SUMMARY AND EXPLANATION OF THE TEST
Measurement of serum thyroxine 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 assay methodology that has occurred in the past. The advent of monospecific antiserum and the discovery of blocking agents to the T4 binding serum proteins has enabled the development of procedurally simple radioimmunoassay (1,2).

This monospecific enzyme immunoassay methodology provides the technician with an increased capacity for accurate results while requiring few technical manipulations. In this method a sample (Calibrators, Controls and Patient Specimen) is added to the streptavidin coated microwells of a 96 well microtiter plate followed by Enzyme (HRP) labeled T4 analog and biotin labeled purified Anti-T4 specific antibody. The competition occurs between the varying amounts of T4 in the sample and fixed amount of T4-derivative for a fixed number of binding sites on the antibody.

After the completion of the required incubation period, the antibody bound T4-analog is separated from the unbound T4-enzyme conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known thyroxine 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 T4 concentration.

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate, native antigen and a substrate that produces color.

Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition results between the native antigen and the enzyme-antigen conjugate for a limited number of antibody binding sites. The interaction is illustrated by the following equation:

\[
\frac{E_A^*}{k_A} + \frac{A}{k_A} + \frac{Ag_{Biotin}}{k_D} \rightarrow \text{ES}
\]

Where:
- \( E_A^* \) = Enzyme-antigen Complex
- \( A \) = Native Antigen
- \( Ag_{Biotin} \) = Biotinylated antigen
- \( k_A \) = Rate Constant of Association
- \( k_D \) = Rate Constant of Dissociation

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effects the separation of the antibody bound fraction after decantation or aspiration.

AgAbBtn + EnzAgAbBtn \rightarrow \text{immobilized complex}

Streptavidin + Streptavidin immobilized on well

**Materials Required:**

- Human Serum References – 1ml/vial - Icons A-F
- One (1) vial of T4 Analog-horseradish peroxidase (HRP) conjugate in-a-stabilizing matrix. A preservative has been added. Store at 2-8°C.
- ctS/T4 Buffer – 13ml - Icon B
- One (1) bottle contains hydrogen peroxide (H₂O₂) in a preservative with a pH adjustment to 4.5. Store at 2-8°C.
- T4 Biotin Reagent – 7ml - Icon C
- One (1) vial of T4 Analog-horseradish peroxidase (HRP) conjugate in-a-stabilizing matrix. A preservative has been added. Store at 2-8°C.
- T4 Biotin Reagent – 7ml - Icon C
- One (1) vial contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
- Stop Solution – 8ml - Icon C
- One (1) bottle contains hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C.
- Wash Solution Concentrate – 20ml - Icon B
- One (1) vial contains buffer containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.
- Wash Buffer – 37ml - Icon C
- One (1) bottle contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
- Wash Concentrate – 5ml - Icon C
- One (1) bottle contains tetramethylbenzidine (TMB) in a preservative.
- Wash Buffer – 37ml - Icon C
- One (1) bottle contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
- Wash Concentrate – 5ml - Icon C
- One (1) bottle contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
- Wash Buffer – 37ml - Icon C
- One (1) bottle contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
- Wash Concentrate – 5ml - Icon C
- One (1) bottle contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
- Wash Buffer – 37ml - Icon C
- One (1) bottle contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
- Wash Concentrate – 5ml - Icon C
- One (1) bottle contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
- Wash Buffer – 37ml - Icon C
- One (1) bottle contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

**PRINCIPLE**

Competitive Enzyme Immunoassay (T4A) – Type 7

The competitive enzyme immunoassay procedure employs the principle of a reactant and conjugate reacting with a limited number of antibody binding sites. The competition occurs between the varying amounts of T4 in the sample and fixed amount of T4-derivative for a fixed number of binding sites on the antibody.

After the competition is complete, the antibody bound fraction is separated from the unbound T4-enzyme conjugate by aspiration or decantation. The antibody bound fraction is quantitated by a suitable substrate to produce color.

