For semi-quantitative determination of thyroid peroxidase autoantibodies in human serum

For “In Vitro Diagnostic” use within the United States of America. This product is for “Research Use Only” outside of the United States of America.

Catalog Number: 21-TPOHU-E01
Size: 96 wells
Version: March 2005 - ALPCO 1/4/08
I. INTENDED USE

The ALPCO Diagnostics Anti-TPO EIA test kit is intended for in vitro semi-quantitative determination of the levels of thyroid peroxidase autoantibodies in human serum. The test assists in the diagnosis of certain autoimmune thyroid diseases including: Hashimoto’s thyroiditis, Graves disease and goiter.

II. INTRODUCTION

For more than two decades, antibodies against thyroid microsomal antigen have been observed and studied in patients with autoimmune thyroid disease, in particular Hashimoto’s thyroiditis \(^{(1,2)}\). In recent years, based on the immunological findings and the molecular cloning of the antigen, it has been shown that thyroid peroxidase (TPO) is a component of thyroid microsomal antigen \(^{(3-7)}\). TPO is a poorly glycosylated heme-containing enzyme and plays a key role in thyroid function, involved in the synthesis of thyroid hormones and the iodination of thyroglobulin. Unlike other thyroid autoantibodies, TPO antibodies fix complement and may have a deleterious effect on thyroid functions \(^{(8)}\).

TPO autoantibodies are characteristically present in the serum from patients with thyroiditis and primary thyrotoxicosis. High concentrations have been associated with Hashimoto’s goiter. Many patients with thyroiditis also produce anti-thyroglobulin antibodies. The levels of these antibodies vary independently and sometimes only one of the two antibodies are present in patients with thyroiditis. It is therefore appropriate to measure the levels of both antibodies (anti-TPO and anti-thyroglobulin) in thyroid autoimmune patients \(^{(9,10)}\). Detection of Anti-TPO antibodies will assist in diagnosis of thyroid related disorders such as Hashimoto’s disease and Graves disease along with the clinical assessment of the disease. This test also helps in early diagnosis of the disease before the patient is clinically ill.

III. PRINCIPLE OF THE ANTI-TPO EIA METHOD

The Anti-TPO EIA procedure is a semi-quantitative method based on the specific binding of purified thyroid peroxidase, which is immobilized on the microwells of a microplate, with autoantibodies to thyroid peroxidase in the patient serum. In the first step, human autoantibodies to thyroid peroxidase in the patient serum bind to the purified thyroid peroxidase on the microwells. After incubation and a washing step to eliminate the unbound material, an enzyme labeled conjugate (anti-human IgG-HRP) is added to the microwells. The conjugate binds to the antigen-antibody complex in the microwells by virtue of its ability to bind to IgG antibodies. After incubation, excess enzyme is eliminated by a second washing step. Subsequent color is developed by the addition of a substrate (TMB). The intensity of the color is directly proportional to the amount of thyroid peroxidase autoantibodies present in the patient sample. The color developed is measured and quantitated by reading against a dose response curve (DRC).

IV. WARNING AND PRECAUTIONS

1. Potential Biohazardous Material

The matrix of the Calibrators and Controls is human serum. The human serum used has been found non-reactive to HbsAg, anti-HIV 1/2 and anti-HCV when tested with FDA licensed reagents. Because there is no test method that can offer complete assurance that HIV, Hepatitis B virus or other infectious agents are absent, these reagents should be handled as if potentially infectious.

2. Sodium Azide

Some reagents contain sodium azide as a preservative. Sodium azide may react with lead, copper or brass to form explosive metal azides. When disposing of these materials, always flush with large volumes of water to prevent azide buildup.

3. Stopping Solution

Stopping Solution consists of 1N H\textsubscript{2}SO\textsubscript{4}. This is a strong acid and should be handled with caution. It can cause burns and should be handled with gloves while wearing eye protection and appropriate protective clothing. Avoid inhalation. Dilute a spill with water before absorbing the spill with paper towels.

V. REAGENTS AND MATERIALS

**Materials Supplied**

- The Anti-TPO EIA kit contains sufficient reagents for 96 tests.
- 1. Thyroid Peroxidase Coated Microwells ..........1 x 96 tests
- 2. Anti-Human IgG-HRP Conjugate .........................1 x 10 ml
- 3. Serum Diluent (concentrate) ...............................1 x 10 ml
- 4. Wash Buffer (concentrate) .................................1 x 22 ml
- 5. Anti-TPO Calibrators
  - (0, 10, 40, 100, 400 U/ml) .................................5 x 1 ml
  - 6. Substrate Solution A (TMB) .............................1 x 8 ml
  - 7. Substrate Solution B (Hydrogen Peroxide).........1 x 8 ml
  - 8. Stopping Solution (1N H\textsubscript{2}SO\textsubscript{4}) ..............1 x 6 ml

All reagents (except #3, 4) are supplied in ready to use form.

