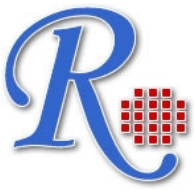
RayBio®
Human/Mouse/Rat Nesfatin Enzyme Immunoassay Kit

Please Read the Manual Carefully Before Starting your Experiment

User Manual 2.2
(Revised March 15, 2012)

RayBio® Nesfatin Enzyme Immunoassay Kit Protocol

(Cat#: EIA-NES-1)



RayBiotech, Inc.

We Provide You With Excellent Protein Array System and Service

Tel: (Toll Free) 1-888-494-8555 or 770-729-2992; Fax: 770-206-2393;
Web: www.raybiotech.com Email: info@raybiotech.com

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I. INTRODUCTION

Obesity, which is characterized by excessive accumulation of adipose tissue in the body, has become one of the greatest public health challenges. Obesity is not only associated with health problems linked to increased weight-dependent pressure overload on lung, joints and bones, but also a important risk factor for life-threatening diseases such as cardiovascular diseases, type 2 diabetes and certain cancers.

Nesfatin is a naturally occurring hormone produced by brain cells. Japanese scientists discovered in 2006 that it is responsible for regulating appetite and producing body fat. Excess Nesfatin in the brain leads to a loss of appetite, less frequent hunger, a 'sense of fullness', and a drop in body fat and weight. A lack of Nesfatin in the brain leads to an increase of appetite, more frequent episodes of hunger, an increase of body fat and weight, and the inability to 'feel full.' This latter condition can be artificially induced by injecting an anti-Nesfatin antibody into the brain.

The receptors of Nesfatin within the brain are not completely understood, although they are thought to be contained in the hypothalamus and in the solitary nucleus, where Nesfatin is believed to produced via peroxisome proliferator-activated receptors (PPARs).

