

RayBio[®]
Human/Mouse/Rat Neuropeptide Y
Enzyme Immunoassay Kit

**Please Read the Manual Carefully
Before Starting your Experiment**

**User Manual 3.2
(Revised April 1, 2013)**

**RayBio[®] Neuropeptide Y
Enzyme Immunoassay Kit Protocol**

(Cat#: EIA-NPY-1)



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RayBiotech, Inc.

**RayBio® Human/Mouse/Rat Neuropeptide Y Enzyme
Immunoassay Kit Protocol**

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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.

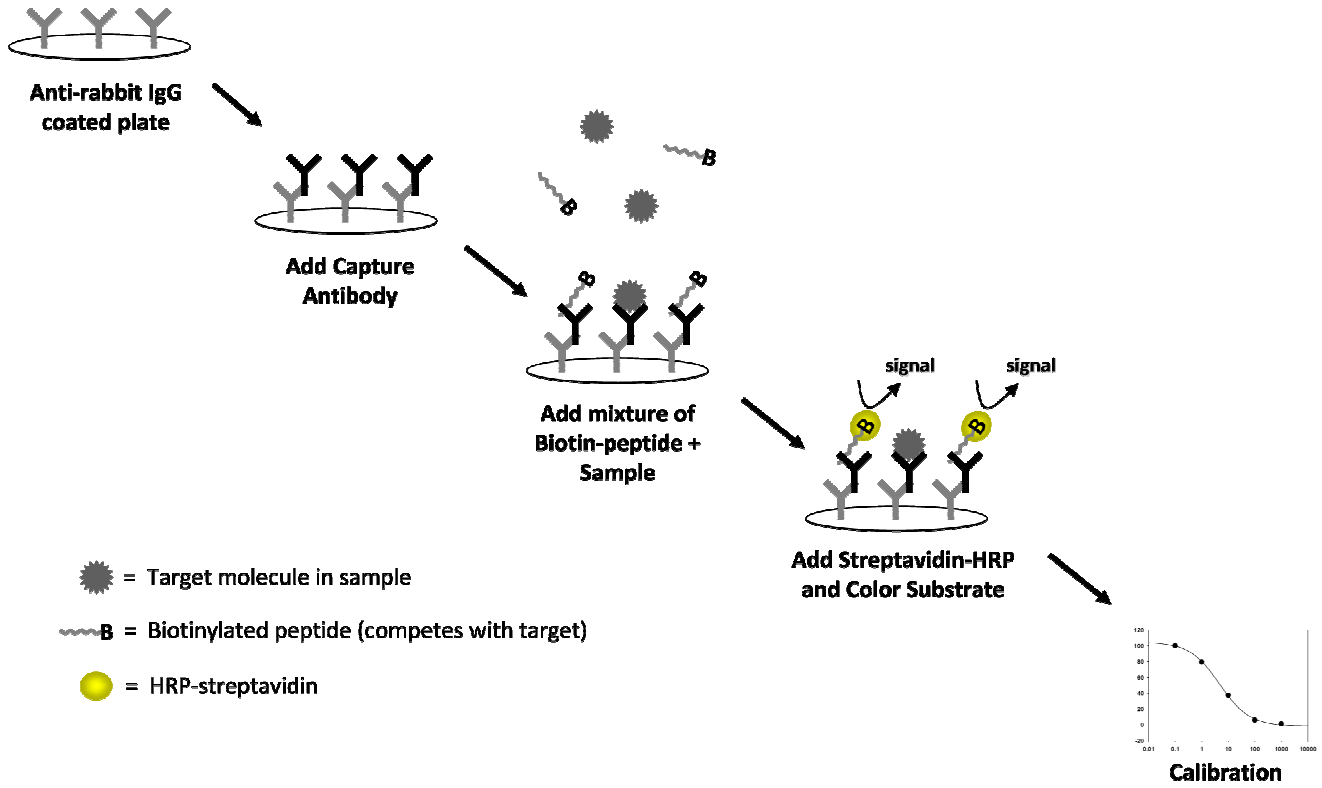
Animal studies strongly demonstrate that the stimulation of Neuropeptide Yergic activity via the administration of certain Neuropeptide Y agonists increases food intake compared to control animals. The effects of Neuropeptide Yergic activity on food intake is also demonstrated by the blockade of certain 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 glucocorticosteroids through directly activating type II glucocorticosteroids 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.

