Nesfatin-1 (1-82)/NUCB-2 (Human) ELISA KIT
Assay Protocol
(Catalog No. EK-003-26)
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CAUTION:

Investigational device. Limited by law to investigational use. For research use only. Not for use in diagnostic procedures.
**INTRODUCTION**

NUCB-2 corresponds to Nesfatin-1, a secreted protein, which is expressed in the appetite-control hypothalamic nuclei. Intracerebrotricular injection of Nesfatin-1 (1-82) reduces food intake, and chronic injection of Human Nesfatin-1 reduces body weight. Also, Nesfatin-1 is a satiety molecule that is associated with melanocortin signaling in the hypothalamus.

Phoenix Pharmaceutical’s Human Nesfatin-1 (1-82) ELISA Kit is designed to measure the concentration of Human Nesfatin-1 (1-82) from human serum/plasma, or conditioned medium.
PROTOCOL OVERVIEW

The immunoplate in this kit is precoated with Anti-Human Nesfatin-1 (1-82) Capture Antibody and the non-specific binding sites are blocked. The Human Nesfatin-1 (1-82) in the sample or in the standard solution can bind to the capture antibody immobilized in the wells. After washing procedure, the Biotinylated Anti-Human Nesfatin-1 (1-82) Detection Antibody can bind to the Human Nesfatin-1 (1-82) trapped in the wells is added. After washing, the Streptavidin-Horseradish Peroxidase (SA-HRP) which catalyzes the substrate solution (TMB) is added. The enzyme-substrate reaction is terminated by the addition of a stop solution. The intensity of the color is directly proportional to the amount of Human Nesfatin-1 (1-82) in the standard solutions or samples. A standard curve of Human Nesfatin-1 (1-82) with known concentration can be established accordingly. The Human Nesfatin-1 (1-82) with unknown concentration in samples can be determined by extrapolation to this standard curve.

ASSAY CONDITIONS

Plasma, serum, culture media, tissue homogenate, CSF, urine or any biological fluid can be assayed as long as the level of the sample is high enough for the sensitivity of the kit to detect it.

Phoenix Pharmaceuticals guarantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for its particular use and establish optimum sample concentrations.
**ASSAY PRINCIPLE**

Human Nesfatin-1 (1-82) Standards or samples

- Anti-Nesfatin-1 (1-82) (H) capture antibody
- Biotinylated anti-Nesfatin-1 (1-82) detection antibody
- Streptavidin-HRP
- Color Development

**Substrate (TMB)**
LIST OF COMPONENTS

Store all components at 4°C. DO NOT FREEZE.

1. 20x Assay Buffer Concentrate (50ml).................Catalog No. EK-BUF
2. 96 Well anti-Nesfatin-1 (1-82) ..........Catalog No. EK-Plate-003-26
Capture Antibody-Coated Plate (1 plate)
3. Human Nesfatin-1 (1-82) Standard..........Catalog No. EK-S-003-26
   (500ng/vial)
   Detection Antibody (1 vial)
5. Human Nesfatin-1 (1-82) Positive Control.Catalog No. EK-PC-03-26
   (2 vials)
6. Streptavidin-Horseradish......................... Catalog No. EK-SA-HRP
   Peroxidase (SA-HRP) (30μl)
7. Substrate Solution (TMB) (12ml)....................Catalog No. EK-SS
8. Stop Solution 2N HCl (15ml) ......................Catalog No. EK-HCL
9. Acetate Plate Sealer (APS) (3 pieces)..............Catalog No. EK-APS
10. Assay Diagram (1 sheet)

MATERIALS REQUIRED BUT NOT SUPPLIED

- Micropipettor(s) and disposable pipette tips
- Multi-channel pipette capable of dispensing 50-100μl
- Solution Reservoir (recommended)
- Microtiter plate washer (recommended)
- Orbital plate shaker capable of 300-500 rpm (recommended)
- Microtiter plate reader capable of absorbance measurement 450nm
- Well-closed containers (15ml tubes or more in capacity)
- Absorbent material for blotting
**REAGENT PREPARATION**

Note: The kit should be equilibrated to room temperature (20-23°C) before opening any vials and starting the assay. It is highly recommended that the solutions be used as soon as possible after rehydration.

1. **1x Assay Buffer:** Dilute the 20x Assay Buffer Concentrate with 950ml of distilled water. This assay buffer will be used to wash the plate and reconstitute all of the other components in this kit. If crystals are observed in the 20x Assay Buffer warm the bottle in a 37°C water bath for approximately 30 minutes or until the crystals disappear. After preparation, store 1x Assay Buffer at 4°C.

