Monobind Inc.
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AccuBind
ELISA Microwells

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

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of TBG Thyroxine Binding Globulin in Human (Neonates) 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 hormones for proper development beginning inside the womb till at least two years of age and has even shown effects through adolescence.[1,2,3,4] The need for its 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, TBG levels in neonates is also important to consider when dealing with hypothyroidism. In certain disease states, T4 and TSH levels will still appear normal.[1,2,3,4] An X-chromosomal linked recessive trait, TBG deficiency of varying degrees will display different clinical symptoms, but will not always show variation in T4 and/or TSH levels. Males will show a much more profound effect as compared to females with the recessive gene. It has been shown that cases occur in males 9:1 to females.[5,6] TBG does not affect metabolic status directly, but will over time lead to a decreased amount of T4 and T3 in the blood stream, which does directly affect metabolism and even development.[5]

"Because thyroid hormone is essential for brain development, the major risk of congenital hypothyroidism (CH) is irreversible cognitive and motor impairment."[7] Recent studies have found that 1 out of every 3017 infants has a form of congenital hypothyroidism. Pituitary deficiencies are also common among three-quarters of children with CH, creating an increased need for detection.[7] At birth and for a few days after, TBG levels are the lowest since the free thyroid hormone concentrations are increased. Only with age will the TBG levels change and no significant decrease can be seen until after adolescence. Though TBG concentrations do not vary as widely as the other thyroid hormones in a euthyroid individual, an abnormal value is indicative of a thyroid disease.[8]

This microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, calibrators, patient specimen, or controls, all made and dried in whole blood, are first added to a microplate well. A buffer containing essential ingredients to isolate TBG from blood proteins is added. The blood from the filter paper dots is allowed to elute in the buffer. In the process, TBG (Thyroxine Binding Globulin) dissociates from the serum (blood) proteins. Biotin conjugate is added directly on top followed 30 minutes later by the enzyme conjugate. After the completion of the required incubation period, excess reagents are removed using a wash step. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several references, made in whole blood, of known TBG concentration permits construction of a dose response curve (DRC-graph) of activity and concentration. An unknown specimen's activity can be extrapolated from the DRC.

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 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 polyclonal anti-TBG 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 the native antigen. During another incubation step, a reaction results between the antigen and the antibody as illustrated in the following equation:

$$Ag + Ab_{Btn} \rightleftharpoons AgAb_{Btn}$$

Ab_{Btn} = Biotinylated antibody
Ag = Antigen (Variable Quantity) $AgAb_{Btn}$ = Immune Complex

The enzyme-labeled antigen is then added, upon which a competition results between the native antigen and the enzyme labeled antigen for a limited number of 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 on it and the streptavidin that is present on the plastic. The interaction is illustrated by the following equation:

$$^{Enz}Ag + Ag_{TBG} + {}^{Btn}Ab_{(p)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ag - {}^{Btn}Ab_{(p)} - Ag_{TBG}$$

$^{Btn}Ab_{(p)}$ = Biotinylated Polyclonal Antibody (Excess Quantity)
Ag_{TBG} = Native Antigen (Variable Quantity)
^{Enz}Ag = Enzyme labeled Antigen (Excess Quantity)
$^{Enz}Ag - {}^{Btn}Ab_{(m)} - Ag_{TBG}$ =Antibody-Antigens Complex
k_a = Rate Constant of Association
k_{-a} = 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 below:
$^{Enz}Ag - Ag_{TBG} - {}^{Btn}Ab_{(p)} + Streptavidin_{C.W.} \Rightarrow$ Immobilized complex
$Streptavidin_{C.W.}$ = Streptavidin immobolized on well
Immobilized complex = complex bound to the solid surface

After sufficient time for quantitation, the excess serum proteins, antibodies and the enzyme labeled antigen are removed via a wash step. The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

4.0 REAGENTS

Materials Provided:

A. N-TBG Calibrators – Dried Blood Spots (Two rows by six dots levels - 2 x 6)
Six (6) levels of N-TBG Calibrators in dried blood spots at approximate concentrations of 0(**A**), 50 (**B**), 100(**C**), 200(**D**), 375(**E**) and 750(**F**) nmol/L placed on S&S type 903 filter paper. Store at 2-8°C. A preservative has been added.
Note 1: The exact values are printed on the outside of the aluminum pouch.
Note 2: The ***Lot Specific*** calibrators, whole human blood based, were calibrated using analytically pure TBG (greater than 99% by weight). This material exceeds the specifications set by USP.