II. GENERAL DESCRIPTION

The RayBio® Neuropeptide Y Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Neuropeptide Y peptide based on the principle of Competitive Enzyme Immunoassay.

The microplate in the kit is pre-coated with anti-rabbit secondary antibody. After a blocking step and incubation of the plate with anti-Neuropeptide Y antibody, both biotinylated Neuropeptide Y peptide and peptide standard or targeted peptide in samples interacts competitively with the Neuropeptide Y antibody. Uncompeted (bound) biotinylated Neuropeptide Y peptide then interacts with Streptavidin-horseradish peroxidase (SA-HRP), which catalyzes a color development reaction. The intensity of colorimetric signal is directly proportional to the amount of biotinylated peptide-SA-HRP complex and inversely proportional to the amount of Neuropeptide Y peptide in the standard or samples. This is due to the competitive binding to Neuropeptide Y antibody between biotinylated Neuropeptide Y peptide and peptides in standard or samples. A standard curve of known concentration of Neuropeptide Y peptide can be established and the concentration of Neuropeptide Y peptide in the samples can be calculated accordingly.

Principle of Competitive EIA



III. REAGENTS

1. Neuropeptide Y Microplate (Item A): 96 wells (12 strips x 8 wells) coated with secondary antibody.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml.
3. Lyophilized standard Neuropeptide Y peptide (Item C): 2 vials.
4. Lyophilized anti-Neuropeptide Y polyclonal antibody (Item N): 2 vials.
5. 1X Assay Diluent E (Item R): 2 vials, 25 ml/vial. Diluent for both standards and samples including serum or plasma, cell culture media or other sample types.
6. Lyophilized biotinylated Neuropeptide Y peptide (Item F): 2 vials.
7. HRP-Streptavidin concentrate (Item G): 600 μ l 200x concentrated HRP-conjugated Streptavidin.
8. Lyophilized positive control (Item M): 1 vial.
9. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'- tetramethylbenzidine (TMB) in buffered solution.
10. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.
11. Assay Diagram (Item J).
12. User Manual (Item K).

IV. STORAGE

- Standard, Biotinylated Neuropeptide Y peptide, and Positive Control should be stored at -20°C after arrival. **Avoid multiple freeze-thaws.**
- The remaining kit components may be stored at 4°C.
- Opened Microplate Wells and antibody (Item N) may be stored for up to 1 month at 2° to 8°C. Return unused wells to the pouch containing desiccant pack and reseal along entire edge.
- If stored in this manner, RayBiotech warrants this kit for 6 months from the date of shipment.

V. ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450nm.
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. Saran Wrap

VI. REAGENT PREPARATION

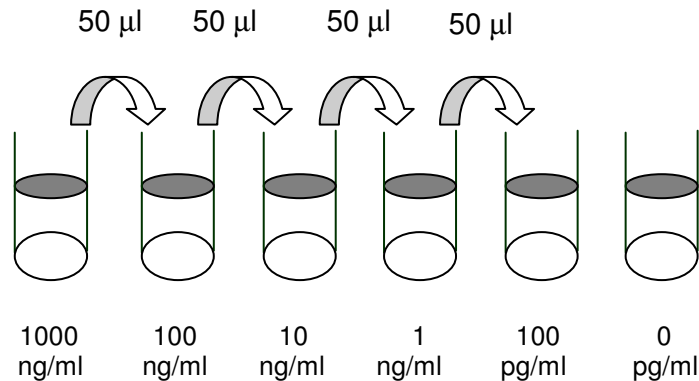
For sample and positive control dilutions, refer to steps 5, 6, 7 and 9 of Reagent Preparation.

1. Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.
2. Briefly centrifuge the NPY Antibody vial (Item N) and reconstitute with 5 μ l of ddH₂O before use. Add 50 μ l of 1x Assay Diluent E into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently.
3. The antibody concentrate should then be diluted 100-fold with 1x Assay Diluent E. This is your anti-Neuropeptide Y antibody working solution, which will be used in step 2 of the Assay Procedure.

