RayBio® Human/Mouse/Rat Pro-Neuropeptide Y/CPON Enzyme Immunoassay Kit

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

User Manual
Last revised August 7, 2017

Caution:
Extraordinarily useful information enclosed

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Please read the entire manual carefully before starting your experiment

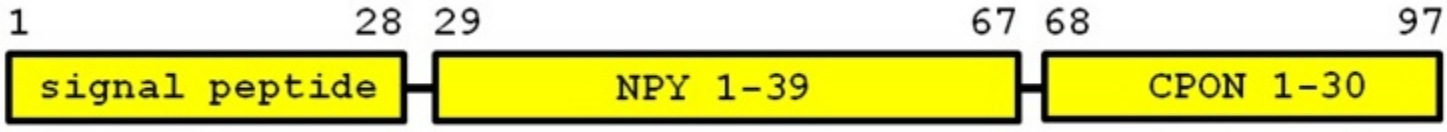
I. Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide hormone found in the neural system and has an important role in obesity.

The main effect of Neuropeptide Y is increased food intake and decreased physical activity. It also increases the proportion of energy stored as fat and blocks nociceptive signals to the brain. In addition to its role in obesity, Neuropeptide Y has been associated with a number of other physiologic processes in the brain, including the regulation of energy balance, memory and learning, and epilepsy.

Animal studies strongly demonstrate that the stimulation of Neuropeptide Yergic activity via the administration of certain Neuropeptide Y agonists increases food intake compared to control animals. The effects of Neuropeptide Yergic activity on food intake is also demonstrated by the blockade of certain Neuropeptide Y receptors (Y1 and Y5 receptors), which expectedly inhibited Neuropeptide Yergic activity; thus, decreases food intake. For its role in obesity, an increase in Neuropeptide Y is caused by high levels of glucocorticosteriods through directly activating type II glucocorticosteriods receptors and indirectly, by abolishing the negative feedback of CRF on Neuropeptide Y synthesis and release. Meanwhile, obesity-induced insulin resistance and the mutation of the leptin receptor (ObRb) results in the abolishment of other negative feedback mechanisms to regulate Neuropeptide Yergic activity and ultimately food intake. High levels of Neuropeptide Y were also found in the cerebrospinal fluid of patients with anorexia nervosa.

The 291 bp coding sequence of the NPY gene synthesizes the pre-pro form of NPY, a 97 aa precursor peptide (see diagram below). Cleavage of the n-terminal 29 aa results in pro-NPY (69 aa), which then undergoes a further cleavage, yielding NPY 1-39 along with the 30-aa C-terminal peptide known as C-flanking peptide of NPY (CPON). NPY 1-39 is further processed to the mature form (NPY 1-36). The function of CPON is not currently understood.

