The enzyme activity 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 antibody, enzyme-antigen conjugate, and a serum containing the native antigen, a competition reaction with the antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

\[ \text{AbBtn} + \text{AgAbBtn} + \text{Ag} \rightarrow \text{AbBtnAgAbBtn} + \text{Ag} \]

\[ \text{AbBtn} = \text{Biotinylated Monoclonal Antibody} \]
\[ \text{AgAbBtn} = \text{Enzyme-Antigen Complex} \]
\[ \text{Ag} = \text{Native Antigen} \]
\[ \text{AbBtnAgAbBtn} = \text{Antigen-Antibody Complex Sandwich Complex} \]

This interaction is illustrated above:

\[ k_a = \text{Rate Constant of Association} \]
\[ k_a = \text{Rate Constant of Dissociation} \]

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated above:

\[ \text{StreptavidinCW} + \text{BtnAb(m)} \rightarrow \text{immobilized complex} \]
\[ \text{Streptavidin immobilized on well} \]

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction, measured by reaction with luminol, is directly 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.

### 4.0 REAGENTS

**Material provided:**

- CombiCalbi T3/T4/TSH Calibrators - 1ml/vial - icons A-F
- Six (6) of Thyroid Hormone human serum calibrators distributed in vials with the concentrations as listed in the table.
- Store at 2-8°C. A preservative has been added.

### 5.0 PRECAUTIONS

- **Required But Not Provided:**
  - Note 3:
  - Kit and component stability are identified on the label.
  - For In Vitro Diagnostic Use
  - 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 HCV 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 SPECIFICATIONS AND PREPARATION

- **The specimens shall be blood serum or plasma in type and the testing of samples outside of their normal range shall be observed.**
- **For accuracy comparison to established normal values, a fastening morning serum sample should be obtained.**
- **The blood should be collected in a plain red top vacutainer tube without additives or anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin.**
- Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

### 7.0 QUALITY CONTROL

- **The laboratory shall perform assays at 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.**
- **Poor control charts should be maintained to follow the performance of the control system.**
- **The laboratory shall set acceptable assay performance limits.**
- **In addition, maximum RL should be employed to ascertain trends.**
- **Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents.**
- **Fresh reagents should be used to determine the reason for the variations.**

### 8.0 REAGENT PREPARATION

1. **Working Reagent A = tT4 or (tT3) - Tracer Solution**
   - Dilute the T4-Tracer (or T3) 1:1 with s-T3/T4 buffer in a suitable storage container. For example, dilute 0.090ml (80µl) of conjugate with 0.800ml (620µl) of buffer for 16 wells. (A slight excess of solution is made.) This reagent should be used within twenty-four hours from time of preparation of the assay. Store at 2-8°C.

2. **B. Strept T4 Tracer Reagent - 1ml/vial - Icon SC**
   - One (1) vial containing thyroxine-horseradish peroxidase (HRP) conjugate in a serum albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.

3. **C. Strept T3 Tracer Reagent - 1ml/vial - Icon SC**
   - One (1) vial containing triiodothyronine-horseradish peroxidase (HRP) conjugate in a serum albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.

4. **D. s-T3/T4 Buffer - 13ml/vial - Icon SCR**
   - 100µl (100µl) for tT3 and TSH.

5. **E. TSH Tracer Reagent - 20ml/vial - Icon SC**
   - One (1) vial containing biotinylated anti-thyroxine (sheep) reagent in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.

6. **F. Strept T4 Biotin Reagent - 1ml/vial - Icon Y**
   - One (1) containing biotinylated anti-thyroxine (sheep) reagent in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.

7. **G. Strept T3 Biotin Reagent – 7ml/vial – Icon CB**
   - One (1) vial containing biotinylated anti-triiothyronine (sheep) reagent in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.

8. **H. Wash Buffer Concentrate – 2 x 96 wells – Icon SC**
   - Two (2) vials containing hydrogen peroxide in buffer. Store at 2-8°C.

9. **I. Wash Buffer**
   - Store vials of Wash Concentrate to 100ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C.

