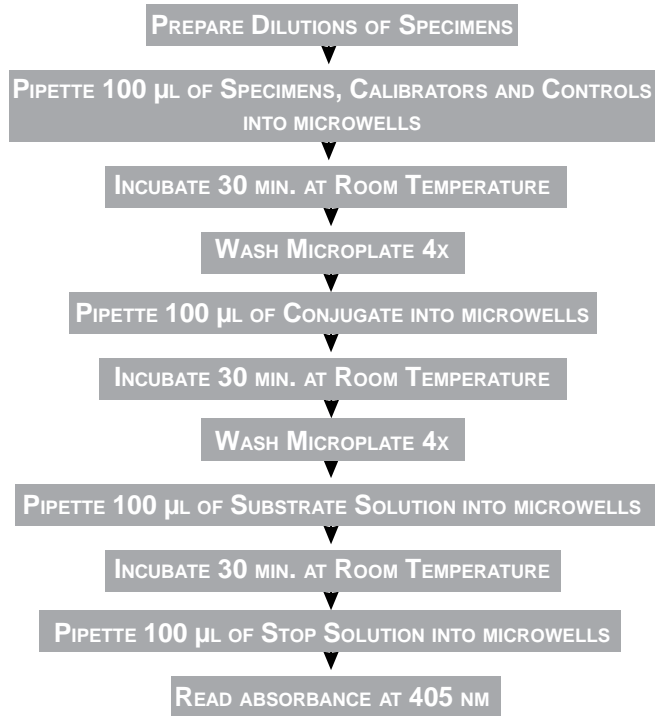


ImmuLisa™ PROCEDURE AT A GLANCE



For technical assistance please contact:



IMMCO Diagnostics, Inc.
60 Pineview Drive
Buffalo, NY 14228-2120
Telephone: (716) 691-0091
Fax: (716) 691-0466
Toll Free USA/Canada: 1-800-537-TEST
E-Mail: info@immcodiagnostics.com

or your local product distributor



EU Authorized Representative/Autorisierter Repräsentant/Rappresentante
Autorizzato/Representante Autorizado/Représentant Autorisé
EMERGO Group, Inc.
Molenstraat 15, 2513 BH, The Hague,
The Netherlands
Tel (+31) 345 8570, Fax (+31) 346 7299
www.emergogroup.com



ImmuLisa™ Anti-Proteinase 3 (PR3) Antibody ELISA

IVD

PRODUCT INSERT

Catalog No. 1162

96 Determinations

INTENDED USE

An enzyme linked immunosorbent assay (ELISA) for the detection and quantitation of antibodies against proteinase 3 (PR3) in human serum.

SUMMARY AND EXPLANATION

Anti-neutrophil cytoplasmic antibodies (ANCA) are a group of antibodies directed against proteins in the granules of neutrophils and in the peroxidase-positive lysosomes of peripheral blood monocytes. The presence of ANCA in patients with vasculitis was first observed in 1982 by Davies¹. These antibodies can be detected by indirect immunofluorescence on ethanol-fixed neutrophils, producing a characteristic cytoplasmic staining pattern (cANCA). cANCA are elicited against several proteins like Cathepsin G, Elastase and Proteinase 3 (PR3). Of these, PR3 is the major autoantigen with a molecular weight of 29kD. PR3 is a neutral serine proteinase localized in the azurophilic granules of the neutrophils². Antibodies against the PR3 antigen serve as a marker for *Wegener's Granulomatosis* (WG)³, a systemic necrotising vasculitides which elicits in two forms, extended and limited³. Extended WG is characterized by granulomatous inflammation of the respiratory tract and crescentic glomerulonephritis with cANCA reactivity in 90% of patients^{4,5}. Limited WG is characterized without renal involvement, and cANCA reactivity is detected in 67% of patients. The onset of the disease can be at any age and men are twice as frequently affected as women. Several studies have established a direct correlation between PR3 antibody levels and the active phase of WG. The concentration of serum anti-PR3 rises dramatically during disease exacerbations (90% frequency), and relapses are usually accompanied by significant titer increases^{6,7}. The presence of cANCA is also indicative of other diseases like idiopathic immune necrotizing glomerulonephritis and inflammatory bowel disorders like ulcerative colitis^{8,9}.

