FT3 ELISA
Direct immunoenzymatic determination of free triiodothyronine (FT3) in human serum or plasma.

INTENDED USE
Immunoenzymatic colorimetric method for quantitative determination of free triiodothyronine (FT3) concentration in human serum and plasma. FT3 ELISA kit is intended for laboratory use only.

1. CLINICAL SIGNIFICANCE
The thyroid hormone, triiodothyronine (T3), is produced by the thyroid gland. An important component in the synthesis is iodine. Thyroxine is converted to the active T3 (three to four times more potent than T4) within cells by deiodinases (5'-iodinase).

Thyroxine-binding globulin (TGB) is the major carrier protein for circulating thyroid hormone. Only a very small fraction of the circulating hormone is free (unbound) 0.3%; this fraction is biologically active. Thus, measurements of free triiodothyronine concentrations correlate more reliably with clinical status than total triiodothyronine levels. For example, the increase in total triiodothyronine levels associated with pregnancy, oral contraceptives and oestrogen therapy result in higher total T3 levels while the free T3 (FT3) concentration remains basically unchanged.

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 triiodothyronine level changes so that the free triiodothyronine concentration remains constant.

The binding of T3 plays a key role in the feedback control of the thyroid, with FT3 acting on the pituitary to inhibit thyroid hormone secretion. The thyronines act on the body to increase the basal metabolic rate, affect protein synthesis and increase the body's sensitivity to catecholamine (such as adrenaline) by permissiveness. The thyroid hormones are essential to proper development and differentiation of all cells of the human body. These hormones also regulate protein, fat, and carbohydrate metabolism, affecting how human cells use energetic compounds. Numerous physiological and pathological stimuli influence thyroid hormone synthesis.

Thyrotoxicosis or hyperthyroidism is the clinical syndrome caused by an excess of circulating free thyroxine, free triiodothyronine, or both. Both T3 and T4 are used to treat thyroid hormone deficiency (hypothyroidism).

Since conditions such as pregnancy, oestrogen therapy and other non-thyroid factors alter TBG concentrations, assessment of thyroid function through total T3 measurement may result in an erroneous diagnosis, because FT3 levels, are unaffected by binding protein changes.

2. PRINCIPLE
The free T3 (FT3, antigen) in the sample competes with the antigenic T3 conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti T3 coated on the microplate (solid phase) (the enzyme conjugate should have no measurable binding to serum proteins especially TBG and albumin).

The interaction is illustrated by the following equation:

\[ \text{Enz} \text{Ag} + \text{Ag} + \text{Ab}_{cw} \rightleftharpoons \text{AgAb}_{cw} + \text{EnzAgAb}_{cw} \]

\[ K_a \text{Ab}_{cw} \]

\[ K_a \text{AgAb}_{cw} \]

Ab_{cw}: monospecific immobilised antibody (constant quantity)
Ag: native antigen (variable quantity)
EnzAg: antigen conjugated to enzyme HRP (constant quantity)
AgAb_{cw}: antigen-antibody complex
EnzAgAb_{cw}: antigen-HRP-antibody complex
K_a: rate constant of association
K_{a-}: rate constant of disassociation
K= K_a / K_{a-}: equilibrium constant

After incubation, the bound/free separation is performed by a simple solid-phase washing. Then, the enzyme HRP in the bound-fraction reacts with the Substrate (H_2O_2) and the TMB Substrate and develops a blu color that changes into yellow when the Stop Solution (H_2SO_4) is added.

The colour intensity is inversely proportional to the FT3 concentration in the sample. FT3 concentration in the sample is calculated through a calibration curve.
3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagents and materials supplied in the kit
1. Calibrators (6 vials, 1 mL each)
   - CAL0
   - CAL1
   - CAL2
   - CAL3
   - CAL4
   - CAL5
   
2. Conjugate (1 vial, 12 mL)
   T3 conjugated with horseradish peroxidase (HRP)

3. Coated Microplate (1 breakable microplate)
   Antibody anti T3 adsorbed on microplate

4. TMB Substrate (1 vial, 15 mL)
   H₂O₂-TMB 0.26 g/L (avoid any skin contact)

5. Stop Solution (1 vial, 15 mL)
   Sulphuric acid 0.15 mol/L (avoid any skin contact)

6. 50X Conc. Wash Solution (1 vial, 20 mL)
   NaCl 45 g/L, Tween-20 55 g/L

3.2. Reagents necessary not supplied
   Distilled water.

3.3. Auxiliary materials and instrumentation
   Automatic dispenser.
   Microplates reader (450 nm, 620-630 nm).

Note
   Store all reagents between 2-8°C in the dark.
   Open the bag of reagent 3 (Coated Microplate) only when it is at room temperature and close it immediately after use.

