

**REF**

GD7185 00

FT4**Enzyme-immunoassay for the quantitative determination of free Thyroxine in human serum****IVD****INDICATION**

The thyroxine (T4) is produced by the thyroid gland and is the major form of thyroid hormone in the blood. Thyroxine important component in the synthesis is iodine.

Thyroxine-binding globulin (TGB) is the major carrier protein for circulating thyroid hormone. Only a very small fraction of the circulating hormone is free (0.03%) and constitutes the active form.

The concentration of free thyroid hormones in the blood is regulated by a negative feedback mechanism involving TSH. The binding of T4 by TBG plays a key role in this feedback mechanism and the most significant changes that occur in T4 binding capacity are the result of alterations in TBG. Changes in the circulating levels of TBG will result in a proportional increase or decrease in the concentration of total T4. However, measurement of serum free T4 is unaffected by changes in T4 protein binding levels and therefore correlates well with the functional thyroid state in most individuals. Factors responsible for discrepancies between serum total T4 levels and true thyroid states include TBG concentration, estrogenic hormones (pregnancy, oral contraceptives and estrogen) and drugs that bind to TBG preventing its binding to free T4.

Thyroid hormones act on the body to increase the basal metabolic rate, affect protein synthesis and increase the body's sensitivity to catecholamines (such as adrenaline). The thyroid hormones are essential to proper development and differentiation of all cells of the human body. These hormones also regulate protein, fat, and carbohydrate metabolism, affecting how human cells use energetic compounds. Numerous physiological and pathological stimuli influence thyroid hormone synthesis. Thyrotoxicosis or hyperthyroidism is the clinical syndrome caused by an excess of circulating free thyroxine, free triiodothyronine, or both. Both T3 and T4 are used to treat thyroid hormone deficiency (hypothyroidism).

PRINCIPLE OF THE ASSAY

This test is based on "one step" competition enzyme immunoassay principle (ELISA). Tested specimen is placed into the microwells coated by specific anti-FT4 antibodies simultaneously with FT4 conjugated to Horseradish peroxidase (HRP). FT4 from the specimen competes with the conjugated antigen for coated antibodies. After washing procedure, the remaining enzymatic activity bound to the microwell surface is detected and quantified by addition of chromogen-substrate solution. The developed colour, detected at 450 nm, is inversely related to the quantity of FT4 present in the specimen.

FT4 concentration in the sample is calculated based on a series of standards.

KIT CONTENT

- 1. Reagent A – Microplate**
12x8 strips.
8 wells breakable strips, coated with anti-FT4 monoclonal antibodies. The strips are assembled on a plastic frame and contained in a sealed bag with desiccant. Bring the strips to room temperature before use, to prevent any moisture formation inside the bag.
- 2. Reagent B – Enzymatic Tracer**
1 vial of 12 ml.
FT4, conjugated with Horseradish peroxidase (HRP) in a proteic stabilized matrix with 0.004% Gentamycin sulphate and 0.1% ProClin 300 as preservatives.
- 3. Reagent C – Washing Solution 25x**
1 vial of 50 ml.
Concentrated solution to be diluted 1:25 with distilled water. It contains a detergent in Phosphate buffer.
- 4. Reagent D/E – Chromogen/Substrate**
1 vial of 12 ml.
Ready to use solution containing Tetramethylbenzidine (TMB) and H₂O₂ in Citric acid buffer.
Avoid any skin contact and light exposure.
- 5. Reagent F – Stop Solution**
1 vial of 15 ml.
Ready to use solution containing Sulphuric acid 0.2 M.
Avoid any skin contact.
- 6. FT4 Standards:**
6 vials of 0.5 ml each.
Ready to use human serum based liquids containing FT4 and 0.1% ProClin 300 as preservative.
Approximately FT4 concentrations are the following:
S₀: 0 pmol/l, S₁: 8 pmol/l, S₂: 15 pmol/l, S₃: 25 pmol/l, S₄: 50 pmol/l, S₅: 90 pmol/l.
For SI units: pmol/l x 0.777 = pg/ml.
Actual concentrations to be used for calculation are stated on the labels of the vials.
- 7. FT4 Control:**
1 vial of 0.5 ml.
Ready to use liquid containing human serum with a defined quantity of FT4 and 0.1% Proclin 300 as preservative.
Refer to the vial label for acceptable range.
- 8. Cardboard sealers**
2 cardboard sealers to be used to cover the plate during the incubations.
- 9. Package insert:** instruction for use GD7185 00 it/ing.