PRINCIPLES OF PROCEDURE

The ELISA is performed as a solid phase immunoassay. Microwells are coated with purified PR3 antigen followed by blocking the unreacted sites to reduce non-specific binding. Controls, calibrators and patient serum samples are incubated in the antigen coated wells which allows specific antibodies present in the serum to bind to the PR3 antigen.

Unbound antibody and other serum proteins are removed by washing the microwells. Bound antibodies are detected by adding an enzyme labeled anti-human IgG conjugate to the microwells. Unbound conjugate is removed by washing.

Specific enzyme substrate (pNPP) is then added to the wells and the presence of antibodies is detected by a color change produced by the conversion of pNPP substrate to a colored reaction product. The reaction is stopped and the intensity of the color change, which is proportional to the concentration of antibody, is read by a spectrophotometer at 405 nm. Results are expressed in enzyme units per milliliter (EU/ml).

REAGENTS

Storage and Preparation

Store all reagents at 2°-8°C. **Do not freeze.**

Do not use if reagent is not clear or if a precipitate is present. All reagents must be brought to room temperature (20°-25°C) prior to use.

When stored at 2°-8°C, the reconstituted wash buffer is stable until the kit expiration date. Reconstitute the wash buffer to 1 liter with distilled or deionized water. Coated microwell strips are for one time use only.

Precautions

All human derived components used have been tested for HBsAg, HCV, HIV-1 and 2 and HTLV-I and found negative by FDA required tests. However human blood derivatives and patient specimens should be considered potentially infectious. Follow good laboratory practices in storing, dispensing and disposing of these materials¹⁰.

WARNING - Sodium azide (NaN₃) may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal of liquids, flush with large volumes of water to prevent azide buildup. Sodium azide may be toxic if ingested. If ingested, report incident immediately to laboratory director or poison control center.

Instructions should be followed exactly as they appear in this kit insert to ensure valid results. Do not interchange kit components with those from other sources other than the same catalog number from IMMCO DIAGNOSTICS. Follow good laboratory practices to minimize microbial and cross contamination of reagents when handling. Do not use beyond expiration date on the label.

Results obtained with the Immulisa™ Anti-PR3 ELISA were also compared with an immunofluorescence assay (IFA). The results tabulated below, indicate a high degree of correlation of the Immulisa™ anti-PR3 assay to the gold standard IF method:

Immulisa™ Anti-PR3

| | Positive | Negative | Total |
|-------------|----------|----------|-------|
| IFA (cANCA) | | | |
| Positive | 60 | 4 | 64 |
| Negative | 0 | 68 | 68 |
| Total | 60 | 72 | 132 |

