Free Thyroxine (fT4) CLIA

REF CAN-fT4-6040 Version: 5.0

Effective: May 18, 2011

INTENDED USE
For the direct quantitative determination of free thyroxine (fT4) in human serum by a chemiluminescence immunoassay (CLIA). For *in vitro* use only.

PRINCIPLE OF THE TEST
The principle of the following chemiluminescence immunoassay (CLIA) test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, control and patient samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. The washing and decanting procedures remove unbound materials. After the washing step, the luminescence substrate solution is added. The relative luminescence units (RLUs) are measured on a microtiter plate luminometer. The RLU values are inversely proportional to the concentration of fT4 in the sample. A set of calibrators are used to plot a standard curve from which the amount of fT4 in patient samples and controls can be directly read.
The labeled T4 (conjugate) employed in this assay system has shown no binding properties towards thyroxine-binding globulin (TBG) and human serum albumin (HSA). The binding sites on the microwell plates are designed to be of a low binding-capacity in order not to disturb the equilibrium between T4 and its carrying proteins. The assay is carried out under normal physiological conditions of pH, temperature and ionic strength.

CLINICAL APPLICATIONS
Thyroxine (T4), the principal thyroid hormone, circulates in blood almost completely bound to carrier proteins. However, only the free (unbound) fraction of thyroxine is considered to be biologically active. The main carriers of thyroxine are thyroxine-binding globulin (TBG), pre-albumin and albumin. The measurement of free thyroxine (fT4) levels correlate better with the clinical status than total thyroxine levels
The dbc free T4 assay is a one step competitive LIA system that is rapid and easy to perform compared to equilibrium dialysis and ultrafiltration methods, which are cumbersome and time-consuming. This system employs a highly specific monoclonal antibody and a non-analog tracer that was proved experimentally to have no significant binding to TBG and albumin.
In the euthyroid, normal population the free T4 concentration is 7-22 pg/mL. The level of free T4 is decreased in hypothyroidism while in thyrotoxic patients the level of free T4 is increased.
This assay is used at times with other thyroid tests for *in vitro* diagnostic purposes and for assessing patients who are receiving thyroid treatments (follow-up).

PROCEDURAL CAUTIONS AND WARNINGS
1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Control materials or serum pools should be included in every run at a high and low level for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.
5. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
6. A calibrator curve must be established for every run.
7. The kit controls should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the controls do not reflect established ranges.
9. The luminescence substrate solutions (A and B) are sensitive to light and should be stored in the original dark bottle away from direct sunlight.
11. When dispensing the substrate, do not use pipettes in which these liquids will come into contact with any metal parts.
12. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
13. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
14. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS
1. All the reagents within the kit are calibrated for the determination free T4 in human serum. The kit is not calibrated for the determination of free T4 in saliva, plasma or other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
4. Samples reading higher than the highest calibrator should be reported as such and should not be diluted. Dilution will alter the existing equilibrium and may lead to false results.
5. 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. Consequently, the clinical diagnosis should include all aspects of a patient's background including the frequency of exposure to animals/products if false results are suspected.
6. Some individuals may have antibodies to mouse protein that can possibly interfere in this assay. Therefore, the results from any patients who have received preparation of mouse antibodies for diagnosis or therapy should be interpreted with caution.

SAFETY CAUTIONS AND WARNINGS
POTENTIAL BIOHAZARDOUS MATERIAL
Human fluids that may be used in the preparation of the standards and control have been tested and found to be non-reactive for Hepatitis B surface antigen and have also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. However no test method can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any human specimen.

CHEMICAL HAZARDS
Avoid direct contact with reagents. In case of contact, wash with plenty of water.

