Xenin-25 (Human)

FLUORESCENT ENZYME IMMUNOASSAY PROTOCOL

(Cat.# FEK-046-74)
(range: 0-10,000 pg/ml)

PHOENIX PHARMACEUTICALS, INC.

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CAUTION:

Investigational device. Limited by law to investigational use.

For research use only. Not for use in diagnostic procedures.

KIT CONTENTS

1. EIA assay buffer concentrate (*20x, 50m*l).....**Catalog no. EK-BUF**
2. Pre-coated fluorescent EIA plate (*96 wells*)
3. Acetate plate sealer (APS) (*3 pieces*)
4. Primary antibody (*1 vial*)
5. Standard peptide (*1 vial*)
6. Biotinylated peptide (*1 vial*)
7. Positive control (*1 vial*)
8. Streptavidin-horseradish peroxidase (SA-HRP) concentrate (*30µl*)
9. Substrate solution (*12ml*)
10. Stable peroxide solution (*1.5ml*)
11. Stop solution (*12ml*)

ADDITIONAL MATERIALS, NOT INCLUDED

1. Fluorescence microtiter plate reader (325nm to 420nm) (required)
2. Micropipette with disposable pipette tips (required)
3. Absorbent material for blotting (required)
4. Vortex (required)
5. Curve-fitting software capable of 4 parameter logistics (recommended)
6. Orbital plate shaker (300-400rpm) (recommended)
7. Microtiter plate washer (recommended)
8. Multi-channel pipette (50-100µl) (recommended)
9. Solution reservoir (recommended)
10. Centrifuge (optional)
11. EDTA Lavender Vacutainer blood collection tubes (optional).....**Catalog no. VT-6450**
12. Aprotinin (30 TIU) (optional) ..... **Catalog no. RK-APRO**
13. C18 SEP-COLUMN (optional)..... **Catalog no. RK-SEPCOL-1**
14. Buffer A (optional) .....**Catalog no. RK-BA-1**
15. Buffer B (optional) .....**Catalog no. RK-BB-1**

STORAGE

1. Store the kit at 4°C upon receipt. Do not freeze.
2. It is highly recommended that all solutions be used as soon as possible after reconstitution.
3. Any unused strips/columns may be removed from the pre-coated immunoplate. Please place strips back in the original zip-lock foil pouch with a dessicant, reseal, and store at 4°C. Do not allow moisture to accumulate on the wells.
4. If necessary, store the 1x assay buffer at 4°C.
5. If necessary, store any reconstituted solutions of standard peptide, biotinylated peptide, primary 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).

INTRODUCTION

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

GENERAL DESIGN OF THIS KIT

The immunoplate in this kit is pre-coated with a secondary antibody, whose nonspecific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the primary antibody added to each well of the plate. This primary antibody's Fab fragment will then be competitively bound by both the biotinylated peptide and the targeted peptide in either the standard peptide solution or the unknown sample. The biotinylated peptide interacts with streptavidin-horseradish peroxidase (SA-HRP) which catalyzes the substrate solution. The resulting fluorescence intensity is directly proportional to the amount of biotinylated peptide-SA-HRP complex, but inversely proportional to the amount of the targeted peptide (in either the standard peptide solution or the unknown sample). This is due to competition between the biotinylated peptide and the target peptide for binding with the primary antibody. A standard curve can be established by plotting the measured O.D. as a function of the various known standard peptide concentrations. Unknown peptide concentration in samples can then be determined via extrapolation based on this standard curve.