Relative Agreement: 97%
Relative Sensitivity: 94%
Relative Specificity: 100%

REFERENCES

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2. Kao RC, Wehner NG, Stubitz KM et. al. Proteinase 3: a distinct human polymorphonuclear leucocyte proteinase that produces emphysema in hamster. J Clin Invest; 1988, 82:1963-1973.
3. van der Woude FJ, Rasmussen N, Lobatto S et. al. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. Lancet; 1985, 1:425-429.
4. Cohen Tervaert JW, van der Woude FJ, Fauci AS et. al. Association between active Wegener's granulomatosis and anti-cytoplasmic antibodies. (ACPA). Arch Int Med; 1989, 149: 2461-2465.
5. Gross WL, Schmitt WH, Csernok E. ANCA and associated diseases: immunodiagnostic and pathogenetic aspects. Clin Exp Imm; 1993, 91:1-12.
6. Nölle B, Specks U, Lüdemann J et. al. Anticytoplasmic autoantibodies, their immunodiagnostic value in Wegener's granulomatosis. Ann Int Med; 1989, 111:28-40.
7. Cohen Tervaert J W, Huitema MG, Hene RJ et. al. Prevention of relapses in Wegener's granulomatosis by treatment based on antineutrophil cytoplasmic antibody titre; Lancet; 1990, 336:709-711.
8. Kallenberg CG, Mulder AH, Cohen Tervaert JW. Antineutrophil cytoplasmic antibodies: a still-growing class of autoantibodies in inflammatory disorders. Am J Med; 1992, 93:675-682.
9. Hagen EC, Ballieux BE, van Es LA et al. Anti-neutrophil cytoplasmic autoantibodies: a review of the antigens involved, the assays, and the clinical and possible pathogenetic consequences. Blood; 1993, 81:1996-2002.
10. Biosafety in Microbiological and Biomedical Laboratories. Center for Disease Control, National Institute for Health; 1993, HHS Pub. No {CDC} 93-8395).
11. Stoffel MP, Csernok C, Herzberg T et. al. Anti-neutrophil cytoplasmic antibodies (ANCA) directed against bactericidal/permeability increasing protein (BPI): a new seromarker for inflammatory bowel disease and associated disorders. Clin Exp Immunol; 1996, 104:54-59.
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PERFORMANCE CHARACTERISTICS

Precision:

Two different anti-PR3 antibody positive sera were tested with the ImmuLisa™ anti-PR3 ELISA to determine the inter- and intra-assay variation. The results are as follows:

| | inter-assay %CV | intra-assay %CV |
|-----------------|--------------------|--------------------|
| Sample 1 | 8.4 | 10.6 |
| Sample 2 | 8.7 | 6.5 |

Recovery:

Three samples with known anti-PR3 antibody concentrations were mixed with appropriate dilutions of another positive sample. Anti-PR3 antibody levels of the mixed samples were determined and the percent recovery calculated. The results are as follows:

| | PR3-Ab conc. added (EU/ml) | PR3-Ab conc. obtained (EU/ml) | % Recovery |
|-----------------|-------------------------------|----------------------------------|------------|
| Sample 1 | 144 | 153 | 107 |
| Sample 2 | 120 | 134 | 112 |
| Sample 3 | 72 | 82 | 113 |

Results obtained with the ImmuLisa™ Anti-PR3 ELISA were compared with another commercially manufactured ELISA. The results are as follows:

| | | ImmuLisa™ Anti-PR3 | | |
|-------------|----------|--------------------|----------|-------|
| | | Positive | Negative | Total |
| Other ELISA | Positive | 57 | 1 | 58 |
| | Negative | 3 | 9 | 12 |
| | Total | 60 | 10 | 70 |

Relative Agreement: 94%
Relative Sensitivity: 98.3%
Relative Specificity: 75%

Materials provided

ImmuLisa™ Anti-Proteinase 3 (PR3-ANCA) Antibody ELISA

Catalog No. 1162

Kit contains sufficient reagents to perform 96 determinations.

- 12 x 8** Ready to use **Microplate** with individual breakaway microwells coated with PR3 antigen.
- 1 x 1.5 ml** *Ready to use **Positive Control** (*red cap*). Contains human serum positive for anti-PR3 antibodies. The expected concentration range in EU/ml is printed on the label.
- 1 x 1.5 ml** *Ready to use **Negative Control** (*white cap*). Contains human serum.
- 4 x 1.5 ml** *Ready to use **set of 4 Calibrators**; Calibrator A (*green cap*), Calibrator B (*violet cap*), Calibrator C (*blue cap*) and Calibrator D (*yellow cap*). Human serum containing antibodies to PR3 antigen. Concentrations in EU/ml are printed on the labels.
- 1 x 12 ml** *Ready to use **anti-human IgG Alk. Phos. Conjugate**. Color coded pink.
- 1 x 60 ml** *Ready to use **Serum Diluent**. Color coded blue.
- 1 x 12 ml** *Ready to use **Enzyme Substrate**. Contains pNPP. **Protect from light.**
- 1 x 12 ml** Ready to use **Stop Solution**.
- 2 vials** Powder **Wash Buffer**. Reconstitute to one liter each.
- 1 x extra** Frame Holder
- 2 x** Protocol Sheets
- *CAUTION - Contains <0.1% NaN₃

