TRUSTwell®

# Free T4 (fT4) ELISA Kit

**IVD | REF AE1026**

**Instructions for Use**

- 96-well ELISA kit for the quantitative determination of Free Thyroxine (fT4) concentration in human serum
- Store at 2-8°C upon receipt

## INTENDED USE

The Free Thyroxine (fT4) ELISA is a competitive solid-phase enzyme-linked immunosorbent assay for the quantitative determination of fT4 levels in human serum. It is intended to be used by trained professionals as an aid in the diagnosis of thyroid dysfunction.

## INTRODUCTION

Thyroid disease is very common on a global level and studies have shown that the prevalence of undiagnosed thyroid disorder is high[1-3]. Thus, quantification of markers that reflect thyroid function is very important. Thyroid function and metabolism are controlled by the human hypothalamic-pituitary-thyroid (HPT) axis, which is made up of the hypothalamus, the pituitary gland, and the thyroid. Thyroid stimulating hormone (TSH) controls the production of the thyroid hormones thyroxine (T4) and 3, 5, 3' triiodothyronine (T3), which play an important role in the regulation of metabolism[4-6].

The majority of T3 and T4 that circulates in the blood is protein-bound, and attaches to plasma proteins such as Thyroxine-Binding Globulin (TBG). The small percentage that is not protein-bound is considered "free" (fT4/fT3), and can readily enter target tissue. Importantly, the binding protein concentration of each individual can be affected by factors such as pregnancy, hormonal therapy, and certain medications, which will, therefore, affect T4/T3 concentrations[6-8]. Due to the general stability of the "free" thyroid hormone concentrations, fT4/fT3 test results may be considered more useful in many cases[3,6,7,8].

Testing of thyroid function commonly incorporates TSH testing along with a combination of T3, T4, fT3, and fT4 assays[3,7,8]. Generally, and depending on the status of the individual's TSH concentration, elevated fT4 results may indicate hyperthyroidism, and low fT4 results may indicate hypothyroidism. Free T4 tests are also commonly used to monitor pregnancy and certain treatments for thyroid disorder, pituitary disease, and thyroid cancer[8,9]. Combining fT4 with other thyroid hormone results and any other necessary test results will help provide enough information for the healthcare provider to design a proper plan of care.

## TEST PRINCIPLE

The Free Thyroxine (fT4) ELISA is a competitive solid-phase enzyme-linked immunosorbent assay for the quantitative measurement of fT4 concentration in human serum.

The Free Thyroxine (fT4) ELISA is comprised of three key components:
1) Solid microwells pre-coated with anti-fT4 antibody,
2) fT4 Calibrators,
3) fT4 Enzyme Reagent comprised of horseradish peroxidase conjugated to T4(T4-HRP)

During the assay, the calibrator, control, or test specimen as well as the T4-HRP are added to the T4-antibody coated microwell. During the 60 minute incubation, the fT4 in the test specimen and in the T4-HRP compete for binding to the antibody coated on the microwell surface.

Unbound material is then removed by washing. In the next step, the TMB Substrate is added and the presence of the HRP bound to microwell surface is shown by the development of a blue color. The reaction is then terminated with the Stop Solution and the optical density (OD) is determined using a spectrophotometer at 450/610-650 nm. The color intensity reflects the amount of T4-HRP bound to the microwell surface, and is inversely proportional to the amount of fT4 in the control or test specimen.

A standard curve can then be developed by plotting the fT4 Calibrator concentrations on the x-axis against the relative OD values on the y-axis. The fT4 concentration of each assay control or test specimen can then be interpolated from the standard curve.

## MATERIALS AND REAGENTS

**Materials and reagents provided:**

| Item | Description | | Quantity | Catalog |
|---|---|---|---|---|
| 1 | Anti-fT4 Ab Coated Microwells | | 8 wells x 12 strips | AE1026W |
| 2 | fT4 Calibrators*: | C1 (0.0 ng/dL) | 1 mL | AE1026C1 |
| 3 | | C2 (0.4 ng/dL) | 1 mL | AE1026C2 |
| 4 | | C3 (1.0 ng/dL) | 1 mL | AE1026C3 |
| 5 | | C4 (2.5 ng/dL) | 1 mL | AE1026C4 |
| 6 | | C5 (5.0 ng/dL) | 1 mL | AE1026C5 |
| 7 | | C6 (8.0 ng/dL) | 1 mL | AE1026C6 |
| 8 | fT4 Assay Control* | | 1 mL | AE1026AC |
| 9 | fT4 Enzyme Reagent | | 12 mL | AE1026H |
| 10 | Wash Buffer Concentrate (40X) | | 20 mL | AWE3002 |
| 11 | TMB Substrate | | 12 mL | ATME2000 |
| 12 | Stop Solution | | 7 mL | ASE1000 |
| 13 | Product Insert | | 1 | PI-AE1026 |
| 14 | ELISA Working Sheet | | 2 | AE0001ES |
| 15 | Microplate Sealers | | 3 | |
| 16 | Desiccant | | 4 | |

