

RayBio[®] Human/Mouse/Rat Thyrotropin-releasing hormone Enzyme Immunoassay Kit

Catalog #: EIA-TRH, EIAM-TRH, EIAR-TRH

User Manual
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Caution:
Extraordinarily useful information enclosed



ISO 13485 Certified

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Please read the entire manual carefully before starting your experiment

I. Introduction

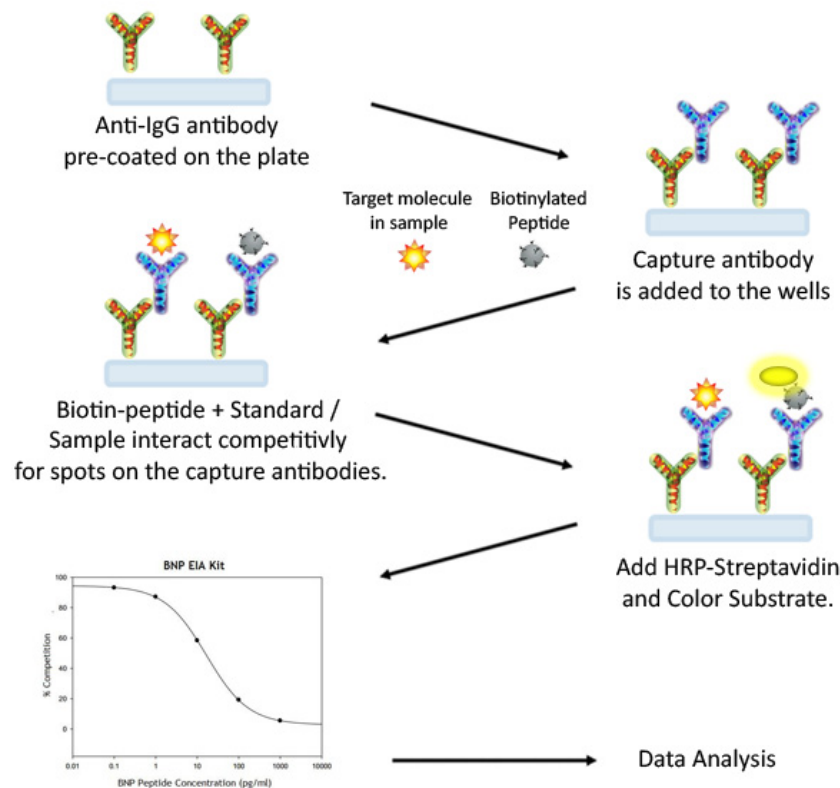
Thyrotropin-releasing hormone (TRH), also called thyrotropin-releasing factor (TRF) or thyroliberin, is a releasing hormone, produced by the hypothalamus, that stimulates the release of thyrotropin (thyroid-stimulating hormone or TSH) and prolactin from the anterior pituitary. It is a tripeptidal hormone produced from a precursor protein. TRH has been used clinically for the treatment of spinocerebellar degeneration and disturbance of consciousness in humans. It has been used to test the response of the anterior pituitary gland. TRH has anti-depressant and anti-suicidal properties, and has been shown in mice to be an anti-aging agent.

II. General Description

The RayBio® Thyrotropin-releasing Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Thyrotropin-releasing peptide based on the competitive enzyme immunoassay principle.

In this assay, a biotinylated Thyrotropin-releasing peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated Thyrotropin-releasing peptide competes with endogenous (unlabeled) Thyrotropin-releasing for binding to the anti-Thyrotropin-releasing antibody. After a wash step, any bound biotinylated Thyrotropin-releasing then interacts with horseradish peroxidase (HRP)-streptavidin, which catalyzes a color development reaction. The intensity of the colorimetric signal is directly proportional to the amount of captured biotinylated Thyrotropin-releasing peptide and inversely proportional to the amount of endogenous Thyrotropin-releasing in the standard or samples. A standard curve of known concentration of Thyrotropin-releasing peptide can be established and the concentration of Thyrotropin-releasing peptide in the samples can be calculated accordingly.

