Thyroxine Binding Globulin (TBG) concentration in Human Serum

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Thyroxine Binding Globulin (TBG) concentration in Human Serum, Plasma or Whole Blood by a Microplate Enzyme Immunoassay, Chemiluminescence

2.0 SUMMARY AND EXPLANATION OF THE TEST

Thyroxine Binding Globulin (TBG), a 54 kD liver glycoprotein, is the principal binding protein for T4 and T3 in circulation. TBG has a high affinity for T3 and a moderate affinity for T4. It is bound by TBG and is bound by TBG more firmly than is T4. As a consequence, the equilibrium in which the majority of the hormone is bound and a very small portion (≤ 0.05%) is free. TBG is not bound to TBPA and is bound by TBPA to a lesser extent than is TBG. As a result, proportion of free T3 is normally 8-10 times greater than T4. Only free (T3/T4) hormones are available to the tissues, therefore the metabolic state of the patient will correlate more closely with the free than with the total concentration of the hormones.

The diagnostic accuracy of the total hormone measurements would be equal to the free hormone if all the patients had similar binding patterns. Unfortunately, several TBG abnormalities that distort the total-free relationship are commonly encountered in clinical practice. Additionally, the presence of antibodies to TBH/Gc in some patients renders total hormone measurements unreliable. Considerable confusion still exists regarding the clinical significance of free hormone testing. There is controversy regarding the clinical utility of free hormone testing in conditions associated with binding protein abnormalities of pregnancy and thyroidal illness. Methods that are sensitive to albumin concentrations, the effect of certain drugs, high fatty acid and levels of hormones binding inhibitors are considered inadequate by some researchers. However, ELISA techniques for physically separating the exceedingly small amounts of free hormones from the dominant bound moiety are too technically demanding, inconvenient and expensive for a routine clinical laboratory. Such methods that employ equilibrium dialysis, ultrafiltration and protein A sepharose are sensitive to albumin concentrations, the effect of certain drugs, high fatty acid and levels of hormones binding inhibitors are considered inadequate by some researchers. In routine analyses the clinical laboratories rely on direct measurements of free and total hormones and their binding inhibitors.

Based on their serum concentrations, familial TBG variants are divided into four major categories: excess, normal, partial deficiency and complete absence. The studies show that in patients with thyroiditis, pernicious anemia and chronic liver disease increase TBG concentrations, while idiopathic and anabolic steroids, large doses of glucocorticoids and nephrosis decrease TBG levels.

In this method, TBG calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated polyclonal antibody (highly specific for TBG) and enzyme labeled TBG are added in sequence, and the reactants mixed. Reaction between the TBG antibody and labeled TBG and native TBG forms a complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the excess enzyme conjugate is separated from the bound fraction via a wash step. The bound enzyme complex on the surface of the well is quantitated by reaction with a suitable signal reagent to produce light.

The employment of several serum references of known TBG levels permits calculation of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen’s activity can be correlated with TBG concentration.

3.0 PRINCIPLE

Competitive Enzyme Immunoassay (TYPE 7):

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction 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:

$$\frac{\text{AgAbBtn} + \text{EnzAgAbBtn}}{\text{EnzAgAbBtn}} = \text{Enzyme-antigen Conjugate (Constant Quantity)}$$

$$= \text{Enzyme-antigen Conjugate -Antibody Complex}$$

$$= \text{Antigen-antibody complex}$$

$$= \text{Free antigen}$$

$$= \text{TBG}$$

4.0 REAGENTS

Materials Provided:

A. TBG Calibrators – 0.5 ml/vial - Icons A-F
B. TBG Tracer Reagent - 5.5 ml/vial – Icon
C. Kit and component stability are identified on the kit label.

Note 1:

A preservative has been added. Store at 2-8°C.

Note 2:

Labeling of the kit and reagents is critical. Store the kit and reagents at 2-8°C. Avoid freezing and thawing. When assayed in duplicate, 0.020 ml (20 µl) of Signal Reagent B into Signal Reagent A and label.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, medium and high range of the dose response curve for each test. Controls for each test 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. Significant deviation from established performance can indicate uncontrolled 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. Wash Buffer

Dilute contents of Wash Concentrate to 1000 ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-3°C for up to 60 days.

2. Working Signal Reagent Solution - Store at 2 -8°C

Dilute contents of Signal Concentrate to 100 ml by preparing by mixing equal portions of Signal Reagent A and Signal Reagent B into 10 ml of 0.9% saline. For example, add 1 ml of A and 1 ml of B per (30 ± 2) ml (A slight excess of solution is made). Discard the unused portion if not used within 36 hours. The calibration reagents are anticipated, within the above time constraint, for the contents of Signal Reagent B into Signal Reagent A and label.

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 references 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 reference control, calibrator and patient specimen to be assayed in duplicate. Use any unused microwell strips for the assembly and store at 2-8°C

2. Pipette 0.010 ml (10µl) of the appropriate serum reference calibrator, control or specimen into the assayed well.

3. Add 0.050 ml (50µl) of the TBG Tracer Reagent to each well. Mix well the contents of the microwell. It is very important to dispense all reagents close to the bottom of the coated well.