**The Calibrators and Assay Controls provided are lot-specific. Please see vial labels for exact concentrations or ranges, respectively*

**Materials and reagents required but not provided in the kit**

1. Pipette capable of delivering 50 µL,100 µL, and 1 mL
2. Microplate reader, washer and incubator
3. Vortex mixer or equivalent
4. Absorbent paper for blotting the microwells
5. Graph paper
6. Timer
7. Distilled or de-ionized water

## STORAGE AND STABILITY

All reagents except the concentrated wash buffer are ready to use as supplied. Store all components at 2-8°C. Do not freeze. Avoid strong light. Ensure that the reagents are brought to room temperature before opening. After removing the desired number of wells, place unused wells in the resealable foil pouch provided with desiccant and return to 2-8°C. All reagents are stable through the expiration date printed on the label if not opened. Once opened, the kit is stable for 8 weeks at 2-8°C, or until the labeled expiration date, whichever is earlier.

## SPECIMEN COLLECTION AND PREPARATION

- Serum specimens should be prepared from whole blood obtained by acceptable venipuncture technique.
- If not tested immediately, the specimens can be stored at 2-8°C for up to 7 days. The specimens should be frozen at -20°C for longer storage. Avoid multiple freeze-thaw cycles. If a specimen is to be shipped, pack in compliance with federal regulations covering the transportation of etiologic agents.
- Specimens containing precipitants may give inconsistent test results. Clarify such specimens by centrifugation before testing.
- Do not use specimens demonstrating gross lipemia, gross hemolysis or turbidity.
- Specimens containing sodium azide may interfere with test results.

## PREPARATION OF THE REAGENTS

1. Bring all reagents to room temperature (22-28°C).
2. **Preparation of working Wash Buffer:**
   If precipitants are visible, warm up the Wash Buffer (40X concentrate) at 37°C. Dilute concentrated Wash Buffer 40 fold with water as follows:

| Plate | DI water | 40X wash buffer | Final volume |
|---|---|---|---|
| Full plate | 195 mL | 5 mL | 200 mL |
| Half plate | 97.5 mL | 2.5 mL | 100 mL |
| Quarter plate | 48.75 mL | 1.25 mL | 50 mL |

3. Mix each reagent before adding to the test wells.
4. Determine the number of strips needed and mark on the ELISA working sheet with the appropriate information. **Calibrators should be run in duplicate to ensure accuracy.**

## ASSAY PROCEDURE

1. Calculate the desired number of microwells. Remove the remaining microwells and place them with desiccant into the resealable foil bag, seal and store at 2- 8°C for later use.
2. Add 50 µL of fT4 calibrators, fT4 Assay Controls, and test specimens into the assigned wells, respectively.
3. Add 100 µL of fT4 Enzyme Reagent into all wells.
4. Cover the plate with a microplate sealer, and then shake gently for 10 seconds.
5. Incubate the microplate at room temperature (22-28°C) for 60 minutes.
6. Wash Step (Can be performed manually or with automated washing):
   **Manual washing:** Carefully remove the incubation mixture by disposing the solution into a waste container. Fill each well with 350 µL of working wash buffer and mix gently for 20-30 seconds. Discard the wash solution completely. Repeat 2 more times. After completing the last wash step, tap the plate on absorbent paper to remove residual liquid.
   **Automated washing:** Automatic plate washer must be calibrated to ensure efficient washing. Fill each well with 350 µL of diluted wash buffer. Aspirate all wells completely. Repeat 2 more times.
7. Add 100 µL of TMB Substrate into each well.
8. Cover the microplate and incubate at room temperature (22-28°C) for 15 minutes.
9. Stop the reaction by adding 50 µL of Stop Solution to each well. Gently mix for 10 seconds. Add the Stop Solution in the same sequence as substrate addition. **It is important to make sure that all the blue color changes completely to a yellow color.**
10. Set the microplate reader wavelength at 450 nm with a filter of 610-650 nm as reference wavelength to optimize the assay result. Measure the OD value of each well within 30 minutes after adding Stop Solution.

