

Read entire protocol before use.

INS-Elisa

Bio-Line S.A. - Rue André Fauchille.17 - B-1150 Bruxelles – Belgium

I. INTENDED USE

Immunoenzymetric assay for the *in vitro* quantitative measurement of human Insulin (INS) in serum and plasma.

II. GENERAL INFORMATION

- A. Name: Bio-Line **Insulin Elisa Kit**
- B. Catalogue number: **BL-28-E**: 96 tests
- C. Manufactured by: Bio-Line S.A.
Rue André Fauchille.17 - B-1150 Bruxelles - Belgium
- D. For technical assistance or ordering information contact:
Tel: +32-2-736.62.18. Fax: +32-2-742.13.15.

III. CLINICAL BACKGROUND

A *Biological activities of insulin*

Insulin, a polypeptide hormone with a molecular weight of 5800 Da, is secreted by the beta cells of the islets of Langerhans from the pancreas. Insulin possesses a wide spectrum of biological actions. It stimulates cellular glucose uptake, glucose oxidation, glycogenesis, lipogenesis, proteogenesis and the formation of DNA and RNA. Insulin plays a key role in the regulation of plasma glucose levels (hepatic output inhibition, stimulation of peripheral glucose utilisation). The resulting hypoglycemic effects of insulin are counterbalanced by hormones with hyperglycemic effects (glucagon, growth hormone, cortisol, epinephrin). Insulin secretion is mainly controlled by the plasma glucose levels : hyperglycemia induces a prompt and important increase in circulating insulin levels. Neural influences, as well as various metabolic and hormonal factors (amino acids, glucagon, gastro intestinal hormone) also participate to the control of insulin secretion. Type I (insulin dependent : "juvenile") diabetes is due to a destruction of the beta cells, with a consequence of absolute lack of insulin. In type II (non-insulin-dependent : "maturity onset") diabetes, insulin resistance may play an important role; however after several years of evolution, beta-cells failure may occur, leading to a relative insulinopenia requiring, in some cases, insulin administration. Insulin resistance is associated with high circulation levels of the hormone. The most common case of insulin resistance is represented by obesity. Various endocrinopathies (acromegaly, Cushing syndrome) as well as rare cases of insulin receptor defects or cases with anti-insulin receptor antibodies are associated with glucose intolerance or even diabetes due to insulin resistance. The determination of plasma insulin levels is an important parameter in the diagnosis of hypoglycemia. Insulin levels are high in cases of insulinoma (beta-cell tumor). Functional postprandial hypoglycemia may also be associated with inappropriate insulin release to carbohydrate intake. Insulin levels are determined either in the fasting state or during dynamic test :

- a) stimulation test : carbohydrate rich meal, oral glucose tolerance test (OGTT), arginin infusion, tolbutamide or other sulfonylureas administration.
- b) inhibition test : fasting, somatostatine infusion

B. *Clinical application of insulin determination*


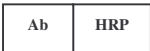

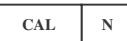





- . Determination of the beta-cell reserve during glucose tolerance test or after a carbohydrate rich meal, as a guide for the instauration of insulin therapy;
- . Contribution to the diagnosis of insulin and non-insulin-dependent diabetes;
- . Characterisation and follow-up of states of glucose intolerance;
- . Diagnosis and study of cases of insulin resistance;
- . Diagnosis of insulinoma and other causes of hypoglycemia.

IV. PRINCIPLES OF THE METHOD

The Bio-Line INS-ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiterplates. The assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of insulin. Calibrators and samples react with the capture monoclonal antibody (MAB 1) coated on microtiter well and with a monoclonal antibody (MAB 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated MAB 1 – human insulin – MAB 2 – HRP, the microtiterplate is washed to remove unbound enzyme labelled antibody. Bound enzyme-labelled antibody is measured through a chromogenic reaction. Chromogenic solution (TMB ready for use) is added and incubated. The reaction is stopped with the addition of Stop Solution and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance, which is proportional to the insulin concentration.

A calibration curve is plotted and INS concentration in samples is determined by interpolation from the calibration curve. The use of the ELISA reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in a high sensitivity in the low range and in an extended calibration range.

