1. INTRODUCTION

Anti-neutrophilic-cytoplasm antibodies (ANCA) represent a group of autoantibodies directed towards the cytoplasmatic components of the neutrophilic granulocytes and monocytes. The classical methods for the determination of ANCA are the immunofluorescent methods. With these indirect immunofluorescence techniques two main patterns are recognized, a cytoplasmatic (c-ANCA) and a perinuclear (p-ANCA) type. The main antigen for the c-ANCA is the proteinase 3 (PR3), which is a serine proteinase present in primary granules. Antibodies of p-ANCA positive sera are mainly directed to myeloperoxidase (MPO). Antibodies to other antigens e.g. lactoferrin, elastase, cathepsin-G and also lysozyme often result in a similar p-ANCA pattern. Beside different untypical variants of p-ANCA IF patterns granulocyte specific antinuclear antibodies (GS-ANA) is indistinguishable from p-ANCA. This makes a clear interpretation and classification of the IF patterns difficult. Therefore every positive IF-ANA findings esp. p-ANCA should be differentiated by ELISA techniques using purified antigens. A survey of documented clinical indications of specific ANCA is given in the table below. PR3-ANCA and MPO-ANCA are reliable serologic markers in the diagnostics of vasculitides. PR3-ANCA is the classical autoantigen in Wegener’s granulomatosis with a clinical specificity of more than 95%. c-ANCA is documented to be present in different diseases. Anti-MPO antibodies are highly specific for idiopathic and vasculitis associated crescentic glomerulonephritis and also for classic polyarteritis nodosa, Churg-Strauss syndrome and the polyangitis overlap syndrome without renal involvement. With respect to sensitivity, either MPO or PR-3 antibodies were found in 77 to 100% of patients with idiopathic and vasculitis associated crescentic glomerulonephritis. In WG, anti-MPO antibodies were detected only occasionally and generally in patients negative for PR-3 antibodies. The MPO and PR-3 specific ELISA methods can provide an important confirmatory result for two of the more important of the identified antigens. ELISA is also useful for interpreting “difficult” samples by IFA such as those which exhibit several antibodies simultaneously or those with high background fluorescence.

2. INTENDED USE

Anti PR3 is an indirect solid phase enzyme immunoassay (ELISA) for the quantitative measurement of IgG class autoantibodies against proteinase 3 (PR3) in human serum or plasma. The assay is intended for laboratory research use only. Anti PR3 (c-ANCA) kit is intended for laboratory use only.

3. PRINCIPLE OF THE ASSAY

The anti-PR3 IgG test is based on the binding of present antibodies on the human neutrophil proteinase 3 coated into the microplates. Any present antibodies in calibrators, controls or pre-diluted patient samples bind to the inner surface of the wells. After a 60 minutes incubation the microplate is washed with wash buffer for removing non-reactive Serum or Plasma components. An anti-human-IgG horseradish peroxidase conjugate solution recognize IgG class antibodies bound to the immobilized antigens. After a 30 minutes incubation any excess enzyme conjugate, which is not specifically bound is washed away with wash buffer. A chromogenic substrate solution containing TMB is dispensed into the wells. After 15 minutes of incubation the color development is stopped by adding the stop solution. The solution color change into yellow. The amount of color is directly proportional to the concentration of IgG antibodies present in the original sample.

4. MATERIALS

4.1. Reagents supplied

- **Anti PR3 Coated Wells**: 12 break apart 8-well snap-off strips coated with human neutrophil PR3; in re-sealable aluminum foil.
- **Stop Solution**: 1 bottle containing 15 ml sulphuric acid, 0.25 mol/l (avoid any skin contact), ready to use.
- **Conjugate**: 1 bottle containing 15 ml with anti h-IgG conjugated with horseradish peroxidase (HRP).
- **TMB Substrate Solution**: 1 bottle containing 15 ml 3, 3’, 5, 5’-tetramethylbenzidine (H2O2:TMB 0.26 g/l) (avoid any skin contact), ready to use.
- **Sample diluent**: 1 bottle containing 100 ml, Phosphate buffer.
- **Wash solution**: 1 bottle containing 50 ml (10x conc.)
- **anti-PR3 Standards**: 5 bottles, 1.2 ml each, ready to use.
  - Standard 0: 0 AU/ml
  - Standard 1: 10 AU/ml
  - Standard 2: 20 AU/ml
  - Standard 3: 40 AU/ml
  - Standard 4: 160 AU/ml
- **Negative Control**: 1 bottle containing 1.2 ml, ready to use.
- **Positive Control**: 1 bottle containing 1.2 ml, ready to use.
4.2. Materials supplied
- 1 Strip holder
- 1 Cover foils
- 1 Test protocol
- 1 Distribution and identification plan

4.3. Materials and Equipment needed
- ELISA microwell plate reader, equipped for the measurement of absorbance at 450 nm
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Vortex tube mixer
- Distilled water
- Disposable tubes
- Timer

5. STABILITY AND STORAGE
The reagents are stable up to the expiry date stated on the label when stored at 2…8 °C in the dark.

