# REVERSE T3 (rT3) ELISA

Diagnostics Biochem Canada

| EU: IVD CE | **USA**: For Research Use Only. Not for Use in Diagnostic Procedures. | **CANADA**: For *in vitro* Diagnostic Use. |
|---|---|---|

REF CAN-RT3-100 Version: 2.0 Effective: August 11, 2016

## INTENDED USE

For the direct quantitative determination of Reverse Triiodothyronine (rT3) in human serum and plasma by an enzyme immunoassay.

## PRINCIPLE OF THE TEST

The DBC rT3 ELISA is a competitive enzyme immunoassay, where the antigen (rT3 present in calibrators, controls and patient samples) competes with a biotin-labelled antigen (rT3-Biotin conjugate) for a limited quantity of antibody which is coated on the microplate wells. After one hour incubation followed by the first washing, unbound materials are removed and a Streptavidin-HRP conjugate is added and incubated for 30 minutes. Following a second washing, the TMB substrate is added. The enzymatic reaction is terminated by addition of the stopping solution, upon which the color intensity is measured with a microplate reader. The color intensity is inversely proportional to the concentration of rT3 in the sample. The set of kit calibrators that are run simultaneously with the samples is used to plot a calibration curve and determine the concentration of rT3 in samples and controls.

## CLINICAL APPLICATIONS

3,3',5'-Triiodo-L-thyronine also known as reverse triiodothyronine or reverse T3 (rT3), differs from 3,3',5-Triiodo-L-thyronine (T3) in the positions of the iodine atoms in the molecule. The majority of circulatory rT3 is synthesized by peripheral deiodination of thyroxine (T4).

Both T3 and rT3 bind to thyroid hormone receptors, but in contrast to T3, rT3 has not been found yet to stimulate receptor metabolic activity; it blocks receptor sites from T3 activation. The ratio of rT3 to T3 is a valuable biomarker of the metabolism and function of thyroid hormones because the process of 5' monodeiodination that converts T4 to T3 and rT3 to 3,3'-T2 is inhibited in a number of non-thyroidal conditions such as fasting, anorexia nervosa, malnutrition, diabetes mellitus, stress, severe trauma or infection, hemorrhagic shock, hepatic dysfunction, pulmonary diseases and others. This scenario is known as "Sick euthyroid" syndrome or "Low T3" syndrome.

An elevated ratio of rT3 over T3 is therefore indicative of "sick euthyroid" syndrome and helps to exclude a diagnosis of hypothyroidism, particularly in critically ill patients[1-9].

The concentration of rT3 could be high in patients on the following medications: amiodarone, dexamethasone, propylthiouracil, ipodate, propranolol, and the anesthetic halothane. The concentration of rT3 could be low in patients on Dilantin, which decreases rT3 due to its displacement from thyroxine-binding globulin and therefore generates an excessive clearance of rT3.

## PROCEDURAL CAUTIONS AND WARNINGS

1. This kit is intended for in vitro use only.
2. Practice good laboratory practices when handling kit reagents and specimens. This includes:
   - Do not pipette by mouth.
   - Do not smoke, drink, or eat in areas where specimens or kit reagents are handled.
   - Wear protective clothing and disposable gloves.
   - Wash hands thoroughly after performing the test.
   - Avoid contact with eyes; use safety glasses; in case of contact with eyes, flush eyes with water immediately and contact a doctor.
3. 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.
4. Avoid microbial contamination of reagents.
5. A calibrator curve must be established for every run.
6. It is recommended to all customers to prepare their own control materials or serum pools which should be included in every run at a high and low level for assessing the reliability of results.
7. The controls (included in kit) must be included in every run and their results must fall within the ranges stated in the quality control certificate; a failed control result might indicate improper procedural techniques or pipetting, incomplete washing or inadequate reagent storage.
8. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
9. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of specimens.
10. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the control do not reflect established ranges.
11. When reading the microplate, the presence of bubbles in the microplate wells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
12. 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.
13. The Biotin-rT3 conjugate solution is sensitive to light and should be of a light yellow color if stored properly; do not use if the solution appears dark green or black in color.
14. When dispensing the substrate and stopping solutions, do not use pipettes in which these liquids will come into contact with any metal parts.
15. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, calibrator and control.
16. Do not use kit components from different kit lots within a test and do not use any component beyond the expiration date printed on the label.
17. Kit reagents must be regarded as hazardous waste and disposed of according to local and/or national regulations.

