

## Rat Insulin Like Protein 3 (INSL3) CLIA Kit

**Catalog No.:** abx490511

**Size:** 96T

**Range:** 39.1 pg/ml - 10000 pg/ml

**Sensitivity:** < 14.4 pg/ml

**Storage:** Store standard, detection reagent A, detection reagent B and the 96-well plate at -20°C, and the rest of the kit components at 4°C.

**Application:** For quantitative detection of INSL3 in Rat Serum, Plasma, Tissue Homogenates, Cell Lysates, Cell Culture Supernatants and other biological fluids.

**Introduction:** Insulin-Like Protein 3 (INSL3) is a protein thought to be involved in the construction of the male and female urogenital tract. It binds to Relaxin Receptor 2 (RXFP2, or LGR8) to mediate the development of the gubernaculum. Mutations in the INSL3 gene are associated with cryptorchidism, where the testicles fail to form or descend properly; similar issues may occur in Klinefelter (XXY) syndrome, where the expression of INSL3 is reduced. Male INSL3-knockout mice had a similar phenotype.

### Principle of the Assay

This kit is based on competitive chemiluminescent immunoassay technology. INSL3 antibody is pre-coated onto a 96-well plate. A competitive inhibition reaction is launched between biotin labeled INSL3 and unlabeled INSL3 with the pre-coated antibody specific to INSL3. After washing away the unbound conjugates, avidin conjugated to Horseradish Peroxidase is added to each microplate well and incubated. After the mixture of substrate A and B is added only wells that contain biotin labeled INSL3 will produce chemiluminescence. The intensity of the emitted light is inversely proportional to the amount of INSL3 in the sample or standard.

### Kit components

1. One pre-coated 96-well microplate (12 × 8 well strips)
2. Standard: 2 tubes
3. Standard Diluent Buffer: 20 ml
4. Wash Buffer (30X): 20 ml. Dilution: 1:30
5. Detection Reagent A (100X): 120 µl
6. Detection Reagent B (100X): 120 µl
7. Diluent A: 12 ml
8. Diluent B: 12 ml
9. Substrate A: 10 ml
10. Substrate B: 2 ml
11. Plate sealer: 4

### Material Required But Not Provided

1. 37°C incubator
2. Luminometer capable of reading 96-well microplates (lag time 30.0 secs and read time 1.0 sec/well)
3. Multi and single channel pipettes and sterile pipette tips
4. Squirt bottle or automated microplate washer
5. Deionized or distilled water
6. Tubes to prepare standard or sample dilutions
7. Absorbent filter papers
8. 100 ml and 1 L volume graduated cylinders

## Protocol

### A. Preparation of sample and reagents

#### 1. Sample

Isolate the test samples soon after collecting, analyze immediately or store at 4°C for up to 5 days. Otherwise, store at -20°C for up to one month or -80°C for up to two months to avoid loss of bioactivity. Avoid multiple freeze-thaw cycles.

- **Serum:** Samples should be collected into a serum separator tube. Coagulate the serum by leaving the tube undisturbed in a vertical position overnight at 4°C or at room temperature for up to 60 minutes. Centrifuge at approximately 1000 × g for 20 min. Analyze the serum immediately or aliquot and store at -20°C or -80°C.
- **Plasma:** Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 × g within 30 minutes of collection. Assay immediately or aliquot and store at -20°C or -80°C. Avoid hemolysis and high cholesterol samples.
- **Tissue homogenates:** The preparation of tissue homogenates will vary depending upon tissue type – this is just an example. Rinse tissues with ice-cold PBS to remove the excess of blood. Weigh before homogenization. Finely mince tissues and homogenize with a tissue homogenizer on ice in PBS and sonicate the cell suspension. Centrifuge the homogenates at 5000 × g for 5 min and collect the supernatant. Assay immediately or aliquot and store at -20°C.
- **Cell lysates:** Detach adherent cells with trypsin and collect by centrifugation and remove the supernatant. Wash the cells three times in ice-cold PBS and re-suspend cells in PBS. Lyse the cells by ultra-sonification 4 times or freeze at -20°C and thaw to room temperature 3 times. Centrifuge at 1500 × g for 10 min at 2-8°C to remove cellular debris. Collect the supernatant and assay immediately.
- **Cell culture supernatants:** Centrifuge at approximately 1000 × g for 20 min to remove precipitant. Analyze immediately or aliquot and store at -20°C or -80°C.
- **Other biological fluids:** Centrifuge at approximately 1000 × g for 20 min to remove precipitant. Analyze immediately or aliquot and store at -20°C or -80°C.

#### Note:

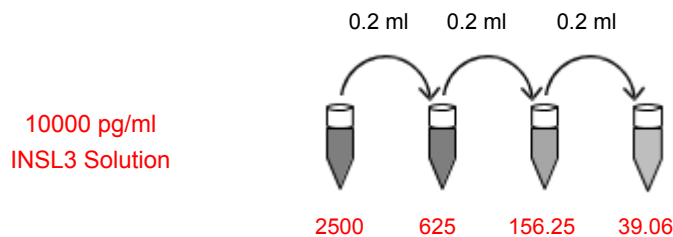
- » Bring sample slowly to room temperature. Sample hemolysis will influence the result. Hemolyzed specimen should not be used.
- » Samples must be diluted so that the expected concentration falls within the kit's range. Sample should be diluted in 0.01 mol/L PBS (PH=7.0-7.2).
- » If the sample are not indicated in the manual's applications, a preliminary experiment to determine the validity of the kit will be necessary.
- » Fresh sample or recently obtained samples are recommended to prevent protein degradation and denaturalization that may lead to erroneous results. For better detection, it is highly recommended to use serum instead of plasma.
- » Always use non-pyrogenic, endotoxin-free tubes for blood collection.

