Visfatin C-Terminal (Human)
Enzyme Immunoassay Kit Protocol

(Cat. No.:EK-003-80)

(Range: 0.1-1000ng/ml)

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 a secondary antibody and the non-specific 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 biotinlyated peptide interacts 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 hydrochloric acid (HCl) and the solution turns yellow. The intensity of the yellow is directly proportional to the amount of biotinylated peptide-SA-HRP complex buy inversely proportional to the amount of the pepide in standard solutions or samples. Thi is due to the competitive binding of the biotinylated 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 insamples can be determined by extrapolation to this standard curve.

KIT MATERIALS

1. **20x** assay buffer concentrate (50ml).....**Catalog no. EK-BUF**
2. 96 well immunoplate (1plate).....**Catalog no. EK-Plate**
3. Acetate plate sealer (APS), (3 peices).....**Catalog no. EK-APS**
4. Primary anitbody concentrate.....**Catalog no. EK-RAB-003-80** (rabbit anti-IgG) (1 vial).
5. Standard peptide (1vial).....**Catalog no. EK-S-003-80**
6. Positive Control (2 vials).....**Catalog no. EK-PC-003-80**
7. Biotinylated peptide concentrate..... **Catalog no. EK-B-003-80** (1 vial)
8. Streptavidin-horseradish peroxidase..... **Catalog no. EK-HRP**
9. Substrate solution (TMB) (12ml)..... **Catalog no. EK-TMB**
10. 2N HCl (15ml)..... **Catalog no. EK-HCl**
11. Assay diagram (1 sheet).
12. General protocol (1 book).

Note:
A concentrated form of the the primary antibody, biotinylated peptide, and standard peptide has been packaged. Please read protocol booklet carefully before performing the assay.

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 (Receommended)
4. Multi-channel pipette capable of dispensing 50-100µl (Recommended)
5. Solution reservoir (Recommended)
6. Absorbent material for blotting.
7. EDTA Lavender Vacutainer tubes (optional)..**Catalog no. VT-6450**
8. Aprotinin (0.6TIU/ml of blood)(optional)...**Catalog no. RK-APR0**

Phoenix Pharmaceuticals Inc. guarantees that its protducts conform to the information contained in this publication. The purchaser must determine the suitablity of the product for its particular use and establish optimum sample concentrations.

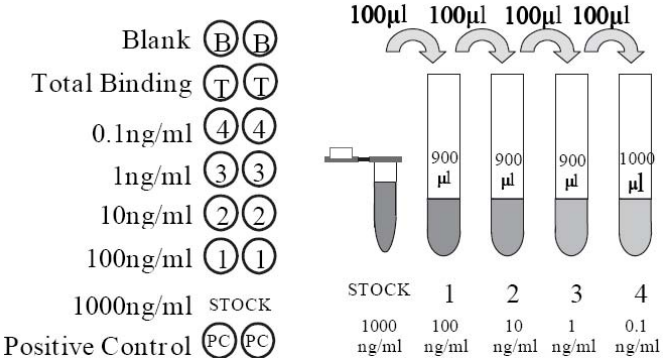
NOTE: This kit should be equilibrated to room temperature (20-23°C) before opening any vials and starting the assay. **It is highly recommended that the solutions be used as soon as possible after rehydration.** Each kit contains sufficient reagents for 96 wells and capable of assaying 40 duplicate samples.

ASSAY PROCEDURE

1. **Thoroughly read this protocol before performing an assay and please allow all kit components to return to room temperature before use(25-45 minutes).**
2. Dilute the **20x** assay buffer concentrate with 950ml of distilled water. This will be the **1x** assay buffer solution and used to dilute or dissolve all other reagents in this kit and samples.
   Note: If crystals appear in the 20 assay buffer, the buffer can be placed in a warm water bath for approximately 30 minutes or until not crystal are visible. Mix thoroughly before use.
3. Centrifuge and dilute the standard peptide with 1ml of **1x** 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 competely dissolve in solution. Cnetri fuge 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



4. Remove immunoplate from the foil pouch and wash each well with 300µl of **1x** assay buffer. Allow it to sit for at least five min utes. Discard the buffer, invert and blot dry plate. Do not let wells dry before proceeding to the next step.
5. Centrifuge and rehydrate the positive control with 200µl of **1x** as say buffer. Allow the solution to sit at least 5 minutes to com pletely dissolve. Mix rhoroughly.
6. Leave wells empty as **Blank**.
7. Add 50µl of **1x** assay buffer into wells B-1 and B-2 as Total Binding.
8. Add 50µl of prepared peptide standard from #4 to Stock (in reverse order of serial dilution into wells from C-1 to C-2 and G-1 to G-2 respectively.
   Note: Peptide standards should be assayed in duplicate.
9. Add 50µl of rehydrated positive controls into wells H-1 and H-2.
   Note: Positive contorls should be assayed in duplicate.
10. Add 50µl of samples into their designated wells in duplicate.
11. Rehydrate the primary antibody concentrate (rabbit anti-peptide IgG) vial provided in this kit with 100µl of **1x** assay buffer. Dilute the primary antibody concentrate 1:25 with **1x** assay buffer. To make primary antibody working solution that is sufficient for 96 wells, pipette 100µl of the primary antibody concnetrate into 2.5ml of **1x** assay buffer, vortex thoroughly. Add 25µl of this working solution to each well except the Blank well.