The employment of several serum references of known thyroxine 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 T4 concentration.

**REAGENT PREPARATION**

1. Working Enzyme Reagent (T4A):
   - Dilute the T4 Enzyme Reagent 1:1 with T3/T4 buffer in a suitable container. For example, dilute 80µl of conjugate with 0.8ml of buffer for 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:
     \[
     \text{Amount of Buffer required} = \text{Number of wells} \times 0.005
     \]
   - Example:
     - 3 wells = 0.015 ml
     - 8 wells = 0.040 ml
     - 16 wells = 0.080 ml
     - 32 wells = 0.160 ml
   - Wash Buffer:
     - Dilute contents of Wash Concentrate to 100ml with distilled or deionized water in a suitable storage container.
     - Store the diluted solution at room temperature (20-27°C) for up to 60 days.

**QUALITY CONTROL**

Each laboratory should assay controls at levels in the hypothyroid, euthyroid and hyperthyroid 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 methods should be employed to ascertain trends.
The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from expected absorbance values may indicate the need for reagents from different batches.

**RESULTS**

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

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding standard in ug/ml on linear graph paper (do not average the duplicates of the serum reference before plotting).
3. Connect the points with a best-fit curve (Figures 1).
4. To determine the concentration of T4 for an unknown, locate the absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the x-axis, and read the concentration (in ug/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the absorbance 1.407 (intersects the calibrator curve at 7.3 ug/ml [Figure 1]).

The data presented in Example 1 is illustrative only and should not be used in lieu of calibration curve prepared with each assay.

**EXAMPLE 1**

### Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mean (X)</th>
<th>Std Dev (σ)</th>
<th>Expected Ranges (±2σ)</th>
</tr>
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<tbody>
<tr>
<td>A1</td>
<td>2.451</td>
<td>2.419</td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td>2.387</td>
<td>1.839</td>
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</tr>
<tr>
<td>C1</td>
<td>1.222</td>
<td>1.221</td>
<td>5</td>
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<tr>
<td>D1</td>
<td>0.811</td>
<td>0.795</td>
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<tr>
<td>E1</td>
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<td>0.581</td>
<td>15</td>
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<tr>
<td>F1</td>
<td>0.444</td>
<td>0.419</td>
<td>25</td>
</tr>
<tr>
<td>G1</td>
<td>0.965</td>
<td>0.963</td>
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### Table 2

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<th>Sample</th>
<th>Mean (X)</th>
<th>CV</th>
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<tr>
<td>Low</td>
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<tr>
<td>Normal</td>
<td>8.9</td>
<td>0.27</td>
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<td>High</td>
<td>16.5</td>
<td>0.73</td>
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### Table 3

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<tr>
<th>Sample</th>
<th>Mean (X)</th>
<th>CV</th>
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<tbody>
<tr>
<td>Low</td>
<td>3.0</td>
<td>0.25</td>
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<tr>
<td>Normal</td>
<td>8.7</td>
<td>0.32</td>
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<td>High</td>
<td>16.3</td>
<td>0.69</td>
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</tbody>
</table>

### Table 4

**REFERENCES**

1. Barker, SB. *Determination of Protein Bound Iodine* [Journal of Biological Chemistry, 173, 175 (1948)].
2. Chopra, LJ, Solomon, DH, and Ho, RS. *Radioimmunoassay of Thyroxine.* [J. Clinical Endocrinol, 33, 865 (1971)].

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**Cat #** 8225-300

<table>
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<th>Size</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>96(A)</td>
<td>1mL set</td>
<td>1mL set</td>
<td>2mL set</td>
<td>2mL set x2</td>
<td>1 (5mL)</td>
<td>1 (5mL)</td>
<td>1 (10mL)</td>
<td>2 (10mL)</td>
<td>1 (20mL)</td>
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</tbody>
</table>

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