Free Tri-iodothyronine ELISA Kit (Rat) (OKCA00175)

Instruction for Use

For the quantitative measurement of Rat Free Tri-iodothyronine in serum, plasma, tissue homogenates, cell lysates.

This product is intended for research use only.

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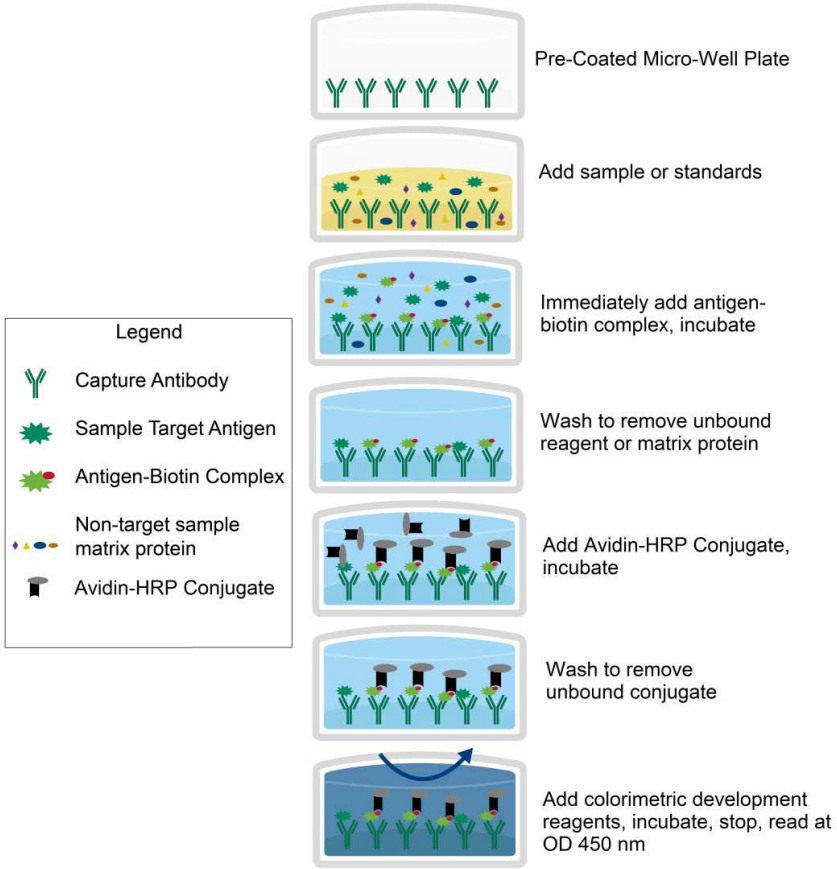
1. Background

Principle

Aviva Systems Biology Free Tri-iodothyronine ELISA Kit (Rat) (OKCA00175) is based on a competitive enzyme immunoassay technique. The microtiter well-plate in this kit has been pre-coated with an anti-Rat Free Tri-iodothyronine antibody. Sample or standards are added to the wells along with a fixed quantity of biotinylated Free Tri-iodothyronine and incubated. The Free Tri-iodothyronine found in the sample or standards competes with the biotinylated Free Tri-iodothyronine for limited binding sites on the immobilized anti-Rat Free Tri-iodothyronine antibody. Excess unbound biotinylated Free Tri-iodothyronine and sample or standard Free Tri-iodothyronine is washed from the plate. Avidin-HRP conjugate is added, incubated and washed. An enzymatic reaction is then produced through the addition of substrate which is catalyzed by the immobilized HRP to generate a blue color product that changes yellow after adding acidic stop solution. The density of yellow coloration is measured by reading the absorbance at 450 nm which is quantitatively proportional to the amount of biotinylated Free Tri-iodothyronine captured in the well and inversely proportional to the amount of Free Tri-iodothyronine which was contained in the sample or standard.

2. Assay Summary



3. Precautions

• Read instructions fully prior to beginning use of the assay kit.
• Any deviations or modifications from the described method or use of other reagents could result in a reduction of performance.
• Reduce exposure to potentially harmful substances by wearing personal protective lab equipment including lab coats, gloves and glasses.
• For information on hazardous substances included in the kit please refer to the Material Safety Data Sheet (MSDS).
• Kit cannot be used beyond the expiration date on the label.

4. Storage and Stability

• Upon receipt store kit at 4°C for 6 months or -20°C for 12 months. Avoid multiple freeze/thaw cycles.

5. Kit Components

• The following reagents are the provided contents of the kit.

