Triiodothyronine (T3) Procedure

**Intended Use:** The Quantitative Determination of Total Triiodothyronine Concentration in Human Serum or Plasma by a Microparticle Chemiluminescence Immunoassay (CLIA)

**SUMMARY AND EXPLANATION OF THE TEST**

Measurement of serum triiodothyronine concentration is generally regarded as a valuable tool in the diagnosis of thyroid dysfunction. This information has provided the impetus for the significant improvement in assay methodology that has occurred in the last two decades. The advent of monoclonal antisera and the discovery of blocking agents to the T3 binding serum proteins have enabled the development of a broadly simple radioimmunoassay (RIA).

This microparticle enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, serum reference, patient specimen, or control is first added to a microparticle well. Enzyme-T3 conjugate is added, and then the reactants are mixed. A competition reaction results between the enzyme conjugate and the native triiodothyronine for a limited number of antibody combining sites immobilized on the well.

After the completion of the required incubation period, the antibody bound T3-enzyme conjugate is separated from the unbound T3-enzyme complex by centrifugation or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce a chemiluminescent signal.

The employment of several serum references of known triiodothyronine concentration permits construction of a graph of activity and concentration. From comparison to the dose response curve, an unknown specimen’s activity can be correlated with T3 concentration.

**PRINCIPLE**

Competitive Chemiluminescence Immunoassay

The essential reagents required for a solid phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen. Upon mixing immobilized antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate. A limited number of immobilized binding sites. The interaction is illustrated by the following equation:

$$k_a = \frac{\text{Monospecific Immobilized Antibody (Constant Quantity)}}{\text{Native Antigen (Variable Quantity)}}$$

$$K = \frac{k_a}{k_a} = \text{Rate Constant of Association}$$

$$K = \frac{k}{k_a} = \text{Equilibrium Constant}$$

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity, determined by reaction with a substrate that generates light, in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

**REAGENTS**

**Materials Provided:**

A. Human Serum References – 1ml/vial - Icons A-F
   - Six (6) vials of serum reference for triiodothyronine at concentrations of 0 (A), 0.5 (B), 1.0 (C), 2.5 (D), 5.0 (E) and 7.5 (F) ng/ml. Store at 2-8°C. A preservative has been added.
   - Store at 2-8°C.

B. Total T3-Tracer – 1.5ml/vial - Icon
   - One (1) vial containing triiodothyronine (HTP) conjugates in an albumin-stabilizing matrix.
   - A preservative has been added.
   - Store at 2-8°C.

C. Total T3/T4 Tracer Buffer – 13ml - Icon
   - One (1) bottle containing buffer, red, preservative, and binding protein inhibitors.
   - Store at 2-8°C.

D. T3 Light Reaction Wells – 96 wells - Icon
   - One (1) vial containing coated with sheep anti-T3 antibody and packaged in a dry bag with a drying agent.
   - Store at 2-8°C.

E. Wash Solution Concentrate – 20ml - Icon
   - One (1) vial containing Luminol in buffer and a preservative.
   - A preservative has been added.
   - Store at 2-30°C.

F. Signal Reagent A – 7ml/vial - Icon 5µl
   - One (1) bottle containing hydrogen peroxide (H2O2) in buffer.
   - Store at 2-8°C.

G. Signal Reagent B – 7ml/vial - Icon 5µl
   - One (1) bottle containing hydrogen peroxide (H2O2) in buffer.
   - Store at 2-8°C.

H. Product Insert.

**PRECAUTIONS**

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

All 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 human sera are involved, no testing can offer complete assurance that infectious agents are absent, all human sera used in production should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, “Biosafety in Microbiological and Biomedical Laboratories,” 2nd Edition, 1988, HHS Publication No. (CDC) 88-8535.

**SPECIMEN COLLECTION AND PREPARATION**

Collec samples by venipuncture in ten (10) ml silicone evacuated tube(s) or evacuated tube(s) containing EDTA or heparin. The usual precautions in the collection of venipuncture samples should be observed. Separate the red blood cells by centrifugation use serum or plasma for the total T4 procedure. Specimen(s) may be refrigerated at 2-8°C for a maximum period of 48 hours. If not to be assayed within 48 hours, the sample(s) may be stored at temperatures of -20°C for several days. Before assay, allow the specimens to equilibrate to ambient temperature 20°C – 27°C. When assayed in duplicate, 0.100 ml of the specimen is required.

**Materials ([Required But Not Provided]:**

1. Pipette capable of delivering 50µl with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100µl and 0.350µl volumes with a precision of better than 1.5%.
3. Adjustable volume (300-500µl) and (200-1000µl) dispenser(s) for conjugate and substrate dilutions.
4. Microplate washers or a squeeze bottle (optional).
5. Microplate Luminometer.
6. Test tubes for dilution of enzyme conjugate and substrate A and B.
7. Absorbent Paper for blotting the microplate wells.
8. Plastic wrap or microplate cover for incubation steps.
9. Vacuum aspirator (optional) for wash steps.
10. Timer.
11. Quality control materials.

**REAGENT PREPARATION**

1. **Working Tracer - T3-enzyme Conjugate Solution**
   Dilute the T3-Tracer 1:11 with Total T3/T4 Tracer buffer in a clean container. For example, dilute 160µl of conjugate with 1.6ml of buffer to make 16 wells (A slight excess of solution is made). This reagent should be used within twenty-four hours for maximum performance of the assay. Store at 2-8°C.
   - General Formula:
     - Amount of Buffer required = Number of wells + 0.1 Volume of T3-enzyme necessary = # of wells *0.1µl + 0.1ml (160µl) for T3 enzyme conjugate

2. **Wash Buffer**
   Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at room temperature 20-27°C.

