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RAT INSULIN
ENZYME IMMUNOASSAY KIT

catalogue # A05105

96 wells

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U.S. patent # 50 47 330
European patent # 89 139 552

THE RAT INSULIN ENZYME IMMUNOASSAY HAS BEEN DEVELOPED AND VALIDATED BY SPI-BIO.

For research laboratory use only.
Not for human diagnostic use.

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September 2005

RAT INSULIN EIA KIT

96 wells
Storage: -20°C
Expiry date: stated on the package

This kit contains:

- ☞ A covered 96 well Microtiter plate, pre-coated with Goat anti-Guinea-Pig IgG, ready to use after thawing
- ☞ One vial of Rat insulin tracer, lyophilised
- ☞ Two vials of Rat insulin standard, lyophilised
- ☞ One vial of Rat insulin antiserum, lyophilised
- ☞ One vial of EIA buffer, lyophilised
- ☞ One vial of concentrated Wash buffer, liquid
- ☞ One vial of tween 20, liquid
- ☞ Two vials of Quality Control sample, lyophilised
- ☞ Two vials of Ellman's reagent, lyophilised
- ☞ One instruction booklet
- ☞ One template sheet
- ☞ One well cover sheet

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 33 samples in duplicate.

PRECAUTIONS FOR USE

Users are recommended to read all instructions for use before starting work.

Each time a new pipet tip is used, aspirate a sample or reagent and dispense it back into the same vessel. Repeat this operation two or three times before distribution.

For research laboratory use only.
Not for human diagnostic use.
Do not pipet liquids by mouth.
Do not use kit components beyond the expiration date.
Do not eat, drink or smoke in area in which kit reagents are handled.
Avoid splashing.

The QC samples provided in this kit have been prepared by diluting rat plasma (Sprague Dawley rat) in EIA buffer. A sanitary control has been completed on Sprague Dawley rats following the Felasa Health Monitoring Recommendations. However, handle the CQ samples as a possible source of infection.

The total amount of reagents contains less than 100 µg of sodium azide. Flush the drains thoroughly to prevent the production of explosive metal azides.

PRINCIPLE OF THE ASSAY

This Enzyme Immunoassay (EIA) is based on the competition between unlabelled rat insulin and acetylcholinesterase (AChE) linked to rat insulin (tracer) for limited specific Guinea-Pig anti-rat insulin antiserum sites.

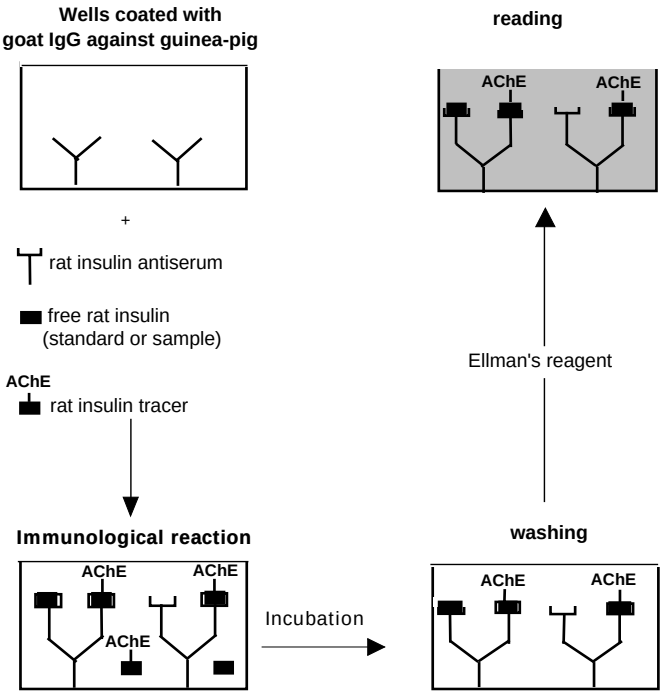
The complex Guinea-Pig antiserum-rat insulin (free insulin or tracer) binds to the Goat anti-Guinea-Pig antibody that is attached to the well.

The plate is then washed and Ellman's Reagent (enzymatic substrate for AChE and chromogen) is added to the wells.

The AChE tracer acts on the Ellman's Reagent to form a yellow compound.

