FREE THYROXINE (fT4) ENZYME IMMUNOASSAY TEST KIT
Catalog Number: 6107220

CE

Enzyme Immunoassay for the Quantitative Determination of Free Thyroxine (fT4) Concentration in Human Serum

FOR IN VITRO DIAGNOSTIC USE ONLY

Store at 2 to 8°C.

PROPRIETARY AND COMMON NAMES

Free T4 Enzyme Immunoassay

INTENDED USE

For the quantitative determination of Free Thyroxine (fT4) concentration in Human serum.
Levels of fT4 are thought to reflect the amount of T4 available to the cells and may therefore determine the clinical metabolic status of T4.

INTRODUCTION

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.
For example, 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. Again, the free thyroxine concentration typically uncovers the patient's actual clinical status.
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 (analog method) is added, then 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 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 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 OF THE TEST

Competitive Enzyme Immunoassay 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 insolubulized binding sites. The interaction is illustrated by the followed equation:

$$Enz_{Ag} + Ag + Ab_{C.W.} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{C.W.} + Enz_{AgAb_{C.W.}}$$

$Ab_{C.W.}$ = Monospecific Immobilized Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
Enz_{Ag} = Enzyme-antigen Conjugate (Constant Quantity)
$AgAb_{C.W.}$ = Antigen-Antibody Complex
$Enz_{Ag}\ Ab_{C.W.}$ = 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 with the kit:

- Microwell plate. 12x8 well strips, Individually separable wells. Coated with sheep anti-thyroxine serum, packaged in an aluminum bag with a drying agent.
- Calibrators. 6 Vials x 1 mL. Human Serum References forthyroxine at approximate* concentrations of 0 (A), 0.6 (B), 1.4 (C), 2.4 (D), 4.0 (E) and 7.4 (F) ng/dL. 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

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- Enzyme-antigen Conjugate. Thyroxine-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added. Ready-to-use. 10.5 mL.
- Wash Solution Concentrate. Surfactant in phosphate buffered saline. A preservative has been added. 20mL.
- TMB Substrate. H_2O_2-TMB 0.25 g/L (avoid any skin contact). 12 mL
- Stop Solution. Sulphuric acid 0.15 mol/L (corrosive: avoid any skin contact). 12 mL

PRECAUTIONS

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

Use fresh serum. Samples can be stored at 2-8°C for 2 days. For longer periods, samples should be frozen (-20°C). Avoid repeated freezing and thawing.

Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of the test and should not be used. Neither Bilirubin nor Hemolysis have significant effect on the procedure.

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 tracer.

Substance	Cross Reactivity	Concentration
l–Thyroxine	1.1.0000	---
d-Thyroxine	0.9800	10 µg/dL
d-Triiodothyronine	0.0150	100 µg/dL
l–Triiodothyronine	0.0300	100 µg/dL
Iodothyrosine	0.000	100 µg/mL
Diiodothyrosine	0.0001	100 µg/mL
Diiodothyronine	0.0001	100 µg/mL

Materials required but not provided:

- Multichannel pipettes and micropipettes (Precision ≥1.5%) and disposable tips.
- Microplate reader with a 450 nm filter. Reference filter of 620 or 655 nm is advisable.
- Manual or automated wash system.
- Absorbent paper of blotting the microplate wells.
- Distilled or deionised water.
- Timer.

REAGENT PREPARATION

- Wash Buffer. Dilute contents of Wash Concentrate to 1000 mL with distilled or deionized water in a suitable storage container. Store at room temperature until expiration date printed on concentrate label. It is essential that all the contents of the wash buffer concentrate dissolve. Crystal formation in the Wash Concentrate can be eliminated by briefly (approx. 5 minutes) heating in a water bath at 37°C or storing the Wash Concentrate at room temperature.

ASSAY 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 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 **300 µL** 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 TMB-Substrate solution to all wells. 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 0.100mL (100µL) 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.