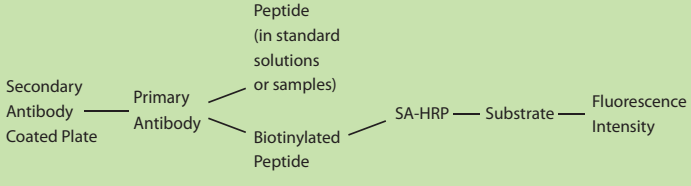

Figure 1. Diagram of the molecular interactions used in this kit

SUMMARY OF ASSAY PROTOCOL

Add 50µl/well of standard, sample, or positive control, along with 25µl/well of primary antibody to each well

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Incubate overnight (16-24 hours) at 4°C

▼

Add 25µl/well of biotinylated peptide

▼

Incubate at room temperature (20-23°C) for 1.5 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 prepared substrate solution

▼

Incubate at room temperature (20-23°C) for 15-20 minutes

▼

Terminate reaction with 100µl/well of stop solution

▼

Measure relative fluorescence intensity of each well and calculate results

A fluorescent microplate reader (325nm - 420nm) capable of top reading is required to measure fluorescence during this assay. Please consult the fluorescence microtiter plate reader's user manual for specific instrument capabilities and settings prior to performing the assay.

Note: Read this protocol in its entirety before starting the assay. Each kit contains reagents sufficient for 96 wells and is capable of assaying 40 duplicate samples.

ASSAY PROTOCOL

Note: The kit and all its components should be equilibrated to room temperature (20-23ºC) before opening any vials and starting the assay. Before opening any Eppendorf tubes for reconstitution, briefly centrifuge at ~3,000rpm for 5 seconds to ensure that all the lypholized material is at the bottom of the tube.

1. Dilute the 20x EIA buffer concentrate with 950ml of distilled water. Mix thoroughly before use. This will be the 1x assay buffer solution used to dilute or reconstitute all other samples and reagents during the assay.

   Note: If crystals appear in the 20x assay buffer, the bottle can be placed in a warm water bath for approximately 30 minutes or until no crystals are visible.

2. Reconstitute the standard peptide in 1ml of the 1x assay buffer and vortex thoroughly. Allow the solution to sit at least 10 minutes at room temperature (20-23°C) to completely dissolve in solution. This will be the standard stock solution.

   Note: Vortex immediately before use.

3. Reconstitute the primary antibody with 1x assay buffer and vortex thoroughly. **Refer to the QC data sheet for specific instructions on rehydrating the primary antibody.** Allow the solution to sit for at least 5 minutes at room temperature to completely dissolve.

4. Reconstitute the positive control with 1x assay buffer and vortex thoroughly. **Refer to the QC data sheet for specific instructions on rehydrating the positive control.** Allow the solution to sit for at least 5 minutes at room temperature to completely dissolve.

5. Prepare the standard dilutions from the rehydrated standard peptide as shown in Figure 2 and Figure 4 below. Vortex the tube thoroughly after each serial dilution.
6. Leave wells A1 and A2 on the immunoplate empty as blanks.
7. Add 50µl of 1x assay buffer into wells B1 and B2. These will represent total binding (of the biotinylated peptide).

Prepare peptide standard solutions as follows:

Standard ID / Number	1x Assay Buffer Volume	Standard Peptide Volume	Concentration
Stock	1000µl	(powder)	1,000,000pg/ml
#1	900µl	100µl of stock	100,000pg/ml
#2	900µl	100µl of #1	10,000pg/ml
#3	900µl	100µl of #2	1,000pg/ml
#4	900µl	100µl of #3	100pg/ml
#5	900µl	100µl of #4	10pg/ml
#6	900µl	100µl of #5	1pg/ml

Figure 2. Table of the standard dilutions

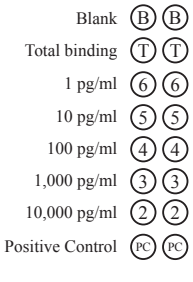


Figure 3. Immunoplate loading map

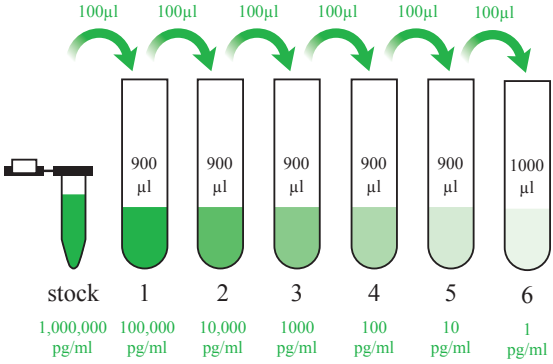


Figure 4. Visual guide of the standard dilutions

8. Add 50µl of the least concentrated peptide standard solution (#6) to wells C1 and C2. Next, add peptide standard #5 into wells D1 and D2, and so forth, continuing in the opposite order of the standard dilution.