The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of tracer bound to the well and is inversely proportional to the amount of free rat insulin present in the well during the immunological incubation.

The principle of the assay is summarised below:



MATERIALS AND EQUIPMENT REQUIRED

In addition to standard laboratory equipment, the following material is required:

☞ Precision micropipettes (20 to 1000 µL)

☞ Spectrophotometer plate reader (405 or 414 nm filter)

☞ Microplate washer (or washbottles)

☞ Microplate shaker

☞ Distilled or deionized water

☞ Polypropylene tubes

SAMPLE COLLECTION & PREPARATION

This assay may be used to measure insulin in rat plasma or serum sample. To do so, blood samples are collected in tubes containing heparin or EDTA. The samples are centrifuged at 1 600 g for 20 minutes. Plasmas are collected and kept at -20°C until assay.

No prior extraction procedure is necessary to measure insulin in plasma samples. However, hemolysis interferes with the assay by degrading insulin. SPI-BIO has developed an inhibitor cocktail and a procedure presented hereafter to prevent hemolysis consequences. Users are recommended to follow it in such a case.

☞ Inhibitor cocktail
Dilute 226.1 mg of EDTA in 500 µL of distilled or deionized water and 65 mg of phenanthroline in 500 µL of methanol. Mix them together.

☞ Inhibitor buffer
Dilute 250 µL of the inhibitor cocktail in 25 mL of EIA buffer provided in the kit.

Afterwards, prepare rat insulin standards, quality control and samples as follows:

☞ Rat insulin standard
Reconstitute the vial with 1 mL of distilled or deionized water. Allow it to stand 5 minutes until the content is completely dissolved and then mix thoroughly by gentle inversion. Then, add 10 µL of the inhibitor cocktail. Prepare seven propylene tubes (for the seven other standards) and add 500 µL of the inhibitor buffer in each tube. Add 500 µL of the first tube (containing the first standard) to the second tube. Continue this procedure for the other tubes.

☞ Quality control
Reconstitute one vial with 1 mL of distilled or deionized water. Allow it to stand 5 minutes until the content is completely dissolved and then add 10 µL of the inhibitor cocktail. Mix thoroughly by gentle inversion.

☞ Sample
Prior dispatching, add 10 µL of inhibitor cocktail for 1 mL of plasma (or 5 µL for 500 µL, 2 µL for 200 µL, etc..). If necessary, dilute the sample with the inhibitor cocktail buffer.

If no hemolysis is observed in plasma sample, prepare the above-mentioned reagents as indicated in the next section: Reagent preparation.

REAGENT PREPARATION

The coated microtiter plates and reagents are provided ready to use.

☞ EIA buffer
Reconstitute one vial with 50 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 month.

☞ Rat insulin standard
Reconstitute the vial with 1 mL of distilled water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the first standard is 10 ng/mL. Prepare seven propylene tubes (for the seven other standards) and add 500 µL of EIA buffer into each tube. Add 500 µL of the first tube (containing the first standard) to the second tube. Continue this procedure for the other tubes. Thus, standard concentrations are: 10 (S1), 5 (S2), 2.5 (S3), 1.25 (S4), 0.63 (S5), 0.31 (S6), 0.16 (S7) and 0.08 ng/mL (S8), respectively. Stability at 4°C : 1 day.

☞ Quality Control
Reconstitute one vial with 1 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 day.

☞ Rat insulin-AChE tracer
Reconstitute one vial with 5 mL of EIA buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 month.

☞ Rat insulin antiserum
Reconstitute one vial with 5 mL of EIA buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 month.

☞ Wash buffer
Dilute 1 mL of the concentrated Wash buffer to 400 mL with distilled or deionized water. Add 200 µL of tween 20 (Use a magnetic stirrer to mix the contents). Stability at 4°C: 1 week.

☞ Ellman's Reagent
Five minutes before use, reconstitute with 50 mL of distilled or deionized water. The tube contents should be thoroughly mixed. Stability at 4°C and in the dark: 4 days.

ASSAY PROCEDURE

It is recommended to perform the assays in duplicate and to follow the instructions hereafter.

