rInsulin ELISA KIT

(REF: EK-547)

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

The rInsulin ELISA system provides direct quantitative *in vitro* determination of rat insulin in plasma and serum. Rat insulin can be measured in the range 0.15-10 ng/mL. Each kit contains materials sufficient for 96 determinations permitting the construction of one standard curve and the assay of 41 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

The technology uses two high affinity monoclonal antibodies in an immunometric assay system. This assay is based on a two-step procedure. In the first step the standards and samples are incubated in streptavidin coated wells with biotin labelled monoclonal antibody (capture antibody). During a 1-hour incubation period with continuous agitation the capture antibody - antigen complex is developed and immobilized on the reactive surface of wells. After incubation wells are washed repeatedly. In the second step the horseradish peroxidase (HRP) labelled monoclonal antibody (signal antibody) is added. It binds to an epitope of the insulin molecule different from that recognised by the capture-antibody, developing the formation of a capture antibody - antigen - signal antibody complex, also referred to as a 'sandwich'. After the one-hour-incubation period with continuous agitation the reaction mixture is washed repeatedly.

After the addition of a ready-to-use tetramethyl-benzidine (TMB) peroxide substrate the signal is measured in an ELISA photometer at 450 nm and 405 nm wavelength (620 nm as reference wavelength is recommended).

The concentration of antigen is directly proportional to the optical density measured in the wells. The unknown concentration of rat insulin in samples is read off a calibration curve constructed by plotting binding values against a series of calibrators containing known amount of rat insulin.

Contents of the kit

1. 1 bottle CONJUGATE (13 mL), ready to use, containing anti-rat insulin antibodies conjugated to horseradish peroxidase in buffer with blue dye and 0.01% merthiolate.
Store at 2-8 °C.

2. 7 vials STANDARDs (S0-S6), (S0 2 mL, S1-S6 0.5 mL per vial), ready to use, containing 0.15 (S1); 0.4 (S2); 1 (S3); 2 (S4); 5 (S5); 10 (S6) ng/mL rat Insulin in buffer with yellow dye and 0.1 % sodium azide.
Store at -20 °C upon arrival for long term. If used within 1 month store at 2-8 °C.

3. 1 bottle ANTISERUM (11 mL), ready to use, containing anti-rat insulin antibodies in buffer with red dye and 0.01 % merthiolate**.**
Store at 2-8 °C.

4. 1 bottle SUBSTRATE (25 mL), ready to use, in brown plastic bottle. Do not expose to direct light!
Store at 2-8 °C.

5. 1 piece MICROTITER PLATE, ready to use. 12 strips, packed in an air-tight foil.
Store at 2-8 °C.

6. 1 bottle WASH BUFFER CONCENTRATE (50 mL), with 0.1 % merthiolate.
Store at 2-8 °C.
See *Preparation of reagents.*

7. 1 bottle STOP REAGENT (6 mL), ready to use, 1M sulfuric acid.
Store at 2-8 °C.

Plate map
Cover sticks
Pack leaflet

Materials, tools and equipment required

Precision pipettes with disposable tips (20, 100, 125, 200 and 300 µL), distilled water, vortex mixer, shaker, plastic trays (separate for each reagent), disposable polypropylene or polystyrene tubes (12 x 75 mm); absorbent tissue, ELISA photometer.

Recommended tools and equipment
Repeating pipettes, multi-channel ELISA pipettes.

Specimen collection

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

Serum samples
Serum samples can be prepared according to common procedures used routinely in clinical laboratory practice. Samples should be prepared and stored deep frozen (-20°C). Frozen samples should be thawed and thoroughly mixed before assaying.

Plasma samples
It is advised that if measurements are to be made on plasma samples, blood should be collected into tubes containing EDTA. Blood should be centrifuged immediately to remove cells and the plasma stored below -15°C prior to analysis.

Samples may need to be diluted depending on the expected concentration. The zero standard, S0 may be used for this purpose.

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.
Wash buffer: Add the wash buffer concentrate (50 mL) to 450 mL distilled water to obtain 500 mL wash solution. Upon dilution, the wash buffer should be stored at 2-8°C where it is stable for at least 3 months.