4. WARNINGS
   - This kit is intended for in vitro use by professional persons only. Not for internal or external use in Humans or Animals.
   - Use appropriate personal protective equipment while working with the reagents provided.
   - Follow Good Laboratory Practice (GLP) for handling blood products.
   - All human source material used in the preparation of reagents for this product has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the Calibrator should be handled in the same manner as potentially infectious material.
   - Some reagents contain small amounts of Proclin 300® as preservative. Avoid the contact with skin or mucosa.
   - The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
   - The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
   - Avoid the exposure of reagent TMB/H₂O₂ to directed sunlight, metals or oxidants. Do not freeze the solution.
   - Several drugs are known to effect the binding of Triiodothyronine to the thyroid hormone carrier proteins or its metabolism to T3 and complicate the interpretation of FT3 results.
   - Circulating autoantibodies to T3 and hormone-binding inhibitors may interfere.
   - Heparin has been reported to have in vivo and in vitro effects on FT3 concentration. Therefore, do not obtain samples in which this anti-coagulant has been used.
   - In severe no thyroidal illness (NTI), the assessment of thyroid status becomes very difficult. TSH measurements are recommended to identify thyroid dysfunction.
   - Familial dysalbuminemic conditions may yield erroneous results on direct FT3 assays.
   - Not intended for newborn screening.

5. PRECAUTIONS
   - Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
   - All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
   - Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
   - Do not interchange kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
   - If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested. To improve the performance of the kit on ELISA automatic systems, it is recommended to increase the number of washes.
   - The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
   - It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate.
   - Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
   - Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.
• Maximum precision is required for reconstitution and dispensation of the reagents.
• Samples microbiologically contaminated, highly lipemic or haemolysed should not be used in the assay.
• Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1. Preparation of the Calibrators (C₀…C₅)
The Calibrators are ready to use, are calibrated against Human Serum Reference for free triiodothyronine and have approximate concentrations of:

<table>
<thead>
<tr>
<th>pg/mL</th>
<th>C₀</th>
<th>C₁</th>
<th>C₂</th>
<th>C₃</th>
<th>C₄</th>
<th>C₅</th>
</tr>
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<tr>
<td></td>
<td>0</td>
<td>0.4</td>
<td>1.2</td>
<td>4.5</td>
<td>8.0</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Exact levels are given on the labels on a lot specific basis.
For SI units: 1 pg/mL x 1.536 = pmol/L
The Calibrators are stable until the expiry date printed on the label. Once opened, the Calibrators are stable 6 months at 2-8°C.

6.2. Preparation of Wash Solution
Dilute the contents of each vial of the "50X Conc. Wash Solution" with distilled water to a final volume of 1000 mL prior to use. For smaller volumes respect the 1:50 dilution ratio. The diluted wash solution is stable for 30 days at 2-8°C.

6.3. Preparation of the Sample
The determination of FT3 should be performed in human serum or plasma.
Specimens may be refrigerated at 2÷8°C (for a maximum period of 48 hours). If the specimens cannot be assayed within 48 hours, the samples may be stored at temperatures of −20°C for up to 30 days. Avoid repetitive freezing and thawing of samples. When assayed in duplicate, 0.10 mL of the specimen is required.

6.4. Procedure
• Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes. At the end of the assay, store immediately the reagents at 2-8°C: avoid long exposure to room temperature.
• Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
• To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
• As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C₀-C₅), two for each sample, one for Blank.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibrator</th>
<th>Sample/Control</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator C₀-C₅</td>
<td>50 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>50 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conjugate</td>
<td>100 µL</td>
<td>100 µL</td>
<td></td>
</tr>
</tbody>
</table>

Shake gently the microplate for 20-30 seconds to mix and cover it.
Incubate 1 h at room temperature (22±28°C).
Remove the content from each well, wash the wells 3 times with 300 µL of diluted Wash Solution (if you use automated equipment, wash the wells at least 5 times)

Important note: during each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel.

