Monobind Inc.
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SBS™
Streptavidin-Biotin System
ELISA Microwells

Total Triiodothyronine (tT3)
Product Code: 8125-300

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

SUMMARY AND EXPLANATION OF THE TEST

Measurement of serum triiodothyronine 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 T3 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 microplate followed by Enzyme (HRP) labeled T3 derivative and biotin labeled purified anti-T3 specific sheep IgG. A competition occurs between the varying amounts of T3 in the sample and fixed amount of T3 analog for a fixed number of binding sites on the antibody.

After the completion of the required incubation period, the antibody bound T3-enzyme conjugate is separated from the unbound T3-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 color.

The employment of several serum references of known triiodothyronine 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 T3 concentration.

PRINCIPLE

Competitive Enzyme Immunoassay (tT3) – Type 7

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate, 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-T3-IgG labeled with biotin (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)
$AgAb_{Btn}$ = Antigen-Antibody Complex

$^{Enz}Ag\ Ab_{Btn}$ = 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.

$$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin_{CW}} \Rightarrow \underline{\text{immobilized complex}}$$

$\underline{Streptavidin_{CW}}$ = 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 color, 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.

MATERIALS

Materials Provided:

A. T3 Calibrators – 1ml/vial - Icons A-F
Six (6) vials of serum reference for triiodothyronine at concentrations of 0 (**A**), 0.5 (**B**), 1.0 (**C**), 2.5 (**D**), 5.0(**E**) and 7.5(**F**) ng/ml. Store at 2-8°C. A preservative has been added.

For SI units: ng/ml x 1.536 = nmol/L

B. T3 Enzyme Reagent – 1.5ml/vial - Icon (E)
One (1) vial of T3 Analog-horseradish peroxidase (HRP) conjugate in an albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C

C. sT3/T4 Buffer – 13ml - Icon (B)
One (1) bottle reagent contains buffer, red dye, preservative, and binding protein inhibitors. Store at 2-8°C.

D. T3 Biotin Reagent – 7 ml/vial
One (1) vial contains biotinylated anti-triiothyronine (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 - Icon
One (1) vial contains a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

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

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

I. Stop Solution – 8ml/vial - Icon (STOP)
One (1) bottle of stop solution contains a strong acid (1N HCL). Store at 2-30°C.

J. 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.

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.

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.
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, 0.1ml of the specimen is required for tT3.

MATERIALS

Required But Not Provided:

1. Pipette capable of delivering 25µl , 50µ and 100µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml 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.

REAGENT PREPARATION

1. **Working Tracer** - **T3-enzyme Conjugate Solution**

Dilute the T3Enzyme Reagent 1:11 with sT3/T4 buffer in a suitable container. For example, dilute 80µl of conjugate with 0.8ml 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 T3 Enzyme necessary = # of wells * 0.005

i.e. = 16 x 0.05 = 0.8ml (sT3/T4 Buffer) and 16 x 0.005 = 0.08ml (80µl) (T3 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 room temperature (20-27°C) for up to 60 days.

3. **Working Substrate Solution**

Pour the contents of the vial labeled Solution 'A' into the vial labeled Solution 'B'. Mix and store at 2-8°C. Use within 60 days. Or for longer periods of usage determine the amount of reagent needed and prepare by mixing equal portions of Substrate A and Substrate B in a suitable 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).

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).

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 0.050 ml (50µl) of the appropriate serum reference, control or specimen into the assigned wells.
3. Add 0.050 ml (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 0.050 ml (50µl) of biotinylated tT3 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 300µl 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 25µl tT3of the specimen and 25µl tT3of 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.

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.

RESULTS

A dose response curve is used to ascertain the concentration of tT3 in unknown specimens.

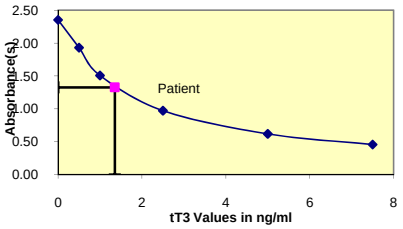
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding tT3 in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Connect the points with a best-fit curve (Figures 1-3).
4. To determine the concentration of tT3 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 from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance 1.328 (intersects the calibrator curve at 1.35 ng/ml (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.

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (ng/ml)
Cal A	A1	2.302	2.352	0
	B1	2.401		
Cal B	C1	1.978	1.930	0.5
	D1	1.930		
Cal C	E1	1.551	1.507	1.0
	F1	1.462		
Cal D	G1	0.972	0.972	2.5
	H1	0.966		
Cal E	A2	0.634	0.619	5.0
	B2	0.604		
Cal F	C2	0.465	0.455	7.5
	D2	0.447		
Patient	E2	1.305	1.328	**1.35**
	F2	1.350		

Figure 1



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.

