

DRG® Free Thyroxine (fT4) (192 wells) (EIA-4109)


Revised 10 Jan. 2011 rm (Vers. 2.1)


INTENDED USE

The Quantitative Determination of Free Thyroxine Concentration in Human Serum by a Microplate Enzyme Immunoassay.

SUMMARY AND EXPLANATION OF THE TEST

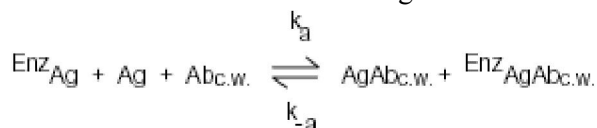
Thyroxine, the principal thyroid hormone, circulates in blood almost completely bound to carrier proteins. The main carrier is thyroxine-binding globulin (TBG). However, only the free (unbound) portion of thyroxine is responsible for the biological action. Further, the concentrations of the carrier proteins 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 (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 followed equation:



Abc.w. = Monospecific Immobilized Antibody (Constant Quantity)

Ag = Native Antigen (Variable Quantity)

Enz_{Ag} = Enzyme-antigen Conjugate (Constant Quantity)

AgAbc.w. = Antigen-Antibody Complex

$\text{Enz}_{\text{Ag}} \text{ Abc.w.}$ = Enzyme-antigen Conjugate -Antibody Complex

k_a = Rate Constant of Association

k_{-a} = Rate Constant of Disassociation

$K = k_a / k_{-a}$ = Equilibrium Constant

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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**

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.

*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 – (2X) 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 -- (2X) 96 wells

One (1) 96-well microplate coated with sheep 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 –(2X) 7 ml/vial

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

F. Substrate B – (2X) 7 ml/vial

One (1) bottle containing hydrogen peroxide (H₂O₂) in acetate buffer. Store at 2-8°C.

G. Stop Solution – (2X) 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 one 96-well microplate.

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**Materials Required But Not Provided**

1. Pipette capable of delivering 50µl and 100 µl 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.

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 nonreactive 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 specimen to separate the serum from the cells. Specimen(s) may be refrigerated at 2-8 ° C for a maximum period of 48 hours. If the specimen can not be assayed within 48 hours, the sample may be stored at temperature of -20° C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100 ml of the specimen is required.

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'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2-8°C.

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.

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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 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. 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.

CALCULATION OF 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 the absorbance for each duplicate serum reference versus the corresponding fT4 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 fT4 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).

Note 1: Computer data reduction software designed for (ELISA) assays may also be used for the data reduction. Duplicates of the unknown may be averaged as indicated.

Note 2: DRG can assist the laboratory in the purchase and implementation of equipment/software to measure and interpret (ELISA) data.

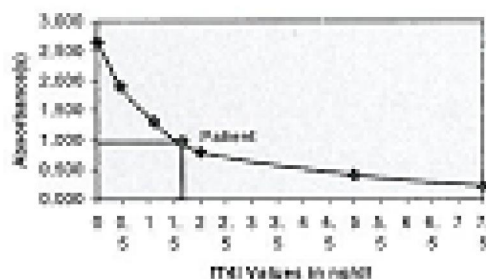
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Figure 1



*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.**

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	0.40
	D1	1.880		
Cal C	E1	1.339	1.306	1.25
	F1	1.273		
Cal D	G1	0.769	0.790	2.10
	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		

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**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 (X) values, standard deviations (σ) 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 (89 specimens)	Pregnancy (31 specimens)
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 tests, 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.

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 ≥ 1.3 .
2. Four out of six quality control pools should be within the established ranges.

RISK ANALYSIS**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. 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 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.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from DRG's instructions may yield inaccurate results.
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.

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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 98/79EC – for this and other devices, made by DRG, can be requested from your distributor.

Interpretation

1. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for determining therapy, particularly if the results conflict with other determinants.
2. For valid tests results, adequate controls and other parameters must be within the listed ranges and assay requirements.
3. If test kits are altered, such as by mixing parts of different kits, which could produce false test result, or if results are incorrectly interpreted, DRG shall have no liability.
4. 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.
5. 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.***
6. 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.
7. Serum free-Thyroxine values may be elevated under conditions such as pregnancy or administration of oral contraceptives.
8. 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.
9. 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.
10. 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.
11. Circulating antibodies to T4 and hormone binding inhibitors may interfere in the performance of the assay.
12. 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”.

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 (s) and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

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In order to validate the intra-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

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

	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	Coefficient
RG EIA “X”	1.56	$Y = 0.1034 + 0.9525 \cdot X$	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. The least square regression equation and correlation coefficient indicates excellent method agreement.

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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 σ (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 μ g/dl
d-Triiodothyronine	0.0150	100 μ g/dl
l-Triiodothyronine	0.0300	100 μ g/dl
Iodothyrosine	0.0001	100 μ g/ml
Diiodotyrosine	0.0001	100 μ g/ml
Diiodothyronine	0.0001	100 μ g/ml
TBG	N/D	40 mg/ml
Albumin	N/D	40 mg/ml
Phenylbutazone	N/D	10 mg/ml
Phenytoin	N/D	40 mg/ml
Salicylates	N/D	500 mg/ml

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