SPECIMEN COLLECTION AND STORAGE
Approximately 0.2 mL of serum is required per duplicate determination. Collect 4-5 mL of blood into an appropriately labelled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

SPECIMEN PRETREATMENT
This assay is a direct system; no specimen pretreatment is necessary.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED
1. Precision pipettes to dispense 25, 100, 150 and 300 µL
2. Disposable pipette tips
3. Distilled or deionized water
4. A 37 °C incubator
5. Plastic wrap or microtiter plate cover.
6. Microwell plate luminometer

REAGENTS PROVIDED AND PREPARATION
1. Mouse Anti-fT4 Antibody Coated Microwell Plate-Break Apart Wells - Ready To Use.
Contents: One 96 well (12x8) monoclonal antibody-coated microwell plate in a resealable pouch with desiccant.
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

2. fT4-Horseradish Peroxidase (HRP) Conjugate Concentrate - X50
Contents: fT4-HRP conjugate in a protein-based buffer with a non-mercury preservative.
Volume: 0.3 mL/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation of conjugate working solution: Dilute conjugate concentrate 1:50 in assay buffer before use (example: 40 µL of conjugate concentrate in 2 mL of assay buffer). If the whole plate is to be used dilute 240 µL of conjugate concentrate in 12 mL of assay buffer. Discard any that is left over.

3. fT4 Calibrators - Ready To Use.
Contents: Five vials containing fT4 in a human serum-based matrix with a non-mercury preservative. Prepared by spiking serum with an exact quantity of T4.
*Listed below are approximate concentrations, please refer to vial labels for exact concentrations.

Calibrator	Concentration	Volume
Calibrator A	0 pg/mL	0.5 mL
Calibrator B	2 pg/mL	0.5 mL
Calibrator C	6 pg/mL	0.5 mL
Calibrator D	20 pg/mL	0.5 mL
Calibrator E	80 pg/mL	0.5 mL

Storage: Refrigerate at 2-8°C
Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

4. Controls - Ready To Use.
Contents: Two vials containing fT4 in a human serum-based matrix with a non-mercury preservative. Prepared by spiking serum with a defined quantity of T4. Refer to vial label for acceptable range.
Volume: 0.5 mL/vial
Storage: Refrigerate at 2-8 °C
Stability: 12 months in unopened vial or as indicated on label. Once opened, the control should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. Wash Buffer Concentrate - X10
Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 50 mL/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation of wash buffer working solution: Dilute wash buffer concentrate 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 mL of wash buffer concentrate in 450 mL of water.

6. Assay Buffer - Ready To Use.
Contents: One bottle containing a protein-based buffer with a non-mercury preservative.
Volume: 15 mL/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

7. Rinse Solution - Ready To Use.
Contents: Two bottles containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 2 x 50 mL/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

8. CLIA Substrate Reagent A - Requires Preparation.
Contents: One vial containing luminol enhancer.
Volume: 0.8 mL/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: See preparation of LIA working substrate solution.

9. CLIA Substrate Reagent B - Requires Preparation.
Contents: One vial containing peroxide solution.
Volume: 1.6 mL/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: See preparation of LIA working substrate solution.

10. CLIA Substrate Reagent C - Requires Preparation.
Contents: One bottle containing buffer with a non-mercury preservative.
Volume: 15 mL/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

CE - European Conformity | Consult Instructions For Use | IVD - In Vitro Diagnostic Use | LOT - Lot Number | Temperature Limit | - Use By
- Manufacturer | REF - Catalogue Number | X# - Dilute 1:# Before Use | + # mL - Reconstitute With # mL H_2O | EC REP - Authorized Representative

Preparation: See preparation of LIA working substrate solution.

Preparation of CLIA Working Substrate Solution

In a clean container mix 1 part of CLIA substrate reagent A with 2 parts of CLIA substrate reagent B and 20 parts of CLIA substrate reagent C. This gives the ready to use substrate solution. If the whole plate is to be used prepare working substrate solution as follows: Combine 0.6 mL of LIA substrate reagent A with 1.2 mL of LIA substrate reagent B and 12 mL of LIA substrate reagent C. It is suggested to wait at least 30 minutes prior to use after preparation of the working substrate solution. The working substrate solution is stable for up to 8 hours at room temperature. Discard the leftovers.

