Quantikine® ELISA

Human/Canine/Porcine Insulin Immunoassay

Catalog Number DINS00

For the quantitative determination of human, canine, or porcine Insulin concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Insulin is a peptide hormone of the insulin-like peptide family that also includes insulin-like growth factors (IGFs), relaxins, and other insulin-like peptides (1-3). Its production by pancreatic β cells is essential for glucose metabolism and regulation of energy balance. Failure of insulin control causes diabetes mellitus (DM), which can either be of Type I (T1D, 5% of diagnosed DM), previously called juvenile or insulin-dependent diabetes, or Type II (T2D, 95% of diagnosed DM), previously called adult-onset or insulin-independent diabetes (4). T1D is a primary insufficiency of β cell insulin production while T2D is a functional insulin deficiency caused mainly by insulin resistance of the target cells. Mutations of the insulin gene are a cause of neonatal diabetes (2, 5, 6). DM has become more frequent over time, and in 2011 affected 79 million adults in the US (8% of the population). Frequency increases with age (27% of adults >65 years) and obesity, and can vary with ethnic background (4). DM is the seventh leading cause of death in the US (2, 4). Complications include heart disease, stroke, hypertension, blindness, kidney disease, and neuropathy (4).

Insulin is synthesized as a 110 amino acid (aa) preproprotein. A 24 aa signal sequence is cleaved to form the 86 aa proinsulin peptide, which undergoes further proteolysis to generate the 30 aa B chain (aa 25–54 of the preproprotein), the 21 aa A chain (aa 90-110), and the 34 aa intervening C-peptide (1, 2). Mature human insulin is the disulfide-linked heterodimer of A and B chains, which shares 98% aa sequence identity (50/51 aa) with porcine and canine insulin, 94% (48/51 aa) with bovine insulin, and 92% (47/51 aa) with mouse, rat, feline and ovine insulin. Insulin is stored in β cells as a zinc-coordinated hexamer (2). It is released as a zinc-free monomer into the hepatic portal vein, thus achieving its highest concentration in the liver (2, 7). Basal levels are continuously secreted, with higher secretion stimulated by food ingestion (7). In diabetics requiring insulin replacement or supplementation (26% of those diagnosed with diabetes), natural insulin or a synthetic analogue is delivered subcutaneously via injection or an implanted pump (4, 7).

Insulin acts through the insulin receptor (Ins R), a receptor tyrosine kinase that is present in varying quantities on all cells. Insulin initiates downstream signaling by binding and crosslinking two receptor molecules to form a trimeric receptor/ligand complex (2, 3, 8). Signals facilitate the cellular uptake of glucose by regulating the appearance of membrane glucose transporters. The Ins R is present in two isoforms, A and B, which may homodimerize or heterodimerize with the IGF-I receptor (3, 6). All receptor combinations bind insulin, IGF-I or IGF-II, but with differing affinities (3, 6). This system allows fine tuning of insulin-mediated signaling pathway activation according to the concentrations of insulin, IGF-I and IGF-II, and expression of receptor subunits on the cell surface (6).

The Quantikine Human/Canine/Porcine Insulin Immunoassay is a 4.5 hour solid-phase ELISA designed to measure Insulin in cell culture supernates, serum, and plasma. It contains *S. cerevisiae*-expressed recombinant human Insulin and has been shown to accurately quantitate the recombinant factor. Results obtained using natural Insulin showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring Insulin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Insulin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Insulin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for Insulin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Insulin bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Insulin Microplate	894214	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for Insulin.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Insulin Conjugate	894215	21 mL of a monoclonal antibody specific for Insulin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Insulin Standard	894216	5.0 pmol of recombinant human Insulin in a buffered protein base with preservatives; lyophilized.	
Assay Diluent RD1-105	895958	18 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-62	895998	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	2 vials (21 mL/vial) of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 1000 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Human Insulin Controls (optional; available from R&D Systems).

PRECAUTIONS

Insulin is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Grossly hemolyzed and icteric samples are not suitable for use in this assay.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

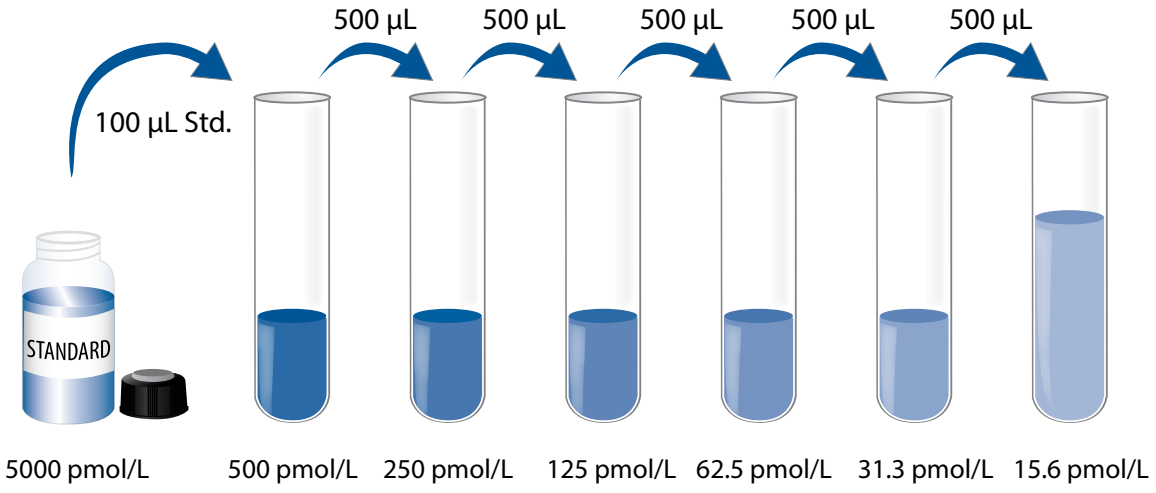
Note: *Insulin is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 40 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 1000 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Insulin Standard - Reconstitute the Insulin Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 5000 pmol/L. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-62 into the 500 pmol/L tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 500 pmol/L standard serves as the high standard. Calibrator Diluent RD5-62 serves as the zero standard (0 pmol/L).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *Insulin is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Aspirate and wash each well two times with Wash Buffer prior to assay. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 150 µL of Assay Diluent RD1-105 to each well.
5. Add 50 µL of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
6. Aspirate each well and wash as in step 3, repeating the process three times for a total of four washes.
7. Add 200 µL of Insulin Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
8. Repeat the aspiration/wash as in step 6.
9. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
10. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

