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IVD



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2°C-8°C



Σ=96 tests

REF

#1445-2

## PROTEINASE-3 IgG ELISA TEST SYSTEM

For *In Vitro* Diagnostic Use

Cat. No. 1445-2

### INTENDED USE

The Diagnostic Automation, Inc. Proteinase-3 IgG ELISA test system is intended for the qualitative and semi-quantitative detection of anti-Proteinase-3 IgG antibody in human serum. The test system is intended to be used as an aid in the diagnosis of various autoimmune vasculitic disorders characterized by elevated levels of anti-neutrophil cytoplasmic antibodies (ANCA). PR-3-ANCA may be associated with autoimmune disorders such as Wegener's granulomatosis, ICGN, MPA, and PRS. This test is for *in vitro* diagnostic use.

### SIGNIFICANCE AND BACKGROUND

Anti-neutrophil cytoplasmic antibody (ANCA) was initially described by Davies, *et al* in 1982 (1). Since this initial discovery, ANCA has been found to be associated with a number of Systemic Vasculitides (SV). ANCA is now recognized to include two primary specificities: C-ANCA directed against Proteinase-3 (PR-3), and P-ANCA directed against Myeloperoxidase (MPO). Testing for both P-ANCA and C-ANCA is highly recommended in the laboratory workup of patients who present with clinical features suggestive of SV. The clinical syndromes most frequently associated with ANCA are as follows:

- Wegener's granulomatosis (2)
- Polyarteritis (3)
- "Overlap" Vasculitis (4)
- Idiopathic Crescentic Glomerulonephritis (ICGN) (5)
- Kawasaki Disease (6)

Although the initial identification of C-ANCA and P-ANCA was based on the indirect immunofluorescence procedures, further identification and purification of PR-3 and MPO has resulted in the development of enzyme immunoassays (ELISA) for both PR-3 and MPO.

### PRINCIPLE OF THE ELISA ASSAY

The Diagnostic Automation, Inc. PR-3 IgG ELISA test system is designed to detect IgG class antibodies to PR-3 in human sera. The test procedure involves three incubation steps:

1. Test sera (properly diluted) are incubated in microwells coated with PR-3 (antigen). Anti-PR-3 specific IgG antibodies in the sample will bind to the immobilized antigen. The plate is washed to remove unbound antibody and other serum components.

2. Peroxidase conjugated goat anti-human IgG is added to the wells and the plate is incubated. The conjugate will react with antibody immobilized on the solid phase in step 1. The wells are washed to remove un-reacted conjugate.
3. The microtiter wells containing immobilized peroxidase conjugate are incubated with peroxidase substrate solution. Hydrolysis of the substrate by peroxidase produces a color change. After a period of time, the reaction is stopped and the color intensity of the solution is measured photometrically. The color intensity of the solution depends upon the antibody concentration in the test sample.

## **KIT COMPONENTS**

### **Reactive Reagents**

1. Twelve, 1 x 8-well strips coated with Proteinase-3 (antigen). The strips are packaged in a strip holder and sealed in an envelope with desiccant.
2. Horseradish peroxidase conjugated goat anti-human IgG. Ready to use. One 15mL vial.
3. Human positive serum control. One 0.4mL vial.
4. Human positive calibrator. One 0.4mL vial.
5. Human negative serum control. One 0.4mL vial.
6. Sample diluent. One 30mL bottle containing Tween-20, BSA, and PBS, pH  $7.2 \pm 0.2$ . Ready to use. (Green solution with a green cap; Product Number 005N). **NOTE:** This reagent may be used with any Diagnostic Automation, Inc. ELISA test system utilizing Product Number 005N.
7. Substrate solution. One 15mL bottle containing 3,3',5,5'-tetramethylbenzidine (TMB). Ready to use
8. Stop solution. One vial containing 15mL of 1.0M H<sub>2</sub>SO<sub>4</sub>, 0.7M HCl. Ready to use. (Clear solution with a red cap).
9. Wash Buffer. One 50mL bottle containing phosphate-buffered-saline and Tween-20. 10X concentrate. (Blue solution with a clear cap). **NOTE:** 1X solution will have a pH of  $7.2 \pm 0.2$ .

