DRG® Insulin (rat/mouse) (EIA-4127)

Revised 1 Feb. 2011 rm (Vers. 2.1) **RUO**

Please use only the valid version of the package insert provided with the kit.

This kit is intended for Research Use Only.

Not for use in diagnostic procedures.

INTENDED USE

This Rat / Mouse Insulin ELISA kit is used for the non-radioactive determination of insulin in mouse and rat sera. Plasma samples may also be used but application to samples of other biological fluids may need validation by the user. One kit is sufficient to measure 39 unknown samples in duplicate.

PRINCIPLES OF PROCEDURE

This assay is a Sandwich ELISA based, sequentially, on: 1) capture of insulin molecules from samples to the wells of a microtiter plate coated by pre-titered amount of a monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin, 2) wash away of unbound materials from samples, 3) binding of horseradish peroxidase to the immobilized biotinylated antibodies, 4) wash away of free enzyme conjugates, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat insulin.

REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well plate including, in duplicates, background, 6 rat insulin standards, 2 quality controls and 39 unknown samples.

A. **Rat/Mouse Insulin ELISA Plate**
Coated with mouse monoclonal anti-rat insulin antibodies.
Quantity: 1 plate
Preparation: Ready to use.
Note: Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C.

B. Adhesive **Plate Sealer**
Quantity: 1 sheet
Preparation: Ready to use.

C. **10X HRP Wash Buffer Concentrate**
10X concentrate of 50 mM Tris Buffered Saline containing Tween-20.
Quantity: Two bottles containing 50 mL each
Preparation: Dilute 10 times with de-ionized water.

D. **Rat/Mouse Insulin Standards**
Rat insulin in Assay Buffer: 0.2, 0.5, 1, 2, 5 and 10 ng/mL.
Quantity: 0.25 mL/vial
Preparation: Ready to use.

E. **Rat/Mouse Insulin Quality Controls 1 and 2**
Rat insulin in QC buffer.
Quantity: 0.25 mL/vial
Preparation: Ready to use.

F. **Matrix Solution**
Charcoal stripped pooled mouse serum
Quantity: 0.5 mL
Preparation: Ready to use.

G. **Assay Buffer**
0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide, and 1% BSA.
Quantity: 20 mL
Preparation: Ready to use.

H. **Rat/Mouse Insulin Detection Antibody**
Pre-titered biotinylated anti-insulin antibody.
Quantity: 10 mL
Preparation: Ready to use.

I. **Enzyme Solution**
Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.
Quantity: 12 mL
Preparation: Ready to use.

J. **Substrate** (Light sensitive, avoid unnecessary exposure to light)
3, 3', 5, 5'-tetramethylbenzidine in buffer.
Quantity: 12 mL
Preparation: Ready to use.

K. **Stop Solution**
0.3 M HCl
Quantity: 12 mL
Preparation: Ready to use.

STORAGE AND STABILITY

Prior to use, all components in the kit can be stored up to 2 weeks at 2 -8°C.
For longer storage (> 2 weeks), freeze diluted Wash Buffer, Insulin Standards, Quality Controls, and Matrix Solution at <-20 °C.
Minimize repeated freeze and thaw of the Insulin Standards, Quality Controls and Matrix Solution.
Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8 °C.
Refer to expiration dates on all reagents prior to use.
Do not mix reagents from different kits unless they have the same lot numbers.

REAGENT PRECAUTIONS

Sodium Azide
Sodium Azide has been added to certain reagents as a preservative. Although the concentrations are low, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. Flush with a large volume of water to prevent azide build-up.

Hydrochloric Acid
Hydrochloric Acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eye. Do not swallow or ingest.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipette with tips, 10 µL -100 µL.
2. Multi-channel Pipette: 50 µL ~ 300 µL
3. Reagent Reservoirs
4. Vortex Mixer
5. Refrigerator
6. De-ionized Water
7. Microtiter Plate Reader capable of reading absorbency at 450 nm and 590 nm
8. Orbital Microtiter Plate Shaker
9. Absorbent Paper or Cloth

SAMPLE COLLECTION AND STORAGE

To prepare serum, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Let blood clot at room temperature for 30 min.
Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at 4 ± 2 °C.
Transfer and store serum samples in separate tubes. Date and identify each sample.
Use freshly prepared serum or aliquot and store samples at –20 ± 5 °C for later use. For long-term storage, keep at -70 °C. Avoid freeze/thaw cycles.

To prepare plasma sample, whole blood should be collected into centrifuge tubes containing enough K_3EDTA to achieve a final concentration of 1.735 mg/mL and centrifuged immediately after collection. Observe same precautions in the preparation of serum samples.
If heparin is to be used as anti-coagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.

Avoid using samples with gross hemolysis or lipemia.

ASSAY PROCEDURE

Pre-warm all reagents to room temperature prior to setting up assay.

