Rat Thyroxine (T4) ELISA
Cat No: K11-0338C

Principle:
The Rat Thyroxine (T4) ELISA is competitive enzyme-linked immunosorbent assay (ELISA) to assay the level of T4 in samples. Standards or Samples competes with the T4 antigen coated microtiter well to form a complex with the biotinylated Anti-T4. Wells are washed to remove the excess conjugate and Streptavidin:HRP Conjugate is added to the microplate and incubated. After incubation and a washing step TMB Substrate, are added. Blue color develops on incubation and the reaction is stopped with a Stop Solution to form a yellow color. The concentration of the T4 in the samples is inversely proportional to the yellow color developed (absorbance) in the wells.

Intended Use:
This Kit is used to assay the level of Rat Thyroxine (T4) in Rat serum and plasma samples. The Kit is For Laboratory / Research Use Only.

Materials provided in the Kit:
1. Rat T4 Coated Microtiter Plate (96 wells) - 1 no
2. Lyophilized Rat T4 Standard (concentrated, 120ng) - 2 vials
3. Biotinylated Anti-T4 (concentrated) - 60 ul
4. Streptavidin-HRP Conjugate - 120 ul
5. Sample Diluent - 20 ml
6. Biotin Antibody Dilution Buffer - 10 ml
7. HRP Conjugate Dilution Buffer - 10 ml
8. (25X) Wash Buffer - 30 ml
9. TMB Substrate - 10 ml
10. Stop Solution - 10 ml
11. Instruction Manual

Materials to be provided by the End-User:
1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Clean tubes and Eppendorf tubes
6. Precision single and multi-channel pipette and disposable tips.
7. 37°C incubator
8. Timer.

Storage Information:
1. All reagents should be stored at 2ºC to 8ºC.
2. All the reagents and wash solutions are stable until the expiration date of the kit.
3. 20 minutes prior before use, bring all components to room temperature (18-25ºC). Store all the components of the kit at its appropriate storage condition after use.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:
1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details.
2. To reduce the likelihood of blood-borne transmission of infectious agents, handle all samples in accordance with NCCLS regulations.

Specimen Collection and Handling:
1. **Serum-** Place whole blood sample at room temperature for 2 hours or put it at 2-8°C overnight and centrifugation for 20 minutes at approximately 1000×g, Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.
2. **Plasma-** Collect plasma using EDTA-Na2 or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2-8°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Avoid hemolysis, high cholesterol samples.

Note: Samples to be used within 5 days can be stored at 2-8°C, besides that, samples must be stored at -20°C (assay ≤1 month) or -80°C (assay≤2 months) to avoid loss of bioactivity and contamination. Avoid multiple freeze-thaw cycles. The hemolytic samples are not suitable for this assay.

Sample Dilution

The user should estimate the concentration of target protein in the test sample, and select a proper dilution factor to make the diluted target protein concentration fall in the optimal detection range of the kit. Dilute the sample with the provided diluent, and several trials may be necessary. The test sample must be well mixed with the diluent. And also standard curves and sample should be making in pre-experiment. If samples with very high concentrations, dilute samples with PBS first and then dilute the samples with Sample Dilution.

Reagent Preparation (all reagents should be diluted immediately prior to use):

Bring all reagents to Room Temperature for 20 minutes prior to use.

1. **Wash Buffer**:
   If crystals have formed in the concentrate, you can warm it with 40°C water bath (Heating temperature should not exceed 50°C) and mix it gently until the crystals have completely been dissolved. The solution should be cooled to room temperature before use.
   Dilute 30 ml (25X) Wash Buffer into 720 ml Deionized or Distilled Water to obtain 750 ml (1X) Wash Buffer.

2. **Standards:**
   Add 1 ml Sample Diluent to the Rat T4 Standard vial provided in the kit. Keep the tube at room temperature for 10 minutes and mix thoroughly before making further dilutions. Prepare the **Standards** by serially diluting the standard stock solution as per the below table. The sample diluent is the Standard No. 1 or blank for this competitive assay.

Standard Concentration	Standard Vial	Dilution Particulars
120 ng/ml	Standard No.8	Reconstitute with 1ml Sample Diluent
60 ng/ml	Standard No.7	300 ul Standard No.8 + 300 ul Sample Diluent
30 ng/ml	Standard No.6	300 ul Standard No.7 + 300 ul Sample Diluent
15 ng/ml	Standard No.5	300 ul Standard No.6 + 300 ul Sample Diluent
7.5 ng/ml	Standard No.4	300 ul Standard No.5 + 300 ul Sample Diluent
3.75 ng/ml	Standard No.3	300 ul Standard No.4 + 300 ul Sample Diluent
1.875 ng/ml	Standard No.2	300 ul Standard No.3 + 300 ul Sample Diluent
0 ng/ml	Standard No.1	300 ul Sample Diluent only

Note: It is best to use Standard Solutions within 2 hours.