**Note: Serum containers may contain excess volume.**

### **ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED**

1. Sample dilution plate. One 96 well microtiter plate for preparing serum dilutions.
2. Two mylar plate sealers.
3. Wash bottle.
4. Micropipettes, including multi-channel
5. One liter graduated cylinder
6. Paper towels
7. Test tube for serum dilution
8. Reagent reservoirs for multi-channel pipettes
9. Pipette tips
10. Distilled or deionized water
11. Timer capable of measuring to an accuracy of +/- 1 second (0-60 minutes)
12. Disposal basin and disinfectant, (Example: 0.5% sodium hypochlorite, 10% household bleach).
13. Single or dual wavelength microplate reader with 450nm filter. If dual wavelength is used, set the reference filter to 600-650 nm.

**Note: Only use dry, clean glassware.**

### **PRECAUTIONS**

1. For *In Vitro* Diagnostic Use.
2. The microwell strips do not contain any viable, infectious agents. However, the strips should be considered potentially infectious and handled accordingly. Wash solutions should be collected in a

disposable basin and treated with 0.5% sodium hypochlorite (10% household bleach) at the end of the days run.

3. Do not use the ELISA plate if the indicator strip on the desiccant pouch has turned from blue to pink.
4. Wipe bottom of plate free of residual liquid and/or fingerprints which can alter optical density (OD) readings.
5. Control sera, conjugate, and wash buffer contain preservative which may be toxic if ingested; Thimerosal at a concentration of 0.04% (w/v). The sample diluent contains sodium azide at a concentration of 0.01% (w/v)
6. Sodium azide has been reported to form lead or copper azides in laboratory plumbing which may cause explosions on hammering. To prevent, rinse sink thoroughly with water after disposing of solution containing sodium azide.
7. Dilution or adulteration of these reagents may result in loss of sensitivity.
8. Do not substitute reagents from kits with different lot numbers or from other manufacturers.
9. Each donor unit used in the preparation of the controls was found to be negative when tested by an FDA approved method for the presence of HBsAg, and for antibodies to HIV-1, HIV-2, and HCV.

#### **WARNING - POTENTIAL BIOHAZARDOUS MATERIAL**

Because no test method can offer complete assurance that human immunodeficiency virus, hepatitis B virus, or other infectious agents are absent, these specimens/reagents, as well as patient samples, should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiology and Biomedical Laboratories", 1984, p.12-16, 3rd edition- 1993, and OSHA Standard for Bloodborne Pathogens (9).

10. Never pipette by mouth. Avoid contact of reagents and patient specimens with skin or mucous membranes.
  11. Avoid microbial contamination of reagents. Incorrect results may occur.
  12. Cross contamination of reagents and/or samples could cause false results.
  13. Contamination of the TMB substrate solution with conjugate or other oxidants will cause the solution to change color prematurely. Do not use substrate solution if it has begun to turn blue. To help eliminate the possibility of contamination, refer to Test Procedure, Section D.1.
  14. Reusable glassware must be washed out and thoroughly rinsed free of all detergents.
  15. Strict adherence to the specified time and temperature of incubations is essential for accurate results. All reagents must be brought to 20-25°C before starting the assay.
  16. Improper washing will cause false positive or false negative results. Be sure to blot the plates free of any residual wash solution before adding conjugate or substrate solution. Do not allow the wells to dry out between incubations.
  17. Do not allow the stop solution to contact skin or eyes. If contact occurs, immediately flush with water.
  18. Caution: Liquid waste at acid pH should be neutralized before adding to sodium hypochlorite (bleach).
  19. Avoid splashing or generation of aerosols.
  20. Do not expose reagents to strong light during storage or incubation.
  21. Allowing the microwell strips and holder to equilibrate to room temperature prior to opening the protective envelope will protect the wells from condensation.
  22. Do not allow the conjugate to come in contact with containers which may have previously contained a solution utilizing sodium azide as a preservative. Residual amounts of sodium azide may destroy the conjugate's enzymatic activity.
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23. Do not expose any of the reactive reagents to bleach-containing solutions or to any strong odors from bleach containing solutions. Trace amounts of bleach (sodium hypochlorite) may destroy the

biological activity of many of the reactive reagents within this kit.