1. Dilute the 10X Wash Buffer concentrate 10 fold by mixing the entire content of each bottle of Wash Buffer with 450 mL de-ionized water. (dilute both bottles with 900 mL deionized water)
2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C.
   Assemble strips in an empty plate holder and wash each well 3 times with 300 µL of diluted Wash Buffer per wash. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. **Do not let wells dry before proceeding to the next step**.
   If automated machine is used for assay, follow the manufacturer's instructions for all washing steps described in this protocol.
3. Add **10 µL Assay Buffer** to the NSB wells and to each of the sample wells.
   Refer to Section 9 for suggested well orientations.
4. If samples to be assayed are serum or plasma, add **10 µL Matrix Solution** to the NSB, Standard, and Control wells (Option A).
   If samples are free of significant serum matrix components, add **10 µL Assay Buffer** instead (Option B).
5. Add in duplicate 10 µL Rat Insulin Standards in the order of ascending concentration to the appropriate wells.
6. Add **10 µL QC1** and **10 µL QC2** to the appropriate wells.
7. Add sequentially **10 µL samples** of the unknown samples in duplicates to the remaining wells.
8. Add **80 µL Detection Antibody** to all wells. **For best result all additions should be completed within one hour**.
   Cover the plate with plate sealer and incubate at room temperature for 2 hours on a orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.
9. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.
10. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash.
    Decant and tap after each wash to remove residual buffer.
11. Add **100 µL Enzyme Solution** to each well.
    Cover plate with sealer and incubate with moderate shaking at room temperature for 30 min on the microtiter plate shaker.
12. Remove sealer, decant solutions from the plate and tap plate to remove the residual fluid.
13. Wash wells 6 times with diluted Wash Buffer, 300 µL per well per wash.
    Decant and tap after each wash to remove residual buffer.

14. Add **100 µL of Substrate Solution** to each well, cover plate with sealer and shake in the plate shaker for **approximately** 5 to 20 minutes. Blue color should be formed in wells of Insulin Standards with intensity proportional to increasing concentrations of insulin.

    NOTE: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time. One can measure the color development using 370 nm filter, if available on the spectrophotometer. When absorbance is between 1.2 and 1.8 at 370 nm, the stop solution can be added to terminate color development.

15. Remove sealer and add **100 µL Stop Solution** [CAUTION: CORROSIVE SOLUTION] and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn into yellow after acidification. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well. Record the difference of absorbance units.

Option A: For Samples with significant Serum Matrix Effect

	Step 1	Step 2	Step 3	Step 4	Step 5-7	Step 8	Step 8-10	Step 11	Step 11-13	Step 14	Step 14	Step 15	Step 15
Well #	Dilute each bottle 10X Wash Buffer with 450mL Deionized Water.	Wash plate 3X with 300 µL Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels	Assay Buffer	Matrix Solution	Standards/ Controls/ Samples	Detection Ab	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µL Wash Buffer	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature . Wash 6X with 300 µL Wash Buffer	Substrate	Seal, Agitate, Incubate 15 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, B1			10 µL	10 µL	----------	80 µL		100 µL		100 µL		100 µL	
C1, D1			------	10 µL	10 µL of 0.2 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
E1, F1			------	10 µL	10 µL of 0.5 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
G1, H1			------	10 µL	10 µL of 1 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
A2, B2			------	10 µL	10 µL of 2 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
C2, D2			------	10 µL	10 µL of 5 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
E2, F2			------	10 µL	10 µL of 10 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
G2, H2			------	10 µL	10 µL of QC I	80 µL		100 µL		100 µL		100 µL	
A3, B3			------	10 µL	10 µL of QC II	80 µL		100 µL		100 µL		100 µL	
C3, D3 ↓			10 µL	------	10 µL of Sample	80 µL		100 µL		100 µL		100 µL	

Option B: For Samples without significant Serum Matrix Effect

	Step 1	Step 2	Step 3-4	Step 5-7	Step 8	Step 8-10	Step 11	Step 11-13	Step 14	Step 14	Step 15	Step 15
Well #	Dilute each bottle 10X Wash Buffer with 450mL Deionized Water.	Wash plate 3X with 300 µL Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels	Assay Buffer	Standards/ Controls/ Samples	Detection Ab	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µL Wash Buffer	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature . Wash 6X with 300 µL Wash Buffer	Substrate	Seal, Agitate, Incubate 15 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, B1			20 µL	----------	80 µL		100 µL		100 µL		100 µL	
C1, D1			10 µL	10 µL of 0.2 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
E1, F1			10 µL	10 µL of 0.5 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
G1, H1			10 µL	10 µL of 1 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
A2, B2			10 µL	10 µL of 2 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
C2, D2			10 µL	10 µL of 5 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
E2, F2			10 µL	10 µL of 10 ng/mL Standard	80 µL		100 µL		100 µL		100 µL	
G2, H2			10 µL	10 µL of QC I	80 µL		100 µL		100 µL		100 µL	
A3, B3			10 µL	10 µL of QC II	80 µL		100 µL		100 µL		100 µL	
C3, D3 ↓			10 µL	10 µL of Sample	80 µL		100 µL		100 µL		100 µL	

MICROTITER PLATE ARRANGEMENT

Rat / Mouse Insulin ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	2 ng/mL	QC 2									
B	Blank	2 ng/mL	QC 2									
C	0.2 ng/mL	5 ng/mL	Sample									
D	0.2 ng/mL	5 ng/mL	Sample									
E	0.5 ng/mL	10 ng/mL	Sample									
F	0.5 ng/mL	10 ng/mL	Sample									
G	1 ng/mL	QC 1	Sample									
H	1 ng/mL	QC 1	Etc.									

CALCULATIONS

The dose-response curve of this assay fits best to a sigmoidal 4-or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4-or 5-parameter logistic function. Graph the reference curve for sample interpretation by plotting the absorbance unit of 450nm, less that of 590nm, on the Y-axis against the concentration of rat insulin standards on the X-axis.

Note:

When sample volumes assayed differ from 10 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor
(e.g., if 5 µL of sample is used, then calculated data must be multiplied by 2).
When sample volume assayed is less than 10 µL, compensate the volume deficit with either matrix solution or assay buffer, whichever is appropriate.

TROUBLE SHOOTING GUIDE

1. To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure that all reagents and samples are added to the bottom of each well.
6. Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with TBS.