## LIMITATIONS

1. All the reagents within the kit are calibrated for the direct determination of rT3 in human serum and plasma. The kit is not calibrated for the determination of rT3 in other specimens of human or animal origin.
2. Do not use grossly haemolysed, lipemic, icteric or improperly stored serum or plasma samples.
3. Samples or control sera containing azide or thimerosal are incompatible with this kit and will lead to false results.
4. Do not use the results of this kit as the sole basis for a clinical diagnosis. For example, medications and heterophilic antibodies (in patients exposed to animals or animal products) can interfere with immunoassays. Consequently, the clinical diagnosis should include all aspects of patients background including the frequency of exposure to animals/products and medications.

## SAFETY CAUTIONS AND WARNINGS

### POTENTIAL BIOHAZARDOUS MATERIAL

Human serum that may be used in the preparation of the calibrators 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. However no test method can offer complete assurance that HIV, HCV and Hepatitis B virus or any other infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions applied to blood specimens.
All human specimens should be considered a potential biohazard and handled as if capable of transmitting infections and in accordance with good laboratory practices.

## 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

Serum: 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 for longer time.

Plasma: Approximately 0.2 mL of plasma is required per duplicate determination. Collect 4-5 mL of blood into EDTA plasma tubes. Store at 4°C for up to 24 hours or at -10°C or lower for longer time.

Consider all human specimens as possible biohazardous materials and take precautions when handling.

## SPECIMEN PREPARATION

Serum and plasma samples are loaded directly to the microplate wells; no specimen pretreatment is necessary.

## REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipettes to dispense 25, 50, 100, 150 and 350 µL
2. Disposable pipette tips
3. Distilled or deionized water
4. Incubator set to 37°C.
5. Microplate well reader with a filter set at 450nm and an upper OD limit of 3.0 or greater

## REAGENTS PROVIDED

| **1. Anti-Reverse T3 Polyclonal Antibody Coated Microplate -Break Apart Wells** – Ready to Use | |
|---|---|
| Contents: | One 96 well (12x8) polyclonal antibody-coated microplate in a re-sealable pouch with desiccant. |
| Storage: | Refrigerate at 2-8°C |
| Stability: | 12 months or as indicated on label |

| **2. Reverse T3-Biotin Conjugate** – Ready to Use | |
|---|---|
| Contents: | Reverse T3-Biotin conjugate in a protein-based buffer with a non-mercury preservative. |
| Volume: | 13 mL/bottle |
| Storage: | Refrigerate at 2-8°C |
| Stability: | 12 months in unopened bottle or as indicated on label. |

| **3. Streptavidin-Horse Radish Peroxidase (HRP) Conjugate** – Ready to Use | |
|---|---|
| Contents: | Streptavidin-HRP conjugate in a protein-based buffer with a non-mercury preservative. |
| Volume: | 20 mL/bottle |
| Storage: | Refrigerate at 2-8°C |
| Stability: | 12 months in unopened bottle or as indicated on label. |

| **4. Reverse T3 Calibrators** – Ready to Use | |
|---|---|
| Contents: | Six vials containing rT3 in a protein-based buffer with a non-mercury preservative.<br>Prepared by spiking buffer with rT3 to the concentrations in labels.<br>Typical calibrator concentrations*: 0; 0.02; 0.1; 0.4; 1 and 2 ng/mL.<br>* Approximate value — please refer to vial labels for exact concentrations. |
| Volume: | Calibrators A-F: 1 mL/vial |
| Storage: | Refrigerate at 2-8°C |
| Stability: | 12 months in unopened vials or as indicated on label |

