

Rat Insulin (INS) ELISA

Cat No: K11-0708

Principle:

The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Rat (INS) in samples. Rat (INS) is added to wells pre-coated with monoclonal antibody. After incubation, add Rat (INS) antibodies labeled with HRP to form immune complex. Unbound HRP Conjugate is removed by washing step. Then addition of TMB Substrate develops blue colour, and stop solution is added to stop the reaction. The concentration Rat (INS) is directly proportional to the colour developed.

Intended Use:

This kit is used to assay the level of (INS) in rat serum, blood, plasma, and tissues. The Kit is For Laboratory / Research Use Only.

Materials provided in the Kit:

1. Rat Insulin monoclonal antibody coated microtiter plate (12x8 wells) - 1 no
2. Recombinant Rat Insulin Standard lyophilized, 24 mIU/ml - 1 vial
3. (1X) Standard Diluent - 10 ml
4. Rat Insulin Detection HRP Conjugate - 12ml
5. (20X) Wash Buffer - 25ml
6. (1X) Assay Diluent - 12 ml
7. TMB Substrate - 12ml
8. Stop Solution - 12ml
9. Instruction Manual

Materials to be provided by the End-User:

1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Graph paper or software for data analysis.
6. Tubes to prepare standard/sample dilutions.
7. Timer.
8. Absorbent paper.
9. Incubator

Storage Information:

1. All reagents should be stored at 2°C to 8°C. Store recombinant lyophilized standard at 2-8°C. Upon reconstitution aliquot standards into polypropylene vials and store at -20°C as per assay requirements. Do not freeze thaw for more than two times.
2. 30 minutes prior before use, bring all components to room temperature (18-25°C). Store all the components of the kit at its appropriate storage condition after use.
3. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details.
2. To reduce the likelihood of blood-borne transmission of infectious agents, handle all samples in accordance with NCCLS regulations.

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

1. The kit cannot test samples which contain NaN_3 , because NaN_3 inhibits HRP activity.
2. Extract as soon as possible after specimen collection as per relevant procedure. The samples should be tested as soon as possible after the extraction. Alternately the extracted samples can be kept in -20°C. Avoid repeated freeze-thaw cycles.
3. **Serum-** Coagulate at room temperature for 10-20 minutes; centrifuge for 20-min at 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.

- Plasma-** Use EDTA or citrate plasma as an anticoagulant, mix for 10-20 minutes; centrifuge for 20-min at the 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.

Samples should be diluted 1:100 (v/v) for optimal recovery, (for example 1 ul sample + 99 ul Assay Diluent (1X) prior to assay. In cases where matrix interferences is under or over observed, the samples may be diluted with Assay Diluent accordingly.

The samples may be kept at 2 - 8°C for up to three days. For long-term storage please store at -20°C.

Note: Grossly hemolyzed samples are not suitable for use in this assay

Reagent Preparation (all reagents should be diluted immediately prior to use):
Please refer to lot specific instructions for preparation of the reagents

Procedural Notes:

- In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
- High Dose Hook Effect may be observed in samples with very high concentrations of Rat Insulin. High Dose Hook Effect is due to excess of antibody for very high concentrations of Rat Insulin present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent. Thus if the Rat Insulin concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
- Avoid assay of Samples containing Sodium Azide (NaN₃), as it could destroy the HRP activity resulting in under-estimation of the amount of Rat Insulin.
- It is recommended that all Standards and Samples be assayed in duplicates.
- Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
- If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to poor sensitivity of the assay.
- The plates should be read within 30 minutes after adding the Stop Solution.
- Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:

- Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.
- Standards Preparation: Reconstitute the recombinant protein by adding 100 µl of Standard Diluent to give a concentration of 24 mIU/ml. Keep the standard for 15 mins with gentle agitation before making further dilutions. Add 40 ul of reconstituted standard (24mIU/ml) to 460 ul Standard Diluent (1X) to prepare 1920 uIU/ml standard solution. This is the top standard. Thus the Rat Insulin Standards concentrations are 1920uIU/ml, 960uIU/ml, 480uIU/ml, 240uIU/ml, 120uIU/ml, 60uIU/ml and 30uIU/ml. Standard Diluent (1X) serves as the zero standard (0 uIU/ml).