V. REAGENTS PROVIDED

Reagents	96 tests Kit	Color Code	Reconstitution
 Microtiterplate with 96 anti INS (monoclonal antibodies) coated wells	96 wells	blue	Ready for use
 Conjugate: HRP labelled anti-INS (monoclonal antibodies) in TRIS-HCl buffer with bovine serum albumin and thymol	1 vial 6 ml	red	Ready for use
 Zero calibrator in human serum and thymol	1 vial lyophilized	yellow	Add 2.0 ml distilled water
 Calibrator N = 1 to 5 (see exact values on vial labels) in human serum and thymol	5 vials lyophilized	yellow	Add 0.5 ml distilled water
 Wash Solution (Tris-HCl)	1 vial 10 ml	brown	Dilute 200 x with distilled water (use a magnetic stirrer).
 Controls - N = 1 or 2 in human serum with thymol	2 vials lyophilized	silver	Add 0.5 ml distilled water
 Chromogen TMB (Tetramethylbenzidine) in Dimethylformamide	1 vial 1 ml	green	Dilute 0.2 ml into 1 vial of substrate buffer
 Substrate buffer: H ₂ O ₂ in acetate / citrate buffer	3 vials 21 ml	white	Ready for use
 Stopping reagent: H ₂ SO ₄ 1.8N	1 vial 6 ml	black	Ready for use

Note: 1. Use the zero calibrator for sample dilutions.
2. 1 µIU of the calibrator preparation is equivalent to 1 µIU of 2nd IRP 66/304.

VI. SUPPLIES NOT PROVIDED

The following material is required but not provided in the kit:

- High quality distilled water
- Pipettes for delivery of: 50 µl, 200 µl, 500 µl and 2 ml (the use of accurate pipettes with disposable plastic tips is recommended)
- Vortex mixer
- Magnetic stirrer
- Horizontal microtiterplate shaker capable of 700 rpm ± 100 rpm
- Washer for Microtiterplates
- Microtiterplate reader capable of reading at 450 nm, 490 nm and 650 nm (in case of polychromatic reading) or capable of reading at 450 nm and 650 nm (monochromatic reading)
- Optional equipment: The ELISA-AID™ necessary to read the plate according to polychromatic reading (see paragraph XI.A.) can be purchased from Robert Maciels Associates, Inc. Mass. 0.2174 USA.

VII. REAGENT PREPARATION

- Calibrators** : Reconstitute the zero calibrator with 2.0 ml distilled water and other calibrators with 0.5 ml distilled water.
- Controls** : Reconstitute the controls with 0.5 ml distilled water.
- Working Wash solution** : Prepare an adequate volume of Working Wash solution by adding 199 volumes of distilled water to 1 volume of Wash Solution (200x). Use a magnetic stirrer to homogenize. Discard unused Working Wash solution at the end of the day.
- Revelation Solution**: pipette 0.2 ml of the chromogen TMB into one of the vials of substrate buffer (H₂O₂ in acetate/citrate buffer). Extemporaneous preparation is recommended.

VIII. STORAGE AND EXPIRATION DATING OF REAGENTS

- Before opening or reconstitution, all kits components are stable until the expiry date, indicated on the vial label, if kept at 2 to 8°C.
- Unused strips must be stored, at 2-8°C, in a sealed bag containing a desiccant until expiration date.
- After reconstitution, calibrators and controls are stable for 1 week at 2 to 8°C. For longer storage periods, aliquots should be made and kept at -20°C. Avoid successive freeze thaw cycles.
- The concentrated Wash Solution is stable at room temperature until expiration date.
- Freshly prepared Working Wash solution should be used on the same day.
- After its first use, the conjugate is stable until expiry date, if kept in the original well-closed vial at 2 to 8°C.
- The freshly prepared revelation solution is stable, before use, for maximum 15 minutes at room temperature and must be discarded afterwards.
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.

IX. SPECIMEN COLLECTION AND PREPARATION

- Serum and plasma must be kept at 2 - 8°C.
- If the test is not run within 24 hours, storage in aliquots at -20°C is recommended. Avoid subsequent freeze thaw cycles.
- Prior to use, all samples should be at room temperature. It is recommended to vortex the samples before use.
- Serum or EDTA plasma provides similar results. Heparin plasma gives lower results.

$$Y(\text{serum}) = 1.08 \times (\text{EDTA plasma}) - 0.7 \quad r=0.98 \quad n=53$$

$$Y(\text{serum}) = 1.21 \times (\text{Heparin plasma}) + 0.5 \quad r=0.98 \quad n=53$$
- Do not use haemolysed samples.

