DRG

DRG® ANCA Screen (EIA-2777)

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Revised 20 July 2006 (Vers. 1.1) **IVD**

INTENDED USE

The ANCA Screen ELISA test system is intended for the qualitative detection of anti-Myeloperoxidase and/or 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). MPO and/or PR-3 may be associated with autoimmune disorders such as Wegener's Granulamatosis, 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 beable to do 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 FOR THE ELISA ASSAY

1. The ANCA Screen ELISA test system is designed to detect IgG class antibodies to MPO and/or PR-3 in human sera. The test procedure involves three incubation steps:
2. Test sera (properly diluted) are incubated in microwells coated with a mixture of MPO and PR-3 (antigen). Anti-MPO and/or 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.
3. 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.
4. 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.

MATERIALS PROVIDED

1. **Plate**. 96 wells configured in twelve 1x8-well strips coated with a mixture of Myeloperoxidase (MPO) and Proteinase-e (PR3) enzymes (antigen). The strips are packaged in a strip holder and sealed in an envelope with desiccant.
2. **Conjugate**. Conjugated (horseradish peroxidase) goat anti-human IgG (y chain specific). Ready to use. One, 15 ml vial with a white cap. Preservative added.
3. **Positive Control** (Human Serum). One 0.35 mL vial with a red cap. Preservative added.
4. **Calibrator** (Human Serum). One, 0.5 mL vial with a blue cap. Preservative added.
5. **Negative Control** (Human Serum). One, 0.35 mL vial with a green cap. Preservative added.
6. **Sample Diluent**. One 30 mL bottle (green cap) containing Tween-2-, bovine serum albumin and phosphate-buffered-sline, (pH 7.2 +/- 0.2), Green solution, ready to use. Note: Shake Well Before Use. Preservative added.
7. **TMB**: One 15 mL amber bottle (amber cap) containing 3,3',5,5'-tetramethylbenzidine (TMB). Ready to use. Contains DMSO $\leq$ 15% (w).
8. **Stop Solution**: One 15mL bottle (red cap) containing 1M H_2SO_4, 0.7M HCI. Ready to use.
9. **Wash buffer concentrate** (10X): dilute 1 part concentrate + 9 parts deionized or distilled water. One 100 ml bottle (clear cap) containing a 10X concentrated phosphate-buffered saline and Tween 20 solution (blue solution). Contains preservative NOTE: 1X solution will have a pH of 7.2 +/- 0.2.

PRECAUTIONS

1. For *In Vitro* Diagnostic Use.
2. Normal precautions exercised in handling laboratory reagents should be followed. In case of contact with eyes, rinse
3. immediately with plenty of water and seek medical advice. Wear suitable protective clothing, gloves, and eye/face
4. protection. Do not breathe vapor. Dispose of waste observing all local, state, and federal laws.
5. The wells of the ELISA plate do not contain viable organisms. However, the strips should be considered

POTENTIALLY BIOHAZARDOUS MATERIALS and handled accordingly. Source materials from which these products were derived were found negative for HIV-1 antigen, HBsAg and for antibodies against HCV and HIV by approved test methods. However, since no test method can offer complete assurance that infectious agents are absent, these products 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 Microbiological and Biomedical Laboratories": current edition; and OSHA's Standard for Bloodborne Pathogens (9).

6. Adherence to the specified time and temperature of incubations is essential for accurate results. All reagents must be allowed to reach room temperature (20-25°C) before starting the assay. Return unused reagents to refrigerated temperature immediately after use.
7. Improper washing could cause false positive or false negative results. Be sure to minimize the amount of any residual wash solution; (e.g., by blotting or aspiration), before adding Conjugate or Substrate. Do not allow the wells to dry out between incubations.
8. The human serum controls, Sample Diluent, Conjugate, and Wash Buffer concentrate contain a preservative (thimerosal, 0.04% (w/v) which may be toxic if ingested.
9. The Stop Solution is TOXIC. Causes burns. Toxic by inhalation, in contact with skin and if swallowed. In case of accident or if you feel unwell, seek medical advice immediately.
10. The TMB Solution is HARMFUL. Irritating to eyes, respiratory system and skin.
11. The Wash Buffer concentrate is an IRRITANT. Irritating to eyes, respiratory system and skin.
12. Wipe bottom of plate free of residual liquid and/or fingerprints, which can alter optical density (OD) readings.
13. Dilution or adulteration of these reagents may generate erroneous results.
14. Reagents from other sources or manufacturers should not be used.
15. TMB solution should be colorless, very pale yellow, very pale green, or very pale blue when used. 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 is noticeably blue in color. To help reduce the possibility of contamination, refer to Test Procedure, Substrate Incubation section to determine the amount of Substrate Solution to be used.
16. Never pipette by mouth. Avoid contact of reagents and patient's specimens with skin or mucous membranes.
17. Avoid microbial contamination of reagents. Incorrect results may occur.
18. Cross contamination of reagents and/or samples could cause erroneous results.
19. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
20. Avoid splashing or generation of aerosols.
21. Do not expose reagents to strong light during storage or incubation.
22. Allowing the microwell strips and holder to equilibrate to room temperature prior to opening the protective envelope will protect the wells from condensation.
23. Wash solution should be collected in a disposal basin. Treat the waste solution with 10% household bleach (0.5% sodium hypochlorite). Avoid exposure of reagents to bleach fumes.
24. Caution: Liquid waste at acid pH should be neutralized before adding to bleach solution.
25. Do not use ELISA plate if the indicator strip on the desiccant pouch has turned from blue to pink.
26. 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.
27. 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.

