



## DIAGNOSTIC AUTOMATION, INC.

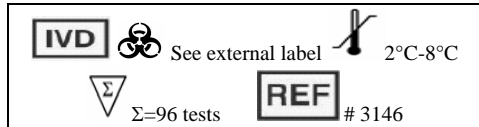
23961 Craftsman Road, Suite E/F,  
Calabasas, CA 91302

Tel: (818) 591-3030 Fax: (818)  
591-8383

[onestep@rapidtest.com](mailto:onestep@rapidtest.com)

[technicalsupport@rapidtest.com](mailto:technicalsupport@rapidtest.com)

[www.rapidtest.com](http://www.rapidtest.com)



### Free Thyroxine (fT4) Catalog No. 3146

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

#### 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.

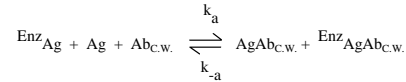
The employment of several serum references of known free thyroxine concentration permits construction of a graph of activity and concentration.

From comparison to the dose response curve, an unknown specimen's activity can be correlated with free thyroxine concentration.

#### PRINCIPLE

##### Competitive enzyme immunoassay - Analog method for free T4

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 followed equation:



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

$\text{Ag}$  = Native Antigen (Variable Quantity)

$\text{EnzAg}$  = Enzyme-antigen Conjugate (Constant Quantity)

$\text{AgAb}_{c.w.}$  = Antigen-Antibody Complex

$\text{EnzAg Ab}_{c.w.}$  = 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.

#### REAGENTS

##### Materials Provided:

A. Human Serum References -- 1 ml/vial -  
Icons A-F

Six (6) vials of human serum based reference calibrators for free thyroxine at approximate\* concentrations of 0 (A), 0.4 (B), 1.1 (C), 2.2 (D), 4.1 (E) and 8.0 (F) ng/dl. Store at 2-8°C. A preservative has been added.

\* Exact levels are given on the labels on a lot specific basis.

For SI units: 1ng/dl x 12.9 =  
pmol/L

B. fT4- Enzyme Reagent – 13 ml/vial

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. Antibody Coated Microplate -- 96  
wells

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

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

E. Substrate A –7 ml/vial

One (1) bottle containing tetramethylbenzidine (TMB) in acetate buffer. Store at 2-8°C.

F. Substrate B – 7 ml/vial

One (1) bottle containing hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in acetate buffer. Store at 2-8°C.

#### G. Stop Solution – 8 ml/vial

One (1) bottle containing a strong acid (1N HCl). Store at 2-8°C.

#### H. 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.

**Note 3:** Above reagents are for a single 96-well microplate.

#### Required But Not Provided:

1. Pipette capable of delivering 50µl 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. 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.

#### 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.

#### 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 red-top venipuncture tube without additives or anti-coagulants. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

#### REAGENT PREPARATION:

##### 1. Wash Buffer

Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store at room temperature 20-27°C for up to 60 days.

##### 2. 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 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 Conjugate solution 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 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.**

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. 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.**

#### 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.

## RESULTS

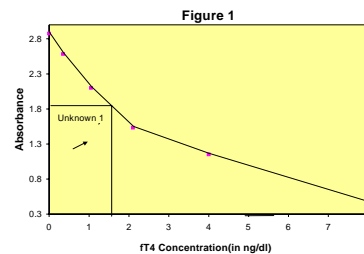
A dose response curve is used to ascertain the concentration of free thyroxine in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot a dose response curve (DRC) using the mean absorbance for each duplicate serum reference versus the corresponding free T4 concentration in ng/dl on linear graph paper.
3. Draw the best-fit curve through the plotted points (See Figure 1).
4. Interpolate the concentrations of unknown control or patient serum from the dose response curve. (See Figure 1).

### EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value* (ng/dl)
Cal A	A1	2.652	2.651	0.00
	B1	2.649		
Cal B	C1	1.991	1.990	0.40
	D1	1.988		
Cal C	E1	1.392	1.390	1.25
	F1	1.387		
Cal D	G1	0.866	0.861	2.10
	H1	0.857		
Cal E	A2	0.396	0.392	5.00
	B2	0.388		

Cal F	C2	0.156	0.161	7.40
	D2	0.167		
Ctrl 1	E2	2.088	2.113	0.34
	F2	2.138		
Ctrl 2	G2	0.671	0.656	3.25
	H2	0.641		
Patient	A3	1.326	1.334	1.36
	B3	1.341		



\*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.**

## 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 0 ng/dl should be  $\geq 1.3$ .
2. Four out of six quality control pools should be within the established ranges.

## LIMITATIONS OF PROCEDURE

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, which 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.

## CLINICAL LIMITATIONS OF FT4 EIA

1. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
2. 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.**

3. 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.
4. Serum free-Thyroxine values may be elevated under conditions such as pregnancy or administration of oral contraceptives.
5. 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.
6. The interpretation of FT4 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.
7. 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.

8. Circulating antibodies to T4 and hormone binding inhibitors may interfere in the performance of the assay.
9. 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"**

**EXPECTED RANGES OF VALUES**

A study of euthyroid adult population was undertaken to determine expected values for the Free T4 EIA Test System. The mean (R) values, standard deviations ( $\sigma$ ) and expected ranges ( $\pm 2\sigma$ ) are presented in Table 1.

