Protocol for Catalog # EK-018-28

Humanin, Nuclear-encoded / HN(N) (Rat) EIA Kit

(range: 0-100 ng/ml)

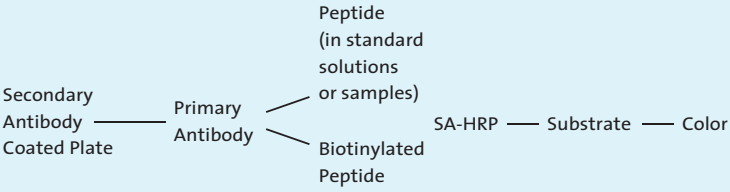
PHOENIX PHARMACEUTICALS, INC.

INTRODUCTION

This Enzyme Immunoassay kit is designed to detect a specific peptide and its related peptides based on the principle of "competitive" enzyme immunoassay.

PRINCIPLE OF ENZYME IMMUNOASSAY WITH THIS KIT

The immunoplate in this kit is pre-coated with secondary antibody and the nonspecific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the primary antibody (peptide antibody) whose Fab fragment will be competitively bound by both biotinylated peptide and peptide standard or targeted peptide in samples. The biotinylated peptide interacts with streptavidin-horseradish peroxidase (SA-HRP) which catalyzes the substrate solution. The intensity of the yellow is directly proportional to the amount of biotinylated peptide-SA-HRP complex but inversely proportional to the amount of the peptide in standard solutions or samples. This is due to the competitive binding of the biotinylated peptide with the standard peptide or samples to the peptide antibody (primary antibody). A standard curve of known concentration can be established accordingly. The unknown concentration in samples can be determined by extrapolation to this standard curve.



CAUTION: INVESTIGATIONAL DEVICE. LIMITED BY LAW TO INVESTIGATIONAL USE. FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

KIT MATERIALS

1. **20**x assay buffer concentrate *(50ml)*.
2. 96 well immunoplate *(1 plate)*.
3. Acetate plate sealer (APS), *(3 pieces)*.
4. Primary antiserum (rabbit anti-peptide IgG) *(1 vial)*.
5. Standard peptide *(1 vial)*.
6. Biotinylated peptide *(1 vial)*.
7. Streptavidin-horseradish peroxidase (SA-HRP) (*30µl)*.
8. Positive control *(2 vials)*.
9. Substrate solution (TMB) *(12ml)*.
10. 2N HCl *(15ml)*.
11. Assay diagram *(1 sheet)*.
12. General protocol *(1 book)*.

NOTE: *Phoenix Pharmaceuticals, Inc. guarantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for their particular needs and establish optimum sample concentration.*

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microtiter plate reader capable of absorbance measurement of 450nm.
2. Orbital plate shaker capable of 300-400rpm *(recommended)*
3. Microtiter plate washer *(recommended)*
4. Multi-channel pipette capable of dispensing 50-100µl *(recommended)*
5. Solution reservoir *(recommended)*
6. Absorbent material for blotting.
7. EDTA Lavender Vacutainer blood collection tubes *(optional)* ..... **Catalog no. VT-6450**
8. Aprotinin (0.6TIU/ml of blood) *(optional)* ..... **Catalog no. RK-APRO**
9. Buffer A *(optional)* ..... **Catalog no. RK-BA-1**
10. Buffer B *(optional)* ..... **Catalog no. RK-BB-1**
11. C18 SEP-COLUMN *(optional)* ..... **Catalog no. RK-SEPCOL-1**

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. Recommended blood collection protocol is provided on page 9. Each kit contains sufficient reagents for 96 wells and is capable of assaying 40 duplicate samples.*

