rInsulin [^{125}I] RIA KIT

(REF: RK-547)

For Research Use Only. Not for use in diagnostic procedures.

The [^{125}I]rInsulin RIA system provides direct quantitative *in vitro* determination of rat Insulin in plasma, tissue and cell culture. Rat insulin can be measured in the range 0.1-25 ng/ml. Each kit contains materials sufficient for 120 determinations permitting the construction of one standard curve and the assay of 48 unknowns in duplicate.

Introduction

Rat insulin is a pancreatic hormone whose molecular weight is about 6000. It is a protein composed of two polypeptide chains, a shorter A-chain of twenty-one residues and a longer B-chain of thirty. The two chains are connected by two disulphide (-S-S-) linkages, while a third such linkage forms an intra-chain precursor called pro-insulin , in which the future A- and B-chains are linked end to end by a peptide strand, C-peptide, before being joined by their –S-S-bonds. It is found in the ß-cell granules in the pancreatic Islets of Langerhans. Specific proteases act on pro-insulin to release the C-peptide and insulin within the granule. On stimulation the C-peptide and insulin are released into the bloodstream in approximately equimolar amounts.

Rat insulin differs from most other species in that it has two forms that are products of non-allelic genes. Translation of the two insulin mRNAs results in the synthesis of two preproinsulins differing by 7 amino acids. Processing of these peptides involves removal of the pre region and formation of proinsulins differing in 4 of 86 amino acids. The proinsulins are cleaved to mature insulins 1 and 2 which have identical A chains but differ by 2 amino acids in the B chain (positions 9 and 29). They are found roughly in the proportion 60% insulin 1 and 40% insulin 2 in the pancreas.

Several factors can effect the release of insulin. One of the main regulators of insulin release is the amount of glucose in the blood. A rise in blood glucose stimulates the release of insulin while a fall in blood glucose suppresses its secretion. Amino acids also stimulate insulin-release to allow their uptake into muscle cells. Insulin is considered to be an anabolic hormone in that it promotes the synthesis of protein, lipid and glycogen and it inhibits the degradation of these compounds. The key target tissues of insulin are liver, muscle and adipose tissue. In promotes cell growth in many different cell types and is an absolute requirement for normal growth in all immature animals. Insulin exerts its effect through a receptor complex comprising two a sub-units of molecular weight 135 kDa and two ß sub-units of molecular weight 90 kDa. It is also well known for its involvement in diabetes, where insulin deficiency results in aberrant blood glucose homeostasis.

Principle of method

This assay is based on the competition between unlabelled insulin and a fixed quantity of ^{125}I-labelled bovine insulin for a limited number of binding sites on a specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound will be inversely proportional to the concentration of added non-radioactive ligand.

The antibody bound insulin is then reacted with the magnetic immunosorbent. Separation of the antibody bound fraction is effected by either magnetic separation or centrifugation of the separating reagent suspension and decantation of the supernatant.

Measurement of the radioactivity in the pellet enables the amount of labelled insulin in the bound fraction to be calculated. The concentration of unlabelled insulin in the samples is then determined by interpolation from a standard curve.

Contents of the kit

1. 1 vial TRACER, lyophilized, reconstitution with 12.5 ml assay buffer, containing ~ 72 kBq , 1.95 µCi [^{125}I]bovine Insulin. The final solution contains [^{125}I]bovine Insulin in 0.025M phosphate buffer pH7.5 containing 0.1% (w/v) sodium azide. Store at 2-8 °C.

2. 1 vial STANDARD, lyophilized, reconstitution with assay buffer, volume stated on the vial label. The final solution contain rInsulin at a concentration of 50 ng/ml. Store at 2-8 °C.

3. 1 vial ANTISERUM (12.5 ml), ready for use, containing guinea pig anti-insulin serum in 0.025 M phosphate buffer pH7.5 containing 0.1% (w/v) sodium azide. Store at 2-8 °C.

4. 1 vial ASSAY BUFFER concentrate (10 ml), dilution to 100 ml. On dilution to 100 ml, this will give 0.025 M phosphate buffer, pH 7.5, containing 0.1% (w/v) sodium azide. Store at 2-8 °C.

