



Cat# 3148

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

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See external label



2°C-8°C



Σ=96 tests



#3148

Free Triiodothyronine (fT3) Microplate EIA

Cat. No. 3148

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

SUMMARY AND EXPLANATION OF THE TEST

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.

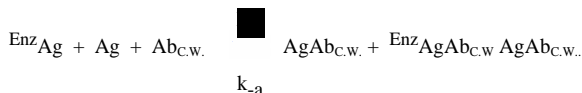
The employment of several serum references of known free triiodothyronine concentration permits construction of a graph of activity and concentration. From

PRINCIPLE

Competitive Enzyme Immunoassay – Analog Method 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 insolubilized binding sites. The interaction is illustrated by the following equation:



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

Ag = Native Free Antigen (Variable Quantity)

EnzAg = Enzyme-T3 antigen Conjugate (Constant Quantity)

$\text{AgAb}_{\text{C.W.}}$ = Antigen-Antibody Complex

$\text{EnzAg Ab}_{\text{C.W.}}$ = 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 FOR 96-well MICROPLATE

A. Human Serum References -- 1.0 ml/vial - Icon A-F

Six (6) vials of serum reference for free triiodothyronine at approximate* concentrations of 0 (A), 0.4 (B), 1.2 (C), 4.5 (D), 8.0 (E) and 18.0 (F) pg/ml. 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: 1pg/ml x 1.536 = pmol/L

B. fT3 –Enzyme reagent 13ml/vial - Icon

One (1) vial of triiodothyronine-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.

C. Antibody Coated Microplate -- 96 wells - Icon

One 96-well microplate coated with sheep anti-triiodothyronine serum and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. Wash Solution Concentrate -- 20ml - Icon

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

E. Signal Reagent A --7.0ml/vial - Icon S^A

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

F. Signal Reagent B -- 7.0ml/vial - Icon S^B

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

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

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 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

The specimens shall be blood serum in type and the usual precautions in the Collection of venipuncture samples should be observed. The blood should be collected in a plain red-top venipuncture tube without additives or gel barrier. Allow blood to clot. Separate the red blood cells by centrifugation use serum for the free T3 procedure. Specimen(s) may be refrigerated at 2-8°C for a maximum period of 48 hours. If the specimen(s) cannot be assayed within 48 hours, the sample(s) may be stored at temperatures of -20°C for up to 30 days. When assayed in duplicate, 0.10ml of the specimen is required.

MATERIALS

Provided:

1. Six (6) vials of free triiodothyronine human serum references.
2. One (1) vial of T3-enzyme reagent.
3. One 96-well Antibody Coated Microplate.
4. One (1) bottle of Wash buffer concentrate.
5. One (1) bottle of Substrate A.
6. One (1) bottle of Substrate B.
7. One (1) bottle of Stop solution.
8. Instructions.

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. Adjustable volume (200-1000µl) dispenser(s) for conjugate and substrate dilutions.
4. Microplate washer or a squeeze bottle (optional).
5. Microplate luminoreader.
6. Test tubes for dilution of enzyme conjugate and substrate A and B.
7. Absorbent Paper for blotting the microplate wells.
8. Plastic wrap or microplate cover for incubation steps.
9. Vacuum aspirator (optional) for wash steps.
10. Timer.
11. Quality control materials.

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 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 in at 37° or storing the wash concentrate at room temperature.

2. Working Substrate Solution - Prepare daily

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).

Use within 24 hours of preparation for maximum performance of the assay.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 -27°C).

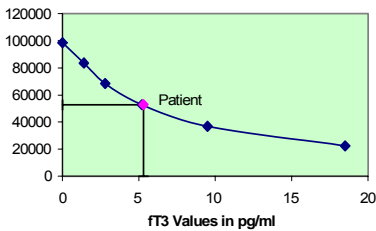
1. Format the microplates wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C
2. Pipette 0.050 ml (50µl) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 ml (100µl) of T3-enzyme conjugate solution to all wells.
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 45 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 (4) additional times for a total of three (5) 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 (4) 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 for 5 minutes in the dark.
10. Read the relative light units in each well for 0.2 - 1.0 seconds. **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 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.

RESULTS

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



- Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
- Plot the RLU (Relative Response Unit) 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).
- Draw the best-fit curve through the plotted points.
- To determine the concentration of ft3 for an unknown, locate the average RLU adequately

EXAMPLE 1

Well	Label	Avg. O.D.	ft3 Concentration (pg/ml)
Cal A	A1	98328	98479
	B1	98631	
Cal B	C1	84030	83523
	D1	83017	
Cal C	E1	68372	68475
	F1	68578	
Cal D	G1	52933	52852
	H1	52771	
Cal E	A2	37445	36902
	B2	36359	
Cal F	C2	22471	22372
	D2	22273	
Pat 1	E2	52177	52178
	F2	52179	

A. Assay Performance

Sample(s), which are contaminated microbiologically, should not be used in the assay. Highly lipemic 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 in the aspiration or decantation wash step(s) may result in poor replication and spurious results.

B. 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.

If a patient, for some reason, reads higher than the highest calibrator report as such (e.g. > 200 pg/ml). **Do not try to dilute the sample. TBG variations in different matrices will not allow FT3 hormone to dilute serially.**

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.

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 RANGES OF 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.

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

TABLE I
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

The data presented in Example 1 and Figure 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

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.

7. Lalloz M.R., et al, *Clin Endocrinol*, **18**, 11 (1983).

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	1.45	0.14	9.7%
Normal	20	4.55	0.22	4.8%
High	20	8.24	0.46	2.4%

TABLE 3
Between Assay Precision (Values in pg/ml)

Sample	N	X	S.D.	C.V.
Low	10	1.51	0.15	9.9%
Normal	10	4.73	0.34	7.3%
High	10	7.81	0.52	6.6%

*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 0.1pg/ml – 14pg/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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