MICROBIOLOGICAL STATE AND CLEANING GRADE

1. All the materials of human origin resulted negative to HbsAg, HIV 1&2 and HCV FDA approved tests. Anyhow, as no test can guarantee the absolute absence of infective agents, handle reagents as potentially infected, especially standards, controls and samples. All objects come in direct contact with samples and all residuals of the assay should be treated or eliminated as potentially infected. Best procedures for inactivation are treatments with autoclave at 121°C for 30 minutes or with sodium hypochlorite at a final concentration of 2.5 % for 24 hours.
2. Avoid any contact with skin and mucous membrane, in particular for Chromogen/Substrate and Stop Solutions.
3. Use protective disposable talk-free gloves.
4. Avoid contaminating reagents when taking them from the vials. We recommend to use automatic pipettes with disposable tips. When dispensing reagents, do not touch with tips the wall of wells in order to avoid cross-contaminations.
5. For the washing step, use only the Washing Solution provided in the kit and follow carefully the indications reported in "WASHING INSTRUCTION".
6. Avoid the substrate/chromogen to come in contact with oxidizing agents or metallic surfaces; avoid intense light exposure during incubation or reagent preparation.

STORAGE AND STABILITY OF THE KIT

1. The kit has to be stored at 2-8 °C and used before the expiry date stated on the label.
2. Unused strips have to be placed in the bag containing the desiccant and firmly sealed before restore at 2-8 °C. After opening the strips are stable up to the expiry date stated on the label.
3. All other reagents can be repeatedly used up to exhaustion if stored at 2-8 °C, provided that they are handled carefully to avoid any environment contamination. Under these conditions the reagents are stable up to the expiry date stated on the labels.

AUXILIARY MATERIALS

- Semi automatic pipettes of 25, 100 and 150 µl
- Vortex mixer and absorbent paper
- Chronometer
- Ultrapure Elisa grade water
- Microplate thermoshaker at 37 (±0.5) °C.
- Photometric reader of microplates or microstrips, linear up to at least 2 OD and supplied with filter of 450 nm.
- Automatic microplates washing device or manual apparatus capable of aspirating and dispensing volumes of 300 µl.

SAMPLES

Serum only may be used. The kit is not calibrated for the determination of FT4 in plasma, saliva or other specimens of human or animal origin. The blood should be collected in plain redtop venipuncture tube without additives and gel barrier. Separate serum as soon as possible to avoid any hemolysis. Samples can be stored at 2-8 °C for a short time (max three days). For longer storage the specimen should be frozen. Avoid repeated freezing and thawing. Highly lipemic, hemolysed, preserved by sodium azide or microbiologically contaminated samples should not be used in the assay.

REAGENTS PREPARATION

- WASHING SOLUTION: dilute 1:25 with distilled or ELISA grade water (e.g.: 20 ml of Reagent C + 480 ml of distilled water) and mix carefully before use. The diluted washing solution can be stored for 3 days at 2-8 °C. The concentrated solution may present a sediment that can be dissolved at 35-39 °C and shaking. It is recommended to store washing solution at room temperature for immediate use.

WASHING INSTRUCTION

A good washing procedure is essential to obtain correct and precise analytical results.

We therefore recommend to use a good quality ELISA microplate washer, maintained at a good level of washing mechanical performances.

Generally, 5 automatic washing cycles of 0.3 ml/well are sufficient to avoid false positive reactions and remove high background. Anyhow we recommend to calibrate the washing system on the kit itself so to match the declared analytical performances.

In case of manual washing, we suggest to perform 5 washing cycles, dispensing and aspirating 0.3 ml/well per cycle.

In any case the liquid washed out from the plates must be inactivated with a sodium hypochlorite solution at a final concentration of 2.5%, before being thrown away or autoclaved, as it must be considered as potentially infected.

ASSAY PROCEDURE

1. At least one hour before use, bring all reagents, standards, control and samples to room temperature (18-30 °C), mixing them carefully on vortex.
2. Do not mix reagents from different lots.
3. We recommend to distribute standards, control and samples in duplicate.
4. Distribution and incubation times must be the same for all wells in the same analysis.
5. Avoid long interruptions between each step of the assay procedure.
6. It is suggested to eliminate the excess of washing solution from the microplate after washing by blotting it gently on an absorbent paper pad.
7. The colour developed in the last incubation is stable for a maximum of 20 minutes. Otherwise, in case of reading after 10-15 min after dispensing stop solution, immediately place the strips **in the dark**.