<table>
<thead>
<tr>
<th>TMB Substrate</th>
<th>100 µL</th>
<th>100 µL</th>
<th>100 µL</th>
</tr>
</thead>
</table>

Incubate at room temperature (22÷28°C) for 15 minutes in the dark.

<table>
<thead>
<tr>
<th>Stop Solution</th>
<th>100 µL</th>
<th>100 µL</th>
<th>100 µL</th>
</tr>
</thead>
</table>

Shake the microplate gently.
Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.

7. 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. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the calibration curve for run-to-run reproducibility. In addition, maximum absorbance should be consistent with past experience. 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.
8. RESULTS

8.1. Mean Absorbance
Calculate the mean of the absorbance (Em) for each point of the calibration curve \((C_0-C_5)\) and of each sample.

8.2. Calibration curve
Plot the mean value of absorbance (Em) of the Calibrators \((C_0-C_5)\) against concentration. Draw the best-fit curve through the plotted points. (es: Four Parameter Logistic).

8.3. Calculation of Results
Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in pg/mL.

9. REFERENCE VALUES
A study of euthyroid adult population was undertaken to determine the expected values for FT3 ELISA kit.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Triodo-thyronine</td>
<td>-</td>
<td>1.0000</td>
</tr>
<tr>
<td>L-Thyroxine</td>
<td>10 µg/mL</td>
<td>&lt; 0.0002</td>
</tr>
<tr>
<td>D-Thyroxine</td>
<td>10 µg/mL</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Iodo-thyrosine</td>
<td>10 µg/mL</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Diodo-thyrosine</td>
<td>10 µg/mL</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Triiodothyroacetic Acid</td>
<td>10 µg/mL</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>10 µg/mL</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Sodium Salicylate</td>
<td>10 µg/mL</td>
<td>N/D</td>
</tr>
<tr>
<td>Phenyltoin</td>
<td>10 µg/mL</td>
<td>N/D</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>10 nmol/L</td>
<td>N/D</td>
</tr>
<tr>
<td>Albumin</td>
<td>50 mg/mL</td>
<td>N/D</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>10 µL/mL of pace red cells added to the serum</td>
<td>N/D</td>
</tr>
</tbody>
</table>

Please pay attention to the fact that the determination of a range of expected values for a “normal” population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacurer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.

10. PERFORMANCE AND CHARACTERISTICS

10.1. Precision

10.1.1. Intra Assay Variation
Within run variation was determined by replicate measurements (24x) of three different control sera in one assay. The within assay variability is ≤ 4.94%.

10.1.2. Inter Assay Variation
Between run variations was determined by replicate measurements (12x) of three different control sera in different lots of kit. The between assay variability is ≤ 13.19%.

10.2. Sensitivity
The lowest detectable concentration of FT3 that can be distinguished from the Calibrator 0 is 0.05 pg/mL at the 95% confidence limit.

10.3. Specificity
The cross-reactivity of the triiodothyronine antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between doses of interfering substance to dose of Triiodothyronine needed to displace the same amount of tracer.

10.4. Correlation with RIA
Diametra FT3 ELISA was compared to another commercially available FT3 assay. 151 serum samples were analysed according in both test systems. The linear regression curve was calculated: \((FT3 \text{ Diametra}) = 0.923^* (FT3 \text{ RIA}) + 0.350\)
\(r^2= 0.903\)

11. WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

BIBLIOGRAPHY
<table>
<thead>
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<th>DiaMetra</th>
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SUGGERIMENTI PER LA RISOLUZIONE DEI PROBLEMI/TROUBLESHOOTING

ERRORI POSSIBILI CAUSE / SUGGESTIONS

**No colorimetric reaction**
- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

**Too low reaction (too low ODs)**
- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

**Too high reaction (too high ODs)**
- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

**Unexplainable outliers**
- contamination of pipettes, tips or containers
- insufficient washing (conjugates not properly removed) too high within-run
- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)
- too high between-run - incubation conditions not constant (time, CV % temperature)
- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation