Monobind Inc.
Lake Forest, CA 92630, USA

SBS™
Streptavidin-Biotin System
ELISA Microwells

Total Thyroxine Streptavidin (Total T4 SBS) Test System

Product Code: 8225-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Total Thyroxine in Human Serum or Plasma sample by a Microplate Enzyme Immunoassay, Colorimetric

2.0 SUMMARY AND EXPLANATION OF THE TEST

Measurement of serum thyroxine concentration is generally regarded as a valuable tool in the diagnosis of thyroid dysfunction. This importance has provided the impetus for the significant improvement in assay methodology that has occurred in the past. The advent of monospecific antiserum and the discovery of blocking agents to the tT4 binding serum proteins have enabled the development of procedurally simple radioimmunoassay.[1,2]

This microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method a sample (Calibrators, Controls and Patient Specimen) is added to the streptavidin coated microwells of a 96 well microtiter plate followed by Enzyme (HRP) labeled tT4 analog and biotin labeled purified Anti-T4 specific sheep IgG. The competition occurs between the varying amounts of tT4 in the sample and fixed amount of tT4-derivative for a fixed number of binding sites on the antibody.

After the completion of the required incubation period, the antibody bound tT4-enzyme analog is separated from the unbound tT4-enzyme conjugate by aspiration or decantation. 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 thyroxine concentration permits construction of a graph of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with tT4 concentration.

3.0 PRINCIPLE

Competitive Enzyme Immunoassay (Total T4) – Type 7
The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen derivative, native antigen and a substrate that produces color.

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} = Anti-T4-IgG labeled with biotin (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.
$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin}_{CW} \Rightarrow$ <u>immobilized complex</u>
$\underline{Streptavidin}_{CW}$ = Streptavidin immobilized on well
<u>Immobilized complex</u> = sandwich complex bound to the solid surface

The enzyme activity in the antibody-bound fraction, measured by reaction with luminol, 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. Strept T4 Calibrators – 1ml/vial - Icons A-F
Six (6) vials of serum reference for Thyroxine at concentrations of 0 (**A**), 2.0 (**B**), 5.0 (**C**), 10.0 (**D**), 15.0(**E**) and 25.0(**F**)µg/dl. A preservative has been added. Store at 2-8°C.
For SI units: µg/dl x 12.9 = nmol/L

B. Strept T4 Enzyme Reagent – 1ml/vial - Icon Ⓔ
One (1) vial of tT4 Analog-horseradish peroxidase (HRP) conjugate in an albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C

C. S-T3/T4 Buffer – 13ml/vial - Icon Ⓑ
One (1) vial reagent contains buffer, red dye, preservative, and binding protein inhibitors. Store at 2-8°C.

D. Strept T4 Biotin Reagent – 7 ml/vial – Icon ∇
One (1) vial contains biotinylated anti-thyroxine (sheep) reagent in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.

E. Streptavidin Coated Plate – 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.

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

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

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

I. Stop Solution – 8ml/vial - Icon STOP
One (1) vial of stop solution contains a strong acid (1N HCL). Store at 2-8°C.

J. 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 2-8°C. 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 0.025, 0.050 & 0.100ml (25, 50 & 100µl) volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100 & 0.350ml (100 & 300µl) volumes with a precision of better than 1.5%.
3. Adjustable volume (20-200µl) and (200-1000µl) dispenser(s) for conjugate and substrate dilutions.
4. Microplate washer or a squeeze bottle (optional).
5. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
6. Test tubes for dilution of enzyme conjugate and substrate A and B.
7. Absorbent Paper for blotting the microplate wells.
8. Plastic wrap or microplate covers for incubation steps.
9. Vacuum aspirator (optional) for wash steps.
10. Timer.
11. 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 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 specimens shall be blood, serum or plasma 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 redtop venipuncture tube with or without additives (for serum) or evacuated tube(s) containing EDTA or heparin (for plasma). Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma 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, 0.050ml (50µl) of the specimen is required for tT4.

7.0 QUALITY CONTROL

Each laboratory should assay 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. **Working Enzyme Reagent (Total T4 SBS)**
   Dilute the Total T4 SBS Enzyme Reagent 1:11 with sT3/T4 buffer in a suitable container. For example, dilute 0.080ml (80µl) of conjugate with 0.8ml (800µl) of buffer for 16 wells (a slight excess of solution is made). This reagent should be used within twenty-four hours for maximum performance of the assay. Store at 2-8°C.
   General Formula:
   Amount of Buffer required = Number of wells ∗ 0.05
   Quantity of tT4 Enzyme necessary = Number of wells ∗ 0.005
   i.e. = 16 x 0.05 = 0.8ml (sT3/T4 Buffer) and
   16 x 0.005 = 0.08ml (80µl) (tT4 Enzyme Reagent).
2. **Wash Buffer**
   Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store the diluted solution at 2-30°C for up to 60 days.
3. **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, serum 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 calibrator, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.025ml (25µl) of the appropriate serum calibrator, control or specimen into the assigned well for Total T4.
3. Add 0.050ml (50µl) of Working Enzyme Reagent solution to the appropriate wells (see Reagent Preparation Section).
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Add 0.050ml (50µl) of biotinylated Total T4 specific antibody conjugate solution to the appropriate wells.
6. Swirl the microplate gently for 20-30 seconds to mix and cover.
7. Incubate 60 minutes at room temperature.
8. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
9. 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.**
10. Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). **Always add reagents in the same order to minimize reaction time differences between wells.**

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

11. Incubate at room temperature for fifteen (15) minutes.
12. Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds. **Always add reagents in the same order to minimize reaction time differences between wells.**
13. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm. **The results should be read within thirty (30) minutes of adding the stop solution.**

Note: For reassaying specimens with concentrations greater than highest calibrator, dilute 0.0125ml (12.5µl) tT4 of the specimen and 0.0125ml (12.5µl) tT4 of the 0 serum reference into the sample well (this maintains a uniform protein concentration). Multiply the readout value by 2 to obtain the thyroxine concentration.