Materials Required But Not Provided

- Deionized or distilled water
- Squeeze bottle to hold diluted wash buffer or Automatic microplate washer capable of dispensing 300 µl
- Pipettes capable of delivering 5 µl to 1000 µl
- Disposable pipette tips
- Clean test tubes 12 x 75 mm and test tube rack
- Timer
- Absorbent paper towels
- Microplate reader capable of reading absorbance values at 405 nm. If dual wavelength microplate reader is available, the reference filter should be set at 600-650 nm

SPECIMEN COLLECTION AND HANDLING

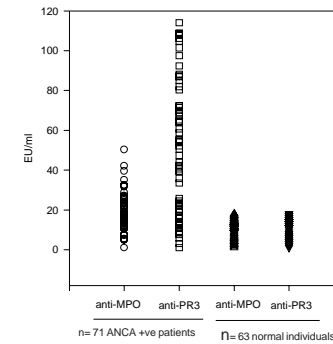
Only serum specimens should be used in this procedure. Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of the test and should not be used. Store specimens at 2°- 8°C for no longer than one week. For longer storage, serum specimens should be frozen. Avoid repeated freezing and thawing of samples. Do not expose the substrate to light during storage. Do not smoke, eat, drink or apply cosmetics in area where kits or serum/plasma samples are handled. Any skin complaints, cuts, abrasions and other skin lesions should be suitably protected.

PROCEDURE

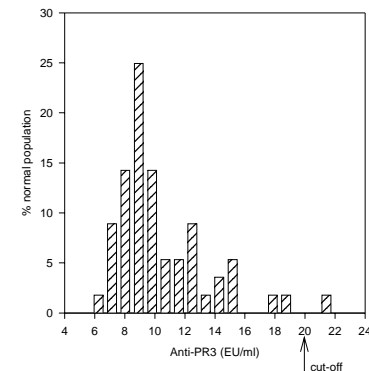
Procedural Notes

- Before starting with the assay read carefully the product insert.
- Let serum specimens and test reagents equilibrate at room temperature before starting with the test procedure. Return all unused specimens and reagents to refrigerator immediately after use.
- All dilutions of the patient samples should be prepared prior to starting with the assay.
- Good washing technique is critical. If washing is performed manually, adequate washing is accomplished by directing a forceful stream of wash buffer with a wide tip wash bottle across the entire microplate. **An automated microplate washer is recommended.**
- Use a multichannel pipette capable of delivering 8 wells simultaneously. This speeds the process and provides for a more uniform incubation time.
- For all steps, careful control of timing is important. The start of all incubation periods begins with the completion of reagent addition.
- Addition of all samples and reagents should be performed at the same rate and in the same sequence.
- Remove required microwell strips from the pouch and carefully reseal the pouch to prevent condensation in the unused wells. Return pouch immediately to refrigerator.

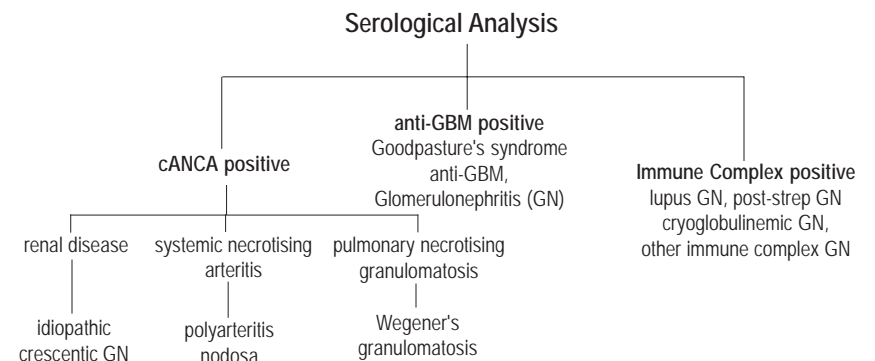
Distribution of anti-MPO and anti-PR3 reactivity by Immulisa™ in ANCA positive patients and normal population.