MATERIALS REQUIRED BUT NOT PROVIDED:

1. ELISA microwell reader capable of reading at a wavelength of 450 nm.
2. Pipettes capable of accurately delivering 10 to 200 uL.
3. Multichannel pipette capable of accurately delivering (50-200 uL)
4. Reagent reservoirs for multichannel pipettes.
5. Wash bottle or microwell washing system.
6. Distilled or deionized water.
7. One liter graduated cylinder.
8. Serological pipettes.
9. Disposable pipette tips.
10. Paper towels.
11. Laboratory timer to monitor incubation steps.
12. Disposal basin and disinfectant. (Example: 10% household bleach, 0.5% sodium hypochlorite.)

STORAGE CONDITIONS:

1. Store the unopened kit at 2-8^{O}C.
2. Coated microwell strips: Store between 2° and 8°C. Extra strips should be immediately resealed with desiccant and returned to proper storage. Strips are stable for 60 days after the envelope has been opened and properly resealed and the indicator strip on the desiccant pouch remains blue.
3. Conjugate: Store between 2° and 8 ^{O}C. DO NOT FREEZE.
4. Calibrator, Positive Control and Negative Control: Store between 2 and 8 ^{O}C.
5. TMB: Store between 2 and 8^{O}C.
6. Wash Buffer concentrate (10X): Store between 2 and 25^{O}C. Diluted wash buffer (1X) is stable at room temperature (20° to 25°C) for up to 7 days or for 30 days between 2 and 8 ^{O}C.
7. Sample Diluent: Store between 2 and 8 ^{O}C.
8. Stop Solution: Store between 2 and 25^{O}C.

SPECIMEN COLLECTION

1. It is recommended that specimen collection be carried out in accordance with NCCLS document M29: Protection of Laboratory Workers from Infectious Disease.
2. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood derivatives should be considered potentially infectious.
3. 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.
4. 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^{O}C for no longer than 48 hours. If delay in testing is anticipated, store test sera at -20^{O}C or lower. Avoid multiple freeze/thaw cycles, which may cause loss of antibody activity and give erroneous results.

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GENERAL PROCEDURE

1. Remove the individual components from storage and allow them to warm to room temperature (20-25°C).
2. Determine the number of microwells needed. Allow six Control/Calibrator determinations (one Blank, one Negative Control, three Calibrators and one Positive Control) per run. A Reagent Blank should be run on each assay. Check software and reader requirements for the correct Controls/Calibrator configurations. Return unused strips to the resealable pouch with desiccant, seal, and returned to storage between 2 and 8°C.

EXAMPLE PLATE SET-UP		
	1	2
A	Blank	Patient 3
B	Neg. Control	Patient 4
C	Calibrator	Etc.
D	Calibrator	
E	Calibrator	
F	Pos. Control	
G	Patient 1	
H	Patient 2	

3. Prepare a 1:21 dilution (e.g.: 10 uL of serum + 200 uL of Sample Diluent. NOTE: Shake Well Before Use) of the Negative Control, Calibrator, Positive Control, and each patient serum.
4. To individual wells, add 100 uL of each diluted control, calibrator and sample. Ensure that the samples are properly mixed. Use a different pipette tip for each sample.
5. Add 100 uL of Sample diluent to well A1 as a reagent blank. Check software and reader requirements for the correct reagent blank well configuration.
6. Incubate the plate at room temperature (20-25°C) for 25 +/- 5 minutes.
7. Wash the microwell strips 5X.