ASSAY PROCEDURE

Important Notes:
1. All reagents must reach room temperature before use.
2. Once the procedure has been started, all steps should be completed without interruption to ensure equal elapsed time for each pipetting step.

1. Prepare working solutions of the conjugate, wash buffer and LIA substrate (refer to reagents provided and preparation section).
2. Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 25 µL of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.
4. Pipette 100 µL of the conjugate working solution into each well (We recommend using a multichannel pipette).
5. Cover plate and incubate for 1 hour in a 37 °C incubator.
6. Wash the wells 5 times, each time with 300 µL of diluted wash buffer per well and on the last washing tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is recommended).
7. Pipette 150 µL of LIA working substrate solution into each well (We recommend using a multichannel pipette).
9. Measure the RLUs in each well on a microplate luminometer between 5 and 20 minutes after addition of the substrate.

CALCULATIONS

1. Calculate the mean RLU of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean RLUs on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter curve is recommended.
3. Calculate the mean RLU of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.

5 Samples reading higher than the highest calibrator should be reported as such and should not be diluted. Dilution will alter the existing equilibrium and may lead to false results.

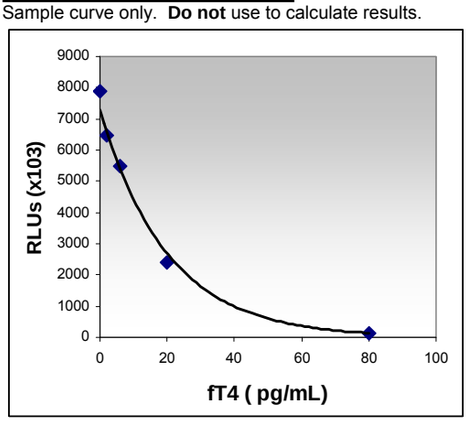
TYPICAL TABULATED DATA**

Calibrator	RLU 1 x 10^3	RLU 2 x 10^3	Mean RLU x 10^3	RLU/RLU_{MAX} (%)
A, 0 pg/mL	8136	7644	7890	100
B, 2 pg/mL	6825	6160	6492	82
C, 6 pg/mL	5679	5990	5835	74
D, 20 pg/mL	2079	1999	2039	26
E, 80 pg/mL	143	133	138	2

**- It is recommended to use the RLU/RLU_{MAX} values for comparative purposes since luminometers vary considerably between manufacturers. Results from different luminometers will show quite different RLU values, however, the RLU/RLU_{MAX} values remain consistent.

TYPICAL CALIBRATOR CURVE

Sample curve only. **Do not** use to calculate results.



PERFORMANCE CHARACTERISTICS

SENSITIVITY

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean RLU of calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the dbc fT4 LIA kit is **1.0 pg/mL.**

SPECIFICITY (CROSS-REACTIVITY)

The following compounds were tested for cross-reactivity with the dbc fT4 LIA kit with T4 cross-reacting at 100%:

Compound	%Cross-Reactivity
L-Thyroxine	100
D-Thyroxine	94
3,3',5'-Triiodo-L-Thyronine (Reverse T3)	86
3,3',5-Triiodo-L-Thyronine (T3)	3.3
3,3',5'-Triiodo-D-Thyronine	1.8
3,3',5'-Triiodothyropropionic acid	0.6

The following compounds were tested but cross-reacted at less than 0.04%: Acetylsalicylic acid, 3,5-Diiodo-L-Thyronine, 3,5-Diiodo-L-Tyrosine and 3-Iodo-L-Tyrosine.

