# RayBio® Human/Mouse/Rat Neuropeptide W-23 Enzyme Immunoassay Kit

Catalog #: EIA-NPW23, EIAM-NPW23, EIAR-NPW23

User Manual
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Caution:
Extraordinarily useful information enclosed


ISO 13485 Certified

3607 Parkway Lane, Suite 100
Norcross, GA 30092
Tel: 1-888-494-8555 (Toll Free) or 770-729-2992, Fax:770-206-2393
Web: www.RayBiotech.com, Email: info@raybiotech.com

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Please read the entire manual carefully before starting your experiment

# I. Introduction

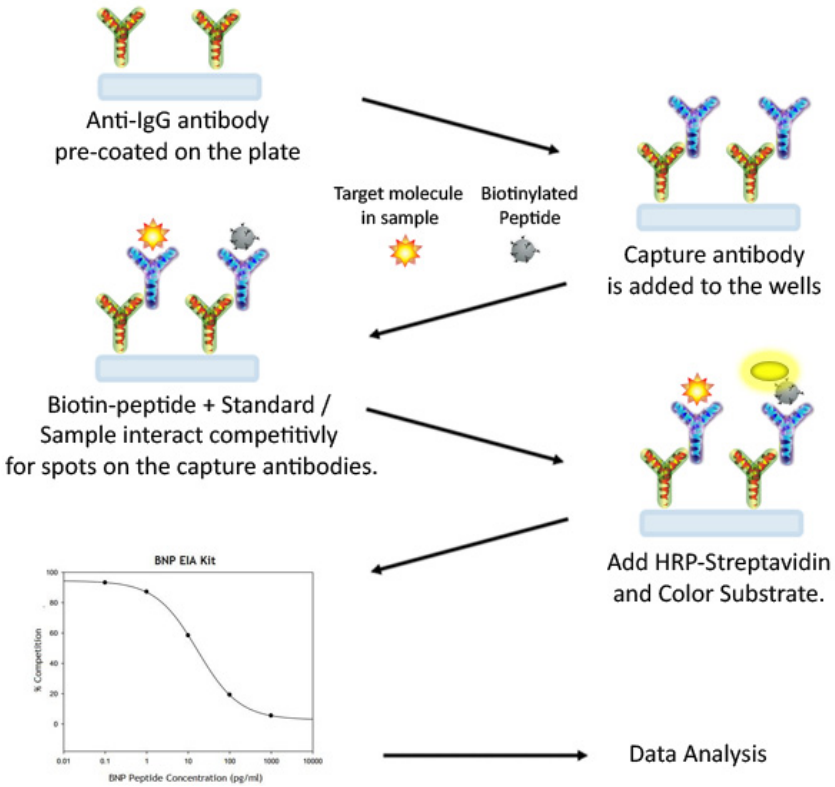
Neuropeptide W plays a regulatory role in the organization of neuroendocrine signals accessing the anterior pituitary gland. It stimulates water drinking and food intake. It may also play a role in the hypothalamic response to stress (By similarity). Neuropeptide W is cleaved into two forms: 23 amino acids (NPW23) and 30 amino acids (NPW30) neuropeptide. These neuropeptides bind to one of two NPW receptors, either NPBWR1 (otherwise known as GPR7) or NPBWR2 (GPR8), which belong to the G protein-coupled receptor family.

# II. General Description

The RayBio® NPW23 Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting NPW23 peptide based on the competitive enzyme immunoassay principle.

In this assay, a biotinylated NPW23 peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated NPW23 peptide competes with endogenous (unlabeled) NPW23 for binding to the anti-NPW23 antibody. After a wash step, any bound biotinylated NPW23 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 NPW23 peptide and inversely proportional to the amount of endogenous NPW23 in the standard or samples. A standard curve of known concentration of NPW23 peptide can be established and the concentration of NPW23 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 NPW23 Peptide (Item C) | 2 vials of Lyophilized NPW23 Peptide. 1 vial is enough to run each standard in duplicate. | Do not store and reuse |
| Anti-NPW23 Polyclonal Antibody (Item N) | 2 vials of Lyophilized anti-NPW23. | 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 NPW23 Peptide (Item F) | 2 vials of Lyophilized Biotinylated NPW23 Peptide, 1 vial is enough to assay the whole plate. | Do not store and reuse |
| HRP-Streptavidin Concentrate (Item G) | 600 µl 100X 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-NPW23 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-NPW23 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-NPW23 antibody working solution, which will be used in step 2 of Assay Procedure (Section VIII).