## **SPECIMEN COLLECTION**

Only freshly drawn and properly stored blood sera obtained by approved aseptic venipuncture procedures should be used in this assay (7, 8). No anticoagulants or preservatives should be added. Avoid using hemolyzed, lipemic, or bacterially contaminated sera.

Store sample at room temperature for no longer than 8 hours. If testing is not performed within 8 hours, sera may be stored at 2-10° C for no longer than 48 hours. If delay in testing is anticipated, store test sera at -20°C or lower. Avoid multiple freeze/thaw cycles which may cause loss of antibody activity and give erroneous results.

## **STORAGE CONDITIONS**

1. Store the unopened kit at 2-8°C.
2. Proteinase-3 coated microwell strips: Extra strips should be immediately resealed with desiccant and returned to storage at 2-8°C. Strips are stable for 60 days after the envelope has been opened and properly resealed and the indicator remains blue.
3. Peroxidase conjugated goat anti-human IgG: Store at 2-8°C. DO NOT FREEZE.
4. Human control and calibrator sera: Store at 2-8°C.
5. TMB substrate solution: Store at 2-8°C.
6. Wash buffer: Store at 2-25° C. Stable for 30 days at 2-8°C after diluting to 1X, or 7 days when stored at room temperature.
7. Sample diluent: Store at 2-8°C.
8. Stop solution: Store at 2-25°C.

**NOTE:** All kit components are stable until the expiration date printed on the label provided the recommended storage conditions are strictly followed. Do not use beyond the expiration date.

## **PROCEDURE**

### **PREPARATION OF REAGENTS**

1. Wash Buffer: Dilute the 50mL of 10X concentrate with distilled water. Mix well.
2. Sample diluent, stop solution, conjugate, and substrate solutions are ready to use.

### **TEST PROCEDURE**

#### **A. Set-Up of the Assay**

Remove the individual kit components and allow them to warm to room temperature (21-25°C). Determine the total number of samples and controls to be tested. The positive calibrator should be run in triplicate. Also, a positive control, negative control, and a reagent blank must be included each time the assay is run. Controls and samples should be run in duplicate until the laboratory becomes proficient with the test procedure. Each sample, calibrator, control, and reagent blank requires one antigen coated microwell. All samples should be vortexed before use.

Determine the number of microwells needed. After the strips and holder have warmed to room temperature cut open the protective envelope and remove the plate containing the antigen coated microwell strips. Strips that are not needed for the assay should be placed into the re-sealable pouch, sealed and returned to storage at 2-8° C.

#### **B. Serum Incubation**

Prepare a 1:21 dilution of the positive and negative controls, the positive calibrator and each patient serum as follows:

1. Add 10 $\mu$ L of each sample to a separate well of the dilution plate provided. Add 200 $\mu$ L of sample diluent to each well containing a sample.
2. Using a multichannel pipette, transfer 100 $\mu$ L of each diluted sample and control from the dilution plate to the test plate. Withdraw and expel the samples several times before the final transfer to ensure that the samples are properly mixed. Use a different pipette tip for each sample. Add 100 $\mu$ L of sample diluent to a well as a reagent blank.
3. Cover the wells with a plate sealer and incubate the plate at room temperature (20-25°C) for 30 minutes.
4. Aspirate or shake out liquid from all wells. Wash the microwell strips 5X.
  - a. Vigorously shake out the liquid from the wells.
  - b. Fill each well with wash buffer. Make sure no air bubbles are trapped in the wells.
  - c. Repeat steps **a.** and **b.** for a total of five washes.
  - d. Shake out the wash solution from all the wells. Invert the plate over a paper towel and tap firmly to remove any residual wash solution from the wells. Visually inspect the plate to ensure that no residual wash solution remains. Collect wash solution in a disposable basin and treat with 0.5% sodium hypochlorite (10% household bleach) at the end of the days run.