6. REAGENT PREPARATION
It is very important to bring all reagents, samples and standards to room temperature (22…28°C) before starting the test run!

6.1. Coated snap-off Strips
The ready to use break apart snap-off strips are coated with human neutrophil PR3 antibodies. Store at 2…8 °C. Open the bag only when it is at room temperature. Immediately after removal of strips, the remaining strips should be resealed in the aluminum foil along with the desiccant supplied and stored at 2…8 °C; stability until expiry date. Do not remove the adhesive sheets on the unused strips.

6.2. anti-PR3 Standards/controls
Since no international reference preparation for Anti-proteinase 3 antibodies is available, the assay system is calibrated in relative arbitrary units. The standard have approximately the following concentration:

<table>
<thead>
<tr>
<th></th>
<th>S0</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
</tr>
</thead>
<tbody>
<tr>
<td>AU/mL</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>40</td>
<td>160</td>
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</tbody>
</table>

6.3. TMB Substrate Solution
The bottle contains 15 ml of a tetramethylbenzidine/hydrogen peroxide system. The reagent is ready to use and has to be stored at 2…8°C in the dark. The solution should be colorless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away.

6.4. Stop Solution
The bottle contains 15 ml 0.15 M sulphuric acid solution (R 36/38, S 26). This ready to use solution has to be stored at 2…8°C.

6.5. Wash Solution
Dilute the contents of each vial of the buffered wash solution concentrate (10X) with distilled water to a final volume of 500 mL prior to use. Store refrigerated: stable at 2-8°C for at least 30 days after preparation or until the expiration date printed on the label.

7. SPECIMEN COLLECTION AND PREPARATION
Either human serum or plasma samples can be used for the test execution. Test samples should be clear. Contamination by lipemia is best avoided, but does not interfere with this assay. Specimens may be refrigerated at 2-8°C for up to five days or stored at -20°C up to six months. Avoid repetitive freezing and thawing of serum or plasma samples. This may result in variable loss of autoantibody activity. Testing of heat-inactivated serum or plasma samples is not recommended. All serum or plasma samples must be pre-diluted 1:100 with sample diluent. Therefore 10 µL of sample may be diluted with 990 µL of sample diluent. The Controls are ready to use.
8. ASSAY PROCEDURE

8.1. Test Preparation

Please read the test protocol carefully before performing the assay. Result reliability depends on strict adherence to the test protocol as described. Prior to commencing the assay, the distribution and identification plan for all specimens and controls should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder. Please allocate at least:

- 1 well (e.g. A1) for the substrate blank
- 2 wells (e.g. B1+C1) control negative
- 2 wells (e.g. D1+E1) for standard 0
- 2 wells (e.g. F1+G1) for standard 1
- 2 wells (e.g. H1+A2) for standard 2
- 2 wells (e.g. B2+C2) for standard 3
- 2 wells (e.g. D2+E2) for standard 4
- 2 wells (e.g. F2+G2) control positive

8.2. Measurement

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<tr>
<th>Reagent</th>
<th>Standard</th>
<th>Sample or Controls</th>
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</thead>
<tbody>
<tr>
<td>Standard S0-S4</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>100 µL</td>
<td></td>
</tr>
<tr>
<td>Diluted Sample</td>
<td></td>
<td>100 µL</td>
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</tbody>
</table>

Incubate 60 minutes at room temperature (22-28°C). Remove the contents from each well, wash the wells three times with 300 µL of diluted wash solution.

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<tr>
<td>Conjugate</td>
<td>100 µL</td>
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Incubate 30 minutes at room temperature (22-28°C). Remove the contents from each well, wash the wells three times with 300 µL of diluted wash solution.

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<tbody>
<tr>
<td>TMB substrate</td>
<td>100 µL</td>
<td>100 µL</td>
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</table>

Incubate 15 minutes in the dark at room temperature (22-28°C).

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<th>Sample or Controls</th>
<th>Blank</th>
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</thead>
<tbody>
<tr>
<td>Stop solution</td>
<td>100 µL</td>
<td>100 µL</td>
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</table>

Shake the microplate gently Read the absorbance (E) at 450 nm against Blank.

9. Results

9.1. Quality Control

The PR3 IgG Positive and the Negative Control should be run with every batch of samples to ensure that all reagents and procedures perform properly. Because Positive and the Negative Control are prediluted, they do not control for procedural methods associated with dilution of specimens. Additional suitable control sera may be prepared by aliquoting pooled human Serum or Plasma specimens and storing at <-20°C. In order for the test results to be considered valid, all of the criteria listed below must be met. If any of these are not met, the test should be considered invalid and the assay repeated.