X. PROCEDURE

A. Handling notes

Do not use the kit or components beyond expiry date.
Do not mix materials from different kit lots.
Bring all the reagents to room temperature prior to use.
Thoroughly mix all reagents and samples by gentle agitation or swirling.
Perform calibrators, controls and samples in duplicate. Vertical alignment is recommended.
Use a clean plastic container to prepare the Wash Solution.
In order to avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.
For the dispensing of the Revelation Solution and the Stop Solution avoid pipettes with metal parts.

High precision pipettes or automated pipetting equipment will improve the precision.

Respect the incubation times.

To avoid drift, the time between pipetting of the first calibrator and the last sample must be no longer than 30 minutes.

Prepare a calibration curve for each run, do not use data from previous runs.

Dispense the Revelation Solution within 15 minutes following the washing of the microtiterplate.

During incubation with Revelation Solution, avoid direct sunlight on the microtiterplate.

B. Procedure

- Select the required number of strips for the run. The unused strips should be resealed in the bag with a desiccant and stored at 2-8°C.
- Secure the strips into the holding frame.
- Pipette 50 µl of each Calibrator, Control and Sample into the appropriate wells.
- Pipette 50 µl of anti-INS-HRP conjugate into all the wells.
- Incubate for 30 minutes at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
- Aspirate the liquid from each well.
- Wash the plate 3 times by:
 - dispensing 0.4 ml of Wash Solution into each well
 - aspirating the content of each well
- Pipette 200 µl of the freshly prepared revelation solution into each well within 15 minutes following the washing step.
- Incubate the microtiterplate for 15 minutes at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm, avoid direct sunlight.
- Pipette 50 µl of Stop Reagent into each well.
- Read the absorbances at 450 nm and 490 nm (reference filter 630 nm or 650 nm) within 1 hour and calculate the results as described in section XI.

XI. CALCULATION OF RESULTS

A. Polychromatic Reading:

- In this case, the ELISA-AID™ software will do the data processing.
- The plate is first read at 450 nm against a reference filter set at 650 nm (or 630 nm).
- A second reading is performed at 490 nm against the same reference filter.
- The ELISA-AID™ Software will drive the reader automatically and will integrate both readings into a polychromatic model. This technique can generate OD's up to 10.
- The principle of polychromatic data processing is as follows:
 - $X_i = OD$ at 450 nm
 - $Y_i = OD$ at 490 nm
 - Using a standard unweighted linear regression, the parameters A & B are calculated : $Y = A * X - B$
 - If $X_i < 3$ OD units, then X calculated = X_i
 - If $X_i > 3$ OD units, then X calculated = $(Y_i - B) / A$
 - A 4 parameter logistic curve fitting is used to build up the calibration curve.
 - The insulin concentration in samples is determined by interpolation on the calibration curve.

B. Bichromatic Reading

- Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
- Calculate the mean of duplicate determinations.
- On semi-logarithmic or linear graph paper plot the OD values (ordinate) for each calibrator against the corresponding concentration of INS (abscissa) and draw a calibration curve through the calibrator points by connecting the plotted points with straight lines.
- Read the concentration for each control and sample by interpolation on the calibration curve.
- Computer assisted data reduction will simplify these calculations. If automatic result processing is used, a 4 parameter logistic function curve fitting is recommended.

XII. TYPICAL DATA

The following data are for illustration only and should never be used instead of the real time calibration curve.

INS-ELISA		OD units Polychromatic model
Calibrator	0 µIU/ml	0.011
	5 µIU/ml	0.105
	15 µIU/ml	0.295
	50 µIU/ml	0.926
	150 µIU/ml	2.709
	500 µIU/ml	4.662

XIII. PERFORMANCE AND LIMITATIONS

A. Detection Limit

Twenty zero calibrators were assayed along with a set of other calibrators. The detection limit, defined as the apparent concentration two standard deviations above the average OD at zero binding, was 0.15 µIU/ml.

B. Specificity

Cross-reactive hormones were added to a high value calibrator (100 µIU/ml or 4 ng/ml). The apparent INS response was measured.

As shown hereafter, animal insulins (except rat insulin) cross-react whereas human, pork and beef proinsulins present no cross-reaction.

Added analyte to a high value serum		Theoretical INS values	Observed INS values	Cross-reaction
		(ng/ml)	(ng/ml)	(%)
Porcine insulin	8 ng/ml	4.2	17.4	> 100
Bovine insulin	8 ng/ml	3.8	17.8	> 100
Dog insulin	16 ng/ml	4.2	17.2	81
Rabbit insulin	16 ng/ml	4.2	14.1	62
Rat insulin	16 ng/ml	3.8	3.7	0
Human proinsulin	32 ng/ml	4.3	4.4	0.3
Porcine proinsulin	16 ng/ml	4.3	4.7	2.5
Bovine proinsulin	16 ng/ml	4.3	4.4	0.6

C. Precision

INTRA ASSAY				INTER ASSAY			
Serum	N	<X> ± SD (µIU/ml)	CV (%)	Serum	N	<X> ± SD (µIU/ml)	CV (%)
A	20	17.5 ± 0.9	5.3	A	20	19.6 ± 1.9	9.5
B	20	84.9 ± 2.6	3.0	B	20	86.8 ± 3.9	4.5

SD : Standard Deviation; CV: Coefficient of variation

D. Accuracy

RECOVERY TEST

Sample	Added INS (µIU/ml)	Recovered INS (µIU/ml)	Recovery (%)
Serum	82	83	101
	48	48	100
	27	26.4	98
	18.8	16.4	87
	327.0	337	103
Plasma	82	85	104
	48	50	104
	27	28.3	104
	18.8	17.7	94

DILUTION TEST

Sample	Dilution	Theoretical Concent. (µIU/ml)	Measured Concent. (µIU/ml)
Serum	1/1	-	56
	1/2	28	29
	1/4	14	13
	1/8	7	7
	1/16	3.5	2
Plasma	1/1	-	220
	1/2	110	106
	1/4	55	48
	1/8	27	26
	1/16	14	13

Samples were diluted with zero calibrator.

E. Time delay between last calibrator and sample dispensing

As shown hereafter, assay results remain accurate even when a sample is dispensed 40 minutes after the calibrators have been added to the coated wells

TIME DELAY					
	T0	10 min	20 min	30 min	40 min
C I	21.3	21.8	20.5	19.3	20.1
C II	94.7	97.2	94	90.8	93.6
818 3	410.2	410.6	436	395.3	405.6

F. Hook effect

A sample spiked with INS up to 10000 µIU/ml gives higher OD's than the last calibrator point.

XIV. INTERNAL QUALITY CONTROL

- If the results obtained for Control 1 and/or Control 2 are not within the range specified on the vial label, the results cannot be used unless a satisfactory explanation for the discrepancy has been given.
- If desirable, each laboratory can make its own pools of control samples, which should be kept frozen in aliquots.
- Acceptance criteria for the difference between the duplo results of the samples should rely on Good Laboratory Practises
- It is recommended that Controls be routinely assayed as unknown samples to measure assay variability. The performance of the assay should be monitored with quality control charts of the controls.
- It is good practise to check visually the curve fit selected by the computer.

XV. REFERENCE INTERVALS

The range of insulin levels in 29 subjects with normal oral glucose tolerance tests, was 5 to 19 µIU/ml, the range is based on 2.5 to 97.5 percentiles of the dataset. These values are given only for guidance; each laboratory should establish its own normal range of values.

XVI. PRECAUTIONS AND WARNINGS

Safety

For *in vitro* diagnostic use only.

The human blood components included in this kit have been tested by European approved and/or FDA approved methods and found negative for HBsAg, anti-HCV, anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum or plasma specimens should be in accordance with local safety procedures.

All animal products and derivatives have been collected from healthy animals. Bovine components originate from countries where BSE has not been reported. Nevertheless, components containing animal substances should be treated as potentially infectious.

Avoid any skin contact with all reagents, Stop Solution contains H₂SO₄, the chromogen contains TMB in Dimethylformamide, Substrate buffer contains H₂O₂. In case of contact, wash thoroughly with water.

Do not smoke, drink, eat or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

XVII. BIBLIOGRAPHY

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XVIII. SUMMARY OF THE PROTOCOL

	CALIBRATORS (µl)	SAMPLE(S) CONTROLS (µl)
Calibrators (0-5) Samples, Controls Anti-INS-HRP conjugate	50 - 50	- 50 50
Incubate for 30 min at room temperature with continous shaking at 700 rpm. Aspirate the contents of each well. Wash 3 times with 400 µl of Wash Solution and aspirate.		
Revelation Solution	200	200
Incubate for 15 min at room temperature with continous shaking at 700 rpm.		
Stop Solution	50	50
Read on a microtiterplate reader and record the absorbance of each well at 450 nm (versus 630 or 650 nm) and 490 nm (versus 630 or 650 nm)		

Bio-Line Catalogue Nr : BL-28-E	Version : 040702-BL	Revision nr : 030710/1
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