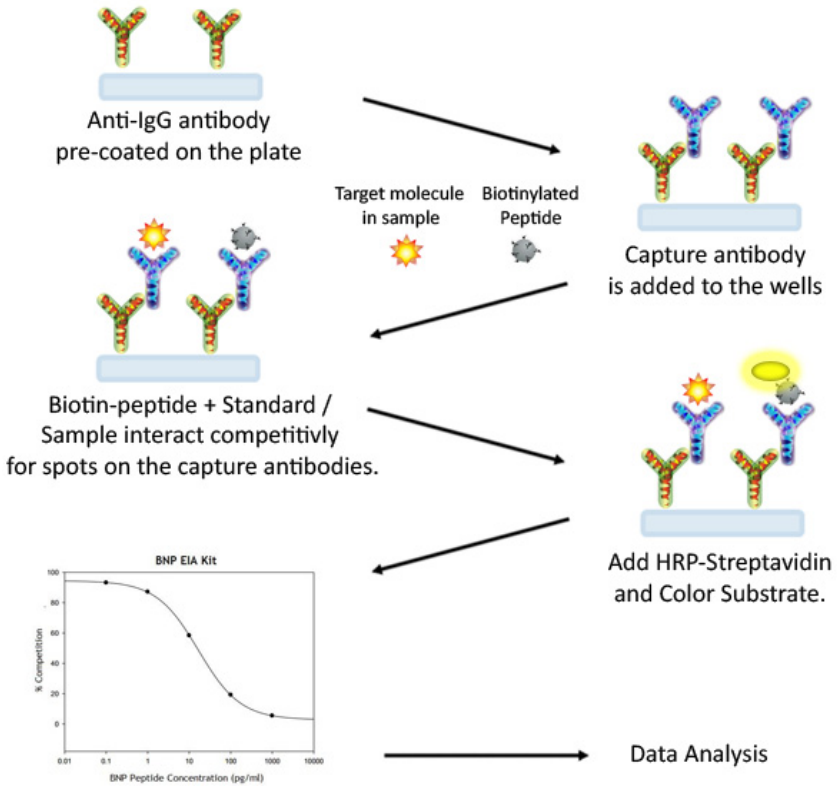


II. General Description

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

In this assay, a biotinylated ProNPY peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated ProNPY peptide competes with endogenous (unlabeled) ProNPY for binding to the anti-ProNPY antibody. After a wash step, any bound biotinylated ProNPY 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 ProNPY peptide and inversely proportional to the amount of endogenous ProNPY in the standard or samples. A standard curve of known concentration of ProNPY peptide can be established and the concentration of ProNPY 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 ProNPY Peptide (Item C)	2 vials of Lyophilized ProNPY Peptide. 1 vial is enough to run each standard in duplicate.	Do not store and reuse
Anti-ProNPY Polyclonal Antibody (Item N)	2 vials of Lyophilized anti-ProNPY.	Do not store and reuse
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 ProNPY Peptide (Item F)	2 vials of Lyophilized Biotinylated ProNPY Peptide, 1 vial is enough to assay half the plate.	Do not store and reuse
HRP-Streptavidin Concentrate (Item G)	600 µl 400X concentrated HRP-conjugated streptavidin.	Do not store and reuse
Positive Control (Item M)	1 vial of Lyophilized Positive Control.	Do not store and reuse
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 µl 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-ProNPY 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-ProNPY antibody vial (Item N) and reconsititute with 55 µl of 1X Assay Diluent B 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-ProNPY antibody working solution, which will be used in step 2 of Assay Procedure (Section VIII).

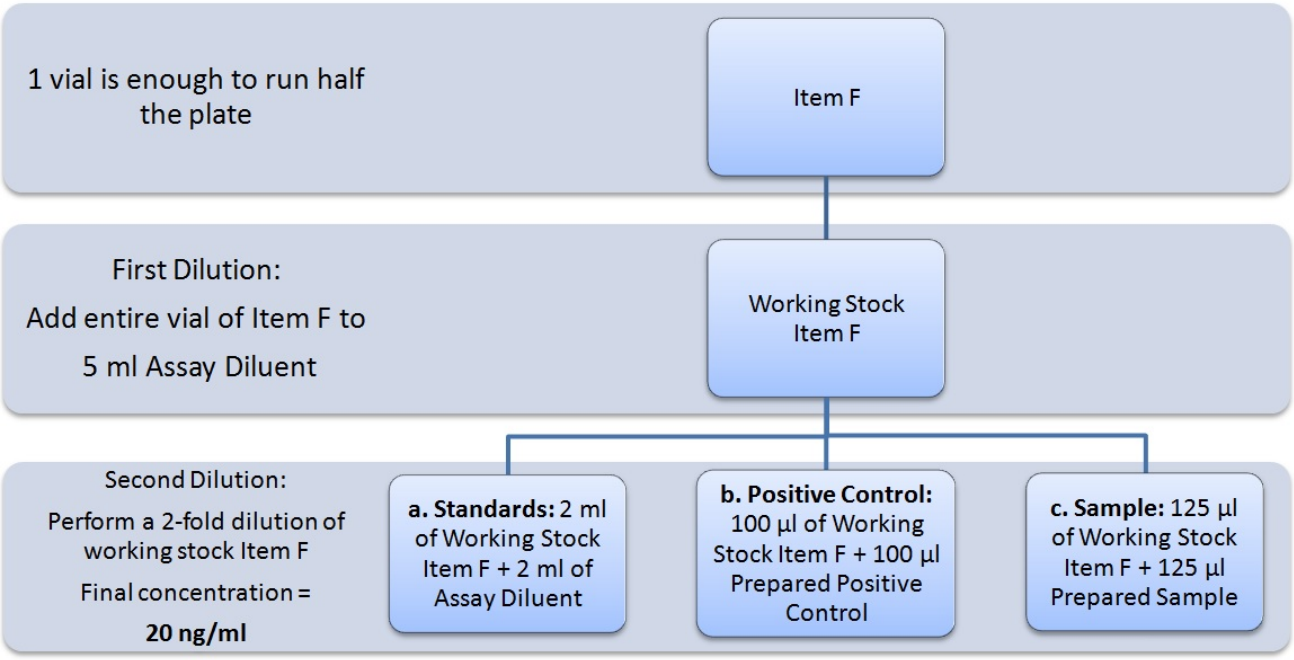
Note: The following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure)