A. Manual Wash Procedure:

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 (bleach) at the end of the days run.

B. Automated Wash Procedure:

If using an automated wash system, set the dispensing volume to 300-350 µL/well. Set the wash cycle for 5 washes with no delay between washes. If necessary, the microwell plate may be removed from

washer, inverted over a paper towel and are tapped firmly to remove any residual wash solution from the microwells.

8. Add 100 µL of the conjugate solution to each well at the same rate and in the same order as the specimens were added.
9. Incubate the plate at room temperature (20-25 ^{O}C) for 25 +/- 5 minutes.
10. Wash the microwells by following the procedure as described in step 7.
11. Add 100 uL of TMB Susbstrate to each well at the same rate and in the same order as the specimens were added.
12. Incubate the plate at room temperature (20-25 ^{O}C) for 10 to 15 minutes.
13. Stop the reaction by adding 50 uL of Stop Solution to each well, including reagent blank well, at the same rate and in the same order as the TMB was added. Positive samples will turn from blue to yellow. After adding the Stop Solution, tap the plate several times to ensure that the samples are thoroughly mixed.
14. Set the microwell reader to read at a wavelength of 450nm and measure the optical density (OD) of each well against the reagent blank. 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 low positive standard (LPS) should be run in triplicate. A reagent blank, negative control, and high positive control must also be included in each assay.
2. Calculate the mean of the three low positive determinations. If any of the three low positive values differ by more than 15% from the mean, discard that value and calculate the mean of the remaining two wells.
3. The mean OD value for the LPS and the OD values for the high positive and negative controls should fall within the following ranges:

OD RANGE	
Negative Control	$\leq$ 0.250
Low Positive Standard	$\geq$ 0.300
High Positive Control	$\geq$ 0.500

a) The OD of the negative control divided by the mean OD of the LPS should be $\leq$ 0.9.
b) The OD of the high positive control divided by the mean LPS value 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 Positive Control and Negative Control are 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.
6. Refer to NCCLS document C24: Statistical Quality Control for Quantitative Measurements for guidance on appropriate QC practices.

INTERPRETATION OF RESULTS

A. CALCULATIONS

1. Correction Factor

A cutoff OD value for positive samples has been determined by the manufacturer and correlated to the Calibrator. The corre4ction factor (CF) will allow you to determine the cutoff value for positive samples and to correct for slight day-to-day variations in test results. The correction factor is determined for each lot of kit components and is printed on the data label located in the kit box.

2. Cutoff OD Value

To obtain the cutoff OD value, multiply the correction factor by the mean OD of the Calibrator.

(CF x mean OD of LPS determined by you = cutoff OD value)

3. Index Values of OD Ratios

Calculate an OD ratio for each specimen by dividing its OD value by the cutoff OD from Step. 2.

Example:	Mean OD of LPS	=	0.793
	CF for LPS	=	0.25
	Cutoff	=	0.793 x 0.25 = 0.198
	Unknown Specimen OD	=	0.432
	Specimen OD Ratio	=	0.432/0.198 = 2.18

B. INTERPRETATIONS:

Index Values or OD ratios are interpreted as follows:

	Index Value or OD RATIO
Non-Reactive Specimens	$\leq$ 0.90
Equivocal Specimens	0.91 - 1.09
Positive Specimens	$\geq$ 1.10

1. An OD ratio $\leq$ 0.90 indicates no detectable IgG antibodies to Myeloperoxidase or Proteinase -3.
2. An OD ratio $\geq$ 1.10 is reactive for IgG antibodies to Myeloperoxidase and/or Proteinase-3. The results of this test system are qualitative; ratio values in the reactive range are not indicative of the amount of antibody present.
3. Specimens with OD ratio values in the equivocal range (0.91 - 1.09) should be re-tested. Specimens that remain equivocal after repeat testing should be tested by an alternate serologic procedure.

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LIMITATIONS OF THE ASSAY

1. A diagnosis should not be made on the basis of ANCA Screen ELISA results alone. Test results for ANCA Screen should be interpreted in conjunction with the clinical evaluation and the results of other diagnostic procedures.
2. 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.
3. Although the ANCA Screen will detect antibodies to both MPO and PR-3, the assay will not differentiate between the two. Positive ANCA Screen specimens should be tested on the individual MPO and PR-3 ELISAs to determine which antibody is present.
4. 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.