LIMITATIONS OF PROCEDURE

A. Assay Performance

1. It is important that the time of reaction in each well is held constant for reproducible results.
2. Pipetting of samples 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. Addition of the substrate solution initiates a kinetic reaction, 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.
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.

B. Interpretation

1. 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.
2. Total serum T3 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 triiodothyronine concentration alone is not sufficient to assess clinical status.
3. Total serum T3 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 tT3 is caused by TBG variation.
4. A decrease in total T3 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 T3 values, has been compiled by the Journal of the American Association of Clinical Chemists.

"NOT INTENDED FOR NEWBORN SCREENING"

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 I - Expected Values

Mean (X)	1.250
Standard Deviation (σ)	0.375
Expected Ranges (±2σ)	0.50 – 2.00
Number	105

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 Monobind tT3 SBS™ AccuBind™ ELISA procedure 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 (in ng/ml)

Sample	N	X	σ	C.V.
Low	16	0.78	0.06	7.9%
Normal	16	1.92	0.10	5.4%
High	16	3.55	0.14	3.9%

TABLE 3
Between Assay Precision (in ng/ml)

Sample	N	X	σ	C.V.
Low	10	0.76	0.07	8.9%
Normal	10	1.85	0.13	6.7%
High	10	3.43	0.16	4.5%

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

B. Accuracy

The Monobind tT3 SBS™ AccuBind™ ELISA was compared with a reference immunometric method. The least square regression equation and the correlation coefficient were computed for the ELISA in comparison with the reference methods. The data obtained are displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method	1.62	y = 3.8 + 0.947(x)	0.987
Reference	1.68		
Range of values	0.15 – 8.0		
Number: 120			

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.

C. Sensitivity

The tT3 procedure has a sensitivity of 0.04 ng/ml. The sensitivity was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

D. 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	
I-Triiodothyronine	1.0000	-
I-Thyroxine	< 0.0002	10µg/ml
Iodothyrosine	< 0.0001	10µg/ml
Diiodothyrosine	< 0.0001	10µg/ml
Diiodothyronine	< 0.0001	10µg/ml
Phenylbutazone	< 0.0001	10µg/ml
Sodium Salicylate	< 0.0001	10µg/ml

REFERENCES

1. Gharib H., Ryan R.J, Mayberry W.E, & Hockett T., "Radioimmunoassay for Triiodothyronine (T3): Affinity and Specificity of Antibody for T3", *J Clinical Endocrinol*, **33,**509 (1971).
2. Chopra I.J., Ho R.S.,& Lam R. "An improved radio-immunoassay of triiodothyronine in human serum", *J. Lab Clinical Med,* **80,** 729 (1971).
3. Young DS, Pestaner LC and Gilberman , "Effects of Drugs on Clinical Laboratory Tests", *Clinical Chemistry* **, 21,** 3660 (1975).
4. Sterling L., "Diagnosis and Treatment of Thyroid Disease", *Cleveland CRC Press*, **p. 9-51** (1975).
5. Braverman LE.:"Evaluation of thyroid status in patients with thyrotoxicosis", *Clin Chem*, **42,** 174-178 (1996).
6. Braverman LE, Utigen RD, Eds.: Werner and Ingbar's "The Thyroid – *'A Fundamental and Clinical Text'",* 7th Ed. Philadelphia, Lippinscott-Raven (1996).
7. Comeau L, Pianan U, Leo-Mensah T, et al,"An automated chemiluminescent immunoassay test for total triiodothyronine", *Clin Chem*, **37,** 941 (1991).
8. Chopra IJ.:"Radioimmunoassay of iodothyronines-*Handbook of Radioimmunoassay*", G.E. Abraham Ed, New York, Marcel Dekker, Inc**.** (1977).
9. Kozwich D, Davis G, Sockol C,"Development of total triiodothyronine enzyme immunoassay in microtiter plate format", *Clin Chem***, 37,** 1040 (1991).
10. Papanastasiou-Diamandi A., Khosravi M.:"Total T3 (triiodothyronine) measurement in serum by time resolved fluorescence immunoassay", *Clin Chem***, 7,** 1029 (1991).

Revision: 1 **Date: 070709** **DCO: 0153**

Cat #: 8*125-300*

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)	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)
	I)	1 (8ml)	2 (8ml)	1 (30ml)	2 (30ml)

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