- The absorbance of the prediluted PR3 IgG Positive must be greater than the absorbance of the prediluted Negative Control.
- The Negative and Positive Control are intended to monitor for substantial reagent failure and they will not ensure precision at the assay cutoff.
- This test is only valid if the optical density at 450 nm for Negative Control (1) and Positive Control (2) as well as for the Calibrator S0-S4 complies with the respective range indicated on the Quality Control Certificate enclosed to each test kit; if any of these criteria is not met, the results are invalid and the test should be repeated.

9.2. Calculation of results

For Anti-PR3 IgG a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice. Smoothed-Spline Approximation and log-log coordinates are also suitable. We recommend using a Lin-Log curve. First calculate the averaged optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration.
Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

In a normal range study with Serum or Plasma samples from healthy blood donors the following ranges have been established with the Anti-PR3 IgG tests:

Anti-PR3 (c-ANCA) IgG [AU/mL] Cut-Off: 20

The values shown below are suggested values only. Each laboratory should establish its own normal range based upon its own techniques, controls, equipment and patient population according to their own established procedures. Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken individually. It is recommended that each laboratory establishes its own normal and pathological ranges of Serum or Plasma Anti-PR3.

9.3. LIMITATIONS OF PROCEDURE
The presence of immune complexes or other immunoglobulin aggregates in the patient sample may cause an increased level of non-specific binding and produce false positives in this assay.

10. SPECIFIC PERFORMANCE CHARACTERISTICS

10.1. Precision and reproducibility
Precision and reproducibility are evaluated by eight reply of two positive samples by two different runs with two different lots. Dispensing and washing operations were performed manually by an operator. The results in terms of standard deviation and coefficient of variation were below:

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<tr>
<th>Sample</th>
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<th>2</th>
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<tr>
<td></td>
<td>SD</td>
<td>CV%</td>
</tr>
<tr>
<td>Intra-test</td>
<td>0,16</td>
<td>4,2</td>
</tr>
<tr>
<td>Inter-test</td>
<td>0,35</td>
<td>7,5</td>
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10.2. Detection Limits
The lowest concentration of anti-PR3 that can be distinguished from zero standard is about 0.13 AU/mL with a confidence limit of 98%.

10.3. Analytic Sensitivity / Specificity
Comparison test against a commercial reference kit, performed on 32 sera (3 of them positive sera and 29 negative sera) showed a 100% specificity. Comparison test against a commercial reference kit, performed on 32 sera (3 of them positive sera and 29 negative sera) showed a 100% sensibility.

11. PRECAUTIONS AND WARNINGS

Warnings
This kit is intended for in vitro use by professional persons only. Use appropriate personal protective equipment while working with the reagents provided. All human source material used in the preparation of standards and controls for this product has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the Standard and the Controls should be handled in the same manner as potentially infectious material. Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious. Some reagents contain small amounts of Sodium Azide (NaN3) or Proclin 300R as preservatives. Avoid the contact with skin or mucosa. Sodium Azide may be toxic if ingested or absorbed through the skin or eyes; moreover it may react with lead or copper plumbing to form potentially explosive metal azides. If you use a sink to remove the reagents, allow scroll through large amounts of water to prevent azide build-up. The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes. The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes. Avoid the exposure of reagent TMB/H2O2 to directed sunlight, metals or oxidants.

PRECAUTIONS
Please adhere strictly to the sequence of pipetting steps provided in this protocol. All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to use. Do not interchange kit components from different lots. The expiry dates printed on the labels of the box and of the vials must be observed. Do not use any kit component beyond their expiry date. WARNING: the conjugate reagent is designed to ensure maximum dose sensitivity and may be contaminated by external agents if not used properly; therefore, it is recommended to use disposable consumables (tips, bottles, trays, etc.). For
divided doses, take the exact amount of conjugate needed and do not re-introduce any waste product into the original bottle. In addition, for doses dispensed with the aid of automatic and semi-automatic devices, before using the conjugate, it is advisable to clean the fluid handling system, ensuring that the procedures of washing, deproteinization and decontamination are effective in avoiding contamination of the conjugate; this procedure is highly recommended when the kit is processed using analyzers which are not equipped with disposable tips. If you use automated equipment is your responsibility to make sure that the kit has been appropriately tested. The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background. It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate. Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction. Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. Maximum precision is required for reconstitution and dispensation of the reagents. Samples microbiologically contaminated should not be used in the assay. Highly lipemic or haemolysed specimens should similarly not be used. Plate readers measure vertically. Do not touch the bottom of the wells.

12. LITERATURE


13. ORDERING INFORMATION

GenWay Biotech, Inc.
Protein and Antibody Solutions
6777 Nancy Ridge Drive
San Diego, CA 92121
Phone: 858-458-0866
Fax: 858-458-0833
sales@genwaybio.com
www.genwaybio.com
SCHEME OF THE ASSAY

Anti-PR3

Test Preparation

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