### 9.0 WORKING SIGNAL REAGENT SOLUTION - Store at 2-8°C

- **Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container.**
- **For example, add 1 ml of A and 1 ml of B per two (2) eight well strips (A slight excess of solution is made).**
- **Discard the unused portion if not used within 36 hours after mixing.**
- **Complete utilization of the reagents is anticipated within the above time constraint, 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 microplates’ 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 tT4. Pipette 0.050ml (50µl) for tT3 and TSH.
3. Add 0.050 ml (50µl) of Working Reagent A, tT4 or tT3 Tracer solution to the appropriate wells (see Reagent Preparation Section). For TSH, add 0.100 of TSH Tracer Reagent and see step 4.
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 45 minutes at room temperature.
6. Swirl the contents of the microplate for 20-30 seconds to mix to cover.
7. Incubate for five (5) minutes at room temperature in the dark.
8. Discard the contents of the microplate by decantation or aspirate.
9. Add 0.100ml (100µl) of working signal reagent solution to all wells (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times.
10. Add 0.050ml (50µl) of specimen into the assigned well for tT4 or tT3, or 0.100ml (100µl) of control or control into the assigned well for tT4 or tT3. The calibrator, control or specimen into the assigned well for TSH.
11. Incubate for five (5) minutes at room temperature in the dark.
12. Discard the content of the microplate gently for 20-30 seconds to mix and cover.

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

### 10.0 CALCULATION OF RESULTS

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

1. Record the RLUs obtained from the printout of the luminometer as outlined in Example 1- tT4, Example 2- tT3 or Example TSH.
2. Calculate the RLUs for each duplicate serum reference versus the corresponding thyroid hormone concentration in the appropriate units on linear graph paper (do not average the duplicates of the serum reference before plotting).
3. Connect the points with a best-fit curve (Figure 1-3).
4. To determine the concentration of tT4 (tT3 – TSH) for an unknown, locate the average RLUs of the duplicates for each unknown on the vertical axis of the graph, read the concentration in µg/L (µg/ml) TSH from the horizontal axis of the graph, and multiply the result by 2 to obtain the thyroxine concentration.

### 11.0 Q.C. PARAMETERS

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

1. The dose response curve should be within established parameters.
2. Four out of six quality control pools should be within the established ranges.

### 12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product are available on request from Monobind Inc.

#### 12.1 Assay Performance

1. The time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly hemolyzed, or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of signal reagent initiates a kinetic reaction; therefore, the signal reagent should be added in the same sequence to eliminate any time-deviation during reaction.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents is permitted.
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. All applicable national standards, regulations and laws, including but not limited to the procedures described in this manual, should be strictly followed to ensure compliance and proper device usage.
10. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.

### 12.2 Interpretation

1. Measurements and interpretation of results must be performed by a skilled individual or trained professional.
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. The reagents for the test system procedure have been formulated to eliminate matrix interference. However, potential interference interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems for all kinds of immunoreassays.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.

### 13.0 EXPECTED RANGES OF VALUES

A study of euthyroid adult population was undertaken to determine expected values. The mean (±) values, standard deviations (±) and expected ranges (±) are presented in Table 1 for tT4 and Table 2 for tT3 and Table 3 for TSH. A nonparametric method (95% Percentile Estimate) was used for TSH in Table 3.

---

**EXAMPLE 1 – tT4**

<table>
<thead>
<tr>
<th>Sample LD.</th>
<th>Well Number</th>
<th>RLU</th>
<th>Mean RLU</th>
<th>Value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>98034</td>
<td>100000</td>
<td>0</td>
</tr>
<tr>
<td>Cal B</td>
<td>B1</td>
<td>101966</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal C</td>
<td>C1</td>
<td>98412</td>
<td>100000</td>
<td>0</td>
</tr>
<tr>
<td>Cal D</td>
<td>D1</td>
<td>73906</td>
<td>157827</td>
<td>1.0</td>
</tr>
<tr>
<td>Cal E</td>
<td>E1</td>
<td>71906</td>
<td>100000</td>
<td>0</td>
</tr>
<tr>
<td>Cal F</td>
<td>F1</td>
<td>48214</td>
<td>100000</td>
<td>0</td>
</tr>
</tbody>
</table>

