



**PHOENIX PHARMACEUTICALS,
INC.**
The Peptide Elite

ASSAY PROTOCOL (Range:0-100ng/ ml)

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INTRODUCTION

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

CONTENTS:

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 (1µg)
6. Biotinylated peptide (1 vial)
7. Streptavidin-horseradish peroxidase (SA-HRP) (30µl)
8. Substrate solution (12ml)
9. 2N HCl (15ml)
10. Assay diagram (1 sheet)
11. General protocol (1 booklet)

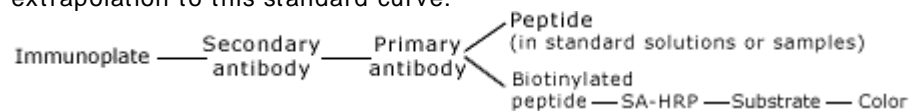
Note: It is recommended that the solutions be used on the same day of rehydration. Blood collection protocol and extraction procedure for plasma is provided for your information. (Materials for extraction not included).



STORAGE: Store the kit at 2°-4°C upon receipt. The kit will be stable for 6 months. The kit should be equilibrated to room temperature before assay.

GENERAL INFORMATION:

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 is able to interact with streptavidin-horseradish peroxidase (SA-HRP) which catalyzes the substrate solution composed of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide to produce a blue colored solution. The enzyme-substrate reaction is stopped by hydrogen chloride (HCl) and the solution turns to yellow. 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 and the peptide in standard solutions or samples to the peptide antibody (primary antibody). A standard curve of a peptide with known concentration can be established accordingly. The peptide 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.

Blood Collection: [Click here](#)

required. It is up to the discretion of the paper reviewers.

Tissue Extraction Method:



GENERAL PROCEDURE FOR UTILIZATION OF THE EIA KIT:

1. Thoroughly read this protocol before performing an assay.
2. Dilute the assay buffer concentrate with 950ml of distilled water. This assay buffer will be used to reconstitute all of the other compounds in this kit and the extract of plasma samples.
3. Rehydrate standard peptide with 1 ml assay buffer, vortex. The concentration of this stock solution is 1,000ng/ml.
4. Prepare peptide standard solutions as follows:

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- including the Blank well.
25. Reseal the immunoplate with APS.
 26. Incubate for 1 hour at room temperature.
 27. Add 100 μ l 2N HCl into each well (including the Blank) to stop the reaction. Go to the next step within 20 minutes.
 28. Clean the immunoplate bottom with 70% ethanol.
 29. Remove APS and load the immunoplate onto a Microtiter Plate Reader.
 30. Read absorbance O.D. at 450nm.

CALCULATION OF RESULTS:

Plot the standard curve on semi-log graph paper. Known concentrations of standard peptide and its corresponding O.D. reading is plotted on the log scale (X-axis) and the linear scale (Y-axis) respectively. The standard curve shows an inverse relationship between peptide concentrations and the corresponding O.D. absorbances. As the standard concentration increases, the intensity of the yellow color, and in turn the O.D. absorbance, decreases.

The concentration of peptide in 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 peptide concentration in the unknown sample.

SUMMARY OF ASSAY PROTOCOL:

1. Add 50 μ l/well of standard or sample, 25 μ l primary antiserum and 25 μ l biotinylated peptide.
2. Incubate at room temperature for 2 hours
3. Wash immunoplate 5 times with 300 μ l/well of assay buffer
4. Add 100 μ l/well of SA-HRP solution
5. Incubate at room temperature for 1 hour
6. Wash immunoplate 6 times with 300 μ l/well of assay buffer
7. Add 100 μ l/well of substrate solution
8. Incubate at room temperature for 1 hour
9. Terminate reaction with 100 μ l/well of 2N HCl
10. Read absorbance O.D. at 450nm and calculate results

SUGGESTED METHOD FROM THE EXTRACTION OF PEPTIDES FROM PLASMA:

Blood Withdrawal: Collect blood samples into the Lavender Vacutaner tubes (#VT6450) which contain EDTA and can collect 7ml blood/tube. Gently rock the Lavender Vacutaner tubes several times immediately after collection of blood for anti-coagulation. Transfer the blood from the Lavender Vacutaner tubes to 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.

Elution Solvents:

1. Buffer A (Code RK-BA-1): 1% trifluoroacetic acid (TFA, HPLC Grade) in H₂O.
2. Buffer B (Code RK-BB-1): 60% acetonitrile (HPLC Grade) in 1% TFA.

Extraction of Peptide from Plasma:

1. Acidify the plasma with an equal amount of buffer A. 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 (Code RK-SEPCOL-1) by washing with buffer B (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-treated C18 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 eluant in a polypropylene 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 assay as early as possible. Reconstitute the dried extract with assay buffer before performing assay. If the peptide value exceeds or does not fall in the range of detection, dilute or concentrate the samples accordingly.

TIPS FOR EXTRACTION OF PLASMA:

When using SEP-COLUMN for the first time, use the enclosed bulb to apply pressure to the column after addition of 1ml of buffer B to facilitate the flow through the column. From step 3-5, no pressure should be applied.

Drying Sample After Extraction:

Ideally, using combination of centrifugal concentrator (i.e. Speedvac) and lyophilization (freeze dryer) produce best results. First, use a Speedvac to dry sample for approximately 15 min to remove the organic layer, then freeze the remaining sample, freeze-drying overnight using a lyophilizer. This produces a more consistent and fluffy powder that is easier to rehydrate than the sample dried with a Speedvac.

REFERENCES:

1. Enzyme Immunoassay Techniques, An Overview. Porstmann, T.

- and Kiessig, S.T., Journal of Immunological Methods, 150(1992) 5-21.
2. Amplification Systems in Immunoenzymatic Techniques, Avrameas, S., Journal of Immunological Methods, 150(1992)23-32.



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