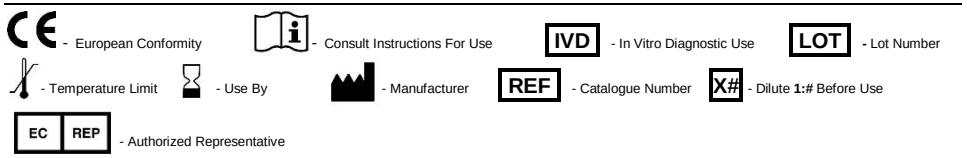
| **5. Reverse T3 Controls** – Ready to Use | |
|---|---|
| Contents: | Two vials containing rT3 in a protein-based buffer with a non-mercury preservative.<br>Prepared by spiking buffer with rT3 to the target concentration in QC certificate.<br>Refer to vial labels for acceptable ranges. |
| Volume: | 1 mL/vial |
| Storage: | Refrigerate at 2-8°C |
| Stability: | 12 months in unopened vials or as indicated on label |

| **6. 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: | Dilute the wash buffer concentrate 1:10 in distilled or deionized water to prepare the working wash buffer. If one whole plate is to be used dilute 50 mL of the wash buffer concentrate in 450 mL of water. |

| **7. TMB Substrate** – Ready to Use | |
|---|---|
| Contents: | One bottle containing tetramethylbenzidine and hydrogen peroxide in buffer. |
| Volume: | 16 mL/bottle |
| Storage: | Refrigerate at 2-8°C |
| Stability: | 12 months or as indicated on label. |

| **8. Stopping Solution** – Ready to Use | |
|---|---|
| Contents: | One bottle containing 1M sulfuric acid. |
| Volume: | 6 mL/bottle |
| Storage: | Refrigerate at 2-8°C |
| Stability: | 12 months or as indicated on label. |

## SYMBOLS



## ASSAY PROCEDURE

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

| | |
|---|---|
| 1. | After all kit components have reached room temperature, mix gently by inversion. Prepare the working wash buffer (see wash buffer concentrate under the section "Reagents Provided"). |
| 2. | Remove the required number of strips from the microplate and assemble into a plate frame. Reseal the bag and return any unused strips to the refrigerator. |
| 3. | Pipette 25 µL of each calibrator, control and specimen sample (serum or plasma) into correspondingly labelled wells in duplicate. |
| 4. | Pipette 100 µL of the Reverse T3-Biotin conjugate into each well (the use of a multichannel pipette is recommended). Gently shake the microplate by hand for ten seconds to ensure complete mixing of the conjugate solution with the calibrators, controls and samples. |
| 5. | Incubate the plate at 37°C for 1 hour. Do not cover the microplate. |
| 6. | Wash the wells with 350 µL/well of working wash buffer solution **3 times**. After washings tap the plate firmly against absorbent paper to remove any residual liquid (the use of an automatic strip washer is strongly recommended).<br>—The accuracy of this assay depends on the correct execution of the washing procedure— |
| 7. | Pipette 150 µL of the Streptavidin-HRP conjugate into each well.<br>(the use of a multichannel pipette is recommended). |
| 8. | Incubate the plate at 37°C for 30 minutes. Do not cover the microplate. |
| 9. | Wash the wells **3 times** using the same procedure as stated in step 6. |
| 10. | Pipette 150 µL of the TMB substrate into each well at timed intervals<br>(the use of a multichannel pipette is recommended). |
| 11. | Incubate at 37°C for 15 minutes. Do not cover the microplate. |
| 12. | Pipette 50 µL of stopping solution into each well at the same timed intervals as in step 10.<br>(the use of a multichannel pipette is recommended). Gently shake the microplate by hand for ten seconds to ensure complete mixing of the stopping solution in the wells. |
| 13. | Measure the absorbance at 450 nm with a microplate reader, within 20 minutes after addition of the stopping solution. |

### CALCULATIONS

1. Calculate the mean optical density of each calibrator, control and specimen sample duplicate.
2. Use a 4-parameter or 5-parameter curve with immunoassay software to generate the control and sample concentration results or draw a calibration curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis and read the concentration of controls and samples off the calibrator curve.
3. If a sample reads greater than 2 ng/mL report the result as ">2 ng/mL".
4. To convert from ng/mL to ng/dL multiply the result by 100; to convert to nmol/L, multiply the ng/dL result by 0.01536 or the ng/mL result by 1.536.

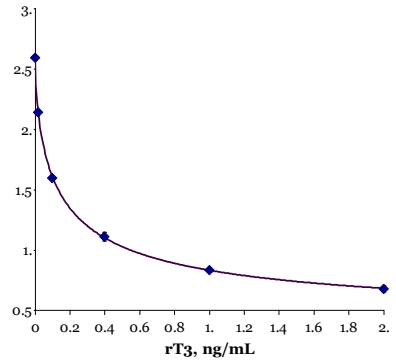
### TYPICAL TABULATED DATA

Sample data only. Do not use to calculate results

| Calibrator | rT3 (ng/mL) | Mean OD (450 nm) |
|---|---|---|
| A | 0 | 2.594 |
| B | 0.02 | 2.140 |
| C | 0.1 | 1.597 |
| D | 0.4 | 1.113 |
| E | 1 | 0.834 |
| F | 2 | 0.678 |
| Unknown | 0.15 | 1.458 |

### TYPICAL CALIBRATION CURVE

Sample curve only. Do not use to calculate results.