**Flow chart of assay procedure**

| | | |
|---|---|---|
| 1. | Secure strips in microwell frame | Number of strips |
| 2. | Add fT4 Calibrators, fT4 Assay Controls, or specimens | 50 µL |
| 3. | Add fT4 Enzyme Reagent | 100 µL |
| 4.<br>5. | Shake<br>Incubate | 10 seconds<br>22-28°C, 60 minutes |
| 6. | Wash: manual or automatic | 3 times |
| 7. | Add TMB Substrate | 100 µL |
| 8. | Incubate in dark | 22-28°C, 15 minutes |
| 9. | Add Stop Solution<br>Gently mix | 50 µL<br>10 seconds |
| 10. | Read result | 450/610-650 nm<br>within 30 minutes |

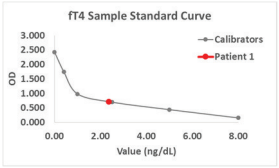
## CALCULATION OF RESULTS

1. Calculate the mean OD value (A450/610-650) for each set of calibrators.
2. Construct a standard curve by plotting the mean OD value obtained for each calibrator against its concentration on graph paper with OD values on the vertical *y*-axis, and concentrations on the horizontal *x*-axis.
3. Interpolate concentrations for the assay controls and test specimens by plotting their mean OD values on the curve.
   *Alternatively, if software is used, calculate the concentration of fT4 following the software menu.*
4. If the OD value of a specimen is greater than that of the highest calibrator, it is recommended to dilute the specimen with C1 in a 1:1 or 1:3 ratio and test again. Any values obtained for a diluted sample must be further converted by applying the appropriate dilution factor in the calculation.

## INTERPRETATION

1. Results of a typical standard curve are shown below:

| Cal ID | Conc. (ng/dL) | OD |
|---|---|---|
| C1 | 0.0 | 2.422 |
| C2 | 0.4 | 1.747 |
| C3 | 1.0 | 0.978 |
| C4 | 2.5 | 0.695 |
| C5 | 5.0 | 0.436 |
| C6 | 8.0 | 0.152 |
| **Patient ID** | **Conc. (ng/dL)** | **OD** |
| 1 | 2.35 | 0.709 |




*The above data and figure are for example purposes and should not be used to calculate your result.*

## QUALITY CONTROL

Good laboratory practice requires that quality control specimens (controls) be run with each calibration curve to check assay performance. Any material used should be assayed repeatedly to establish mean values and acceptable ranges to assure proper performance.

The following criteria should be met to consider assay results to be valid:

- The mean OD of the '0' calibrator should be ≥ 1.3.
- CVs for duplicates of calibrators should be ≤ 15%.
- fT4 Assay Control concentrations should be within the specified range on the vial labels.

## NORMAL REFERENCE

It is recommended that each laboratory establish its own normal ranges based on a representative sampling of the local patient population and its own assay technique. The following values for the Free Thyroxine (fT4) ELISA can be used as initial guideline ranges only:

**Normal Values for fT4 (ng/dL)**

| | |
|---|---|
| Sample Number (n) | 109 |
| Average | 1.51 |
| Standard Deviation (SD) | 0.34 |
| Range (±2SD) | 0.82 - 2.19 |

## PEFORMANCE CHARACTERISTICS

**1. Analytical Sensitivity**

Twenty (20) replicates of the '0' calibrator were run in a single assay on each of three lots of the Free Thyroxine (fT4) ELISA, and the mean and standard deviation (SD) of the OD values were calculated. The analytical sensitivity of the test was interpolated from the standard curve. The analytical sensitivity of the Free Thyroxine (fT4) ELISA was determined to be .202 ng/dL at 2SD.

**2. Specificity**

Specificity of the Free Thyroxine (fT4) ELISA was evaluated by spiking potential interfering substances into a human serum matrix.Specificity was calculated by deriving a ratio between the concentrationof each tested metabolite and the concentration of fT4 needed to displace the same amount of conjugate. There was no cross-reactivity observed at the concentrations listed below:

| | |
|---|---|
| d-Thyroxine | 10 µg/mL |
| d-Triiodothyronine | 100 µg/mL |
| Iodotyrosine | 100 µg/mL |
| Diiodotyrosine | 100 µg/mL |
| TBG | 50 µg/mL |
| Human Albumin | 50 mg/mL |
| Phenylbutazone | 25 µg/mL |
| Phenytoin | 50 µg/mL |
| ASA | 500 µg/mL |
| Acetaminophen | 500 µg/mL |

**3. Accuracy**

The accuracy of the Free Thyroxine (fT4) ELISA was determined by external comparison of 126 specimens with varying concentrations of fT4 with a reference commercial ELISA. The results are listed in the following table:

| Method | Mean (ng/dL) | Least Square Regression | Corr. Coef. ( r ) |
|---|---|---|---|
| (y) | 1.26 | y=0.0354+0.9514(x) | 0.964 |
| Reference (x) | 1.24 | | |