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

4. Briefly centrifuge the vial of biotinylated Neuropeptide Y peptide (Item F) and reconstitute with 20 μ l of ddH₂O before use. Add 10 μ l of Item F to 5 m 1X Assay Diluent E. Pipette up and down to mix gently. *The final concentration of biotinylated Neuropeptide Y will be 20 ng/ml.* This solution will only be used as the diluent in step 5 of Reagent Preparation.
5. Preparation of Standards: Label 6 microtubes with the following concentrations: 1000 ng/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml and 0 pg/ml. Pipette 450 μ l of biotinylated Neuropeptide Y solution into each tube, except for the 1000 ng/ml (leave this one empty). *It is very important to make sure the concentration of biotinylated Neuropeptide Y is 20 ng/ml in all standards.*
 - a. Briefly centrifuge the vial of standard Neuropeptide Y peptide (Item C) and reconstitute with 10 μ l of ddH₂O. In the tube labeled 1000 ng/ml, pipette 8 μ l of Item C and 792 μ l of 20 ng/ml biotinylated Neuropeptide Y solution (prepared in step 4 above). This is your Neuropeptide Y stock solution (1000 ng/ml Neuropeptide Y, 20 ng/ml biotinylated Neuropeptide Y). Mix thoroughly. This solution serves as the first standard.
 - b. To make the 100 ng/ml standard, pipette 50 μ l of Neuropeptide Y stock solution the tube labeled 100 ng/ml. Mix thoroughly.
 - c. 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 Neuropeptide Y and 50 μ l of the prior concentration until 100 pg/ml is reached. Mix each tube thoroughly before the next transfer.

- d. The final tube (0 pg/ml Neuropeptide Y, 20 ng/ml biotinylated Neuropeptide Y) serves as the zero standard (or total binding).



6. Prepare a 10-fold dilution of Item F. To do this, add 2 µl of Item F to 18 µl of 1X Assay Diluent E. This solution will be used in steps 7 and 9.
7. Positive Control Preparation: Briefly centrifuge the positive control vial and reconstitute with 100 µl of ddH₂O before use (Item M). To the tube of Item M, add 101 µl 1x Assay Diluent E. Also add 4 µl of 10-fold diluted Item F (prepared in step 6) to the tube. This is a 2-fold dilution of the positive control. Mix thoroughly. The positive control is a cell culture medium sample with an expected signal between 10% and 30% of total binding (70-90% competition) if diluted as described above. It may be diluted further if desired, but be sure the final concentration of biotinylated Neuropeptide Y is 20 ng/ml.
8. If Item B (20X Wash Concentrate) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.

9. Sample Preparation: Use 1X Assay Diluent E + biotinylated NPY to dilute samples, including serum/plasma, cell culture medium and other sample types.

It is very important to make sure the final concentration of the biotinylated Neuropeptide Y is 20 ng/ml in every sample.
EXAMPLE: to make a 4-fold dilution of sample, mix together 5 μ l of 10-fold diluted Item F (prepared in step 6), 182.5 μ l of 1X Assay Diluent E, and 62.5 μ l of your sample; mix gently. The total volume is 250 μ l, enough for duplicate wells on the microplate.

Do not use Item F diluent from Step 5 for sample preparation. If you plan to use undiluted samples, you must still add biotinylated Neuropeptide Y to a final concentration of 20 ng/ml. EXAMPLE: Add 5 μ l of 10-fold diluted Item F to 245 μ l of sample. 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 ranges for serum or plasma.

10. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use. The HRP-Streptavidin concentrate should be diluted 200-fold with 1X Assay Diluent E.

VII. 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 anti-Neuropeptide Y antibody (see Reagent Preparation step 3) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycles/sec). You may also incubate overnight at 4 degrees C.

3. Discard the solution and wash wells 4 times with 1x Wash 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 step 5), positive control (see Reagent Preparation step 7) and sample (see Reagent Preparation step 9) into 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) or overnight 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 10) to each well. Incubate with gentle shaking for 45 minutes at room temperature. 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 absorbances at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.