B. Preparation of Biotinylated ProNPY (Item F)

6. Briefly centrifuge the vial of Biotinylated ProNPY (Item F) and reconstitute with 20 µl of ddH_2O 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 5 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 ProNPY will be **40 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 ProNPY will be **20 ng/ml**.
   b. Second Dilution of Item F for Positive Control: Add 100 µl of Working Stock Item F to 100 µl of the prepared Positive Control (Item M). (See section D for Positive Control preparation) The final concentration of biotinylated ProNPY will be **20 ng/ml**.
   c. Second Dilution of Item F for samples: Add 125 µl of Working Stock Item F to 125 µl of prepared sample (see section E for sample preparation). This is a 2-fold dilution of your sample. The final concentration of biotinylated ProNPY will be **20 ng/ml**.



C. Preparation of Standards

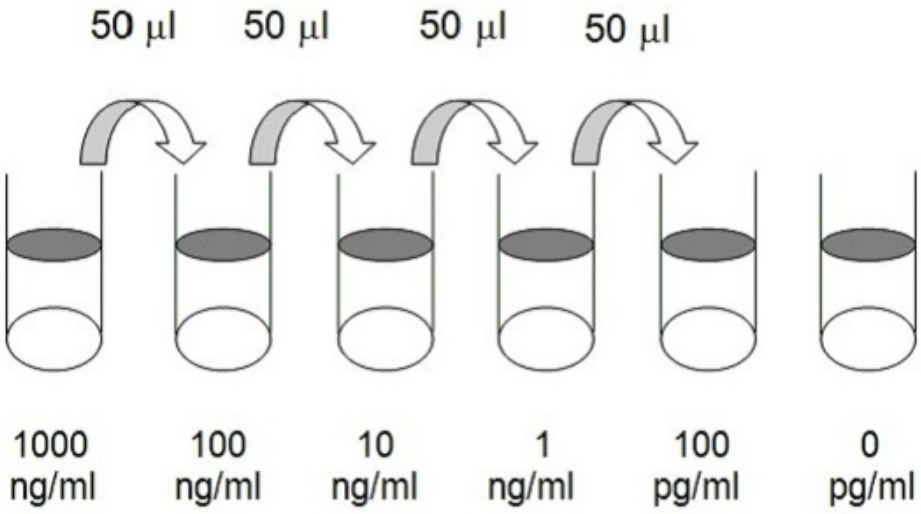
8. Label 6 microtubes with the following concentrations: 1,000 ng/ml, 100 ng/ml, 10ng/ml, 1 ng/ml, 100 pg/ml and 0 pg/ml. Pipette 450 µl of biotinylated ProNPY Item F working solution (prepared in step 7a) into each tube, except the 1,000 ng/ml (leave this one empty).

It is very important to make sure the concentration of biotinylated ProNPY is 20 ng/ml in all standards.

9. Briefly centrifuge the vial of ProNPY Standard (Item C). Reconstitute with 10 µl of ddH_2O and briefly vortex if desired. Pipette 8 µl of Item C and 792 µl of 20 ng/ml biotinylated ProNPY 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 ProNPY standard, 20 ng/ml biotinylated ProNPY).

10. To make the 100 ng/ml standard, pipette 50 µl of the 1000 ng/ml ProNPY standard into the tube labeled 100 ng/ml. Mix thoroughly.