12. Rehydrate the biotinylated peptide concentrate vial provided in this kit with 100µl of **1x** assay buffer. Dilute the biotinylated peptide concentrate 1:25 with **1x** assay buffer. To make biotinylated peptide working solution that is sufficient for 96 wells, pipette 100µl of the bioti nylated peptide concentrate into 2.5ml of **1x** assay buffer, vortex thor oughly. Add 25µl of this working solution to each well except the Blank well.
13. Seal the immunoplate with acetate plte sealer (APS). Incubate the immunoplate for 2 hours at room temperature (20-23C). Orbital shaking at 300-400rpm is recommended for the duration of the incubation.
14. 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 assay buffer to make SA-HRP solution, vortex thoroughly.
15. Remove APS from immunoplate. Discard contents of wells.
16. Wash each well with 350µl of 1x assay buffer, discard the buffer, invert and blot dry plate. Repeat 4 times.
17. Add 100µl SA-HRP solution into each well.
18. Reseal the immunoplate with APS. Incubate for 1 hour at room temperature (20-23°C). Orbital shaking at 300-400rpm is recommended for the duration of the incubatiom.
19. Remove APS from the immunoplate. Wash and blot dry the immunoplate 4 times with 1x assay buffer as described above in step 13.
20. Add 100µl of TMB sustrate solution provided in this kit into each well. Orbital shaking at 300-400rpm 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.
21. Reseal the immunoplate with APS. Incubate for 1 hour at room temperature (20-23°C).
22. 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. Go to the next step within 20 minutes.
23. Load the immunoplate onto a Microtiter Plate Reader. Read absorbance O.D. at 450nm.

ADDITIONAL RECOMMENDED PROCEDURAL NOTES:

- Reagents of different lot numbers shoud 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 dessicant 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 appropeate 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.

CALCULATION OF RESULTS

Plot the standard curve on semi-log graph paper. A standard curve is contructed 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 realtionship between peptide concentrations and the corresponding O.D. absorbance. As the standard concentration increases, the intensity of the yellow color decreases, thereby reducing the O.D. absorbance.

The concentration of the 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 standrd curve. A vertical line drawn from this point will intersect the X-axis at a coordinate corresponding to the peptide concnetration in the sample. If samples have been diluted prior to the assay, the measured concentration must be multiplied by thier respective dilution factors.

The standard curve will be a reverse sigmoidal shape.

Refer to the QC Data Sheet for acceptable values of the Positive Control.

STORAGE

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

SUMMARY OF ASSAY PROTOCOL

Add 50µl /well of Standard, Sample, or Positive Control, 25µl primary antibody and 25µl biotinlyated peptide

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

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Wash immunoplate 4 times with 300-350µl/well of **1x** assay buffer

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Add 100µl/well of SA-HRP solution

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

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Wash immunoplate 4 times with 300-350µl/well

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Add 100µl/well of substrate solution (TMB)

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

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Terminate the reaction with 100µl/well of Stop Solution

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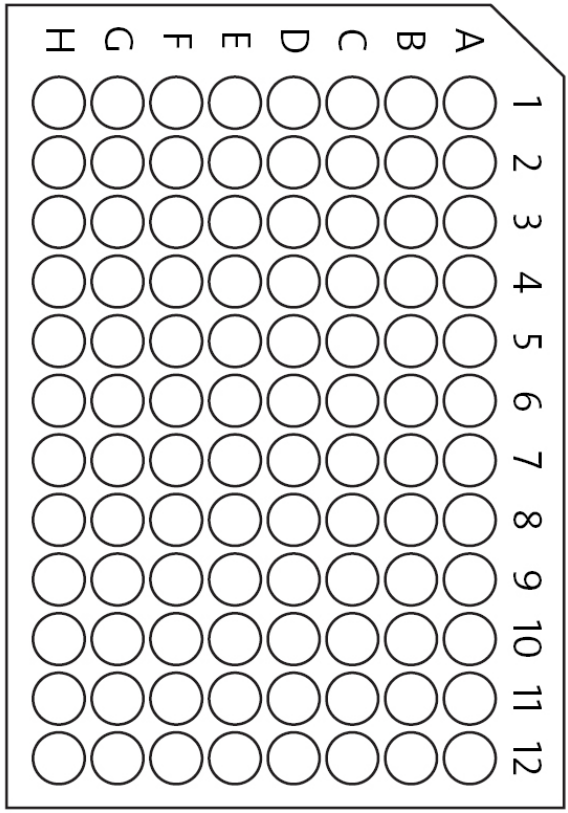
Read absorbance O.D. at 450nm and calculate results

REFERENCES

1. Portsmann, T., and Kiessig, S.T., Enzyme Immunoassay Tech niques, An Overview, *Journal of Immunological Methods, 150:5-21(1992).*

2. Avrameas, S., Amplification Systems in Immunoenzymeatic Tech niques, *Journal of Immunologial Methods, 150: 23-32(1992).*

ASSAY DIAGRAM



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