Total Thyroxine (tT4) Test System
Product Code: 275-300

1.0 INTRODUCTION

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

2.0 SUMMARY AND EXPLANATION OF THE TEST

Measurement of serum thyroxine concentration is generally regarded as an integral component of a metabolic test for assessing thyroid function. This importance has provided the impetus for the significant improvement in assay methodology that has occurred in the last three decades. This procedural evolution has been traced from the empirical protein bound iodine (PBI) test (1) to the more sophisticated radioimmunoassay (2).

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

After the completion of the required incubation period, the antibody-bound enzyme-antibody conjugate is separated from the unbound antibody-antigen 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 reference sera of known thyroxine concentration and reagents designations of the activity of antibody and concentration. From comparison to the dose response curve, an unknown specimen’s activity can be correlated with thyroxine concentration.

3.0 PRINCIPLE

Competitive Enzyme Immunoassay (Type 5)
The essential reagents required for a solid phase enzyme immunoassay are: antibody, enzyme 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 insolubulized binding sites. The interaction is illustrated by the following equation:

\[
\text{Ag} + \text{Ag-AbC.W.} + \text{EnzAgAbC.W.} \rightarrow \text{Ag-AbC.W.} + \text{EnzAgAbC.W.}
\]

4.0 REAGENTS

Materials Provided:
A. Human Serum References – 1.0 ml/vial - Icon A
B. 10x Native Antigen vials – 10 ml - Icon B
C. 10x Antibody vials – 10 ml - Icon C
D. 10x Enzyme conjugate vials – 10 ml - Icon D

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 and HBV Antibodies by FDA test. Since no known test can offer complete assurance that infectious agents are absent, all human serum reagents should be treated as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control. "A Guide to State Laboratories, Diagnostic and Biomedical Laboratories," 2nd Edition, 1988, HHHS Publication No. (CDC) 88-8395.

Note: 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 (20-27 °C).

1. Format the microplates’ wells for each serum reference, control and patient specimen to be assayed in duplicate. Remove any unused microtiter 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 or specimen into the assigned well (see Reagent Preparation Section).
3. Swirl the microplate gently for 20-30 seconds to mix and cover.
4. Incubate at room temperature.
5. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
6. Add 350µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or 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 instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the bulb (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.
7. Add 0.100 ml (100µl) of working signal reagent solution to all wells (see Reagent Preparation Section). Add 0.100 ml (100µl) of working signal reagent solution to all wells (see Reagent Preparation Section). Decant (tap and blot) or 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 instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the bulb (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). Decant (tap and blot) or 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 instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the bulb (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.
9. Incubate at room temperature for five (5) minutes.
10. Read the relative light units in each well with a Chemiluminescence microplate reader for 0.5-1.0 seconds. The results should be read within 30 minutes after adding signal reagent.

Note: For reasaying specimens with concentrations greater than 25 µg/dl, pipet 12.5µl of the specimen and 12.5µl of the 0 serum reference into each well (this maintains a uniform protein concentration). Multiply the readout value by 2 to obtain the thyroxine concentration.

11.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of thyroxine in unknown specimens.
1. Record the RLU’s obtained from the printout of the microplate reader as outlined in Ex. 1.
2. Plot the RLU’s for each duplicate serum reference versus the corresponding T4 concentration in µg/dl on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of T4 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 T4 concentration 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 (49979) of the unknown intersects the calibration curve at (6.6µg/dl) T4 concentration. Multiply the readout value by 2 to obtain the thyroxine concentration.

Note 1: Computer data reduction software designed for chemiluminescence assays may also be used for the data reduction. If such software is utilized the software should be ascertained.
6. Failure to remove adhering solution adequately in the aspiration of T4 causing contamination or wash step(s) may result in poor replication and spurious results.
7. If reagents from the same lot are used, no intermixing of reagents from different batches.
8. 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.
9. Patient Specimens with T4 concentrations greater than 25 µg/dl can be diluted by pipetting 12.5 µl of the specimen and 12.5 µl of the zero reference into the sample well as this maintains a uniform precision of concentration. Multiply the readout value by 2 to obtain the thyroxine concentration.
10. 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.

11. It is important to calibrate all the equipment e.g. Pipetters, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative 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

13. EXPECTED RANGES OF VALUES

A study of euthyroid adult population was undertaken to determine expected values for the T4 AccuLite™ CLIA method. The mean (R) values, standard deviations (σ) and expected ranges (±2σ) are presented in Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross Reactivity</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>I-Thyroxine</td>
<td>1.0000</td>
<td>-</td>
</tr>
<tr>
<td>d-Thyroxine</td>
<td>0.9800</td>
<td>10µg/dl</td>
</tr>
<tr>
<td>d-Triiodothyronine</td>
<td>0.0150</td>
<td>100µg/dl</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>0.0300</td>
<td>100µg/dl</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>Diodothyronine</td>
<td>0.0001</td>
<td>10µg/ml</td>
</tr>
</tbody>
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Only slight amounts of bias between this method 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

The cross-reaction (Specificity) of the thyroxine antibody to selected substances was evaluated by adding the interfering substance to the Immunoassay in concentrations ranging from 10µg/dl to 100µg/dl. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of thyroxine needed to displace the same amount of tracer.

14.5 REFERENCES

4. Sterling, L., Diagnosis and Treatment of Thyroid Disease, Cleveland CRC Press P. 19-51. (1975)