AccuDiag™
Free Thyroxine
Free T4
ELISA Kit

REF 3146-15

IVD 2°C 8°C Σ 96 Tests

Test	Free T4 ELISA
Method	Enzyme Linked Immunosorbent Assay
Principle	Competitive Enzyme Immunoassay
Detection Range	0-7.40ng/dL
Sample	50µL
Specificity	97%
Sensitivity	0.314ng/dL
Total Time	~75 min
Shelf Life	12 Months from the manufacturing date

INTENDED USE

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

SUMMARY AND EXPLANATION

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.

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. The free thyroxine concentration can help in uncovering the patient's actual clinical status.

In this method, serum reference, patient specimen, or control is first added to a microplate well. Enzyme-T4 conjugate (analog method) is added and 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 via a wash 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 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.

TEST PRINCIPLE

Competitive enzyme immunoassay – Analog method for free T4
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 insolubilized binding sites. The interaction is illustrated by the followed equation:

$$Enz_{Ag} + Ag + Ab_{C.W.} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{C.W.} + {}^{Enz}AgAb_{C.W.}$$

$Ab_{C.W.}$ = Monospecific Immobilized Antibody (Constant Quantity)

Ag = Native Antigen (Variable Quantity)

${}^{Enz}Ag$ = Enzyme-antigen Conjugate (Constant Quantity)

$AgAb_{C.W.}$ = Antigen-Antibody Complex

${}^{Enz}Ag\ Ab_{C.W.}$ = 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.

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 redtop venipuncture tube without additives or anti-coagulants. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

Specimen(s) may be refrigerated at 2-8°C for a maximum period of (5) days. If the specimen(s) cannot be assayed within this time, the sample (s) may be stored at temperatures of -20 °C for up to 30 days. Avoid the use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100 ml of the specimen is required.

MATERIALS AND COMPONENTS

Materials provided with the test kits

1. **Human Serum References -- 1 ml/vial - Icons A-F**
   Six (6) vials of human serum based reference calibrators for free thyroxine at **approximate*** concentrations of 0 (**A**), 0.40 (**B**), 1.25 (**C**), 2.10 (**D**), 5.00 (**E**) and 7.40 (**F**) ng/dl. 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: ng/dL x 12.9 = pmol/L
2. **fT4- Enzyme Reagent – 13 ml/vial**
   One (1) vial of thyroxine-horseradish peroxidase (HRP) conjugate in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.
3. **Antibody Coated Microplate -- 96 wells**
   One 96-well microplate coated with anti-thyroxine serum and packaged in an aluminum bag with a drying agent. Store at 2-8°C.
4. **Wash Solution Concentrate -- 20ml**
   One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.
5. **Substrate A –7 ml/vial**
   One (1) bottle containing tetramethylbenzidine (TMB) in acetate buffer. Store at 2-8°C.
6. **Substrate B – 7 ml/vial**
   One (1) bottle containing hydrogen peroxide (H_2O_2) in acetate buffer. Store at 2-8°C.
7. **Stop Solution – 8 ml/vial**
   One (1) bottle containing a strong acid (1N HCl). Store at 2-30 °C.
8. **Product Instructions**

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. Kit and component stability are identified on the label.
Note 3: See end of this product insert for various configurations of reagents by kit size.

Materials required but not provided

1. Pipette capable of delivering 50µl & 100µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.350ml volumes with a precision of better than 1.5%.
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent Paper for blotting the microplate wells.

REAGENT PREPARATION

1. **Wash Buffer**
   Dilute contents of Wash solution to 1000ml with distilled or deionized water in a suitable storage container. Dilute buffer can be store at 2-30°C for up to 60 days.
2. **Working Substrate Solution**
   Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly Store at 2-8 °C.

Note 1: Do not use the working substrate if it looks blue.
Note 2: Do not use reagents that are contaminated or have bacteria growth.

ASSAY PROCEDURE

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

** Test Procedure should be by a skilled individual or trained professional**

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 0.050 ml (50µl) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 ml (100µl) of fT4-Enzyme Reagent 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 350µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) 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 two (2) 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.**
   DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION
   Incubate at room temperature for fifteen (15) minutes.
9. Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds. **Always add reagents in the same order to minimize reaction time differences between wells**
10. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. **The results should be read within thirty (30) minutes of adding the stop solution.**

RESULTS

A dose response curve is used to ascertain the concentration of free T4 in unknown specimens.