5. 1 vial MAGNETIC IMMUNOSORBENT (55 ml), ready for use, containing paramagnetic particles in buffer with 0.1% (w/v) sodium azide. Store at 2-8 °C.

Pack leaflet

Materials, tools and equipment required

Pipettes or pipetting equipment with disposable tips (50 µl, 100 µl, 200 µl, 400 µl, 2.0 ml and 12.5 ml); disposable polypropylene or polystyrene tubes (12 x 75 mm); refrigerator; glass measuring cylinder (100 ml); test tube rack; vortex mixer; plastic foil; separators, comprising magnetic base and assay rack - these are for use in the magnetic separation protocol; adsorbent tissue; gamma counter.

Note: For the centrifugal protocol, the following additional equipment will be required:

Decantation racks; refrigerated centrifuge capable of >1500 xg.

Specimen collection

This section is provided for guidance only. It remains the investigator's responsibility to validate the chosen sample collection technique.

Blood samples

It is advised that if measurements are to be made on plasma samples, blood should be collected into tubes containing heparin or EDTA. Blood should be centrifuged immediately to remove cells and the plasma stored below -15°C prior to analysis. Serum samples can also be assayed with this kit. Samples may need to be diluted prior to assay depending on the expected concentration.

Tissue samples

Pancreatic samples should be frozen immediately after removal and stored at -80°C until required. Prior to analysis, glands should be homogenised or sonicated in appropriate buffer. Buffers described in the literature include Hank's-Wallace buffer and collagenase. Krebs-Ringer bicarbonate buffer (KRB) containing HEPES, BSA and glucose. Krebs-Henselet bicarbonate buffer containing glucose and BSA. Samples may need to be diluted depending on the expected concentration. Assay buffer remaining after reconstitution of assay components may be used for this.

Cell culture

Cell culture media should be collected and stored below -15°C prior to analysis, though they may be stored overnight at 2–8°C. Samples may need to be diluted depending on the expected concentration. Assay buffer remaining after reconstitution of assay components may be used for this preparation.

Preparation of reagents, storage

Storage: *see Contents of the kit*. At these temperatures each reagent is stable until expiry date. The actual expiry date is given on the package label.

Preparation: Equilibrate all reagents and samples to room temperature prior to use.

Assay buffer: Warm the bottle containing assay buffer concentrate to 40°C or until the gel-like material melts. Temperatures above 60°C should be avoided. Transfer the contents of the bottle, with washings, to a 100 ml measuring cylinder and dilute to 100 ml with distilled or deionised water. Mix well. Assay buffer is used to reconstitute the standard and the tracer.

Tracer and standard: Carefully add the required volume of assay buffer and replace stopper. Mix the contents of the bottles by inversion and swirling, taking care to avoid foaming.

Preparation of working standards

1. Label 9 polystyrene or polypropylene tubes 0.1, 0.2, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5 and 25.

2. Pipette 500 µl of assay buffer into all tubes.

3. Into the 25 tube pipette 500 µl of stock standard (50 ng/ml) and vortex thoroughly.

4. Transfer 500 µl from the 25 tube to the 12.5 tube and vortex thoroughly.

5. Repeat this doubling dilution successively with the remaining tubes.

6. 50 µl aliquots from each serial dilution give rise to 9 standard levels of rat insulin ranging from 0.1 to 25 ng/ml.

Assay procedure

(*For a quick guide , refer to Table 1.*)

1. Equilibrate all reagents to room temperature.
2. Prepare reagents and assay standards as described in the previous section.
3. Label polystyrene or polypropylene tubes in duplicate for total counts (TC), non-specific binding (NSB), zero standard (B0), standards and samples.
4. Pipette 200 µl assay buffer into the NSB tubes.
5. Pipette 100 µl assay buffer into the B0 tubes.
6. Pipette 50 µl assay buffer into the tubes labelled for standards and samples.
7. Starting with the most dilute, pipette 50 µl of each standard into the appropriately labelled tubes.
8. Pipette 50 µl unknown sample into appropriately labelled tubes.
9. Pipette 100 µl antiserum into all tubes except NSB and TC.
10. Vortex mix the tubes thoroughly. Cover the tubes and incubate for 2 hours at room temperature (15–30°C).
11. Uncover the tubes and pipette 100 µl of tracer into all tubes. The TC tubes should be stoppered and put aside for counting.
12. Vortex mix all tubes thoroughly. Cover the tubes and incubate for 1 hours at room temperature (15–30°C).
13. Gently shake and swirl the bottle containing the magnetic immunosorbent to ensure a homogeneous suspension. Add 400 µl into each tube except the TC. Vortex mix all tubes thoroughly and incubate at room temperature for 15 minutes.
14. Separate the antibody bound fraction using either magnetic separation or centrifugation as described below.

