EAGLE
BIOSCIENCES

# Free Thyroxine (fT4) LIA Assay Kit

Catalog Number:
T4F31-L01 (1 x 96 wells)
For Research Use Only. Not for use in diagnostic procedures.
*v. 8.0 (effective 19Apr23)*

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## INTENDED USE

The Eagle Biosciences Free Thyroxine (fT4) LIA Assay Kit is for the direct quantitative determination of free thyroxine in human serum by a chemiluminescence immunoassay (LIA). The Eagle Biosciences Free Thyroxine (fT4) LIA assay kit is for research use only and not to be used in diagnostic procedures.

## INTRODUCTION

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 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.

## PRINCIPLE OF THE ASSAY

The principle of the following chemiluminescence immunoassay (LIA) test follows the typical competitive binding scenario. Competition occurs between an unlabelled antigen (present in standards, control and patient samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microplate. 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 labelled 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 microplates 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.

## 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. The performance of this assay is markedly influenced by the correct execution of the washing procedure!
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.
10. When dispensing the substrate, do not use pipettes in which these liquids will come into contact with any metal parts.
11. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
12. 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.
13. 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. This kit is intended for research use only and should not be used in diagnostic procedures.
6. Some individuals may have antibodies to mouse protein that can possibly interfere in this assay. Therefore, the results form any specimens that 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 controls has been tested and found to be nonreactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. No test method however, 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 microplate cover
6. Microplate luminometer

## REAGENTS PROVIDED AND PREPARATION

1. Mouse Anti-fT4 Antibody-Coated Break-Apart Well Microplate — Ready To Use

   Contents: One 96-well (12x8) monoclonal antibody-coated microplate 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 — Requires Preparation 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 Testosterone 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 bottle labels for exact concentrations.

| Calibrator | Concentration | Volume/Vial |
|---|---|---|
| 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 precise quantity of T4. Refer to vials labels for expected value and acceptable range.
   Volume: 0.5 mL/vial
   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.

5. Wash Buffer Concentrate – Requires Preparation 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: Dilute 1:10 in distilled or deionized water before use. If one whole plate is to be used dilute 50 mL of the wash buffer concentrate in 450 mL of water.

6. Assay Buffer – Ready to Use
   Contents: One bottle containing 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. LIA Substrate Reagent A – Requires Preparation
   Contents: One vial containing luminol plus 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.

8. LIA Substrate Reagent B – Requires Preparation
   Contents: One vial containing stabilized 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.

9. LIA 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. |
| Preparation: | See preparation of LIA working substrate solution. |

## PREPARATION OF LIA WORKING SUBSTRATE SOLUTION

In a clean plastic container (glass is not suitable) mix 1 part of LIA substrate reagent A with 2 parts of LIA substrate reagent and 20 parts of LIA substrate reagent C. This gives the ready to use substrate solution.

If the whole plate is to be used prepare working substrate soslution as follows:
Combine 0.7 mL of LIA substrate reagent A with 1.4 mL of LIA substrate reagent B and 14 mL of LISA substrate reagent C. The working substrate solution is stable for up to 4 hours at room temperature. Discard the leftovers.

## ASSAY PROCEDURE

Important Notes:

- All reagents must reach room temperature before use.
- Once the procedure has been started, all steps should be completed without interruption to ensure equal elapsed time for each pipetting step.
- The washing procedure influences the precision markedly; it is essential to ensure the washing is effective and thorough

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 well 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 the 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).
8. Measure the RLUs in each well on a microplate luminometer between 20-30 minutes after addition of the substate.

## 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 not be diluted. Dilution will alter the existing equilibrium and may lead to false results.

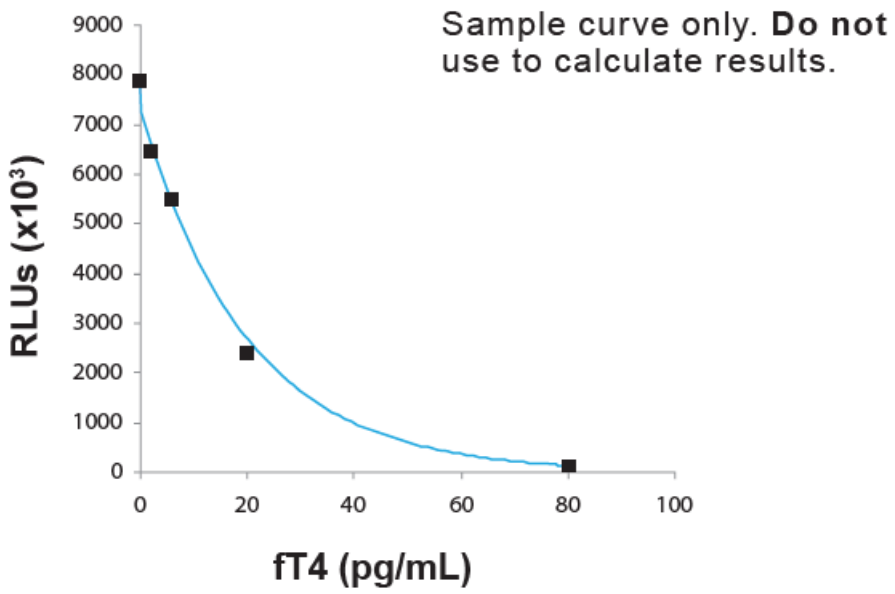
## TYPICAL TABULATED DATA**

Sample data only. Do not use to calculate results.

| 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 purposed since luminometers vary considerably between manufacturers. Results from different luminometers will show quite different RLI values, however, the RLU/$RLU_{MAX}$ values remain consistent.

## TYPICAL CALIBRATOR CURVE



## 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 fT4 LIA kit is **1.0 pg/mL.**

### SPECIFICITY (CROSS-REACTIVITY)

The following compounds were tested for cross-reactivity with the 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 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 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 25200 µg/mL and assayed with the 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 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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## Warranty Information

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For further information about this kit, its application or the procedures in this kit, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.