Distribution of Normal Population with anti-PR3 Immulisa™



The presence of cANCA distinguishes certain characteristic diseases from other glomerulonephritic conditions as is illustrated below¹²:



Interpretation

The following serves only as a guide in the interpretation of the laboratory results. The values depicted below were determined by testing 63 normal blood donors and represent the mean of the normals plus 3SD. Each laboratory must determine its own normal values.

| Anti-PR3 values | Interpretation |
|-----------------|----------------|
| ≤20 EU/ml | Negative |
| 20-25 EU/ml | Borderline |
| >25 EU/ml | Positive |

LIMITATIONS OF THE PROCEDURE

Test results obtained by this assay alone, are not diagnostic and should be considered in conjunction with the clinical presentation of the patient.

EXPECTED VALUES

The expected values in a normal population are negative (<20 EU/ml). However, 2-4% of apparently healthy, asymptomatic individuals may test positive for PR3 antibodies. In contrast, some patients with active disease may have undetectable levels of these antibodies. Immunosuppressive therapy, initiation or alteration in treatment should not be started on the basis of just positive PR3 antibody results, but rather on careful clinical observations.

The following table depicts the frequency of PR3 and MPO specific ANCA in sera from 112 ANCA associated vasculitides patients¹¹.

Incidence of anti-PR3 and anti-MPO in ANCA associated vasculitides

| | Wegener's granulomatosis | Microscopic polyangitis | Churg-Strauss syndrome |
|------------------------------|--------------------------|-------------------------|------------------------|
| ANCA positive by IFA | 78% | 59% | 67% |
| anti-PR3 positive | 90% | 0% | 10% |
| anti-MPO positive | 0% | 62% | 17% |
| unknown specificity positive | 40% | 31% | 73% |

The following figures depict the incidence of PR3 antibodies in normals and selected group of ANCA positive individuals in the ant-PR3 Immulisa™.

Test Method

- Step 1** Let all reagents and specimens equilibrate at room temperature.
- Step 2** Label protocol sheet to indicate sample placement in the wells. It is good laboratory practice to run samples in duplicate.
- Step 3** For a **qualitative determination** use only the Ready to Use Low Calibrator D (*vial with yellow cap*).
- or** For a **semi-quantitative determination** use the Ready to Use Calibrators A through D as depicted in the sample layout below.

QUALITATIVE DETERMINATION

| | | | | |
|---|-------|-----|---|---|
| A | BLANK | S5 | | |
| B | NEG | S6 | | |
| C | POS | S7 | | |
| D | CAL D | S8 | | |
| E | S1 | S9 | | |
| F | S2 | S10 | | |
| G | S3 | S11 | | |
| H | S4 | S12 | | |
| | 1 | 2 | 3 | 4 |

SEMI-QUANTITATIVE DETERMINATION

| | | | | |
|---|-------|----|---|---|
| A | BLANK | S2 | | |
| B | NEG | S3 | | |
| C | POS | S4 | | |
| D | CAL A | S5 | | |
| E | CAL B | S6 | | |
| F | CAL C | S7 | | |
| G | CAL D | S8 | | |
| H | S1 | S9 | | |
| | 1 | 2 | 3 | 4 |

- Step 4** Prepare a **1:101** dilution of the patient samples by mixing **5 µl** of the patient sera with **0.5 ml** of Serum Diluent.
- Step 5** Remove the required microwells from pouch and return unused strips in the sealed pouch to refrigerator. Securely place the microwells into the extra provided holder .
- Step 6** Pipette **100 µl** of Ready to use Calibrators, Positive and Negative controls and diluted patient samples to the appropriate microwells as per protocol sheet.
- Note:** Include one well which contains **100 µl** of the Serum Diluent as a reagent blank. Zero the ELISA reader against the reagent blank.
- Step 7** Incubate **30 minutes** (± 5 min) at room temperature.

