Neonatal Thyroïd Binding Globulin (N-TBG) Test System
Product Code: 8925-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of TBG (Thyroxine Binding Globulin) from whole blood by a Microplate Enzyme Immunoassay, Colorimetric.

2.0 SUMMARY AND EXPLANATION OF THE TEST

Determination of hypothyroidism within the first few days of birth has been recognized as the single most important diagnostic test in neonates by the American Thyroid Association. The nervous system depends on thyroid hormone for its growth and development. Inadequate thyroid hormone at any point during the first 2 years of life can result in irreversible neurologic damage. Early detection and treatment has resulted in the establishment of screening centers by federal and state health departments.

Currently most laboratories test only T4 and TSH levels; however, measurement of TBG concentrations does not vary as widely as the other thyroid controls, all made and dried in whole blood, are first added to a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated polyclonal anti-TB antibody.

First, the elution buffer and a dried blood spot containing native antigen are mixed so the antigen is accessible for further reaction. After incubation, polyclonal biotinylated antibody is then added directly to the eluted sample containing few native antigen. During another incubation step, a reaction results between the antigen and the antibody as illustrated in the following equation:

$$ Ag + AbBtn \rightarrow AgAbBtn $$

The enzyme-labeled antigen is then added, upon which a competition results between the native antigen and the enzyme labeled antigen to detect sample containing of the specific binding sites on the antibody not consumed in the first incubation. After incubation, the antigen bound antibody is attached to the surface of the plastic wells because of the biotin label and the streptavidin that is present on the plastic. The interaction is illustrated by the following equation:

$$ Ag + AbBtn \rightarrow AgAbBtn $$

$Ag = Antigen (Variable Quantity) AgAbBtn = Immune Complex

3.0 PRINCIPLE

Delayed Competitive Enzyme Immunoassay (TYPE 9)

The essential reagents required for an enzyme immunoassay include high affinity and specificity antibody in, enzyme labeled antigen and the native antigen. In this procedure, the immunobilization takes place during the assay at the surface of the microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated polyclonal anti-TB-antibody.

The specimen should be collected 3-7 days post partum. Physical data including age and weight of the infant, whether a multiple birth, or a premature birth etc should accompany the sample. It is important for the clinician to know these facts in order to properly assess the thyroid status of the infant.

The dried blood samples are stable at 2-8°C for 2-3 weeks if stored in zip-lock, moisture resistant bags with desiccants. In patients receiving therapy with high biotin doses (i.e. >15 mg/day) no sample should be taken at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sampling.

7.0 QUALITY CONTROL

Each laboratory should assay external controls at levels in the hypothyroid, euthyroid and hyperthyroid range for monitoring assay performance. These reagents are 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 clinical and statistical methods should be employed to ascertain trends. The individual laboratories are to set acceptable performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can be an indication of 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 1000ml with distilled or deionized water. Place diluted buffer at 2-8°C for up to 60 days.

Note 1: Do not use the substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents and patient samples to room temperature (20 - 27°C).

**Test** should be performed by a skilled individual or trained professional.

1. Assemble the required number of microwells for each calibrator, control, patient sample to be assayed in duplicate. For each assay use once unmissioned microwell strips.

2. Punch out one (1) blood dot out of each calibrator, control, specimen. Place dots in a suitable storage container. Store diluted buffer at 2-3°C for up to 60 days.

3. Place the microplate gently for 20-30 seconds to mix.

4.1 Required But Not Provided:

1. Laboratory Shaker capable of 150rpm rotation.

2. Dispensers (2) for reagent addition. (Note: Do not puncture blood dots from areas that are printed or that are near the edge of the blood spot.)

3. 0.050ml µl volume of N-TBG Elution Buffer to all wells.

4. Place the microplate gently for 20-30 seconds to mix. (Note: Make sure that all blood dots are fully submerged in the liquid. Do not punch blood dots)

5. Cover with a microplate cover and rotate for 60 minutes at ambient temperature using a laboratory rotator set @ 150rpm. (Note: see alternative overnight incubation).

6. Add 50 µl of N-TBG Biotin Reagent directly to each well (do NOT discard any reagents already in well) and shake the plate gently for 20-30 seconds to mix.

7. Cover the microplate and incubate for 30 minutes at ambient temperature. (Note: Do not puncture blood dots)

8. Add 50 µl of N-TBG Enzyme Reagent directly to each well (do NOT discard any reagents already in well) and shake the plate gently for 20-30 seconds to mix.

9. Cover the microplate and incubate for 30 minutes at ambient temperature. (Note: Do not puncture blood dots)

10. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

11. Add 350µl of wash buffer (see Reagent Preparation Section), decant (tart and blot) and 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 disperse the wash. Discard the waste and repeat four (4) additional times.

12. Add 0.100 ml (100µl) of substrate solution to each well.
13. Cover the microplate and incubate for 15 minutes at ambient temperature. No rotation is required for this step.
14. Add 0.050 ml (50 µl) of stop solution to each well and gently mix (15 minutes of adding the stop solution).

4. To determine the concentration of N-TBG for an unknown, the absorbance obtained from the printout of the microplate reader as outlined in Example 1.

