



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

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

SUMMARY AND EXPLANATION OF THE TEST

TBG (Thyroxine Binding Globulin) 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, render 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, ultrafiltration 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 decreases 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.

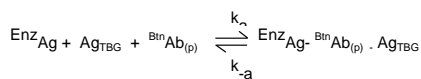
After the completion of the required incubation period, the excess enzyme conjugate is separated from the bound fraction via a wash step. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

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.

PRINCIPLE

Immunoenzymometric assay:

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. Upon mixing polyclonal biotinylated antibody, the enzyme-labeled antigen and a serum containing the native antigen, a competition results between the native antigen and the enzyme labeled antigen for a limited number of specific binding sites on the antibody. The antigen bound antibody attaches 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:



$\text{B}^{\text{in}}\text{Ab}_{(\text{p})}$ = Biotinylated Polyclonal Antibody (Excess Quantity)

Ag_{TBG} = Native Antigen (Variable Quantity)

EnzAg = Enzyme labeled Antigen (Excess Quantity)

$\text{EnzAg} \cdot \text{B}^{\text{in}}\text{Ab}_{(\text{p})} \cdot \text{Ag}_{\text{TBG}}$ = 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:



$\text{Streptavidin}_{\text{C.W.}}$ = Streptavidin immobilized on well

Immobilized complex = complex bound to the solid surface

After equilibrium is attained, 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.

REAGENTS

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) $\mu\text{g/ml}$. Store at 2-8°C. A preservative has been added.

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 - 5.5 ml/vial.

One (1) vial containing Enzyme (HRP) labeled TBG in buffer, dye, and preservative. Store at 2-8°C.

C. Antibody Biotin Reagent – 5.5 ml.

One (1) vial of Biotin labeled Anti-TBG polyclonal IgG in buffer, dye and preservatives. Store at 2-8°C.

D. Streptavidin Coated Microplate – 96 wells - Icon^U

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 – 20 ml - Icon^U

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

F. Substrate A –7ml/vial - Icon S^A

One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

G. Substrate B – 7ml/vial - Icon S^B

One (1) bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H. Stop Solution – 8ml/vial - Icon^{STOP}

One (1) bottle containing a strong acid (1N HCl). Store at 2-30°C.

I. Product Insert.

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.

Note 3: Above reagents are for a single 96-well microplate.

Required But Not Provided:

- Pipette capable of delivering 10 & 50 μl volumes with a precision of better than 1.5%.
- Multi-channel dispenser(s) for deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5%.
- Microplate washers or a squeeze bottle (optional).
- Microplate Reader with 450nm and 620nm wavelength absorbance capability.
- Absorbent Paper for blotting the microplate wells.
- Plastic wrap or microplate cover for incubation steps.
- Vacuum aspirator (optional) for wash steps.
- Timer.
- Quality control materials.

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.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood; serum in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established 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.

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 repetitive freezing and thawing. When assayed in duplicate, 0.020ml of the specimen is required.

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 20-27°C for up to 60 days.

2. Working Substrate Solution

Determine the amount of solution needed and prepare by mixing equal portions of Substrate Solution A and Substrate Solution B in a clean container. For example, add 1 ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). **Discard the unused portion if not used within 60 days after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Substrate Solution B into Signal Reagent A and label accordingly.

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

TEST PROCEDURE

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

- Format the microplates' wells for each serum reference, 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.**
- Pipette 0.010 ml (10 μl) of the appropriate serum reference, diluted control or specimen into the assigned wells.
- Add 0.050 ml (50 μl) 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.**
- Add 0.050 ml (50 μl) of the biotin antibody reagent to each well.
- Swirl the microplate gently for 20-30 seconds to mix and cover.
- Incubate 30 minutes at room temperature.
- Add 300 μl 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.**
- Add 0.100 ml (100 μl) of working substrate solution to all wells (see Reagent Preparation Section).
- Incubate at room temperature for fifteen (15) minutes.
- Add 0.050ml (50 μl) of stop solution to each well and mix gently for 15-20 seconds.
- 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.

