
Instructions for use
Reverse T3 (rT3) ELISA

REF

TF E-2500R



96

RUO

For research
use only –
Not for use
in diagnostic
procedures

Reverse T3 (rT3) ELISA

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 rT3 ELISA is a competitive enzyme immunoassay, where the antigen (rT3 present in standards, controls and 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 Standards that are run simultaneously with the samples is used to plot a standard curve and determine the concentration of rT3 in samples and controls.

PROCEDURAL CAUTIONS AND WARNINGS

1. This kit is intended for research 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 Standard 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 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, standard 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.

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 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 bio-hazard 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.

Plasma:

Approximately 0.2 ml of plasma is required per duplicate determination. Collect 4–5 ml of blood into EDTA plasma tubes. Centrifuge and carefully remove the plasma layer.



Do not test samples the same day of the blood draw. The serum or plasma specimen samples must be stored at the recommended storage conditions for at least 20 hours prior to being tested.

Upon serum or plasma collection, the samples must be stored:

- Refrigerated (2–8°C) for a period of no longer than 5 days, or
- Frozen ($\leq -20^{\circ}\text{C}$) for a period of no longer than 3 months.

Avoid multiple freeze/thaw cycles.

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

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

- Precision pipette to dispense 25, 50, 100, 150 and 350 μl
- Disposable pipette tips
- Distilled or deionized water
- Microplate shaker
Recommendation: Orbital (3 mm diameter) set to 600 rpm
Note: Other shakers may be used, provided the test results obtained with those shakers can meet the kit QC certificate criteria and the criteria of any end-user controls.
- Microplate reader with a filter set at 450 nm and an upper OD limit of 3.0 or greater
- Microplate washer (recommended)

REAGENTS PROVIDED

- AA E-0030** **WASH-CONC 10x** **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 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.
- AA E-0055** **SUBSTRATE** **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.

3. AA E-0080 **STOP-SOLN** **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.
Hazards identification:



H315 Causes skin irritation.
H319 Causes serious eye irritation.

4. Standards and Controls – Ready To Use.

Cat. no.	Symbol	Standard	Concentration*	Volume/Vial
TF E-2501	STANDARD A	Standard A	0 ng/ml	1 ml
TF E-2502	STANDARD B	Standard B	0.02 ng/ml	1 ml
TF E-2503	STANDARD C	Standard C	0.1 ng/ml	1 ml
TF E-2504	STANDARD D	Standard D	0.4 ng/ml	1 ml
TF E-2505	STANDARD E	Standard E	1 ng/ml	1 ml
TF E-2506	STANDARD F	Standard F	2 ng/ml	1 ml
TF E-2551	CONTROL 1	Control 1	Refer to vial labels for acceptable range!	1 ml
TF E-2552	CONTROL 2	Control 2		1 ml

*Approximate value — please refer to vial labels for exact concentrations

Contents: rT3 in a protein based buffer with a non-mercury preservative. Prepared by spiking buffer with rT3 to the concentrations in label/QC certificate.
Storage: Refrigerate at 2 - 8 °C
Stability: 12 months in unopened vials or as indicated on label.

5. TF E-2531 **96** **Anti-Reverse T3 Polyclonal Antibody-Coated Break-Apart Well Microplate – Ready To Use.**

Contents: One 96 well (12x8) polyclonal antibody-coated microplate in a resealable pouch with desiccant.
Storage: Refrigerate at 2 - 8 °C
Stability: 12 months or as indicated on label.

6. TF E-2510 **BIOTIN-AB** **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 or as indicated on label.

7. TF E-2540 **CONJUGATE** **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 vial or as indicated on label.

ASSAY PROCEDURE

Specimen Pretreatment: Refer to **SPECIMEN COLLECTION AND STORAGE** section.

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.	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 standard, 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. (<i>The use of a multichannel pipette is recommended.</i>)
5.	Incubate the microplate on a microplate shaker** for 1 hour at room temperature.
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. (<i>The use of an automatic strip washer is strongly recommended.</i>) 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. (<i>The use of a multichannel pipette is recommended.</i>)
8.	Incubate the microplate on a microplate shaker** for 30 minutes at room temperature.
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. (<i>The use of a multichannel pipette is recommended.</i>)
11.	Incubate the microplate on a microplate shaker** for 10-20 minutes at room temperature or until Standard A attains dark blue colour for desired OD.
12.	Pipette 50 µl of Stopping Solution into each well at the same timed intervals as in step 10 (<i>the use of a multichannel pipette is recommended</i>). 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.
** See REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED (#4) .	