- Step 8** Wash **4x** with wash buffer. For manual washing, fill each microwell with reconstituted wash buffer. Discard the fluid by inverting and tapping out the contents of each well or by aspirating the liquid from each well. To blot at the end of the last wash, invert strips and tap the wells vigorously on absorbent paper towels. For automatic washers, program the washer as per manufacturer's instructions.
- Step 9** Pipette **100 µl** of Conjugate into microwells.
- Step 10** Incubate **30 minutes** (± 5 min) at room temperature.
- Step 11** Wash all microwells as in Step 8.
- Step 12** Pipette **100 µl** of Enzyme Substrate into each microwell in the same order and timing as for the Conjugate.
- Step 13** Incubate **30 minutes** (± 5 min) at room temperature.
- Step 14** Pipette **100 µl** of Stop Solution into each microwell using the same order and timing as for the addition of the Enzyme Substrate. Read absorbance values within 1 hour from adding Stop Solution.
- Step 15** Read absorbance of each microwell at **405 nm** using a single or 405/630nm dual wavelength microplate reader against the reagent blank set at zero absorbance.

Quality Control

Calibrators, Positive and Negative Controls and a reagent blank must be run with each assay to verify the integrity and accuracy of the test. The absorbance reading of the reagent blank should be <0.4. The Calibrator A should have an absorbance reading of not less than 1.0, otherwise the test must be repeated. The negative control must be < 20 EU/ml. If the test is run in duplicate, the mean of the two readings should be taken for determining EU/ml. While performing Qualitative determinations, the optical density of the Calibrator D must be greater than that of the negative control and lesser than the absorbance of the positive control. For semi-quantitative determinations, the positive control must give values in the range stated on the vial.

RESULTS

Calculations

The concentrations of the patient samples can be determined by either of two methods:

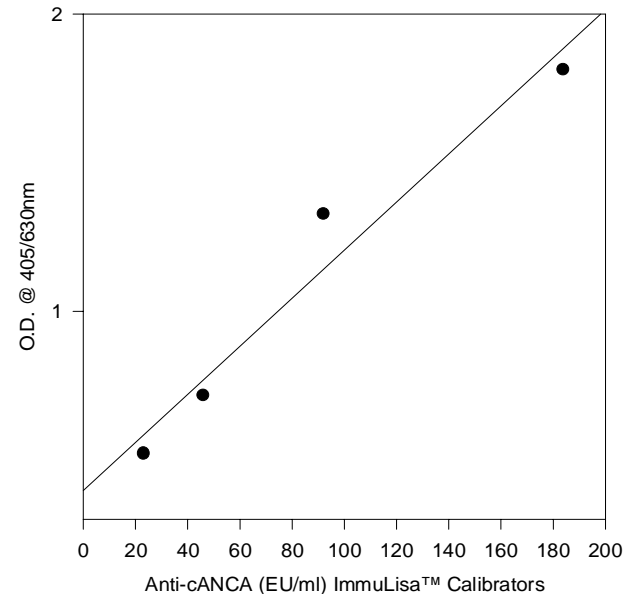
1. QUALITATIVE DETERMINATION

$$\frac{\text{Abs. of Test Sample}}{\text{Abs. of Calibrator D}} \times \text{EU/ml of Calibrator D} = \text{EU/ml Test Sample}$$

2. SEMI-QUANTITATIVE DETERMINATION

Plot absorbance of Calibrator A through D against their respective concentration on a linear-linear graph paper. Plot the concentration in EU/ml on the X-axis against the absorbance on the Y-axis and draw the best fit curve. Determine the concentrations of the patient samples from the curve against its corresponding absorbance value.

Anti-cANCA Immulisa™ Standard Curve



Calibrator

The Ready to Use Calibrators are included to provide semi-quantitation and must be used with each run. Patient samples containing higher antibody levels may give absorbance values greater than that of the Calibrator A. For determining accurate semi-quantitative values such serum sample should be further diluted so they fall within the range of the calibrator curve when retested. For determining EU/ml, multiply the units obtained by the dilution factor.