B. Whole Blood Controls – (I, II & III)
Three (3) controls for TBG at varying concentrations (batch specific) made in whole blood spotted on S&S filter paper (Cat# 903) supplied in a zip-lock foil bag with a desiccant. Please see the bag label for ranges for different controls. Store at 2-8°C. A preservative has been added.

C. N-TBG Elution Buffer – 6.0 ml:
One (1) vial containing 6.0 ml of buffer with preservatives. Store at 2-8ºC.

D. N-TBG Biotin Reagent – 6.0 ml --Icon Ⓑ
One (1) vial containing of x-TBG in buffer with blue dye, surfactants and preservatives. Store at 2-8°C.

E. N-TBG Enzyme Reagent – 6.0ml/vial- Icon Ⓔ
One (1) vial of TBG-horseradish peroxidase (HRP) conjugate in a protein-stabilizing matrix. A preservative and red dye has been added. Store at 2-8°C.

F. Streptavidin Coated Microplate – 96 wells - Icon ⇓
One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

G. Wash Solution Concentrate – 20ml/vial – Icon-
One (1) vial containing surfactant, buffer and saline. Store at 2-8°C.

H. Substrate Solution – 13ml/vial - Icon S[c]
One (1) vial containing tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

I. Stop Solution – 8ml/vial - Icon STOP
One (1) vial containing a strong acid (1N H_2SO_4). Store at 2-8°C.

J. Product Instructions.

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C. **Opened reagents are stable for sixty (60) days when stored at 2-8ºC. Kit and component stability are identified on the label.**
Note 3: Do not exchange reagents between different batches**.**

4.1 Required But Not Provided:
1. Laboratory Shaker capable of 150rpm rotation.
2. Dispenser(s) for repetitive deliveries of 0.050ml, 0.100ml and 0.350ml volumes with a precision of better than 1.5%.
3. Adjustable volume (20-200µl) and (200-1000µl) dispenser(s) for conjugate dilutions.
4. 1/8th inch hole punch.
5. Microplate washer or a squeeze bottle (optional).
6. Microplate Reader capable of absorbance readings at 450nm and 620nm.
7. Absorbent Paper for blotting the microplate wells.
8. Plastic wrap and microplate cover for incubation steps.
9. Vacuum aspirator (optional) for wash steps.
10. Timer.
11. External 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 blood 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 SPECIMEN COLLECTION AND PREPARATION

The sampling from neonates is performed by lancing the heels of the infants and then spotting enough whole blood on S&S filter paper card (Cat# 903) to fill the marked circle. Allow the filter paper to dry at room temperature overnight away from heat and moisture. Enclose the dry blood specimen (DBS) in a moisture barrier plastic bag with desiccant and send to the laboratory.
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. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

7.0 QUALITY CONTROL

Each laboratory should assay external controls at levels in 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. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. 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. **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 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 Procedure should be performed by a skilled individual or trained professional*****

1. Assemble the required number of microwells for each calibrator, control and patient sample to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8ºC.**
2. Punch out 1/8" blood dot out of each calibrator, control and specimens into the assigned wells. ***(NOTE:* 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 (50µl) of N-TBG Elution Buffer to all the wells.
4. Shake the microplate gently for 20-30 seconds to mix. **(NOTE: Make sure that all blood dots are fully submerged in the liquid and not stuck to the walls of the microwells).**
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 reactants already in well)* and shake the microplate gently for 20-30 seconds to mix.
7. Cover the microplate and incubate for 30 minutes at ambient temperature. **No rotation is required for this step.**
8. Add 50 µl of N-TBG Enzyme Reagent directly to each well *(do **NOT** discard any reactants already in well)* and shake the microplate gently for 20-30 seconds to mix.
9. Cover the microplate and incubate for 30 minutes at ambient temperature. **No rotation is required for this step.**
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 (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. Decant the wash 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 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds.
    NOTE: Always add reagents in the same order to minimize reaction time differences between wells.
15. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. **The results should be read within fifteen (15) minutes of adding the stop solution.**

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 any unused 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 specimens into the assigned wells. ***(NOTE:* 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 (50µl) of N-TBG Elution Buffer to all the wells.
4. Shake the microplate gently for 20-30 seconds to mix. **(NOTE: Make sure that all blood dots are fully submerged in the liquid and not stuck to the walls of the microwells).**
5. Cover with a microplate cover and rotate for 45 minutes at ambient temperature using a laboratory rotator set @ 150rpm.
6. Seal with a microplate film and incubate overnight at ambient room 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 Neo-natal TBG in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding N-TBG concentration in nmol/L on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of N-TBG for an unknown, locate the average absorbance 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 nmol/L) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.39) intersects the dose response curve at (127.27 nmol/L) N-TBG concentration (See Figure 1).