III. How It Works



IV. Storage

The entire kit may be stored at -20°C to -80°C for up to 6 months from the date of shipment. For extended storage, it is recommended to store at -80°C. **Avoid repeated freeze-thaw cycles.** For prepared reagent storage, see table below.

V. Reagents

| Component | Size / Description | Storage / Stability After Preparation |
|---|--|---|
| EIA Microplate (Item A) | 96 wells (12 strips x 8 wells) coated with secondary antibody. | 1 month at 4°C* |
| Wash Buffer Concentrate (20X) (Item B) | 25 ml of 20X concentrated solution. | 1 month at 4°C |
| Standard Thyrotropin-releasing Peptide (Item C) | 2 vials of Thyrotropin-releasing Peptide. 1 vial is enough to run each standard in duplicate. | The first standard: 2-3 days at 4°C Additional dilutions: Do not store |
| Anti-Thyrotropin-releasing Polyclonal Antibody (Item N) | 2 vials of anti-Thyrotropin-releasing . | 1 month at 4°C |
| 5X Assay Diluent B (Item E) | 15 ml of 5X concentrated buffer. Diluent for both standards and samples including serum, plasma, cell culture media or other sample types. | 1 month at 4°C |
| Biotinylated Thyrotropin-releasing Peptide (Item F) | 2 vials of Biotinylated Thyrotropin-releasing Peptide, 1 vial is enough to assay the whole plate. | 2-3 days at 4°C |
| HRP-Streptavidin Concentrate (Item G) | 600 µl 50x concentrated HRP-conjugated streptavidin. | Do not store and reuse |
| Positive Control (Item M) | 1 vial of Positive Control. | 2-3 days at 4°C |
| TMB One-Step Substrate Reagent (Item H) | 12 ml of 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution. | N/A |
| Stop Solution (Item I) | 8 ml of 0.2 M sulfuric acid. | N/A |

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

VI. Additional Materials Required

1. Microplate reader capable of measuring absorbance at 450 nm
2. Precision pipettes to deliver 2 μ l to 1 ml volumes
3. Adjustable 1-25 ml pipettes for reagent preparation
4. 100 ml and 1 liter graduated cylinders
5. Absorbent paper
6. Distilled or deionized water
7. SigmaPlot software (or other software which can perform four-parameter logistic regression models)
8. Tubes to prepare standard or sample dilutions
9. Orbital shaker
10. Aluminum foil
11. Plastic wrap

VII. Reagent Preparation

Keep kit reagents on ice during reagent preparation steps.

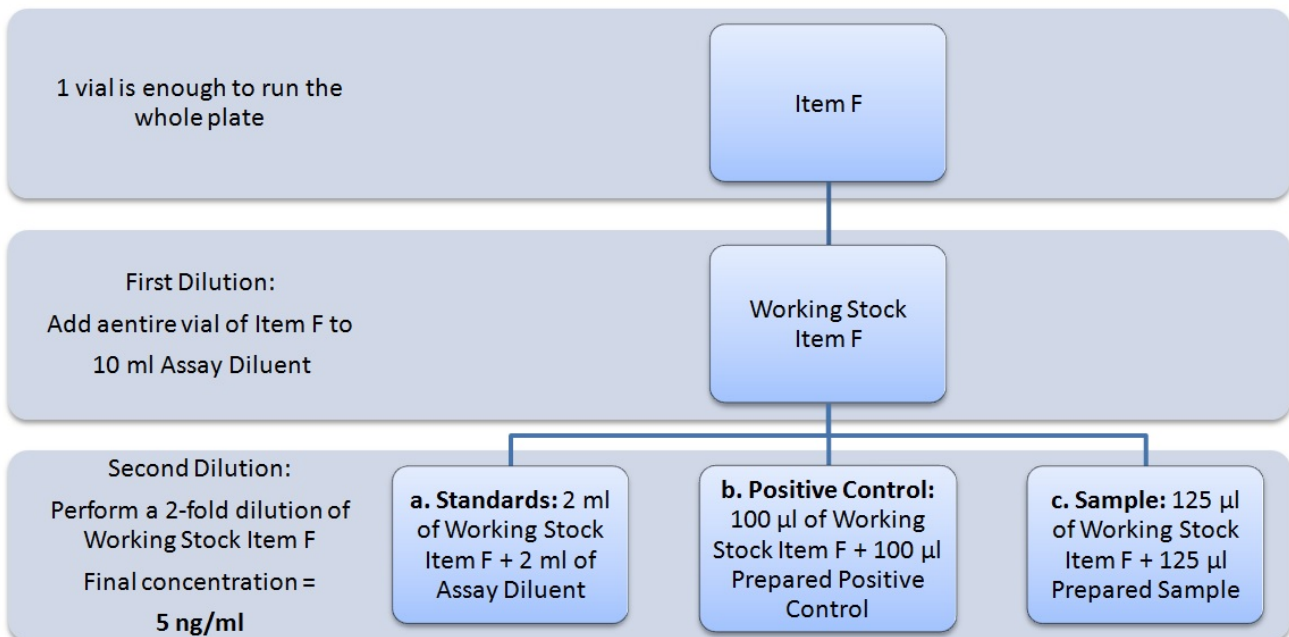
A. Preparation of Plate and Anti-Thyrotropin-releasing Antibody