**Materials Required but not Supplied**

1. Microwell plate reader capable of reading at 450 nm.
2. Calibrated semi-automatic micropipets to deliver 10, 50, 100 µl and 1.0 ml.
3. Disposable 13 x 100 borosilicate glass tubes for sample dilutions.
4. Automated microplate washer or squeeze bottle for plate washing.

VI. SPECIMEN COLLECTION AND STABILITY

Collect whole blood by venipuncture using evacuated tubes. Allow the blood clot to form at room temperature, centrifuge immediately and separate the serum from the clot. Store at 2-8°C if the test is to be run on the same day, otherwise, store frozen at -20°C until assay time.
Reagent Preparation and Storage

1. **Serum Diluent**
   Pour the contents of the vial into a 100 ml graduated container, q.s. to the 100 ml mark with distilled water. Label as Working Serum Diluent. Working Serum Diluent is stable until kit expiration when stored at 2-8°C.

2. **Wash Buffer Solution**
   Note: The wash buffer concentrate when stored at 2-8°C may have some crystals at the bottom of the vial. Add the entire contents of the concentrate to 1200 ml of distilled water in an appropriate container (rinse out all crystals). Mix thoroughly. Store at 2-8°C until kit expiration.

3. **Substrate Solution**
   Mix Substrate Solution A and B in the ratio of 1:1. For 6 microwell strips (48 wells) mix 3 ml of Substrate Solution A with 3 ml of Substrate Solution B. Mix well before use. Keep in the dark and use within one hour after preparation.

4. **Patient Sample Dilution**
   Accurately pipet 50 µl of each patient sample into 1 ml of serum sample diluent solution (see number 1 above). Mix thoroughly by inversion or vortexing. The diluted patient sample is stable for 48 hours at 2-8°C. Keep the patient serum sample frozen for any future reference.

   NOTE: If a sample reads more than 400 U/ml, it can be further diluted 1:10 and 1:100 using the original patient sample dilution already prepared as described above. The value obtained should be multiplied by the dilution factor.

VII. TEST PROTOCOL

Preparation and Precautions

1. Bring all reagents and patient samples to room temperature before use.
2. It is recommended that all calibrators, patient samples, and controls be assayed in duplicate.
3. External controls should be run with the test to verify the precision and accuracy of the method.
4. All incubation times, temperatures, and reagent volumes are critical to achieve accurate results.
5. Return all unused reagents to the refrigerator immediately after use.
6. The supplied reagents in each kit must be used together. No interchanging of reagents from different kit lots should be attempted.
7. Do not use reagents that are past the expiration date.

VIII. ASSAY PROCEDURE

1. Assemble the required number of microwell strips into the plate holder.
2. Pipet 100 µl of Calibrators, Controls, and diluted Patient Samples into the microwells.
3. Cover the microwells and incubate at 37°C for 60 minutes.
4. Using an automatic or manual plate washing technique aspirate and wash the contents of the wells three times with Wash Buffer Solution. If an automatic plate washer is used, wash the wells three times with 300 µl of wash buffer solution according to the instrument manufacturer's instructions. If manual washing is used, fill a squeeze bottle with Wash Buffer Solution. Discard the contents of all the wells into a sink by quick decantation. Carefully fill the wells one by one with the wash solution (avoid air bubbles in the wells during washing), discard the wash solution, blot dry with a paper towel, and then repeat the procedure two more times. Make sure that the plate is blotted dry each time between washes.
5. Pipet 100 µl of enzyme conjugate solution into all wells.
6. Cover the microwells and incubate at 37°C for 30 minutes.
7. Wash the microwells as in step number 4 above.
8. Pipet 100 µl of working substrate solution into all wells.
9. Cover the microwells and incubate at 37°C for 15 minutes.
10. Stop the reaction by adding 50 µl of stopping solution to all wells. The blue color in the wells will turn yellow. The yellow color in the wells is stable for 30 minutes, and the wells should be read within this time.
11. Read the well contents on a microplate reader at 450 nm.

Calculation of Results

1. Record the optical density readings (O.D.) for all wells.
2. Calculate the mean O.D. value of the duplicates.
3. Plot the Dose Response Curve using the mean O.D. (on the Y axis against the Calibrator concentration units (on the X axis) using semi-log graph paper.
4. Extrapolate the patient results from the Dose Response Curve.
**TABLE 1**

