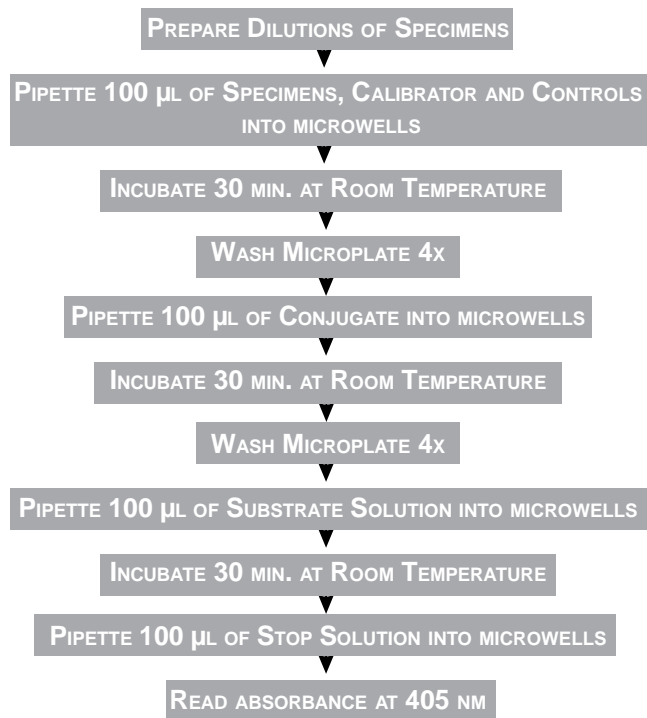


## ImmuLisa™ PROCEDURE AT A GLANCE



*For technical assistance please contact:*



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*or your local product distributor*



## ImmuLisa™ ANCA Screen ELISA

For Investigational Use only

### PRODUCT INSERT

Catalog No. 1160

96 Determinations

### INTENDED USE

Enzyme linked immunosorbent assay (ELISA) for the detection of antibodies to myeloperoxidase (MPO) and proteinase 3 (PR3) in human serum.

### SUMMARY AND EXPLANATION

The presence of anti-neutrophil cytoplasmic antibodies (ANCA) in patients with vasculitis was first observed in 1982 by Davies<sup>1</sup>. ANCA are a group of autoantibodies directed against proteins in the granules of neutrophils and in peroxidase-positive lysosomes of peripheral blood monocytes. These antibodies can be detected by indirect immunofluorescence on ethanol-fixed neutrophils, producing a characteristic perinuclear staining pattern<sup>2,3</sup>. pANCA occur in vasculitis, glomerulonephritis, Churg-Strauss syndrome, polyarteritis nodosa, systemic lupus erythematosus, and rheumatoid arthritis<sup>4</sup>. A major antigen of pANCA is myeloperoxidase (MPO), which constitutes a potent microbicidal system within the neutrophil granulocytes. Additional target antigens such as human leukocyte elastase, and lactoferrin have also been associated with the pANCA fluorescence pattern<sup>6,7</sup>. Antibodies to MPO can also be induced by drugs such as hydralazine, clozapine, and L-tryptophan<sup>8</sup>. Occupational exposure to environmental factors such as silica dust may provoke a anti-MPO positive progressive glomerulonephritis<sup>8</sup>. Measurement of MPO-specific ANCA is an important aid in the evaluation of clinical subtypes within systemic vasculitides.

cANCA are directed against several proteins like Cathepsin G, Elastase and Proteinase 3 (PR3). PR3, the major antigen in this group is a neutral serine proteinase localized in the azurophilic granules of the neutrophils<sup>9</sup>. Antibodies against the PR3 antigen serve as a marker for Wegener's Granulomatosis (WG)<sup>10</sup>, a systemic necrotising vasculitides which elicits in two forms, extended and limited<sup>10</sup>. Extended WG is characterized by granulomatous inflammation of the respiratory tract and crescentic glomerulonephritis with cANCA reactivity in 90% of patients<sup>11,12</sup>. Limited WG is characterized without renal involvement, and cANCA reactivity is detected in 67% of patients. Disease onset can occur at any age. 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<sup>13,14</sup>.

