

INSL 5 (Human)

ENZYME IMMUNOASSAY KIT PROTOCOL

(Cat. # EK-035-70)
(Range: 0-100ng/ml)



PHOENIX PHARMACEUTICALS, INC.

TABLE OF CONTENTS

| | |
|--|-----------|
| 1. Kit Contents | 2 |
| 2. Additional Materials, Not Included | 2 |
| 3. Storage | 3 |
| 4. References | 3 |
| 5. Introduction | 4 |
| 6. General Design of the Kit | 4 |
| 7. Summary of Assay Protocol | 5 |
| 8. Assay Protocol | 6 |
| A. Protein and Antibody Rehydration..... | 6 |
| B. Standard Protein Dilutions | 7 |
| C. Immunoplate Loading | 8 |
| D. Immunoplate Incubations..... | 8 |
| 9. Additional Recommendations | 9 |
| 10. Calculation of Results | 10 |

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, 50ml)..... **Catalog no. EK-BUF**
2. Pre-coated EIA plate (96 wells, 1 plate).....**Catalog no. EK-Plate**
3. Acetate plate sealer (APS) (3 pieces).....**Catalog no. EK-APS**
4. Primary INSL 5 antibody (1 vial).....**Catalog no. EK-RAB-051-60**
5. Standard INSL 5 protein (500 μ l).. **Catalog no. EK-S-051-60**
6. Biotinylated INSL 5 (1 vial)..... **Catalog no. EK-B-051-60**
7. Positive control (2 vials)..... **Catalog no. EK-PC-051-60**
8. Streptavidin-horseradish peroxidase (SA-HRP) concentrate (30 μ l) **Catalog no. EK-SA-HRP**
9. Substrate solution (12ml).....**Catalog no. EK-TMB**
10. 2N HCl (Stop solution) (15ml).**Catalog no. EK-HCl**
11. Protocol Booklet (1 book).

ADDITIONAL MATERIALS, NOT INCLUDED

1. Microtiter plate reader (450nm) (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 (50-100 μ l) (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**

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 desiccant, 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 protein, biotinylated protein, 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).

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 concentrations.

INTRODUCTION

This kit is designed to measure the concentration of INSL 5 and its related proteins 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. This primary antibody's Fab fragment will then be competitively bound by both the biotinylated protein and the targeted protein in either the standard protein solution or the unknown sample. The biotinylated protein interacts with streptavidin-horseradish peroxidase (SA-HRP) which catalyzes the substrate solution. The intensity of the resulting yellow color is directly proportional to the amount of biotinylated protein-SA-HRP complex, but inversely proportional to the amount of the targeted protein (in either the standard protein solution or the unknown sample). This is due to competition between the biotinylated protein and the target protein 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 protein concentrations. Unknown protein concentration in samples can then be determined via extrapolation based on this standard curve.

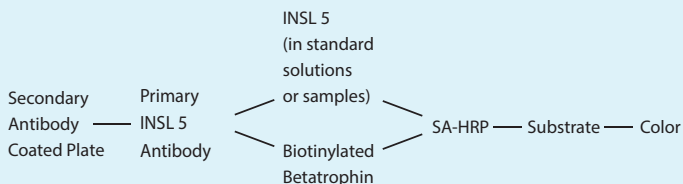


Figure 1. Diagram of the molecular interactions used in this kit

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.

SUMMARY OF ASSAY PROTOCOL

Add 50 μ l/well of standard, sample, or positive control, along with 25 μ l/well of primary INSL 5 antibody



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



Add 25 μ l/well of biotinylated INSL 5



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



Wash immunoplate 4 times with 350 μ l/well of 1x assay buffer



Add 100 μ l/well of SA-HRP solution



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



Wash immunoplate 4 times with 350 μ l/well of 1x assay buffer



Add 100 μ l/well of TMB substrate solution



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



Terminate reaction with 100 μ l/well of 2N HCl



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

ASSAY PROTOCOL

Note: The kit and all its components should be equilibrated to room temperature (20-23°C) 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 lyophilized material is at the bottom of the tube.

1. Dilute the 20x EIA assay 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. Centrifuge and dilute the INSL 5 standard 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 completely dissolve in solution. Vortex and centrifuge immediately before use.
3. Reconstitute the primary INSL 5 antibody in 5ml of 1x assay buffer and vortex thoroughly. Allow the solution to sit for at least 5 minutes at room temperature to completely dissolve.
4. Reconstitute the positive control in 200µl of 1x assay buffer and vortex thoroughly. 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 protein as shown in Figure 2 and Figure 4 on the next page. 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 protein).
8. Add 50µl of the least concentrated protein standard solution (#5) to wells C1 and C2. Next, add protein standard #4 into wells D1 and D2, and so forth, continuing in the opposite order of the standard dilution.

Note: Standard proteins should always be assayed in duplicate.

