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3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555

ISO 13485:2016

Neuromedin-U ELISA Kit

Catalog Number: EIA-NMU
User Manual
Last Revised: February 12, 2024

Please read entire manual carefully before starting experiment.

Introduction

The RayBio® Neuromedin-U Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Neuromedin-U peptide based on the competitive ELISA principle.

In this assay, a biotinylated Neuromedin-U peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated Neuromedin-U peptide competes with endogenous (unlabeled) Neuromedin-U for binding to the anti-Neuromedin-U antibody. After a wash step, any bound biotinylated Neuromedin-U 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 Neuromedin-U peptide and inversely proportional to the amount of endogenous Neuromedin-U in the standard or samples. A standard curve of known concentration of Neuromedin-U peptide can be established and the concentration of Neuromedin-U peptide in the samples can be calculated accordingly.

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 the 'Reagents' table on the next page.

Reagents

Component	Size / Description	Storage / Stability After Preparation
Microplate	96 wells (12 strips x 8 wells) coated with secondary antibody.	1 month at 4°C*
Wash Buffer	25 ml of 20X concentrated solution.	1 month at 4°C
Standard Neuromedin-U Peptide	2 vials of Lyophilized Neuromedin-U Peptide. 1 vial is enough to run each standard in duplicate.	Do not store and reuse
Anti-Neuromedin-U Polyclonal Antibody	2 vials of Lyophilized anti-Neuromedin-U.	Do not store and reuse
Assay Diluent G	30 ml of 1X concentrated buffer. Diluent for standards and samples.	1 month at 4°C
Assay Diluent B	15 ml of 5X concentrated buffer. Diluent for anti-Neuromedin-U antibody and HRP-Streptavidin.	1 month at 4°C
Biotinylated Neuromedin-U Peptide	2 vials of Lyophilized Biotinylated Neuromedin-U Peptide, 1 vial is enough to assay the whole plate.	Do not store and reuse
HRP-Streptavidin	600 µl 200x concentrated HRP-conjugated streptavidin.	Do not store and reuse
Positive Control	1 vial of Lyophilized Positive Control.	Do not store and reuse
TMB One-Step Substrate Reagent	12 ml of 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution.	N/A
Stop Solution	8 ml of 0.2 M sulfuric acid.	N/A

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

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

Reagent Preparation

Keep kit reagents on ice during reagent preparation steps.

Note: **Assay Diluent G** should be used for dilution of samples, biotinylated peptide, and standard peptide when testing **plasma or serum samples**. **1X Assay Diluent B** should be used for dilution of samples, biotinylated peptide, and standard peptide when testing **cell culture media or other sample types**.

A. Preparation of Plate and Anti-Neuromedin-U 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 should be diluted 5-fold with deionized or distilled water.
4. Briefly centrifuge the anti-Neuromedin-U antibody vial 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-Neuromedin-U antibody working solution, which will be used in step 2 of Assay Procedure.