The presence of cANCA is also indicative of other diseases like idiopathic immune necrotizing glomerulonephritis and inflammatory bowel disorders like ulcerative colitis<sup>15,16</sup>. The Immulisa™ ANCA Screen ELISA offers a convenient method of screening patient sera for the presence of ANCA before further identifying positive samples for c-or pANCA with the Immulisa™ anti-PR3, and anti-MPO ELISA.

### PRINCIPLES OF PROCEDURE

The test is performed as a solid phase immunoassay. Microwells are coated with purified MPO and PR3 antigen. Controls, calibrator and patient serum samples are incubated in the microwells allowing antibodies present in the serum to bind the antigen. Unbound antibody and other serum proteins are removed by washing the microwells. Bound antibodies are incubated with an enzyme labeled anti-human IgG conjugate. Unbound conjugate is removed by washing the microwells. 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 the 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 materials<sup>17</sup>.

**WARNING** - Sodium azide ( $\text{NaN}_3$ ) 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.

#### Materials provided

## REFERENCES

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- 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.

ImmuLisa™ ANCA Screen ELISA

Catalog No. 1160

Kit contains sufficient reagents to perform 96 determinations.

- 12 x 8** Ready to use **Microplate** with individual breakaway microwells coated with antigen.
- 1 x 1.5 ml** \*Ready to use **Positive Control** (*red cap*). Contains human serum positive for ANCA 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.
- 1 x 1.5 ml** \*Ready to use **Calibrator** (*green cap*). Human serum containing antibodies to ANCA antigen. Concentrations in EU/ml are printed on the label.
- 1 x 12 ml** \*Ready to use **anti-human 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

\*Contains <0.1% NaN<sub>3</sub>

### Materials Required But Not Provided

- Deionized or distilled water
- Squeeze bottle to hold diluted wash buffer
- 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
- Automatic microplate washer capable of dispensing 300 µl

### Other ANCA ELISA available from IMMCO Diagnostics:

ImmuLisa™ Anti-MPO Test System

Catalog No. 1161

ImmuLisa™ Anti-PR3 Test System

Catalog No. 1162

## 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.

## PROCEDURE

### Procedural Notes

- Before starting with the assay read carefully the product insert.
- Let serum specimens and test reagents equilibrate at room temperature for at least 30 minutes 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.

## PERFORMANCE CHARACTERISTICS

### Precision:

Two ANCA positive sera were tested with the Immulisa™ ANCA Screen to determine inter-and intra-assay variability. The results are as follows:

	inter-assay %CV	intra-assay %CV
<b>Sample 1</b>	3.6	8.1
<b>Sample 2</b>	4.0	3.7

### Recovery:

Three samples with known ANCA concentrations were mixed with another positive sample. ANCA levels of the mixed samples were determined and the percent recovery calculated. The results are as follows:

:

	ANCA conc. added (EU/ml)	ANCA conc. obtained (EU/ml)	% Recovery
<b>Sample 1</b>	135.9	132.5	97.5
<b>Sample 2</b>	110.9	114.4	103.2
<b>Sample 3</b>	65.7	71.5	108.8

Results obtained with the Immulisa™ ANCA Screen ELISA were compared with the Immulisa™ Anti-MPO and Anti-PR3 ELISA. The results are as follows:

		Immulisa™ ANCA Screen		
		Positive	Negative	Total
Immulisa™ Anti-MPO & PR3	Positive	64	3	67
	Negative	0	4	4
	Total	64	7	71

Relative Agreement: 95.8%  
 Relative Sensitivity: 95.5%  
 Relative Specificity: 100%

## EXPECTED VALUES

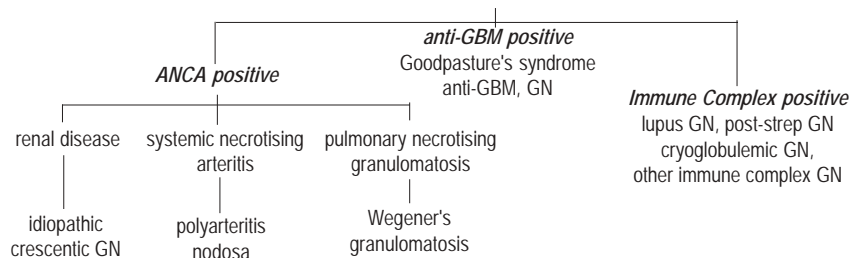
Expected values in a normal population are negative. However, 4% of apparently healthy, asymptomatic individuals may test positive for ANCA. Recently reported studies show patients diagnosed for Rheumatoid Arthritis, chronic polyarthritis, undifferentiated connective tissue disease and SLE contain anti-MPO antibodies<sup>5</sup>. Some patients with active WG may have undetectable anti-PR3 antibodies.

The following table depicts the frequency of PR3 and MPO specific ANCA in sera from 112 ANCA associated vasculitides patients<sup>18</sup>.

	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 presence of ANCA distinguishes certain characteristic diseases from other glomerulonephritic conditions, as is illustrated below<sup>12</sup>:

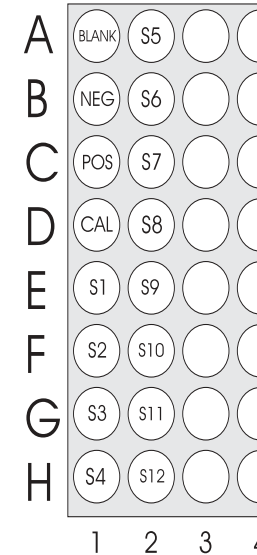
### Serological Analysis



## Test Method

- Step 1** Let all reagents and specimens equilibrate at room temperature.
- Step 2** Label protocol sheet to indicate sample placement and serial dilutions in the wells according to the following figure. The user has the option to run samples in duplicate.

Sample Layout



- Step 3** Prepare with Serum Diluent a **1:101** dilution of patient serum samples by mixing **5 µl** of each of the above with **0.5 ml** of Serum Diluent.
- Step 4** 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 5** Pipette **100 µl** of Ready to Use Calibrator, Positive and Negative controls and diluted patient samples to the appropriate microwells as per above sample layout.
- Note:** Include one well which contains **100 µl** of the Serum Diluent as a reagent blank. Zero the ELISA reader against the reagent blank. The absorbance of the reagent blank should not be more than 0.3 when read against air.

- Step 6** Incubate **30 minutes** ( $\pm$  5 min) at room temperature.
- Step 7** 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. Do not dry wells completely. For automatic washers, program the washer as per manufacturer's instructions.
- Step 8** Pipette **100  $\mu$ l** of Conjugate into microwells.
- Step 9** Incubate **30 minutes** ( $\pm$  5 min) at room temperature.
- Step 10** Wash all microwells as in Step 7.
- Step 11** Pipette **100  $\mu$ l** of Enzyme Substrate into each microwell in the same order and timing as for the Conjugate.
- Step 12** Incubate **30 minutes** ( $\pm$  5 min) at room temperature.
- Step 13** Pipette **100  $\mu$ l** 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 14** Read absorbance of each microwell at **405 nm** using a single or dual wavelength microplate reader against the reagent blank set at zero absorbance.

### Quality Control

Calibrator, 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  $<0.3$ . The calibrator 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. The absorbance of the positive control should be greater than the negative control and lesser than that of the calibrator.

## RESULTS

### Calculations

The following method should be used to determine whether the specimen is positive or negative for ANCA:

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

### Interpretation

The following serves only as a guide in the interpretation of the laboratory results. The values depicted below were determined by testing 62 normal blood donors and represent the mean of the normals plus 3 SD. Each laboratory must determine its own normal values.

ANCA Screen values	Interpretation
$\leq 20$ 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. Any test with borderline reactivity be retested to confirm the result. It is also recommended that patients with borderline results be retested at appropriate intervals. Immunosuppressive therapy, initiation or alteration in treatment should not be started on the basis of just positive ANCA results, but rather on careful clinical observations.