CE

AccuDiag™
CHEMILUMINESCENCE ENZYME IMMUNOASSAY (CLIA)
Free Thyroxine (fT4)

REF 9004-15

IVD See external Label 2°C 8°C Σ 96 Tests

Sensitivity	0.28 ng/dl

INTENDED USE

The Quantitative Determination of Free Thyroxine Concentration in Human Serum by a Microplate Chemiluminescence Immunoassay (CLIA).

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 light.

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 Chemiluminescence Immunoassay – Analog Method for free T4 (Type 5).

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. The interaction is illustrated by the following equation:

$$^{Enz}Ag + Ag + Abc.w. \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAbc.w. + {}^{Enz}AgAbc.w.$$

Abc.w.= Monospecific Immobilized Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
^{Enz}Ag= Enzyme-antigen Conjugate (Constant Quantity)
$AgAb_{c.w.}$ = Antigen- Antibodies complex
$^{Enz}AgAb_{c.w.}$ = 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, determined by reaction with a substrate that generates light, 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 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.

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

MATERIALS AND COMPONENTS

Materials provided with the test kits

A. **Human Serum References – 1ml/vial**
Six (6) vials of human serum reference calibrators for free thyroxine at approximate * concentrations of 0(**A**), 0.4 (**B**), 1.0 (**C**), 1.85(**D**), 3.5 (**E**) and 7.2 (**F**) ng/dl. Store at 2-8 ºC. A preservative has been added.
For SI units: 1ng/dl x 12.9 = pmol/L.
***Exact levels are given on the labels on a lot specific basis.**

B. **fT4 Tracer Reagents – 13ml/vial**

One (1) vial of thyroxine- – horseradish peroxidase (HRP) conjugate in a protein-stabilized matrix. A preservative has been added. Store at 2-8 ºC.

C. **Light Reaction – 96 wells**
One 96-well white microplate coated with anti-thyroxine serum and packaged in an aluminum bag with a drying agent. Store at 2-8 ºC.

D. **Wash Concentrate -20 ml**
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8 ºC (see Reagent Preparation Section).

E. **Signal Reagent A – 7ml/vial**
One (1) bottle containing lumenol in buffer. Store at 2-8 ºC (see Reagent Preparation Section)

F. **Signal Reagent B – 7ml/vial**
One (1) bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8 ºC.(see Reagent Preparation Section).

G. **Product Insert**

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. **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: Above reagents are for a single 96-well microplate

Materials required but not provided

1. Pipette(s) capable of delivering 50µl 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 washer or a squeeze bottle (optional).
4. Microplate luminometer.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer
9. Quality Control Materials.

REAGENT PREPARATION

1. **Wash Buffer**
Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at room 2-30 ºC. for up to 60 days.
2. **Working Signal Reagent Solution**
Store at 2-8. ºC. Determine the amount of reagent needed and prepared by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1ml of A 1ml of B per two (2) eight well strips (A slight excess of solution is made). **Discard the unused portion if not used within 36 hours after mixing**. If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

Note: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20-27 ºC).
*****Test Procedure should be performed 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 ºC.**
2. Pipette 0.050 ml (50 µl) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 ml (100 µl) of fT4-Tracer Reagents to all wells.
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 45 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 350 µl of wash buffer (see Reagent Preparation Section) decant (tab and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instructions 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 (100 µl) of working signal reagent 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
9. Incubate for five (5) minutes in the dark.
10. Read the relative light units in each well for 0.2 – 1.0 seconds.
The results should be read within thirty (30) minutes of adding the signal solution.

RESULTS

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

1. Record the RLU's (Relative Light Units) obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the RLU's for each duplicate serum reference versus the corresponding fT4 concentration in ng/dl on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of fT4 for an unknown, locate the average RLU's for each unknown on the vertical axis of the graph, find the intersecting point on the curve , and read the concentration (in pg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average RLU's (53513) of the unknown intersects the calibration curve at (1.08ng/dl) fT4 concentration (See Figure 1)*

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

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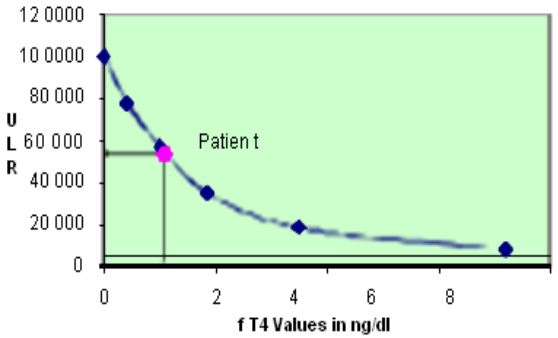
Sample ID	Well Number	Mean RLU (A)	Mean RLU (B)	Value (pg/ml)
Cal A	A1	99385	100000	0.00
	B1	100615		
Cal B	C1	78937	77670	0.40
	D1	76403		
Cal C	E1	56645	57078	1.00
	F1	57511		
Cal D	G1	34449	35218	1.85
	H1	35806		
Cal E	A2	18830	18678	3.50
	B2	18526		
Cal F	C2	8191	8156	7.20
	D2	8120		
Ctrl 1	E2	61664	60668	**0.86**
	F2	59671		
Ctrl 2	G2	38592	37577	**1.73**
	H2	36563		
Patient	A3	52742	53513	**1.08**
	B3	54283		