Standard Concentration	Standard No	Dilution Particulars
24 mIU/ml	Standard, concentrated	Original Standard (lyophilized) + 100ul Standard Diluent (1X)
1920uIU/ml	Standard No.7	40 ul Original Standard + 460 ul Standard Diluent (1X)
960uIU/ml	Standard No.6	250 ul Standard No. 7 + 250 ul Standard Diluent (1X)
480uIU/ml	Standard No.5	250 ul Standard No. 6 + 250 ul Standard Diluent (1X)
240uIU/ml	Standard No.4	250 ul Standard No. 5 + 250 ul Standard Diluent (1X)
120uIU/ml	Standard No.3	250 ul Standard No. 4 + 250 ul Standard Diluent (1X)
60uIU/ml	Standard No.2	250 ul Standard No. 3 + 250 ul Standard Diluent (1X)
30uIU/ml	Standard No.1	250 ul Standard No. 2 + 250 ul Standard Diluent (1X)
0 uIU/ml	Standard No.0	250 ul Standard Diluent (1X)

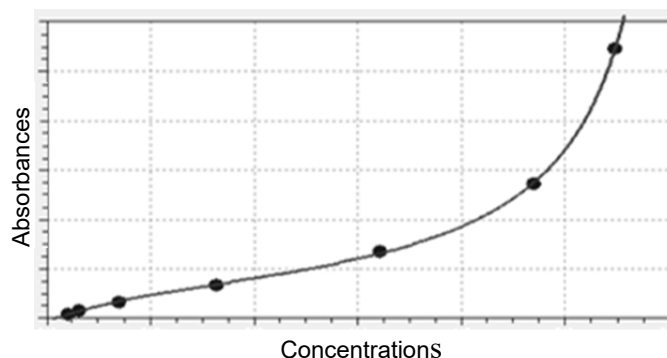
- Add 100ul/well of **Standards** and **Samples** to the plate, Seal plate and incubate for 1 hour at RT.

4. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.
5. Add 100µl of diluted **Detection Antibody** solution to each well, Seal plate and incubate for 1 hour at RT.
6. Wash plate 4 times with **Wash Buffer (1X)** as in step 4.
7. Add 100ul of **TMB Substrate** solution and incubate in the dark for 30 minutes at RT. Positive wells should turn bluish in color. It is not necessary to seal the plate during this step.
8. Stop reaction by adding 100ul of **Stop Solution** to each well. Positive wells should turn from blue to yellow.
9. Read absorbance at 450 nm within 30 minutes of stopping reaction.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Use the Net Absorbance (Absorbance of Standard/Sample - Absorbance of Blank) to calculate the Mean Absorbances. Using standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the concentration. If samples were diluted, multiply by the appropriate dilution factor.

Software which is able to generate a cubic spline curve-fit, 4PL or a polynomial regression to the 2nd order is best recommended for automated results.

Typical Graph**Precautions:**

Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

Performance Characteristics:

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

Sensitivity:

Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. 10 replicates of '0' standards were evaluated and the LOD was found to **30uIU/ml**.

Specificity:

The antibodies used in the kit for capture and detection are specific for Rat Insulin.

Assay Range:
30 - 1920 uIU/ml

Precision:
Intra-Assay: CV<10%
Inter-Assay: CV<12%

Dilutional Linearity:

The Linearity of the kit was assayed by testing samples spiked with 1920 uIU/ml concentration of Rat Insulin and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1:2	1:4	1:8
serum (n=5)	85-105%	86-109%	83-112%
EDTA plasma (n=5)	84-106%	85-117%	83-118%
heparin plasma (n=5)	83-99%	80-95%	82-93%

Note: The kit has not been validated for concentrations and dilutional linearity / recovery beyond the concentration of 1920uIU/ml. In case your samples have expected concentrations beyond this range, you may validate the same using the Standard Diluent provided in the kit. However, we do not warrant for linearity beyond the range indicated above.

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Rat Insulin ELISA

ASSAY PROCEDURE

1	Bring all reagents to room temperature before use.	
2	Pipette Standards 1 - 8 Samples	100 ul
3	Incubate 60 minutes (RT)	
4	1X Wash Buffer Decant, 4 x 300 ul	
5	Pipette Detection antibody	100 ul
6	Incubate 60 minutes (RT)	
7	1X Wash Buffer Decant, 4 x 300 ul	
9	Pipette TMB Substrate	100 ul
10	Incubate in the dark 30 minutes (RT)	
11	Pipette Stop Solution	100 ul
12	Measure 450nm within 30 mins	

Troubleshooting:

