Free Thyroxine (fT4)

ENZYME IMMUNOASSAY TEST KIT

Enzyme Linked Immunosorbent Assay (ELISA) for the Quantitative Determination of Free Thyroxine (fT4) in Human Serum

FOR IN VITRO DIAGNOSTIC USE ONLY

Store at 2°C to 8°C

INTENDED USE

fT4 Competitive ELISA test is intended for the quantitative determination of Free Thyroxine (fT4) in human serum. For In Vitro Diagnostic Use only.

INTRODUCTION

Thyroxine, the principle 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.

PRINCIPLE OF THE ASSAY

fT4 Quantitative Test Kit is a competitive enzyme-linked immunosorbent assay. In this method, Standards & patient specimen is first added to a microplate well followed by the Enzyme-T4 conjugate & 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 the washing 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 reaction is stopped after specified time with stop solution and absorbance is determined for each well using an ELISA reader. The enzyme activity in the antibody-bound fraction is inversely proportional to the native free antigen concentration. From the curve, an unknown specimen's activity can be correlated with free thyroxine concentration.

MATERIALS AND COMPONENTS

Materials provided with the test kits:

1. Coated Microwells: Antibody-coated microtiter wells.
2. Free T4 HRPO Conjugate Diluent
3. Free T4 HRPO Enzyme Conjugate concentrate (20X).
4. TMB Substrate. Ready to use
5. Stop Solution. Ready to use
6. fT4 Standard set of 6 standards labeled as A to F in liquid form. Ready to use. For standard concentrations refer vial label.
7. Wash Buffer Concentrate (20X).
8. Control set
9. Pack Insert
10. Plate sealers
11. Protocol Sheet
12. Microwell Holder

Materials required but not provided

1. Precision pipettes: 10-100µl, 50-200µl, 100-1000µl
2. Disposable pipette tips
3. Distilled water
4. Disposable Gloves
5. ELISA reader
6. ELISA washer

STORAGE AND STABILITY

1. **fT4** kit is stable at 2-8°C upto expiry date printed on the label.
2. Coated microwells should be used within one month upon opening the pouch provided that once opened, the pouch must be resealed to protect from moisture. If the colour of the desiccant has changed from blue to pink at the time of opening the pouch, another coated microwells pouch should be used.
3. Diluted Wash Buffer is stable for upto one week when stored at 2-8°C.

SPECIMEN COLLECTION

1. Collect Blood specimen by venipuncture according to the standard procedure.
2. Only serum should be used.
3. Preferably use fresh samples. However, Specimens can be stored up to 48 hours at 2-8°C, for short duration.
4. For longer storage, specimens can be frozen at -20°C. Thawed samples must be mixed prior to testing.
5. Do not heat inactivate before use.
6. Specimen containing precipitate or particulate matter should be clarified by centrifugation prior to use.
7. Specimen should be free from particulate matter and microbial contamination.

PRECAUTIONS

1. Bring all reagents and specimen to room temperature before use.
2. Do not pipette any material by mouth.
3. Do not eat, drink or smoke in the area where testing is done.
4. Use protective clothing and wear gloves when handling samples.
5. Use absorbent sheet to cover the working area.
6. Immediately clean up any spills with sodium hypochlorite.
7. All specimens, standards and controls should be considered potentially infectious and discarded appropriately.
8. Neutralize acid containing waste before adding hypochlorite.
9. Do not use kit after the expiry date.
10. Do not mix components of one kit with another.
11. Always use new tip for each specimen and reagent.
12. Do not allow liquid from one well to mix with other wells.
13. Do not let the strips dry in between the steps.

REAGENT PREPARATION

1. All reagents should be brought to room temperature (18-25°C) and mixed by gently inverting or swirling prior to use. Do not induce foaming.
2. Dilute Wash Buffer 20 times (for example add 5ml concentrated buffer to 95 ml distilled or deionized water). Mix well before use.
3. Dilute Enzyme Conjugate with Conjugate Diluent according to the requirement as shown below. Prepare a fresh dilution for each assay.

No. of Strips	0.5	1	2	3	4	5	6	7	8	9	10	11	12
Enzyme Conjugate(µl)	12.5	25	50	75	100	125	150	175	200	225	250	275	300
Conjugate Diluent (µl)	250	500	1000	1500	2000	2500	3000	3500	4000	4500	5000	5500	6000

TEST PROCEDURE

1. Secure the desired number of coated wells in the holder. Dispense **25 µl** of standards, controls and sera into the appropriate wells.
2. Dispense **50 µl** of diluted Conjugate into each well. Incubate at room temperature (18-25 °C) for **45 minutes**.
3. After incubation, empty the microtitre wells and wash the plate **5 times** with **350 µl** of diluted wash buffer. Strike the microtitre plate sharply onto the absorbent paper towel to remove all residual droplets.
4. Dispense **100 µl** of TMB Substrate into each well. Incubate at room temperature (18-25 °C), in the dark, for **20 minutes**.
5. Stop the reaction by adding **100 µl** of Stop Solution to each well. Gently mix for 10 seconds until the blue color completely changes to yellow.
6. Read the optical density at 450/630 nm with a microtiter plate reader within **15 minutes**.