**TABLE I**  
Expected Values for the Free T4 EIA Test System (in ng/dl)

	Adult Pregnancy (89 specimens)	
Mean (X)	1.40	1.50
Standard Deviation ( $\sigma$ )		0.30
Expected Ranges ( $\pm 2\sigma$ )		0.8 – 2.0

Each laboratory is advised to establish its own ranges for normal and abnormal populations. These ranges are always dependent upon locale, population,

laboratory, technique and specificity of the method.

**PERFORMANCE CHARACTERISTICS**

**A. Precision**

The *inter* and *intra* assay precision of the FT4 Microplate EIA Test System were determined by analyses on three different levels of pooled patient sera. The number, mean values, standard deviation ( $\sigma$ ) and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

**Intra-Assay Precision**

In order to validate the within-assay precision of Free Thyroxine (FT4) EIA twenty replicates of each of three pooled sera (low medium and high ranges of the dose response curve) were assayed in the same assay. An intra-assay precision of 3.25 to 10.98% was obtained.

**TABLE 2**

	Low [ng/dl]	Medium [ng/dl]	High [ng/dl]
Number (n)	20	20	20
Mean	0.550	1.740	3.250
1 S.D.	0.061	0.074	0.106
% CV	10.98	4.26	3.25

**Inter-Assay Precision:**

In order to validate the inter-assay precision of Free Thyroxine (FT4) EIA 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.

**TABLE 3**  
Inter Assay Precision

	Low [ng/dl]	Medium [ng/dl]	High [ng/dl]
Number (n)	10	10	10
Mean	0.480	1.410	3.490
1 S.D.	0.052	0.085	0.279
% CV	10.81	6.01	7.90

**B. Method Comparison:**

The FT4 Microplate EIA 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 FT4 EIA in comparison with the predicate method (Table 4).

**TABLE 4**  
Linear Regression Analysis.

Method	Mean(x)	Equation
Diagnostic Automation EIA "X"	1.63	Y = 0.0917 + 0.9339*X
Predicate RIA "Y"	1.61	

0.37 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 free thyroxine procedure has a sensitivity of 0.05 ng/dl. The sensitivity was ascertained by determining the variability of the 0 ng/dl serum calibrator and using the  $2\sigma$  (95% certainty) statistics to calculate the minimum dose.

**D. Specificity:**

The cross-reactivity of the thyroxine antibody, used for Free T4 EIA, 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	
l-Thyroxine	1.0000	----
d-Thyroxine	0.9800	10 $\mu$ g/dl
d-Triiodothyronine	0.0150	100 $\mu$ g/dl
l-Triiodothyronine	0.0300	100 $\mu$ g/dl
Iodothyrosine	0.0001	100 $\mu$ g/ml
Diiodotyrosine	0.0001	100 $\mu$ g/ml
Diiodothyronine	0.0001	100 $\mu$ g/ml
TBG	N/D	40 $\mu$ g/ml
Albumin	N/D	40 mg/ml
Phenylbutazone	N/D	10 $\mu$ g/ml
Phenytoin	N/D	40 $\mu$ g/ml
Salicylates	N/D	500 $\mu$ g/ml

## REFERENCES

1. Barker, S.B., "Determination of Protein Bound Iodine." *Journal Biological Chemistry* **173**, 175. (1948)
2. Chopra, I.J., Solomon, D.H., and Ho, R.S., "A Radioimmunoassay of Thyroxine", *J. Clinical Endocrinol* **33**, 865. (1971)
3. Young, D.S., Pestaner, L.C., and Gilberman, U., "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. 19-51*. (1975)
5. Halpern, E.P and Bordens, RW. "Microencapsulated antibodies in radioimmunoassay. Determination of free Thyroxine". *Clinical Chemistry* **Vol. 25**, 1561-1563. (1979)
6. Stjernholm, MR, Alsever, RN and Rudolph, MC. "Thyroid function tests in diphenylhydantoin-treated patients". *Clin. Chem. Vol. 21*, 1388-1392. (1977)
7. Nelson J.C. and Wilcox, RB. "Analytical performance of Free and Total thyroxine assays". *Clin. Chem. Vol. 42*, 146-154. (1996)
8. Midgeley John, EM. "Direct and Indirect Free Thyroxine Assay Methods. Theory and Practice". *Clin. Chem. Vol. 47*, 1353-1363. (2001)
9. Bayer, MF and McDougall, IR. "Radioimmunoassay of free thyroxine in serum: comparison with clinical findings and results of conventional thyroid-function tests". *Clin. Chem. Vol. 26*, 1186-1192. (1980)
10. Anthony, GW, Jackson, RA et.al. "Misleading results from immunoassays of serum free thyroxine in the presence of rheumatoid factor". *Clin. Chem. Vol. 43*, 957-962. (1997)
11. Wosilait, WD. "A theoretical analysis of the distribution of thyroxine among sites on the thyroxine binding globulin, thyroid binding prealbumin and serum albumin". *Res. Comm. Chem. Pathology-Pharmacology* **16**, 541-548. (1977)

Revision Date: 3/15/06