Description	Quantity	Storage Conditions
Anti- Free Tri-iodothyronine Microplate	96 Well (12 x 8 Well Strips)	2-8°C for 6 Months
Free Tri-iodothyronine Standard	5 x 1 mL	2-8°C for 6 Months -20°C long term
Free Tri-iodothyronine Antibody-Biotin Complex	1 x 6 mL	
Avidin-HRP Conjugate	1 x 6 mL	2-8°C for 6 Months
20X Wash Buffer	1 x 15 mL	
Substrate A	1 x 7 mL	
Substrate B	1 x 7 mL	
Stop Solution	1 x 7 mL	

6. Required Materials Not Supplied

• Microplate reader capable of reading absorbance at 450 nm.
• Automated plate washer (optional).
• Pipettes capable of precisely dispensing 0.5 µL through 1 mL volumes of aqueous solutions.
• Pipettes or volumetric glassware capable of precisely measuring 1 mL through 100 mL of aqueous solutions.
• New, clean tubes and/or micro-centrifuge tubes for the preparation of standards or samples.
• Absorbent paper or paper toweling.
• Distilled or deionized ultrapure water.
• 37°C Incubator (optional)

7. Technical Application Tips

• Do not mix or substitute components from other kits.
• To ensure the validity of experimental operation, it is recommended that pilot experiments using standards and a small selection of sample dilutions to ensure optimal dilution range for quantitation.
• Samples exhibiting OD measurements higher than the highest standard should be diluted further in the appropriate sample dilution buffers.
• Prior to using the kit, briefly spin component tubes to collect all reagents at the bottom.
• Replicate wells are recommended for standards and samples.
• Cover microplate while incubating to prevent evaporation.
• Do not allow the microplate wells dry at any point during the assay procedure.
• Do not reuse tips or tube to prevent cross contamination.
• Avoid causing bubbles or foaming when pipetting, mixing or reconstituting.
• Completely remove of all liquids when washing to prevent cross contamination.
• Prepare reagents immediately prior to use and do not store, with the exception of the top standard.
• Equilibrate all materials to ambient room temperature prior to use (standards exception).
• For optimal results in inter- intra-assay consistency, equilibrate all materials to 37°C prior to performing assay (standards exception) and perform all incubations at 37°C.
• Pipetting less than 1 µL is not recommended for optimal assay accuracy.
• Once the procedure has been started, all steps should be completed without interruption. Ensure that all reagents, materials and devices are ready at the appropriate time.
• Incubation times will affect results. All wells should be handled in the same sequential order and time intervals for optimal results.
• Samples containing precipitates or fibrin strands or which are hemolytic of lipemic might cause inaccurate results due to interfering factors.
• TMB Substrate is easily contaminated and should be colorless or light blue until added to plate. Handle carefully and protect from light.

8. Reagent Preparation

• Equilibrate all materials to room temperature prior to use and use prepare immediately prior to use.

8.1 1X Free Tri-iodothyronine-Biotin Complex

Provided at ready to use concentration.

8.2 1X Avidin-HRP Conjugate

Provided at ready to use concentration.

8.3 Rat Free Tri-iodothyronine Assay Standards

8.3.1 Prepare the Rat Free Tri-iodothyronine **standards** no greater than 2 hours prior to performing experiment. Standards should be held on ice until use in the experiment.

8.3.2 Reconstitute one vial of the provided 32 pmol/L **Free Tri-iodothyronine Standard** for each experiment. Prepare the stock 32 pmol/L **Free Tri-iodothyronine Standard** by reconstituting one tube of **Lyophilized Free Tri-iodothyronine Standard** as follows:

8.3.2.1 Gently spin or tap the vial at 6,000 – 10,000 rpm for 30 seconds to collect all material at the bottom.

8.3.2.2 Add 1 mL of **Sample Diluent** to the vial.

8.3.2.3 Seal the vial then mix gently and thoroughly.

8.3.2.4 Leave the vial at ambient temperature for 15 minutes.

8.3.3 Prepare a set of seven serially diluted standards as follows:

8.3.3.1 Label tubes with numbers 2 – 6.

8.3.3.2 Use the undiluted 32 pmol/L **Free Tri-iodothyronine Standard** as the high standard point (Tube #1).

8.3.3.3 Add 300 µL of **Sample Diluent** to Tube #'s 2 – 6.

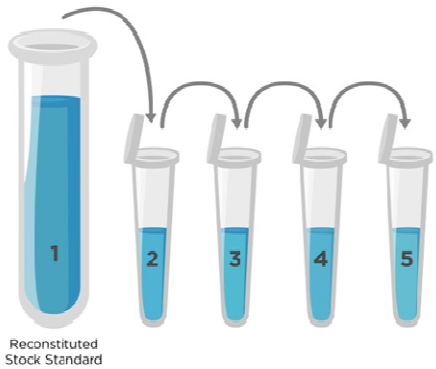
8.3.3.4 Prepare **Standard #2** by adding 300 µL of 32 pmol/L **Free Tri-iodothyronine** (Tube #1) to Tube #2. Mix gently and thoroughly.

