

Instruction Manual No. M-0030

Human Insulin

For Quantitative Determination of
Insulin In Human Serum or Plasma

For In Vitro Research Use Only

Human Insulin ELISA KIT # 030, Kit Contents

Components	96 tests
Monoclonal Anti-Insulin coated microwell strip plate (96 wells), Cat. # 031	1 plate
Human Insulin Std. A , 0.75 ml, 0 uIU/ml	Cat. # 032
Human Insulin Std. B , 0.75 ml, 5 uIU/ml	Cat. # 033
Human Insulin Std. C , 0.75 ml; 25 uIU/ml	Cat. # 034
Human Insulin Std. D , 50 uIU/ml, 0.75 ml	Cat. # 035
Human Insulin Std. E , 100 uIU/ml, 0.75 ml	Cat. # 036
Human Insulin Std. F , 200 uIU/ml, 0.75 ml	Cat. # 037
Stds are calibrated to 1 st WHO IRP Insulin (66/304)	
Anti-Insulin-HRP Conjugate , 11 ml	Cat. # 038
Wash Buffer (20X) ; 50 ml, Cat# WB-20	1 bottle
HRP substrate Soln A , Cat. # 30SA	1 bottle
HRP substrate Soln B , Cat. # 30SB	1 bottle
Stop solution, 10 ml, Cat. # T-10	1 bottle
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Introduction

Insulin is the principal hormone responsible for glucose metabolism. It is synthesized in the cells of the islets of Langerhans as the precursor, proinsulin, which is processed to form C-peptide and insulin and both are secreted in equimolar amounts into the portal circulation. The mature insulin molecule comprises two polypeptide chains, the A chain (21 amino acids) and the B chain (30 amino acids), which are linked by two inter-chain disulphide bridges. There is, in addition, a single intra-chain disulphide bridge in the A chain. The sequence of insulin is highly conserved in mammalian species, and is homologous with the insulin-like growth factors IGF-I and IGF-II. Secretion of insulin is mainly controlled by plasma glucose concentration and the hormones have a number of important metabolic actions. Its principal function is to control the uptake and utilization of glucose in peripheral tissues via the glucose transporter. This and other hypoglycemic activities, such as the inhibition of hepatic gluconeogenesis and glycogenolysis are counteracted by the hyperglycemic hormones including glucagons, epinephrine (adrenaline), growth hormone and cortisol. Insulin concentrations are severely reduced in insulin-dependent diabetes (DDM) and some other conditions such as hypopituitarism. Insulin concentrations may be raised in non-insulin-dependant diabetes (NIDDM), obesity, insulinoma and some endocrine dysfunctions such as Cushing's Syndrome and Acromegaly^{1, 2} The main clinical utility measurement is in the investigation of hypoglycemia. Insulin assay have been used in the following applications:

PERFORMANCE CHARACTERISTICS

Precision:

Intra-assay: three pool sera were assayed of 8 in a single run

Inter-assay: three pool sera were assayed in duplicate in three days

Serum Sample	Mean (mU/mL)	Intra-assay		Inter-assay	
		S.D.	CV%	S.D.	CV%
1	15.2	1.59	10.40	1.54	11.8
2	51.4	3.23	6.29	3.6	7.67
3	124	10.53	8.50	6.87	5.80

Accuracy

A serum containing 200 uU/mL of insulin was diluted with series of insulin free serum. The dilutions were tested and the insulin recoveries were compared with the expected concentrations.

Sample Dilution	Insulin Level Expected (mU/mL)	Insulin Level Measured (mU/mL)	Recovery %
Undiluted	200		
1:0.6	125	125.3	100.2
1:1	100	104.4	104.4
1:2	66.7	60.8	91.2
1:4	40	43.0	107.5
1:6	28.6	28.5	99.7
1:7	25	27.3	109.2
1:9	20	20.4	102
1:12	15.4	15.4	100

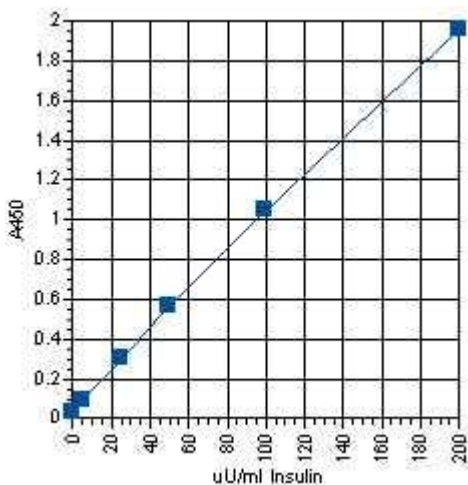
Known insulin samples were spiked with different concentrations of insulin. Samples were then tested and the insulin recoveries compared with the expected concentrations as illustrated: (Unit uIU/mL)

