1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Thyroid Peroxidase (TPO) Autoantibodies in Human Serum or Plasma by a Microparticle Enzyme Immunoassay.

Measurements of TPO autoantibodies may aid in the diagnosis of certain thyroid diseases such as Hashimoto’s and Grave’s as well as nontoxic goiter.

2.0 SUMMARY AND EXPLANATION OF THE TEST

Antibodies to thyroid peroxidase have been shown to be characteristically present in the sera of patients with Hashimoto’s thyroiditis (95%), idiopathic myositis (95%) and Graves Disease (80%). In fact 72% of patients positive for anti-TPO exhibit some degree of thyroid dysfunction. The clinical measurement becomes a valuable tool in the diagnosis of thyroid dysfunction.

Measurements of antibodies to TPO have been done in the past by Passive Hemaglutination (PHA). PHA tests do not have the sensitivity of enzyme immunoassays and are limited by subjective interpretation. This procedure, with the enhanced sensitivity of EIA, permits the detectability of subclinical levels of antibodies to TPO. In addition, the results are quantitated by a spectrophotometer, which eliminates subjective interpretation.

Monobind’s microparticle enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, serum reference, diluted patient specimen, or control is first added to a microwell. Biotinylated Thyroid Peroxidase Antigen (TPO) is added, and then the reactants are mixed. Reaction results between the autoantibodies to TPO and the biotinylated TPO to form an immune complex, which is deposited to the surface of streptavidin coated wells through the high affinity reaction of biotin and streptavidin.

After the completion of the required incubation period, aspiration or decantation separates the reactants that are not attached to the wells. An enzyme conjugate is then added to permit quantification of reaction through interacting with human IgG of the immune complex. After washing, the enzyme activity is determined by reaction with substrate to produce color.

The employment of several serum references of known antibody activity permits construction of a graph of enzyme and antibody activities. From comparison to the dose response curve, an unknown specimen’s enzyme activity can be correlated with antibody activity level.

3.0 PRINCIPLE

A Sequential ELISA Method (TYPE 1)

The reagents required for the sequential ELISA assay include immobilized antigen, circulating autoantibody and enzyme-linked species-specific antibody. In this procedure, the immobilization takes place during the assay at the surface of a microwell plate through the interaction of the antigen on the well and exogenously added biotinylated thyroid peroxidase antigen.

Upon mixing the biotinylated antigen and a serum containing the autoantibody, a reaction results between the antigen and the antibody to form an immune-complex. The interaction is illustrated by the following equation:

\[
\text{h-Ab(X-TPO) + BtnAg(TPO) = h-Ab(X-TPO)-BtnAg(TPO)}
\]

\[
\text{h-Ab(X-TPO) = Human Auto-Antibody (Variable Quantity)}
\]

\[
\text{BtnAg(TPO) = Biotinylated Antigen (Constant Quantity)}
\]

\[
\text{h-Ab(X-TPO)-BtnAg(TPO) = Immune Complex (Variable Quantity)}
\]

\[
\text{k_a = Rate of Association}
\]

\[
\text{k_s = Rate of Disassociation}
\]

The complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antigen. This interaction is illustrated below:

\[
\text{h-Ab(X-TPO)-BtnAg(TPO) + Streptavidin = Immobilized complex (IC)}
\]

\[
\text{Streptavidin immobilized on well}
\]

\[
\text{Immunocomplex (IC) = sandwich complex bound to the solid surface}
\]

After the incubation time, the well is washed to separate the unbound components by aspiration and/or decantation. The enzyme-linked conjugates (anti-h-igG) is then added to the micro wells. This conjugate binds to the immune complex that formed.

\[
\text{IC. \text{h-Ab(X-TPO)-BtnAg(TPO)} + \text{Enzyme-antibody Conjugate} (Constant Quantity)}
\]

\[
\text{Ag-Ab Complex (Variable Quantity)}
\]

The anti-higG enzyme conjugate that binds to the immune complex in a secondary incubation is separated from unreacted material by a wash step. The enzyme activity in this fraction is directly proportional to the amount of antibody-complex formed in the specimen. By utilizing several different serum references of known antibody activity, a reference curve can be generated from which the antibody activity of an unknown can be ascertained.