PLATE PREPARATION

Prepare the wash buffer as indicated in the reagent preparation section. Open the plate packet and select the sufficient strips for your assay and place the unused strips back in the packet (stored at 4°C). Rinse each well five times with the wash buffer (300 µL/well).

Just before distributing reagents and samples, remove the buffer from the wells by inverting the plate and shaking out the last drops.

DISTRIBUTION OF REAGENTS AND SAMPLES

A plate set-up is suggested on the following page. The contents of each well may be recorded on the sheet provided with the kit.

PIPETTING THE REAGENTS

Note that the first column should be left empty for blanking Ellman's reagent. All samples and reagents must reach room temperature prior to performing the assay. Use different tips to pipet the buffer, standard, sample, tracer, antiserum and other reagents.

	A	B	C	D	E	F	G	H
12	*	*	*	*	*	*	*	*
11	*	*	*	*	*	*	*	*
10	*	*	*	*	*	*	*	*
9	*	*	*	*	*	*	*	*
8	*	*	*	*	*	*	*	*
7	*	*	*	*	*	*	*	*
6	*	*	*	*	*	*	*	*
5	*	*	*	*	*	*	*	*
4	S5	S5	S6	S6	S7	S7	S8	S8
3	S1	S1	S2	S2	S3	S3	S4	S4
2	NSB	NSB	Bo	Bo	Bo	Bo	*	*
1	B	B	B	B	B	B	B	B

B : Blank
NSB : Non-Specific Binding
Bo : Maximum Binding
S1-S8 : Standards 1-8
*: Samples or Quality controls

- EIA buffer: Dispense 100 µL to Non Specific Binding (NSB) wells and 50 µL to Maximum Binding (Bo) wells.

- Rat insulin standard: Dispense 50 µL of each of the eight standards (S1 to S8) in duplicate to appropriate wells. Start with the lowest concentration standard (S8) and equilibrate the tip in the next higher standard before pipetting.

- Quality Control and samples: Dispense 50 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in EIA buffer.

- Rat insulin AChE tracer: Dispense 50 µL to each well, except Blank (B) wells.

- Rat insulin antiserum: Dispense 50 µL to each well except Blank (B) wells and the Non Specific Binding (NSB) wells.

INCUBATING THE PLATES

Cover the plate with a plastic film and incubate for 16-20 hours at 4°C (optimal temperature).

DEVELOPING AND READING THE PLATE

Reconstitute the wash buffer and Ellman's Reagent as indicated in reagent preparation section. Empty the plate by turning over and shaking. Then, wash each well five times with the wash buffer (300 µL/well). Dispense 200 µL of Ellman's Reagent to the 96 wells. Incubate in the dark (plate covered with an aluminium sheet) at room temperature. Optimal development is obtained using an orbital shaker. The plate should be read between 405 and 414 nm (yellow colour) when the Maximum Binding (Bo) wells reach an absorbance of 0.2-0.8 unit.

Enzyme Immunoassay Protocol (Volume are in µL)					
	Blank	Non specific binding	Maximum binding	Standard	Sample
Buffer	-	100	50	-	-
Standard	-	-	-	50	-
Sample	-	-	-	-	50
Tracer	-	50	50	50	50
Antiserum	-	-	50	50	50
Cover the plate, incubate at 4°C for 16-20h					
Wash the plate 5 times					
Ellman's reagent	200	200	200	200	200
Incubate the plate with an orbital shaker in the dark at room temperature					
Read the plate between 405 and 414 nm					

DATA ANALYSIS

Make sure that your plate reader has subtracted the absorbance readings of the blank well (absorbance of Ellman's reagent) from the absorbance readings of the rest of the plate. If not, do it now.

- Calculate the average absorbance for each NSB, Bo, standards and samples.
- Calculate the B/Bo (%) for each standard and sample: (average absorbance of standards or sample - average absorbance of NSB) divided by (average absorbance of Bo - average absorbance of NSB) & multiplied by 100.
- Using a semi-log graph paper, plot the B/Bo (%) for each standard point (y axis) versus the concentration (x axis). Draw a best-fit line through the points.
- To determine the concentration of your samples, find the B/Bo (%) value on the y axis. Read the corresponding value on the x axis which is the concentration of your unknown sample. Samples with a concentration greater than 4 ng/mL should be re-assayed after dilution in EIA buffer.
- Most plate readers are supplied with curve-fitting software capable of graphing this type of data (logit/log or 4-parameter). If you have this type of software, we recommend using it. Refer to it for further information.

