

Orexin A (Human, Rat, Mouse)

Extraction Free EIA Kit Protocol

(Cat. No.:EKE-003-30)
(Range: 0-100ng/ml)



PHOENIX PHARMACEUTICALS, INC.

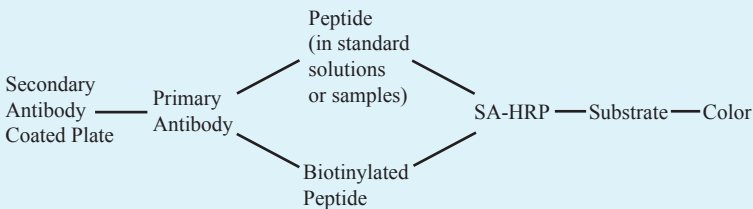
OREXIN A (H, R, M) EXTRACTION FREE EIA KIT PROTOCOL

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. 20x EIA Assay Buffer Concentrate (50ml).... Catalog no. EK-BUF
2. 96 well immunoplate (1 plate)..... Catalog no. EK-PLATE
3. Acetate plate sealer (APS), (3 pieces)Catalog no. EK-APS
4. Primary antibody (rabbit anti-peptide IgG) (1 vial).

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- 5. Standard peptide (1 vial).
- 6. Biotinylated peptide (1 vial).
- 7. Streptavidin-horseradish peroxidase (SA-HRP) (30µl) Catalog no. EK-SA-HRP
- 8. Positive control (2 vials).
- 9. Substrate solution (TMB) (12ml).....Catalog no. EK-SS
- 10. 2N HCl (15ml).....Catalog no. EK-HCl
- 11. Diluent Buffer (23 ml).....Catalog no. EKE-DB-03
- 12. General protocol with Assay diagram (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 plasma 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 (0.6 TIU/ml of blood) (optional)Catalog no. RK-APRO

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. Each kit contains sufficient reagents for 96 wells and is capable of assaying 40 duplicate plasma samples.

REAGENT PREPARATION

1. **1x EIA Assay Buffer:** Dilute the 20x EIA assay buffer concentrate with 950 ml of distilled water. This will be the **1x EIA assay buffer** solution. **Read Protocol booklet thoroughly to determine which buffer to use in order to rehydrate or dilute reagents.**
NOTE: If crystals appear in the 20x EIA 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.
2. **Primary Antibody:** Rehydrate primary antibody with **1ml of 1x EIA assay buffer**. Allow to sit for at least 5-10 minutes to completely dissolve. Mix thoroughly. Next, add **4ml of diluent buffer** to the primary antibody. Mix thoroughly.
3. **Biotinylated Peptide:** Rehydrate biotinylated peptide with **1ml 1x EIA assay buffer**. Allow to sit for at least 5-10 minutes to completely dissolve. Next, add **4ml of diluent buffer** to biotinylated peptide. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.
4. **Positive Control:** Centrifuge and rehydrate the positive control with **200µl of diluent buffer**. Allow it to sit at least 5-10 minutes to completely dissolve. Mix thoroughly.
5. **Streptavidin Horseradish Peroxidase (SA-HRP):** 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 **1x EIA assay buffer** to make SA-HRP solution, vortex thoroughly.
6. **Sample Preparation:** **Dilute plasma sample to 1:1 with diluent buffer.** Vortex and centrifuge before use.

Note: It is highly recommended that normal plasma samples be used in comparison with patient plasma samples to establish a baseline value. If further dilutions are required, dilute plasma samples in Diluent Buffer

ASSAY PROCEDURE

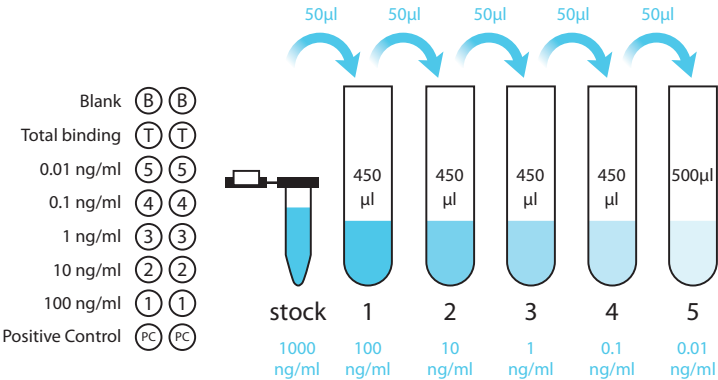
1. Thoroughly read this protocol before performing an assay. Please allow all kit components to return to room temperature before use (25-45 minutes).

2. Centrifuge and dilute the standard peptide with **1ml of 1x EIA 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. Vortex and centrifuge immediately before use.