CALCULATIONS

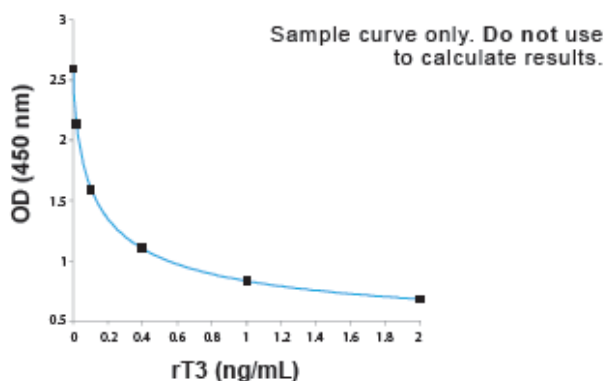
- Calculate the mean optical density of each standard, control and specimen sample duplicate.
- Use a 4-parameter or 5-parameter curve with immunoassay software to generate the control and sample concentration results or draw a standard curve on semi-log paper with the mean optical densities on the Y-axis and the standard concentration on the X-axis and read the concentration of controls and samples off the standard curve.
- If a sample reads greater than 2 ng/ml then dilute it with Standard A at a dilution of not more than 1:5. The result obtained must be multiplied by the dilution factor.
- 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.

Standard	rT3 (ng/ml)	Mean OD (450 nm)
A	0	2.527
B	0.02	2.232
C	0.1	1.563
D	0.4	0.785
E	1	0.431
F	2	0.270
Unknown	0.15	1.289

TYPICAL STANDARD CURVE



PERFORMANCE CHARACTERISTICS

SENSITIVITY

The limit of detection (LoD) was determined from the analysis of at least 60 samples of the blank and a low value sample in two independent experiments and it was calculated as follows:

$LoD = \mu_B + 1.645\sigma_B + 1.645\sigma_S$, where σ_B and σ_S are the standard deviation of the blank and low value sample and μ_B is the mean value of the blank.

The Limit of Detection (LoD) was determined to be **0.014 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
T4	< 0.1
T2	0

INTERFERENT SUBSTANCES

The following substances did not show significant interference with the assay: haemoglobin up to 2 g/l, free and conjugated bilirubin up to 200 mg/l, triglycerides up to 5.0 mg/ml and Biotin up to 2.4 µg/ml.

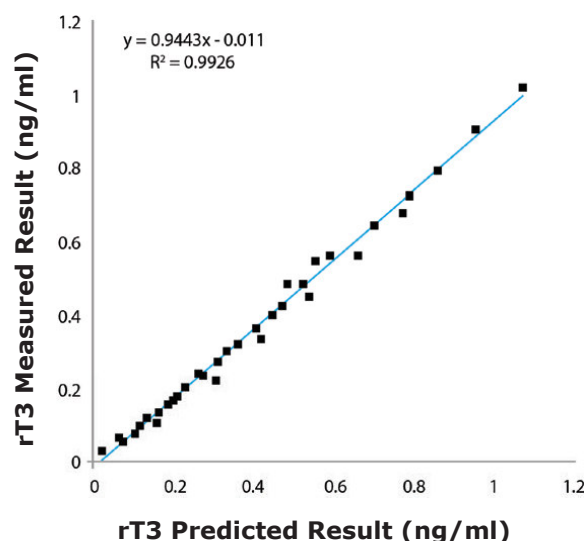
PRECISION

The experimental protocol used a nested components-of-variance design with 2 testing days, two lots and five scientists per day, each scientist ran 2 tests (one test with each lot) per day, and two replicate measurements per run (a 2 x 5 x 2 x 2 design) for each sample. The results were analyzed with a two-way nested ANOVA and summarized in the table below.

Sample	Mean (ng/ml)	Within Run SD	Within Run CV (%)	Total SD	Total CV (%)
1	0.233	0.01	3.6	0.02	7.0
2	0.535	0.01	2.4	0.05	9.5
3	1.174	0.06	4.9	0.13	10.7
4	0.102	0.01	6.0	0.01	10.4
5	0.082	0.00	5.8	0.01	8.7
6	0.094	0.01	8.1	0.01	11.5
7	0.257	0.01	4.3	0.02	9.7
8	0.480	0.02	4.2	0.04	9.2

LINEARITY

The linearity study was performed with four human serum samples covering the range of the assay and following CLSI guideline EP6-A. The samples were diluted in Standard A up to a 1:5 dilution, tested in duplicate, and the results compared to the predicted concentration. The statistical analysis shows that the assay is sufficiently linear.



COMPARATIVE STUDIES

The rT3 ELISA kit (y) was tested manually, as well as with automated technology. The comparison of 40 samples yielded the following linear regression results:

$$y = 0.8452x + 0.0195, r = 0.96$$

REFERENCES VALUES

References 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 % Range (ng/ml)	Total Range (ng/ml)
Serum	120	0.15	0.098 - 0.218	0.069 - 0.262
Plasma	120	0.15	0.098 - 0.26	0.072 - 0.309













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CHANGE HISTORY

Previous Version:	6.0-r	New Version:	6.0a-r
Changes:	REAGENTS PROVIDED Hazard labelling for component AA E-0080 updated		

Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Use-by date		Batch code		
	Consult instructions for use		Content		
	Caution		Catalogue number		Distributor
	Date of manufacture				For research use only!