11. Repeat this step with each successive concentration, preparing a dilution series as shown in the illustration below. Each time, use 450 µl of biotinylated ProNPY and 50 µl 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) and reconstitute with 100 µl of ddH_2O.

13. Refer to step 7b. This is a 2-fold dilution of the Positive Control. The final concentration of biotinylated ProNPY should still be 20 ng/ml.

The Positive Control is a cell culture media 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 ProNPY is 20 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 µl of sample + 62.5 µl of 1X Assay Diluent B.).
    b. Perform step 7c (125 µl of working solution Item F + 125 µl of sample prepared above).

    The total volume is 250 µl, enough for duplicate wells on the microplate.
    It is very important to make sure the final concentration of the biotinylated ProNPY is **20 ng/ml**.

    Note: Optimal sample dilution factors should be determined empirically, however you may contact technical support (888-494-8555; techsupport@raybiotech.com) to obtain recommended dilution factors for serum.

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 400-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 µl of Anti-ProNPY 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 4ºC.

3. Discard the solution and wash wells 4 times with 1X Wash Solution Buffer (200-300 µl 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 µl 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 4ºC.

5. Discard the solution and wash 4 times as directed in Step 3.

6. Add 100 µl 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 µl 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 µl 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 µl anti-ProNPY to each well. Incubate 1.5 hours at room temperature or overnight at 4ºC.

3. Add 100 µl standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4ºC.

4. Add 100 µl prepared Streptavidin solution. Incubate 45 minutes at room temperature.

5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.

6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

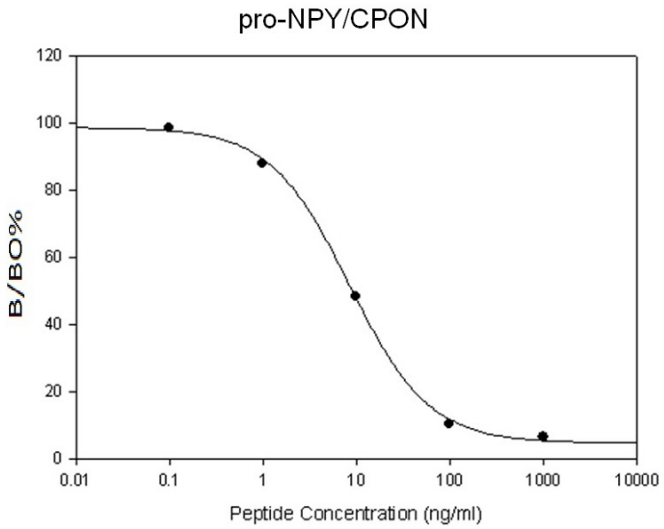
X. Calculation of Results

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-blank OD)/(B_0-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 ProNPY is 0.19 ng/ml or 16.59 pM.

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- ProNPY 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 kit was designed to recognize the C-terminus of pro-NPY and therefore will detect full-length pro-NPY and CPON. However, it does not recognize active forms including NPY 1-36, 2-36, 3-36 or 3-35.

This kit detects both CPON and the 69 aa pro-form of NPY.

Cross Reactivity: This EIA kit shows no cross-reactivity with any of the cytokines tested: Ghrelin, Nesfatin, Angiotensin II and APC.

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)
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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	○ Inaccurate pipetting ○ Improper standard dilution	○ Check pipettes ○ Briefly centrifuge Item C and dissolve the powder thoroughly by gently mixing
Low signal	○ Improper preparation of standard and/or biotinylated antibody ○ Too brief incubation times ○ Inadequate reagent volumes or improper dilution	○ 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	○ Inaccurate pipetting ○ Air bubbles in wells	○ Check pipettes ○ Remove bubbles in wells
High background	○ Plate is insufficiently washed ○ Contaminated wash buffer	○ Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. ○ Make fresh wash buffer
Low sensitivity	○ Improper storage of the ELISA kit ○ Stop solution	○ Follow storage recomendations in sections IV and V. Keep substrate solution protected from light. ○ Add stop solution to each well before reading plate

RayBio® ELISA Kits

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