

**Free Thyroxine (fT4)  
ELISA Kit Protocol**

(Cat. No.:EK-310-08)

**PHOENIX PHARMACEUTICALS, INC.**  
330 Beach Road, Burlingame CA  
Tel: 650-558-8898 Fax: 650-558-1686  
E-Mail: [info@phoenixpeptide.com](mailto:info@phoenixpeptide.com)  
[www.PhoenixPeptide.com](http://www.PhoenixPeptide.com)

## **INTENDED USE**

For the quantitative determination of Free Thyroxine (fT4) concentration in Human serum. **FOR RESEARCH ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES**

## **WARNINGS AND PRECAUTIONS FOR USERS**

Test methods are not available which can offer complete assurance that Hepatitis B virus, Human Immunodeficiency Virus (HIV/HTLV-III/LAV), or other infectious agents are absent from the reagents in this kit. Therefore, all human blood products, including patient samples, should be considered potentially infectious. Handling and disposal should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation, where it exists (e.g., USA Center for Disease Control/National Institute of Health Manual, "Biosafety in Microbiological and Biomedical Laboratories," 1984) (22).

## **LIMITATIONS OF THE PROCEDURE**

1. Reliable and reproducible results will be obtained when the as say procedure is carried out with a complete understanding of the package insert instructions and with adherence to good laboratory practice.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
3. Serum samples demonstrating gross lipemia, gross hemolysis, or turbidity should not be used with this test.

## TABLE OF CONTENT

Introduction and Protocol Overview	4
Principle of the Test	5
Storage	6
List of Components	6
Specimen Collection and Preparation	6
Reagent Preparation	7
Assay Procedure	7
Calculation of Results	8
Expected Values and Sensitivity	9
Performance Characteristics	9
Reference Levels	12
References	12

## INTRODUCTION AND PROTOCOL OVERVIEW

L-Thyroxine (T4) or 3,5,3',5'-tetraiodothyronine is the most commonly measured thyroid hormone for the diagnosis of thyroid function. T4 has its primary influence on protein synthesis and oxygen consumption in virtually all tissues but it is also important for growth, development, and sexual maturation.

T4 is synthesized by the thyroid gland and is secreted into the bloodstream. Here the T4 becomes bound to serum proteins for transport to the cells.

The major transport protein is Thyroxine Binding Globulin (TBG) which normally accounts for 80% of the bound T4. Other thyroid hormone binding proteins are Thyroxine Binding Prealbumin and Albumin. Most of the serum T4 is bound to these transport proteins leaving only about 0.03% free to exert its effect on cells. It is the free T4 (fT4) that represents the metabolically active fraction; for this reason the measurement of fT4 concentration is considered to be an indicator of patient thyroid status.

Primary hypothyroidism results in underproduction of T4 by the thyroid gland and consequently an abnormally low circulating fT4 concentration in the blood. Primary hyperthyroidism leads to excessive thyroid production on T4 and resulting elevated fT4 concentration.

Total serum T4 concentrations are dependent on the level of circulating TBG as well as the patient's thyroid status. The concentration of TBG can be affected by certain drugs, steroid hormones, pregnancy, and by various nonthyroid illnesses. In an earlier generation of thyroid function tests, the effect of variable TBG concentration was dealt with by calculating a Free Thyroxine Index (FTI). This FTI is the product of Total T4 concentration and Thyroid Uptake (TU), which assesses the number of available binding sites on the TBG. This approach requires carrying out two separate assay determinations (total T4 and TU), but does provide a better indicator of thyroid status than total T4 alone.

fT4 tests are designed to directly reflect the equilibrium existing in serum between T4 and TGB-bound T4. These methods, including this fT4 test, can generally reflect thyroid status in a single assay.

**CAUTION:** Phoenix Pharmaceuticals guarantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for its particular use and establish optimum sample concentrations.

### **PRINCIPLE OF THE TEST**

The fT4 test is a solid phase competitive enzyme immunoassay. Patient serum samples, standards, and Thyroxine-Enzyme Conjugate Working Reagent are added to wells coated with monoclonal T4 antibody. fT4 in the patient specimen and the T4 labeled conjugate compete for available binding sites on the antibody. After a 60 minutes incubation at room temperature, the wells are washed with water to remove unbound T4 conjugate. A solution of H<sub>2</sub>O<sub>2</sub>/TMB is then added and incubated for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of 3N HCl, and the absorbance is measured spectrophotometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled fT4 in the sample. By reference to a series of fT4 standards assayed in the same way, the concentration of fT4 in the unknown sample is quantified.