**4. Precision**

a. **Intra-assay precision:** Twenty replicates of each of three pooled human serum controls (low, medium, and high concentrations) were tested and the mean, SD, and coefficient of variation (CV%) were determined. The results are shown in the following table:

| Sample | N | Mean (ng/dL) | SD | CV |
|---|---|---|---|---|
| Low | 20 | 0.551 | 0.329 | 7.56 |
| Medium | 20 | 1.044 | 0.052 | 4.66 |
| High | 20 | 4.37 | 0.061 | 12.50 |

b. **Inter-Assay Precision:** Three human serum pooled controls (low, middle, and high concentrations) were assayed in 20 separate runs over 3 lots. The mean, SD, and CV% were determined. The results are shown in the following table:

| Sample | N | Mean (ng/dL) | SD | CV |
|---|---|---|---|---|
| Low | 20 | 0.967 | 0.084 | 8.736 |
| Medium | 20 | 2.140 | 0.243 | 11.355 |
| High | 20 | 3.829 | 0.488 | 12.746 |

## WARNINGS AND PRECAUTIONS

**For *in Vitro* Diagnostic Use**

1. Read these Instructions for Use completely before performing the test. Failure to follow the instructions could lead to inaccurate test results.
2. Do not use expired test kits.
3. Bring all reagents to room temperature (22-28°C) before use.
4. Do not use a component from any other test kit as a substitute for the components in this kit. Do not re-use the used microwells to test new samples.
5. Do not use serum derived from hemolyzed blood specimens for testing.
6. Do not ingest the reagents. Avoid contact with eyes, skin and mouth. Wear protective clothing and disposable gloves while handling the kit reagents and clinical specimens. Wash hands thoroughly after performing the test.
7. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
8. Follow the US CDC Universal Precautions for prevention of transmission of HIV, HBV and other blood-borne pathogens.
9. Dispose of all specimens and materials used to perform this test fT4 as bio- hazardous waste.
10. Prior to the first incubation, and after addition of the Stop Solution, gently shake the microwells to ensure thorough mixing. Avoid splashing liquid while shaking. Do not allow formation of air bubbles in the microwell.
11. Do not allow the microwells to dry between the end of the washing operation and the reagent distribution.
12. The enzyme substrate reaction is very sensitive to metal ions. Thus, do not allow any metal elements to come into contact with the conjugate or TMB Substrate.
13. The enzyme-substrate reaction is temperature dependent. Ensure that the room temperature is between 22-28˚C during the incubation.
14. The TMB substrate must be colorless. The appearance of blue color indicates that the reagent cannot be used and must be replaced. The TMB Substrate must be stored in the dark.
15. Do not pipette directly from the TMB Substrate container. Pour into a clean glass or plastic secondary container for further use.
16. Use a new dispensing tip for each specimen. Never use the specimen container to distribute conjugate and TMB Substrate.
17. **The wash procedure is critical. Wells must be aspirated completely before adding the Wash buffer or liquid reagents. Automatic washers must be validated with the test kit prior to use. Insufficient washing will result in poor precision and falsely elevated OD values.**
18. **Microplate reader must be calibrated per manufacturer's instruction to ensure accurate OD readings. A non-calibrated reader may lead to invalid test results.**
19. Avoid exposure to strong light during color development.

## LIMITATIONS OF TEST

1. The Assay Procedure and the Interpretation of Results must be followed closely when testing for fT4 concentration in human specimens. Failure to follow the procedure may lead to inaccurate results.
2. The test is limited to the detection of fT4 concentration in human serum.
3. A clinical diagnosis should not be based on the results of a single test and should only be made by the physician after all clinical and laboratory findings have been evaluated.
4. Normal thyroid hormone levels do not exclude thyroid disease.
5. If the OD value of a specimen is greater than that of the highest calibrator, it is recommended to dilute the specimen with fT4 Calibrator C1 and test again.
6. Any interpretation or use of this test result must also integrate other clinical findings as well as on the professional judgment of health care providers.

## REFERENCES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**Index of Symbols**

| | | |
|---|---|---|
| Consult instructions for use | REF Catalogue number | Tests per kit |
| IVD For *in vitro* diagnostic use only | LOT Batch code | Use-by date |
| 2 °C – 8 °C Temperature limit 2-8 °C | Do not re-use | Keep dry |
| Manufacturer | Date of manufacture | Keep away from sunlight |
| NON STERILE If device is non-sterile | Warnings / Precautions | |


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