8.3.3.5 Prepare **Standard #3** by adding 300 µL of **Standard #2** from Tube #2 to Tube #3. Mix gently and thoroughly.

8.3.3.6 Prepare further serial dilutions through Tube #5. Reference the table below as a guide for serial dilution scheme.

8.3.3.7 Tube #6 is a blank standard (only **Sample Diluent**), which should be included with every experiment.

Standard Number (Tube)	Standard To Dilute	Volume Standard to Dilute (µL)	Volume Sample Diluent Buffer (µL)	Total Volume (µL)	Final Concentration
Tube #1	32 pmol/L Reconstituted Free Tri-iodothyronine Standard	NA	NA	NA	32 pmol/L
Tube #2	32 pmol/L	300	300	600	16 pmol/L
Tube #3	16 pmol/L	300	300	600	8 pmol/L
Tube #4	8 pmol/L	300	300	600	4 pmol/L
Tube #5	4 pmol/L	300	300	600	2 pmol/L
Tube #6	NA	0	300	300	0.0 (Blank)



8.4 1X Wash Buffer

8.4.1 If crystals have formed in the **20X Wash Buffer** concentrate, equilibrate to room temperature and mix gently until crystals have completely dissolved.

8.4.2 Add the entire 15 mL contents of the **20X Wash Buffer** bottle to 285 mL of ultra-pure water to a clean > 300 mL bottle or other vessel.

8.4.3 Seal and mix gently by inversion. Avoid foaming or bubbles.

8.4.4 Store the **1X Wash Buffer** at room temperature until ready to use in the procedure. Store the prepared **1X Wash Buffer** at 4°C for no longer than 1 week. Do not freeze.

8.5 Microplate Preparation

- Micro-plates are provided ready to use and do not require rinsing or blocking.
- Unused well strips should be returned to the original packaging, sealed and stored at °4C.
- Equilibrate microplates to ambient temperatures prior to opening to reduce potential condensation.

9. Sample Preparation

9.1 Sample Preparation and Storage

- Store samples to be assayed at 2-8°C for 24 hours prior being assayed.
- For long term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.
- Samples not indicated in the manual must be tested to determine if the kit is valid.
- Prepare samples as follows:

  - **Serum** - Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1,000 x g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
  - **Plasma** - Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1,000 x g at 2-8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
  - **Tissue Homogenates** – 100 mg tissue was rinsed with 1X PBS, homogenized in 1 ml of 1X PBS and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5,000 x g, 2-8°C. The supernatant was removed and assayed immediately. Alternatively, aliquot and store samples at -20°C or -80°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles.
  - **Cell Lysates** – (1) Adherent Cell: Remove media and rinse cells once with ice-cold PBS (PH7.2-7.4). Scrape cells off the plate and transfer to an appropriate tube. Dilute cell suspension with 1xPBS, until cell concentration reached 100 million/ml. Then store overnight at -20°C. After two freeze-thaw cycles to break up the cell membranes, the cell lysates were centrifuged for 5 minutes at 5000 g, 2 - 8°C. Collect the supernatant. Cell lysates should be assayed immediately or aliquotted and stored at -20°C. Centrifuge the sample again after thawing before the assay. Avoidrepeated freeze-thaw cycles. (2) Suspension Cell: Collect cells with appropriate tube, centrifuge for 5 minutes at 1000 g, 2 - 8°C. Remove the supernatant and resuspend cells with 1xPBS. Centrifuge for 5 minutes at 1000 g, 2 - 8°C. Remove the supernatant. Dilute cell with 1xPBS, until cell concentration reached 100 million/ml. Then store overnight at -20°C. After two freeze-thaw cycles to break up the cell membranes, the cell lysates were centrifuged for 5 minutes at 5000 g, 2 - 8°C. Collect the supernatant. Cell lysates should be assayed immediately or aliquotted and stored at -20°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles.

10. Assay Procedure

• Equilibrate all reagents and materials to ambient room temperature prior to use in the procedure.
• To control for small potential variations in micro well-plate and day to day ambient temperature fluctuations, equilibrate all reagents prior to use and perform all incubation steps at 37°C for optimal consistency and reproducibility.

10.1 Determine the required number of wells and return any remaining unused wells and desiccant to the pouch.

10.2 Retain at least one well as an absolute Blank without any samples or reagents.

10.3 Add 50 µL of serially titrated standards, diluted samples or blank into wells of the Anti- Free Tri-iodothyronine Microplate. At least two replicates of each standard, sample or blank is recommended.

10.4 Immediately add 50 µL of **Free Tri- iodothyronine Biotin-Complex** to each well (excluding absolute Blank).

10.5 Cover the plate with the well plate lid and incubate for 60 minutes at 37°C.

10.6 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.