ASSAY SCHEME

- Put the desired number of microstrips into the frame.
- Follow the scheme:

	Microplate wells coated with anti-FT4 antibody		
	REAGENTS	Standards, Control	Sample
Immunological reaction	Standards, Control	25 µl	-
	Sample	-	25 µl
	Reagent B (Enzymatic Tracer)	100 µl	100 µl
	- Cover the strips with cardboard sealer - Incubate on a microplate thermoshaker (approximately 500-800 rpm) 30 minutes at 37 (± 0.5) °C*		
	- Peel out the cardboard sealer and aspirate the reaction solution from all wells - Rinse 5 times with 300 µl of diluted washing solution, carefully aspirating off the remaining liquid		
Colorimetric reaction	Reagent D/E (Chromogen-Substrate)	100 µl	100 µl
	- Cover the strips with cardboard sealer - Incubate 20-30 minutes at room temperature (22-28 °C), avoiding light exposure		
	Reagent F (Stop Solution)	150 µl	150 µl
	- Gently mix for 5-10 seconds - Read the absorbance of each well at 450 nm.		

*Alternatively, incubate 60 minutes at 37 (± 0.5) °C.

CALCULATION OF RESULTS

- Calculate the mean of the absorbance values for each point of the standard curve, control and of each sample.
- Draw a calibration curve on a linear graph paper with the mean optical densities on the Y-axis and the standards concentrations on the X-axis. If immunoassay software is being used, a 4-parameter curve is recommended.
- Interpolate the values of the samples on the standard curve to obtain the corresponding values of concentration expressed in pmol/l.

VALIDITY OF THE TEST

For the test to be valid the following criteria must be met:

- Standard 0 pmol/l OD 450 nm: ≥ 1.3
- Calculated value of Control should be within the established range stated on the label.

EXPECTED VALUES

From data obtained by testing serum specimens from 216 individuals determined as normal by EIA thyroid T3 free and TSH assays the following reference range is suggested. Otherwise, it is recommended that each laboratory establishes its own normal and abnormal values according to the examined population.

Subjects	Range (pmol/l)
Adults:	9.0 - 22.2

Note:

- The results obtained with this kit should never be used as the sole basis for clinical diagnosis. For example, the occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products has the potential of causing interferences in immunological tests. For diagnostic purpose, results should be used in conjunction with other data; e.g. symptoms, results of other thyroid tests, clinical impressions, etc.
- Serum free T4 values may be elevated under conditions such as pregnancy or administration of oral contraceptives.
- The interpretation of free T4 is complicated by a variety of drugs that can affect the binding of T4 to the thyroid hormone carrier proteins. In severe non thyroidal illness (NTI) the assessment of thyroid becomes especially difficult. Since the patient in this category may suffer from concomitant primary hypothyroidism or from compensatory secondary hypothyroidism. In case like these a sensitive TSH evaluation of the patient may be recommended.
- In rare conditions associated with extreme variations in albumin binding capacity for T4, such as familiar disalbuminemic hyperthyroxinemia, directed assessment of free T4 may be misleading.
- Circulating antibodies to T4 and hormone binding inhibitors may interfere in the performance of the assay.

ANALYTICAL PERFORMANCES

Analytical Sensitivity

The lower detection limit is 1.0 pmol/l. The sensitivity was calculated by determining the variability of Standard 0 pmol/l and using the 2 SD (95% certainty) statistics.

Precision

a. Intra Assay Variation

Two samples were assayed 32 times each on the same calibration curve. The results are tabulated below:

Sample	Mean, pmol/l	SD	%CV
1	21.4	0.854	4.0
2	34.8	1.024	2.9

b. Inter Assay Variation

Two samples were assayed 12 times 4 runs a day on the same calibration curve. The results are tabulated below:

Sample	Mean, pmol/l	SD	%CV
1	21.3	1.18	5.5
2	34.4	1.06	3.1

Specificity

The following compounds were tested for cross-reactivity:

Substance	Cross-reactivity (%)
L-Thyroxine	100
Triiodothyronine	0.004
Diiodothyronine	0.002
Tetraiodothyroacetate	0.001

Accuracy

The present kit was compared with a Chemiluminescent microparticle immunoassay as a reference test. 114 specimens were tested.

The following linear regression curve was calculated:

$$y = 0.95x + 0.6 \text{ pmol/l} \quad r = 0.97$$

PRECAUTIONS IN USE

The reagents are not considered harmful according to the 67/548/EEC and 88/379/EEC directives about classification, packaging and labelling of dangerous substances. However, the reagents should be handled with caution, avoiding swallowing and contact with skin, eyes and mucous membranes.

The use of laboratory reagents according to good laboratory practice is recommended.

Waste Management

Please refer to local legal requirements.

REFERENCES

1. Sterling L., "Diagnosis and treatment of Thyroid Disease", CRC Press, 19-51 (1975)
2. Nelson J.C. and Wilcox, RB., "Analytical performance of Free and Total thyroxine assay". Clin. Chem. 42, 146-154 (1996)
3. Midgeley J., "Direct and Indirect Free Thyroxine Assay Methods in Theory and Practice". Clin. Chem. 47, 1353-1363 (2001)