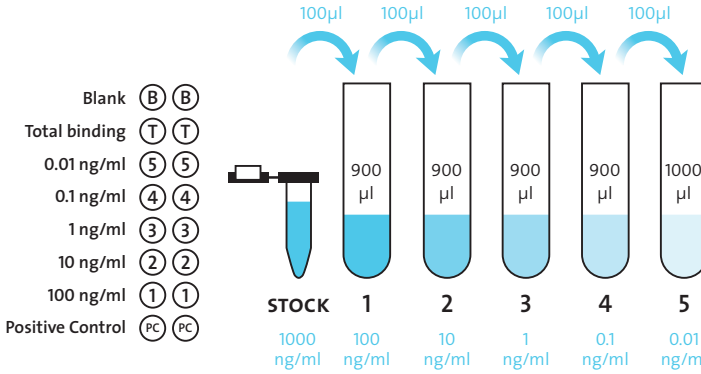
ASSAY PROCEDURE

1. **Thoroughly read this protocol before performing an assay. Please allow all kit components to return to room temperature before use (25-45 minutes).**
2. Dilute the **20**x assay buffer concentrate with 950ml of distilled water. This will be the **1**x assay buffer solution and used to dilute or reconstitute all other reagents in this kit and samples.

   Note: If crystals appear in the **20**x assay buffer, the buffer can be placed in a warm water bath for approximately 30 minutes or until no crystals are visible. Mix thoroughly before use.
3. Centrifuge and dilute the standard peptide with 1ml of **1**x assay buffer, vortex. The concentration of this stock solution is 1,000ng/ml. Allow the solution to sit at least 10 minutes at room temperature (20-23°C) to completely dissolve in solution. Centrifuge and vortex immediately before use.

Prepare peptide standard solutions as follows:

Standard No.	Std. volume	1x Assay Buffer	Concentrations
Stock	1000µl	----	1,000ng/ml
#1	100µl of stock	900µl	100ng/ml
#2	100µl of #1	900µl	10ng/ml
#3	100µl of #2	900µl	1ng/ml
#4	100µl of #3	900µl	0.1ng/ml
#5	100µl of #4	900µl	0.01ng/ml



4. Rehydrate primary antibody with 5ml of **1**x assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.
5. Rehydrate biotinylated peptide with 5ml of **1**x assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.
6. Centrifuge and rehydrate the positive control with 200µl of **1**x assay buffer. Allow it to sit at least 5 minutes to completely dissolve. Mix thoroughly.
7. Leave wells A-1 and A-2 empty as **Blank.**
8. Add 50µl of **1**x assay buffer into wells B-1 and B-2 as **Total Binding**.
9. Add 50µl of prepared peptide standards from **#5** to **#1** (in reverse order of serial dilution) into wells from C-1 and C-2 to G-1 and G-2 respectively.

   Note: Peptide standards should be assayed in duplicate.
10. Add 50µl of rehydrated positive control into wells H-1 and H-2.

    Note: Positive controls should be assayed in duplicate.
11. Add 50µl of prepared samples into their designated wells in duplicate.
12. Add 25µl of rehydrated primary antibody into each well **except** the **Blank** well.

13. Add 25µl of rehydrated biotinylated peptide into each well **except** the **Blank** well.

    Note: A multi-channel pipette is **NOT** recommended to load the biotinylated peptide or primary antiserum.

14. Seal the immunoplate with acetate plate sealer (APS). Incubate the immunoplate for 2 hours at room temperature (20-23°C). Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.
15. Centrifuge the SA-HRP vial provided in this kit (3,000-5,000 rpm, 5 seconds) and pipette 12µl of SA-HRP into 12ml of **1**x assay buffer to make SA-HRP solution, vortex thoroughly.
16. Remove APS from the immunoplate. Discard contents of the wells.
17. Wash each well with 350µl of **1**x assay buffer, discard the buffer, invert and blot dry plate. Repeat 4 times.
18. Add 100µl of SA-HRP solution into each well.
19. Reseal the immunoplate with APS. Incubate for 1 hour at room temperature (20-23°C). Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.
20. Remove APS from the immunoplate.
21. Wash and blot dry the immunoplate 4 times with **1**x assay buffer as described above in step 17.
22. Add 100µl of TMB substrate solution provided in this kit into each well. Orbital shaking at 300-400 rpm is recommended for the duration of the incubation. After the addition of TMB solution, it is strongly recommended to cover the immunoplate to protect from light.
23. Reseal the immunoplate with APS. Incubate for 1 hour at room temperature (20-23°C).
24. Remove APS from the immunoplate. Add 100µl 2N HCl 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.
25. Load the immunoplate onto a Microtiter Plate Reader. Read absorbance O.D. at **450nm**.

