Free THYROXINE (fT4)

ENZYME IMMUNOASSAY TEST KIT
Catalog Number: 10306

Enzyme Immunoassay for the Quantitative Determination of Free Thyroxine (FT4) in Human Serum

INTENDED USE

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

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. After equilibrium is attained, the antibodybound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibodybound 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.

MATERIALS AND COMPONENTS

Materials provided with the test kits:

- Antibody-coated microtiter wells. 96 wells per pouch.
- Reference standard set, 6 x 1mL, ready to use.
- Exact levels are given on the labels on a lot specific basis.
- Free T4 HRPO Conjugate Diluent, 15mL.
- Free T4 HRPO Conjugate Concentrate (20X), 0.8 mL
- TMB Substrate Reagent, 12 mL.
- 50x Wash Buffer Concentrate, 15 mL
- Stop Solution , 12 mL.

Materials required but not provided:

- Precision pipettes: 50µL~200µLand 1.0mL
- Disposable pipette tips.
- Distilled water.
- Vortex mixer or equivalent.
- Absorbent paper or paper towel.
- Graph paper.
- Microtiter well reader.

SPECIMEN COLLECTION AND PREPARATION

Serum should be prepared from a whole blood specimen obtained by acceptable medical techniques. This kit is for use with serum samples without additives only.

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 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.Prepare the washing solution by diluting 1 part of the 50X Wash buffer concentrate to 49 parts of distilled water.

2.To prepare Free T4-HRPO conjugate reagent, add 0.10 mL of Free T4-HRPO conjugate concentrate to 2.0 mL of Free T4 conjugate diluent (1:20 dilution), and mix well. The amount of conjugate diluted is depend on your assay size. The conjugate reagent is stable at 4°C at least for two weeks.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (18-22°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 0.05 mL(50µL) of the appropriate serum reference, control or specimen into the assigned well.

3.Add 0.1 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 four (4) 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 four (4) additional times.

8.Add 0.100 mL (100µL) of TMB substrate regeant to all wells. Always add reagents in the same order to minimize reaction time differences between wells

9.Incubate at room temperature for 20 minutes.

10.Add 0.1 mL of Stop Solution, and Mix well.

11.Read the wells at 450 nm in a Microtiter well 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

1.Calculate the average absorbance values (A450) for each set of Referenc standards, control, and samples.

2.We recommend to use a proper software to calculate the results, 4-parameters is the most preferred. If the software is not available, construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/dL on linear graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.

3.Using the mean absorbance value for each sample, determine the corresponding concentration of Free T4 in ng/dl from the standard curve.

EXAMPLE OF STANDARD CURVE

Results of typical standard run with optical density reading at 450nm shown in the Y axis against Free T4 concentrations shown in the X axis. This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her own data and standard curve.

Free T4 (ng/dL)	Absorbance (450nm)
0	2.728
0.5	2.221
1.0	1.571
2.0	0.943
4.0	0.424
8.0	0.214

EXPECTED RANGES OF VALUES

A study of euthyroid adult population was undertaken to determine expected values for the Free T4 EIA System. The mean (X) values, standard deviations (SD) and expected ranges (±2 SD) are presented below:

	Normal Adult (90 specimens)	Pregnancy (50 specimens)
Mean (X)	1.31	1.46
Standard Deviation (SD)	0.33	0.36
Expected Ranges (±2 SD)	0.65 – 1.97	0.61 – 2.09

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

Precision

The inter and intra assay precision of the fT4 Microplate Test System were determined by analyses on three different levels of pooled patient sera. The number, mean values, standard deviation (SD) and coefficient of variation for each of these control sera are presented below:

Intra-Assay Precision:			
	S1	S2	S3
Number (n)	20	20	20
Mean	0.65	1.83	2.98
SD	0.07	0.086	0.111
CV	10.8%	4.7%	3.7%

Inter-Assay Precision:			
	S1	S2	S3
Number (n)	10	10	10
Mean	0.64	1.89	3.07
1SD	0.071	0.116	0.167
CV	11.1%	6.1%	5.4%

SENSITIVITY

The free thyroxine procedure has a sensitivity of 0.25 ng/dL.The sensitivity was ascertained by determining the variability of the 0 ng/dL serum calibrator and using the SD (95% certainty) statistics to calculate the minimum dose.

SPECIFICITY

The crossreactivity of the thyroxine antibody, used for Free T4 Assay, 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	1.00	----
d-Thyroxine	0.99	10µg/dL
dTriiodothyronine	0.014	100µg/dL
l–Triiodothyronine	0.028	100µg/dL
Iodothyrosine	0.0001	100µg/mL
Diiodotyrosine	0.0001	100µg/mL
Diiodothyronine	N/D	100µg/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

LIMITATIONS OF THE PROCEDURE

There are some limitation of the assay. We should let our customers know about that.

1)As with all diagnostic tests, a definite clinical diagnosis should not be based on the results of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.

2)Studies have implicated possible interference in immunoassay results in some patients with known rheumatoid factor and antinuclear antibodies. Serum samples from patients who have received infusions containing mouse monoclonal antibodies for diagnostic or therapeutic purposes, may contain antibody to mouse protein (HAMA). Although we have added some agents to avoid the interferences, we cannot guarantee to eliminate all the effects of that.

3)The wash procedure (steps 6-8) is critical. Insufficient washing will result in poor precision and falsely elevated absorbance. The use of tap water for washing could result in a higher background absorbance.

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Chemux Bioscience,Inc.

Website: www.chemux.com

385 Oyster Point Blvd Suite5-6.,South San Francisco,CA94080
Tel:+1-650-872-1800