**4.0 REAGENTS**

**Materials Provided:**
A. Human Serum References – 1ml/vial - Icons A-F
B. Total T3/T4 Tracer Buffer – 13ml - Icon
C. Total Light Reaction Wells – 96 wells - Icon
D. Wash Concentrate – 20ml - Icon
E. Signal Reagent A – 1ml - Icon
F. Signal Reagent B – 1ml - Icon
G. T3 Light Reaction Wells – 96 wells - Icon

**2.0 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 importance has provided the impetus for the significant improvement in the triiodothyronine test, which has occurred in the last two decades. The advent of monoclonal antisera and the discovery of blocking agents to the T3 binding serum proteins and their corresponding development has enabled the development of procedurally simple radioimmunoassay (1,2).

This microtiter 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 microtiter well. Enzyme-T3 conjugate is added, and then the reagents are mixed. A competition 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 conjugate by decantation or aspiration. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce light.

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.

**3.0 PRINCIPLE**

**Competitive Chemiluminescence Immunoassay (Type 5)**

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 for a limited number of immobilized binding sites. The interaction is illustrated by the following equation:

\[
\text{AbC}_{\text{W}} + \text{Monospecific Immobilized Antibody (Constant Quantity)} \rightarrow \text{AbC}_{\text{W}} \cdot \text{Ab}
\]

\[
\text{Ag} + \text{Native Antigen (Variable Quantity)} \rightarrow \text{Ag} \cdot \text{Ab}
\]

\[
\text{EnzAg} \cdot \text{Antigen-Antibody Complex} \rightarrow \text{EnzAg} \cdot \text{AbC}_{\text{W}}
\]

\[
k = \frac{k_{\text{a}} \cdot k_{-\text{a}}}{k_{\text{a}}}
\]

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.

**5.0 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 known test can offer complete assurance that infectious agents are absent, all human serum reagents 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 and Prevention’s “Guide for the Care and Use of Laboratory Animals,” 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

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

**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 should be observed. For comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain red-top Vacutainer tube without additive (anticoagulants 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 from plasma prior to the test.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml of the specimen is required.

**7.0 QUALITY CONTROL**

Each laboratory should assay external controls at levels in the hypothyroid, euthyroid and hyperthyroid range for triiodothyronine. The external control must be from a different source than that of the reagents. The external control should be assayed at the same time, on the same instrument, with the same diluent, and with the same batch as the unknown sera. Each laboratory shall assay its own controls.

**8.0 REAGENT PREPARATION**

1. **Working Tracer - T3-enzyme Conjugate Solution**

   Dilute 1ml of T3-enzyme conjugate solution to 10ml in a clean container. For example, dilute 160µl of conjugate with 1.6ml of buffer for 16 wells (A slight excess of solution is anticipated). This reagent should be used within twenty-four hours for maximum performance of the assay. Store at 2-8°C.

   General Information:
   - Amount of Buffer required = Number of wells * 0.1
   - Quantity of T3-Enzyme necessary = # of wells * 0.01
   - i.e. 16 x 0.1 = 1.6ml for Total T3/T4 Conjugate Buffer

2. **Wash Buffer**

   Dilute contents of Wash Concentrate to 100ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml of the specimen is required.

3. **Working Signal 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 1ml 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 36 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.

   **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 ± 2°C).**

1. **T3 Microtiter Plate Preparation**

   - 1. Pipette the microtiter’s wells for each serum reference, control and patient specimen to be assayed in duplicate. Reagent and wash steps are blocked with non-fat dry milk strips back into the aluminum bag, seal and store at 2-8°C.
   - 2. Pipette 0.050 ml (50µl) of the appropriate serum reference, control and patient specimen into the appropriate wells.
   - 3. Add 0.100 ml (100µl) of Working Tracer, T3-enzyme conjugate solution to all wells (see Reagent Preparation Section).
   - 4. Incubate the微型titer plate for 20-30 seconds to mix and cover.
   - 5. Incubate 45 minutes at room temperature.
   - 6. Discard the contents of the microtiter by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
   - 7. Add 350µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) and aspirate. Repeat four (4) additional times for a total of five (5) washes. An automatic or manual plate washer can be used. Follow the manufacturer’s instructions for proper operation. If a vacuum bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.
   - 8. Add 0.100 ml (100µl) of working signal reagent solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.
   - 9. Incubate for five (5) minutes in the dark at room temperature.
   - 10. Read the relative light units in each well, for minimum 0.5 – 1.0 seconds, using a microplate luminometer. The results should be read within thirty (30) minutes of adding the signal solution.

   **Note:** For re-assaying specimens with concentrations greater than 7.5 ng/ml, pipette 25µl of the specimen and 25µl of the 0 ng/ml standard solution into the sample well (this maintains a uniform protein concentration). Multiply the readout value by 2 to obtain the triiodothyronine concentration.

**10.0 CALCULATION OF 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 microtiter plate as shown in Figure 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 intersecting 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).**
5. **In the following example, the average RLU’s (63817) for 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. If such software is utilized, the validation of the software should be ascertained. Duplicates of the unknown may be averaged as indicated (See Figure 1).
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 aspirated test results, adequate controls and other parameters may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches should be used.
8. Patient specimens with T3 concentrations above 7.5 ng/mL may be diluted ½ with ‘0’ serum reference. The sample’s concentration is obtained by multiplying the result by the dilution factor, 2.
9. 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.
10. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance with device usage.
11. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventive maintenance.
12. Risk Analysis: as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 Interpretation
1. Measurements and interpretation of results must be performed by a skilled individual or trained professional.
2. Laboratory results alone are not the sole aspect for interpreting patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
5. 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.
6. Total serum triiodothyronine concentration is dependent upon a multiplicity of factors: thyroid gland function and its regulation, thyroxine 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.
7. A decrease in total triiodothyronine values is found with protein-wasting diseases, certain liver diseases and administration of testosterone, diphenylhydantoin 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‘.

13.0 EXPECTED RANGES OF VALUES

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

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross Reactivity</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-Triiodothyronine</td>
<td>1.0000</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>L-Thyroxine</td>
<td>0.0002</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>Iodothyronine</td>
<td>0.0001</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>Diiodothyronine</td>
<td>0.0001</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>0.00001</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>0.00001</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>Sodium Salicylate</td>
<td>0.0002</td>
<td>10µg/ml</td>
</tr>
</tbody>
</table>

5.1 REFERENCES