Note: Standard peptides should always be assayed in duplicate.

9. Add 50µl of rehydrated positive control into wells H1 and H2.

Note: Positive controls should always be assayed in duplicate.

10. Add 50µl of any unknown/prepared samples into their designated wells, again in duplicate.

Note: Each laboratory must determine the appropriate dilution factors and preparation for their samples to ensure that peptide levels are detectable and within the linear range of the standard curve.

11. Add 25µl of rehydrated primary antibody into each well **except** the blank wells (A1 and A2).

Note: A multi-channel pipette is **NOT** recommended to load the primary antibody.

12. Seal the immunoplate with an acetate plate sealer (APS). Incubate the immunoplate overnight (approximately 16-24 hours) at 4°C.

Note: Please wait 16 to 24 hours before continuing with the assay protocol.

13. Reconstitute the biotinylated peptide with 1x assay buffer and vortex thoroughly. **Refer to the QC data sheet for specific instructions on rehydrating the biotinylated peptide.** Allow the solution to sit for at least 5 minutes at room temperature to completely dissolve.

14. Remove the APS from the immunoplate. Do **NOT** wash or the immunoplate or discard the contents of the wells.

15. Add 25µl of rehydrated biotinylated peptide into each well **except** the blank wells (A1 and A2).

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

16. Reseal the immunoplate with an APS. Incubate for 1.5 hours at room temperature (20-23°C).

Note: Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.

17. Centrifuge the SA-HRP vial (3,000-5,000 rpm for 5 seconds) to mix. Pipette 12µl of SA-HRP into 12ml of 1x assay buffer and vortex the solution thoroughly.

18. Remove the APS from the immunoplate and discard the contents of the wells. Wash each well with 350µl of 1x assay buffer, discard the buffer, invert the immunoplate, and blot the plate dry. Repeat 4 times.

19. Add 100µl of SA-HRP solution into each well.

20. Reseal the immunoplate with an APS. Incubate for 1 hour at room temperature (20-23°C).

Note: Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.

21. Mix 9 parts substrate solution with 1 part stable peroxide solution. This working substrate solution is stable for 24 hours at room temperature (20-23°C).

22. Remove the APS from the immunoplate and discard the contents of the wells. Wash each well with 350µl of 1x assay buffer, discard the buffer, invert the immunoplate, and blot the plate dry. Repeat 4 times.

23. Add 100µl of the working substrate solution into each well. Gently tap the immunoplate to ensure thorough mixing.

24. Reseal the immunoplate with an APS. Incubate for 15-20 minutes at room temperature (20-23°C).

Note: Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.

25. Remove the APS from the immunoplate. Do **NOT** wash or the immunoplate or discard the contents of the wells.

26. Add 100µl of stop solution into each well to stop the reaction. Gently tap the plate to ensure thorough mixing.

Note: Proceed to the next step within 20 minutes.

27. Load the immunoplate onto a fluorescence microtiter plate reader and measure the relative fluorescence units (RFU) of each well.

Note: The excitation and emission maxima for the working substrate solution are 325nm and 420nm, respectively. Wavelengths between 315nm and 340nm for excitation, and 370nm and 470nm for emission can also be used for measurements.