**NOTE: Autowash** - If using an automated wash system, set the dispensing volume to 300-350 $\mu$ L/well. Set the wash cycle for 5 washes with no delay between washes. Remove microtiter plate from washer, invert plate over paper towel and tap firmly to remove any residual wash solution from the wells.

**NOTE: Insufficient or excessive washing will result in assay variation and will affect validity of results. Therefore, for best results the use of semi-automated or automated washing equipment set to deliver a volume to completely fill each well (250-300 $\mu$ L) is recommended. Complete removal of the wash buffer after the last wash is critical for the accurate performance of the test. Also, visually ensure that no bubbles are remaining in the wells.**

#### C. Conjugate Incubation

1. Add 100 $\mu$ L of the conjugate solution to each well at the same rate and in the same order as the specimens were added.
2. Cover the plate with the plate sealer provided and incubate at room temperature (21-25°C) for 30 +/-2 minutes.
3. Wash the plate by following the procedure in Step **B.4., a.** through **d.**

#### D. Substrate Incubation

1. Add 100 $\mu$ L of the TMB substrate solution to each well (including the reagent blank well) at the same rate and in the same order as the conjugate was added. (1mL of TMB substrate is sufficient for 8 wells).
2. Incubate the plate at room temperature (21-25°C) for 15 +/- 2 minutes.
3. Add 100 $\mu$ L of stop solution to each well at the same rate and the same order as the TMB solution was added. Positive samples will turn from blue to yellow. After adding stop solution, **tap plate several times to ensure that the samples are thoroughly mixed.**
4. Set the microplate reader to read at a wavelength of 450nm and measure the optical density (OD) of each well against the reagent blank. Wait a minimum of 5 minutes and read. The plate should be read within 30 minutes after the addition of the stop solution.

#### QUALITY CONTROL

1. Each time the assay is run, the positive calibrator should be run in triplicate. A positive and negative control, and reagent blank must also be included in each assay.
2. Calculate the mean of the three positive calibrator determinations. If any of the three positive calibrator values differ by more than 15% from the mean, discard that value and calculate the mean of

the remaining two values.

3. The mean OD value for the positive calibrator and the OD values for the positive and negative controls should fall within the following ranges:

OD RANGE

Negative Control	$\leq 0.25$
Positive Calibrator	$\geq 0.30$
Positive Control	$\geq 0.50$

- a. The OD of the negative control divided by the mean OD of the positive calibrator should be  $\leq 0.9$ .
  - b. The OD of the positive control divided by the mean OD of the positive calibrator should be  $\geq 1.25$ .
  - c. If the control values are not within the above ranges, the test should be considered invalid and the test should be repeated.
4. The HPC is intended to monitor for substantial reagent failure and will not ensure precision at the assay cut-off.
  5. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.

## INTERPRETATION OF RESULTS

### A. Calculations

1. Positive Calibrator

Based upon testing of normal and disease-state specimens, a maximum normal unit (AAU) value has been determined by the manufacturer and correlated to the positive calibrator. The calibrator will allow you to determine the unit value of test samples, and to correct for slight day-to-day variations in test results. The calibrator unit value is determined for each lot of kit components and is printed on the vial label.

2. Conversion of Optical Density to AAU/mL

The conversion of OD to unit value (AAU/mL) can be represented by the following equation:

$$\text{Test Specimen AAU/mL} = (A \times B)/C$$

**Where:** AAU/mL = Unknown unit value to be determined  
A = OD of test specimen in question.  
B = Unit value of calibrator (AAU/mL).  
C = The mean OD of calibrator.