NOTE: Prepare peptide standard dilutions with Diluent Buffer

Prepare peptide standard dilutions as follows:

Standard No.	Std. volume	Diluent Buffer	Concentrations
#1	50µl of stock	450µl	100ng/ml
#2	50µl of #1	450µl	10ng/ml
#3	50µl of #2	450µl	1ng/ml
#4	50µl of #3	450µl	0.1ng/ml
#5	50µl of #4	450µl	0.01ng/ml



3. Leave wells A-1 and A-2 empty as Blank.
4. Add 50µl of diluent buffer into wells B-1 and B-2 as Total Binding.
5. 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.

6. Add 50µl of rehydrated positive control into wells H-1 and H-2.
- Note:** Positive controls should be assayed in duplicate.

7. Add 50µl of prepared plasma samples into their designated wells in duplicate.
8. Add 25µl of rehydrated primary antibody into each well except the Blank well.
9. 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 antibody.
10. 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.
11. Remove APS from the immunoplate. Discard contents of the wells.
12. Wash each well with 350µl of 1x EIA assay buffer, discard the buffer, invert and blot dry plate. Repeat 4 times.
13. Add 100µl of prepared SA-HRP solution into each well.
14. 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.
15. Remove APS from the immunoplate.
16. Wash and blot dry the immunoplate 4 times with 1x EIA assay buffer as described above in step 13.
17. 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.
18. Reseal the immunoplate with APS. Incubate for 1 hour at room temperature (20-23°C).
19. 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.
20. Load the immunoplate onto a Microtiter Plate Reader. Read absorbance O.D. at 450nm.

SUMMARY OF ASSAY PROTOCOL

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



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 1 hour



Wash immunoplate 4 times with 350µl/well of 1x 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 **450nm** and calculate results

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 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 plasma samples to be measured to ensure that the plasma samples are within the dynamic range of the standard curve.
- High levels of interfering proteins may cause variations within plasma sample results; therefore, it is imperative to select the appropriate plasma 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. Because plasma samples have been diluted prior to the assay, the measured concentration must be multiplied by their respective dilution factors (e.g. 1ng/ml (from standard curve) x 2 (dilution factor) = 2ng/ml). 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 1x EIA assay and diluent buffer at 4°C.
4. Remove any unneeded strips from antibody-coated plate, reseal them in zip-lock foil and at 4°C.
5. Keep rehydrated solution of Standard, Biotinylated peptide, Antibody and SA-HRP at 4°C.

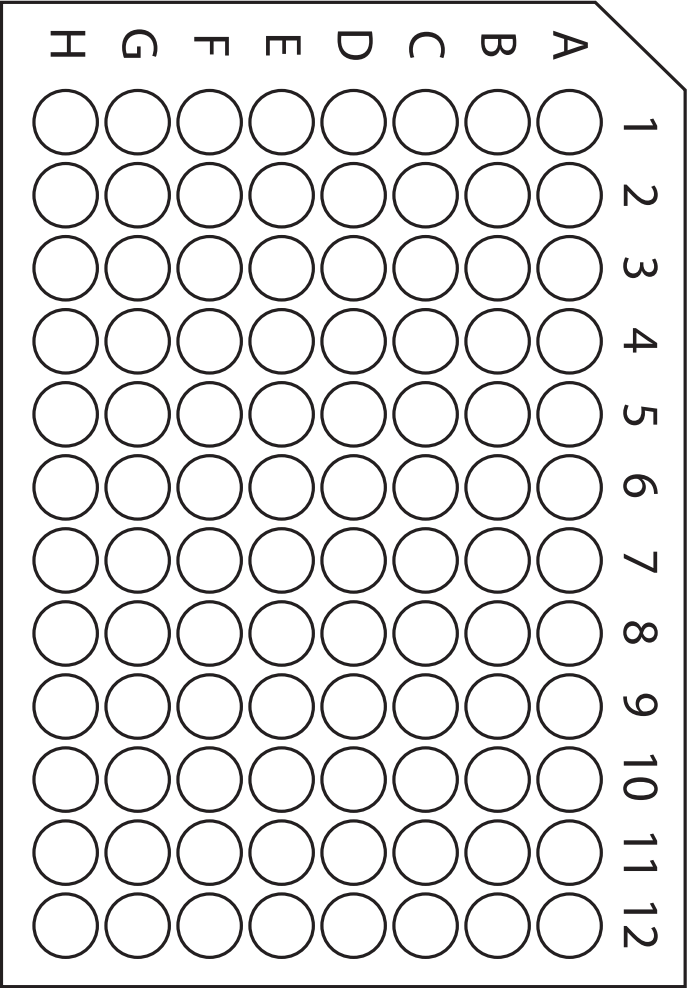
REFERENCES

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).

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ASSAY DIAGRAM



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