**EXAMPLE 2 – tT3**

<table>
<thead>
<tr>
<th>Sample LD.</th>
<th>Well Number</th>
<th>RLU</th>
<th>Mean RLU</th>
<th>Value (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>32771</td>
<td>100000</td>
<td>0</td>
</tr>
<tr>
<td>Cal B</td>
<td>B1</td>
<td>25583</td>
<td>100000</td>
<td>0</td>
</tr>
<tr>
<td>Cal C</td>
<td>C1</td>
<td>14616</td>
<td>100000</td>
<td>0</td>
</tr>
<tr>
<td>Cal D</td>
<td>D1</td>
<td>72597</td>
<td>100000</td>
<td>0</td>
</tr>
<tr>
<td>Cal E</td>
<td>E1</td>
<td>70692</td>
<td>100000</td>
<td>0</td>
</tr>
<tr>
<td>Cal F</td>
<td>F1</td>
<td>50067</td>
<td>100000</td>
<td>0</td>
</tr>
<tr>
<td>Cal G</td>
<td>G1</td>
<td>20368</td>
<td>100000</td>
<td>0</td>
</tr>
<tr>
<td>Cal H</td>
<td>H1</td>
<td>59846</td>
<td>100000</td>
<td>0</td>
</tr>
<tr>
<td>Cal I</td>
<td>I1</td>
<td>101966</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**EXAMPLE 3 – TSH**

<table>
<thead>
<tr>
<th>Sample LD.</th>
<th>Well Number</th>
<th>RLU</th>
<th>Mean RLU</th>
<th>Value (µU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>248</td>
<td>269</td>
<td>0</td>
</tr>
<tr>
<td>Cal B</td>
<td>B1</td>
<td>291</td>
<td>319</td>
<td>0.9</td>
</tr>
<tr>
<td>Cal C</td>
<td>C1</td>
<td>392</td>
<td>411</td>
<td>0.9</td>
</tr>
<tr>
<td>Cal D</td>
<td>D1</td>
<td>1539</td>
<td>1551</td>
<td>0.5</td>
</tr>
<tr>
<td>Cal E</td>
<td>E1</td>
<td>7823</td>
<td>7750</td>
<td>2.5</td>
</tr>
<tr>
<td>Cal F</td>
<td>F1</td>
<td>7677</td>
<td>7823</td>
<td>2.5</td>
</tr>
<tr>
<td>Cal G</td>
<td>G1</td>
<td>20587</td>
<td>20777</td>
<td>7.0</td>
</tr>
<tr>
<td>Cal H</td>
<td>H1</td>
<td>50567</td>
<td>50877</td>
<td>7.0</td>
</tr>
<tr>
<td>Cal I</td>
<td>I1</td>
<td>50067</td>
<td>50267</td>
<td>7.0</td>
</tr>
</tbody>
</table>

---

**Figure 1**

**Figure 2**

**Figure 3**

---

**NOT INTENDED FOR NEWBORN SCREENING**
It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: specifically of the method, population tested and precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values obtained by the manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

14.9 PERFORMANCE CHARACTERISTICS

14.1 Precision

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

### Table 1 - Expected Values – (tT3) - In ng/ml

<table>
<thead>
<tr>
<th>Number</th>
<th>M</th>
<th>H</th>
<th>Mean</th>
<th>Standard Deviation (σ</th>
<th>Expected Ranges (±2σ</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td></td>
<td></td>
<td>1.45</td>
<td>0.965(x)</td>
<td>0.959</td>
<td></td>
</tr>
</tbody>
</table>

### Table 9 (tT4)

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean (x)</th>
<th>Standard Deviation (σ</th>
<th>Expected Ranges (±2σ</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0000</td>
<td>0.0000</td>
<td>100ng/ml</td>
<td>1000ng/ml</td>
<td></td>
</tr>
</tbody>
</table>

### Table 10 (TSH)

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean (x)</th>
<th>Standard Deviation (σ</th>
<th>Expected Ranges (±2σ</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.65</td>
<td>0.28+0.964(0</td>
<td>0.967</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Only slight amounts of bias between this method and the reference method is 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-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>
<thead>
<tr>
<th>Substance</th>
<th>Cross Reactivity</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tT3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-1.0000</td>
<td>1000ng/ml</td>
<td></td>
</tr>
<tr>
<td>d-Thyroxine</td>
<td>10µg/dl</td>
<td></td>
</tr>
<tr>
<td>d-Thyroidoxyne</td>
<td>10µg/ml</td>
<td></td>
</tr>
<tr>
<td>I-Thyroxine</td>
<td>10µg/ml</td>
<td></td>
</tr>
<tr>
<td>Thyroxine</td>
<td>10µg/ml</td>
<td></td>
</tr>
<tr>
<td>Lutroin Hormone</td>
<td>1000ng/ml</td>
<td></td>
</tr>
<tr>
<td>Chorionic Gonadotropin</td>
<td>1000ng/ml</td>
<td></td>
</tr>
</tbody>
</table>

15.0 REFERENCES

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