1. Equilibrate plate to room temperature before opening the sealed pouch.
2. Label removable 8-well strips as appropriate for your experiment.
3. 5X Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.
4. Briefly centrifuge the anti-Thyrotropin-releasing antibody vial (Item N) . Then add 50 μ l of 1X Assay Diluent B to the vial to prepare the antibody concentrate. Pipette up and down to mix gently.
5. The antibody concentrate should then be diluted 100-fold with 1X Assay Diluent B. This is your anti-Thyrotropin-releasing antibody working solution, which will be used in step 2 of Assay Procedure (Section VIII).

Note: The following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure)

B. Preparation of Biotinylated Thyrotropin-releasing (Item F)

6. Briefly centrifuge the vial of Biotinylated Thyrotropin-releasing (Item F) before use.
7. See the image below for proper preparation of Item F. Transfer the entire contents of the Item F vial into a tube containing 10 ml of 1X Assay Diluent B. This is your Working Stock of Item F. Pipette up and down to mix gently. *The final concentration of biotinylated Thyrotropin-releasing will be **10 ng/ml**.*
 - a. Second Dilution of Item F for Standards: Add 2 ml of Working Stock Item F to 2 ml of 1X Assay Diluent B. The final concentration of biotinylated Thyrotropin-releasing will be **5 ng/ml**.
 - b. Second Dilution of Item F for Positive Control: Add 100 μ l of Working Stock Item F to 100 μ l of the prepared Positive Control (Item M). (See section D for Positive Control preparation) The final concentration of biotinylated Thyrotropin-releasing will be **5 ng/ml**.
 - c. Second Dilution of Item F for samples: Add 125 μ l of Working Stock Item F to 125 μ l of prepared sample (see section E for sample preparation). This is a 2-fold dilution of your sample. The final concentration of biotinylated Thyrotropin-releasing will be **5 ng/ml**.

