Monobind Inc.
Lake Forest, CA 92630, USA

AccuBind
ELISA Microwells

Thyroxine Binding Globulin (TBG) Test System
Product Code: 3525-300

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, Colorimetric

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. Electrophoretic analyses indicate that T4 is bound, in decreasing order, to TBG, to a T4 binding prealbumin (TBPA) and to albumin. By virtue of its intense affinity for T4, TBG is by far the major determinant of overall binding capacity. The interaction between T4 and its binding proteins conforms to a reversible binding equilibrium in which the majority of the hormone is bound and a very small portion ($\leq$ 0.05%) is free. T3 is not bound by TBPA and is bound by TBG less firmly than is T4. As a consequence, 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 protein concentrations. Unfortunately, serum TBG abnormalities that distort the total:free relationship are commonly encountered in clinical practice. Additionally, the presence of antibodies to thyroid hormones in some patients renders total hormone measurements unreliable. Considerable confusion still exists regarding the validity 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 non-thyroidal illness. Methods that are sensitive to albumin concentrations, the effect of certain drugs, high free fatty acid and levels of hormones binding inhibitors are considered inadequate by some researchers. However, the techniques for physically separating the exceedingly small amounts of free hormones from the dominant protein bound moiety are too technically demanding, inconvenient and expensive for a routine clinical laboratory. Such methods that employ equilibrium dialysis, ultrafilteration and gel-filtration are typically used by researchers. In routine analysis, the clinical laboratories rely on direct measurements of free and total hormones and their binding proteins, mainly TBG.

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 estrogens (pregnancy and oral contraceptives), acute intermittent porphyria and chronic liver disease increase TBG concentrations, while androgenic 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 antibodies, enzyme 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 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 serum references of known TBG levels permits construction 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 followed equation:

$$^{Enz}Ag + Ag + Ab_{Btn} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{Btn} + {}^{Enz}AgAb_{Btn}$$

Ab_{Btn} = Biotinylated Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)
$AgAb_{Btn}$ = Antigen-Antibody Complex
$^{Enz}AgAb_{Btn}$ = Enzyme-antigen Conjugate -Antibody Complex
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Disassociation
$K = k_a / k_{-a}$ = Equilibrium Constant

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effects the separation of the antibody bound fraction after decantation or aspiration.

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin}_{CW} \Rightarrow$ immobilized complex

$\underline{Streptavidin}_{CW}$ = Streptavidin immobilized on well
Immobilized complex = sandwich complex bound to the solid surface

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 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. TBG Calibrators – 0.5 ml/vial - Icons A-F
Six (6) vials of references **TBG** Antigen at levels of 1(**A**), 4(**B**), 8(**C**), 16(**D**), 32(**E**) and 64(**F**) µg/ml. A preservative has been added. Store at 2-8°C.
Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the international reference material (IS 88/638).

B. TBG Enzyme Reagent – 5.5ml/vial – Icon
One (1) vial containing Enzyme (HRP) labeled TBG in buffer, dye, and preservative. Store at 2-8°C.

C. TBG Antibody Biotin Reagent – 5.5ml/vial - ∇
One (1) vial of Biotin labeled Anti-TBG polyclonal IgG in buffer, dye and preservatives. Store at 2-8ºC.

D. 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.

E. Wash Solution Concentrate – 20ml/vial - Icon
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

F. Substrate A – 7ml/vial - Icon S^A
One (1) vial containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C. See "Reagent Preparation."

G. Substrate B – 7ml/vial - Icon S^B
One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C. See "Reagent Preparation."

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

I. Product Insert

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. **Opened reagents are stable for sixty (60) days when stored at 2-8ºC. Kit and component stability are identified on the label.**
Note 3: Above reagents are for a single 96-well microplate.

4.1 Required But Not Provided:

1. Pipette capable of delivering 0.010 & 0.050ml (10 & 50µl) volumes with a precision of better than 1.5%.
2. Multi-channel dispenser(s) for deliveries of 0.100 & 0.350ml (100 & 350µl) volumes with a precision of better than 1.5%.
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. 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 HCV Antibodies by FDA licensed reagents. 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 and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants. Allow the blood to clot for samples. Centrifuge the specimen to separate the serum from the cells.

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(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.020ml (20µl) of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, medium and elevated ranges of the dose response curve 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. 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 at room temperature 2-30°C for up to 60 days.
2. **Working Substrate Solution –** Stable for one (1) year
   Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note 1: Do not use the working 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, 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 reference calibrator, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8ºC.**
2. Pipette 0.010ml (10µl) of the appropriate serum reference calibrator, diluted control or specimen into the assigned wells.
3. Add 0.050ml (50µl) of the TBG enzyme reagent to each well. Mix well the contents of the microwells. **It is very important to dispense all reagents close to the bottom of the coated well.**
4. Add 0.050ml (50µl) of the biotin antibody reagent to each well.
5. Swirl the microplate gently for 20-30 seconds to mix and cover.
6. Incubate 30 minutes at room temperature.
7. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
8. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) 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 two (2) additional times.**
9. Add 0.100ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section).
10. Incubate at room temperature for fifteen (15) minutes.
11. Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds.
12. 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 thirty (30) minutes of adding the stop solution.**

Note: Always add reagents in the same order to minimize reaction time differences between wells.