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

B. Preparation of Biotinylated Neuromedin-U Peptide

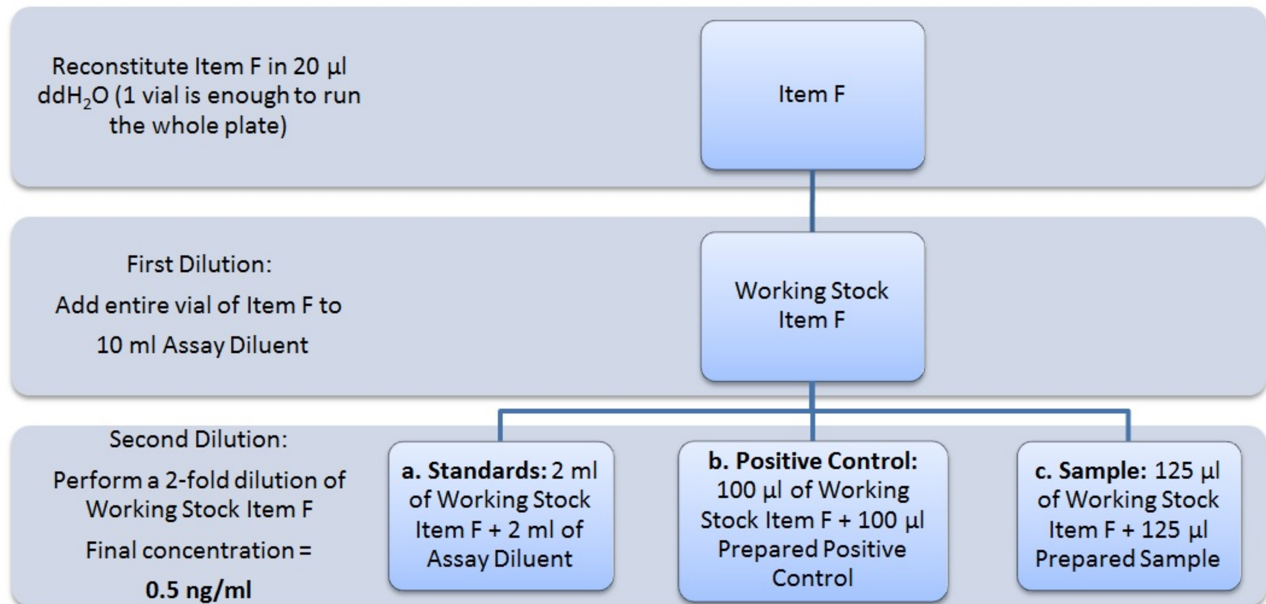
6. Briefly centrifuge the vial of Biotinylated Neuromedin-U Peptide and reconstitute with 20 µl of ddH_2O before use.
7. See the image below for proper preparation of the Biotinylated Neuromedin-U Peptide. Transfer the entire contents of the biotinylated peptide vial into a tube containing 10 ml of the appropriate Assay Diluent. This is your working stock. Pipette up and down to mix gently. *The final concentration of biotinylated Neuromedin-U will be **1 ng/ml.***

   a. Second Dilution of Biotinylated Neuromedin-U Peptide for Standards: Add 2 ml of working stock biotinylated peptide to 2 ml of the appropriate Assay Diluent. The final concentration of biotinylated Neuromedin-U will be **0.5 ng/ml.**

   b. Second Dilution of Biotinylated Neuromedin-U Peptide for Positive Control: Add 100 µl of working stock biotinylated peptide to 100 µl of the prepared Positive Control. (See section D for Positive Control preparation) The final concentration of biotinylated Neuromedin-U will be **0.5 ng/ml.**

   c. Second Dilution of Biotinylated Neuromedin-U Peptide for samples: Add 125 µl of working stock biotinylated peptide 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 Neuromedin-U will be **0.5 ng/ml**.

"Item F" = Biotinylated Neuromedin-U Peptide



C. Preparation of Standards

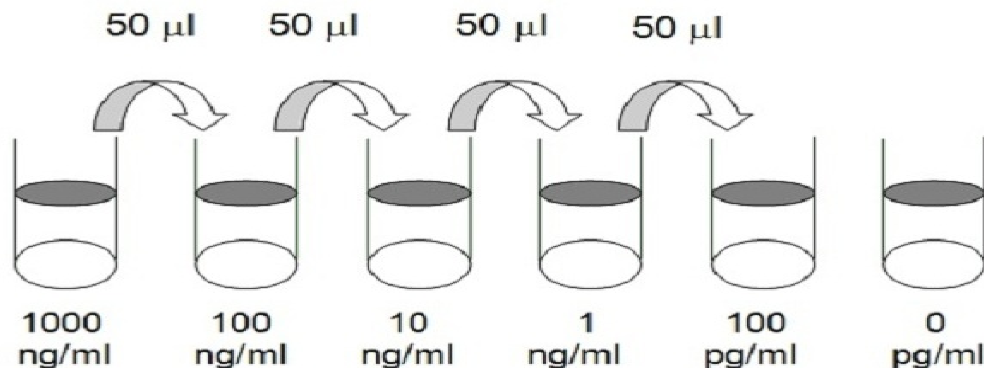
8. Label 6 microtubes with the following concentrations: 1,000 ng/ml, 100 ng/ml, 10ng/ml, 1 ng/ml, 100 pg/ml and 0 pg/ml. Pipette 450 µl of biotinylated Neuromedin-U peptide working solution (prepared in step 7a) into each tube, except the 1,000 ng/ml (leave this one empty).