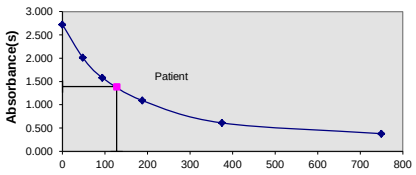
Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Well Number	Abs.	Mean	Value (nmol/L)
Cal A	A1	2.805	2.72	0
	B1	2.636		
Cal B	C1	1.919	2.01	48
	D1	2.124		
Cal C	E1	1.689	1.58	94
	F1	1.461		
Cal D	G1	1.009	1.09	188
	H1	1.177		
Cal E	A2	0.580	0.61	375
	B2	0.641		
Cal F	C2	0.389	0.38	750
	D2	0.362		
Cont - I	E2	2.091	1.96	52.75
	F2	1.833		
Cont – II	G2	1.433	1.49	108.12
	H2	1.549		
Cont - III	A3	0.940	0.97	223.98
	B3	0.990		
Patient	C3	1.250	1.39	127.27
	D3	1.575		

*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.

Figure 1



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 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. 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, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.
5. Plate readers measure vertically. Do not touch the bottom of the wells.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication 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 are essential Any deviation from Monobind 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 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.
11. Risk Analysis- as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

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 have been formulated to eliminate maximal interference; however, potential 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 immunoassays (Boscato LM, Stuart MC. 'Heterophilic antibodies: a problem for all immunoassays' Clin. Chem. 1988:3427-33). For diagnostic purposes, the results from this assay should be 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

In agreement with established reference intervals for a "normal" pediatric population the expected ranges for N-TBG AccuBind® Test System are detailed in Table 1. The units can be converted using a conversion factor of 18.5.

TABLE 1
Age Dependent Reference Intervals for TBG[9]

Age	N-TBG – in nnmol/L			N-TBG – in µg/mL		
1 – 7 days	116.6	-	399.6	6.3	-	21.6
8 – 15 days	220.0	-	442.9	11.9	-	23.9
1 mo – 3 yrs	235.1	-	571.7	12.7	-	30.9
4 – 6 yrs	272.5	-	608.7	14.7	-	32.9
7 – 8 yrs	301.7	-	568.0	16.3	-	30.7
9 – 10 yrs	291.6	-	519.9	15.8	-	28.1
11 yrs	285.6	-	506.9	15.4	-	27.4
12 yrs	273.4	-	483.6	14.8	-	26.1
13 yrs	255.3	-	466.8	13.8	-	25.2
14 yrs	226.1	-	166.2	12.2	-	9.0
15 yrs	199.8	-	451.4	10.8	-	24.4
16 yrs	185.0	-	440.3	10.0	-	23.8
17 yrs	157.3	-	427.4	8.5	-	23.1
18 – 19 yrs	142.3	-	392.2	7.7	-	21.2

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 until 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 analyses on three different levels of dried blood controls. The number (N), mean values (X), standard deviation (σ) and coefficient of variation (C.V.) for each of these controls are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in nmol/L)

Sample	N	X	σ	C.V.%
Low	20	66.97	7.57	11.3
Normal	20	131.15	16.86	12.9
High	20	268.94	19.29	7.2

TABLE 3
Between Assay Precision (Values in nmol/L)

Sample	N	X	σ	C.V.%
Low	10	72.76	11.52	15.8
Normal	10	120.76	12.11	10.0
High	10	227.69	22.74	10.0

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

14.2 Sensitivity
The N-TBG AccuBind® ELISA test system has a sensitivity of 28.6 nmol/L. The sensitivity was ascertained by determining the variability of the 0 nmol/L calibrator and using the 2 σ (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy
The N-TBG AccuBind® ELISA test system was compared with a reference methodology. 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 specimens was 87. The least square regression equation and the 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

Method	Mean	Least Square Regression Analysis	Correlation Coefficient
Monobind (y)	118.06	y=1.01(x)-4.35	0.999
Reference (x)	121.18		

Only slight amounts of bias between this method and the reference method are 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 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 thyroxine needed to displace the same amount of the conjugate.

Substance	Cross Reactivity
Bilirubin	ND
Lipids	ND
Triglycerides	ND
Human IgG	ND

15.0 REFERENCES

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Please visit our website to learn more about our products and services.

Glossary of Symbols
(EN 980/ISO 15223)

IVD – In Vitro - Diagnostic Medical Device
Temperature Limitation Storage Condition (2-8° C)
Consult Instructions for Use
REF – Catalogue Number
Contains Sufficient Test for Σ
LOT – Batch Code
Used By (Expiration Day)
Date of Manufacturer
Manufacturer
EC REP – Authorized Rep in European Country
CE – European Conformity