INTRA-ASSAY PRECISION

Three samples were assayed ten times each on the same calibrator curve. The results (in pg/mL) are tabulated below:

Sample	Mean	SD	CV%
1	3.79	0.16	4.8
2	23.26	1.14	4.9
3	70.60	3.04	4.3

INTER-ASSAY PRECISION

Three samples were assayed ten times over a period of four weeks. The results (in pg/mL) are tabulated below:

Sample	Mean	SD	CV%
1	4.27	0.53	12.3
2	20.54	2.36	11.5
3	67.34	6.67	9.9

EFFECT OF BILIRUBIN

Bilirubin was added to a patient sample at concentrations of 50 and 100 µg/mL and assayed with the dbc fT4 LIA kit. Results are tabulated below:

Sample	fT4 (pg/mL)
Unspiked	8.78
+ 50 µg/mL bilirubin	10.68
+100 µg/mL bilirubin	9.72

No significant effect was observed at these concentrations.

EFFECT OF HUMAN SERUM ALBUMIN (HSA)

Purified human serum albumin (HSA) was added to a patient sample at concentrations of 10, 20 and 40 mg/mL. Samples were assayed with the dbc fT4 LIA kit. Results are tabulated below:

Sample	fT4 (pg/mL)
Unspiked	8.78
+10 mg/mL	8.81
+20 mg/mL	9.46
+40 mg/mL	9.90

No binding of labelled fT4 to HSA was found at these concentrations.

EFFECT OF THYROXINE-BINDING GLOBULIN (TBG)

The zero calibrator was spiked precisely with purified TBG at concentrations ranging from 25-200 µg/mL and assayed with the dbc fT4 LIA kit. Results are tabulated below:

Sample	TBG Added (µg/mL)	RLU (x10^6)
1	0	1.294
2	25	1.390
3	50	1.472
4	100	1.490
5	200	1.542

No significant binding of labelled fT4 to TBG was found at these concentrations.

EFFECT OF NON-ESTERIFIED FATTY ACIDS

Oleic acid was added to a patient sample at concentrations of 0.5, 5 and 20 mmol/L and assayed with the dbc fT4 LIA kit. Results are tabulated below:

Sample	fT4 (pg/mL)
Unspiked	24.83
+0.5 mmol/L	20.53
+5 mmol/L	26.06
+20 mmol/L	83.64

At high concentrations of oleic acid, the free T4 level was significantly increased. This is due to the well-known effect that non-esterified fatty acids can dissociate T4 from its carrier proteins.

EXPECTED NORMAL VALUES

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values. The following reference range was established with 80 apparently healthy adults:

Group	N	Range (pg/mL)
Normal Euthyroid Samples	80	7-22

REFERENCES

1. Ingbar,S.H.et at. J. Clin. Invest. 44:1679, 1965.
2. Robins, J. Metabolism 22(8):1021, 1973.
3. Schall, R.F. J. Clin. Chem. 24(10):1801, 1978.
4. Selenkow,H.A. and Robin, N.I. J.Maine Med. Assoc. 61:199, 1970.
5. Oppenheimer, J.H. et. al. J. Clin. Invest. 42:1769, 1963.
6. Young, D.S., et. al. Clin. Chem. 21:3640, 1975.
7. Sterling, K., and Hegedus, A. J. Clin. Invest. 41:1031, 1962.
8. Cavalieri, R.R., et. al. Clin. Res. 15:124, 1967.
9. Comoglio, S. and Celada, F.J. Immunol. Meth. 10:161-170, 1976
10. McComb, R. B., Bowers, G.N., Posen, S. Alkaline Phosphatase, 1st Ed., Chap. 9, pg. 525-704, Plenum Press, New York, 1979

OTHER RELATED dbc KITS

Also available from stock are the following dbc kits:

- dbc TSH LIA Kit, REF CAN-TSH-6030
- dbc Total T4 ELISA Kit, REF CAN-T4-4240
- dbc Total T3 ELISA Kit, REF CAN-T3-4220
- dbc Free T3 ELISA Kit, REF CAN-fT3-4230
- dbc TSH ELISA Kit, REF CAN-TSH-4080
- dbc TSH LIA Kit, REF CAN-TSH-6030
- dbc Anti-Thyroglobulin Autoantibodies, REF CAN-TGAb-4000
- dbc Anti-TPO Autoantibodies, REF CAN-TPOAb-4700

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