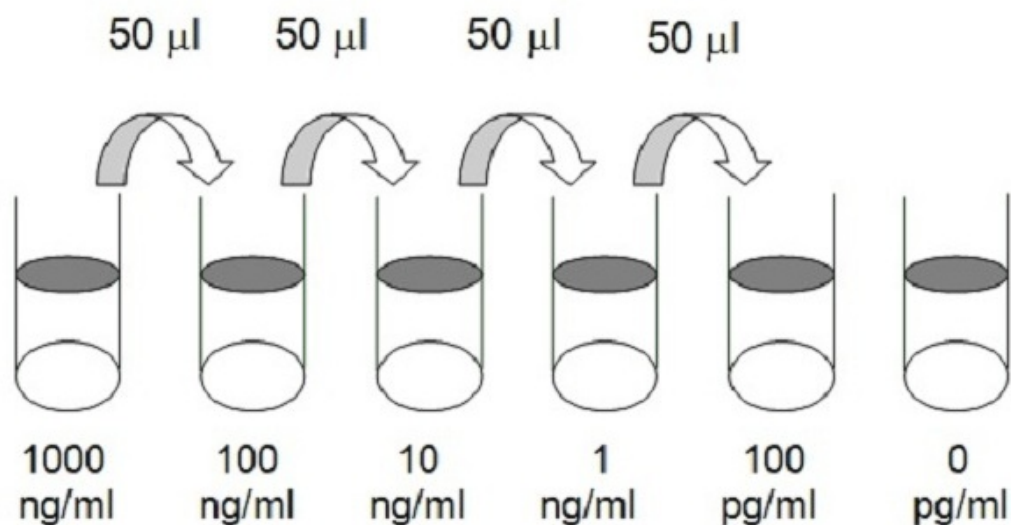


C. Preparation of Standards

- Label 6 microtubes with the following concentrations: 1,000 ng/ml, 100 ng/ml, 10ng/ml, 1 ng/ml, 100 pg/ml and 0 pg/ml. Pipette 450 μ l of biotinylated Thyrotropin-releasing Item F working solution (prepared in step 7a) into each tube, except the 1,000 ng/ml (leave this one empty).

It is very important to make sure the concentration of biotinylated Thyrotropin-releasing is 5 ng/ml in all standards.

- Briefly centrifuge the vial of Thyrotropin-releasing Standard (Item C). Pipette 8 μ l of Item C and 792 μ l of 5 ng/ml biotinylated Thyrotropin-releasing working solution (prepared in step 7a) into the tube labeled 1000 ng/ml. Mix thoroughly. This solution serves as the first standard (1,000 ng/ml Thyrotropin-releasing standard, 5 ng/ml biotinylated Thyrotropin-releasing).
- To make the 100 ng/ml standard, pipette 50 μ l of the 1000 ng/ml Thyrotropin-releasing standard into the tube labeled 100 ng/ml. Mix thoroughly.
- Repeat this step with each successive concentration, preparing a dilution series as shown in the illustration below. Each time, use 450 μ l of biotinylated Thyrotropin-releasing and 50 μ l of the prior concentration until the 100 pg/ml is reached. Mix each tube thoroughly before the next transfer.



D. Positive Control Preparation

12. Briefly centrifuge the Positive Control vial (Item M).
13. Refer to step 7b. This is a 2-fold dilution of the Positive Control. The final concentration of biotinylated Thyrotropin-releasing should still be 5 ng/ml.

The Positive Control is a mouse serum sample that serves as a system control to verify that the kit components are working. The resulting OD will not be used in any calculations; if no positive competition is observed please contact RayBiotech Technical Support. The Positive Control may be diluted further if desired, but be sure the final concentration of biotinylated Thyrotropin-releasing is 5 ng/ml.

E. Sample Preparation

14. If you wish to perform a 2-fold dilution of your sample, proceed to step 7c. If you wish to perform a higher dilution of your sample, dilute your sample with 1X Assay Diluent B before performing step 7c.

EXAMPLE (to make a 4-fold dilution of sample):

- a. Dilute sample 2-fold (62.5 μ l of sample + 62.5 μ l of 1X Assay Diluent B.).
- b. Perform step 7c (125 μ l of working solution Item F + 125 μ l of sample prepared above).

The total volume is 250 μ l, enough for duplicate wells on the microplate. It is very important to make sure the final concentration of the biotinylated Thyrotropin-releasing is **5 ng/ml**.

Note: Optimal sample dilution factors should be determined empirically, however you may reference below for recommended dilution factors for serum:

Human=8x Mouse=8x Rat=8x.

If you have any questions regarding the recommended dilutions you may contact technical support at 888-494-8555 or techsupport@raybiotech.com.

F. Preparation of Wash Buffer and HRP

15. If Item B (20X Wash Concentrate) contains visible crystals, warm to room temperature and mix gently until dissolved.
16. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
17. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use.
18. Dilute the HRP-Streptavidin concentrate 50-fold with 1X Assay Diluent B.