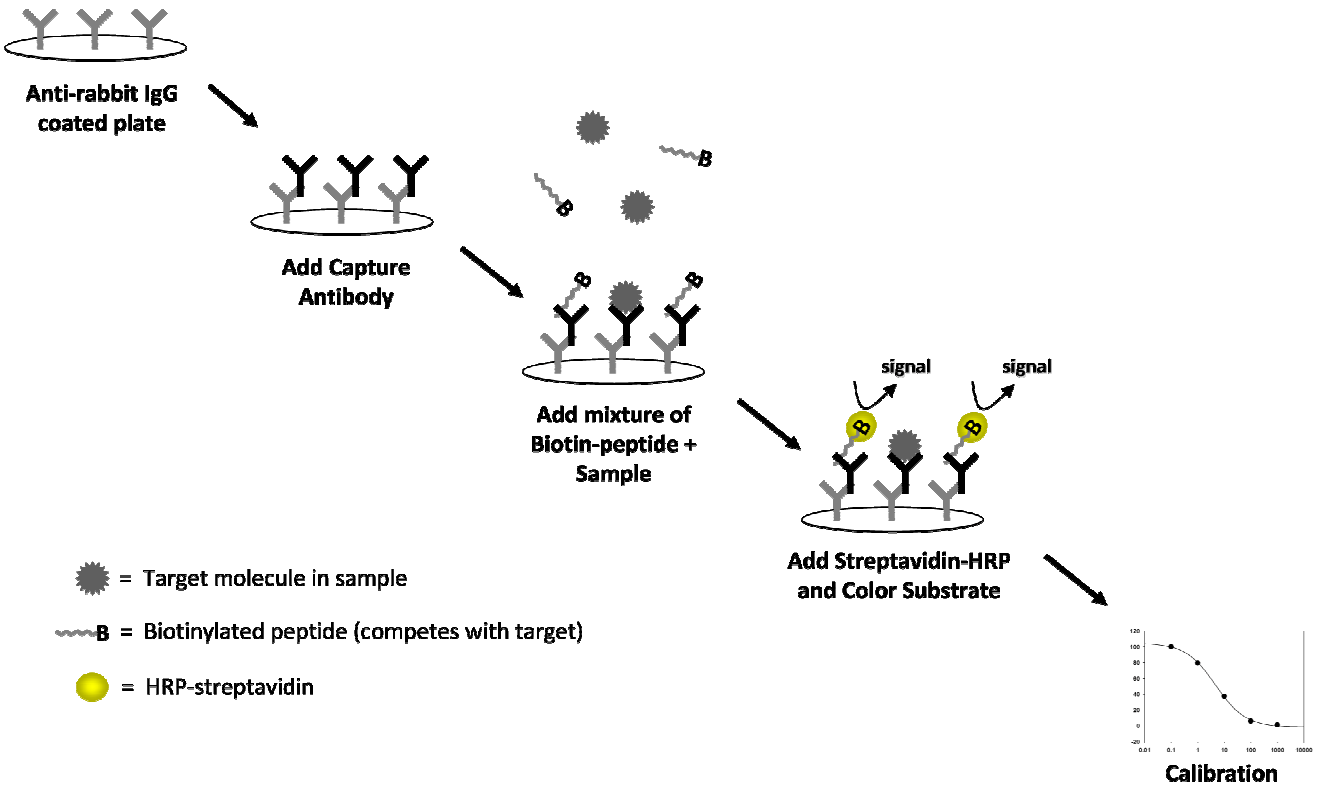
II. GENERAL DESCRIPTION

The RayBio® Nesfatin Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Nesfatin 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-Nesfatin antibody, both biotinylated Nesfatin peptide and peptide standard or targeted peptide in samples interacts competitively with the Nesfatin antibody. Uncompeted (bound) biotinylated Nesfatin 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 Nesfatin peptide in the standard or samples. This is due to the competitive binding to Nesfatin antibody between biotinylated Nesfatin peptide and peptides in standard or samples. A standard curve of known concentration of Nesfatin peptide can be established and the concentration of Nesfatin peptide in the samples can be calculated accordingly.

EIA-NES-1 detects 420aa Nesfatin protein. No other active isoforms have been reported.

Principle of Competitive EIA



III. REAGENTS

1. Nesfatin Microplate (Item A): 96 wells (12 strips x 8 wells) coated with secondary antibody.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml
3. Standard Nesfatin Peptide (Item C): 2 vials, 10 µl/vial
4. Anti-Nesfatin polyclonal antibody (Item N): 2 vials, 5 µl/vial
5. Assay Diluent A (Item D): 30 ml, contains 0.09% sodium azide as preservative. Diluent for standards and serum or plasma samples.
6. Assay Diluent B (Item E): 15 ml of 5x concentrated buffer. Diluent for standards and cell culture media or other sample types.
7. Biotinylated Nesfatin peptide, (Item F): 2 vials, 20 µl/vial
8. HRP-Streptavidin concentrate (Item G): 600 µl 200x concentrated HRP-conjugated Streptavidin.
9. Positive control (Item M): 1 vial, 100 µl
10. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'- tetramethylbenzidine (TMB) in buffered solution.
11. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.
12. Assay Diagram (Item J).
13. User Manual (Item K)

IV. STORAGE

- Standard, Biotinylated Nesfatin peptide, and Positive Control should be stored at -20°C or -80°C (recommended at -80°C) after arrival. **Avoid multiple freeze-thaws.**
- The remaining kit components may be stored at -20°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 warranties 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 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. Saran Wrap

VI. REAGENT PREPARATION

If testing plasma or serum samples, use Assay Diluent A to dilute Item F and Item C. If testing cell culture media or other sample types, use Assay Diluent B to dilute Item F and Item C. For sample and positive control dilutions, refer to steps 6, 7, 8 and 10 of Reagent Preparation.

1. Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.

2. Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.

3. Briefly centrifuge the Anti-Nesfatin Antibody vial (Item N) before use. Add 50 µl of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently.

4. The antibody concentrate should then be diluted 100-fold with 1x Assay Diluent B. This is your anti-Nesfatin antibody working solution, which will be used in step 2 of the Assay Procedure.

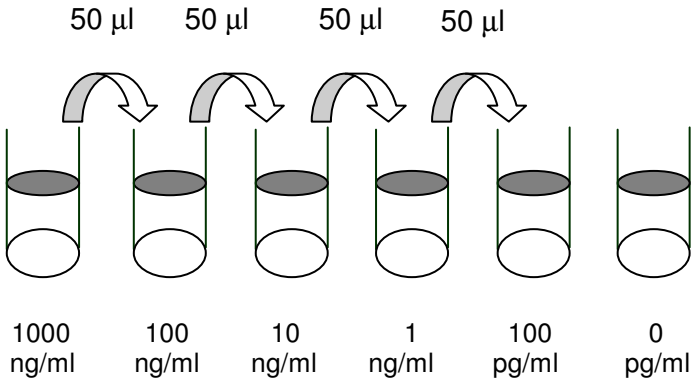
NOTE: the following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure).

