Practical course: Basic biochemical methods and ischemic heart models

Determination of serum insulin level by ELISA

A practical manual

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Supported by: HURO/0901/069/2.3.1
BACKGROUND

Insulin is a peptide hormone composed of 51 amino acids (6 kDa) and is produced in the islets of Langerhans in the pancreas. Insulin secretion is triggered by elevated glucose concentration in the blood. This condition usually occurs in healthy individuals after having a meal. Insulin plays a major role in the regulation of carbohydrate and fat metabolism in the body. Insulin stimulates cells in the liver, muscle, and fat tissue to take up glucose from the blood, thereby leading to a decrease in blood glucose level. Intracellular effects of insulin includes stimulation of glycolysis, glycogen synthesis, fatty acid and triglyceride synthesis. Disturbances in synthesis and secretion of insulin or in cellular responsiveness for insulin may lead to the development of chronic metabolic diseases.

Diabetes mellitus, often simply referred to as diabetes, is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced. The high blood glucose produces the classical symptoms of diabetes: i) polyuria (frequent urination), ii) polydipsia (increased thirst) and iii) polyphagia (increased hunger). Chronic elevation of blood glucose level leads to serious damage to many tissues and organs including the heart, blood vessels, eyes, kidneys, and nerves. Approximately 350 million people have diabetes worldwide.

There are three main types of diabetes:

Type 1 diabetes
Type 1 diabetes (previously known as insulin-dependent, IDDM, juvenile or childhood-onset) is characterized by deficient insulin
production and requires daily administration of insulin. The cause of type 1 diabetes is not known and it is not preventable with current knowledge.

*Type 2 diabetes*

Type 2 diabetes (formerly called non-insulin-dependent, NIDDM or adult-onset) results from insulin resistance, a condition in which cells fail to respond to insulin properly, sometimes combined with an absolute insulin deficiency. Type 2 diabetes comprises 90% of people with diabetes around the world, and is largely the result of excess body weight and physical inactivity. Until recently, this type of diabetes was seen only in adults but it is now also occurring in children.

*Gestational diabetes*

Gestational diabetes is hyperglycaemia with onset or first recognition during pregnancy.

**THE AIM OF THE PRACTICAL**

Quantitative determination of insulin level in rat serum or plasma after oral glucose administration by means of an enzyme-linked immunosorbent assay (ELISA) kit (Ultrasensitive Rat Insulin ELISA from Mercodia).

**PRINCIPLE OF THE ASSAY**

This ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies
are directed against separate antigenic determinants on the insulin molecule (Figure 1). During incubation insulin in the sample reacts with anti-insulin antibodies bound to the microtitration well and with peroxidase-conjugated anti-insulin antibodies. A washing step removes unbound enzyme labeled antibody. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB, a frequently used chromogenic in ELISAs). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at a wavelength of 450 nm using a microplate reader.

Figure 1. Direct sandwich ELISA for insulin determination
MATERIALS AND REAGENTS REQUIRED

**Pipettes** for 25, 50, 100 and 200 μL (Repeating pipettes preferred for addition of enzyme conjugate solution, Substrate TMB and Stop Solution.)

*Microplate reader* with 450 nm filter

**Tube** (10–100 mL) for preparation of enzyme conjugate solution

**Bottle** 1000 mL

*Redistilled water* (ultra pure water) for making solutions

*Plate shaker* (The recommended velocity is 700-900 cycles per minute, orbital movement)

**Insulin control** (high, low) Mercodia Diabetes-Antigen Control Rat, Mouse (L, M, H) (10-1220-01)

**Reagent kit** for 1 X 96 kit
Each Mercodia Ultrasensitive Rat Insulin ELISA kit (10-1251-01) contains reagents for 96 wells, sufficient for 42 samples and one Calibrator (standard) curve in duplicate. The expiry date for the complete kit is stated on the outer label. The recommended storage temperature is 2–8°C.

**Coated Plate** Mouse monoclonal anti-insulin
1 plate, 96 wells, 8-well strips, Ready for use
For unused microplate strips, reseal the bag using adhesive tape, store at 2–8°C and use within 8 weeks.

**Calibrators 1, 2, 3, 4, 5**  Color coded yellow.
Concentration of insulin stated on the vial label.
5 vials, 1000 μL each. Ready for use

**Calibrator 0**  Color coded yellow
1 vial 5 mL. Ready for use

**Enzyme Conjugate 11X**
Peroxidase conjugated mouse monoclonal anti-insulin
1 vial, 1.3 mL.

**Enzyme Conjugate Buffer**  Color coded blue.
1 vial 13 mL Ready for use

**Preparation of enzyme conjugate 1X:**
Prepare the needed volume of enzyme conjugate solution by dilution of Enzyme Conjugate 11X in Enzyme Conjugate Buffer (Table 1).

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<tr>
<th>Number of strips</th>
<th>Enzyme conjugate 11X</th>
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<tr>
<td>12 strips</td>
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<td>8 strips</td>
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<td>4 strips</td>
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Table 1. Preparation of enzyme conjugate 1X solution
When preparing enzyme conjugate 1X solution for the whole plate, pour all of the Enzyme Conjugate Buffer into the Enzyme Conjugate 11X vial. Mix gently.