Insulin	Insulin Spiked	Expected Value	Measured Value	Recovery %
5	100	52.5	47.7	90.9
5	200	102.5	101.2	98.7
50	10	30.0	31.4	104.7
50	25	37.5	35.2	93.9
50	200	125.0	124.9	99.9
50	40	45.0	45.4	100.1

WORKSHEET OF TYPICAL ASSAY

Wells	Stds/samples	Mean A _{450nm}	Calculated Conc. (uIU/ml)
A1, A2	Std. A (0 uIU/ml)	0.002	
B1, B2	Std. B (5.0 uIU/ml)	0.042	
C1, C2	Std. C (20.0 uIU/ml)	0.303	
D1, D2	Std. D (50 uIU/ml)	0.600	
E1, E2	Std. E (100 uIU/ml)	1.180	
F1, F2	Std. F (200 uIU/ml)	2.152	
G1, G2	Sample 1	0.1.30	113.2

NOTE: These data are for **demonstration purpose only**. A complete standard curve must be run in every assay to determine sample values. Each laboratory should determine their own normal reference values.



A typical std. assay curve (do not use this for calculating sample values)

1. To assess the residual cell function, especially in newly diagnosed cases of IDDM.
2. As an aid to the discrimination between IDDM and NIDDM.
3. The diagnosis of insulinoma.
4. In the investigation of the pathophysiology of diabetes mellitus.

Insulin assays are the essentials in various dynamic tests, such as oral of intravenous glucose tolerance tests (OGTT and IVGTT), to determine the insulin response of the pancreas and the degree of insulin resistance. In many applications, insulin measurements may be complicated by cross-reactivity with partially degraded insulin, proinsulin and split forms of proinsulin. Immune complexes of these molecules are essentially problematic in patients who have developed antiinsulin antibodies through animal insulin administration.

PRINCIPLE OF THE TEST

Human Insulin ELISA kit is based on simultaneous binding of human Insulin from samples to two antibodies, one immobilized on microtiter well plates, and other conjugated to the enzyme horseradish peroxidase. After a washing step, chromogenic substrate is added and color developed. The enzymatic reaction (color) is directly proportional to the amount of Insulin present in the sample. Adding stopping solution terminates the reaction. Absorbance is then measured on a microtiter well ELISA reader at 450 nm and the concentration of Insulin in samples and control is read off the standard curve.

MATERIALS AND EQUIPMENT REQUIRED

Adjustable micropipet (5-100 ul) and Multichannel pipet with disposable plastic tips. Reagent troughs, Plate shaker (orbital shaker), Plate washer (recommended) and ELISA plate Reader.

PRECAUTIONS

The Insulin ELISA test is intended for *in vitro* research use only. The reagents contain thimerosal as preservative; necessary care should be taken when disposing solutions. The Control Serum has been prepared from human sera shown to be negative for HBsAg and HIV antibodies. Nevertheless, such tests are unable to prove the complete absence of viruses, therefore, sera should be handled with appropriate precautions.

Applicable **MSDS**, if not already on file, for the following reagents can be obtained from ADI or the web site.

TMB (substrate), H₂SO₄ (stop solution), and Prolcin-300 (0.1% v/v in standards, sample diluent and HRP-conjugates). All waste material should be properly disinfected before disposal. Avoid contact with the stop solution (1N sulfuric acid).

SPECIMEN COLLECTION AND HANDLING

Collect blood by venipuncture, allow clotting, and separating the serum by centrifugation at room temperature. Do not heat inactivate the serum. If sera cannot be immediately assayed, these could be stored at -20°C for up to six months. Avoid repeated freezing and thawing of samples. No preservatives should be added to the serum. Do not use EDTA plasma as it can cause erroneous results.

STORAGE AND STABILITY

The microtiter well plate and all other reagents are stable at 2-8°C until the expiration date printed on the label. The whole kit stability is usually 6 months from the date of shipping. Standards are stable for two month at 2-8°C. The unused portions of the standards can be frozen in suitable aliquots for long-term use. Repeated freezing and thawing is not recommended.

TEST PROCEDURE (ALLOW ALL REAGENTS TO REACH ROOM TEMPERATURE BEFORE USE).