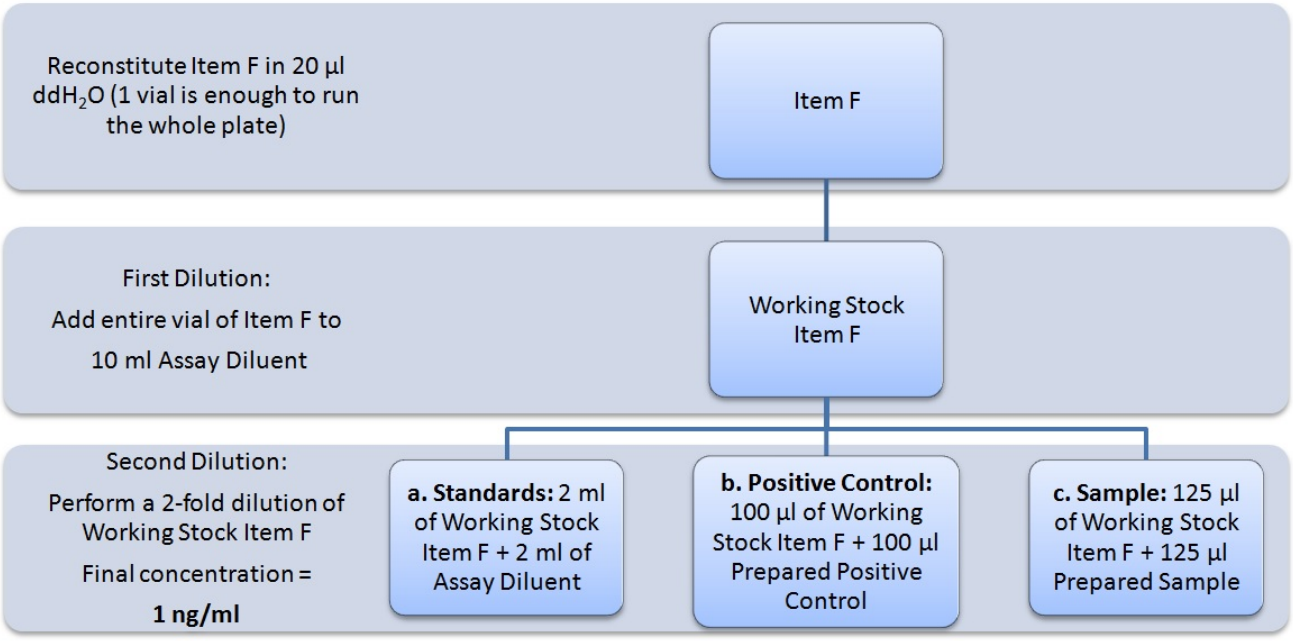
*Note: The following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure)*

## B. Preparation of Biotinylated NPW23 (Item F)

6. Briefly centrifuge the vial of Biotinylated NPW23 (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 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 NPW23 will be **2 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 NPW23 will be **1 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 NPW23 will be **1 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 NPW23 will be **1 ng/ml**.



## C. Preparation of Standards

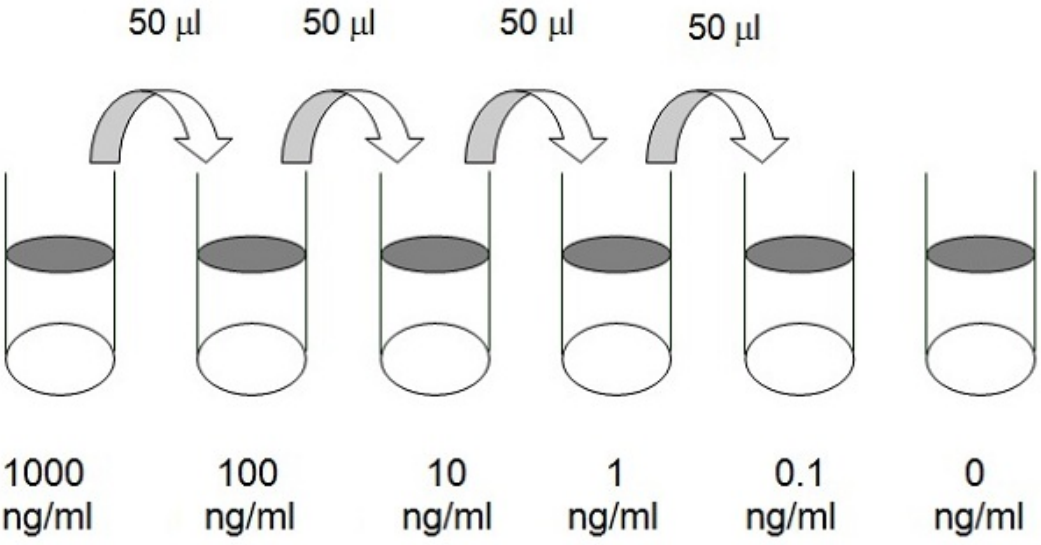
8. Label 6 microtubes with the following concentrations: 1,000 ng/ml, 100 ng/ml, 10ng/ml, 1 ng/ml, 100 pg/ml and 0 pg/ml. Pipette 450 µl of biotinylated NPW23 Item F working solution (prepared in step 7a) into each tube, except the 1,000 ng/ml (leave this one empty).

