Version 26-02/25

User's Manual

Human INSL5, short A- & B-Chains ELISA Kit

REF DEIA-PP9

Σ 96T

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

This kit is designed to measure the concentration of INSL 5 and its related proteins based on the principle of a "competitive" enzyme immunoassay.

Principles of Testing

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 pro-portional 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.

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

Reagents And Materials Provided

1. EIA assay buffer concentrate (20x, 50ml)
2. Pre-coated EIA plate (96 wells, 1 plate)
3. Acetate plate sealer (APS) (3 pieces)
4. Primary INSL5 antibody (1 vial)
5. Standard INSL5 peptide (500ul)
6. Biotinylated INSL5 (1 vial)
7. Positive control (2 vials)
8. Streptavidin-horseradish peroxidase (SA-HRP) concentrate (30µl)
9. Substrate solution (12ml)
10. 2N HCl (Stop solution) (15ml)
11. Protocol Booklet (1 book)

Materials Required But Not Supplied

1. Microtiter plate reader (450nm) (required)
2. Micropipette with disposable pipette tips (required)
3. Absorbent material for blotting (required)
4. Vortex (required)

5. Curve-fi tting 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)
12. Aprotinin (30 TIU) (optional)

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 assay buffer at 4°C.
4. Remove any unneeded strips from antibody-coated plate, reseal them in zip-lock foil and keep at 4°C.
5. Keep rehydrated solution of Standard, Biotinylated peptide, Antibody and SA-HRP at 4°C.

Specimen Collection And Preparation

SUGGESTED METHOD FOR THE EXTRACTION OF PEPTIDES FROM PLASMA:

Blood Withdrawal:

Collect blood samples into the Lavender Vacutainer tubes which contain EDTA and can collect up to 7ml of blood. Gently rock the Lavender Vacutainer tubes several times immediately after collection of blood for anti-coagulation. Transfer the blood from the Lavender Vacutainer tubes to the 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. If Lavender Vacutainer tubes are centrifuge-safe, the aprotinin may be added into the initial collection step.

Extraction of Peptides 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 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-equilibrated C-18 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 the eluant into a polystyrene 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 the assay as soon as possible. Use 1x assay buffer to reconstitute the dried extract. If the peptide value does not fall within the range of detection, dilute or

concentrate the sample accordingly.

Tips for extraction of plasma:

When using a C-18 SEP-COLUMN for the first time, use a bulb (not supplied) to apply pressure to the column after the addition of 1ml of buffer B to facilitate the flow through the column. From steps 3-5, no pressure should be applied.

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

Drying Sample After Extraction:

A combination of a centrifugal concentrator (i.e. Speedvac) and a lyophilizer (freeze-dryer) produces the best results for drying the sample after extraction. First, use a Speedvac to dry sample for approximately 15 minutes to remove the organic layer. Then snap-freeze the remaining sample, and freeze-dry overnight using a lyophilizer. This two-step procedure produces a more consistent fluffy powder that is easier to rehydrate than a sample dried only with a centrifugal concentrator. However, if a centrifugal concentrator is not accessible, freeze-drying overnight using a lyophilizer will be sufficient.

Assay Procedure

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 lypholized material is at the bottom of the tube.

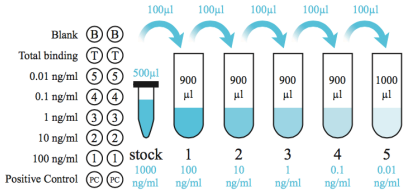
1. Dilute the 20x EIA assay buffer concentrate with 950ml of distilled water. Mix thoroughly before use. This will be the 1x assay buffer solution used to dilute or reconstitute all other samples and reagents during the assay.

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 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 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	--	500µl (Liquid)	1000ng/ml
#1	900µl	100µl of stock	100ng/ml
#2	900µl	100µl of #1	10ng/ml
#3	900µl	100µl of #2	1ng/ml
#4	900µl	100µl of #3	0.1ng/ml
#5	900µl	100µl of #4	0.01ng/ml

9. Add 50µl of rehydrated positive control into wells H-1 and H-2. 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 protein 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.

(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 25µl 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 12µl 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 350µl of 1x assay buffer, discard the buffer, invert the immunoplate, and blot the plate dry. Repeat 4 times.
18. Add 100µl 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 350µl of 1x assay buffer, discard the buffer, invert the immunoplate, and blot the plate dry. Repeat 4 times.
21. Add 100µl 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 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.

- 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

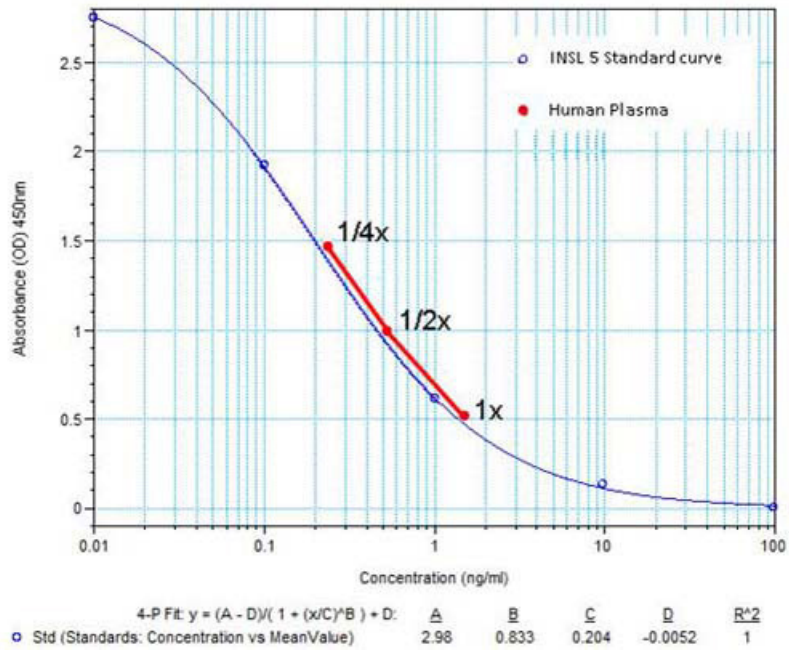
1. Label the X-axis (log scale) with the concentration of standards #5 through #1 (0.01 to 100 ng/ml).
2. Label the Y-axis (linear scale) as absorbance (O.D.) at 450nm.
3. Average all duplicate readings (standards, positive control, samples) and substract the average blank O.D. reading.
4. Plot the O.D. 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.



Precision

Intra-assay Precision (Precision within an assay): CV%<10%

Inter-assay Precision (Precision between assays): CV%<15%

Detection Range

0-100 ng/ml

Specificity

Peptide Cross-reactivity

INSL 5 (Human): 100%

Insulin (Human): 0

INSL 3 (Human): 0

INSL 4 (Human): 0

INSL 6 (Human): 0

INSL 7 (Relaxin 3): (Human): 0

Relaxin 2 (Human): 0

Vesiculin (Human): 0

Ghrelin (Human): 0

GLP-1 (Human): 0

PYY (3-36): (Human): 0