2.0 SUMMARY AND EXPLANATION OF THE TEST
Measurements of thyroid hormones (fT3, fT4 and TSH) are generally regarded as invaluable in vitro diagnostic tests 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 can be traced from the empirical protein bound iodine (PBI) test to the ‘theoretically sophisticated radioimmunoassay’ and currently used ELISA, ELISA, FA and Chemiluminescence.

The Combination Thyroid Panel (CTP) provides the convenience of combination calibrators, universal plate and flexible reagent selection allowing technicians to perform a variety of assay designs. In this method, sensitive calibrator, patient specimen, or control is first added to a microwell plate. Enzyme-fT3 (fT4) conjugate and biotinylated fT4 or fT3 antibody are added, and the reactants are mixed. In the case of TSH, the biotinylated and enzyme conjugate are added in one step. A reaction results between the enzyme conjugate, biotinylated conjugate and the native thyroid hormone (fT3, fT4 or TSH) for the antibody combining sites. Immobilization takes place through the reaction of the incorporated biotin and streptavidin coated on the well. After the completion of the required incubation period, the bound enzyme conjugate is separated from unbound enzyme 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 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 serum references of known thyroid hormone concentration(s) permits construction of a graph of activity vs concentration, measured 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. Combi-Cal® fT3/fT4/TSH Calibration – 1ml vial - Icons A-F
Six (6) vials of Triiodothyronine (T3) human serum reference calibrator dispensed in vials with the concentrations listed in the Table. A preservative has been added. Store at 2-8°C.

B. Strept fT3 Tracer Reagent – 7ml/vial - Icon
One (1) vial contains triiodothyronine-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C

C. Strept fT4 Tracer Reagent – 7ml/vial - Icon
One (1) vial contains enzyme labeled affinity purified polyclonal goat antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

D. TSH Tracer Reagent — 13ml/vial - Icon
One (1) vial contains enzyme labeled affinity purified polyclonal goat antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

E. Strept fT3 Biotonin Reagent – TSH ml/vial
One (1) vial contains biotinylated anti-triiodothyronine (sheep) reagent in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.

F. Strept fT4 Biotonin Reagent – TSH ml/vial
One (1) vial contains biotinylated thyroxine (sheep) reagent in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.

G. Light Reaction Wells – 2 x 96 wells – Icon
Two (2) vials of reaction buffer. 96-well microplates coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

H. Wash Solution Concentrate – 20ml/vial - Icon
One (1) vial contains enzyme labeled antibody, enzyme-antigen conjugate, native antigen and a substrate that emits light.

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

\[
\text{Ab}_\text{Bi} + \text{TSH} + \text{Ab}_\text{Enz} \rightarrow \text{TSH} + \text{Ab}_\text{Enz} \text{Bi} \rightarrow \text{Enzyme-Ab(TSH)Immobilized complex}
\]

The enzymatic reaction in the antibody-bound fraction, measured by reaction with luminol, is inversely proportional to the native antigen concentration. 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.

Immunoenzymometric assay (TSH) - Type 3
The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme conjugated and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-TSH antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, a reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

\[
\text{Ab}_\text{Bi} + \text{TSH} + \text{Ab}_\text{Enz} \rightarrow \text{TSH} + \text{Ab}_\text{Enz} \text{Bi} \rightarrow \text{Enzyme-Ab(TSH)Immobilized complex}
\]

The essential reagents required for a chemiluminescence immunoassay include antibody, enzyme-antigen conjugate, native antigen and a substrate that emits light.

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

\[
\text{Ab}_\text{Bi} + \text{TSH} + \text{Ab}_\text{Enz} \rightarrow \text{TSH} + \text{Ab}_\text{Enz} \text{Bi} \rightarrow \text{Enzyme-Ab(TSH)Immobilized complex}
\]

The enzymatic reaction in the antibody-bound fraction, measured by reaction with luminol, is inversely proportional to the native antigen concentration. 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.