EXPECTED RESULTS

In a study, 90 normal donor sera from Northeastern United States were evaluated for ANCA autoantibodies. Of the 90 tested, one (1.1%) was positive and one (1.1%) was equivocal.

In another study using 105 specimens, which were sent to a reference laboratory in Northeastern United States, fourteen (14/105 = 13.3%) were positive for ANCA antibodies. Taken together, these studies demonstrate that the incidence of ANCA is relatively rare.

PERFORMANCE CHARACTERISTICS

An in-house comparative study was performed to demonstrate the equivalence of the ANCA Screen IgG ELISA test system to another commercially available ANCA IgG ELISA test system. Performance was evaluated using 316 specimens; 196 disease-state specimens, 113 specimens that were sent to a reference laboratory in the Northeastern United States for routine ANCA serology, and 7 specimens, which were previously tested and found to be reactive for ANCA. The results of the investigation have been summarized in Table 1 and 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
7	Information Not Available					Previously tested ANCA positive, no diagnosis available
113	Information Not Available					Specimens sent to a reference lababoratory for routine ANCA serology

Table 2. Calculation of Relative Sensitivity, Specificity, and Agreement
ANCA Screen IgG ELISA Result

		+	-	⌖ *	Totals
Commercial	+	148	8	0	156
ANCA ELISA	-	3	113	4	120
Test System	±*	15	22	3	40
	Totals	166	143	7	316

* Equivocal specimens were excluded from all calculations.
Relative Sensitivity = 148/156 = 94.9% 95% Confidence Interval ** = 91.4 to 98.3%
Relative Specificity = 113/116 = 97.4% 95% Confidence Interval ** = 94.5 to 100%
Relative Agreement = 261/272 = 96.0% 95% Confidence Interval ** = 93.6 to 98.3%
**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. ANCA Screen: Results of Precision Testing

Day 1: Intra-Assay Reproducibility

Specimen	Mean Ratio	Standard Deviation:	Percent CV	Replicates
1	7.40	0.25	3.4	8
2	5.89	0.20	3.4	8
3	1.00	0.07	6.7	8
4	0.97	0.07	7.6	8
5	0.17	0.01	5.3	8
6	0.08	0.01	6.0	8

Day 2: Intra-Assay Reproducibility

Specimen	Mean Ratio	Standard Deviation:	Percent CV	Replicates
1	7.10	0.21	3.0	8
2	5.59	0.30	5.4	8
3	0.90	0.05	5.6	8
4	0.78	0.06	7.7	8
5	0.18	0.02	8.3	8
6	0.06	0.01	20.7	8

Day 3: Intra-Assay Reproducibility

Specimen	Mean Ratio	Standard Deviation	Percent CV	Replicates
1	7.60	0.30	3.9	8
2	5.90	0.32	5.4	8
3	1.14	0.07	6.1	8
4	0.96	0.07	7.3	8
5	0.21	0.03	11.9	8
6	0.08	0.01	12.5	8

Inter-Assay Reproducibility; All Days
Combined:

Specimen	Mean Ratio	Standard Deviation	Percent CV	Replicates
1	7.40	0.34	4.6	24
2	5.80	0.30	5.2	24
3	1.00	0.11	11.0	24
4	0.90	0.11	12.2	24
5	0.18	0.02	11.1	24
6	0.07	0.02	21.4	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 result of this investigation indicates that cross reactivity with other antinuclear antibodies is not likely.

Table 4. Results of the Cross Reactivity Investigation

	ANA HEp-2 IFA Results		ANCA Screen ELISA Results:	
Sample Number	Pattern	Endpoint Titer	Optical Density	Ratio
1	Homogenous	1:1280	0.066	0.36
2	Homogenous	1:640	0.019	0.10
3	Speckled	1:2560	0.044	0.24
4	Nucleolar	1:1280	0.101	0.56
5	Centromere	1:1280	0.050	0.28
6	Centromere	1:1280	0.035	0.19
7	Speckled	1:5120	0.051	0.28
8	Nucleolar	1:10240	0.028	0.15

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ABBREVIATED TEST PROCEDURE

1. Dilute Serum 1:21
2. Add diluted serum to microwell 100 uL/Well
3. ⟶ Incubate 20 to 30 minutes.
4. Wash
5. Add Conjugate – 100 uL/Well
6. ⟶ Incubate 20 to 30 minutes
7. Wash
8. Add TMB 100 uL/Well
9. ⟶ Incubate 10 to 15 minutes
10. Add Stop Solution 50 uL/Well-Mix
11. READ

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