FREE TRIIODOTHYRONINE (fT3) ENZYME IMMUNOASSAY TEST KIT
Catalog Number: 6107210

CE

Enzyme Immunoassay for the Quantitative Determination of Free Triiodothyronine (fT3) Concentration in Human Serum

FOR IN VITRO DIAGNOSTIC USE ONLY

Store at 2 to 8°C.

PROPRIETARY AND COMMON NAMES

Free T3 Enzyme Immunoassay

INTENDED USE

For the quantitative determination of Free Triiodothyronine (fT3) concentration in human Serum.
Levels of fT3 are thought to reflect the amount of T3 available to the cells and may therefore determine the clinical metabolic status of T3.

INTRODUCTION

Triiodothyronine, a thyroid hormone, circulates in blood almost completely bound (>99.5%) to carrier proteins (1,2). The main transport protein is thyroxine-binding globulin (TBG). However, only the free (unbound) portion of triiodothyronine is believed to be 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 triiodothyronine level changes so that the free triiodothyronine concentration remains constant. Thus, measurements of free triiodothyronine concentrations correlate more reliably with clinical status than total triiodothyronine levels.
For example, the increase in total triiodothyronine levels associated with pregnancy, oral contraceptives and estrogen therapy result in higher total T3 levels while the free T3 concentration remains basically unchanged.
This microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations in a direct determination of free T3. In this method, serum reference, patient specimen, or control is first added to a microplate well. Enzyme-T3 conjugate (analog method) is added, then the reactants are mixed. A competition reaction results between the enzyme conjugate and the free triiodothyronine for a limited number of antibody combining sites immobilized on the well.
After the completion of the required incubation period, the antibody bound enzyme-triiodothyronine conjugate is separated from the unbound enzyme-triiodothyronine 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 triiodothyronine 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 triiodothyronine concentration.

PRINCIPLE OF THE TEST

Competitive Enzyme Immunoassay for Free T3.

The essential reagents required for a solid phase enzyme immunoassay include immobilized T3 antibody, enzyme-T3 conjugate and native free T3 antigen. The enzyme-T3 conjugate should have no measurable binding to serum proteins especially TBG and albumin. The method achieves this goal.
Upon mixing immobilized antibody, enzyme-T3 conjugate and a serum containing the native free T3 antigen, a competition reaction results between the native free T3 and the enzyme-T3 conjugate for a limited number of insolubulized binding sites. The interaction is illustrated by the followed equation:

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

$Ab_{C.W.}$ = Monospecific Immobilized Antibody (Constant Quantity)
Ag = Native Free Antigen (Variable Quantity)
^{Enz}Ag = Enzyme-T3 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-triiodothyronine, packaged in an aluminum bag with a drying agent.
- Standards. 6 Vials x 1 mL. Human References Serum for free triiodothyronine at approximate* concentrations of 0 (A), 1.0 (B), 3.0 (C), 5.0 (D), 8.0 (E) and 16.0 (F) pg/mL.

  A preservative has been added. * Exact levels are given on the labels on a lot specific basis.

  For SI units: 1pg/mL x 1.536 = pmol/L
- Enzyme-antigen Conjugate. Triiodothyronine-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added. Ready-to-use. 12 mL.

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- Wash Solution Concentrate. Surfactant in phosphate buffered saline. A preservative has been added. 20 mL.
- TMB Substrate. Ready to use. H_2O_2-TMB 0.25 g/L (avoid any skin contact). 12 mL.
- Stop Solution. 1 N HCl (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

Collect sample(s) by venipuncture in ten (10) mL silicone evacuated tube(s). The usual precautions in the collection of venipuncture samples should be observed. Separate the red blood cells by centrifugation use serum for the free T3 procedure. Specimen(s) may be refrigerated at 2-8ºC for a maximum period of 48 hours. If the specimen(s) can not be assayed within 48 hours, the sample(s) may be stored at temperatures of -20ºC for up to 30 days. When assayed in duplicate, 0.10 mL of the specimen is required.

The cross-reactivity of the triiodothyronine 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 Triiodothyronine needed to displace the same amount of tracer.

Substance	Cross Reactivity	Concentration
I-Triiodothyronine	1.0000	-
I-Thyroxine	< 0.0002	10 µg/mL
Iodothyrosine	< 0.0001	10 µg/mL
Diiodothyrosine	< 0.0001	10 µg/mL
Diiodothyronine	< 0.0001	10 µg/mL
Phenylbutazone	< 0.0001	10 µg/mL
Sodium Salicylate	< 0.0001	10 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.