## STORAGE

Unopened test kits should be stored at 4°C upon receipt and the microtiter plate should be kept in a sealed bag with desiccants to minimize exposure to damp air. Opened test kits will remain stable until the expiration date shown, provided it is stored as described above. A microtiter plate reader with a bandwidth of 10 nm or less and an optical density range of 0-2 OD or greater at 450 nm wavelength is acceptable for use in absorbance measurement. **DO NOT FREEZE.**

## LIST OF COMPONENTS

### Materials Provided with the Kit:

- T4 Antibody-Coated Microplate, 96 wells
- T4-Enzyme Conjugate Reagent, ready to use, 10.5 ml
- Free T4 Reference Standards, 0, 0.3, 0.95, 2.1, 3.6, and 7.0 ng/dl, 1 ml each
- Color Reagent A, 13 ml
- Color Reagent B, 13 ml
- Stop Solution (3N HCl), 10 ml

### Materials Required but not provided:

- Pipette capable of delivering 50 µl volumes with a precision of better than 1.5%.
- Dispenser(s) for repetitive deliveries of 0.050 ml and 0.200 ml volumes with a precision of better than 1.5%.
- Microplate Reader with 450 nm wavelength absorbance capability.
- Test tubes for dilution of enzyme conjugate and for mixing Color Reagent A with Color Reagent B.
- Absorbent paper for blotting the microplate wells.
- Timer.
- Control Sera for quality control.

## 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 sample without additives only. Serum samples may be refrigerated at 2-8°C for a maximum period of 48 hours. If the samples can not be assayed within 48 hours, they may be stored at temperatures of -20°C for up to 30 days.

## REAGENT PREPARATION

### Working Substrate Solution – Prepare immediately before use.

To prepare H<sub>2</sub>O<sub>2</sub>/TMB solution, make an 1:1 mixing of Color Reagent A with Color Reagent B up to 1 hour before use. Mix gently to ensure complete mixing. The prepared H<sub>2</sub>O<sub>2</sub>/TMB reagent should be made at least 15 minutes before use and is stable at room temperature in the drk for up to 3 hours. Discard excess after use.

## ASSAY PROCEDURE

Note: *Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (18-25°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 and specimen into the assigned well.
3. Add 0.100 ml (100 µl) of Free T4 Enzyme Conjugate Reagent to all wells.
4. Swirl the microplate gently for 20-30 seconds to mix.
5. Incubate 60 minutes at room temperature.
6. Remove the incubation mixture by emptying the plate content into a waste container. Rinse and empty the microtiter plate 5 times with distilled water. Strike the microtiter plate sharply onto absorbent paper or paper towels to remove all residual water droplets.
7. Add 0.200 ml (200 µl) 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.** Gently mix for 10 seconds.
8. Incubate at room temperature in the dark for 20 minutes.
9. Stop the reaction by adding 50 µl of 3N HCl (Stop Solution) to each well.
10. Gently mix for 30 seconds. **It is important to make sure that all the blue color changes to yellow color completely.**
11. Read absorbance at 450 nm with a microtiter well reader within 30 minutes.

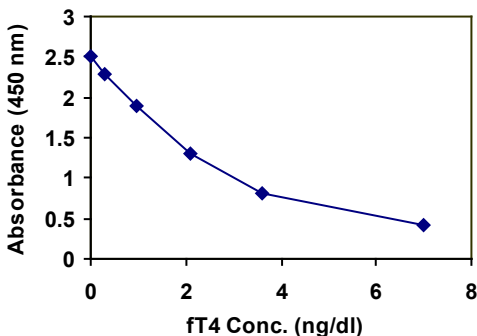
**CALCULATION OF RESULTS**

1. Calculate the mean absorbance value (A450) for each set of reference standards, controls and patient samples.
2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/dl on graph paper, with absorbance values on the vertical or Y axis, and concentrations on the horizontal or X axis.
3. Use the mean absorbance values for each specimen to determine the corresponding concentration of fT4 in ng/dl from the standard curve.

Each laboratory must provide its own data & std. curve in each experiment.

fT4 (ng/dl)	Absorbance (450 nm)
0	2.496
0.3	2.292
0.95	1.903
2.1	1.295
3.6	0.819
7.0	0.410

*Results of a typical standard run with optical density readings at 450 nm shown in the Y axis against fT4 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 in each experiment.*





**EXPECTED VALUES AND SENSITIVITY**

A study of euthyroid adult population was undertaken to determine expected values for the fT4 EIA Test System. The mean (X) values, standard deviations (S.D.) and expected ranges (+/- 2 S.D.) are presented in the table below:

**Expected Values for the Free T4 EIA Test System (in ng/dl):**

	Adult (110 specimens)	Pregnancy (30 specimens)
Mean (X)	1.4	1.5
Std. Dev	0.6	0.7
Expected Ranges (+/- 2 S.D.)	0.8-2.0	0.8-2.2

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 several 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****1. Accuracy**

The fT4 Microplate EIA Test System was compared with a coated tube radio-immunoassay method. Biological specimens from hypothyroid, euthyroid, and hyperthyroid populations were used (Values ranged from 0.1 ng/dl – 8 ng/dl). The total number of such specimens was 85. The least square regression equation and the correlation coefficient were computed for this fT4 Test System EIA in comparison with the reference method. The data obtained is shown in the table below:

Method	Mean (X)	Least Square Regression Analysis	Coefficient
This Method	1.5	$y = 0.10 + 0.952(x)$	.978
Reference	1.4		

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.