10.7 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.8 Wash plate five times with **1X Wash Buffer** as follows:

10.8.1 Add 300 µL of **1X Wash Buffer** to each assay well.

10.8.2 Incubate for 2 minutes.

10.8.3 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.

10.8.4 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.8.5 Repeat steps 10.8.1 through 10.8.4 **four** more times.

10.9 Immediately add 50 µL of **Avidin HRP-Conjugate** to each well (excluding absolute Blank).

10.10 Cover the plate with the well plate lid and incubate for 30 minutes at 37°C.

10.11 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.

10.12 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time

10.13 Wash as in step 10.8.

10.14 Add 50 µL of **Substrate A** and 50 µL **Substrate B** to each well and incubate at 37°C in the dark for 15 minutes. Wells should change to gradations of blue. If the color is too deep, reduce the incubation time.

(NOTE: optimal incubation time must be determined by the user. Optimal development can be visualized by blue shading in the top four standard wells, while the remaining standards still apear clearer.)

10.15 Add 50 µL of **Stop Solution** to each well. Well color should change to gradations of yellow immediately. Add the **Stop Solution** in the same well order as done for the **Substrate A** and **Substrate B**.

10.16 Read the O.D. absorbance at 450 nm with a standard microplate reader within 5 minutes of stopping the reaction in step 10.15. If wavelength correction is available, set to 540 nm or 570 nm.

11. Calculation of Results

For analysis of the assay results, calculate the **Relative OD_{450}** for each test or standard well as follows:

$$(\text{Relative } OD_{450}) = (\text{Well } OD_{450}) - (\text{Mean Blank Well } OD_{450})$$

The standard curve is generated by plotting the mean replicate **Relative OD_{450}** of each standard serial dilution point vs. the respective standard concentration. The Free Tri- iodothyronine concentration contained in the samples can be interpolated by using linear regression of each mean sample **Relative OD_{450}** against the standard curve. This is best achieved using curve fitting software.

Note: if wavelength correction readings are available, subtract the readings at 540 nm or 570 nm from the readings at 450 nm. This may provide greater reading accuracy.

Note: if the samples measured were diluted, multiply the derived mean sample concentration by the dilution factor for a final sample concentration.

12. Typical Expected Data

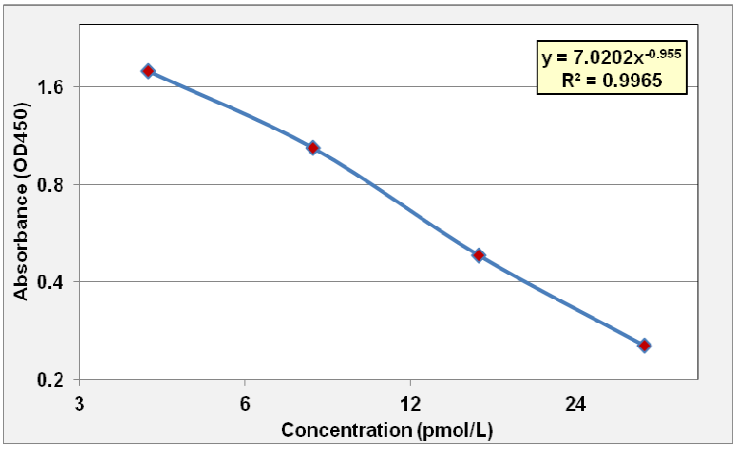
12.1 Typical absorbance values

Expected absorbance for standards when TMB incubation is performed for 20 minutes at 37°C and measured at OD_{450}.

Sample Concentration (pmol/L)	Sample 1 (OD_{450})	Sample 2 (OD_{450})	Mean (OD_{450})
32	0.25	0.26	0.255
16	0.473	0.494	0.484
8	1.081	0.992	1.037
4	1.87	1.722	1.796
2	2.842	2.876	2.859

12.2 Typical standard curve

This standard curve is for demonstration purposes only. An assay specific standard curve should be performed with each assay.



12.3 General Specifications

General Specifications	
Range	2 pmol/L – 32 pmol/L
LOD	< 0.38 pmol/L (Derived by linear regression of OD_{450} of the Mean Blank + 2xSD)
Specificity	Rat Free Tri-iodothyronine
Cross-Reactivity	No detectable cross-reactivity with other relevant proteins

12.4 Reproducibility

Three samples concentrations were measured in replicate within an assay plate and across replicate assays to assess Intra- and Inter-Assay precision.

Intra-Assay Precision: %CV < 15%, n=20

Inter-Assay Precision: %CV < 15%, n=20

13. Technical Resources

Technical Support:

For optimal service please be prepared to supply the lot number of the kit used.

USA

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