4. Add 0.050 ml (50µl) of the TBG Binding Reagent to each well.

5. Swirl the microplate gently for 20-30 seconds to mix and cover with lid.

6. Incubate 30 minutes at room temperature.

7. Discard the contents of the microplate by decantation or aspiration. If a decanting bottle is employed, fill each well by depressing the container (avoiding air bubbles) to disperse the wash. Decant the wash and repeat a further 4 additional times for a total of five (5) washes. An automatic or manual plate washer can be used. Follow the manufacturer’s instructions for use. If a seqequential bottle is employed, fill each well by depresssing the container (avoiding air bubbles) to disperse the wash. Decant the wash and repeat a further 4 additional times.

8. Add 0.350 ml (350µl) of wash buffer (see Reagent Preparation Section) or wash buffer reagents for a single plate. Repeat four (4) additional times for a total of five (5) washes. Attach or trained professional.

9. Add 0.100 ml (100µl) of working Signal Reagent to all wells (separate microplate) and incubate for 10 minutes at room temperature.

**DO NOT SHAKE THE PLATE AFTER SIGNAL ADDITION**

Incubate for five (5) minutes at room temperature in the dark. Using the 415 μL (Relative Light Units) 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 solution.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of free hormone in the patient specimen. The results are visualized at 450 nm.

1. Record the RLU obtained from the printout of the luminometer as outlined in Example 1.

2. Plot the corresponding Signal Concentration versus the corresponding TBG concentration in µg/ml on linear graph paper.

3. Draw the best-fit curve through the plotted points.

4. To determine the concentration of TBG for an unknown, locate the average RLU of the test specimen on the x-axis of the graph. Read the concentration (in µg/ml) from the y-axis of the graph. The y value of the unknown may be averaged as indicated (See Figure 1).

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

**References:**

EXAMPLE 1

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Well</th>
<th>RLU</th>
<th>Mean RLU</th>
<th>Conc (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>94717</td>
<td>100000</td>
<td>1</td>
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<tr>
<td></td>
<td>B1</td>
<td>105283</td>
<td></td>
<td></td>
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<tr>
<td>Cal B</td>
<td>C1</td>
<td>77485</td>
<td>76887</td>
<td>4</td>
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<tr>
<td></td>
<td>D1</td>
<td>76248</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal C</td>
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<td>61865</td>
<td>60151</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>58436</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal D</td>
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<td>39893</td>
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<tr>
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<tr>
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<td>64</td>
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<tr>
<td></td>
<td>D2</td>
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<tr>
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<tr>
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<tr>
<td></td>
<td>B3</td>
<td>38651</td>
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</table>

* The data presented in Example 1 and Figure 1 is for illustration only and should not be used in lieu of a dose response curve prepared with each assay. In addition, the RLU's of the calibrators have been normalized to 100,000 RLU's for the F calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.

Figure 1

1.10 QC PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:
1. The RLU of calibrator F should be ≥ 50,000.
2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

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

12.1 Assay Performance
1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not exceed ten (10) minutes to avoid assay drift.
3. Highly lipemic, 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(s) 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 step(s) may result in poor repulsion and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.

8. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed in this protocol, is essential. Any deviation from Monobind’s IFU may yield inaccurate results.
9. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure reproducibility and proper device usage.
10. It is important to calibrate all equipment e.g. Pipettes, Readers, Washers, and the automated instrument used with this device, and to perform routine preventative maintenance.
11. Risk Analysis of the assay as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from morebind@monobind.com.

12.2 Interpretation
1. Measurements and interpretation of results should 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 AccuLite® CLIA procedure have been formulated to eliminate maximal interference; however, potential interference 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 immunoassays. (Bioscato, LM, Stuart, MC. “Heterophile antibodies: a problem for all immunoassays.” Clin. Chem. 1983;34:273-33). For diagnostic purposes, the results from this assay should be used in combination with clinical examination, patient history and all other clinical findings.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
5. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
6. 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.

13.0 EXPECTED RANGES OF VALUES

Based on a study of an apparent normal population and established references, a normal range for TBG AccuLite® CLIA Test System was established, as mentioned below.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Within Assay Precision (Values in µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1.0 20 19.6 1.60 8.5%</td>
</tr>
<tr>
<td>B1</td>
<td>2.0 19.6 1.19 8.5%</td>
</tr>
<tr>
<td>C1</td>
<td>3.0 19.4 1.06 8.2%</td>
</tr>
</tbody>
</table>

14.2 Sensitivity
The TBG AccuLite® CLIA Test System procedure has a sensitivity of 0.1 µg/ml (lowest calibrator). The sensitivity was ascertained by determining the variability of the 0 µg/ml calibrator and using the 2o (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy
The TBG AccuLite® CLIA test system was compared against a reference method. Biological specimens (n=167) from population (symptomatic and asymptomatic) were used. The values ranged from 0 – 97 µg/ml. The correlation is presented in Table 4.

14.4 Linearity & Hook Effect
The test will not be affected by TBG concentrations up to 3400 µg/ml in serum or plasma.

15.0 REFERENCES


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