**Example:** Test specimen OD for PR-3 = 0.946  
Calibrator OD for PR-3 = 0.435  
Calibrator unit value for PR-3 = 155 AAU/mL

$$\text{Test Specimen AAU/mL} = (0.946 \times 155)/0.435$$
$$\text{Test Specimen} = 337 \text{ AAU/mL for anti-PR-3}$$

### B. Interpretation of Results

1. The patients' index values are interpreted as follows:

Index Value	Results	Importance
≤0.90	Negative	No detectable antibody to PR-3 by the ELISA test
0.91-1.09	Equivocal	Samples should be retested
≥1.10	Positive	Indicated presence of detectable antibody to PR-3 by the ELISA test

2. Index values >8.44 should be reported as >8.44.

### EXPECTED VALUES

1. Antibodies to PR-3 are strongly associated with the c-ANCA pattern (cytoplasmic anti-neutrophil cytoplasmic antibodies) (3,7).
2. Antibodies to PR-3 are found in most cases (> 90%) of Wegener's granulomatosis (4,5). The PR-3 ELISA was tested with 40 patients with Wegener's granulomatosis. Thirty nine patients (97.5%) were found to be positive.
3. Antibodies to PR-3 are found in many cases (approximately one half) of microscopic polyangiitis (7). The PR-3 ELISA assay was tested with 40 patients with microscopic polyangitis. Twenty two patients (55%) were found to be positive.
4. Antibodies to PR-3 are rarely seen in normal populations (4). The
5. PR-3 ELISA was tested with 155 normals. One hundred fifty-five were found to be negative.

In a study conducted by Diagnostic Automation, Inc. 90 normal donor sera from Southwestern United States were evaluated for Proteinase-3 autoantibodies. Of the 90 tested, none were positive.

In another study using 113 specimens which were sent to a reference laboratory in Northeastern United States, eight (8/113 = 7.1%) were positive for anti-Proteinase-3 IgG. Taken together, these studies demonstrate that the incidence of IgG antibody to Proteinase-3 is relatively rare.

### LIMITATION OF THE ASSAY

6. A diagnosis should not be made on the basis of anti-PR-3 ELISA results alone. Test results for anti-PR-3 should be interpreted in conjunction with the clinical evaluation and the results of other diagnostic procedures.
7. The performance characteristics of this device have not been established for lipemic, hemolyzed and icteric specimens; therefore, these specimens should not be tested with this assay.
8. The results of this assay are not diagnostic proof of the presence or absence of disease. Immunosuppressive therapy should not be started based on a positive result.

### PERFORMANCE CHARACTERISTICS

#### Comparative Study:

An in-house comparative study was performed to demonstrate the equivalence of the Diagnostic Automation, Inc. Proteinase-3 IgG ELISA test system to another commercially available PR-3 IgG ELISA test system. Performance was evaluated using 315 specimens; 196 disease-state specimens, 113 specimens which were sent to a reference laboratory in the Northeastern United States for routine ANCA serology, and 6 specimens which were previously tested and found to be reactive for ANCA. A summary of the clinical specimens appears in Table 1 below. The results of the investigation have been summarized in Table 2 below.

**Table 1. Summary of Clinical Specimens**

						AGE	
n	Male	Female	High	Low	Mean	Comments	
45	18	27	82	14	54.7	Disease Category: Wegener's Granulomatosis	
41	21	20	100	22	63.2	Disease Category: Idiopathic Necrotizing and Crescentic Glomerulonephritis	
41	16	25	87	20	63.1	Disease Category: Microscopic Polyarteritis	
39	17	22	94	11	60.8	Disease Category: Pulmonary Renal Syndrome	
30	15	15	78	3	43.4	Vasculitis/Glomerulonephritis Disease Controls, Non-ANCA related vasculitis.	
6	Information Not Available					Previously tested ANCA positive, no diagnosis available	
113	Information Not Available					Specimens sent to a reference laboratory for routine ANCA serology	

**Table 2: Calculation of Relative Sensitivity, Specificity, and Agreement**

**Diagnostic Automation, Inc. PR-3 IgG ELISA Result**

		+	-	±*	Totals
Commercial PR-3 ELISA Test System	+	56	6	0	62
	-	3	211	4	218
	±*	11	23	1	35
	Totals	70	240	5	315

\*Equivocal specimens were excluded from all calculations.