Add **25** µl standards/controls/samples into the respective Microwells

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Add **50** µl diluted Conjugate in each well

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Apply plate sealer and incubate for **45** minutes at **18-25°C**

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Wash Microwells **5 times** with **350** µl **of** diluted wash buffer

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Add **100** µl Substrate in each well

↓

Incubate for **20** Minutes at **18-25°C in dark**

↓

Add **100** µl stop solution in each well

↓

Read results @450nm (Ref.600-700nm) within **15** minutes

CALCULATION OF RESULTS

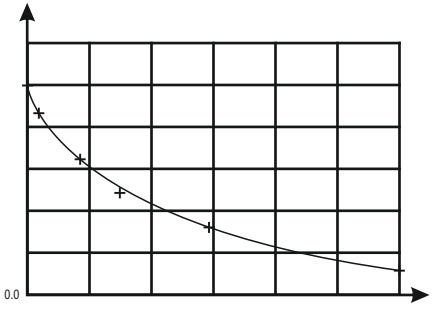
Construct a Standard curve by plotting the absorbance obtained from each reference Standard against its concentration in ng/dl on the graph paper, with absorbance values on the vertical or Y axis and concentrations on the horizontal or X axis. Use the absorbance values for each specimen to determine the corresponding concentration of fT4 in ng/dl from the Standard curve. Any diluted specimens must be corrected by the appropriate dilution factor.

Example of Standard curve

Results of a typical Standard run with optical density reading at 450nm (ref 600-700nm) shown in the Y axis against fT4 concentrations shown in the X axis.

Suggest: Use 4-Parameter Standard curve to calculate sample values.

fT4 Values (ng/dl)	Absorbance (450nm)
A	1.952
B	1.789
C	1.529
D	1.035
E	0.610
F	0.329



This Standard curve is for the purpose of illustration only and should not be used to calculate samples. Each user should obtain his or her own Standard curve and data.

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 (± 2SD)	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.

The minimal detectable concentration of fT4 by this assay is estimated to be 0.25 ng/dl.

PERFORMANCE CHARACTERISTICS

A) Internal Evaluation:

1. Accuracy: In an internal study **fT4** was evaluated against commercially available licensed kit with 90 random clinical samples, & **fT4** has demonstrated 100% clinical correlation with the commercially available licensed kit.

2. Precision: **fT4** was evaluated with licensed external Quality controls for Precision Studies & following is the data:

Controls	No. of testings	Mean Control values with **fT4**	Coefficient of Variation (CV)
Level 1	10	0.804	7.53
Level 2	10	2.236	5.74
Level 3	10	2.845	6.44

B) External Evaluation:

fT4 ELISA has been evaluated by a NABL accredited lab against their reference method. In this evaluation **fT4** ELISA has demonstrated 100% correlation with the reference method.

*Data file: Orchid Biomedical Systems (P) Ltd.

IMPORTANT NOTE

1. The fT4 assay is a temperature sensitive assay. The best temperature condition for this assay is from 18°C to 25°C.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
3. It is recommended to use the multi channel pipettes to avoid time effect. A full plate of 96 wells may be used if automated pipetting is available.
4. Duplication of standards, controls and samples is not mandatory but may provide information on reproducibility & application errors.

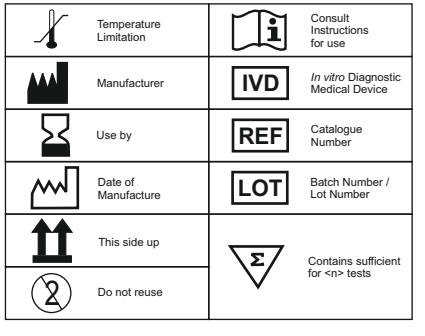
LIMITATIONS OF THE ASSAY

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. The activity of the enzyme used is temperature-dependent and the OD values may vary. The higher the room temperature (+18°C to +25°C) during substrate incubation, the greater will be the OD values. Corresponding variations apply also to the incubation times. However, the standards are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.
3. Adaptation of this assay for use with automated sample processors and other liquid handling devices, in whole or in part, may yield differences in test results from those obtained using the manual procedure. It is the responsibility of each laboratory to validate that their automated procedure yields test results within acceptable limits.
4. Insufficient washing (e.g., less than 5 wash cycles, too small wash buffer volumes, or shortened reaction times) can lead to incorrect OD values.

BIBLIOGRAPHY

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).
5. Halpern, E.P and Bordens, RW. "Microencapsulated antibodies in radioimmunoassay. Determination of free Thyroxine". Clinical Chemistry, Vol 25, 1561-1563 (1979).
6. Stjernholm, MR, Alsever, RN and Rudolph, MC. "Thyroid function tests in diphenylhydantoin-treated patients". Clin. Chem. Vol 21. 1388-1392 (1977).
7. Nelson J.C. and Wilcox, RB. "Analytical performance of Free and Total thyroxine assays". Clin. Chem. Vol 42, 146-154. (1996).
8. Midgeley John, EM. "Direct and Indirect Free Thyroxine Assay Methods. Theory and Practice". Clin. Chem. Vol 47. 1353-1363. (2001).
9. Bayer, MF and McDougall, IR. "Radioimmunoassay of free thyroxine in serum: comparison with clinical findings and results of conventional thyroid-function tests". Clin. Chem. Vol 26. 1186-1192. (1980).
10. Anthony, GW, Jackson, RA et.al. "Misleading results from immunoassays of serum free thyroxine in the presence of rheumatoid factor". Clin. Chem. Vol 43. 957-962. (1997).
11. Wosilait, WD. "A theoretical analysis of the distribution of thyroxine among sites on the thyroxine binding globulin, thyroid binding prealbumin and serum albumin". Res. Comm. Chem. Pathology-Pharmacology. 1977;16: 541-548.
12. Data on file: Orchid Biomedical Systems (P) Ltd.

SYMBOL KEYS



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