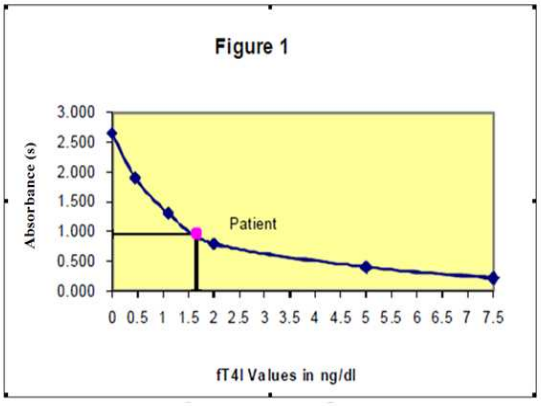
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding Free T4 concentration in ng/dl on linear graph paper (Don't average the duplicates of the serum references before plotting).
3. Connect the points with a best –fit curve.
4. To determine the concentration of Free T4 for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/dl) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (0.964) intersects the dose response curve at (1.65ng/dl) Free T4 concentration (See figure 1).

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value* (ng/dl)
Cal A	A1	2.658	2.612	0.00
	B1	2..566		
Cal B	C1	1.919	1.900	0.45
	D1	1.880		
Cal C	E1	1.339	1.306	1.10
	F1	1.273		
Cal D	G1	0.769	0.790	2.00
	H1	0.811		
Cal E	A2	0.396	0.400	5.00
	B2	0.404		
Cal F	C2	0.215	0.217	7.40
	D2	0.219		
Ctrl 1	E2	1.827	1.835	0.50
	F2	1.843		
Ctrl 2	G2	0.541	0.557	2.70
	H2	0.573		
Patient	A3	0.951	0.964	1.65
	B3	0.976		

Note 1: Computer data reduction software designed for (ELISA) assays may also be used for the data reduction.
If such software is utilized, the validation of the software should be ascertained.

Figure 1



*The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a standard curve prepared with each assay. **Assigned values for calibrators are lot specific.**

Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator 0 µg/dl should be ≥ 1.3.
2. Four out of six quality control pools should be within the established ranges.

A. ASSAY PERFORMANCE

1. It is important that the time of reaction in each well is held constant for reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The additional of substrate solution initiates a kinetic reaction, which terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash steps(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from DAI IFU may yield inaccurate results.
10. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
11. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.

B. INTERPRETATION

1. **Measurements and interpretation of results must be performed by a skilled individual trained professional.**
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, DAI shall have no liability.
5. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
6. If a patient, for some reason reads higher than the highest calibrator report as such (e.g. > 7.4 ng/dl). **Do not try to dilute the sample. TBG variations in different matrices will not allow Free T4 hormone to dilute serially.**
7. Serum free-thyroxine concentration is dependent upon a multiplicity of factors: thyroid gland function and its regulation, Thyroxine binding globulin (TGB) concentration, and the binding of Thyroxine to TBG (3, 4).

Thus, free-Thyroxine concentration alone is not sufficient to assess the clinical status.

8. Serum free-thyroxine values may be elevated under conditions such as pregnancy or administration of oral contraceptives.
9. A decrease in free Thyroxine values is found with protein-wasting diseases, certain liver diseases and administration of testosterone, diphenylhydantoin or salicylates. A table of interfering drugs and conditions, which affect free Thyroxine values, has been compiled by the Journal of the American Association of Clinical Chemists.
10. The interpretation of FT4 is complicated by a variety of drugs that can affect the binding of T4 to the thyroid hormone carrier proteins or interfere in its metabolism to T3. In severe non-thyroidal illness (NTI) the assessment of thyroid becomes especially difficult. Since the patients in this category may suffer from concomitant primary hypothyroidism or from compensatory secondary hypothyroidism. In cases like these a sensitive TSH evaluation of the patient may be recommended.
11. In rare conditions associated with extreme variations in albumin binding capacity for T4- such as familial dysalbuminemic hyperthyroxinemia (FDH) – direct assessment of Free T4 may be misleading.
12. Circulating antibodies to T4 and hormone binding inhibitors may interfere in the performance of the assay.
13. Heparin is reported to have in vivo and in vitro effects on free T4 levels. Samples from patients undegoing heparin therapy should be collected well before the administration of the anticoagulant.