Problem	Possible cause	Investigation/Actions
High Absorbances	<ol style="list-style-type: none"> 1. Cross-contamination from other specimens 2. Insufficient or inefficient washing or reading 3. Wavelength of filter not correct. 4. High assay background. 5. Contaminated TMB 6. Incubation time too long or incubation temperature too high. 7. Incorrect dilution of serum 	<ul style="list-style-type: none"> > Repeat assay taking care when washing and pipetting. > Check washer efficiency > Check that the wavelength is 450nm. If a dual wavelength spectrophotometer is available, set the reference filter between 600-650 nm. > Repeat assay and include a well that contains only sample diluent or sample absorbent (i.e. a blank well). > Check that TMB is colorless or faint blue. > Check incubation time and temperature. > Check incubator is at the correct temperature. > Repeat assay, ensuring correct serum dilution is used.
Low Absorbances	<ol style="list-style-type: none"> 1. Incubation time too short or incubation temperature too low. 2. Incorrect dilution or pipetting of sera 3. Incorrect filter wavelength. 4. Contaminated Conjugate solution. 5. Kit has expired. 6. Air blank reading high. 7. Incorrect storage of kit. 8. Kit reagents not equilibrated at room temperature 9. Incorrect reagents used. 10. Over washing of plate (e.g. inclusion of a long soak step). 	<ul style="list-style-type: none"> > Ensure time and temperature of assay incubation are correct. > Check incubator is set at the correct temperature. > Repeat assay ensuring correct dilutions and volumes are used. > Ensure controls are sufficiently mixed. > Check the wavelength is set at 450nm. If a dual wavelength spectrophotometer is available, set the reference filter between 600-650nm. > Dispense conjugate directly from the bottle using clean pipette tip; avoid transferring Conjugate to another container if possible. > Do not return unused Conjugate to bottle. > Ensure all pipettes and probes used to dispense the Conjugates are clean and free from serum, detergent and bleach. > Check expiration date of kit and do not use if expired. Investigate causes of high background absorbance. > Ensure kit is stored at 2-8°C, plate is sealed in foil pouch and desiccant sachet is blue/purple. > Allow sufficient time for reagents to equilibrate to room temperature prior to assay. > Check the reagents used match those listed on the specification sheet. > Repeat assay using recommended wash procedure.
Poor Duplicates	<ol style="list-style-type: none"> 1. Poor mixing of samples. 2. Poor pipette precision 3. Addition of reagents at inconstant timing intervals; reagent addition takes too long, air bubbles when adding reagents. 4. Inefficient washing - Wash buffer left in wells, inconsistent washing, inadequate washing. 5. Reader not calibrated or warmed up prior to plate reading. 6. Optical pathway not clean 7. Spillage of liquid from wells 8. Serum samples exhibit microbial growth, haemolysis or lipaemia. 9. Uneven well volumes due to evaporation. 	<ul style="list-style-type: none"> > Mix reagents gently and equilibrate to room temperature. > Calibration may need to be checked. > Check pipetting technique-change pipette tip for each sample and ensure excess liquid is wiped from the outside of the tip. > Use consistent timing when adding reagents. > Ensure all dilutions are made before commencing addition to plate. > Improve pipetting technique and skill. > Tap out wash buffer after washing. > Check wells are sufficiently and uniformly filled and aspirated when washing. > Check reader precision > Check reader manual to ascertain warm up time of instrument. > Gently wipe bottom of plate. > Check reader light source and detector are clean. > Repeat assay, taking care not to knock the plate or splash liquid > It is not recommended to use serum samples exhibiting microbial growth, haemolysis or lipaemia. > Cover plate with a lid or plate sealer (not provided).
All wells yellow	<ol style="list-style-type: none"> 1. Contaminated TMB. 2. Contaminated reagents (e.g. Conjugate, Wash buffer). 3. Incorrect dilution of serum. 4. Incorrect storage of kit. 5. Inefficient washing- Wash buffer left in wells, inconsistent washing, inadequate washing. 6. If Conjugate reconstitute is required – Conjugate reconstituted incorrectly. 	<ul style="list-style-type: none"> > Check TMB is colorless or faint blue. > Check reagents for turbidity. > Repeat assay, ensuring correct serum dilution is used. > Ensure kit is stored at 2-8°C, plate is sealed in foil pouch and desiccant sachet is blue / purple. > Tap out wash buffer after washing. > Check wells are sufficiently and uniformly filled and aspirated when washing. > Repeat assay ensuring Conjugate is reconstituted according to assay method.

All wells negative

1. Test not performed correctly – correct reagents not added or not added in the correct sequence.
 - > Check procedure and check for unused reagents.
 - > Ensure that Stop Solution was not added before Conjugate or TMB.
 - > Ensure that serum was diluted in correct Sample diluent; e.g. do not use Sample Absorbent for an IgG ELISA.
2. Contaminated Conjugate solution.
 - > Dispense Conjugate directly from the bottle using a clean pipette tip; avoid transferring Conjugate to another container if possible.
 - > Do not return unused Conjugate to bottle.
 - > Ensure all pipettes and probes used to dispense the Conjugate are clean and free from serum, detergent and bleach.
 - > Repeat assay using recommended wash procedure.
3. Over- washing of plate (e.g. inclusion of a long soak step).
4. Incorrect storage of kit.
 - > Ensure kit is stored at 2-8°C, plate is sealed in foil pouch and desiccant sachet is blue / purple.
5. Wash Buffer made up with Stop Solution instead of Wash Buffer Concentrate
 - > Ensure Wash Buffer is made up correctly.