#### 2. Wash buffer

Dilute the concentrated Wash buffer 30-fold (1/30) with distilled water (i.e. add 20 ml of concentrated wash buffer into 580 ml of distilled water).

#### 3. Standard

Bring samples and all kit components to room temperature. Prepare the Standard with 0.5 ml of Standard Diluent buffer (kept for 10 min at room temperature) to make the 10000 pg/ml Standard Solution. Allow the reconstituted standard to sit for 10 minutes with gentle agitation prior to carrying out the serial dilutions; avoiding foaming or bubbles. Label 4 tubes with 2500 pg/ml, 625 pg/ml, 156.25 pg/ml, 39.06 pg/ml. Aliquot 0.6 ml of the Standard diluent buffer into each tube. Add 0.2 ml of 10000 pg/ml standard solution into the 1st tube and mix thoroughly. Transfer 0.2 ml from 1st tube to 2nd tube, mix thoroughly, and so on.



#### 4. Detection Reagent A and B Preparation

Centrifuge Detection Reagent A and B briefly before use. Detection Reagent A and B should be diluted 100-fold with Diluent A and B respectively, and mixed thoroughly. They are sticky solutions, therefore pipette with a slow, smooth action to reduce volume errors. The solution should be prepared no more than 15 minutes prior to the experiment. Please discard after use.

#### 5. Substrate working solution Preparation

Substrate A and B should be mixed with a ratio of 99:1 respectively and mix thoroughly within 15 minutes of use. For example, prepare 1 ml of substrate working solution by mixing 0.99 ml of Substrate A and 0.01 ml of Substrate B. Discard any unused, prepared Substrate Solution.

#### B. Assay Procedure

Equilibrate the kit components and samples to room temperature before use. It is recommended to plot a standard curve for each test.

1. Set standard, test sample and control (zero) wells on the pre-coated plate and record their positions. It is recommended to measure each standard and sample in duplicate or triplicate. Add the solution at the bottom of each well without touching the side walls. Mix the standards and samples up and down to be homogeneous before adding into the wells but avoid adding bubbles.
2. Add 50  $\mu$ l of the diluted standards into the standard wells. Aliquot 50  $\mu$ l Standard Diluent Buffer to the control (zero) well.
3. Add 50  $\mu$ l of appropriately diluted sample into the test sample wells. Add the solution at the bottom of each well without touching the side wall. Shake the plate mildly to mix thoroughly.
4. Immediately aliquot 50  $\mu$ l of Detection Reagent A working solution (if it appears cloudy mix gently until the solution is uniform) to each well. Shake the plate gently to mix thoroughly (a microplate shaker is recommended).
5. Seal the plate with a cover and incubate for 1 h at 37°C.
6. Remove the cover and discard the solution. Wash the plate 3 times with 1X Wash Buffer. Fill each well completely with Wash buffer (350  $\mu$ l) using a multi-channel Pipette or autowasher (1-2 minute soaking period is recommended). Complete removal of liquid at each step is essential for good performance. After the final wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean absorbent paper towels.
7. Aliquot 100  $\mu$ l of Detection Reagent B working solution into each well, seal and incubate at 37°C for 30 min.
8. Repeat the aspiration/wash process 5 times as explained in step 6.
9. Aliquot 100  $\mu$ l of Substrate working solution into each well. Seal the plate with a cover and incubate at 37°C for 10 min. Avoid exposure to light. The incubation time is for reference only, the optimal time should be determined by the end user.
10. Measure the chemiluminescence signal in a microplate luminometer immediately.

This assay is competitive, therefore there is an inverse correlation between INSL3 concentration in the sample and the RLUs (Relative Light Units) measured. Create a graph with the log of the standard concentration (y-axis) and average absorbance

measured (x-axis). Apply a best fit trendline through the standard points. The INSL3 concentration of the samples can be interpolated from the standard curve.

**Note:** If the samples measured were diluted, multiply the dilution factor by the interpolated concentration of the sample to obtain the concentration before dilution.

## C. Precautions

1. Before using the kit, centrifuge the tubes briefly to bring down the contents trapped in the lid.
2. If crystals have formed in the concentrated Wash Buffer, warm to room temperature and mix gently until the crystals have completely dissolved.
3. Avoid foaming or bubbles when mixing or reconstituting components. Prepare the Standard dilutions within 15 min of use and discard any unused working standards. For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 10 minutes.
4. It is recommended measuring each standard and sample in duplicate.
5. Do not let the wells uncovered for extended periods between incubation. Once reagents are added to the wells, avoid letting the strips dry as this can inactivate the biological material on the plate. Incubation time and temperature must be controlled.
6. Ensure plates are properly sealed or covered during incubation steps.
7. Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings.
8. Do not reuse pipette tips and tubes to avoid cross contamination.
9. Do not vortex the standard during reconstitution, as this will destabilize the protein. Once your standard has been reconstituted, it should be used right away. We do not recommend reusing the reconstituted standard.
10. The Substrate solution is easily contaminated; work under sterile conditions when handling the substrate solution. The Substrate A and B should also be protected from light. Unreacted substrate should be colorless or very light yellow in appearance. Aspirate the dosage needed with sterilized tips and do not dump the residual solution back into the vial.

## D. Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, medium and high levels of INSL3 were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, medium and high levels of INSL3 were tested on 3 different plates, 8 replicates in each plate.

$$CV (\%) = (\text{Standard Deviation} / \text{mean}) \times 100$$

Intra-Assay: CV<10%

Inter-Assay: CV<12%