4.1 Required But Not Provided:
1. Pipette capable of delivering 0.025 & 0.050ml (25 & 50µl) volumes with a precision of better than 1.5%.
2. Dispensers for repetitive deliveries of 0.100ml & 0.350ml (100µl & 350µl) volumes with a precision of better than 1.5%.
3. Adjustable volume (20-200µl) and (200-1000µl) dispensers for 5µl and 50µl.
4. Microplate washer or a squeeze bottle (optional).
5. Pipette Paper for blotting the microplate wells.
6. Test tubes for dilution of enzyme conjugate and Signal reagents A and B.
7. Absorbent Paper for blotting the microplate wells.
8. Plastic wrap or microplate cover for incubation steps.
9. Vacuum aspirator (optional) for wash steps.
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 HBs Antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "BioSafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

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 specimen volume shall be as stated in the kit. The specimen shall be collected in aseptic technique. The specimen shall be separated from unbound antigen 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.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample should be heated to approximately 55°C for 30 minutes. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required for fT4 and TSH and 0.100ml (100µl) for fT3.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the hypothyroid, euthyroid and hyperthyroid range for monitoring assay performance. Fresh reagents should be used to determine the reason for the variations. The individual laboratory should set up appropriate controls to identify the cause of variations. The individual laboratory should set up appropriate controls to identify the cause of variations. The individual laboratory should set up appropriate controls to identify the cause of variations.

8.0 REAGENT PREPARATION

1. Wash Buffer
Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C.

2. Working Signal Reagent Solution - Store at 2-8°C
Determine the amount of reagent needed and prepare by mixing equal parts of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 ml of A and 1ml of B per test. Use Signal Reagent A and B each day. Discard the unused portion if not used within 36 hours after mixing. If complete utilization of the reagents is anticipated, within the above time frame, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

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

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20 - 27°C). **Test Procedure should be performed by a skilled individual or trained professional!**

1. Format the microwells’ wells for each serum calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.
2. Pipette 0.025ml (25µl) of the appropriate serum reference calibrator, control or specimen into the assigned well for fT4, Pipette 0.025ml (50µl) for fT3. Pipette 0.025ml (25µl) for TSH.

3. Add 0.005ml (50µl) of fT4 or fT3 Trazer Reagent to the appropriate wells. For TSH, add 0.010 of fT3 Trazer Reagent and step 4 and 5.

4. Swirl the microplate gently for 20-30 seconds to mix.

5. Add 0.005ml (50µl) of x-t4 or (x-T3) Biotin Reagent Solution to the appropriate wells.

6. Swirl the microplate gently for 20-30 seconds to mix and cover. Incubate 60 minutes at room temperature.

7. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper. Add 0.350ml (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 container (avoiding air bubbles) to dispense the wash.

8. DO NOT SHAKE PLATE AFTER SIGNAL ADDITION

9. Incubate for five (5) minutes at room temperature in the dark.

12. Read the Relative Light Units (RLUs) in each well in a microplate luminometer for at least 0.2 seconds/well. The results can be read within 30 minutes of adding the signal reagent.

Note: For reasaying specimens with concentrations greater than the highest calibrator, dilute 0.0125ml (12.5µl - fT4 & TSH) or 0.025ml (25µl - fT3) of the specimen and 0.0125ml (12.5µl - fT4 & TSH) or 0.025ml (25µl - fT3) of the 0 serum reference into the sample well (this maintains a uniform protein concentration). Multiply the readout value by 2 to obtain the FT4, TSH and FT3 concentration.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of thyroid hormones in unknown specimens. Each well contains 0.025ml of fT3 (fT3) in the printout of the luminometer as outlined in Example 1 – fT3, Example 2 – fT4 or Example 3 – TSH.

1. For the RLUs for each duplicate serum reference versus the corresponding thyroid hormone concentration in the appropriate wells on linear graph paper (do not average the duplicates of the serum references before plotting).

2. Connect the points with a best-fit curve (Figures 1-3).

4. To determine the concentration of fT4 (fT4 – TSH – fT4 for an unknown, locate the average RLUs of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration in ng/dl (fT4 ng/dl) FT4 (fT4 µU/ml TSH) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, in order to obtain an average RLU, 20/223 intersects the calibrator curve at 0.0 ng/dl FT4 concentration (See Figure 2).

Note: Computer data reduction software for CLIA assay may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

The formulas presented in Example 1-3 and Figure 1-3 are for illustration only and should not be used in lieu of a dose response curve prepared with each assay. In addition, the RLUs of the calibrators have been normalized to 100,000 RLUs for the fT4 calibrator for TSH and for the calibrator for T3 and T4 (greatest light output). This conversion minimizes differences caused by efficiency calibrators which have been normalized to 100,000 RLUs for the fT3 Chemi calibration.

