1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Total Triiodothyronine Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay

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 any methodology that has occurred in the last two decades. The advent of nonspecific antisera and the discovery of blocking reagents that bind to the T3 binding serum proteins have enabled the development of procedurally simple radioimmunoassays (1,2). This microplate immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, serum reference, patient specimen, control, and a preservative are added to microplate wells. Enzyme T3 conjugate is added, and then the reagents 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 incubation period, the antibody bound T3-enzyme conjugate is separated from the unbound T3 conjugate by aspiration or decantation. The activity of the enzyme is measured by its conversion of the unbound substrate (TMB) into a blue dye, the absorbance of which is measured at 620 nm.

3.0 PRINCIPLE

Competitive Enzyme Immunoassay (TYPE 5): The essential requirements for a competitive-type phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen.

Upon mixing immunoassay antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction occurs 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:

\[ \text{AgAbC.W.} + \text{EnzAgAbC.W.} \rightarrow \text{AgAbC.W. + EnzAgAbC.W.} \]

\[ k_a = \text{Rate Constant of Association} \]
\[ k_d = \text{Rate Constant of Dissociation} \]
\[ k = k_a = k_d = \text{Equilibrium Constant} \]

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. 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.

4.0 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. T3 Enzyme Reagent – 1.5ml/vial - Icon C

One (1) vial containing T3-horseradish peroxidase (HRP) conjugate in an albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.

C. T3/T4 Conjugate Buffer – 13ml - Icon D

One (1) bottle containing buffer, red dye, preservative, and binding protein inhibitors. Store at -20°C.

D. T3 Antibody Coated Plate – 96 wells - Icon E

One 96-well microplate coated with Sheep anti-T3 serum and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate – 20ml - Icon F

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

F. Substrate A – 7ml/vial - Icon G

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

G. Substrate B – 7ml/vial - Icon H

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

H. Stop Solution – 8ml/vial - Icon I

One (1) bottle containing stop solution containing a strong acid (1N HCL). Store at 2-30°C.

I. Product Instructions

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C. Opened control is stable for sixty (60) days when stored at 2-8°C. Kit and component stability is monitored by the detection of an internal standard.

Note 3: Above reagents are for a single 96-well microplate.

4.1 Materials Required But Not Provided:

1. Pipettes capable of delivering 50µl volumes with a precision of better than 1.5%.
2. Dispensers for repetitive deliveries of 0.100ml and 0.350ml volumes with a precision of better than 1.5%.
3. Adjustable volume (20-200µl) and (200-1000µl) dispensers (for antigen and conjugate and sub grade wash solution).
4. Microplate washers or a squeeze bottle (optional).
5. Microplate readers with 450nm and 620nm wavelength absorbance capability.
6. Test tubes for preparation of enzyme conjugate and sub grade washes.
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.

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 regulatory procedures. Good laboratory procedures for handling blood products can be found in the Center for Disease Control and Prevention, “Biosafety in Microbiological and Biomedical Laboratories,” 2nd Edition, 1988, HHS Publication No. (CDC) 88-8385.

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 accurate comparison to established normal values, a fasting serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anticoagulants (for serum). If the specimen cannot be assayed within two hours of 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 monitoring assay performance. These controls should be treated as test samples. For all test procedures performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to trends in control data. The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past assay performance. Significant deviation or poor performance can indicate uncontrolled change in experimental conditions or degradation of reagent(s). Few reagents should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION

1. Working Reagent A - T3-enzyme Conjugate Solution

Dilute the T3-enzyme conjugate 1:11 with T3/T4 conjugate buffer. For example, dilute 16.5ul of conjugate with 1.66ul of buffer for 16M (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.

2. Wash Buffer

Dilute contents of wash concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-8°C for up to 60 days.

3. Working Substrate Solution

Pour the contents of the vial labeled Solution ‘A’ into the clear, labeled vial labeled ‘B’. Place ETA-DTA in the clear vial to clear vial for easy identification. Mix and label accordingly. Store at 2-8°C.