4.0 REAGENTS

Materials Provided:

- A. TMB Color Reagent – 1.0 ml/vial - Icons A-F
- Six (6) vials of references for anti-TPO at levels of 0(A), 25(B), 50(C), 100(D), 200(E) and 500(F) IU/ml. Store at 2-8°C. A presumptive test result is negative.
- B. TPO Biotin Reagent – 13ml/vial - Icons V

One (1) vial of biotinylated thyroid peroxidase antigen stabilized in a buffering matrix. A preservative has been added. Store at 2-8°C.

- C. Anti-TPO Enzyme Reagent – 13ml/vial - Icons E

One (1) vial of anti-human IgG-horseradish peroxidase (HRP) conjugate stabilized in a buffering matrix. A preservative has been added. Store at 2-8°C.

- D. Streptavidin Coated Plate – 96 wells – Icons U

96-well microwell plates with streptavidin immobilized and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

- E. Serum Diluent – 20ml

One (1) vial of serum diluent concentrate containing buffer and preservatives. Use as prepared.

- F. Wash Solution Concentrate – 20ml - Icons K

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

- G. Substrate A – 7ml/vial - Icons S

One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

- H. Substrate B – 7ml/vial - Icons S

One (1) bottle containing hydrogen peroxide (H2O2) in buffer. Store at 2-8°C.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

The specimens shall be blood; serum or plasma in type and the usual precautions in the collection of venipuncture samples shall be followed. Avoid contamination of specimens with anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assessed within this time, the collected sample(s) may be stored at temperatures of -20°C for up to 18 months. Avoid contaminating devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.05ml of the specimen is required.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood; serum or plasma in type and the usual precautions in the collection of venipuncture samples shall be followed. Normal values, a fasting morning serum sample should be obtained. The blood should be placed in a clean red top venipuncture tube with sodium citrate or ethylenediamine tetra-acetic acid (EDTA) and heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the normal, borderline and elevated range for monitoring assay performance. The repeated testing of controls and unknowns as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate uncontrolled change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION

1. Serum Diluent

Dilute the serum diluent to 200ml in a suitable container with distilled or deionized water. Store at 2-8°C.

2. Wash Buffer

Dilute contents of wash concentrate to 100ml with distilled or deionized water in a suitable storage container. Store at 2-30°C for up to 60 days.

3. Working Substrate Solution

Pour the contents of the vial into a beaker containing 1ml of distilled water. Cover the beaker and mix thoroughly by inversion. Store at 2-8°C for up to forty-eight (48) hours.

Note 1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20-27°C). All tests should be performed by a skilled individual or trained professional!

1. Format the microwells’ plates for each serum reference, control and patient specimen to be assayed in duplicate. Place any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2. Pipette 0.025 ml (25µl) of the appropriate serum reference, control specimen or diluted patient specimen into the assigned well.

3. Add 0.100 ml (100µl) of the TPO Biotin Reagent.

4. Swirl the microwell gently for 20-30 seconds to mix and disperse the contents.

5. Incubate 60 minutes at room temperature.

6. Discard the contents of the microwell by decanting or aspirating, then dry the plate by washing. Discard the wash and repeat two (2) additional times.

7. Add 0.100 ml (100µl) of the x-TPO Enzyme Reagent All wells simultaneously.

8. Add 0.100 ml (100µl) of the x-TPO Enzyme Reagent All wells simultaneously.

9. Incubate for thirty (30) minutes at room temperature. Carefully separate the microwell plates and explain above.

10. Add 1.00 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). Decant (blot and tap) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. The manufacturer’s instruction should be followed for proper usage. If a squeeze bottle is employed, fill each well with depressing the container (avoiding air bubbles) to displace the wash. Decant the wash and repeat two (2) additional times.

11. Add 0.10 ml (100µl) of the x-TPO Enzyme Reagent All wells simultaneously.

12. Incubate at room temperature for fifteen (15) minutes.

13. Add 0.050ml (50µl) of step solution to each well and mix gently for 15-20 seconds. Add wash reagents in the same order to minimize reaction time differences between wells.

14. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm) to minimize well interference and a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

Note: For re-assaying specimens with concentrations greater than 500 IU/ml, dilute the sample an additional 1:5 or 1:10 using the original diluted material. Multiply by the dilution factor to obtain the concentration of the specimen.
10.0 CALCULATION OF RESULTS
A reference curve is used to ascertain the concentration of anti-TPO in unknown specimens.
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding anti-TPO activity in IU/ml on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the level of anti-TPO activity for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in IU/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.323) intersects the dose response curve at 200 IU/ml anti-TPO concentration (See Figure 1).

Note: Computer data reduction software designed for ELISA assays may also be useful for the data reduction. If such software is utilized, the validation of the software should be ascertained.

11.0 Q.C. PARAMETERS
In order for the assay results to be considered valid the following criteria should be met:
1. The absorbance (OD) of calibrator F should be > 1.3.
2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS
The MSDS and Risk Analysis Form for this product are available on request from Monobind Inc.

12.1 Essay Performance
1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated samples should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the assay.
5. The addition of substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Very high concentration of anti-TPO in patient specimens can contaminate samples immediately following these extreme levels. Bad duplicates are indicative of cross contamination. Repeat any sample, which follows any patient specimen with over 3.0 units of absorbance.
10. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind’s IFU may yield inaccurate results.
11. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
12. It is important to calibrate all the equipment e.g. Pipettes, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.
13. Risk Analysis - as required by CE Mark IVD Directive 98/79/EC should not be used in lieu of a standard curve prepared with each assay.

14.0 PERFORMANCE CHARACTERISTICS
14.1 Precision
The within and between assay precisions of the anti-TPO AccuBind™ ELISA test system were determined by analyses on three different levels of pool control sera. The number (N), mean value (X), standard deviation (σ) and coefficient of variation (C.V) for each of these control sera are presented in Table 2 and Table 3.

14.2 Sensitivity
The anti-TPO AccuBind™ ELISA test system has a sensitivity of 0.92 IU/ml. The sensitivity (detection limit) was ascertained by determining the variability of the ‘0 IU/ml’ calibrator and using the 2σ rule.

14.3 Accuracy
The anti-TPO AccuBind™ ELISA test system was compared with a reference anti-TPO ELISA microplate. Biological specimens from normal and disease states populations were used. The disease states included; Hashimoto’s thyroiditis, Graves Disease, thyroid nodules as well as thyroid carcinoma. The total number of such cases is 250.

14.4 Specificity
Interferences from ANA, DNA, thyroglobulin (TPO) and rheumatoid antibodies were found to be insignificant.

15.0 REFERENCES

Revision: 3 Date: 060712 DCO: 0639
Cat #: 1125-300
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Table 1. In excess of 40IU/ml are considered positive for the presence of anti-TPO autoantibodies.

<table>
<thead>
<tr>
<th>Number</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Upper 95% (+2σ) level</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>17.8</td>
<td>10.8</td>
<td>39.2</td>
</tr>
</tbody>
</table>

Table 2. Values in excess of 40IU/ml are considered positive for the presence of anti-TPO autoantibodies.

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td>20</td>
<td>25.5</td>
<td>1.5</td>
<td>5.9%</td>
</tr>
<tr>
<td>Pool 2</td>
<td>20</td>
<td>120.5</td>
<td>4.6</td>
<td>3.8%</td>
</tr>
<tr>
<td>Pool 3</td>
<td>20</td>
<td>352.4</td>
<td>14.8</td>
<td>4.2%</td>
</tr>
</tbody>
</table>

Table 3. Between Assay Precision (Values in IU/ml)

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
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<td>Pool 1</td>
<td>10</td>
<td>28.5</td>
<td>1.8</td>
<td>6.8%</td>
</tr>
<tr>
<td>Pool 2</td>
<td>20</td>
<td>118.5</td>
<td>5.3</td>
<td>4.5%</td>
</tr>
<tr>
<td>Pool 3</td>
<td>10</td>
<td>385.4</td>
<td>22.5</td>
<td>6.2%</td>
</tr>
</tbody>
</table>

Table 4. The data presented in Example 1 and Figure 2 are for illustration only and should not be used in lieu of a standard curve prepared with each assay.

Figure 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
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Table 1


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