5. Briefly centrifuge the vial of Biotinylated Nesfatin (Item F) before use. Add 5 µl of Item F to 5 ml of the appropriate Assay Diluent. Pipette up and down to mix gently. *The final concentration of biotinylated Nesfatin will be 10 ng/ml.* This solution will only be used as the diluent in step 6 of Reagent Preparation.

6. 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 µl of biotinylated Nesfatin 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 Nesfatin is 10 ng/ml in all standards.*

a. Briefly centrifuge the vial of Nesfatin (Item C). In the tube labeled 1000 ng/ml, pipette 8 µl of Item C and 792 µl of 10 ng/ml biotinylated Nesfatin solution (prepared in step 5 above). This is your Nesfatin stock solution (1000 ng/ml Nesfatin, 10 ng/ml biotinylated Nesfatin). Mix thoroughly. This solution serves as the first standard.
b. To make the 100 ng/ml standard, pipette 50 µl of Nesfatin 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 µl of biotinylated Nesfatin and 50 µl 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 Nesfatin, 10 ng/ml biotinylated Nesfatin) serves as the zero standard (or total binding).



7. Prepare a 10-fold dilution of Item F. To do this, add 2 µl of Item F to 18 µl of the appropriate Assay Diluent. This solution will be used in steps 8 and 10.

8. Positive Control Preparation: briefly centrifuge the positive control vial (Item M). To the tube of Item M, add 101 µl 1x Assay Diluent B. Also add 2 µl of 10-fold diluted Item F (prepared in step 7) 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 Nesfatin is 10 ng/ml.

9. 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.

10. Sample Preparation: Use Assay Diluent A + biotinylated Nesfatin to dilute serum/plasma samples. For cell culture medium and other sample types, use 1X Assay Diluent B + biotinylated Nesfatin as the diluent. *It is very important to make sure the final concentration of the biotinylated Nesfatin is 10 ng/ml in every sample.* EXAMPLE: to make a 4-fold dilution of sample, mix together 2.5 µl of 10-fold diluted Item F (prepared in step 7), 185 µl of appropriate Assay Diluent, and 62.5 µl of your sample; mix gently. The total volume is 250 µl, 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 Nesfatin to a final concentration of 10 ng/ml. EXAMPLE: Add 2.5 µl of 10-fold diluted Item F to 247.5 µl 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.

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

    Note: Do not use Assay Diluent A for HRP-Streptavidin preparation in Step 11.

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 µl anti-Nesfatin antibody (see Reagent Preparation step 4) 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 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 step 6), positive control (see Reagent Preparation step 8) and sample (see Reagent Preparation step 10) 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℃.

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 11) 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 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 absorbances at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

