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Instructions for use

FT4 ELISA 2nd Generation

REF TF E-2200

2°C – 8°C

Σ 96

IVD

CE

INTENDED USE

For the direct quantitative determination of Free Thyroxine by an enzyme immunoassay in human serum.
For *in vitro* diagnostic use only.

PRINCIPLE OF THE TEST

The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabelled antigen (present in standards, controls 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 enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the colour formed is inversely proportional to the concentration of fT4 in the sample. A set of standards is 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.

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 free T4 assay is a one step competitive ELISA 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 standard curve must be established for every run.
7. The 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. When reading the microplate, the presence of bubbles in the wells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
10. The substrate solution (TMB) is sensitive to light and should remain colourless if properly stored. Instability or contamination may be indicated by the development of a blue colour, in which case it should not be used.
11. When dispensing the substrate and stopping solution, 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 direct determination of fT4 in human serum. The kit is not calibrated for the determination of fT4 in 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 100 pg/ml should be reported as such and should not be diluted. Dilution will alter the existing equilibrium and may lead to false results.
5. The interpretation of free T4 results can be complicated by a variety of drugs, severe nonthyroidal illness and some rare conditions such as familial dysalbuminemic hyperthyroxinemia (FDH). For diagnostic purposes, the results of this assay should always be used in combination with the clinical examination, medical history and other findings.
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 serum that may be used in the preparation of the standards and controls has been tested and found to be non-reactive 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 blood specimen.

CHEMICAL HAZARDS

Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SPECIMEN COLLECTION AND STORAGE

Approximately 0.1 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 pipette to dispense 25, 100, 150 and 300 µl
2. Disposable pipette tips
3. Distilled or deionized water
4. A 37 °C incubator
5. Microplate reader with a filter set at 450 nm and an upper OD limit of 3.0 or greater (see assay procedure step 11).

REAGENTS PROVIDED

AA E-0030 **WASH-CONC** **10x** **Wash Buffer Concentrate** – Requires Preparation **X10**

Content:	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 the whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of water.

AA E-0055 **SUBSTRATE** **TMB Substrate** – Ready To Use

Content:	One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
Volume:	16 ml/bottle
Storage:	Refrigerate at 2 – 8 °C
Stability:	12 months or as indicated on label.

AA E-0080 STOP-SOLN **Stopping Solution** – Ready To Use

Content: One bottle containing 1 M sulfuric acid.

Volume: 6 ml/bottle

Storage: Refrigerate at 2 – 8 °C

Stability: 12 months or as indicated on label.

Hazards identification:

H315 Causes skin irritation.

H319 Causes serious eye irritation.

Standards and **Controls** – Ready To Use

Listed below are approximate concentrations, please refer to vial labels for exact concentrations:

Cat. no.	Component	Standard	Concentration	Volume/Vial
TF E-2201	STANDARD A	**Standard A**	0 pg/ml	0.5 ml
TF E-2202	STANDARD B	**Standard B**	2 pg/ml	0.5 ml
TF E-2203	STANDARD C	**Standard C**	6 pg/ml	0.5 ml
TF E-2204	STANDARD D	**Standard D**	20 pg/ml	0.5 ml
TF E-2205	STANDARD E	**Standard E**	80 pg/ml	0.5 ml
TF E-2251	CONTROL 1	**Control 1**	Refer to vial labels for expected value and acceptable range!	0.5 ml
TF E-2252	CONTROL 2	**Control 2**		0.5 ml

Content: fT4 in a human serum-based matrix with a non-mercury preservative. Prepared by spiking serum with a defined quantity of T4.

Storage: Refrigerate at 2 – 8 °C

Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards and controls should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

TF E-2213 ASSAY-BUFF **Assay Buffer** – Ready To Use

Content: 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.

TF E-2231 96 **Mouse Anti-fT4 Antibody-Coated Break-Apart Well Microplate** – Ready To Use

Content: 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.