QUALITY CONTROL

Each laboratory should assay controls at levels in the low, medium and high 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.

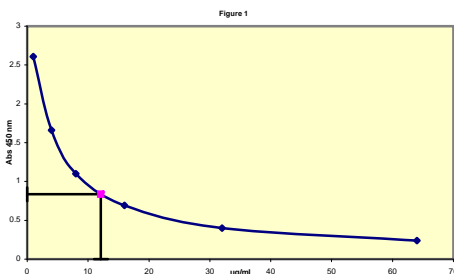
RESULTS

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

- Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
- Plot the absorbance for each duplicate serum reference versus the corresponding TBG concentration in $\mu\text{g/ml}$ on linear graph paper.
- Draw the best-fit curve through the plotted points.
- 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 assays may also be used for the data reduction.

TBG Example DRC.



*The data presented in Example 1 and Figure 1 are for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay.

Q.C. PARAMETERS

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

- The absorbance (OD) of calibrator F should be ≥ 1.3 .
- Four out of six quality control pools should be within the established ranges.

Interpretation

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.

EXAMPLE 1

Sample I.D.	Well Position	Absorbance	Mean Absorbance	Concentration ($\mu\text{g/ml}$)
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		

LIMITATIONS OF PROCEDURE

A. Assay Performance

- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond five (5) minutes to avoid assay drift. Use a multichannel pipet to quickly dispense Enzyme Reagent to avoid drift if the dispensing is to take more than 5 minutes.
- Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time deviation during reaction.
- Plate readers measure vertically. Do not touch the bottom of the wells.
- Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
- Sample(s), which are contaminated microbiologically, should not be used in the assay.
- Each component in one assay should be of the same lot number and stored under identical conditions.

EXPECTED RANGES OF VALUES

Based on a study of an apparent normal population and established references a normal range for AccuBind™ TBG EIA Microplate Test System was established as mentioned below.

Normal Range	
Males	12 - 26 $\mu\text{g/ml}$
Females	11 - 27 $\mu\text{g/ml}$

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.

PERFORMANCE CHARACTERISTICS

A. Precision

The within and between assay precision of the TBG Microplate EIA Procedure 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 3 and Table 4.

TABLE 2
Within Assay Precision (Values in $\mu\text{g/ml}$)

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 $\mu\text{g/ml}$)

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.

B. Sensitivity

The TBG Microplate EIA Procedure has a sensitivity of 1.0 $\mu\text{g/ml}$.

C. Specificity

The cross-reactivity of the TBG Microplate Procedure 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

D. Linearity & Hook Effect

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

E. Method Comparison

The *Monobind* AccuBind™ hTBG Elisa was compared against a predicate automated TBG method. Biological specimens (n=167) from population (symptomatic and asymptomatic) were used. The values ranged from 0 – 97 $\mu\text{g/ml}$. The correlation is presented in Table 4.

TABLE 4

Least Square		Regression Analysis	Correlation Coefficient
Method	Mean (x)		
This Method (x)	15.2767	$y = -0.1997 + 1.0192(x)$	99.1
Reference (y)	15.3709		

REFERENCES

- 'Clinical Guide to Laboratory Tests' N.W. Tietz, 3rd Ed. WB Saunders Company, Philadelphia, PA (1995).
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- 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.
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Instruments & Applications

Monobind's immunoassay products are designed to work in both manual and automated lab environments. AccuBind™ and AccuLite™ are compatible with any open-ended instrumentation, including chemistry analyzers, microplate readers and microplate washers. There may or may not be an application developed for your particular instrument, please visit the instrument section of our website, or contact techsupport@monobind.com

Monobind offers several instruments, including the Impulse 2 Luminometer CLIA Plate Reader designed hand-in-hand with our products and capable of 2-point calibration. Visit our website for more information.