Relative Sensitivity =  $56/62 = 90.3\%$

95% Confidence Interval\*\* = 83 to 97.7%

Relative Specificity =  $211/214 = 98.6\%$

95% Confidence Interval\*\* = 97.0 to 100%

Relative Agreement =  $267/276 = 96.7\%$

95% Confidence Interval\*\* = 94.6 to 98.8%

\*\*95% confidence intervals calculated using the exact method.

**Reproducibility:**

To evaluate both intra-assay and inter-assay reproducibility, six specimens were tested; eight replicates each, on each of three days. These results were then used to calculate mean unit values, standard deviations, and percent CV. Two of the specimens were strong positives, two were clearly negative, and two were near the assay cut off. The results of the study have been summarized below.

**Table 3. Diagnostic Automation, Inc. PR-3 IgG ELISA; Results of Precision Testing**

Day 1: Intra-Assay Reproducibility.

Specimen	Mean Result (AAU/mL)	Standard Deviation	Percent CV	Replicates
1	1077	39	3.6	8
2	468	12	2.6	8
3	38	2	6.6	8
4	195	6	3.3	8

5	28	2	8.5	8
6	1687	61	3.6	8

Day 2: Intra-Assay Reproducibility.

Specimen	Mean Result (AAU/mL)	Standard Deviation	Percent CV	Replicates
1	967	60	6.2	8
2	406	27	6.6	8
3	42	3	8.1	8
4	192	8	4.1	8
5	35	2	6.5	8
6	1702	74	4.3	8

Day 3: Intra-Assay Reproducibility.

Specimen	Mean Result (AAU/mL)	Standard Deviation	Percent CV	Replicates
1	1085	24	2.2	8
2	423	20	4.6	8
3	36	4	9.8	8
4	178	13	7.6	8
5	28	5	17.2	8
6	1628	54	3.3	8

Inter-Assay Reproducibility; All Days Combined:

Specimen	Mean Result (AAU/mL)	Standard Deviation	Percent CV	Replicates
1	1043	69	6.6	24
2	432	33	7.7	24
3	39	4	10.4	24
4	189	12	6.5	24
5	30	5	15.4	24
6	1672	69	4.1	24

**Cross Reactivity:**

To evaluate the test system for potential cross-reactivity to other autoantibodies, eight specimens which were positive for antibodies to nuclear antigens (ANA) on HEP-2 cells were tested. Two of the specimens demonstrated a homogenous pattern, two demonstrated a nucleolar pattern, two demonstrated the centromere pattern, and two demonstrated a speckled pattern. The results of this study have been summarized in Table 4 below. The results of this investigation indicate that cross reactivity with other antinuclear antibodies is not likely.

**Table 4. Results of the Cross Reactivity Investigation.**

ANA HEP-2 IFA RESULTS	PR-3 IgG ELISA RESULTS
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Sample Number	Pattern	Endpoint Titer	Optical Density	AAU/mL
1	Homogeneous	1:1280	0.056	53
2	Homogeneous	1:640	0.014	13
3	Speckled	1:2560	0.030	28
4	Nucleolar	1:1280	0.069	66
5	Centromere	1:1280	0.060	58
6	Centromere	1:1280	0.026	25
7	Speckled	1:5120	0.049	48
8	Nucleolar	1:10240	0.019	48

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**ISO 13485-2003**

Revision Date: 3/24/06