10.0 CALCULATION OF RESULTS

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

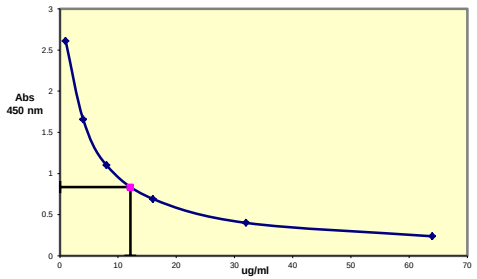
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding TBG concentration in µg/ml on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of TBG for an unknown, locate the average 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 ng/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). (See Figure 1).

Note: Computer data reduction software designed for ELISA 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 Position	Abs (A)	Mean Abs (B)	Value (ug/ml)
Cal A	A1	2.601	2.610	1
	B1	2.619		
Cal B	C1	1.672	1.659	4
	D1	1.646		
Cal C	E1	1.101	1.103	8
	F1	1.105		
Cal D	G1	0.688	0.692	16
	H1	0.697		
Cal E	A2	0.389	0.403	32
	B2	0.412		
Cal F	C2	0.243	0.237	64
	D2	0.231		
Control	E2	0.409	0.410	***31.8***
	F2	0.411		
Patient 1	G2	0.828	0.491	***12.1***
	H2	0.835		
Patient 2	A3	0.267	0.273	***52.0***
	B3	0.280		

Figure 1



Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction.

*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.

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 A should be ≥ 1.3
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. Pipetting of samples should not extend beyond 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 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.
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 replication and spurious 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 time and temperature requirements prescribed are essential. Any deviation from Monobind's IFU may yield inaccurate results.
10. 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.
11. 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. 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.

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 AccuBind® ELISA procedure 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

Based on a study of an apparent normal population and established references, a normal range for TBG AccuBind® ELISA Test System was established as illustrated below:

TABLE 1
Expected Values for TBG AccuBind® ELISA Test System

Normal Range
Males 12 - 26 µg/ml
Females 11 - 27 µg/ml

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: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. 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 analysts 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 precision of the TBG AccuBind® ELISA Test System were determined by analyses on three different levels of control sera. The number, mean value, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in µg/ml)

Sample	N	X	σ	C.V.
Level 1	20	4.3	0.16	3.6%
Level 2	20	11.8	1.10	9.3%
Level 3	20	19.6	1.60	8.2%

TABLE 3
Between Assay Precision* (Values in µg/ml)

Sample	N	X	σ	C.V.
Level 1	10	4.6	0.31	6.7%
Level 2	10	12.1	1.09	9.0%
Level 3	10	21.1	1.01	4.8%

*As measured in ten experiments in duplicate.

14.2 Sensitivity

The TBG AccuBind® ELISA Test System has a sensitivity of 1.0 µg/ml. The sensitivity was ascertained by determining the variability of the '0' calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy

The TBG AccuBind® ELISA Test System was compared against a reference method. Biological specimens (n=167) from population (symptomatic and asymptomatic) were used. The values ranged from 0 – 97µg/ml. The correlation is presented in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method(x)	15.2767	y = -0.1997+1.0192(x)	0.991
Reference (y)	15.3709		

14.4 Specificity

The cross-reactivity of the TBG AccuBind® ELISA Test System to selected substances was evaluated by adding the interfering substance to a pooled serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of TBG needed to produce the same absorbance.

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

14.4 Linearity & Hook Effect

The test will not be affected by TBG concentrations up to 340 mg/dl in serum or plasma.

15.0 REFERENCES

1. 'Clinical Guide to Laboratory Tests' N.W. Tietz, 3rd Ed. WB Saunders Company, Philadelphia, PA (1995).
2. Centers for Disease Control/ NIH manual, "Biosafety in Microbiological and Biomedical Laboratories". (1984).
3. Zinn AB, Marshall JS and Carlson DM: Carbohydrate structures of thyroxine binding globulin and their effect on hepatocyte membrane binding. J.Biol.Chem.253:6768-6773. 1978.
4. Refotoff S.: Inherited thyroxine binding globulin abnormalities in man. Endocr. Rev. 10:275-293. 1989.
5. Grimaldi S, Bartalena L, Rama

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Size	96(A)	192(B)
Reagent (fill) A)	1 (0.5ml set)	1 (0.5ml set)
B)	1 (5.5ml)	2 (5.5ml)
C)	1 (5.5ml)	2 (5.5ml)
D)	1 plate	2 plates
E)	1 (20ml)	1 (20ml)
F)	1 (7ml)	2 (7ml)
G)	1 (7ml)	2 (7ml)
H)	1 (8ml)	2 (8ml)

For Orders and Inquires, please contact

Monobind Inc.
100 North Pointe Drive
Lake Forest, CA 92630 USA

Tel: +1 949.951.2665 Mail: info@monobind.com
Fax: +1 949.951.3539 Fax: www.monobind.com

IVD 2°-8° CE
EC REP **CEpartner4U,** Esdoornlaan 13
3951 DBMaarn, The Neatherlands
www.cepartner4u.eu

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
2°-8° — 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