Magnetic separation

Attach the rack on the magnetic separator base and ensure that all tubes are in contact with the base plate. Leave for 5 minutes. After separation, do not remove the rack from the separator base. Pour off and discard the supernatant. Keeping the separator inverted , place the tubes on a pad of absorbent tissues and allow to drain for 5 minutes.

Centrifugation

Centrifuge all tubes at 4°C for 10 minutes at 1500 xg or greater. After centrifugation, place the tubes carefully into suitable decantation racks, then pour off and discard the supernatant. Keeping the tubes inverted, place the tubes on a pad of absorbent tissues and allow to drain for 5 minutes.
15. On completion of either magnetic or centrifugal separation, firmly blot the rims of any adhering liquid. Do not re-invert the tubes once they have been turned upright.
16. Determine the radioactivity present in each tube by counting for at least 60 seconds in a gamma scintillation counter.

Table 1. Assay Protocol, Pipetting Guide (all volumes are in microlitres)

Tubes	TC	NSB	B0	Stan - dard	Sam- ple
Buffer	**-**	**200**	**100**	**50**	**50**
Stan- dard	-	**-**	**-**	**50**	**-**
Sample	-	**-**	**-**		**50**
Anti- serum	-	**-**	**100**	**100**	**100**
Vortex mix, cover tubes and incubate for 2 hours at room temperature (15-30 °C)					
Tracer	**100**	**100**	**100**	**100**	**100**
Vortex mix, cover tubes and incubate for 1 hours at room temperature (15-30 °C)					
Separa- ting reagent	-	**400**	**400**	**400**	**400**
Vortex mix. Incubate for 15 minutes at room temperature.					
Separate either using magnetic separator for 5 minutes or by centrifugation for 10 minutes at >1500 xg .					
Decant tubes and blot on filter paper.					
Count radioactivity (60 sec/tube).					
Calculate the results.					

Calculation of results

Calculate the average count per minute (cpm) for each pair of assay tubes.
Calculate the percent NSB/TC using the following equation:

$$NSB/TC(\%) = \frac{NSB\ (cpm)}{TC\ (cpm)} \times 100$$

If the counter background is high, it should be subtracted from all counts.

Users may wish to subtract the average NSB cpm from all tubes except TC. If so the appropriate correction should be made. Calculate the percent B0/TC using the following equation:

$$B0/TC(\%) = \frac{B0\ (cpm) - NSB\ (cpm)}{TC\ (cpm)} \times 100$$

Calculate the percent bound for each standard and sample using the following equation:

$$B/B0(\%) = \frac{S1\text{-}9/M_x\ (cpm) - NSB\ (cpm)}{B0\ (cpm) - NSB\ (cpm)} \times 100$$

A standard curve can be generated by plotting the percent B/B0 as a function of the log rInsulin concentration.
Plot B/B0(%) (y-axis) against concentration ng standard per ml (x-axis). The concentration (ng/ml) value of the samples can be read directly from the graph (see Figure 1).

Smoothed spline curve fit is recommended.

Table 2. Typical assay data

Tube	Conc. ng/ml	Mean counts (cpm)	B/TC (%)	B/B0 (%)
TC	-	23135	-	-
NSB	-	564	2.44	-
B0	-	7994	32.1	-
S1	0.1	7654	-	95.4
S2	0.2	7460	-	92.8
S3	0.39	7106	-	88.0
S4	0.78	6589	-	81.0
S5	1.56	4969	-	59.3
S6	3.12	3418	-	38.4
S7	6.25	2520	-	26.3
S8	12.5	2083	-	20.4
S9	25	1639	-	14.5