"NOT INTENDED FOR NEWBORN SCREENING"

EXPECTED VALUES

A study of euthyroid adult population was undertaken to determine expected values for the Free T4 EIA Test System. The mean (X) values, standard deviations (σ) and expected ranges (±2 σ) are presented in Table 1.

TABLE I
Expected Values for the Free T4 ELISA Test System (in ng/dl)

	Adult	Pregnancy
Number of Specimens	**89**	**31**
Mean (X)	**1.40**	**1.50**
Standard Deviation (σ)	**0.30**	**0.37**
Expected Ranges (±2 σ)	**0.8 – 2.0**	**0.76 – 2.24**

It is important to keep in mind that stablishment 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 wich a population indigenous to the area in wich the laboratory is lacated.

PERFORMANCE CHARACTERISTICS

I. Precision

The *inter* and *intra* assay precision of the fT4 Microplate ELISA Test System were determined by analyses on three different levels of pooled patient sera. The number (n), mean values (x), standard deviation (σ) and coefficient of variation (C.V.) for each of these control sera are presented in Table 2 and Table 3.

In order to validate the intra-assay precision of the fT4 ELISA Test System, twenty replicates of each of three pooled sera (low medium and high ranges of the dose response curve) were assayed in the same assay. An intra-assay precision of 3.25 to 10.98% was obtained.

TABLE I
Intra-Assay Precision (in ng/dl)

Sample	N	X	σ	C.V.
Low	20	0.550	0.061	10.98%
Medium	20	1.740	0.074	4.26%
High	20	3.250	0.106	3.25%

In order to validate the inter-assay precision of Free Thyroxine (FT4) ELISA one duplicate of each of three pooled sera (low medium and high ranges of the dose response curve) was assayed in 10 assays done over a period of six months that involved five different sets of reagents and three different technicians. An inter-assay precision of 6.01 to 10.81% was obtained.

TABLE 3
Inter Assay Precision (in ng/dl)

Sample	N	X	σ	C.V.
Low	10	0.480	0.052	10.81%
Medium	10	1.410	0.085	6.01%
High	10	3.490	0.279	7.90%

II. Sensitivity

The free T4 ELISA test system has a sensitivity of 0.314 ng/dl. The sensitivity was ascertained by determining the variability of the 0 ng/dl serum calibrator and using the 2σ (95% certainty) statistics to calculate the minimum dose.

III. Accuracy

The FT4 Microplate EIA Test System was compared with a coated tube radioimmunoassay **(RIA)** method. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (The values ranged from 0.1ng/dl – 8ng/dl). The total number of such specimens was 197. The least square regression equation and the correlation coefficient were computed for this FT4 EIA method in comparison with the predicate method (Table 4).

TABLE4
Linear Regression Analysis

Method	Mean (X)	Equation	Correlation Coefficient
DAI EIA ''X''	1.56	y = 0.1034 + 0.9525X	0.920
Predicate RIA ''Y''	1.59		

Only slight amounts of bias between this method and the reference method are indicated by the closeness of the mean values.

IV. Specificity

The cross-reactivity of the thyroxine antibody, used for Free T4 ELISA, 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
I-Thyroxine	1.0000	---
d-Thyroxine	0.9800	10µg/dl
d-Triiodothyronine	0.0150	100µg/dl
I-Triiodothyronine	0.0300	100µg/dl
Lodothyrosine	0.0001	100µg/ml
Diiodotyrosine	0.0001	100µg/ml
Diiodothyronine	0.0001	100µg/ml
TBG	N/D	40µg/ml
Albumin	N/D	40mg/ml
Phenylbutazone	N/D	10µg/ml
Phenytoin	N/D	40µg/ml
Salicylates	N/D	500µg/ml

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.

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 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.

Safe Disposal of kit components must be according to local regulatory and statutory requirements.

REFERENCES

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* **16,** 541-548. (1977)

ISO 13485
ISO 9001

Diagnostic Automation/
Cortez Diagnostics, Inc.
21250 Califa Street, Suite 102 and 116,
Woodland Hills, California 91367 USA

Date Adopted	**2016-02-04**
REF **3146-15**	**AccuDiag™- Free T4 ELISA-2016**
EC REP	**CEpartner4U, Esdoornlaan 13, 3951DB Maarn. The Netherlands.** www.cepartner4u.eu

Revision Date: 2012-06-07