It is very important to make sure the concentration of biotinylated Neuromedin-U is 0.5 ng/ml in all standards.

9. Briefly centrifuge the vial of Neuromedin-U Standard. Reconstitute with 10 µl of ddH_2O and briefly vortex if desired. Pipette 8 µl of Standard Peptide and 792 µl of 0.5 ng/ml biotinylated Neuromedin-U 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 Neuromedin-U standard, 0.5 ng/ml biotinylated Neuromedin-U).

10. To make the 100 ng/ml standard, pipette 50 µl of the 1000 ng/ml Neuromedin-U 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 Neuromedin-U 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 and reconstitute with 100 µl of ddH $_2$O.

13. Refer to step 7b. This is a 2-fold dilution of the Positive Control. The final concentration of biotinylated Neuromedin-U should still be 0.5 ng/ml.

The Positive Control is a mouse serum sample 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 Neuromedin-U is 0.5 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 the appropriate Assay Diluent 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 the appropriate Assay Diluent.).
    b. Perform step 7c (125 µl of working solution Biotinylated Neuromedin-U Peptide + 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 Neuromedin-U is **0.5 ng/ml**.

Note: Optimal sample dilution factors should be determined empirically, however you may reference the following for recommended dilution factors for serum: Human=16x Mouse=16x Rat=8x .
If you have any questions regarding the recommendended dilutions you may contact technical support at 770-729-2992 or techsupport@raybiotech.com.

F. Preparation of Wash Buffer and HRP

15. If the Wash Buffer 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 before use.

18. Dilute the HRP-Streptavidin concentrate 200-fold with 1X Assay Diluent B.

*Note: do **not** use Assay Diluent G for HRP-Streptavidin preparation in step 18*

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-Neuromedin-U Antibody (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 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 4°C.

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 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 to each well. Read at 450 nm immediately.

Assay Procedure Summary

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

2. Add 100 µl anti-Neuromedin-U 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.

Calculation of Results

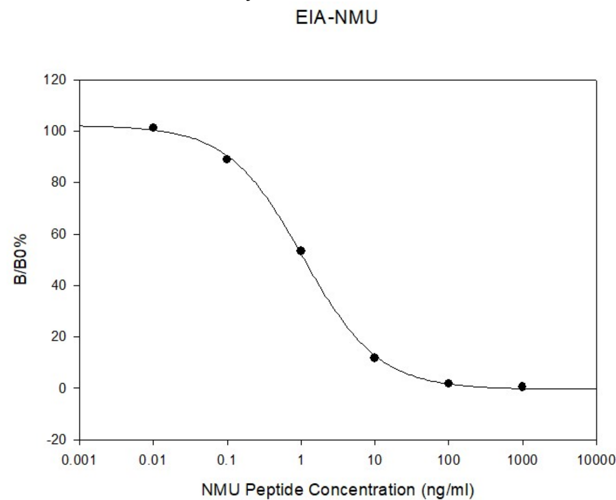
Calculate the mean absorbance for each set of duplicate stands, controls, and samples and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and 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 Neuromedin-U is 0.2 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- Neuromedin-U 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 Positive Control

Specificity

This EIA kit is designed to detect human, mouse, and rat Neuromedin-U

Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	• Inaccurate pipetting • Improper standard dilution	• Check pipettes • Briefly centrifuge Standard Neuromedin-U Peptide 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 manual. Keep substrate solution protected from light. • Add stop solution to each well before reading plate