VIII. Assay Procedure

1. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 μ l of Anti-Thyrotropin-releasing Antibody (Item N) (See Reagent Preparation step 5) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycle/sec). You may also incubate overnight at 4°C.
3. Discard the solution and wash wells 4 times with 1X Wash Solution Buffer (200-300 μ l each). Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 μ l of each standard (see Reagent Preparation Section C), Positive Control (see Reagent Preparation Section D) and sample (see Reagent Preparation Section E) to appropriate wells. Be sure to include a blank well (Assay Diluent only). Cover wells and incubate for 2.5 hours at room temperature with gentle shaking (1-2 cycles/sec) overnight or at 4°C.
5. Discard the solution and wash 4 times as directed in Step 3.
6. Add 100 μ l of prepared HRP-Streptavidin solution (see Reagent Preparation step 18) to each well. Incubate for 45 minutes at room temperature with gentle shaking. It is recommended that incubation time should not be shorter or longer than 45 minutes.

7. Discard the solution and wash 4 times as directed in Step 3.
8. Add 100 μ l of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).
9. Add 50 μ l of Stop Solution (Item I) to each well. Read at 450 nm immediately.

IX. Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.
2. Add 100 μ l anti-Thyrotropin-releasing to each well. Incubate 1.5 hours at room temperature or overnight at 4°C.
3. Add 100 μ l standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.
4. Add 100 μ l prepared Streptavidin solution. Incubate 45 minutes at room temperature.
5. Add 100 μ l TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.
6. Add 50 μ l Stop Solution to each well. Read at 450 nm immediately.

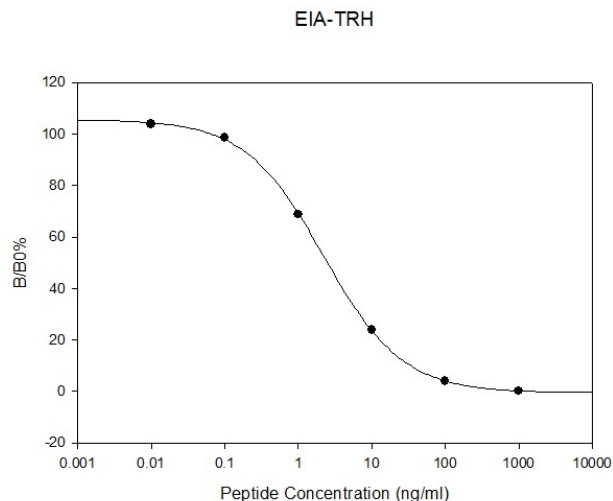
X. Calculation of Results

Calculate the mean absorbance for each set of duplicate stands, controls, and samples and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit curve through the standard points.

Percentage absorbance = $(B - \text{blank OD}) / (B_0 - \text{blank OD})$ where
B = OD of sample or standard and
 B_0 = OD of zero standard (total binding)

A. Typical Data

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. Sensitivity

The minimum detectable concentrations of Thyrotropin-releasing is 0.6 ng/ml.

C. Standard Curve Range

0.1-1,000 ng/ml

D. Reproducibility

Intra-Assay: CV<10%

Inter-Assay: CV<15%

E. Assay Diagram

Recommended Plate Layout:

| | | | | | | | | | | | |
|---------------|---------------|-----|-----|------|------|------|------|------|------|------|------|
| Blank | Blank | SA1 | SA1 | SA9 | SA9 | SA17 | SA17 | SA25 | SA25 | SA33 | SA33 |
| Total Binding | Total Binding | SA2 | SA2 | SA10 | SA10 | SA18 | SA18 | SA26 | SA26 | SA34 | SA34 |
| Standard1 | Standard1 | SA3 | SA3 | SA11 | SA11 | SA19 | SA19 | SA27 | SA27 | SA35 | SA35 |
| Standard2 | Standard2 | SA4 | SA4 | SA12 | SA12 | SA20 | SA20 | SA28 | SA28 | SA36 | SA36 |
| Standard3 | Standard3 | SA5 | SA5 | SA13 | SA13 | SA21 | SA21 | SA29 | SA29 | SA37 | SA37 |
| Standard4 | Standard4 | SA6 | SA6 | SA14 | SA14 | SA22 | SA22 | SA30 | SA30 | SA38 | SA38 |
| Standard5 | Standard5 | SA7 | SA7 | SA15 | SA15 | SA23 | SA23 | SA31 | SA31 | SA39 | SA39 |
| Pos Control | Pos Control | SA8 | SA8 | SA16 | SA16 | SA24 | SA24 | SA32 | SA32 | SA40 | SA40 |

