The employment of several serum references of known antibody activity permits construction of a dose response curve (graph) of enzyme and antibody activities. From comparison to the dose response curve, an unknown specimen's enzyme activity can be correlated with auto-antibody immune level. The intensity of light is directly proportional to the concentration of anti-thyroglobulin antibody in the specimen.

3.0 PRINCIPLE

A Sandwich Sequential CLIA Method (Type 1)
The reagents required for this quantitative CLIA assay include immobilized antigen, circulating anti-antibody 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 streptavidin coated on the well and exogenously added biotinylated thyroglobulin antigen.

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

$$ h-Ab(X-Tg) + BtnAg(Tg) $$

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

$$ h-Ab(X-Tg) + BtnAg(Tg) + I.C. + h-IgG $$

After washing, the enzyme label is added directly to the microwells (variable quantity). The enzyme activity, determined by reaction with a substrate that generates light, in this fraction is directly proportional to the antibody concentration 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. Anti-Thyroglobulin Calibrators – 0.1ml (mIcu) Ions A-F
   (50 µg/ml (1000, 500, 250), 25 µg/ml (500)), 12.5 µg/ml (250), 5 µg/ml (500), 0.5 µg/ml (500)) IUmL. Store at 2-8°C.
   A preservative has been added.

B. Human Thyroglobulin Biotin Conjugate – 10 µml Ions (1) IUmL
   (1) Vial containing lyophilized thyroglobulin in a buffered matrix. A preservative has been added. Store at 2-8°C.

C. Anti-Tg Reagen Tracer – 13 µml Ions (1) IUmL
   (1) Vial containing lyophilized human IgG-horseradish peroxidase (HRP) conjugate in a buffered matrix. A preservative has been added. Store at 2-8°C.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

All Monobind products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA required tests. Since no known test can offer 100% assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous materials.

Good laboratory procedures for handling blood products can be found in the "Center for Disease Control / National Institute of Health, "Biohazard in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

Safe disposal of kit components must be according to local regulatory and statutory requirements.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or plasma in tye and the usual precautions in the collection of venipuncture samples should be observed. A microwell plate is prepared by established normal values, a fasting morning serum sample should be obtained. The blood sample is collected in a plain red top vacutainer tube without additives or anticoagulants. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of 72 hours. If serum(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid continuous freeze-thaw cycles. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100 ml of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, medium and high range for monitoring assay performance. These controls should be treated 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 measures should be employed to ascertain trends. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reasons for the variation.

8.0 REAGENT PREPARATION

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

B. Signal Reagen B
   Dilutes of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Buffer can be stored at 2-30°C for up to 60 days.

C. Working Signal Reagen D - Store at 2 - 8°C. Determine the amount of reagent needed and prepare by mixing reagent A (100µl) with Signal Reagen B in a clean container. For example, add 1 ml of A and 1ml of B per two 0.01µl strips (A slight excess of solution is made to ensure sufficient reagents to cover unneeded use if not used within 36 hours after mixing). If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagen B into Signal Reagen D and label accordingly.

D. Sample Dilution (1/100)
   Dispense 0.100ml (10µl) of each patient specimen into 1ml of serum diluent. Cover and mix thoroughly by inverting the container 50 times.

Note: 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).

"Test procedure should be performed by a skilled individual or a trained professional"

1. Format the microplates' wells for each serum reference, control, and patient specimen to be assayed. Replace any unused microwell strip back into the aluminum bag, seal and store at 2-8°C.

2. Pipette 0.050 ml (50µl) of the appropriate serum reference, control or diluted patient specimen into the assigned well.

3. Add 350µl of wash buffer (see Reagent Preparation Section) to each well. Mix gently to wash the plate. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Discard the was.

4. Discard the contents of the microwell by decantation or aspiration. Repeat this process (2 x). If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Discard the wash.

5. Incubate 30 minutes at room temperature.

6. Discard the contents of the microplate by decantation or aspiration. Replace it with 0.100 ml (10µl) of the appropriate serum reference, control or diluted patient specimen into the assigned well.

7. Discard the contents of the microplate by decantation or aspiration. Replace it with 0.100 ml (10µl) of the appropriate serum reference, control or diluted patient specimen into the assigned well.

8. Add 0.100 ml (100µl) of anti-Tg Reagent to all wells. Always add reagents in the same order to minimize occurrence of differences between wells.

9. Incubate the microplate at room temperature. DO NOT SHAKE THE PLATE AFTER ENZYME ADDITION

10. Discard the contents of the microplate by decantation or aspiration. Repeat this process (2 x). If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Discard the wash.

11. Add 0.100 ml (100µl) of working signal reagent to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.

12. Incubate at room temperature for five (5) minutes in the dark.

13. Read the RLU’s (Relative Light Units) in each well in a microlampmeter for at least 0.2 seconds.

The Intended Use: The Quantitative Determination of Thyroglobulin (Tg) Autoantibodies in Human Serum or Plasma by a Microplate Chemiluminescence Immunoassay (CLIA). Measurements of Tg 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 thyroglobulin have been shown to be characteristically present in patients with thyroiditis and primary thyroidositis. Antibody activity in the diagnosis of thyroid dysfunction. Passive Hemagglutination (PHA) methods have been employed for the past for measurements of antibodies to Tg. PHA tests do not have the sensitivity of enzyme immunoassay and are limited by subjective interpretation. The procedure, with the enhanced sensitivity of chemiluminescence, permits the detectability of subclinical levels of antibodies to Tg. In addition, the results are quantitated by a luminometer, which eliminates subjective interpretation.