10.0 RESULTS

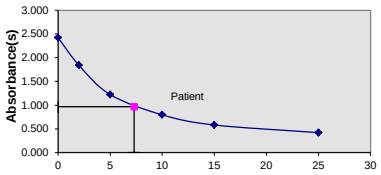
A dose response curve is used to ascertain the concentration of thyroxine in unknown specimens.
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding tT4 in ug/dl on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Connect the points with a best-fit curve (Figures 1).
4. To determine the concentration of tT4 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 ug/dl tT4) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance 0.963 intersects the calibrator curve at 7.3 ug/dl (Figure1).

Note: Computer data reduction software designed for ELISA assay 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 (A)	Mean Abs (B)	Value (µg/dl)
Cal A	A1	2.451	2.419	0
	B1	2.387		
Cal B	C1	1.845	1.839	2
	D1	1.832		
Cal C	E1	1.229	1.221	5
	F1	1.213		
Cal D	G1	0.811	0.795	10
	H1	0.779		
Cal E	A2	0.582	0.581	15
	B2	0.580		
Cal F	C2	0.440	0.419	25
	D2	0.398		
Patient	E2	0.960	0.963	7.3
	F2	0.965		

Figure 1



*The data presented in Example 1 and Figure 1 is illustrative only and **should not** be used in lieu of calibration 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 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 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.
7. Total serum thyroxine concentration is dependent upon a multiplicity of factors: thyroid gland function and its regulation, thyroxine binding globulin (TBG) concentration, and the binding of thyroxine to TBG.[3,4] Thus, total thyroxine concentration alone is not sufficient to assess clinical status.
8. Total serum thyroxine values may be elevated under conditions such as pregnancy or administration of oral contraceptives. A T3U uptake test may be performed to estimate the relative TBG concentration in order to determine if the elevated T4 is caused by TBG variation.
9. A decrease in total thyroxine values is found with protein-wasting diseases, certain liver diseases and administration of testosterone, diphenylhydantoin or salicylates. A table of interfering drugs and conditions, which affect total thyroxine values, has been compiled by the Journal of the American Association of Clinical Chemists.

"NOT INTENDED FOR NEWBORN SCREENING"

13.0 EXPECTED RANGES OF VALUES

A study of euthyroid adult population was undertaken to determine expected values. The mean (R) values, standard deviations (**σ**) and expected ranges (±2**σ**) are presented in Table 1

.

TABLE 1

	Male	Female *
Mean (X)	7.6	8.2
Std Dev (σ)	1.6	1.7
Expected Ranges (±2σ)	4.4 – 10.8	4.8 – 11.6
Number	42	58

*Normal patients with high TBG levels were **not** excluded except if pregnant.

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 Total T4 SBS AccuBind® ELISA Test System were determined by analyses on three different levels of pool control sera. The number, mean values, 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 (in µg/dl)

Sample	N	X	σ	CV
Low	16	3.1	0.21	6.7%
Normal	16	8.9	0.27	3.0%
High	16	16.5	0.73	4.4%

TABLE 3
Between Assay Precision (in µg/dl)

Sample	N	X	σ	CV
Low	10	3.0	0.25	8.3%
Normal	10	8.7	0.32	3.7%
High	10	16.3	0.69	4.2%

*As measured in ten experiments in duplicate over a ten day period.

14.2 Sensitivity

The Total T4 SBS AccuBind® ELISA Test System procedure has a sensitivity of 0.4ug/dl. The sensitivity was ascertained by determining the variability of the 0 ug/dl serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy

The Total T4 SBS AccuBind® ELISA Test System was compared with a reference method. The least square regression equation and the correlation coefficient were computed for the ELISA in comparison with the reference method. The data obtained are displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method	8.07	y = 0.39+0.952(x)	0.934
Reference	8.06		
	Range of values 0.8 – 25		
	Number: 131		

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 antibodies used to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of thyroid hormone needed to displace the same amount of tracer.

Substance	Cross Reactivity	Concentration
l–Thyroxine	1.0000	-
d-Thyroxine	0.9800	10µg/dl
d-Triiodothyronine	0.0150	100µg/dl
l–Triiodothyronine	0.0300	100µg/dl
Iodothyrosine	0.0001	100µg/ml
Diiodothyrosine	0.0001	100µg/ml
Diiodothyronine	0.0001	100µg/ml

15.0 REFERENCES

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Revision: 4 Date: 2019-Jul-16 **DCO: 1353**
MP8225 **Product Code: 8*225-300***

Size		96(A)	192(B)	480(D)	960(E)
Reagent (fill)	A)	1ml set	1ml set	2ml set	2ml set x2
	B)	1 (1.5ml)	1 (1.5ml)	1 (8ml)	2 (8ml)
	C)	1 (13ml)	2 (13ml)	1(60ml)	2 (60ml)
	D)	1 (7ml)	2 (7ml)	1 (30ml)	2 (30ml)
	E)	1 plate	2 plates	5 plates	10 plates
	F)	1 (20ml)	1 (20ml)	1 (60ml)	2 (60ml)
	G)	1 (7ml)	2 (7ml)	1 (30ml)	2 (30ml)
	H)	1 (7ml)	2 (7ml)	1 (30ml)	2 (30ml)
	I)	1 (8ml)	2 (8ml)	1 (30ml)	2 (30ml)

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

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(EN 980/ISO 15223)