9.1 ALTERNATIVE TEST PROCEDURE – OVERNIGHT
1. Assemble the required number of microwells for each calibrator, control and patient sample to be assayed in duplicate. Replace the used microwell strips back into the aluminum bag, seal and store at 2-8°C.
2. Punch out 1/8” blood dot out of each calibrator, control and specimen in the appropriate bands. DO NOT punch blood dots from areas that are printed or that are near the edge of the blood spot.
3. Add 0.050 ml (50 µl) of N-TBG Eton Buffer to all the wells.
4. Shake the microplate gently for 20-30 seconds to mix.
5. Cover the microplate and incubate for 15 minutes at ambient temperature using a laboratory rotator set at 150° rpm.
6. Seal with a microplate film and incubate overnight at ambient temperature.
7. Follow steps 6-15 in the “Test Procedure” above.

10.0 CALCULATION OF RESULTS
A dose response curve is used to ascertain the concentration of N-TBG in unknown specimens.

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

TABLE 1

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Well Number</th>
<th>Abs. Mean</th>
<th>Value (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>2.805</td>
<td>2.72</td>
</tr>
<tr>
<td>Cal B</td>
<td>C1</td>
<td>2.636</td>
<td>2.01</td>
</tr>
<tr>
<td>Cal C</td>
<td>E1</td>
<td>1.889</td>
<td>2.561</td>
</tr>
<tr>
<td>Cal D</td>
<td>G1</td>
<td>1.009</td>
<td>1.09</td>
</tr>
<tr>
<td>Cal E</td>
<td>A2</td>
<td>0.580</td>
<td>0.61</td>
</tr>
<tr>
<td>Cal F</td>
<td>B2</td>
<td>0.514</td>
<td>0.64</td>
</tr>
<tr>
<td>Cont - I</td>
<td>F1</td>
<td>1.833</td>
<td>1.96</td>
</tr>
<tr>
<td>Cont - II</td>
<td>Q1</td>
<td>1.433</td>
<td>1.49</td>
</tr>
<tr>
<td>Cont - III</td>
<td>J1</td>
<td>0.940</td>
<td>0.97</td>
</tr>
<tr>
<td>Patient</td>
<td>C3</td>
<td>1.290</td>
<td>1.39</td>
</tr>
</tbody>
</table>

11.0 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 ‘0’ nmol/L should be ≤ 1.8
2. Four measurements are made at 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. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Dispensing reagents 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. The addition of substrate solution initiates a kinetic reaction, graph paper is the addition of the stop solution.
5. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-temperature during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor assay results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Accurate and precise pipetting, as well as following the exact measurement and interpretation of results must be strictly followed to ensure compliance and proper device usage.

It is important to keep in mind that a normal range establishment is dependent upon a multiplicity of factors like the specificity of the method, the locale, the population tested and the precision of the method in the hands of technicians. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only if an in-house range can be determined by the technicians using the method with a population indigenous to the area in which the laboratory is located.

14.0 PERFORMANCE CHARACTERISTICS
14.1 Precision:
The within and between assay precisions of the N-TBG AccuBind® ELISA test system were determined by analyzing three different levels of dried blood controls. The number (N), mean values (X), standard deviation (σ) and coefficient of variation (CV) for each of these controls are presented in Table 2 and Table 3.

14.2 Specificity:
The cross-reactivity of the antibody used for N-TBG AccuBind® ELISA Test System to selected substances was evaluated by adding massive amounts of the interfering substance to a serum matrix. The cross-reactivity was calculated by deriving a ratio between doses of interfering substance to dose of N-TBG needed to displace the same amount of the conjugate.

15.0 REFERENCES

Revision: 1 Date: 2019-Jul-16 DOI: 1353 MP8925 Product Code: 8925-300

TABLE 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>10</td>
<td>66.97</td>
<td>7.57</td>
<td>11.3</td>
</tr>
<tr>
<td>Normal</td>
<td>20</td>
<td>131.15</td>
<td>16.86</td>
<td>12.9</td>
</tr>
<tr>
<td>High</td>
<td>10</td>
<td>227.69</td>
<td>22.74</td>
<td>10.0</td>
</tr>
</tbody>
</table>

*Measured in ten experiments in duplicate over a ten day period.

The N-TBG AccuBind® ELISA test system was compared with a reference method. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (the values, ranged from 49 nmol/L –200 nmol/L). The total number of such samples was 147. The observed bias of the regression equation and correlation coefficient were computed for this N-TBG AccuBind® ELISA Test System in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean</th>
<th>Least Squares Regression Analysis</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monobind</td>
<td>118.06</td>
<td>y=1.01x+4.35</td>
<td>0.999</td>
</tr>
<tr>
<td>Reference</td>
<td>121.18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Only slight amounts of bias between this method and the reference method indicates 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 antibody used for N-TBG AccuBind® ELISA Test System to selected substances was evaluated by adding massive amounts of the interfering substance to a serum matrix. The cross-reactivity was calculated by deriving a ratio between doses of interfering substance to dose of N-TBG needed to displace the same amount of the conjugate.

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