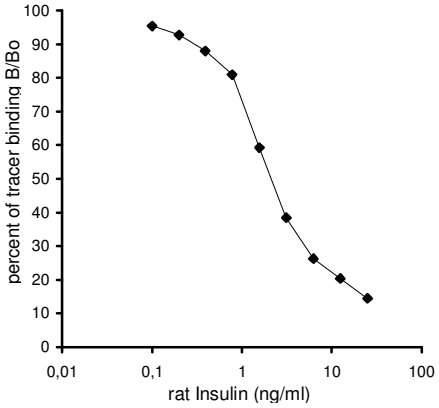


Figure1: A typical standard curve
(*Do not use to calculate unknown samples!)*

Characterization of assay

Stability

The components of this assay system will have a shelf-life of at least 3 weeks from the date of despatch.
Upon arrival, all components should be stored at 2-8°C where they are stable until the expiry date printed on the end pack label.
Once reconstituted, all reagents should de stored at 2-8°C where they are stable for at least 14 days.

Sensitivity

The sensitivity, defined as the amount of rat insulin needed to reduce zero dose binding by two standard deviations was 60 pg/ml.

Additional information

Components from various lots or from kits of different manufacturers should not be mixed or interchanged.

Precautions

Radioactivity

This product contains radioactive material. It is the responsibility of the user to ensure that local regulations or code of practice related to the handling of radioactive materials are satisfied.

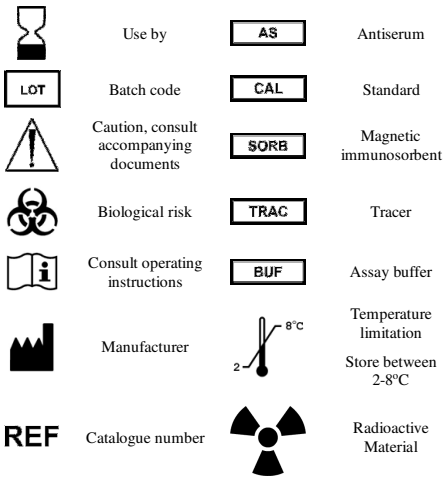
Chemical hazard

Components contain sodium azide as an antimicrobial agent. Dispose of waste by flushing with copious amount of water to avoid build-up of explosive metallic azides in copper and lead plumbing. The total azide present in each pack is 155 mg.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water (see safety data sheet for specific advice).

Safety data sheet

Product name:
Sodium azide
CAS No. 26628-22-8
R: 22-32 Toxic if swallowed. Contact with acids liberates very toxic gas.
S: (1/2)-28-45 (Keep locked up and out of the reach of children). After contact with skin, wash immediately with plenty of water. In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).
Composition:
Sodium azide solution.
Hazards identification:
Toxic if swallowed, inhaled, or absorbed through skin. May cause eye and skin irritation.
First aid measures:
In case of contact, immediately flush eyes or skin with copious amounts of water. If inhaled remove to fresh air. In severe cases seek medical attention.
Fire fighting measures:
Dry chemical powder. Do not use water.
Accidental release:
Wear suitable protective clothing including laboratory overalls, safety glasses and gloves. Mop up spill area, place waste in a bag and hold for waste disposal. Wash spill site area after material pick-up is complete.
Handling and storage:
Wear suitable protective clothing including overalls, safety glasses and gloves. Do not get in eyes, on skin, or on clothing. Wash thoroughly after handling.
Personal protection:
See above instructions for handling and storage.
Physical and chemical properties:
Formula weight: 65.01. Density: 1.850.
Stability and reactivity:
Avoid contact with metals and acid chlorides. This yields a very toxic gas.
Toxicological information:
LD50: 27 mg/kg oral, rat
LD50: 20 mg/kg skin, rabbit
Ecological information:
Not applicable
Disposal consideration:
Up to 5 vials worth of material may be disposed of directly down the sink with water. If 6 or more vials are to be disposed of they should pass through a chemical waste route.
Note: Inorganic azides will react with lead and copper plumbing fixtures to give explosive residues. Disposal of significant quantities of azides via such plumbing is not recommended.
Transport information :
No special considerations applicable.
Regulatory information:
The information contained in this safety data sheet is based on published sources and is believed to be correct. It should be used as a guide only. It is the responsibility of the user of this product to carry out an assessment of workplace risks, as may be required under national legislation.



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