Autoantibodies to Tg are often present in patients with autoimmune thyroid disease. Approximately 10 percent of healthy individuals have autoantibodies to Tg at low levels. Serum concentrations are found in 30% and 85% of patients with Graves' disease and Hashimoto's Thyroiditis respectively. Antibodies to thyroglobulin (Tg) occur more frequently than autoantibodies to Tg in these conditions thus rendering anti-Tg assays without any practical use. However, anti-Tg assays are useful while determining Tg levels in patients with thyroid conditions. The presence of Tg autoantibodies produces false results in determination of Tg levels both by competitive assays and by sandwich immunoassays.

Monobind's microplate chemiluminescence 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 microplate well. Biotinylated thyroglobulin (Tg) is then added, then the reactants are mixed. Reaction results between the auto-antibodies to Tg and the biotinylated thyroglobulin, a microwell coated with a streptavidin protein 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 anti-human IgG-enzyme conjugate is then added to permit quantitation of reaction through interaction with human IgG of the immune complex. After washing, the enzyme activity is determined by reaction with substrate to produce light (luminescence).
results can be read within 30 minutes of adding the substrate solution.  

**Note:** For re-assaying specimens with concentrations greater than 2000 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-Tg (x-Tg) in unknown specimens.

1. Record the RLU’s obtained from the printout of the microplate luminometer as outlined in Example 1.
2. Plot the mean RLU’s for each duplicate serum reference versus the corresponding x-Tg activity in IU/ml on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of x-Tg for an unknown, locate the average RLU’s for each unknown on the vertical axis of the graph, 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 RLU’s of the unknown intersects the calibration curve at 1403 IU/ml x-Tg concentration (See Figure 1).*

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

### 11.0 QC. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The Dose Response Curve should be within established parameters.
2. Plot 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 is available on request from Monobind Inc.

#### 12.1 Assay 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 hemolyzed, hemoglobin or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of signal reagent initiates a kinetic reaction, therefore the signal reagent(s) should be added in the same sequence to eliminate any time-deviation during reaction.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.

#### 12.2 Interpretation

1. Measurements and interpretation of results must be performed by a skilled individual or trained professional.
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. For valid test results, replicate controls and other parameters must be within the listed ranges and assay requirements.
4. If tests kits are altered, such as using different lot numbers, different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
5. If control data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
6. The presence of autoantibodies to Tg is confirmed when the serum level exceeds 125 IU/ml. The clinical significance of the result, coupled with anti-thyroid peroxidase activity, should be used in evaluating the thyroid condition. However, clinical inferences should not be solely based on this test but rather as an adjunct to the clinical manifestations of the patient and other relevant tests.
7. The cost benefits should be considered in the use of thyroglobulin antibodies testing when performed in concert with anti-thyroid peroxidase (TPO). The widespread practice of performing both tests has been questioned.

### 13.0 EXPECTED RANGES OF VALUES

A study of normal population was undertaken to determine expected values for the Anti-Tg AccuLite™ CLIA procedure. The number (n), mean (x) and standard deviation (σ) are given in Table 1. Values in excess of 125 IU/ml are considered positive for the presence of anti-Tg autoantibodies.

#### Table 1

<table>
<thead>
<tr>
<th>Number (n)</th>
<th>Mean (x)</th>
<th>Standard deviation (σ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>74.3</td>
<td>25.2</td>
</tr>
<tr>
<td>Upper 95% (±2σ) level</td>
<td>124.7</td>
<td></td>
</tr>
</tbody>
</table>

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of “normal”-persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

### 14.0 PERFORMANCE CHARACTERISTICS

#### 14.1 Precision

The within and between assay precision of the Anti-Tg AccuLite™ CLIA method were determined by analyses on three different levels of pool control sera. The number, mean, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.*

#### Table 2

<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>63.3</td>
<td>3.3</td>
<td>5.2%</td>
</tr>
<tr>
<td>Pool 2</td>
<td>20</td>
<td>224.3</td>
<td>14.5</td>
<td>6.5%</td>
</tr>
<tr>
<td>Pool 3</td>
<td>20</td>
<td>1498.1</td>
<td>67.4</td>
<td>4.5%</td>
</tr>
</tbody>
</table>

#### Table 3

<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>10</td>
<td>67.0</td>
<td>5.7</td>
<td>8.5%</td>
</tr>
<tr>
<td>Pool 2</td>
<td>10</td>
<td>237.6</td>
<td>18.5</td>
<td>7.8%</td>
</tr>
<tr>
<td>Pool 3</td>
<td>10</td>
<td>1518.3</td>
<td>78.4</td>
<td>5.2%</td>
</tr>
</tbody>
</table>

*As measured in ten experiments in duplicate.

#### 14.2 Sensitivity

This procedure has a sensitivity of 1.95 IU/ml. The sensitivity was ascertained by determining the efficiency of the ‘0 IU/ml’ calibrator and using the 2σ (95% certainty) statistics to calculate the minimum dose.

#### 14.3 Accuracy

The Monobind x-Tg AccuLite™ CLIA was compared with a reference microparticle anti-Tg ELISA. Biologic specimens from normals, 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 specimens was 122. The least square regression equation and correlation coefficient were computed for this assay in comparison with the reference method. The data obtained is displayed in Table 4.

#### Table 4

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean</th>
<th>Least Square Regression</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>This Method</td>
<td>387.6</td>
<td>y = -11.2 + 0.989 (x)</td>
<td>0.992</td>
</tr>
<tr>
<td>Reference</td>
<td>404.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Only slight amounts of bias between the Anti-Tg AccuLite™ CLIA procedure and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

#### 14.4 Specificity

Interferences from ANA, DNA, thyroid peroxidase (TPO) and monomol antibodies were found to be insignificant in the assay system.

### 15.0 REFERENCES


Revision: 3 Date: 060712 DCO: 0653