STORAGE OF TEST KITS

The components will remain stable through the expiration date shown on the label if stored between 2-8°C in dark. Do not frezee. Do not use reagents beyond the kit expiration date.

The bag containing the microplate should be brought to room temperature before opening to avoid condensation in the wells.

Once opened the bag, microplate strips are stable for 1 month at 2-8°C in the plastic bag tightly sealed, with the silicagel.

Opened reagents are stable for 1 month at 2-8°C.

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.

ASSAY PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 30°C).

1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate.
2. Pipette 0.050 mL (50 µL) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 mL (100 µL) of Triiodothyronine-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 (100 µL) 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 0.100 mL (100 µL) 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 450 nm in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

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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 the 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 triiodothyronine 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 fT3 concentration in pg/ml 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 fT3 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 pg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance 1.799 (intersects the standard curve at (1.2 pg/mL) fT3 concentration (See Figure 1).

EXAMPLE 1

WELL	SERUM REFERENCES	ABSORBANCE
1	0.0 pg/mL	2.350
2	0.0 pg/mL	2.453
3	1.7 pg/mL	1.934
4	1.7 pg/mL	1.925
5	3.5 pg/mL	1.624
6	3.5 pg/mL	1.641
7	7.0 pg/mL	1.043
8	7.0 pg/mL	1.000
9	15.0 pg/mL	0.629
10	15.0 pg/mL	0.620
11	20.0 pg/mL	0.460
12	20.0 pg/mL	0.453

Well	Unknown I. D.	O.D.	Avg. O.D.	Value
13	Unknown #1	1.843		
14	Unknown #1	1.866	1.855	2.2 pg/mL

The data presented in Example 1 are for illustration 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 PROCEDURE

1. Assay Performance

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

   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.

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

2. Interpretation

   If computer controlled data reduction is used to calculate the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

   Several drugs are known to effect the binding of Triiodothyronine to the thyroid hormone carrier proteins or its metabolism to T3 and complicate the interpretation of free T3 results (3).

   Circulating autoantibodies to T3 and hormone-binding inhibitors may interfere (4).

   Heparin has been reported to have *in vivo* and *in vitro* effects on free T3 concentration (5). Therefore, do not obtain samples in which this anti-coagulant has been used.

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In severe nonthyroidal illness (NTI), the assessment of thyroid status becomes very difficult. TSH measurements are recommended to identify thyroid dysfunction (6).

Familial dysalbuminemic conditions may yield erroneous results on direct free T3 assays (7).

"NOT INTENDED FOR NEWBORN SCREENING"

EXPECTED VALUES

A study of euthyroid adult population was undertaken to determine expected values for the fT3 EIA Test System. The mean (R) values, standard deviations (S.D.) and expected ranges (±2 S.D.) are presented in Table 1.

TABLE 1

Expected Values for the Free T3 EIA Test System (in pg/mL)

	Adult (110 specimens)	Pregnancy (75 specimens)
Mean (X)	2.8	3.0
Standard Deviation (S.D.)	0.7	0.6
Expected Ranges (±2 S. D.)	1.4 – 4.2	1.8 – 4.2

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 fT3 Microplate EIA Test System were determined by analyses on three different levels of pool control sera. The number, mean values, standard deviation 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 pg/mL)

Sample	N	X	S.D.	C.V.
Low	20	1.37	0.16	11.9%
Normal	20	4.21	0.17	4.1%
High	20	7.1	0.17	2.4%

TABLE 3

Between Assay Precision (Values in pg/ml)

Sample	N	X	S.D.	C.V.
Low	10	1.4	0.15	10.7%
Normal	10	4.4	0.23	5.2%
High	10	7.0	0.30	4.2%

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

B. Accuracy

The fT3 Microplate EIA Test System was compared with a coated tube radioimmunoassay analog method. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (The values ranged from 0.1 pg/mL – 14 pg/mL). The total number of such specimens was 85. The least square regression equation and the correlation coefficient were computed for this fT3 EIA in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method	3.4	y = 0.15+0.925(x)	0.955
Reference	3.5		

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 Triiodothyronine procedure has a sensitivity of 0.05 pg/mL. The sensitivity was ascertained by determining the variability of the 0 pg/mL serum calibrator and using the 2 (95% certainty) statistic to calculate the minimum dose.

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