2. Add 100 μl anti-Neuropeptide Y antibody to each well. Incubate 1.5 hours at room temperature or overnight at 4°C .



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



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

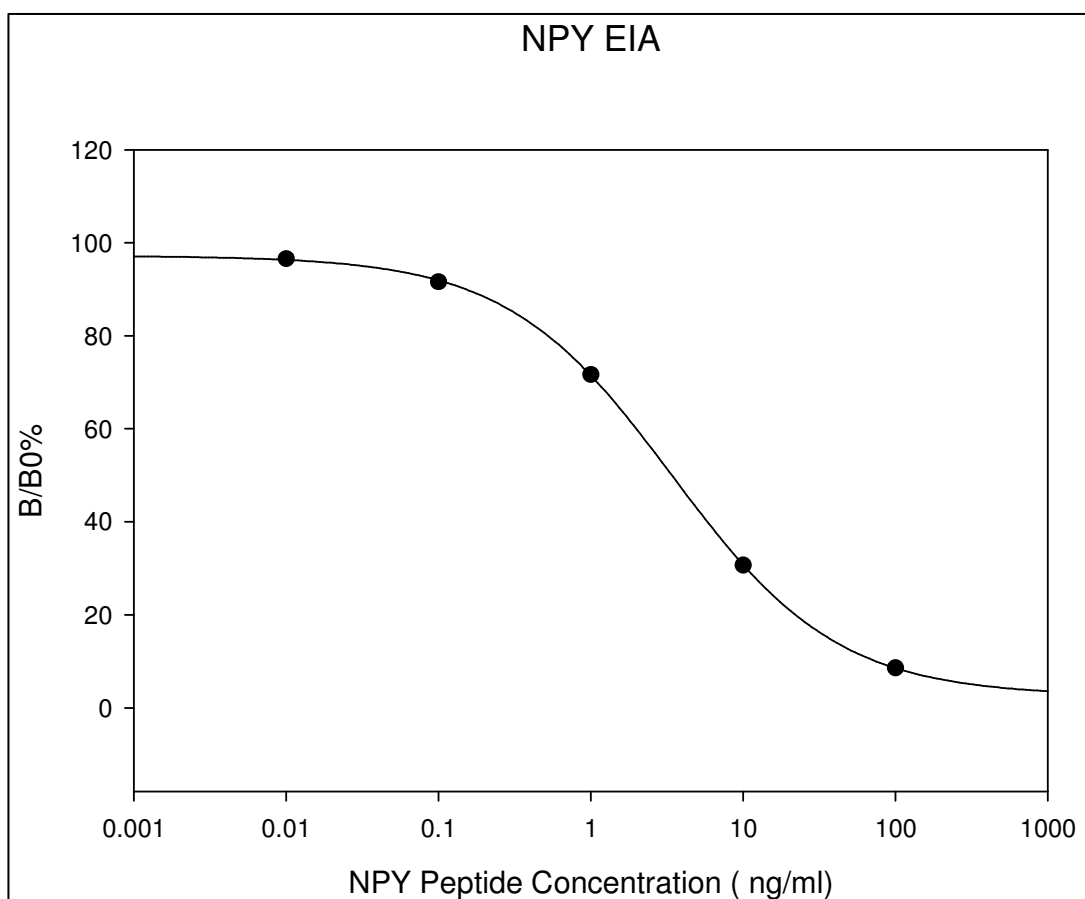
IX. CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, 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
B₀ = 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 concentration of Neuropeptide Y is 180 pg/ml or 16.59pM.

C. DETECTION RANGE

0.1-1,000 ng/ml

D. REPRODUCIBILITY

Intra-Assay: CV<10%

Inter-Assay: CV<15%

X. SPECIFICITY

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

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

XI. REFERENCES

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XII. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Poor standard curve	<ol style="list-style-type: none"> 1. Inaccurate pipetting 2. Improper standard dilution 	<ol style="list-style-type: none"> 1. Check pipettes 2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	<ol style="list-style-type: none"> 1. Too brief incubation times 2. Inadequate reagent volumes or improper dilution 	<ol style="list-style-type: none"> 1. Ensure sufficient incubation time; assay procedure step 2 change to over night 2. Check pipettes and ensure correct preparation
3. Large CV	<ol style="list-style-type: none"> 1. Inaccurate pipetting 	<ol style="list-style-type: none"> 1. Check pipettes
4. High background	<ol style="list-style-type: none"> 1. Plate is insufficiently washed 2. Contaminated wash buffer 	<ol style="list-style-type: none"> 1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed. 2. Make fresh wash buffer
5. Low sensitivity	<ol style="list-style-type: none"> 1. Improper storage of the EIA kit 2. Stop solution 	<ol style="list-style-type: none"> 1. Store your standard at $\leq -20^{\circ}\text{C}$ after receipt of the kit. 2. Stop solution should be added to each well before measure

RayBio® EIA kits:

If you are interested in other EIA kits, please visit www.raybiotech.com for details.

Notes:

This product is for research use only.



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