



Free Thyroxine (Free T4) Test System
Product Code: 1225-300

1.0 INTRODUCTION

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

2.0 SUMMARY AND EXPLANATION OF THE TEST

Thyroxine, the principal thyroid hormone, circulates in blood almost completely bound to carrier proteins. The main carrier is thyroxine-binding globulin (TBG). However, only the free (unbound) portion of thyroxine is responsible for the biological action. Further, the concentrations of the carrier proteins are altered in many clinical conditions, such as pregnancy. In normal thyroid function as the concentrations of the carrier proteins alters, the total thyroxine level changes so that the free thyroxine concentration remains constant. Thus, measurements of free thyroxine concentrations correlate better with clinical status than total thyroxine levels.

The increase in total thyroxine associated with pregnancy, oral contraceptives and estrogen therapy occasionally result in total T4 levels over the limits of normal while the free thyroxine concentration remains in the normal reference range. Masking of abnormal thyroid function can also occur in both hyper and hypothyroid conditions by alterations in the TBG concentration. The total T4 can be elevated or lowered by TBG changes such that the normal reference levels result. The free thyroxine concentration can help in uncovering the patient's actual clinical status.

In this method, serum reference, patient specimen, or control is first added to a microplate well. Enzyme-T4 conjugate (analog method) is added and the reactants are mixed. A competition reaction results between the enzyme conjugate and the free thyroxine for a limited number of antibody combining sites immobilized on the well.

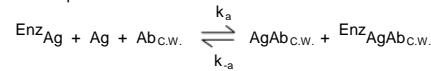
After the completion of the required incubation period, the antibody bound enzyme-thyroxine conjugate is separated from the unbound enzyme-thyroxine conjugate via a wash step. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce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

The employment of several serum references of known free 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 free thyroxine concentration.

3.0 PRINCIPLE

Competitive Enzyme Immunoassay, Analog Method for Free-T4 (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 free antigen, a competition reaction results between the native free antigen and the enzyme-antigen conjugate for a limited number of insolubilized binding sites. The interaction is illustrated by the following equation:



- Ab_{c.w.} = Monospecific Immobilized Antibody (Constant Quantity)
- Ag = Native Antigen (Variable Quantity)
- EnzAg = Enzyme-antigen Conjugate (Constant Quantity)
- AgAb_{c.w.} = Antigen-Antibody Complex
- EnzAgAb_{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 free 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. Free T4 Calibrators – 1 ml/vial - Icons A-F**
Six (6) vials of human serum based reference calibrators for free thyroxine at **approximate*** concentrations of 0 (A), 0.40 (B), 1.25 (C), 2.10 (D), 5.00 (E) and 7.40 (F) ng/dl. Store at 2-8°C. A preservative has been added. For SI units use the conversion factor 12.9 to convert ng/dl to pmol/L.
* Exact levels are given on the labels on a lot specific basis.
- B. Free T4- Enzyme Reagent – 13 ml/vial - Icon E**
One (1) vial of thyroxine-horseradish peroxidase (HRP) conjugate in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.
- C. Free T4 Antibody Coated Plate – 96 wells - Icon Y**
One 96-well microplate coated with anti-thyroxine serum and packaged in an aluminum bag with a drying agent. Store at 2-8°C.
- D. 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.
- E. Substrate A – 7 ml/vial - Icon S^A**
One (1) bottle containing tetramethylbenzidine (TMB) in acetate buffer. Store at 2-8°C.
- F. Substrate B – 7 ml/vial - Icon S^B**
One (1) bottle containing hydrogen peroxide (H₂O₂) in acetate buffer. Store at 2-8°C.
- G. Stop Solution – 8 ml/vial - Icon S^{OP}**
One (1) bottle containing a strong acid (1N HCl). Store at 2-8°C.
- H. Product Instructions.**

- 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. **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 IFU.

4.1 Materials Required But Not Provided:

- 1. Pipette capable of delivering 50µl & 100µl 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. Microplate washers or a squeeze bottle (optional).
- 4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
- 5. Absorbent Paper for blotting the microplate wells.
- 6. Plastic wrap or microplate cover for incubation steps.
- 7. Vacuum aspirator (optional) for wash steps.
- 8. Timer.
- 9. 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 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.

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 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. 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 use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml 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. 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. **Wash Buffer**
Dilute contents of wash concentrate to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.
- 2. **Working Substrate Solution**
Pour the contents of the plastic 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 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**

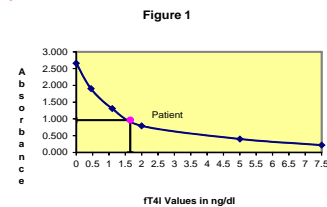
- 2. Pipette 0.050 ml (50µl) of the appropriate serum reference, control or specimen into the assigned well.
- 3. Add 0.100 ml (100µl) of FT4 Enzyme Reagent to all wells.
- 4. Swirl the microplate gently for 20-30 seconds to mix and cover.
- 5. Incubate 60 minutes at room temperature.
- 6. Discard the 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.**
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.**

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of free T4 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 Free T4 concentration in ng/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 Free 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 ng/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 (0.964) intersects the dose response curve at (1.65ng/dl) free T4 concentration (See Figure 1).