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

⇩

2. Add 100 µl anti-Nesfatin 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

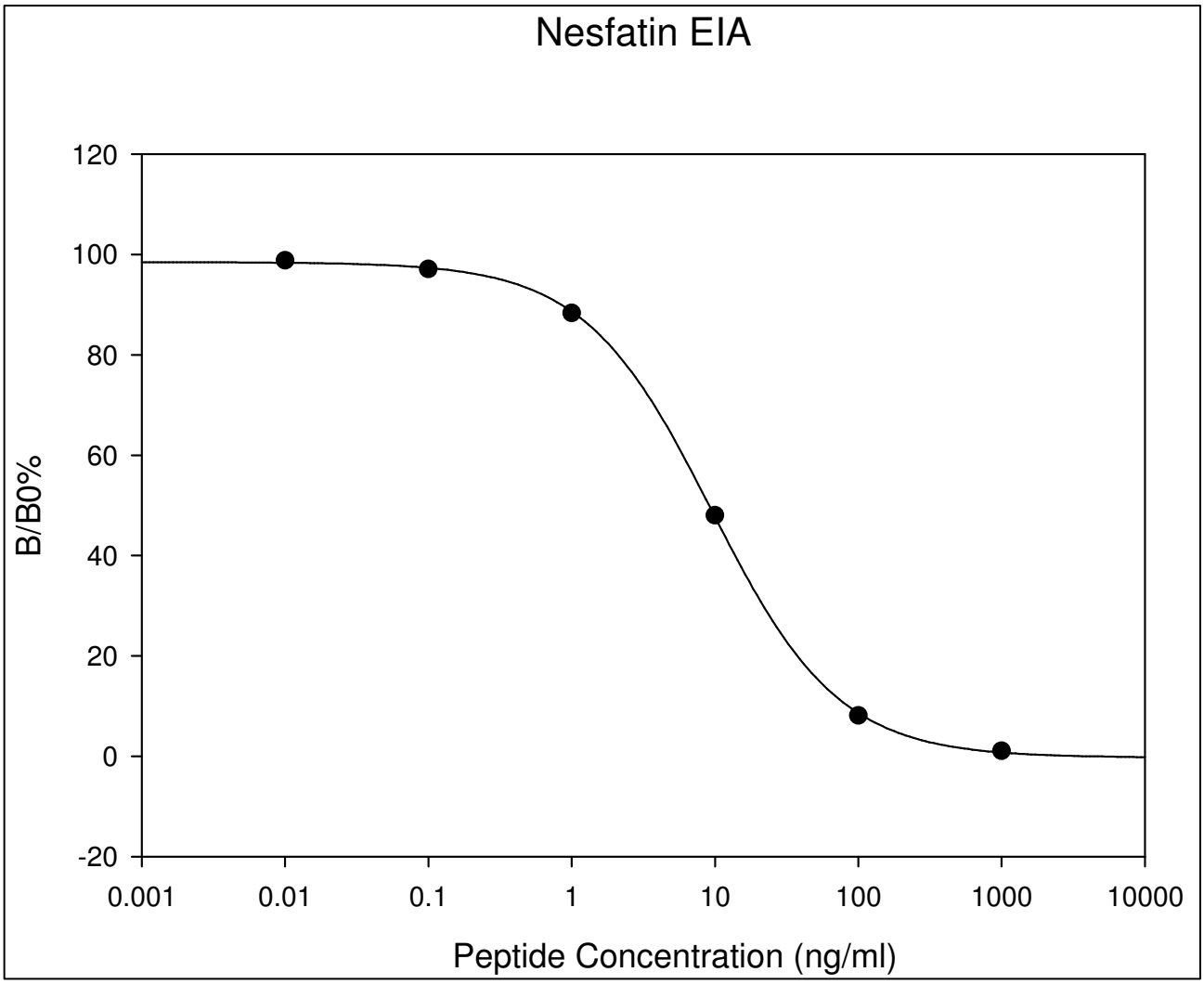
IX. CALCULATION OF RESULTS

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 straight line through the standard points.

Percentage absorbance = (B – blank OD)/(B_o – blank OD) where
B = OD of sample or standard and
B_o = 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 Nesfatin is 147 pg/ml or 3.09pM.

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, Angiotensin II, NPY and APC.

XI. REFERENCES

Oh-I S, Shimizu H, Satoh T, *et al* (2006). "Identification of nesfatin-1 as a satiety molecule in the hypothalamus". *Nature* **443** (7112): 709–12.

XII. TROUBLESHOOTING GUIDE

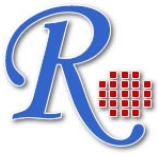
Problem	Cause	Solution
1. Poor standard curve	1. Inaccurate pipetting	1. Check pipettes
	2. Improper standard dilution	2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	1.Too brief incubation times	1. Ensure sufficient incubation time; assay procedure step 2 change to over night
	2. Inadequate reagent volumes or improper dilution	2. Check pipettes and ensure correct preparation
3. Large CV	1. Inaccurate pipetting	1. Check pipettes
4. High background	1. Plate is insufficiently washed	1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	2. Contaminated wash buffer	2. Make fresh wash buffer
5. Low sensitivity	1. Improper storage of the EIA kit	1. Store your standard at ≤ -20°C after receipt of the kit.
	2. Stop solution	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.



3607 Parkway Lane, Suite 200
Norcross, GA 30092
Tel: 770-729-2992, 1-888-494-8555
Fax: 770-206-2393
Web: www.raybiotech.com