ADDITIONAL RECOMMENDATIONS

- Reagents of different lot numbers should never be mixed.
- Plasma, serum, culture media, tissue homogenate, CSF, urine, or any biological fluid can be assayed as long as the level of peptide in the sample is high enough for the sensitivity of the specific kit.
- 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. Please consult the literature for specific methodology.
- When handling the plate, avoid touching the bottom. Any fingerprints or blots may affect the O.D. readings.
- 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.
- 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 its container a few times to wet the pipette walls prior to loading the pipette.
- Avoid submerging the whole pipette tip into reagents and samples. Droplets can accumulate at the end of the tip, causing an excess of solution to be loaded into the well and affecting assay results.
- Performing this procedure outside of the recommended room temperature (20-23°C) may affect assay results.
- Fluorometric units are typically defined as relative fluorescence units (RFU) because the integrated signal is dependent on instrument settings. Please consult the fluorescence microtiter plate reader's user manual for specific instrument capabilities and settings.
- Any modifications to the existing protocol (i.e. standard dilutions, pipetting technique, washing technique, incubation time or temperature, storage conditions, and kit expiration) may affect the sensitivity, specificty and results of the assay.

CALCULATION OF RESULTS

1. Label the X-axis (log scale) with the concentration of standards #6 through #2 (1 to 10,000 pg/ml).
2. Label the Y-axis (linear scale) as relative fluorescence units (RFU).
3. Average all duplicate readings (standards, positive control, samples) and substract the average blank RFU reading.
4. Plot the RFU measurement for each standard peptide concentration directly above its X-axis coordinate.
5. Draw the best fit curve through these data points. This standard curve will have a reverse sigmoidal shape. It should show an inverse relationship between peptide concentration and absorbance. As the standard peptide concentration increases, fluorescence intensity will decrease.

   Note: We strongly recommend using curve-fitting software capable of 4 parameter logistics or log-logit functionality.
6. To determine the peptide concentration in any unknown samples, first locate its RFU measurement on the Y-axis. Draw a horizontal line across the graph from that RFU measurement to the intersection with the standard curve. The X-axis coordinate at this intersection point will correspond to the peptide concentration (pg/ml) in the assayed sample.

   Note: Multiply the calculated peptide concentration by any concentrating or dilution factor(s) used while preparing the original sample.
7. Refer to the QC Data Sheet for acceptable values of the positive controls.

SUGGESTED METHOD FOR PEPTIDE EXTRACTION

General Blood Withdrawal and Plasma Collection:

1. Collect blood samples into Lavendar Vacutainer tubes (Catalog no. VT-6450), which contain EDTA and can hold up to 7ml of blood.
2. Gently rock the Lavendar Vacutainer tubes several times immediately after collection of blood to avoid coagulation.
3. Transfer the blood to centrifuge tubes containing aprotinin (Catalog no. RK-APRO), and gently rock several more times to inhibit the activity of proteases.

   Note: We recommend 0.6 TIU, or 100µl, of Aprotinin per 1ml of blood collected. If the Lavendar Vacutainer tubes are centrifuge-safe, the Aprotinin may be added directly to them.
4. Centrifuge the blood at 1,600 x g for 15 minutes at 4°C and collect the plasma.

   Note: Plasma can be kept at -70°C and will remain stable for up to one month.
5. For peptide extraction from sample, acidify the plasma with an equal amount of Buffer A (Catalog no. RK-BA-1). Mix and centrifuge at 6,000 to 17,000 x g for 20 minutes at 4°C. This will be loaded onto the C-18 SEP-COLUMN.

   Note: We recommend using at least 1ml of plasma for peptide extraction. It may be possible to perform the extraction using smaller volumes as long as volumes of reconstituting and eluting buffer are adjusted accordingly.

General Tissue Preparation:

1. Boil tissue in 75% HoAc (Acetic Acid) for 20 minutes at 100°C.
2. Homogenize tissue in lysis buffer, usually with a low pH.
3. Centrifuge the tissue homogenate at 12,000rpm for 20 to 30 minutes at 4°C.
4. For peptide extraction from sample, take 1ml of supernatant and combine with 1ml of Buffer A (Catalog no. RK-BA-1) to acidify sample. Centrifuge at 6,000 to 17,000 x g for 20 minutes and collect the supernatant. This will be loaded onto the C-18 SEP-COLUMN. Performing the centrifugation on ice helps to inhibit peptidases.

