Monobind Inc.
Lake Forest, CA 92630, USA

SBS
Streptavidin-Biotin System
CLIA Microwells

Total Thyroxine (T4-SBS) Test System
Product Code: *8275-300*

1.0 INTRODUCTION

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

2.0 SUMMARY AND EXPLANATION

Measurement of serum thyroxine concentration is generally regarded as an important *in-vitro* diagnostic test for assessing thyroid function. This importance has provided the impetus for the significant improvement in assay methodology that has occurred in the last three decades. This procedural evolution can be traced from the empirical protein bound iodine (PBI) test[1] to the theoretically sophisticated radioimmunoassay.[2]

This microplate 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 microplate followed by Tracer (HRP) labeled tT4 derivative and biotin labeled purified anti-T4 specific sheep IgG. A competition occurs between the varying amounts of tT4 in the sample and fixed amount of tT4 analog for a fixed number of binding sites on the antibody.

After the completion of the required incubation period, the antibody bound T4-enzyme conjugate is separated from the unbound T4-enzyme derivative 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 light.

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 thyroxine concentration.

3.0 PRINCIPLE

Competitive Enzyme Immunoassay – Type 7
The essential reagents required for a chemiluminescence immunoassay include antibody, enzyme-antigen conjugate, native antigen and a substrate that produces light.

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.

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin}_{CW} \Rightarrow$ 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 measured by reaction with a suitable substrate to produce light, which 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. Combi-CAl® T3/T4/TSH Calibrators -- 1ml/vial - Icons A-F
Six (6) vials of serum reference for triiodothyronine 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: ng/ml x 1.287 = µmol/L

B. T4 Strep Tracer Reagent – 1.5ml/vial - Icon Ⓔ
One (1) vial contains 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 Tracer Buffer - 13ml/vial - Icon Ⓑ
One (1) vial contains buffer, red dye, preservative, and binding protein inhibitors. Store at 2-8°C.

D. T4 Strep 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. Light Reaction Wells – 96 wells – Icon ⇓
One 96-well microplates 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. Signal Reagent A –7 ml/vial - Icon S[A]
One (1) vial contains Luminol in buffer. Store at 2-8°C. See "Reagent Preparation."

H. Signal Reagent 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. 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 96-well microplate. For other kit configurations, please refer to the table at the end of this insert.

4.1 Required but not provided:
1. Pipette capable of delivering 0.025ml (25µl) volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.50, 0.100ml & 0.350ml (50, 100 & 350µl) volumes with a precision of better than 1.5%.
3. Adjustable volume (20-200µl) and (200-1000µl) dispenser(s) for conjugate dilutions.
4. Microplate washer or a squeeze bottle (optional).
5. Microplate Luminometer
6. Test tubes for dilution of enzyme conjugate
7. Absorbent Paper for blotting the microplate wells.
8. Plastic wrap or microplate cover 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. Collect sample(s) by venipuncture in ten (10) ml silicone evacuated tube(s) or evacuated tube(s) containing EDTA or heparin. 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. Separate the red blood cells by centrifugation to use serum or plasma for the t T4 procedure.

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.

Specimen(s) may be refrigerated at 2-8°C for a maximum period of 48 hours. If the specimen(s) can not be assayed within 48 hours, 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. Before assay, allow the specimens to equilibrate to ambient temperature (2-30°C). When assayed in duplicate, 0.100ml (100µl) of the specimen is required.

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. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the standard curve for run-to-run reproducibility. In addition, maximum light intensity 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 Tracer Reagent (T4-SBS)**
Dilute the Strep T4 Tracer Reagent 1:11 with sT3/T4 buffer in a suitable container. For example, dilute 0.080ml (80µl) of conjugate with 0.800ml (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 Total T4 Tracer necessary = # of wells * 0.005
i.e. = 16 x 0.05 = 0.800ml (800µl) (sT3/T4 Buffer) and 16 x 0.005 = 0.08ml (80µl) (Total T4 Tracer Reagent).
2. **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.
3. **Working Signal Reagent Solution -** Store at 2 - 8°C.
Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1ml 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 36 hours after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

Note: 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 reference calibrator, control or specimen into the assigned wells.
3. Add 0.050ml (50µl) of Working Tracer 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 Strep T4 Biotin Reagent to the appropriate wells.
6. Swirl the microplate gently for 20-30 seconds to mix and cover.
7. Incubate 45 minutes at room temperature.
8. Add 0.350ml (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.**
9. Add 0.100ml (100µl) of working signal reagent 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 PLATE AFTER SIGNAL ADDITION