3. **Preparation of Biotinylated Anti-Rat T4 Working Solution**:
   Prepare within 1 hour before your assay.
   Dilute the Biotinylated Anti-T4 with Biotin Antibody Dilution Buffer at 1:100 and mix thoroughly. (for example Add 1 ul Biotinylated Anti-T4 to 99 ul Biotin Antibody Dilution Buffer)

4. **Preparation of Streptavidin:HRP Conjugate Working Solution:**
   Prepare within 30 minutes before your assay.
   Dilute the Streptavidin:HRP Conjugate with HRP Conjugate Dilution Buffer at 1:100 and mix thoroughly. (for example Add 1 ul of Streptavidin:HRP Conjugate to 99 ul of HRP Conjugate Dilution Buffer)

Procedural Notes:

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Rat Thyroxine (T4) High Dose Hook Effect is due to excess of antibody for very high concentrations of Rat Thyroxine (T4) present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent. Thus if the Rat Thyroxine (T4) concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.

3. Avoid assay of Samples containing Sodium Azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of Rat Thyroxine (T4)
4. It is recommended that all Standards and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to poor sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:
It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay.

1) Add **50 ul** of **prepared Standards** or **Samples** to respective wells.

2) Add **50 ul Biotinylated Anti-Rat T4 Working Solution** to each well. Mix well.

3) Cover with the plate and Incubate for 45 minutes at 37°C.

4) Aspirate and wash plate 4 times with diluted Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

5) Add **100 ul Streptavidin:HRP Conjugate Working Solution** to each well.

6) Incubate for 30 minutes at 37°C.

7) Aspirate the contents and wash as per Step (4) indicated herein above.

8) Pipette **90 ul TMB Substrate** into each well.
DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

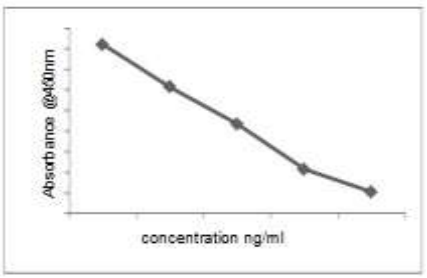
9) Pipette **50 ul** of **Stop Solution** to all wells. The wells should turn from blue to yellow in color.

10) Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

Calculation of Results:
Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Use the Net Absorbance (Absorbance of Standard/Sample - Absorbance of Blank) to calculate the Mean Absorbances. Using standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the concentration. If samples were diluted, multiply by the appropriate dilution factor.

Software which is able to generate a cubic spline curve-fit or a polynomial regression to the 2nd order is best recommended for automated results.

Typical Graph



Stability
The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 10% within the expiration date under appropriate storage condition.

Standard(n=5)	37°C for 1 month	2-8°C for 12 months
Average (%)	80	95-100

To minimize extra influence on performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is strongly suggested that the same operator performs the whole assay from the beginning to the end.

Performance Characteristics:
Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

Sensitivity:
Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. 10 replicates of '0' standards were evaluated and the LOD was found to **0.725 ng/ml**.

Specificity:
This assay has high sensitivity and excellent specificity for detection of T4. No significant cross-reactivity or interference between T4 and analogues was observed.

Recovery
Matrices listed below were spiked with certain level of T4 and the recovery rates were calculated by comparing the measured value to the expected amount of T4 in samples.

Matrix	Recovery Range (%)	Average (%)
Serum(n=5)	86-102	92
EDTA Plasma(n=5)	86-103	94
Heparin Plasma(n=5)	91-103	95

Assay Range:
1.875 ng/ml - 120 ng/ml

Precision:
Intra-Assay: CV<8%
Inter-Assay: CV<10%

Linearity:
The linearity of the kit was assayed by testing samples spiked with appropriate concentration of T4 and their serial dilutions. The results were demonstrated by percentage of calculated concentration to the expectation.

Sample	1:2	1:4	1:8
serum (n=5)	88-102%	87-104%	87-104%
EDTA plasma (n=5)	82-96%	82-91%	87-99%
heparin plasma (n=5)	83-98%	80-99%	81-99%

LIMITED WARRANTY

KinesisDx does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the product; against defects in products or components not manufactured by KinesisDx, or against damages resulting from such non-KinesisDx made products or components. KinesisDx passes on to customer the warranty it received (if any) from the maker thereof of such non-Krishgen made products or components.

This warranty also does not apply to product to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by KinsisDx.

THIS WARRANTY IS EXCLUSIVE. The sole and exclusive obligation of KinesisDx shall be to repair or replace the defective product in the manner and for the period provided above. KinesisDx shall not have any other obligation with respect to the products or any part thereof, whether based on contract, tort, strict liability or otherwise. Under no circumstances, whether based on this Limited Warranty or otherwise, shall KinesisDx be liable for incidental, special, or consequential damages.

This Limited Warranty states the entire obligation of KinesisDx with respect to the product. If any part of this Limited Warranty is determined to be void or illegal, the remainder shall remain in full force and effect.

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Rat T4 (Thyroxine) ELISA

ASSAY PROCEDURE

1	Bring all reagents to room temperature before use.		
2	Pipette Standards 1 - 8 Samples	50 ul	50 ul
3	Pipette Biotinylated Anti-Rat T4: HRP Conjugate	50ul	50ul
4	Incubate	45 minutes (37ºC)	
5	Wash 1X Wash Buffer	Decant, 4 x 300 ul	
3	Pipette Streptavidin: HRP Conjugate	100 ul	100 ul
5	Incubate	30 minutes (37ºC)	
6	Wash 1X Wash Buffer	Decant, 4 x 300 ul	
7	TMB Substrate	90 ul	90 ul
9	Incubate in the dark	10 minutes (37ºC)	
10	Pipette Stop Solution	50 ul	50 ul
11	Measure 450 within 15 mins		