INTENDED USE
For the direct quantitative determination of Triiodothyronine by an enzyme immunoassay in human serum.

PRINCIPLE OF THE TEST
The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabelled antigen (conjugate) for a limited number of antibody binding sites on the microplate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is read by addition of the stopping solution. The absorbance is measured on a microplate reader. The intensity of the colour formed is inversely proportional to the concentration of T3 in the sample. A set of standards is used to plot a standard curve can be directly read.

CLINICAL APPLICATIONS
Triiodothyronine (T3) and thyroxine (T4) are the two active thyroid hormones found in the blood stream. About 80% of T3 is produced by the deiodination of T4 in the peripheral thyroid gland. T3 is transported through the peripheral blood stream bound to proteins, mainly thyroxine binding globulin, thyroxine binding prealbumin and albumin. About 0.3% of the total T3 is unbound and is therefore considered the free fraction. T3 has an influence on oxygen consumption and heat production in virtually all tissues. It also plays a role in growth, development and sexual maturation of growing organisms.

T3 is one parameter used in the clinical diagnosis and differentiation of thyroid disease, particularly hyperthyroidism. In most hyperthyroid patients both serum T3 and serum T4 levels are elevated. Serum T3 levels are a sensitive indicator of the impending hyperthyroid state. Elevated T4 and free thyroxine index values. Serum T3 levels are clinically significant in both the diagnosis of thyroid disease and in the detection of T3 toxicity. It has been demonstrated that T3 levels may be affected by a number of medications, acute and chronic inflammatory diseases, acute and chronic non-thyroidal illnesses. It is therefore necessary to differentiate those results that are due to thyroid dysfunction from those related to non-thyroidal diseases. Safety and Warnings

SAFETY CAUTIONS AND WARNINGS

POTENTIAL BIOHAZARDOUS MATERIAL
Human serum that may be used in the preparation of the standards and controls has been tested and found to be non-reactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV). These tests are, however, not infallible and may be false negative. No test method however, can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any biological material.

CHEMICAL HAZARDS
Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SPECIMEN COLLECTION AND STORAGE
Approximately 0.1 mL of serum is required per duplicate determination. Collect 4–5 mL of blood into an appropriately labelled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store at 4°C for up to 24 hours or at -10°C for longer or if the analysis is not to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

SPECIMEN PRETREATMENT
This assay is a direct system; no specimen pretreatment is necessary.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED
1. Pipette to dispense 50, 100, 150 and 300 μL
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Microplate reader with a filter set at 450 nm and an upper OD limit of 0.3 or greater (see assay procedure step 10)

REAGENTS PROVIDED
1. Rabbit Anti-T3 Antibody-Coated Break-Apart Well Microplate — Ready To Use
   Contents: One 96-well (12x8) polyclonal antibody-coated microwell strip
   Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 μL of the wash buffer concentrate in 450 μL of water.
   Storage: Refrigerate at 2–8°C
2. 3-Horseradish Peroxidase (HRP) Conjugate
   Contents: One vial containing 30 μg HRP in a protein-based buffer with a non-mercury preservative.
   Preparation: Dilute 1:50 in assay buffer before use (eq. 40 μL of HRP in 2 mL assay buffer).
   Stabilization: 12 months or as indicated on label.
   Storage: Refrigerate at 2–8°C
3. Calibration (T3) — Ready To Use
   Contents: Five vials containing T3 in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of T3.
   Preparation: Dilute 1:5 in assay buffer before use (eq. 2 μg of T3 in 10 mL of buffer).
   Stabilization: 12 months or as indicated on label.

CALIBRATORS

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator A</td>
<td>0 mg/mL</td>
<td>2.0 mL</td>
</tr>
<tr>
<td>Calibrator B</td>
<td>0.2 mg/mL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Calibrator C</td>
<td>1 mg/mL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Calibrator D</td>
<td>3 mg/mL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Calibrator E</td>
<td>10 mg/mL</td>
<td>0.5 mL</td>
</tr>
</tbody>
</table>

Storage: Refrigerate at 2–8°C
Stabilization: 12 months or as indicated on label.

5. Wash Buffer Concentrate — Requires Preparation
   Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
   Volume: 50 mL/bottle
   Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 μL of the wash buffer concentrate in 450 μL of water.
   Storage: Refrigerate at 2–8°C
   Stabilization: 12 months or as indicated on label.

6. Assay Buffer — Ready To Use
   Contents: One bottle containing a protein-based buffer with a non-mercury preservative.
   Volume: 15 mL/bottle
   Storage: Refrigerate at 2–8°C
   Stabilization: 12 months or as indicated on label.