Assay procedure

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

1. Equilibrate reagents and samples to room temperature before use.
2. Prepare reagents as described in the previous section.
3. Homogenize all reagents and samples by gentle mixing to avoid foaming.
4. Label the plate map for duplicates of each standards (S0-S6), and samples (SX).
5. Pipette 20 µL each of standards and samples into the appropriate wells.
6. Pipette 100 µL antiserum into each well.
7. Cover the plate by the enclosed foil, place it on the shaker. Incubate the plate for 1 hour, shaking (300 RPM) at room temperature (18-25 °C).
8. Remove the cover and pour the liquid directly over the lab sink. Holding in the upside down position place the plate immediately on an absorbent tissue. *Pay special attention to crossing-over between wells due to droplets backflow!*
9. Add 300 µL wash buffer into each well, decant (tap and blot) or aspirate. Repeat this step 4 times.
   An automatic or manual plate washer can be used. Follow manufacturer's instruction for proper usage.

10. Pipette 125 µL of conjugate into each well.
11. Cover the plate by the enclosed foil, place it on the shaker. Incubate the plate for 1 hour, shaking (300 RPM) at room temperature (18-25 °C).
12. Remove the cover and pour the liquid directly over the lab sink. Holding in the upside down position place the plate immediately on an absorbent tissue. *Pay special attention to crossing-over between wells due to droplets backflow.*
13. Add 300 µL wash buffer into each well, decant (tap and blot) or aspirate. Repeat this step 4 times.
14. Pour the substrate into a plastic tray and pipette 200 µL to each well. Place it into the dark for 30 minutes. *(If less than the whole volume is used in one assay, do not pipette directly from the bottle, and never fill the unused reagent back into its original bottle).*
15. Pipette 50 µL stop reagent into each well, and shake the plate gently for a few seconds.
16. Measurement in the ELISA photometer at 450 nm with 620 nm as reference wavelength, and at 405 nm with 620 nm as reference wavelength.
17. Calculate the rat insulin concentrations of the samples as described in calculation of results or use special software.

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

Tubes	Standard	Sample
Standard	**20**	
Sample		**20**
Antiserum	**100**	**100**
Shake for 1 hour at room temperature		
Decant the fluid and blot on filter paper		
Wash buffer	**300**	**300**
Decant the fluid and blot on filter paper		
Repeat the washing step 4 times		
Conjugate	**125**	**125**
Shake for 1 hour at room temperature		
Decant the fluid and blot on filter paper		
Wash buffer	**300**	**300**
Decant the fluid and blot on filter paper		
Repeat the washing step 4 times		
Substrate	**200**	**200**
30 minutes at room temperature in the dark		
Stop reagent	**50**	**50**
Measurement		
Calculate the results		

Calculation of results

The calculation is illustrated using representative data. Data obtained should be similar to those shown in Table 2 and 3.

Manual calculation

Calculate the average A.U. for each pair of duplicates. Subtract the mean S0 from all (standards and samples) mean A.U.-s. Draw the standard curve on a lin-lin graph paper by plotting calculated A.U. of each standard level (ordinate) against the respective concentration (abscissa). Obtain sample values by interpolation of sample A.U. on the standard curve.

Data evaluation using normalized binding

For computerised calculations and/or quality assessment normalised specific binding values, rather than A.U. values are used. Specific binding values can be calculated for each standard and sample according to the following equation:

$$B/Bmax\ (\%) = \frac{S1\text{-}6/SX\ (AU) - S0\ (AU)}{S6\ (AU) - S0\ (AU)} \times 100$$

S0 is the zero standard, or non-specific binding, SX is the sample.

Smoothed spline curve fit is recommended.

Table 2. Typical assay data at 450/620 nm

Wells	Conc. ng/mL	A.U. 450/620	A.U. mean	B/B_{max}(%)
S0	0	0.045 0.062	0.054	
S1	0.15	0.079 0.096	0.088	1.19
S2	0.4	0.161 0.147	0.154	3.49
S3	1	0.412 0.398	0.405	12.24
S4	2	0.900 0.866	0.883	28.92
S5	5	2.856 2.986	2.921	100.00

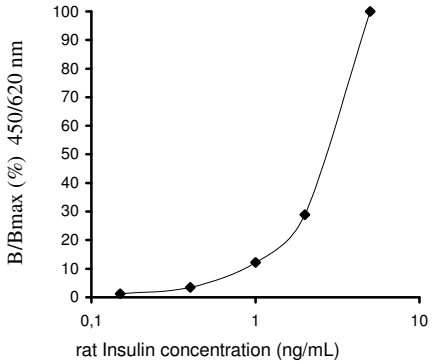

Figure1: A typical standard curve at 450 nm
(*Do not use to calculate unknown samples!)*