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 back in the foil pouch with a desiccant and stored at 4ºC. 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 sample results; therefore, it is imperative to select the appropriate sample preparation procedure to obtain 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 300-400 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.

Calculation of results

Plot the standard curve on semi-log graph paper. A standard curve is constructed by plotting the known concentrations of standard peptide on the log scale (X-axis), and its corresponding O.D. reading on the linear scale (Y-axis). It is strongly recommended to use curve-fitting software capable of 4 parameter logistics or log-logit to quantify the concentration of standard peptide. The standard curve shows an inverse relationship between peptide concentrations and the corresponding absorbance. As the standard concentration increases, the yellow color decreases, thereby reducing the O.D. absorbance.

The concentration of peptide in a sample is determined by locating the sample's O.D. on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line drawn from this point will intersect the X-axis at a coordinate corresponding to the peptide concentration in the sample. If samples have been diluted prior to the assay, the measured concentration must be multiplied by their respective dilution factors.

The standard curve will be a reverse sigmoidal shape.

Refer to QC Data Sheet for acceptable values of the positive controls.

STORAGE

1. Store the kit at 4°C upon receipt.
2. It is highly recommended that solutions be used as soon as possible after rehydration.
3. Store **1**x assay buffer at 4°C.
4. Remove any unneeded strips from antibody-coated plate, reseal them in zip-lock foil and keep at 4°C.
5. Keep rehydrated solution of Standard, Biotinylated peptide, Antibody and HRP at 4°C.

SUMMARY OF ASSAY PROTOCOL

Add 50µl/well of standard, sample, or positive control, 25µl primary antibody and 25µl biotinylated peptide.

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Incubate at room temperature (20-23°C) for 2 hours

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Wash immunoplate 4 times with 350µl/well of **1**x assay buffer

▼

Add 100µl/well of SA-HRP solution

▼

Incubate at room temperature (20-23°C) for 1 hour

▼

Wash immunoplate 4 times with 350µl/well of **1**x assay buffer

▼

Add 100µl/well of TMB substrate solution

▼

Incubate at room temperature (20-23°C) for 1 hour

▼

Terminate reaction with 100µl/well of 2N HCl

▼

Read absorbance O.D. at **450**nm and calculate results

SUGGESTED METHOD FOR THE EXTRACTION OF PEPTIDES FROM PLASMA

Blood Withdrawal:

Collect blood samples into the Lavender Vacutainer tubes (#VT-6450) which contain EDTA and can collect up to 7ml of blood. Gently rock the Lavender Vacutainer tubes several times immediately after collection of blood for anti-coagulation. Transfer the blood from the lavender vacutainer tubes to the centrifuge tubes containing aprotinin (0.6TIU/ml of blood) and gently rock for several times to inhibit the activity of proteinases. Centrifuge the blood at 1,600 x g for 15 minutes at 4°C and collect the plasma. Plasma kept at -70°C may be stable for one month. If Lavender Vacutainer tubes are centrifuge-safe, the aprotinin may be added into the initial collection step.

Extraction of Peptides from Plasma:

1. Acidify the plasma with an equal amount of buffer A (Cat. No. RK-BA-1). For example, if you are using 1ml of plasma, add 1ml of buffer A. Mix and centrifuge at 6,000 to 17,000 x g for 20 minutes at 4°C.
2. Equilibrate a SEP-COLUMN containing 200mg of C18 (Cat. No. RK-SEPCOL-1) by washing with buffer B (Cat. No. RK-BB-1) (1ml, once) followed by buffer A (3ml, 3 times).

Note: From steps 3-5, no pressure should be applied to the column.