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11. Read the absorbance in each well at 450 nm (using a reference wavelength of 620-630 nm 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. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include he 80, 50 and 20% intercepts of the standard curve for run-to-run reproducibility. 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.

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 free T4 concentration in ng/dl on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
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 1.123 (intersects the standard curve at (8.1 ng/dL) fT4 concentration (See Figure 1).

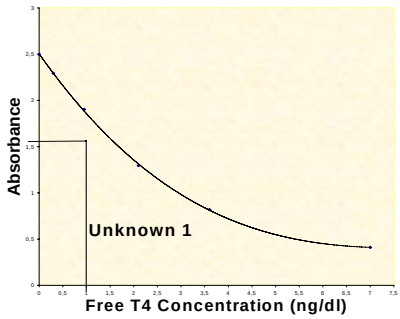
EXAMPLE 1

WELL	SERUM REFERENCES	ABSORBANCE
1	0.0 ng/dL	2.462
2	0.0 ng/dL	2.531
3	0.3 ng/dL	2.330
4	0.3 ng/dL	2.255
5	0.95 ng/dL	1.915
6	0.95 ng/dL	1.892
7	2.1 ng/dL	1.328
8	2.1 ng/dL	1.262
9	3.6 ng/dL	0.834
10	3.6 ng/dL	0.804
11	7.0 ng/dL	0.399
12	7.0 ng/dL	0.42

WELL	Unknown I. D.	O.D	Avg. O.D	Value
13	Unknown #1	1.783		
14	Unknown #1	1.676	1.730	1.3 ng/dL

*The data presented in Example 1 and Figure 1 are for illustration

Figure 1



only and should not be used in lieu of a standard curve prepared with each assay.

Q.C. PARAMETERS
Maximum Absorbance (O calibrator) = 1.5 – 2.7

LIMITATIONS OF THE PROCEDURE

1. Assay Performance

   It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift. If more than one (1) plate is used, it is recommended to repeat the dose response curve.

   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.

   Plate readers measure vertically. Do not touch the bottom of the wells.

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Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.

Sample(s), which are contaminated microbiologically, should not be used in the assay. Highly lipemeic or hemolysed specimen(s) should similarly not be used

2. Interpretation

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.

1. "NOT INTENDED FOR NEWBORN SCREENING"

EXPECTED 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 (σ) and expected ranges (±2 σ) are presented in Table 1.

TABLE 1

Expected Values for the Free T4 EIA Test System (in ng/dL)

	Adult (110 specimens)	Pregnancy (30specimens)
Mean (X)	1.4	1.5
Standard Deviation (σ)	0.6	0.7
Expected Ranges (±2 σ)	0.8 – 2.0	0.8 – 2.2

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 fT4 Microplate EIA Test System were determined by analyses on three different levels of pool control sera. The number, mean values, standard deviation (σ) and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2

Within Assay Precision (Values in ng/dl)

Sample	N	X	S.D.	C.V.
Low	16	0.45	0.035	7.8%
Normal	16	1.45	0.063	4.3%
High	16	3.3	0.215	6.5%

TABLE 3

Between Assay Precision (Values in ng/dL)

Sample	N	X	σ	C.V.
Low	10	0.48	0.052	10.8%
Normal	10	1.41	0.085	6.0%
High	10	3.49	0.279	7.9%

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

B. Accuracy

The T4 Microplate EIA Test System was compared with a coated tube radioimmunoassay method. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (The values ranged from 0.1 ng/dL – 8 ng/dL). The total number of such specimens was 85. The least square regression equation and the correlation coefficient were computed for this fT4 EIA in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

Least Square Regression Correlation

Method	Mean (x)	Analysis	Coefficient
This Method	1.47	y = 0.12+0.962(x)	0.965
Reference	1.42		

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

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The 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 2σ (95% certainty) statistic to calculate the minimum dose.

REFERENCES

1. Barker, S.B., "Determination of Protein Bound Iodine.",*Joumal 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).

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