<table>
<thead>
<tr>
<th>Identification</th>
<th>O.D.1</th>
<th>O.D.2</th>
<th>Mean O.D.</th>
<th>Concentration U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator 0</td>
<td>0.089</td>
<td>0.093</td>
<td>0.091</td>
<td>0.089</td>
</tr>
<tr>
<td>Calibrator 10</td>
<td>0.396</td>
<td>0.408</td>
<td>0.402</td>
<td>0.406</td>
</tr>
<tr>
<td>Calibrator 40</td>
<td>0.909</td>
<td>0.932</td>
<td>0.921</td>
<td>0.924</td>
</tr>
<tr>
<td>Calibrator 100</td>
<td>1.464</td>
<td>1.377</td>
<td>1.421</td>
<td>1.464</td>
</tr>
<tr>
<td>Calibrator 400</td>
<td>2.195</td>
<td>2.334</td>
<td>2.264</td>
<td>2.195</td>
</tr>
<tr>
<td>Control 1</td>
<td>0.705</td>
<td>0.761</td>
<td>0.733</td>
<td>26.2</td>
</tr>
<tr>
<td>Control 2</td>
<td>1.098</td>
<td>1.053</td>
<td>1.076</td>
<td>54.2</td>
</tr>
<tr>
<td>Patient 1</td>
<td>0.346</td>
<td>0.328</td>
<td>0.337</td>
<td>8.0</td>
</tr>
<tr>
<td>Patient 2</td>
<td>1.808</td>
<td>1.834</td>
<td>1.821</td>
<td>200.8</td>
</tr>
</tbody>
</table>

**IX. QUALITY CONTROL**

Control sera used in the assay must fall within their specified ranges.

The laboratory should prepare confirmed negative and positive serum pools to be run each time to validate the assay. Alternatively, a commercially available set of controls can be run each time for validation of the assay.

**X. INTERPRETATION OF RESULTS**

The Anti-TPO EIA Dose Response Curve (DRC) is calibrated against the International Standard (IS) established by the Medical Research Council (66/287).

Values greater than 40 Units/ml are considered to be positive for anti-thyroid peroxidase antibodies. The clinical significance of this test's results along with anti-Thyroglobulin values should be used as an indicator of the thyroid problem; but the clinical evaluation of the patient should be interpreted considering clinical manifestations of the patient and other tests.

The results of this test cannot be interchanged with the PHA (passive hemagglutination assay) test because the ELISA test is a more sensitive test and detects a lower level of autoantibodies to TPO.

**XI. LIMITATIONS AND IMPORTANT NOTES**

1. As in any in vitro diagnostic test, the thyroid peroxidase autoantibody assay data obtained with this procedure should be used as an aid in conjunction with other clinical data available to the physician.

2. Adherence to the assay protocol and accuracy in pipetting the reagents are essential in order to obtain valid test results.

3. Samples which are lipemic or hemolyzed should not be used in the assay. Samples which are contaminated microbiologically likewise should not be used in the assay.

4. Do not mix different lots of any kit component within an individual assay. Do not use components beyond the expiration date shown on the outside label.

5. Failure to obtain the appropriate anti-thyroid peroxidase values for the controls may indicate imprecise manipulations, improper sample handling or reagent deterioration.

**XII. PERFORMANCE CHARACTERISTICS**

**Cross-Reactivity:** Interference from ANA, DNA and rheumatoid factors was found to be negligible.

**Specificity and Sensitivity:**

A total of 84 patients confirmed serum samples were evaluated. Of these, 59 samples were confirmed positive and the remaining 23 samples were confirmed negative. The Anti-TPO ELISA test results of the samples are presented below.

Total Number of Patient Samples Tested = 84

<table>
<thead>
<tr>
<th>Reference Method</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomerica</td>
<td>59</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22</td>
</tr>
</tbody>
</table>

Quantitative Analysis of the Results:

Relative Accuracy = 96.4%

Relative Specificity = 91.6%

Relative Sensitivity = 98.3%

* Reference Method available upon request.

**Recovery Studies:**

Recovery studies were conducted with the Anti-TPO test kit using previously confirmed serum samples of known amounts of TPO antibody. The average recovery was 96.0%. The results are presented in Table 3.

<table>
<thead>
<tr>
<th>Antibody added (U/ml)</th>
<th>Antibody recovered (U/ml)</th>
<th>Percent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>110.2</td>
<td>96.2</td>
<td>87.2%</td>
</tr>
<tr>
<td>55.1</td>
<td>56.4</td>
<td>102.3%</td>
</tr>
<tr>
<td>282.5</td>
<td>311.5</td>
<td>110.3%</td>
</tr>
<tr>
<td>216.7</td>
<td>184.9</td>
<td>85.4%</td>
</tr>
</tbody>
</table>

**Precision:**

The reliability of Anti-TPO EIA test kit was assessed by examining its reproducibility on samples representing various levels of thyroid peroxidase autoantibody.

**Intra-assay (within run)**

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>Mean Value (U/ml)</th>
<th>S.D.</th>
<th>%C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>5.6</td>
<td>0.011</td>
<td>2.95%</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>98.7</td>
<td>0.027</td>
<td>2.70%</td>
</tr>
</tbody>
</table>

**Inter-assay (between runs)**

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>Mean Value (U/ml)</th>
<th>S.D.</th>
<th>%C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>80.0</td>
<td>0.054</td>
<td>3.60%</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>365.0</td>
<td>0.059</td>
<td>3.00%</td>
</tr>
</tbody>
</table>
XIII. REFERENCES