TF E-2240 CONJUGATE-CONC 50x **fT4-Horseradish Peroxidase (HRP) Conjugate Concentrate** – Requires Preparation **X50**

Content: fT4-HRP conjugate in a protein-based buffer with a non-mercury preservative.

Volume: 300 µl/vial

Storage: Refrigerate at 2 – 8 °C

Stability: 12 months or as indicated on label.

Preparation: Dilute 1:50 in assay buffer before use (e.g. 40 µl of HRP in 2 ml of assay buffer). If the whole plate is to be used dilute 240 µl of HRP in 12 ml of assay buffer. Discard any that is left over.

ASSAY PROCEDURE

Specimen Pretreatment: **None.**

All reagents must reach room temperature before use. Standards, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1.	Prepare working solutions of the fT4-HRP conjugate and wash buffer.
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 standard, 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.	Gently shake the plate for 10 seconds.
6.	Incubate the plate at 37 °C for 1 hour.
7.	Wash the wells 3 times with 300 µl of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry. (The use of a washer is recommended).
8.	Pipette 150 µl of TMB substrate into each well at timed intervals.
9.	Incubate the plate at 37 °C for 10 – 15 minutes (or until Standard A attains dark blue colour for desired OD).
10.	Pipette 50 µl of stopping solution into each well at the same timed intervals as in step 8.
11.	Read the plate on a microplate reader at 450 nm within 20 minutes after addition of the stopping solution.
⚠	*If the OD exceeds the upper limit of detection or if a 450 nm filter is unavailable, a 405 or 415 nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of patient/control samples.*

CALCULATIONS

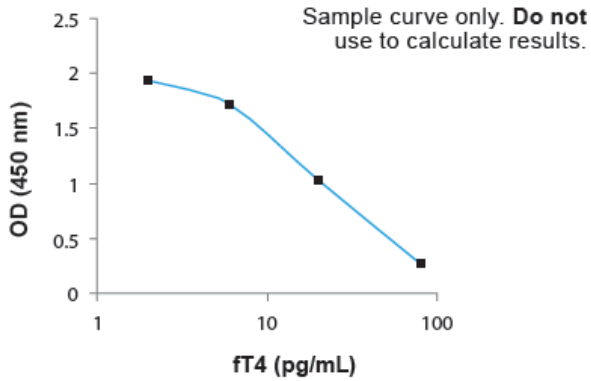
1. Calculate the mean optical density of each standard duplicate.
2. Draw a standard curve on semi-log paper with the mean optical densities on the Y-axis and the standard concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the standard curve.

TYPICAL TABULATED DATA

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

Standard	OD 1	OD 2	Mean OD	Value (pg/ml)
A	2.043	2.094	2.068	0
B	1.886	1.973	1.929	2
C	1.709	1.727	1.718	6
D	1.032	1.049	1.041	20
E	0.266	0.283	0.274	80
Unknown	1.332	1.312	1.322	13.0

TYPICAL STANDARD CURVE



PERFORMANCE CHARACTERISTICS

SENSITIVITY

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Standard A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the Direct fT4 ELISA kit is **1.0 pg/ml**.

SPECIFICITY (CROSS-REACTIVITY)

The following compounds were tested for cross-reactivity with the Direct fT4 ELISA 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 standard 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

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 (pg/ml) was established with 80 apparently healthy adults:

Group	N	Range (pg/ml)
Normal Euthyroid Samples	80	7 – 22

EFFECT OF BILIRUBIN

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

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

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 Direct fT4 ELISA 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 Standard A was spiked precisely with purified TBG at concentrations ranging from 25 – 200 µg/ml and assayed with the Direct fT4 ELISA kit. Results are tabulated below:

Sample	TBG Added (µg/ml)	OD 450 nm
1	0	1.883
2	25	2.030
3	50	2.149
4	100	2.175
5	200	2.251

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 Direct fT4 ELISA 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.

REFERENCES

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Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Use-by date	LOT	Batch code	IVD	For in-vitro diagnostic use only!
	Consult instructions for use	CONT	Content		CE marking of conformity
	Caution	REF	Catalogue number		Distributor
	Date of manufacture				