AVIVA SYSTEMS BIOLOGY

Read entire protocol before use.

INS-EASIA

I. INTENDED USE

Immunoenzymetric assay for the quantitative measurement of human Insulin (INS) in serum.

II. GENERAL INFORMATION

A. Proprietary name : Aviva INS-EASIA Kit

B. Catalogue number : OKDA00009 : 96 tests

C. Manufactured by : Aviva Systems Biology
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III. 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. 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 Aviva INS-EASIA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on breakable 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.

V. REAGENTS PROVIDED

Reagents	96 tests Kit	Color Code	Reconstitution
Microtiterplate with 96 anti INS (monoclonal antibodies) coated breakable wells.	96 wells	blue	**Ready** for use
Ab **HRP** 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
CAL **0** Zero calibrator in human serum and thymol	1 vial lyophilized	yellow	**Add** 2.0 ml distilled water
CAL **N** 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 **SOLN** **CONC** Wash Solution (Tris-HCl)	1 vial 10 ml	brown	**Dilute** 200 x with distilled water (use a magnetic stirrer).
CONTROL **N** Controls - N = 1 or 2 in human serum with thymol	2 vials lyophilized	silver	**Add** 1 ml distilled water
CHROM **TMB** Chromogenic TMB Solution (Tetramethylbenzydine)	1 vial 12 ml	black	**Ready** for use
STOP **SOLN** Stop solution: HCl 1.0 N	1 vial 12 ml	white	**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:
1. High quality distilled water
2. Pipettes for delivery of: 50 µl, 500 µl and 2 ml (the use of accurate pipettes with disposable plastic tips is recommended)
3. Vortex mixer
4. Magnetic stirrer
5. Washer for Microtiterplates
6. Microtiterplate reader capable of reading at 450 nm nm and 650 nm (bichromatic reading)

VII. REAGENT PREPARATION

A. **Calibrators** : Reconstitute the zero calibrator with 2.0 ml distilled water and other calibrators with 0.5 ml distilled water.
B. **Controls** : Reconstitute the controls with 1 ml distilled water.
C. **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.

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 wells 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.
§ Alterations in physical appearance of kit reagents may indicate instability or deterioration.

IX. SPECIMEN COLLECTION AND PREPARATION

§ Serum 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.
§ 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 Chromogenic 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 Chromogenic Solution within 15 minutes following the washing of the microtiterplate.
During incubation with Chromogenic Solution, avoid direct sunlight on the microtiterplate.

B. Procedure

1. Select the required number of wells for the run. The unused wells should be resealed in the bag with a desiccant and stored at 2-8°C.
2. Secure the wells into the holding frame.
3. Pipette 50 µl of each Calibrator, Control and Sample into the appropriate wells.
4. Pipette 50 µl of anti-INS-HRP conjugate into all the wells.
5. Incubate for 30 minutes at room temperature
6. Aspirate the liquid from each well.
7. Wash the plate 3 times by:
   - § dispensing 0.4 ml of Wash Solution into each well
   - § aspirating the content of each well
8. Pipette 100 µl of Chromogenic Solution the into each well within 15 minutes following the washing step.
9. Incubate the microtiterplate for 15 minutes at room temperature .
10. Pipette 100 µl of Stop Reagent into each well.
11. Read the absorbances at 450 nm (reference filter 630 nm or 650 nm) within 1 hour and calculate the results as described in section XI.

XI. CALCULATION OF RESULTS

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
2. Calculate the mean of duplicate determinations.
3. 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.
4. Read the concentration for each control and sample by interpolation on the calibration curve.
5. 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-EASIA		OD units Polychromatic model
Calibrator	0 µIU/ml	0.025
	5.1µIU/ml	0.070
	13.8 µIU/ml	0.13
	44.4 µIU/ml	0.507
	128 µIU/ml	1.313
	250 µIU/ml	2.34

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 0.17 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 (ng/ml)	Observed INS values (ng/ml)	Cross-reaction (%)
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 (µIUml)**	**CV (%)**	**Serum**	**N**	**<X> ± SD (µIUml)**	**CV (%)**
A	23	13.09 ± 0.6	4.8	A	8	13.29 ± 1.08	8.1
B	23	32.9 ± 1.9	6.0	B	7	34.12 ± 3.1	9.0

SD : Standard Deviation; CV: Coefficient of variation

D. Accuracy

RECOVERY TEST

Sample	Added INS (µIU/ml)	Recovered INS (µIU/ml)	Recovery (%)
Serum	182.1	174.5	95.8
	86.7	80	92.3
	39.6	37.4	94.4
	15.1	13.6	90

DILUTION TEST

Sample	Dilution	Theoretical Concent. (µIU/ml)	Measured Concent. (µIU/ml)
Serum 1	1/1	------	82.3
	1/2	41.2	42.21
	1/4	20.6	22.86
	1/8	10.3	11.04
	1/16	5.15	5.9
	1/32	2.58	3.3
Serum 2			
	1/1	-----	57.5
	1/2	28.7	27.7
	1/4	14.4	14.5
	1/8	7.2	8.0
	1/16	3.6	4.4
	1/32	1.8	2.3

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 30 minutes after the calibrators have been added to the coated wells

	T0	10 min	20 min	30 min
C I	12.8	12.7	12.4	12.9
C II	31.3	30.7	30	28.6

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 Research Purposes 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 HCl. 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.

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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. Aspirate the contents of each well. Wash 3 times with 400 µl of Wash Solution and aspirate.		
Revelation Solution	100	100
Incubate for 15 min at room temperature m.		
Stop Solution	100	100
Read on a microtiterplate reader and record the absorbance of each well at 450 nm (versus 630 or 650 nm)		

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