* The data presented in Example 1 and Figure 1 is for illustration only and should not be used in lieu of a dose response curve prepared with each assay. In addition, the RLU's of the calibrators have been normalized to 100,000 RLU's for the A calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.

Figure 1



Q.C. PARAMETERS

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

1. The Dose Response Curve should be within established parameters.
2. Four out of six quality control pools should be within the established ranges.

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.

Interpretation

1. **Measurement and Interpretation of results must be performed by a skilled individual or 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, Diagnostic Automation, Inc. 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 that 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-Thyroxdine concentration is dependent upon a multiplicity of factors: thyroid glad function and its regulation,Thyyroxine binding globulin (TGB) (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-washing 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 complied 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.
11. 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.
12. 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.

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13. Circulating antibodies to T4 and hormone binding inhibitors may interfere in the performance of the assay.
14. Heparin is reported to have in vivo and in vitro effects on free T4 levels. Samples from patients undergoing 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 CLIA Test System. The mean (R) values, standard deviations (σ.) and expected ranges (±2σ.) are presented in Table 1.

Table 1

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

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.

ASSAY PERFORMANCE

1. It is important that the time of reaction in each well is held constant to achieve 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 addition of signal reagent initiates a kinetic reaction, therefore the signal reagent(s) should be added in the same sequence to eliminate any time-deviation during reaction.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash steps(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.
8. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Diagnostic Automation, Inc. IFU may yield inaccurate results.
9. 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.
10. 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.

PERFORMANCE CHARACTERISTICS

Precision

The inter and intra assay precision of the fT4 CLIA method were determined by analyses on three different levels of pooled patient sera. The number, mean values, standard deviation (σ) and coefficient of variation for each of these controls sera are presented in Table 2 and Table 3.

In order to validate the within-assay precision of fT4 CLIA assay, 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 5.32% to 9.34% was obtained.

TABLE 2 Intra-Assay Precision - In ng/dl

Sample	N	X	σ	CV%
Low	20	0.460	0.043	9.34%
Normal	20	1.540	0.082	5.32%
High	20	3.144	0.233	7.09%

In order to validate the inter-assay precision of fT4 CLIA assay, one set in duplicate of each of three pooled sera (low medium and high ranges of the dose response (curve) was assayed in 10 assays performed over a period of six months that involved five different sets of reagents and three differentechnicians. An inter-assay precision of 5.26% to 9.76% was obtained.

TABLE 3 Inter-Assay Precision - In ng/dl

Sample	N	X	σ	CV
Low	10	0.491	0.048	9.76%
Normal	10	1.463	0.077	5.26%
High	10	3.227	0.250	7.75%

Sensitivity

The free thyroxine procedure has a sensitivity of 0.28 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.

Accuracy

The FT4 CLIA method was compared to an enzyme immunoassay. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (The values ranged from 0.11ng/dl – 6.8ng/dl). The total number of such specimens was 108. The least square regression equation and the correlation coefficient were computed for this method in comparison with the predicate assay (Table 4).

TABLE 4
Linear Regression Analysis

	Mean	Equation
Method	(x)	
Accudiag™ CLIA "X"	1.38	y=0.0727+0.987x
Predicate EIA "Y"	1.45	

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.

Specificity

The cross-reactivity of the thyroxine antibody, used for Free T4 CLIA, 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 tracer.

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Substance	Cross Reactivity	Concentration
I-Thyroxine	1.0000	-
d-Thyroxine	0.9800	10µg/ml
d-Triiodothryonine	0.0150	100µg/ml
l-Triiodothryonine	0.0300	100µg/ml
Iodothyrosine	0.0001	100µg/ml
Diiodotryrosine	0.0001	100µg/ml
Diiodothryronine	0.0001	100µg/ml
TBG	N/D	40µg/ml
Albumin	N/D	40µg/ml
Phenylbutazone	N/D	10µg/ml
Phenytoin	N/D	40µg/ml
Salicylates	N/D	100µg/ml

PRECAUTION

1. For in vitro diagnostic use only.
2. 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 required tests. 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.

ISO 13485
ISO 9001

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