



Total Thyroxine (T4) Test System Product Code: 225-300

1.0 INTRODUCTION

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

2.0 SUMMARY AND EXPLANATION OF THE TEST

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 enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, serum reference, patient specimen, or control is first added to a microplate well. Enzyme-T4 conjugate is added, and then the reactants are mixed. A competition reaction results between the enzyme conjugate and the native thyroxine for a limited number of antibody combining sites immobilized on the well.

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

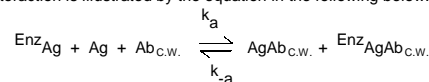
The employment of several serum references of known thyroxine concentration permits construction of a graph of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with thyroxine concentration.

3.0 PRINCIPLE

Competitive Enzyme Immunoassay (TYPE 5)

The essential reagents required for a solid phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen.

Upon mixing immobilized 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 insolubilized binding sites. The interaction is illustrated by the equation in the following below.



$\text{Ab}_{\text{C.W.}}$ = Monospecific Immobilized Antibody (Constant Quantity)

Ag = Native Antigen (Variable Quantity)

EnzAg = Enzyme-antigen Conjugate (Constant Quantity)

$\text{AgAb}_{\text{C.W.}}$ = Antigen-Antibody Complex

$\text{EnzAgAb}_{\text{C.W.}}$ = Enzyme-antigen Conjugate -Antibody Complex

k_a = Rate Constant of Association

k_a = Rate Constant of Disassociation

$K = k_a / k_a$ = Equilibrium Constant

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. 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

A. 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. Store at 2-8°C. A preservative has been added. **For SI units: µg/dl x 12.9 = nmol/L**

B. T4-Enzyme Reagent – 1.5ml/vial - Icon E

One (1) vial of thyroxine-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.

C. T3/T4 Conjugate Buffer – 13 ml - Icon B

One (1) bottle reagent containing buffer, red dye, preservative, and binding protein inhibitors. Store at 2-8°C.

D. T4 Antibody Coated Plate – 96 wells - Icon Y

One 96-well microplate coated with sheep anti-thyroxine serum and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate – 20ml - Icon D

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) 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 (H₂O₂) in buffer. Store at 2-8°C.

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

One (1) bottle containing a strong acid (1.0N 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 2-8°C. Kit and component stability are identified on the label.**

Note 3: Above reagents are for a 96-well microplate. For other kit configurations, see table at the end of this IFU.

4.1 Required But Not Provided:

1. Pipette capable of delivering 25µl & 50µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.350ml volumes with a precision of better than 1.5%.
3. Adjustable volume (20-200µl) and (200-1000µl) dispenser(s) for conjugate and substrate preparation
4. Microplate washer or a squeeze bottle (optional).
5. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
6. Test tubes for preparation 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 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 (for serum) or evacuated tube(s) containing EDTA or heparin. 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 use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml 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. 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 Reagent A = T4-Enzyme Conjugate Solution

Dilute the T4-enzyme conjugate 1:11 with Total T3/T4 conjugate buffer in a suitable container. For example, dilute 160µl of conjugate with 1.6ml 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 * 0.1
Quantity of T4 Enzyme necessary = # of wells * 0.01
i.e. = 16 x 0.1 = 1.6ml for Total T3/T4 conjugate buffer
16 x 0.01 = 0.16ml (160µl) for T4 enzyme conjugate

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 Substrate Solution

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 references and controls to room temperature (20 - 27°C).

****Test Procedure should be performed by a skilled individual or trained professional****

1. Format the microplate's 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.**
2. Pipette 0.025 ml (25µl) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 ml (100µl) of Working Reagent A, T4 Enzyme Reagent to all wells (see Reagent Preparation Section).
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 60 minutes at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
7. Add 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.**
8. 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.**
9. **DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION**
9. Incubate at room temperature for fifteen (15) minutes.
10. 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.**
11. 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: For reassaying specimens with concentrations greater than 25 µg/dl, pipet 12.5µl of the specimen and 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.

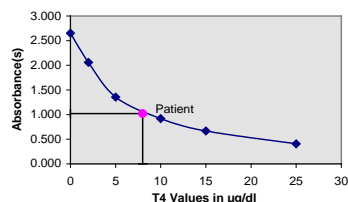
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding T4 concentration in µg/dl on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Connect the points with a best-fit curve.
4. To determine the concentration of T4 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 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 absorbance (1.022) intersects the standard curve at (8 µg/dl) T4 concentration (See Figure 1).

Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. **If such software is utilized, the validation of the software should be ascertained.**

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (µg/dl)
Cal A	A1	2.648	2.650	0
	B1	2.652		
Cal B	C1	2.090	2.060	2
	D1	2.031		
Cal C	E1	1.344	1.355	5
	F1	1.366		
Cal D	G1	0.897	0.918	10
	H1	0.939		
Cal E	A2	0.676	0.668	15
	B2	0.659		
Cal F	C2	0.408	0.406	25
	D2	0.404		
Ctrl 1	E2	1.425	1.435	4.6
	F2	1.383		
Ctrl 2	G2	0.611	0.613	16.3
	H2	0.608		
Patient	A3	0.984	1.022	8.0
	B3	1.060		

EXAMPLE 1

Figure 1



The data presented in Example 1 and Figure 1 are for illustration only and **should not** be used in lieu of a standard 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:

- The absorbance (OD) of calibrator 0 µg/dl should be ≥ 1.3 .
- 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 is available on request from Monobind Inc.

12.1 Assay Performance

- It is important that the time of reaction in each well is held constant to achieve reproducible results.
- Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
- Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
- If more than one (1) plate is used, it is recommended to repeat the dose response curve.
- 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.
- 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.
- Use components from the same lot. No intermixing of reagents from different batches.
- Patient specimens with T4 concentrations greater than 35 µg/dl may be diluted ½ with the '0' serum reference into the sample well; pipet 12.5µl of the specimen and 12.5µl of the '0' serum reference in the sample well to maintain a uniform protein concentration. The sample's concentration is obtained by multiplying the result by the dilution factor, 2.
- 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.
- 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.
- 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.
- 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

- Measurements and interpretation of results must be performed by a skilled individual or trained professional.**
- 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.
- For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
- 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.**
- 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.
- 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.**
- Total serum thyroxine values may be elevated under conditions such as pregnancy or administration of oral contraceptives. A T3 uptake test may be performed to estimate the relative TBG concentration in order to determine if the elevated T4 is caused by TBG variation.
- 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 T4 AccuBind™ ELISA Test System. The mean (X) values, standard deviations (σ) and expected ranges (±2 σ) are presented in Table 1.

Table 1 Expected Values for the T4 ELISA Test System (in µg/dl)			
	Male	Female *	
Number of Specimens	42	58	
Mean (X)	7.6	8.2	
Std.Dev (σ)	1.6	1.7	
Expected Ranges (±2 σ)	4.4 – 10.8	4.8 – 11.6	

*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 precisions of the T4 AccuBind™ ELISA test system were determined by analyses on three different levels of pool control sera. The number (N), mean values (X), standard deviation (σ) and coefficient of variation (C.V.) 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	20	6.87	0.16	2.3
Normal	20	9.95	0.16	1.6
High	20	13.13	0.17	1.3

Table 3
Between Assay Precision (Values in µg/dl)

Sample	N	X	σ	C.V.%
Low	20	5.76	0.37	6.3
Normal	20	9.41	0.57	6.1
High	20	16.18	1.21	7.5

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

14.2 Sensitivity

The T4 AccuBind™ ELISA test system has a sensitivity of 3.2ng/well. This is equivalent to a sample containing a concentration of 0.128 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 tT4 AccuBind™ ELISA method was compared with a coated tube radioimmunoassay method. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (The values ranged from 0.8µg/dl – 25µg/dl). The total number of such specimens was 131. The least square regression equation and the correlation coefficient were computed for the T4 AccuBind™ ELISA method in comparison with the reference method. The data obtained is displayed in 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		

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 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 conjugate.

Substance	Cross Reactivity	Concentration
l-Thyroxine	1.0000	-
d-Thyroxine	0.9800	10µg/dl

d-Triiodothyronin	0.0150	100µg/dl
e		
l-Triiodothyronine	0.0300	100µg/dl
lodothyrosine	0.0001	100µg/ml
Diiodothyrosine	0.0001	100µg/ml
Diiodothyronine	0.0001	100µg/ml

15.0 REFERENCES

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Cat #: 225-300

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

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