10. Incubate for five (5) minutes in the dark at room temperature
11. Read the Relative Light Units (RLUs) in each well, for minimum 0.5 – 1.0 seconds, using a microplate luminometer. **The results should be read within thirty (30) minutes of adding the signal solution.**

Note 1: For reassaying specimens with concentrations greater than 25 µg/dl, pipet 0.0125ml (12.5µl) of the specimen and 0.0125ml (12.5µl) 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 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of thyroxine in unknown specimens.
1. Record the RLUs obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the RLUs for each duplicate serum reference calibrator versus the corresponding tT4 concentration in µg/dl on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of tT4 for an unknown, locate the average RLUs for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µg/dl) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average RLUs (40596) of the patients' sample intersects the calibration curve at (7.4µg/dl) tT4 concentration (See Figure 1).

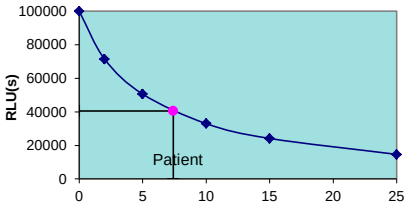
Note 1: Computer data reduction software designed for chemiluminescence assays may also be used for the data reduction. If such software is utilized, the validation of the

software should be ascertained. Duplicates of the unknown may be averaged as indicated (See Figure 1).

EXAMPLE 1 – Total T4

Sample I.D.	Well Number	RLUs	Mean RLUs	Value (µg/ml)
Cal A	A1	100645	100000	0
	B1	99355		
Cal B	C1	72597	71454	2.0
	D1	70311		
Cal C	E1	50446	50567	5.0
	F1	50692		
Cal D	G1	33219	32995	10.0
	H1	32771		
Cal E	A2	23993	24152	15.0
	B2	24313		
Cal F	C2	14616	14650	25.0
	D2	14684		
Ctrl 1	E2	47395	47876	**5.5**
	F2	48357		
Ctrl 2	G2	25583	25228	**14.3**
	H2	24872		
Patient	A3	41113	40596	**7.4**
	B3	40080		

Figure 1



* 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. In addition, the RLUs of the calibrators have been normalized to 100,000 RLUs for the A calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.

11.0 Q.C. PARAMETERS

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

1. The Dose Response Curve should be within established parameters.
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 signal reagent initiates a kinetic reaction, therefore the signal reagent(s) should be added in the same sequence to eliminate any time-deviation during reaction.
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. Multichannel pipettes are recommended for addition of reagents.
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 the test system 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 used 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 tT3 uptake test may be performed to estimate the relative TBG concentration in order to determine if the elevated tT4 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 for the Total T4 SBS AccuLite® CLIA test system method. The mean (R) values, standard deviations (σ) and expected ranges (±2σ) are presented in Table 1.

TABLE 1
Expected Values for the Total T4 SBS Test System (in µg/dl)

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method	7.82	y = 0.28+0.964(x)	0.967
Reference	7.65		
Range of values	0.8 – 25	Number: 86	

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 AccuLite® CLIA test system was 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 values in µg/dl

Sample	N	X	σ	C.V.
Low	16	2.9	0.17	5.8%
Normal	16	9.1	0.22	2.4%
High	16	15.6	0.65	4.2%

TABLE 3
Between Assay Precision values in µg/dl

Sample	N	X	σ	C.V.
Low	10	2.8	0.23	8.2%
Normal	10	8.8	0.52	5.9%
High	10	15.9	0.78	4.9%

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

14.2 Sensitivity

The Total T4 SBS AccuLite® CLIA Test System has a sensitivity of 50 pg. This is equivalent to a sample containing a concentration of 0.2 µg/dl. The sensitivity was ascertained by determining the variability of the 0 µg/dl serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy

The Total T4 SBS AccuLite® CLIA test system method was compared with a reference method. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (the values ranged from 0.5µg/dl – 28µg/dl). The total number of such specimens was 120. The least square regression equation and the correlation coefficient were computed for this method in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
Monobind	8.37	y = 0.18+0.965(x)	0.965
Reference	8.25		

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.

14.3 Specificity

The cross-reactivity (Specificity) of the thyroxine antibody 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 thyroxine 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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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)	2 (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)

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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 Manufacture
Manufacturer
EC REP Authorized Rep in European Country
CE European Conformity