## PERFORMANCE CHARACTERISTICS

### SENSITIVITY

The limit of detection (LoD) was determined from the analysis of 60 samples of the blank and a low value sample in two independent experiments and it was calculated as follows:
LoD = µB + 1.645σB + 1.645σS, where σB and σS are the standard deviation of the blank and low value sample and µB is the mean value of the blank.
The Limit of Detection (LoD) was determined to be **0.009 ng/mL.**

## SPECIFICITY

### CROSS-REACTIVITY

The following compounds were tested for cross-reactivity with rT3 cross-reacting at 100%:

| Steroid | %Cross Reactivity |
|---|---|
| rT3 | 100 |
| T3 | <0.001 |
| T4 | 0.005 |
| T2 | 0.004 |

## INTERFERENT SUBSTANCES

The following substances did not show significant interference with the assay: hemoglobin up to 2 g/L, free and conjugated bilirubin up to 200 mg/L, triglycerides up to 5.5 mg/mL and Biotin up to 40 µg/mL.

## INTRA-ASSAY PRECISION

Four serum samples were assayed 24 times each on the same calibrator curve. The results are tabulated below:

| Sample | Mean (ng/mL) | SD (ng/mL) | %CV |
|---|---|---|---|
| 1 | 0.089 | 0.0024 | 2.7 |
| 2 | 0.250 | 0.020 | 8.0 |
| 3 | 0.455 | 0.019 | 4.2 |
| 4 | 1.018 | 0.140 | 13.8 |

## INTER-ASSAY PRECISION

Four serum samples were assayed in 20 different tests in the span of ten days. The results are tabulated below:

| Sample | Mean (ng/mL) | SD (ng/mL) | CV% |
|---|---|---|---|
| 1 | 0.127 | 0.016 | 12.6 |
| 2 | 0.304 | 0.038 | 12.5 |
| 3 | 0.469 | 0.057 | 12.2 |
| 4 | 0.847 | 0.083 | 9.8 |

## RECOVERY

Spiked samples were prepared by adding defined amounts of rT3 to three patient serum samples. The results are tabulated below:

| Sample | Observed Result (ng/mL) | Expected Result (ng/mL) | Recovery % |
|---|---|---|---|
| Serum Sample 1 | 0.437 | - | - |
| +0.15 ng/mL | 0.586 | 0.587 | 99.8 |
| +0.30 ng/mL | 0.728 | 0.737 | 98.8 |
| +0.45 ng/mL | 0.854 | 0.887 | 96.3 |
| Serum Sample 2 | 0.327 | - | - |
| +0.15 ng/mL | 0.435 | 0.477 | 91.2 |
| +0.30 ng/mL | 0.596 | 0.627 | 95.1 |
| +0.45 ng/mL | 0.777 | 0.777 | 100.0 |
| Serum Sample 3 | 0.149 | - | - |
| +0.15 ng/mL | 0.287 | 0.299 | 96.0 |
| +0.30 ng/mL | 0.460 | 0.449 | 102.4 |
| +0.45 ng/mL | 0.614 | 0.599 | 102.5 |

## COMPARATIVE STUDIES

The DBC rT3 ELISA kit (y) was compared with the leading competing technology: Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) (x). The comparison of 40 serum samples yielded the following linear regression results:

$y = 0.79x + 0.01$, $r = 0.96$

## REFERENCE VALUES

Reference values were obtained from commercial human specimens and calculated using a non-parametric method. Each laboratory must establish the range of reference values for their own population.

| Group | n | Median (ng/mL) | 95% Confidence Range (ng/mL) | Total range (ng/mL) |
|---|---|---|---|---|
| Serum adults | 160 | 0.13 | 0.08-0.31 | 0.06-0.76 |
| Plasma adults | 120 | 0.14 | 0.08-0.29 | 0.049-0.65 |

## LITERATURE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