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

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

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20-25°C).

**Test Procedure should be performed by a skilled individual or trained professional**

1. Format the microplates’ wells for each serum reference, control and patient specimen to be assayed in duplicate. Remember to label the plate with the sample(s) name.** If the specimen is not a whole blood, the microplate should be placed 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 specimen into the assigned well.

3. Add 0.100 ml (100µl) of Working Reagent A, T3 Enzyme Reagent to all wells (see Reagent Preparation Section).

4. Swirl the microplates gently for 20-30 seconds to mix and cover.

5. Incubate 60 minutes at room temperature.

6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

7. Add 350ul of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times.

8. Safe Disposal of kit components must be according to local regulatory and statutory requirement.

9. DO NOT SHARE THE PLATE AFTER SUBSTRATE ADDITION

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

10. Add 0.050ml (50µl) of each reagent into each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

11. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well to well variations) in a microplate reader.

Note: For re-assaying specimens with concentrations greater than 50 ng/ml, dilute (1:1) the serum reference 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. Receive each printed out the reagent packaging, along with the material safety data sheet and the microplate reader as outlined in Example 1.

2. Plot the absorbance for each duplicate serum reference versus the corresponding T3 concentration in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3. Draw the best-fit curve through the plotted points.

4. To determine the concentration of T3 for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis (y-axis) of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) on the horizontal axis (x-axis) of the graph (the duplicates of the unknown may be averaged as indicated). In the event that there are no duplicates, the average absorbance is used to intersect the dose response curve at 1.05g/ml T3 concentration (See Figure 1).

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

AgAbC.W. = Antigen-Antibody Complex
EnzAgAbC.W. = Enzyme-antigen Conjugate - Antibody Complex
\[ k_a = \text{Rate Constant of Association} \]
\[ k_d = \text{Rate Constant of Dissociation} \]
\[ k = k_a = k_d = \text{Equilibrium Constant} \]
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. Patient specimens with T3 concentrations above 7.5 ng/ml may be diluted with 0.0 serum reference. The sample’s concentration is obtained by multiplying the result by the dilution factor.

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, Readers, 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 - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The within and between assay precisions of the tT3 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.

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>Low</td>
<td>16</td>
<td>0.78</td>
<td>0.06</td>
<td>7.9%</td>
</tr>
<tr>
<td>Normal</td>
<td>16</td>
<td>1.92</td>
<td>0.10</td>
<td>5.4%</td>
</tr>
<tr>
<td>High</td>
<td>16</td>
<td>6.55</td>
<td>0.14</td>
<td>3.9%</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>Low</td>
<td>10</td>
<td>0.76</td>
<td>0.07</td>
<td>8.9%</td>
</tr>
<tr>
<td>Normal</td>
<td>10</td>
<td>1.85</td>
<td>0.13</td>
<td>6.7%</td>
</tr>
<tr>
<td>High</td>
<td>10</td>
<td>3.43</td>
<td>0.16</td>
<td>4.5%</td>
</tr>
</tbody>
</table>

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

14.2 Sensitivity

The tT3 AccuBind™ ELISA test system has a sensitivity of 0.04 ng/ml. The sensitivity was ascertained by determining the variability of the 0.0 ng/ml level and using the ln (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy

The tT3 AccuBind™ ELISA method was compared with a reference radioimmunoassay method. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (the values ranged from 0.15 ng/ml – 8.0 ng/ml). The total number of each specimen was 320. The least square regression equation for equation (y = mx+b) and the correlation coefficient computed for the tT3 AccuBind™ ELISA method in comparison with the reference method. The data obtained is displayed in Table 4.

14.4 Specificity

The cross-reactivity of the triiodothyronine antibody to selected substances was evaluated by adding the interfering substance to each serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of triiodothyronine needed to displace the same amount of conjugate.

15.0 REFERENCES