Prepare protein standard solutions as follows:

| Standard ID / Number | 1x Assay Buffer Volume | Standard Protein Volume | Concentration |
|----------------------|------------------------|-------------------------|---------------|
| Stock | -- | 500 μ l (Liquid) | 1000ng/ml |
| #1 | 900 μ l | 100 μ l of stock | 100ng/ml |
| #2 | 900 μ l | 100 μ l of #1 | 10ng/ml |
| #3 | 900 μ l | 100 μ l of #2 | 1ng/ml |
| #4 | 900 μ l | 100 μ l of #3 | 0.1ng/ml |
| #5 | 900 μ l | 100 μ l of #4 | 0.01ng/ml |

Figure 2. Table of the standard dilutions

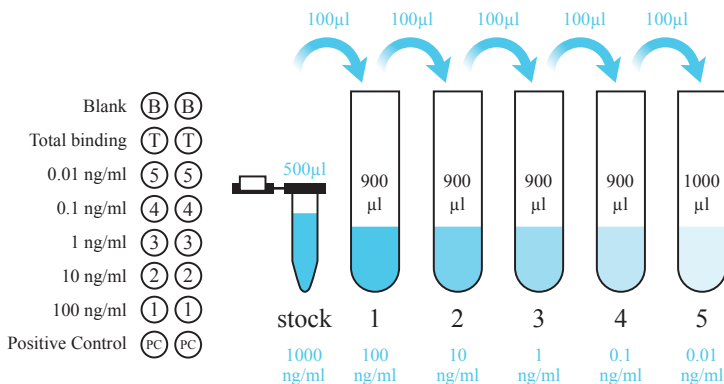


Figure 3. Immunoplate loading map

Figure 4. Visual guide of the standard dilutions

9. Add 50 μ l of rehydrated positive control into wells H-1 and H-2.

Note: Positive controls should always be assayed in duplicate.

10. Add 50 μ l 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 protein levels are detectable and within the linear range of the standard curve.

11. Add 25 μ l 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.

(The next day)

13. Reconstitute the biotinylated INSL 5 in 5ml of 1x assay buffer and vortex thoroughly. Allow the solution to sit for at least 5 minutes at room temperature to completely dissolve.

14. Remove the APS from Immunoplate. **DO NOT WASH THE PLATE.** Add 25 μ l of rehydrated biotinylated protein into each well **except** the blank wells (A1 and A2).

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

15. Seal the immunoplate with an acetate plate sealer (APS). Incubate the immunoplate for 2 hours at room temperature (20-23°C).

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

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

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

18. Add 100 μ l of SA-HRP solution into each well.

19. 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.

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

21. Add 100 μ l of the TMB substrate solution into each well.

Note: TMB is light-sensitive. After the addition of the TMB substrate solution, it is strongly recommended to cover the immunoplate to protect it from light.

22. 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.

23. Remove the APS from the immunoplate. Do **NOT** wash or the immunoplate or discard the contents of the wells.
24. Add 100µl of 2N HCl into each well to stop the reaction. The color in the wells should change from blue to yellow. Gently tap the plate to ensure thorough mixing.

Note: Proceed to the next step within 20 minutes.

25. Load the immunoplate onto a microtiter plate reader and measure absorbance O.D. at 450nm.

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 protein 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.
- 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, specificity and results of the assay.

CALCULATION OF RESULTS

1. Label the X-axis (log scale) with the concentration of standards #5 through #1 (0.01 to 100 ng/ml).
2. Label the Y-axis (linear scale) as absorbance (O.D.) at 450nm.
3. Average all duplicate readings (standards, positive control, samples) and subtract the average blank O.D. reading.
4. Plot the O.D. for each standard protein concentration directly above its X-axis coordinate.
5. Draw the best fit curve through these data points. It should show a direct relationship between protein concentration and absorbance. As the standard protein concentration increases, the yellow color decreases, thereby reducing absorbance (O.D.).

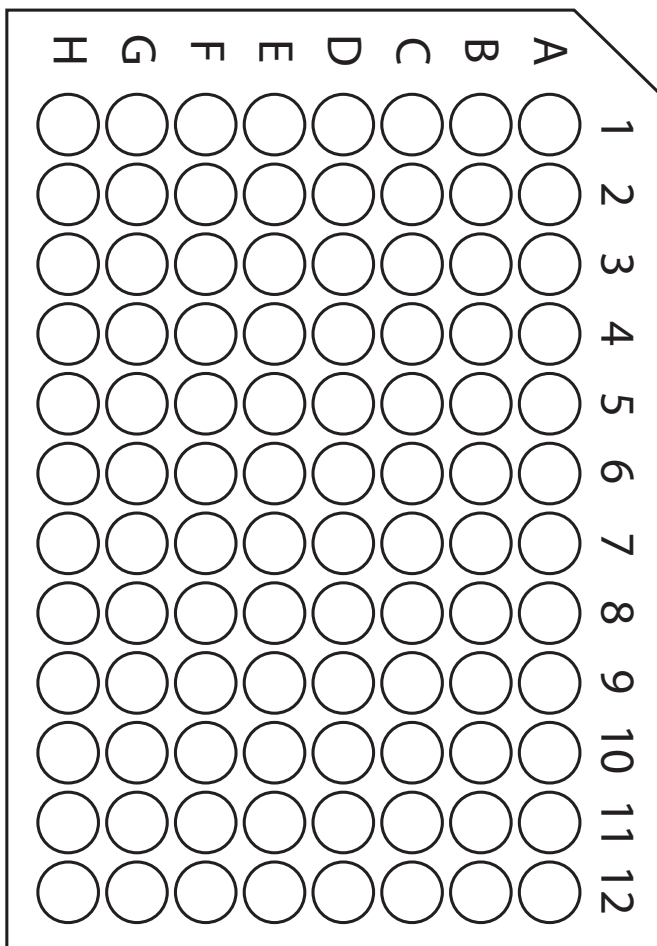
Note: We strongly recommend using curve-fitting software capable of 4 parameter logistics or log-logit functionality.

6. To determine the protein concentration in any unknown samples, first locate its absorbance (O.D.) on the Y-axis. Draw a horizontal line across the graph from that absorbance to the intersection with the standard curve. The X-axis coordinate at this intersection point will correspond to the protein concentration (ng/ml) in the assayed sample.

Note: Multiply the measured protein concentration by any dilution factor(s) used while preparing the original sample.

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

ASSAY DIAGRAM



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