3. **Working Signal Reagent Solution**
   - Store at 2-8°C.
   - Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). Discard the unused portion if not used within 24 hours after mixing. If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

**TEST PROCEDURE**

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C).

1. Format the microplates’ wells for each serum reference, control and patient specimen to be assayed in duplicate.
2. **Note:** Not for use with the laboratory’s equipment/software to measure and interpret chemiluminescence data.
3. **Note:** In the case of the average RLU’s (63817) of the unknown intersects the calibration curve at (1.4 ng/ml) T3 concentration (See Figure 1).

**QUALITY CONTROL**

Each laboratory should assay external controls at levels in the hypothyroid, euthyroid and hyperthyroid range for monitoring assay performance. These controls should be treated as unknowns, therefore, do not use buffer (water) for each test procedure performed. Quality control charts should be maintained to follow the trend line of results with each lot of reagents. Statistic statements and control charts provide a system for comparison of test performance. 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 reason for the variations.

**RESULTS**

A dose response curve is used to ascertain the concentration of triiodothyronine in unknown specimens.

1. Record the RLU’s obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the RLU’s for each duplicate serum reference versus the corresponding T3 concentration in ng/ml on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of T3 for an unknown, locate the average RLU’s for each unknown on the vertical axis of the graph, find the corresponding point on the curve, and read the concentration in ng/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 (63817) of the unknown intersects the calibration curve at (1.4 ng/ml) T3 concentration (See Figure 1).

**Note:** Computer data reduction software designed for chemiluminescence assays may also be used for the data reduction. Duplicates of the unknown may be averaged as indicated (See Figure 1).

**Note:** Microplates are made to be used in the purchase and implementation of equipment/software to measure and interpret chemiluminescence data.
**Q.C. 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. Four out of six quality control pools should be within the established ranges.

**LIMITATIONS OF PROCEDURE**

**A. Assay Performance**

1. It is important that the time of reaction in each well is held constant for reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
4. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
5. Use components from the same lot. No intermixing of reagents from different batches.
6. Multichannel pipettes are recommended for addition of reagents.
7. Sample(s), which are contaminated microbiologically, should not be used in the assay. Highly lipemic or hemolyzed specimen(s) should not be used.

**B. Interpretation**

1. If computer controlled 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.
2. Total serum triiodothyronine concentration is dependent upon a multiplicity of factors: thyroid gland function and its regulation, thymus binding globulin (TBG) concentration, and the binding of triiodothyronine to TBG (3, 4). Thus, total triiodothyronine concentration alone is not sufficient to assess clinical status.
3. A decrease in total triiodothyronine values is found with protein-wasting diseases, certain liver diseases and administration of testosterone, diphénylhydantoin or salicylates. A table of interfering drugs and conditions, which affect total triiodothyronine values, has been compiled by the Journal of the American Association of Clinical Chemists.

**EXPECTED RANGES OF VALUES**

A study of euthyroid adult population was undertaken to determine expected values for T3 AccuLite™ CLIA method. The mean (R) values standard deviations (σ) and expected ranges (±2σ) are presented in Table 1. The total number of samples was 85.

**TABLE I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (X)</th>
<th>Standard Deviation (σ)</th>
<th>Expected Ranges (±2σ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl 1</td>
<td>1.22</td>
<td>0.35</td>
<td>0.52 – 1.98</td>
</tr>
<tr>
<td>Ctrl 2</td>
<td>1.0</td>
<td>2.2</td>
<td>1.0 – 1.6</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 analyst using the method with a population indigenous to the area in which the laboratory is located.

**PERFORMANCE CHARACTERISTICS**

**A. Precision**

The within and between assay precision of the T3 AccuLite™ CLIA assay were determined by analyses on three different levels of pool control sera. The number, mean value, standard deviation (σ) 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>Mean (X)</th>
<th>σ</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0.85</td>
<td>0.058</td>
<td>6.8%</td>
</tr>
<tr>
<td>Normal</td>
<td>2.25</td>
<td>0.123</td>
<td>5.5%</td>
</tr>
<tr>
<td>High</td>
<td>3.20</td>
<td>0.134</td>
<td>4.2%</td>
</tr>
</tbody>
</table>

**TABLE 3**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (X)</th>
<th>σ</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0.81</td>
<td>0.068</td>
<td>8.4%</td>
</tr>
<tr>
<td>Normal</td>
<td>2.19</td>
<td>0.145</td>
<td>6.6%</td>
</tr>
<tr>
<td>High</td>
<td>3.32</td>
<td>0.176</td>
<td>5.3%</td>
</tr>
</tbody>
</table>

*As measured in ten experiments in duplicate over a ten day period.

**B. Accuracy**

The T3 AccuLite™ CLIA test was compared with a reference enzyme immunoassay method. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (The values ranged from 0.010ng/ml – 7.30ng/ml). The total number of such specimens was 110. The least square regression equation and the correlation coefficient were computed for the T3 AccuLite™ CLIA in comparison with the reference method. The data obtained is displayed in Table 4.

**D. Sensitivity**

The Triiodothyronine test system procedure has a sensitivity of 0.04 ng/ml. The sensitivity was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

**REFERENCES**

4. Sterling L, Diagnosis and Treatment of Thyroid Disease, Cleveland, CRC Press, 9, 51 (1975).