**Wash Buffer 21X**
1 bottle, 50 mL
Dilute with 1000 mL redistilled water to make wash buffer 1X solution.

**Substrate TMB**  Colorless solution (*Note: light sensitive*)
1 bottle, 22 mL. Ready for use

**Stop Solution**  0.5 M H$_2$SO$_4$
1 vial, 7 mL. Ready for use

**SAMPLE PREPARATION, COLLECTION AND HANDLING**

**Preparation of animals for sampling**
In order to demonstrate a normal insulin response for glucose loading, two or three wistar rats should undergo fasting for 12 hours. After drawing a venous blood sample, the rats are treated with 1.5 g/kg glucose by oral gavage. Then blood samples are taken 30, 60, 120 min after glucose administration. Alternatively, blood can be taken from ZDF rats or ob/ob mice or streptozotocin-treated rats to demonstrate pathological alterations in insulin level.
Serum
Collect blood from the saphenous vein by venipuncture, allow to clot, and separate the serum by centrifugation (2000 x g).

Plasma
Collect blood from the saphenous vein by venipuncture into tubes containing heparin or EDTA as anticoagulant, and separate the plasma fraction by centrifugation (2000 x g).

Storage
Samples can be stored at 2–8°C up to 24 hours. For longer periods store samples at –20°C. Avoid repeated freezing and thawing.

Preparation of samples for the assay
No dilution is normally required, however, samples containing >1.0 μg/L should be diluted 1/10 v/v with Calibrator 0.

TEST PROCEDURE
Note: Prepare a calibrator curve for each assay run.
1. All reagents and samples must be brought to room temperature before use.
2. Prepare the required amount of enzyme conjugate 1X solution (according to Table 1 on previous pages)
3. Prepare wash buffer 1X solution
4. Prepare the samples, insulin control solutions, and calibrators.
5. Prepare sufficient microplate wells to accommodate Calibrators and samples in duplicate.
6. Make a plate plan (see Table 2)
Table 2. Recommended plate plan. Cal 0-5: calibrator solutions (standards); ICL: insulin control low; ICH: Insulin control high; S: sample

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7. Pipette 25 μL each of Calibrators into appropriate wells.
8. Pipette 25 μL each of samples into appropriate wells.
9. Add 100 μL of enzyme conjugate 1X solution into each well.
10. Incubate on a plate shaker (700-900 rpm) for 2 hours at room temperature (18-25°C).
11. Wash each well 6 times with wash buffer 1X solution. Discard the reaction volume by inverting the microplate over a sink. Add 350 μL wash solution to each well. Discard the wash solution, tap firmly several times against absorbent paper to remove excess liquid. Repeat 5 times. Avoid prolonged soaking during washing procedure.
12. Add 200 μL Substrate TMB into each well.
13. Incubate 15 minutes at room temperature (18-25°C).
14. Add 50 μL Stop Solution to each well.
Place the plate on the shaker for approximately 5 seconds to ensure mixing.
15. Read optical density at 450 nm. Read within 30 minutes.
16. Calculate the results.

*Note! To prevent contamination between the conjugate and substrate, separate pipettes are recommended.*

**INTERNAL QUALITY CONTROL**

It is recommended to record the following information for each assay: lot number of the kit, dilution and reconstitution dates of kit components, OD values for the blank, Calibrators and controls.

**CALCULATION OF RESULTS**

**Manual Calculation**

1. Plot the absorbance values obtained for the Calibrators, except for Calibrator 0, against the insulin concentration on a lin-lin paper and construct a calibrator curve.
2. Read the concentration of the samples from the calibrator curve.

**Computerized calculation**

The absorbance for the Calibrators (except for Calibrator 0) should be plotted against their concentration by using an appropriate computer program (e.g. MS excel, Sigma Plot, Graphpad Prism, etc) and the with a display of the equation of the curve. Then the equation is used for calculating the concentration for the samples based on their OD values.
Conversion factor
1 μg corresponds to 174 pmol.

IMPORTANT NOTES
Performance limitations
Grossly lipemic, icteric or haemolysed samples do not interfere in the assay. Insulin is, however, degraded over time in haemolysed samples. The degradation could give falsely low values and contribute to higher inter assay variation.

Detection limit
The detection limit is ≤0.020 μg/L as determined with the methodology described in ISO11843-Part 4.
Concentration of samples with absorbance below Calibrator 1 should not be calculated, instead expressed less or equal to (≤) the

REFERENCES

1. Mercodia Ultrasensitive Rat Insulin ELISA - Directions for Use. Mercodia AB
PROTOCOL SUMMARY AND CHECKLIST
(Mercodia Ultrasensitive Rat Insulin ELISA)

Ready:

1. Add Calibrators, Controls and Samples 25 μL
2. Add enzyme conjugate 1X solution to all wells 100 μL
3. Incubate for 2 hours at 18-25°C on a plate shaker
4. Wash plate with wash buffer 1X solution 6 times
5. Add Substrate TMB 200 μL
6. Incubate 15 minutes
7. Add Stop Solution 50 μL
8. Shake for 5 seconds to ensure mixing
9. Measure $A_{450}$ with a microplate reader
10. Evaluate results

Date: Time:
Experimenter:
File name for Abs data: