PRINCIPLE
Competitive Enzyme Immunoassay (TYPE 5):
The required components for assessing the binding capacity of human serum are: enzyme-conjugate, thyroxine (T4), binding protein (P), and immobilized thyroxine antibody (Ab).
Upon mixing the enzyme-conjugate and thyroxine with the specimen, a binding reaction results between the patient’s binding proteins and the added thyroxine but not with the enzyme conjugate. This interaction is represented below:

\[
T4 + P \rightarrow T4 + P (1)
\]

The added thyroxine (T4) not consumed in reaction 1 then competes with the enzyme-T3 conjugate for a limited number of immobilized binding sites. The interaction is illustrated by the following equation:

\[
EnzT3 + T4 + AbC.W. \rightarrow EnzT4AbC.W. + EnzT3AbC.W. \ (2)
\]

T4 = Thyroxine added (constant quantity)
P = Specific binding proteins (varying quantity)

Enzyme Reagent A
One (1) bottle (1:11 dilution) of the T3U-enzyme reagent contains tetramethylbenzidine (TMB) in a suitable container. For example, dilute 160µl of the T3U-enzyme reagent with 13 ml of T3U-enzyme conjugate buffer for a total of 14 ml. This reagent should be used within twenty-four hours for maximum performance of the assay. Store at 2-8°C.

Enzyme-T3 Antibody Coated Plate
Each 96-well microplate has been solid-phase coated with RIA-grade thyroxine (T3). After equilibrium is attained, the antibody-bound fraction is separated from unbound enzyme-antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the binding capacity of the specimen. Thus, in hypothyroidism, the binding proteins are relatively unsaturated (due to the low level of thyroxine) resulting in higher consumption of the added thyroxine than an euthyroid specimen. This leads to higher binding of the enzyme-triiodothyronine conjugate caused by the reduced concentration of the available thyroxine. In hyperthyroidism, the reverse is true. The binding proteins are relatively satu rated with thyroxine (due to the high level of thyroid hormone) resulting in lower consumption of the added thyroxine. The remaining thyroxine is relatively much higher than an euthyroid specimen resulting in lower enzyme-antigen antibody binding due to the increased competition of the thyroxine for the limited antibody sites.

SPECIMEN COLLECTION AND PREPARATION
All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 182 and HCV Antibodies by FDA required tests. Since no known test can offer 100% assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory practices for proper usage, if a bottle of solvents is kept in the Center for Disease Control / National Institute of Health, “Biosafety in Microbiological and Biomedical Laboratories,” 2nd Edition, 1989, HHS Publication No. (CDC) 88-8395.

PRECAUTIONS
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 182 and HCV Antibodies by FDA required tests. Since no known test can offer 100% assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory practices for proper usage, if a bottle of solvents is kept in the Center for Disease Control / National Institute of Health, “Biosafety in Microbiological and Biomedical Laboratories,” 2nd Edition, 1989, HHS Publication No. (CDC) 88-8395.

SPECIMEN COLLECTION AND PREPARATION
The specimens shall be blood; serum or plasma from the blood donor site. All products shall be stored at 2-8°C. When assayed in duplicate, 0.05 ml of the diluted specimen is required.

REAGENT PREPARATION:

1. Working Reagent A - T3U-Enzyme Reagent Solution
Dilute the T3U-enzyme reagent 1:11 with T3 Uptake conjugate buffer to make a suitable test tube. For example, dilute 160µl of conjugate buffer with 1.6ml of buffer for 16 wells (A slight excess of solution is advised). 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 solution 1000ml with distilled or deionized water in a suitable storage container. Store at room temperature 20-27°C for up to 60 days.

3. Working Substrate Solution
Prepare the contents of the working vial labeled Solution ‘A’ into the clear vial labeled Solution ‘B’. Place the yellow cap on the clear vial for identification. Mix and label according to 2-8°C.

4. Stop Solution
Prepare the contents of the working vial labeled ‘Stop’ solution into the clear vial labeled ‘Stop’. Place the yellow cap on the clear vial for identification. Mix and label according to 2-8°C.

Note: Do not use the working substrate if it looks blue.
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 %T-Uptake (%U) on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Connect the points with the best-fit curve.
4. To determine the %T-Uptake for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the reference response, and read the %T-Uptake (%U) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.690) intersects the reference curve at 26.6%U (See Figure 1).

**EXAMPLE 1**

<table>
<thead>
<tr>
<th>Sample I.D.</th>
<th>Well Number</th>
<th>Abs (A)</th>
<th>Mean Abs (B)</th>
<th>Value (%U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>2.644</td>
<td>2.622</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>2.600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal B</td>
<td>C1</td>
<td>1.888</td>
<td>1.880</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>D1</td>
<td>1.872</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal C</td>
<td>E1</td>
<td>0.710</td>
<td>0.718</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>0.726</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal D</td>
<td>G1</td>
<td>0.265</td>
<td>0.256</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>H1</td>
<td>0.247</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl 1</td>
<td>A2</td>
<td>1.701</td>
<td>1.690</td>
<td>26.6</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>1.680</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl 2</td>
<td>C2</td>
<td>0.330</td>
<td>0.314</td>
<td>45.5</td>
</tr>
<tr>
<td></td>
<td>D2</td>
<td>0.298</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The data presented in Example 1 and Figure 1 is for illustration only and should not be used in lieu of a calibrator curve prepared with each assay.

![Figure 1](image-url)

**EXAMPLE 2**

The T-Uptake can also be expressed as a T-Uptake Ratio. Divide the %U by 30% to convert into a T-Uptake ratio. See Example 2.

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 %T-Uptake (%U) on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Connect the points with the best-fit curve.
4. To determine the %T-Uptake for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the reference response, and read the %T-Uptake (%U) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.690) intersects the reference curve at 26.6%U (See Figure 1).

**Q.C. PARAMETERS**

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator A should be ≥ 1.3.
2. Four out of six quality control pools should be within the established ranges.

**LIMITATIONS OF PROCEDURE**

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. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time-deviation during reaction.
5. Plate readers measure vertically. Do not touch the bottom of the wells.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.

**B. Interpretation**

1. The T3-Uptake Test is dependent upon a multiplicity of factors: thyroid gland and its regulation, thyroxine binding globulin (TBG) concentration, and the binding of the thyroid hormones to TBG. Thus, the T3-Uptake Test alone is not sufficient to assess clinical status.
2. The free thyroxine index (FTI), which is the product of the T-Uptake Ratio and the total thyroxine concentration, has gained wide clinical acceptance as a more accurate assessment of thyroid status. However, FTI should not be used in lieu of a calibrator curve prepared with each assay.

**EXPECTED RANGES OF VALUES**

A study of an euthyroid adult population (85 specimens) was undertaken to determine expected values for the T-Uptake and are presented in Table 1.

**TABLE 1**

<table>
<thead>
<tr>
<th>Thyroid Status</th>
<th>% T-Uptake</th>
<th>T-Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>25 - 35</td>
<td>0.83 - 1.17</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>&gt;35</td>
<td>&gt; 1.17</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>&lt; 25</td>
<td>&lt; 0.83</td>
</tr>
</tbody>
</table>

**REFERENCES**