*It is very important to make sure the concentration of biotinylated NPW23 is 1 ng/ml in all standards.*

9. Briefly centrifuge the vial of NPW23 Standard (Item C). Reconstitute with 10 µl of dd$H_2O$ and briefly vortex if desired. Pipette 8 µl of Item C and 792 µl of 1 ng/ml biotinylated NPW23 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 NPW23 standard, 1 ng/ml biotinylated NPW23).

10. To make the 100 ng/ml standard, pipette 50 µl of the 1000 ng/ml NPW23 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 NPW23 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 dd$H_2O$.

13. Refer to step 7b. This is a 2-fold dilution of the Positive Control. The final concentration of biotinylated NPW23 should still be 1 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 NPW23 is 1 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 NPW23 is **1 ng/ml**.

    Note: Optimal sample dilution factors should be determined empirically, however you may reference below for recommended dilution factors for serum: Human=sample extraction Mouse=sample extraction Rat=sample extraction.
    If you have any questions regarding the recommendended 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 100-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-NPW23 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-NPW23 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.

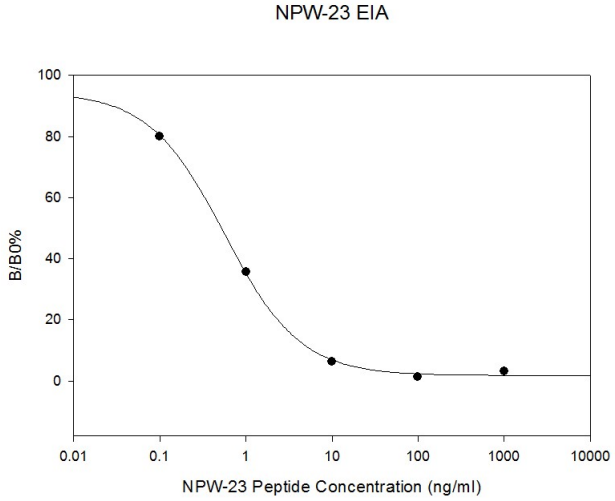
# X. Calculation of Results

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 NPW23 is 0.3 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- NPW23 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 NPW23

## 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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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.
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# XIII. Troubleshooting Guide

| Problem | Cause | Solution |
|---|---|---|
| Poor standard curve | ○ Inaccurate pipetting<br>○ Improper standard dilution | ○ Check pipettes<br>○ Briefly centrifuge Item C and dissolve the powder thoroughly by gently mixing |
| Low signal | ○ Improper preparation of standard and/or biotinylated antibody<br>○ Too brief incubation times<br>○ Inadequate reagent volumes or improper dilution | ○ Briefly spin down vials before opening. Dissolve the powder thoroughly.<br>○ Ensure sufficient incubation time; assay procedure step 2 may be done overnight<br>○ Check pipettes and ensure correct preparation |
| Large CV | ○ Inaccurate pipetting<br>○ Air bubbles in wells | ○ Check pipettes<br>○ Remove bubbles in wells |
| High background | ○ Plate is insufficiently washed<br>○ Contaminated wash buffer | ○ Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed.<br>○ Make fresh wash buffer |
| Low sensitivity | ○ Improper storage of the ELISA kit<br>○ Stop solution | ○ Follow storage recomendations in sections IV and V. Keep substrate solution protected from light.<br>○ Add stop solution to each well before reading plate |

# RayBio® ELISA Kits

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