7. TMB Substrate — Ready To Use
   Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
   Volume: 16 mL/bottle
   Storage: Refrigerate at 2–8°C
   Stabilization: 12 months or as indicated on label.

8. Stopping Solution — Ready To Use
   Contents: One bottle containing 1M sulfuric acid.
   Volume: 6 mL/bottle
   Storage: Refrigerate at 2–8°C
   Stabilization: 12 months or as indicated on label.

1/2
4. Read the values of the unknowns directly off the calibrator

3. Calculate the mean optical density of each unknown

2. Draw a calibrator curve on semi-log paper with the mean concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.

1. Prepare working solutions of the T3-HRP conjugate and wash buffer.

Remove the required number of well strips. Reseal the bag and return any unused strips to the refrigerator.

Pipette 50 μL of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.

Pipette 100 μL of the conjugate working solution into each well. (We recommend using a multichannel pipette.)

Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.

Wash the wells 3 times with 300 μL of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry. (The use of a washer is recommended.)

Pipette 150 μL of TMB substrate into each well at 20 minutes after addition of the stopping solution.

Pipette 100 μL of the conjugate working solution into each well. (We recommend using a multichannel pipette.)

Pipette 50 μL of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.

Incorporate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.

Wash the wells 3 times with 300 μL of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry. (The use of a washer is recommended.)

If the OD exceeds the upper limit of detection or if a 450 nm filter is unavailable, a 405 or 415 nm filter may be substituted. The optical densities will be lower,

Sample data only. Do not use to calculate results.

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>OD 1</th>
<th>OD 2</th>
<th>Mean OD</th>
<th>Value (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.454</td>
<td>2.480</td>
<td>2.467</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>2.009</td>
<td>1.986</td>
<td>1.998</td>
<td>0.2</td>
</tr>
<tr>
<td>C</td>
<td>1.139</td>
<td>1.129</td>
<td>1.134</td>
<td>3.0</td>
</tr>
<tr>
<td>D</td>
<td>0.565</td>
<td>0.565</td>
<td>0.565</td>
<td>3.0</td>
</tr>
<tr>
<td>E</td>
<td>0.220</td>
<td>0.225</td>
<td>0.223</td>
<td>10</td>
</tr>
<tr>
<td>Unknown</td>
<td>1.082</td>
<td>1.056</td>
<td>1.074</td>
<td>1.1</td>
</tr>
</tbody>
</table>

**CALCULATIONS**

1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.
5. If a sample reads more than 10 ng/mL, then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

**EXPECTED VALUES**

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values.

<table>
<thead>
<tr>
<th>Group</th>
<th>Range (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Normal Males and Females</td>
<td>0.7–2.1</td>
</tr>
</tbody>
</table>

**INTER-ASSAY PRECISION**

Three samples were assayed ten times over a period of four weeks. The results (in ng/mL) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.64</td>
<td>0.07</td>
<td>10.4</td>
</tr>
<tr>
<td>2</td>
<td>1.24</td>
<td>0.12</td>
<td>9.7</td>
</tr>
<tr>
<td>3</td>
<td>4.86</td>
<td>0.44</td>
<td>9.0</td>
</tr>
</tbody>
</table>

**RECOVERY**

Spiked samples were prepared by adding defined amounts of T3 to a serum pool. The results (in ng/mL) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Obs. Result</th>
<th>Exp. Result</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>3.8</td>
<td>3.3</td>
<td>115.1</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>4.6</td>
<td>108.7</td>
</tr>
<tr>
<td>4</td>
<td>5.7</td>
<td>6.3</td>
<td>90.5</td>
</tr>
</tbody>
</table>

**LINEARITY**

Three patient serum samples were diluted with calibrator A. The results (in ng/mL) are tabulated below:

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Thyroxine</td>
<td>100</td>
</tr>
<tr>
<td>L-Thyroxine</td>
<td>34</td>
</tr>
<tr>
<td>Triiodothyronic acid</td>
<td>20</td>
</tr>
<tr>
<td>Diido-D-thyronine</td>
<td>0.5</td>
</tr>
<tr>
<td>D-Thyroxine</td>
<td>0.2</td>
</tr>
<tr>
<td>L-Thyroxine</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The following compounds were tested but cross-reacted at less than 0.1%: Diiodothyrosine, lodothyrosine, Phenothin, Sodium Salicylate and r-Thyroidine.