   Note: If a separate protein assay is required, designate and remove an aliquot before addition of Buffer A. This buffer contains materials which may interfere with protein analysis.

Extraction of Peptides from Sample:

1. Equilibrate a SEP-COLUMN containing 200mg of C18 (Catalog no. RK-SEPCOL-1). Wash with 1ml of Buffer B (Catalog no. RK-BB-1) once, followed by 3ml of Buffer A three (3) times.
2. Load the acidified sample (plasma, serum, tissue, etc.) solution onto the pre-equilibrated C-18 SEP-COLUMN.
3. Slowly wash the column with 3ml of Buffer A twice and discard the wash.
4. Elute the peptide slowly with 3ml of Buffer B once and collect the eluant into a polystyrene tube.

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

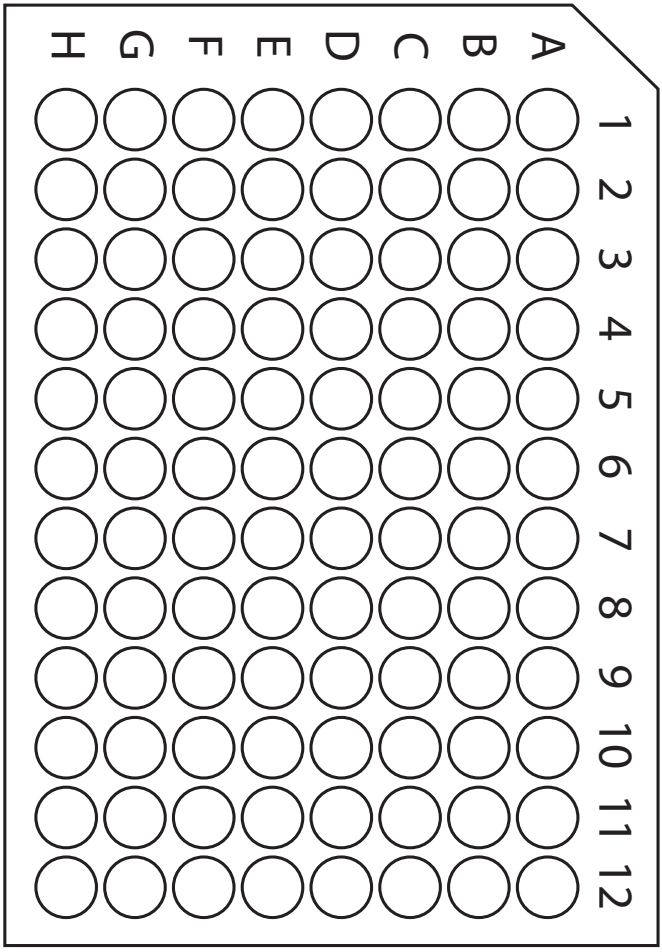
5. Evaporate eluant to dryness in a centrifugal concentrator or by a suitable substitute method.

   Note: A combination of a centrifugal concentrator (i.e. Speedvac) and a lyophilizer (freeze-dryer) produces the best results. First, use a centrifugal concentrator to dry the sample for approximately 15 minutes, removing the organic layer. Snap-freeze the remaining sample and freeze-dry overnight using a lyophilizer. If a centrifugal concentrator is not accessible, freeze-drying overnight using a lyophilizer will be sufficient.

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

   Note: For example, if 1ml of plasma was extracted, dried, and then reconstituted in 250µl of 1x assay buffer, then the original sample would have now have undergone a 4x concentration.

ASSAY DIAGRAM



USA

Phoenix Pharmaceuticals, Inc.

330 Beach Rd.
Burlingame, California 94010
Tel: 650-558-8898, 1-800-988-1205
Fax: 650-558-1686
info@phoenixpeptide.com
www.phoenixpeptide.com

Europe

Phoenix Europe GmbH

Viktoriastrasse 3-5
D-76133 Karlsruhe
Germany
Tel: +49 (721) 12 08 150
Fax: +49 (721) 12 08 15 15
europe@phoenixpeptide.eu