TYPICAL DATA

EXAMPLE DATA

The following data are for demonstration purpose only. Your data may be different and still correct. These data were obtained using all reagents as supplied in this kit under the following conditions: 1.5 hour developing at 20°C, reading at 414 nm. A logit/log curve fitting was used to determine the concentrations.

	mAU	B/Bo (%)
NSB	3	
Bo	453	100
Standard 10 ng/mL	64	13.6
Standard 5 ng/mL	90	19.3
Standard 2.50 ng/mL	126	27.3
Standard 1.25 ng/mL	171	37.3
Standard 0.63 ng/mL	235	51.6
Standard 0.31 ng/mL	293	64.4
Standard 0.16 ng/mL	352	77.6
Standard 0.08 ng/mL	384	84.7
QC	265	

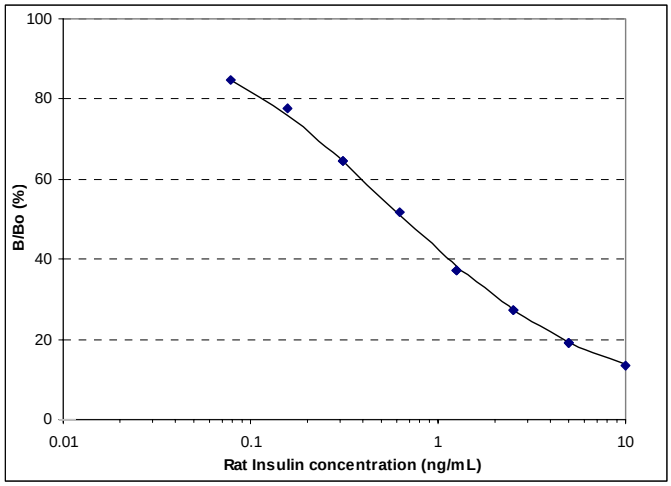
ACCEPTABLE RANGE

- Bo absorbance: > 200 mAU in the conditions indicated above.
- Ratio NSB absorbance / Bo absorbance: < 0.1.
- 50% B/Bo%: 0.50 to 0.76 ng/mL (mean: 0.63 ng/mL).
- QC sample: See the label on the vial.

CROSS-REACTIVITY

Compound	**%**	**Compound**	**%**
Rat Insulin	100	Mouse Insulin	100
Hamster Insulin	100	Porcine Insulin	100
Human Insulin	100	Sheep Insulin	100

RAT INSULIN STANDARD CURVE



ASSAY TROUBLE SHOOTING

☞ Bo value is too low: incubation in wrong conditions (time or temperature) or reading time too short or Rat insulin-AChE tracer, Rat insulin antiserum or Ellman's reagent have not been dispensed.

☞ NSB value too high: contamination of NSB wells with Rat insulin antiserum or inefficient washing.

☞ High dispersion of duplicates: poor pipetting technique or irregular plate washing.

☞ IC_{50} or QC concentrations not within the expected range: wrong preparation of standards.

☞ Analyses of two dilutions of a biological sample do not agree: Interfering substances are present. Sample must be purified prior to EIA analysis (excepting plasma samples).

These are a few examples of trouble shooting that may occur. If you need further explanation, SPI-BIO will be happy to answer any question or information about this assay. Please feel free to contact our technical support staff by letter, phone (33 (0)1 39 30 62 60), fax (33 (0)1 39 30 62 99) or E-mail (sales@spibio.com), and be sure to indicate the lot number of the kit (see outside of the box).

SPI-BIO offers a training workshop in EIA practice & theory. This workshop is given twice a year. For further information, please contact our Customer Relation Representative (33 (0)1 39 30 62 60).

BIBLIOGRAPHY

☞ **Grassi J. & Pradelles Ph.**
Compounds labelled by the acetylcholinesterase of *Electrophorus Electricus*. Its preparation process and its use as a tracer or marquer in enzymo-immunological determinations.
United States patent, N° 1,047,330. September 10, 1991