Table 3. Typical assay data at 405/620 nm

Wells	Conc. ng/mL	A.U. 405/620	A.U. mean	B/B_{max}(%)
S0	0	0.055 0.063	0.059	
S1	0.15	0.067 0.075	0.071	0.56
S2	0.4	0.111 0.096	0.104	2.10
S3	1	0.179 0.189	0.184	5.84
S4	2	0.334 0.321	0.328	12.56
S5	5	0.968 1.024	0.996	43.76
S6	10	2.248 2.153	2.200	100.00

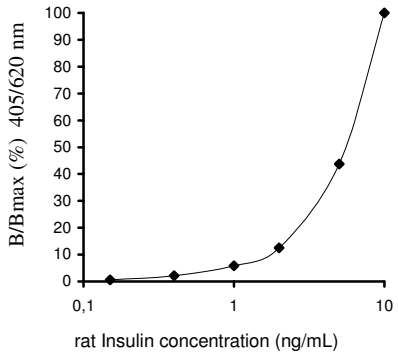

Figure2: A typical standard curve at 405 nm
(*Do not use to calculate unknown samples!)*

Characterization of assay

Sensitivity

For the analytical sensitivity 0.1 ng/mL has been obtained by assaying 16 replicates of the zero standard. The sensitivity has been determined as the concentration corresponding to the sum of the mean A.U. and its double standard deviation.

Specificity

Pig insulin: 31.0 %
Bovine insulin: 76.0 %
Human insulin: 46.0 %

Precision

3 samples were assayed in 16 replicates to determine intra-assay precision. Values obtained (ng/mL) are shown below.

Sample	Number of replicates	Mean value	CV%
1	16	0.65	5.86
2	16	1.96	3.99
3	16	3.65	1.89

Reproducibility

To determine inter-assay precision 3 samples were measured in duplicates in 10 independent assays. Values obtained (ng/mL) are shown below.

Sample	Number of runs	Mean value	CV%
1	10	0.69	9.29
2	10	2.05	6.64
3	10	4.00	4.35

Recovery

Recovery was defined as the measured increase expressed as per cent of expected increase upon spiking serum samples with known amount of rat insulin. 94-110 % was obtained for 3 samples.

Added conc. (ng/mL)	Expected conc. (ng/mL)	Measured conc. (ng/mL)	Recovery (%)
0	-	0.6391	-
2.5	1.5696	1.4812	94
5	2.8196	2.8910	103
10	5.3196	5.5049	103

0	-	1.5904	-
2.5	2.0452	1.9992	98
5	3.2952	3.2966	100
10	5.7952	6.0473	104
0	-	1.9094	-
2.5	2.2047	2.2909	104
5	3.4547	3.6964	107
10	5.952	6.5883	110

Dilution test (linearity)

A serial dilution (1:2 – 1:4) of 2 individual serum samples (1-2), and one plasma sample (3) was carried out with the zero-standard. The recovery was: 88-112%

Samp No.	Dil. factor	Expected (ng/mL)	Measured (ng/mL)	Reco-very(%)
1	1	-	0.7919	-
1	2	0.3960	0.4044	102
1	4	0.1980	0.2216	112
2	1	-	2.3699	-
2	2	1.1850	1.0411	88
2	4	0.5925	0.5194	88
3	1	-	5.1136	-
3	2	2.5568	2.3721	93
3	4	1.2784	1.1380	89

Additional information

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

Precautions

Chemical hazard

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).

Caution! The stop reagent is corrosive. Avoid contact with it because it may cause skin irritations and burns.

	Use by	CAL	Standard
LOT	Batch code	MP	Coated plate
	Caution, consult accompanying documents	CONJ	Conjugate
	Biological risk	WASHB	Wash buffer
	Consult operating instructions	SUB	Substrate
	Manufacturer	STOP	Stop reagent
REF	Catalogue number	AS	Antiserum
2 8	Temperature limitation Store between 2-8ºC		

Website: http://www.izotop.hu
Technical e-mail: immuno@izotop.hu
Commercial e-mail: commerce@izotop.hu

INSTITUTE OF ISOTOPES Ltd.
1535 Budapest. Pf.: 851.
Tel.: (36-1)392-2577, Fax: (36-1)395-9247