## 2. Precision

The within and between assay precision of the fT4 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 shown in the following tables:

<b>Within Assay Precision (Values in ng/dl)</b>				
Sample	N	X	S.D.	C.V.
Low	16	0.3	0.03	9.8%
Normal	16	1.4	0.06	4.5 %
High	16	3.6	0.22	6.2 %

<b>Between Assay Precision (Values in ng/dl)*</b>				
Sample	N	X	S.D.	C.V.
Low	10	0.34	0.04	11.5%
Normal	10	1.35	0.07	3.7 %
High	10	3.69	0.25	4.2 %

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

## 3. Specificity

Results are expressed as the ratio of the fT4 concentration to the concentration of the cross-reactant that will displace 50% of the bound T4 enzyme conjugate x 100%

Cross-Reactant	% Cross-Reactivity
l-Thyroxine (T4)	(100)
d-Thyroxine	100
l-Triiodothyronine (T3)	4.5
d-Triiodothyronine	5.4
Diiodotyronine	<0.06
Diiodotyrosine	<0.05
Iodotyrosine	<0.05
Phenytoin	<0.05
Sodium Salicylate	<0.05

#### 4. Effects of Exogenous Added Drugs

fT4 concentrations were determined in the presence of each of several drugs added to serum at approximately twice the normal therapeutic serum concentration level. The % change in apparent fT4 found in the presence of these drugs was measured, and is shown in the table below.

Substance	Final Concentration Exogenous Drug	% Change in FT4 Concentration
Sodium salicylate	40 mg/dl	+ 1.8
Diphenylhydantoin	30 µg/dl	+ 28.0
Propylthiouracil	4 mg/dl	+ 18.7
Phenylbutazone	30 mg/dl	+ 0.8

##### a. Effects of Endogenous Estrogens

Samples from third trimester pregnant women were analyzed to determine thyroid status by assaying for total T4, Thyroid Uptake, and fT4. In 33% of the samples, the total T4 levels were elevated to borderline hyperthyroid; 67% were euthyroid. The calculated Free Thyroxine index and fT4 concentrations were found to be well within the normal range for all of the samples.

##### b. Sensitivity

The fT4 EIA procedure has a sensitivity of 0.05 ng/dl. The sensitivity was ascertained by determining the variability of the 0 ng/dl serum calibrator and using the  $2\delta$  (95% certainty) statistic to calculate the minimum dose.

## REFERENCE LEVELS

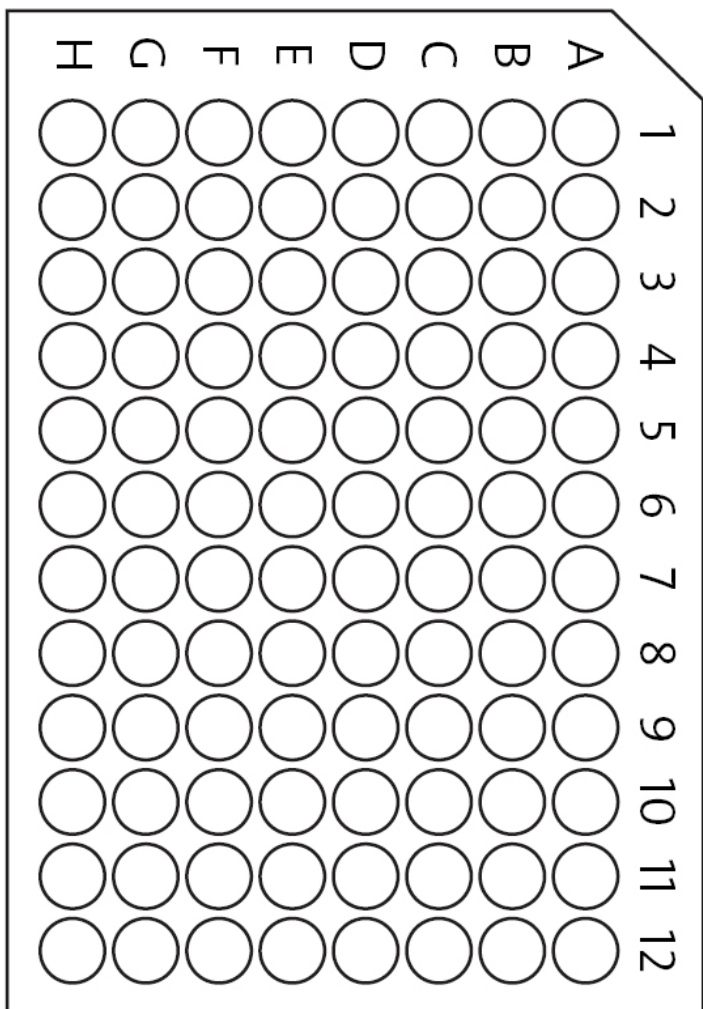
Alterations in the concentration of serum binding proteins will generally result in a corresponding change in total T4 concentrations while the physiologically active fT4 level remains largely unchanged in a euthyroid individual. Therefore, determination of fT4 concentration may provide a more accurate assessment of thyroid status than total T4 measurement. Elevated fT4 Concentrations are indicative of hyperthyroidism and low levels are indicative of hypothyroidism.

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**NOTES**

**ASSAY DIAGRAM**



**NOTES**