11.0 Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:
1. A dose response curve should be established parameters.
2. Four out of six quality control pools should be within the established range.
3. Total serum thyroxine values may be elevated under conditions such as pregnancy or administration of oral contraceptives. A fT3 uptake test may be performed to estimate the relative TBG concentration.
4. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
5. Total serum thyroxine values in newborns may be elevated under conditions such as prematurity or if the newborn is exposed to TBG variation.

13.0 EXPECTED RANGES OF VALUES

A study of euthyroid adult population was undertaken to determine expected values. The mean (R) values, standard deviations (σ) and expected ranges (±2σ) are presented in Table 1 for fT4 and Table 2 for fT3. A nonparametric method (95% Percentile Estimate) was used for TSH in Table 3.

<table>
<thead>
<tr>
<th>TABLE 1 - Expected Values – (fT4 - in µg/ml)</th>
<th>TABLE 2 - Expected Values – (fT3 - in mg/dl)</th>
<th>TABLE 3 - Expected Values – (TSH - in µIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>```</td>
<td>```</td>
<td>```</td>
</tr>
</tbody>
</table>

70% Confidence Intervals for 2.5 Percentile Low Range: 0.28 – 0.53 High Range: 5.60 – 6.82

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The variability between assay precision of the Free T4 & Free T3 test systems were determined by analyses on three different levels of pool control sera. The mean number, standard deviation and coefficient of variation for each of these control sera are...
presented in Table 4 and Table 5. The precisions for TSH CLIA are displayed in Table 6 and 7.

### Table 4

<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>24</td>
<td>0.46</td>
<td>0.430</td>
<td>9.4%</td>
</tr>
<tr>
<td>Pool 2</td>
<td></td>
<td>2.1</td>
<td>0.190</td>
<td>7.1%</td>
</tr>
<tr>
<td>Pool 3</td>
<td></td>
<td>3.12</td>
<td>0.233</td>
<td>7.5%</td>
</tr>
</tbody>
</table>

FT4 values in ng/dl – FT3 values in pg/ml

**Sensitivity:**

The sensitivity was ascertained by determining the variability of the 0 µIU/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

For 1 hour incubation = 0.065 µIU/ml

**Specificity:**

14.4 Specificity

The cross-reactivity of the antibodies used to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of thyroid hormone needed to displace the same amount of tracer.

### Table 5

**Within Assay Precision TSH**

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td>24</td>
<td>0.48</td>
<td>0.028</td>
<td>6.1%</td>
</tr>
<tr>
<td>Pool 2</td>
<td></td>
<td>5.74</td>
<td>0.158</td>
<td>2.75%</td>
</tr>
<tr>
<td>Pool 3</td>
<td></td>
<td>34.54</td>
<td>2.061</td>
<td>5.97%</td>
</tr>
</tbody>
</table>

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

**Within Assay Precision FT4 & FT3**

<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>0.49</td>
<td>0.480</td>
<td>9.8%</td>
</tr>
<tr>
<td>Pool 2</td>
<td></td>
<td>2.11</td>
<td>0.220</td>
<td>10.4%</td>
</tr>
<tr>
<td>Pool 3</td>
<td></td>
<td>3.23</td>
<td>0.250</td>
<td>7.7%</td>
</tr>
</tbody>
</table>

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

### Table 6

**Between Assay Precision TSH**

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td>24</td>
<td>0.48</td>
<td>0.028</td>
<td>6.1%</td>
</tr>
<tr>
<td>Pool 2</td>
<td></td>
<td>5.74</td>
<td>0.158</td>
<td>2.75%</td>
</tr>
<tr>
<td>Pool 3</td>
<td></td>
<td>34.54</td>
<td>2.061</td>
<td>5.97%</td>
</tr>
</tbody>
</table>

**Between Assay Precision FT4 & FT3**

<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>24</td>
<td>0.46</td>
<td>0.430</td>
<td>9.4%</td>
</tr>
<tr>
<td>Pool 2</td>
<td></td>
<td>2.1</td>
<td>0.190</td>
<td>7.1%</td>
</tr>
<tr>
<td>Pool 3</td>
<td></td>
<td>3.12</td>
<td>0.233</td>
<td>7.5%</td>
</tr>
</tbody>
</table>

**References**

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