*The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a standard curve prepared with each assay. **Assigned values for calibrators are lot specific.**



EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value* (ng/dl)
Cal A	A1	2.658	2.612	0.00
	B1	2.566		
Cal B	C1	1.919	1.900	0.45
	D1	1.880		
Cal C	E1	1.339	1.306	1.10
	F1	1.273		

Cal D	G1	0.769	0.790	2.00
	H1	0.811		
Cal E	A2	0.396	0.400	5.00
	B2	0.404		
Cal F	C2	0.215	0.217	7.40
	D2	0.219		
Ctrl 1	E2	1.827	1.835	0.50
	F2	1.843		
Ctrl 2	G2	0.541	0.557	2.70
	H2	0.573		
Patient	A3	0.951	0.964	1.65
	B3	0.976		

Note 1: 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.**

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 ng/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.
- 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.
- If a patient, for some reason, reads higher than the highest calibrator report as such (e.g. > 7.4 ng/dl). **Do not try to**

dilute the sample. TBG variations in different matrices will not allow Free T4 hormone to dilute serially.

- Serum free-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, free-Thyroxine concentration alone is not sufficient to assess the clinical status.
- Serum free-thyroxine values may be elevated under conditions such as pregnancy or administration of oral contraceptives.
- A decrease in free 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 free Thyroxine values, has been compiled by the Journal of the American Association of Clinical Chemists.
- The interpretation of Free T4 is complicated by a variety of drugs that can affect the binding of T4 to the thyroid hormone carrier proteins or interfere in its metabolism to T3. In severe non-thyroidal illness (NTI) the assessment of thyroid becomes especially difficult. Since the patients in this category may suffer from concomitant primary hypothyroidism or from compensatory secondary hypothyroidism. In cases like these a sensitive TSH evaluation of the patient may be recommended. Please see Monobind Cat# 325-300.
- In rare conditions associated with extreme variations in albumin binding capacity for T4- such as familial dysalbuminemic hyperthyroxinemia (FDH) – direct assessment of Free T4 may be misleading.
- Circulating antibodies to T4 and hormone binding inhibitors may interfere in the performance of the assay.
- Heparin is reported to have in vivo and in vitro effects on free T4 levels. Samples from patients undergoing heparin therapy should be collected well before the administration of the anticoagulant.

"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 Free T4 AccuBind® ELISA test system. The mean (X) values, standard deviations (σ) and expected ranges ($\pm 2\sigma$) are presented in Table 1.

	Adult	Pregnancy
Number of Specimens	89	31
Mean (X)	1.40	1.50
Standard Deviation (σ)	0.30	0.37
Expected Ranges ($\pm 2\sigma$)	0.8 – 2.0	0.76 – 2.24

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 *inter* and *intra* assay precisions of the Free T4 AccuBind® ELISA test system were determined by analyses on three different levels of pooled patient 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.

Sample	N	X	σ	C.V.
Low	20	0.550	0.061	10.98%
Medium	20	1.740	0.074	4.26%
High	20	3.250	0.106	3.25%

In order to validate the *inter*-assay precision of FT4 AccuBind® ELISA test system, one duplicate of each of three pooled sera (low medium and high ranges of the dose response curve) was assayed in 10 assays done over a period of six months that involved five different sets of reagents and three different technicians. An inter-assay precision of 6.01 to 10.81% was obtained.

Sample	N	X	σ	C.V.
Low	10	0.480	0.052	10.81%
Medium	10	1.410	0.085	6.01%
High	10	3.490	0.279	7.90%

14.2 Sensitivity

The Free T4 AccuBind® ELISA test system has a sensitivity of 0.162 ng/dl. The sensitivity was ascertained by determining the variability of the 0 ng/dl serum calibrator and using the 2σ (95% certainty) statistics to calculate the minimum dose.

14.3 Accuracy

The Free T4 AccuBind® ELISA test system was compared with a coated tube radioimmunoassay (RIA) method. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (The values ranged from 0.1ng/dl – 8ng/dl). The total number of such specimens was 197. The least square regression equation and the correlation coefficient were computed for this Free T4 AccuBind® ELISA method in comparison with the predicate method (Table 4).

Method	Mean (x)	Equation	Correlation Coefficient
Monobind EIA "X"	1.56	$y = 0.1034 + 0.9525x$	0.920
Predicate RIA "Y"	1.59		

Only slight amounts of bias between this method and the reference method are indicated by the closeness of the mean values.

14.4 Specificity:

The cross-reactivity of the thyroxine antibody used for Free T4 AccuBind® ELISA to selected substances was evaluated by adding massive amounts of the interfering substance to a serum matrix. The cross-reactivity was calculated by deriving a ratio between doses of interfering substance to dose of thyroxine needed to displace the same amount of the conjugate.

Substance	Cross Reactivity	Concentration n
I-Thyroxine	1.0000	----
d-Thyroxine	0.9800	10µg/dl
d-Triiodothyronine	0.0150	100µg/dl
I-Triiodothyronine	0.0300	100µg/dl
Iodothyrosine	0.0001	100µg/ml
Diiodothyrosine	0.0001	100µg/ml
Diiodothyronine	0.0001	100µg/ml
TBG	N/D	40 µg/ml
Albumin	N/D	40 mg/ml
Phenylbutazone	N/D	10 µg/ml
Phenytol	N/D	40 µg/ml
Salicylates	N/D	500 µg/ml

15.0 REFERENCES

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Cat #: 1225-300

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

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