Key:

Blank = Buffer Only

Total Binding = Biotin- Thyrotropin-releasing only

Standard 1 = 1000 ng/ml

Standard 2 = 100 ng/ml

Standard 3 = 10 ng/ml

Standard 4 = 1 ng/ml

Standard 5 = 100 pg/ml

Pos Control = Biotin with Item M

XI. Specificity

This EIA kit is designed to detect human, mouse, and rat Thyrotropin-releasing hormone

XIV. Select EIA Publications

1. Plum L, Lin HV, Dutia R, Tanaka J, Aizawa KS, et al. The Obesity Susceptibility Gene Carboxypeptidase E Links FoxO1 Signaling in Hypothalamic Pro-opiomelanocortin Neurons with Regulation of Food Intake. *Nature Med.* 2009;15(10):1195-1201. (Ghrelin EIA, EIA-GHR-1)
2. Hug C, Lodish HF. Visfatin: a new adipokine. *Science.* 2005; 307(5708):366-7.
3. Kim MK. Crystal structure of visfatin/pre-B cell colony-enhancing factor 1/nicotinamide phosphoribosyltransferase, free and in complex with the anti-cancer agent FK-866. *J Mol Biol.* 2006; 362(1):66-77.
4. Revollo, J.R., et al. The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. *J. Biol. Chem.* 2004; 279: 50754-50763.
5. Oh-I S, Shimizu H, Satoh T, et al. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. *Nature* 2006; 443 (7112): 709-12.
6. Zhang J, Ren P, Avsian-Kretchmer O, Luo C, Rauch R, Klein C, Hsueh A. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* 2005; 310 (5750): 996-9.
7. Cummings D, Weigle D, Frayo R, Breen P, Ma M, Dellinger E, Purnell J. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *N Engl J Med* 2002; 346 (21): 1623-30.
8. Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature* 2002; 407 (6806): 908-913.9. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; 402 (6762): 656-60.

XIII. Troubleshooting Guide

| Problem | Cause | Solution |
|---------------------|--|---|
| Poor standard curve | <ul style="list-style-type: none"> ○ Inaccurate pipetting ○ Improper standard dilution | <ul style="list-style-type: none"> ○ Check pipettes ○ Briefly centrifuge Item C and dissolve the powder thoroughly by gently mixing |
| Low signal | <ul style="list-style-type: none"> ○ Improper preparation of standard and/or biotinylated antibody ○ Too brief incubation times ○ Inadequate reagent volumes or improper dilution | <ul style="list-style-type: none"> ○ Briefly spin down vials before opening. Dissolve the powder thoroughly. ○ Ensure sufficient incubation time; assay procedure step 2 may be done overnight ○ Check pipettes and ensure correct preparation |
| Large CV | <ul style="list-style-type: none"> ○ Inaccurate pipetting ○ Air bubbles in wells | <ul style="list-style-type: none"> ○ Check pipettes ○ Remove bubbles in wells |
| High background | <ul style="list-style-type: none"> ○ Plate is insufficiently washed ○ Contaminated wash buffer | <ul style="list-style-type: none"> ○ Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. ○ Make fresh wash buffer |
| Low sensitivity | <ul style="list-style-type: none"> ○ Improper storage of the ELISA kit ○ Stop solution | <ul style="list-style-type: none"> ○ Follow storage recommendations in sections IV and V. Keep substrate solution protected from light. ○ Add stop solution to each well before reading plate |

RayBio[®] ELISA Kits

Over 3,000 ELISA kits available, visit www.RayBiotech.com/ELISA-Kits.html for details.

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