2. **Biotinylated anti-Human Nesfatin-1 (1-82) Detection Antibody:** Rehydrate Biotinylated anti-Human Nesfatin-1 (1-82) Detection Antibody with 100µl of 1x Assay Buffer, vortex (centrifuge the tube to dislodge powder from the cap or walls). Dilute Biotinylated anti-Human Nesfatin-1 (1-82) Detection Antibody to 1:2000 and mix thoroughly before use.

3. **Streptavidin-Horseradish Peroxidase (SA-HRP):** Centrifuge the SA-HRP vial (30µl) provided in this kit (3,000-5,000 rpm, 5 seconds) and dilute SA-HRP with 1x Assay Buffer to 1:2000 before use. Vortex thoroughly.

4. **Human Nesfatin-1 (1-82) Positive Control:** Rehydrate Human Nesfatin-1 (1-82) Positive Control with 250µl of 1x Assay Buffer (centrifuge the tube to dislodge powder from cap or walls). Vortex thoroughly.

**HUMAN NESFATIN-1 (1-82) STANDARD PREPARATION**

1. Rehydrate recombinant Human Nesfatin-1 (1-82) Standard with 1ml 1x Assay Buffer, vortex. Allow the solution to sit at least 10 minutes at room temperature (20-23°C) to completely dissolve in solution. Vortex and centrifuge before use. The concentration of this stock solution is 500ng/ml.
2. Prepare Standard solutions as follows:

<table>
<thead>
<tr>
<th>Standard No.</th>
<th>Standard Protein Volume</th>
<th>Assay Buffer Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock</td>
<td>Powder</td>
<td>1000 μl</td>
<td>500ng/ml</td>
</tr>
<tr>
<td>#1</td>
<td>100 μl of Stock</td>
<td>900 μl</td>
<td>50ng/ml</td>
</tr>
<tr>
<td>#2</td>
<td>500 μl of #1</td>
<td>500 μl</td>
<td>25ng/ml</td>
</tr>
<tr>
<td>#3</td>
<td>500 μl of #2</td>
<td>500 μl</td>
<td>12.5ng/ml</td>
</tr>
<tr>
<td>#4</td>
<td>500 μl of #3</td>
<td>500 μl</td>
<td>6.25ng/ml</td>
</tr>
<tr>
<td>#5</td>
<td>500 μl of #4</td>
<td>500 μl</td>
<td>3.12ng/ml</td>
</tr>
<tr>
<td>#6</td>
<td>500 μl of #5</td>
<td>500 μl</td>
<td>1.56ng/ml</td>
</tr>
<tr>
<td>#7</td>
<td>500 μl of #6</td>
<td>500 μl</td>
<td>0.78ng/ml</td>
</tr>
</tbody>
</table>

Table of Standard Dilutions

Blank 100μl
50 ng/ml 500μl
25 ng/ml 500 μl
12.5 ng/ml 500 μl
6.25 ng/ml 500 μl
3.12 ng/ml 500 μl
1.56 ng/ml 500 μl
0.78 ng/ml 500 μl

Immunoplate loading map

Visual Guide of the Standard Dilutions
HUMAN NESTFATIN-1 (1-82) ELISA PROTOCOL

1. Thoroughly read this protocol before performing an assay. Allow all reagents to come to room temperature (20-23°C) prior to the start of the assay.

2. Remove Capture Antibody-Coated Plate from its zip-lock foil pouch. Remove unneeded strips from the plate frame, reseal them in the foil pouch, and return the foil pouch to 4°C.

3. Leave wells A-1 and A-2 empty as Blank.

4. Add 100μl of the prepared Human Nesfatin-1(1-82) Standard solutions from #7 to #1 (reverse order of serial dilution) in duplicate to each well.

5. Add 100μl of Human Nesfatin-1(1-82) Positive Control solution in duplicate.

6. Add 100μl diluted samples in duplicate into their designated wells.

7. Seal the immunoplate with Acetate Plate Sealer (APS). Incubate for 2 hours at room temperature (20-23°C) on a plate shaker (300-400 rpm).

8. Before washing the plate, remove the plate sealer carefully. Completely discard the liquid from wells. Wash each well with 300-350μl assay buffer four times. At the end of each wash, discard the buffer, invert the plate, and tap on a clean absorbent towel.

9. Add 100μl Biotinylated anti-Human Nesfatin-1(1-82) Detection Antibody into each well. Reseal the immunoplate with plate sealer and incubate for 2 hours at room temperature (20-23°C) on a plate shaker (300-400 rpm).

10. Wash 4 times with the 1x Assay Buffer as described in step 8.

11. Add 100μl SA-HRP solution into each well. Reseal the immunoplate with plate sealer and incubate the plate for 30 minutes at room temperature (20-23°C) on plate shaker (300-400 rpm).
12. Wash 4 times with the 1x Assay Buffer as described in step 9.

13. Add 100µl Substrate Solution (TMB) provided in this kit into each well. Reseal the plate with plate sealer to protect from light and incubate the plate for 20-30 minutes at room temperature (20-23°C) on a plate shaker (300-400 rpm).

14. Add 100µl Stop Solution (2N Hydrochloric Acid) into each well to stop the reaction. The color in the well should change from blue to yellow. If the color change does not appear to be uniform, gently tap the plate to ensure thorough mixing. Proceed to the next step within 20 minutes.

15. Read Absorbance O.D. at 450nm using a Microtiter Plate Reader.
ADDITIONAL RECOMMENDED PROCEDURAL NOTES:

• Reagents of different lot numbers should not be mixed.
• Recheck the reagent labels when loading the plate to ensure that everything is added correctly.
• Unused microplate strips should be placed in the foil pouch with a desiccant and stored at 4°C. Do not allow moisture to enter the wells.
• When handling the plate, avoid touching the bottom.
• Manual washing may cause high duplicate coefficient variations. To reduce this factor, liquid from the plate should be removed by inverting and blotting the plate on an absorbent material.
• If the room temperature is not within the suggested range (20-23°C), variations in results may occur.
• The same reservoir for the reagents may be reused if the reservoir is washed well with distilled water before each use.
• Each laboratory must determine the appropriate dilution factors for the samples to be measured to ensure that the samples are within the dynamic range of the standard curve.
• High levels of interfering proteins may cause variations within the sample results. Therefore, it is imperative to select the appropriate sample preparation procedure to obtain the optimal results.
• Each time a new tip is used, make sure the tip is secure and free of air bubbles. For better intra-assay variation, aspirate and expel a reagent or sample back into the container a few times prior to loading.
• Avoid submerging the whole tip into reagents because droplets can accumulate at the end of the tip causing an excess of reagent to be loaded into the well. This can lead to poor results.
• For optimal results, an orbital plate shaker capable of 300-500 rpm is recommended for all incubations.
• Modification of the existing protocol (i.e. standard dilutions, pipetting technique, washing technique, incubation time or temperature, storage conditions, and kit expiration) may affect the sensitivity and specificity of the test.
SUMMARY OF ASSAY PROTOCOL

Add 100μl/well of Nesfatin-1(1-82) standard, sample, or positive control except the Blank wells

- Incubate at room temperature (20-23°C) for 2 hours
- Wash immunoplate 4 times with 350μl/well of 1x assay buffer
- Add 100μl/well of Biotinylated anti-Nesfatin-1(1-82) Detection Antibody
- Incubate at room temperature (20-23°C) for 2 hours
- Wash immunoplate 4 times with 350μl/well of 1x assay buffer
- Add 100μl/well of SA-HRP solution
- Incubate at room temperature (20-23°C) for 30 minutes
- Wash immunoplate 4 times with 350μl/well of 1x assay buffer
- Add 100μl/well of Substrate Solution (TMB)
- Incubate at room temperature (20-23°C) for 20-30 minutes
- Terminate reaction with 100μl/well of 2N HCL
- Read absorbance O.D. at **450nm** and calculate results
CALCULATION OF RESULTS

Plot the standard curve on log-log graph paper. Known concentration of Human Nesfatin-1(1-82) Standard and its corresponding O.D. reading is plotted on the log scale (X-axis) and the log scale (Y-axis), respectively. The standard curve shows a correlated relationship between Human Nesfatin-1(1-82) concentrations and the corresponding O.D. absorbance. As the standard concentration increases, the intensity of the yellow color, and in turn the O.D. absorbance, increases.

The concentration of Human Nesfatin-1(1-82) within a sample is determined by plotting the sample's O.D. on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line dropped from this point will intersect the X-axis at a coordinate corresponding to the Human Nesfatin-1(1-82) concentration in the unknown sample.

Refer to QC Data sheet for acceptable values of the positive control.
**STORAGE**

1. Store the kit at 4°C upon receipt. The kit should be equilibrated to room temperature (20-23°C) before assay.
2. Store 1x Assay Buffer at 4°C.
3. Remove any unneeded strips from Human Nesfatin-1(1-82) antibody-Coated plate, reseal them in zip-lock foil and keep at 4°C.

**NOTE:**

1. It is recommended that the solutions be used on the same day of rehydration.
2. Unextracted serum samples of normal subjects are to be diluted with 1x Assay buffer.
3. After adding Stop Solution, read the plate within 20 minutes.

**REFERENCES**

1. Shinsuke Oh et al. Narewe advance online publication 1 October 2006. NUCB2 A satiety molecule associated with melanocortin signalling in the hypothalamus.