3. Load the acidified plasma solution onto the pre-equilibrated C-18 SEP-Column.
4. Slowly wash the column with buffer A (3ml, twice) and discard the wash.
5. Elute the peptide slowly with buffer B (3ml, once) and collect the eluant into a polystyrene tube.
6. Evaporate eluant to dryness in a centrifugal concentrator or by a suitable substitute method.

7. Keep the dried extract at -20°C and perform the assay as soon as possible. Use **1x** assay buffer to reconstitute the dried extract. If the peptide value does not fall within the range of detection, dilute or concentrate the sample accordingly.

Tips for extraction of plasma:

When using a C-18 SEP-COLUMN for the first time, use a bulb (not supplied) to apply pressure to the column after the addition of 1ml of buffer B to facilitate the flow through the column. From steps 3-5, no pressure should be applied.

Ensure there is a constant flow for all solutions during the extraction procedure. Do not allow air bubbles to enter the C-18 matrix for optimal sample processing and recovery.

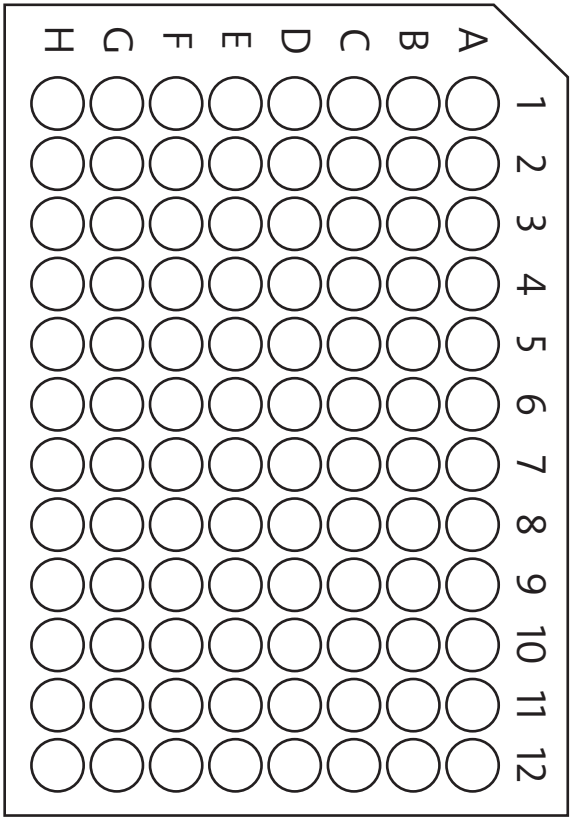
Drying Sample After Extraction:

A combination of a centrifugal concentrator (i.e. Speedvac) and a lyophilizer (freeze-dryer) produces the best results for drying the sample after extraction. First, use a Speedvac to dry sample for approximately 15 minutes to remove the organic layer. Then snap-freeze the remaining sample, and freeze-dry overnight using a lyophilizer. This two-step procedure produces a more consistent fluffy powder that is easier to rehydrate than a sample dried only with a centrifugal concentrator. However, if a centrifugal concentrator is not accessible, freeze-drying overnight using a lyophilizer will be sufficient.

REFERENCES

1. Porstmann, T. and Kiessig, S.T., Enzyme Immunoassay Techniques, An Overview, Journal of Immunological Methods, 150: 5-21 (1992).
2. Avrameas, S., Amplification Systems in Immunoenzymatic Techniques, Journal of Immunological Methods, 150: 23-32 (1992).

ASSAY DIAGRAM



USA

Phoenix Pharmaceuticals, Inc.

330 Beach Rd.
Burlingame, California 94010
Tel: 650-558-8898, 800-988-1205
Fax: 650-558-1686
Info@PhoenixPeptide.com
www.PhoenixPeptide.com

Europe

Phoenix Europe GmbH

Viktoriastrasse 3-5
D-76133 Karlsruhe
Germany
Tel: +49 (721) 16 11 950
Fax: +49 (721) 16 11 952
Germany@PhoenixPeptide.com