Remove required number of coated strips and arrange them on the plate. Store unused strips in the bag. Dispense 200-300 ul of wash buffer to all wells. Mix for 5 seconds and discard or aspirate the solution. The step should be done just before adding the samples, do not allow the wells to dry at any time during the assay.

1. Label or mark the microtiter well strips to be used on the plate. Dilute Wash buffer 1:20 (50 ml in 950 ml water).
2. Pipet **25 ul of standards** and serum samples into appropriate wells in *duplicate*. Dispense **100 ul** of Antibody-Enzyme Conjugate into each well. Gently mix the samples, cover the plate and incubate at room temp for **30 min**.
3. Wash the plate **5X** with **wash buffer (300 ul/wash)**. We recommend using an automated ELISA plate Washer for better consistency. Failure to wash the wells properly will lead to high blank or zero values. If washing manually, plate must be tapped over paper towel between washings to ensure proper washing
4. Pre-mix equal volume of Soln. A and Soln. B (for 96 wells, mix 10 mls of Soln A and 10 ml of Soln B in separate clean tube or dispensing tray). Add **200 ul of HRP-substrate mix** at timed intervals into each well (prepared by mixing. Prepare substrate mix as needed. Substrate mix must not be stored). Mix gently for 5-10 seconds, cover the plate and **incubate for 15 min at room temp**. Blue color develops into standard and all positive wells. Note: Substrate solution must be at room temp.
5. Stop the reaction by adding **50 ul of stop solution** to all wells. Mix gently for 5-10 seconds. Blue color turns yellow. Measure the **absorbance at 450 nm** using an ELISA reader within 30 min.

NOTES- Read instructions carefully before the assay. Do not allow reagents to dry on the wells. Careful aspiration of the washing solution is essential for good assay precision. Since timing of the incubation steps is important to the performance of the assay, pipet the samples without interruption and it should not exceed 5 minutes to avoid assay drift. If more than one plate is being used in one run, it is recommended to include a standard curve on each plate. The unused strips should be stored in a sealed bag at 4°C.

Addition of the HRP substrate solution starts a kinetic reaction, which is terminated by dispensing the stopping solution. Therefore, keep the incubation time for each well the same by adding the reagents in identical sequence. Plate readers measure absorbance vertically. Do not touch the bottom of the wells.

CALCULATION OF RESULTS

Calculate the mean absorbance for each duplicate. Subtract the absorbance of the zero standard from the mean absorbance values of standards and samples. Draw the standard curve on a graph paper by plotting net absorbance values of standards against appropriate protein concentrations. Read off the Insulin concentrations of the control and samples.

EXPECTED VALUES

Fasting: 5-35 uU/mL
30 min. after oral glucose 30-230 uU/mL
120 min. after oral glucose 14-160 uU/mL

It is recommended that each laboratory determine its own normal and abnormal range.

Testing of other Biological Fluids Species Crossreactivity

This kit is primarily designed to test human serum samples. It is possible to use the plasma and other biological fluids. However, the sample volume and dilutions must be adjusted according to the expected concentrations or unknown samples be tested at several dilutions to determine the optimum range.

Crossreactivity of human insulin antibodies used in the kit with insulin from other species (mouse, rat, and monkey) has not been established.

SPECIFICITY

There is no cross reactivity with C-peptide at the concentration of 5000 pmo/mL, with intact human proinsulin (biosynthetic) 0.3%. High concentrations of lipid or bilirubin do not interfere in the insulin assay. Purified hemoglobin up to 50 ug/mL does not interfere in the test. No interference for rheumatoid factor or human anti-mouse antibodies (HAMA) was observed.

DETECTION LIMIT - Based on sixteen replicates determinations of the zero standards, the minimum concentration of human Insulin detected using this assay is ~ 0.5 uU/ml. The detection limit is defined as the value deviating by 2 SD from the zero standards. The sensitivity of the assay is 1.5 uU/ml.

References: 1. Clark PMS & Hales CN (1991) Assay of Insulin. In P.C. Pickup and G. Williams eds. Textbook of Diabetes, Vol 1, 335-347, Blackwell Scientific Publications; 2. Clark PMS and Hales CN (1994) How to Measure Plasma Insulin. Diabetes/Metabolism Reviews, 10:79-90; 3. Andersen L, Dinesen B, Jorgensen PN, Poulsen F and Roder MF (1993) Enzyme Immunoassay for Intact Human Insulin in Serum or Plasma. Clin Chem 38:578-582; 4. Volund A (1993) Conversion of Insulin units to SI units. American Journal of Clinical Nutrition 58:714-715