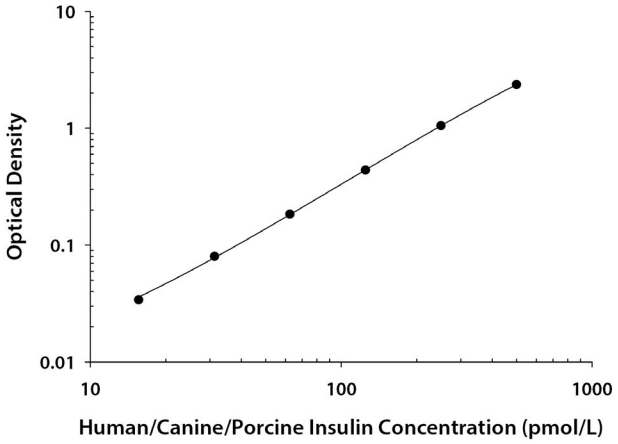
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Insulin concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pmol/L)	O.D.	Average	Corrected
0	0.007 0.007	0.007	—
15.6	0.040 0.042	0.041	0.034
31.3	0.083 0.091	0.087	0.080
62.5	0.177 0.204	0.191	0.184
125	0.425 0.465	0.445	0.438
250	1.020 1.093	1.057	1.050
500	2.342 2.399	2.371	2.364

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pmol/L)	66.8	158	299	62.2	148	288
Standard deviation	2.58	5.89	11.9	4.60	10.2	21.5
CV (%)	3.9	3.7	4.0	7.4	6.9	7.5

RECOVERY

The recovery of Insulin spiked to levels throughout the range of the assay in various matrices was evaluated.

Human Samples	Average % Recovery	Range
Cell culture media (n=4)	95	85-108%
Serum (n=4)	96	87-103%
EDTA plasma (n=4)	95	86-102%
Heparin plasma (n=4)	92	85-105%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Insulin were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

Human Samples		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	101	100	99	100
	Range (%)	95-104	94-104	93-106	95-104
1:4	Average % of Expected	100	102	103	104
	Range (%)	95-105	98-108	89-104	99-108
1:8	Average % of Expected	100	106	107	112
	Range (%)	93-107	98-114	98-115	106-114
1:16	Average % of Expected	110	107	104	108
	Range (%)	105-119	103-114	100-110	95-121

Note: *Canine & porcine samples were evaluated and no significant difference in linearity or recovery was observed from the data above.*

SENSITIVITY

Twenty-eight assays were evaluated and the minimum detectable dose (MDD) of Insulin ranged from 0.270-2.15 pmol/L. The mean MDD was 0.881 pmol/L.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *S. cerevisiae*-expressed recombinant human Insulin.

The NIBSC/WHO Insulin preparations 66/304, 1st International Reference Preparation for immunoassay, and 83/500, WHO International Standard for bioassay, were evaluated in this kit. The dose response curves of the standards 66/304 and 83/500 parallel the Quantikine standard curve. To convert sample values obtained with the Quantikine Human/Canine/Porcine Insulin kit to approximate NIBSC/WHO 66/304 or 83/500 Units, use the equations below.

NIBSC/WHO (66/304) approximate value (IU/mL) = 0.098 x 10^{-6} x Quantikine Insulin value (pmol/L)

NIBSC/WHO (83/500) approximate value (IU/mL) = 0.150 x 10^{-6} x Quantikine Insulin value (pmol/L)

Note: *Based on data generated in August 2013.*

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy, fasting volunteers were evaluated for the presence of Insulin in this assay. No medical histories were available for the donors used in this study.

Human Samples	Mean (pmol/L)	Range (pmol/L)	Standard Deviation (pmol/L)
Serum (n=34)	58.0	17.2-218	41
EDTA plasma (n=34)	60.3	15.8-208	39
Heparin plasma (n=34)	59.4	16.4-246	45

Canine Samples	Mean (pmol/L)	Range (pmol/L)	Standard Deviation (pmol/L)
Serum (n=10)	58.8	16.7-190	52
Heparin plasma (n=5)	78.3	21.5-146	56

Canine Samples	Mean of Detectable (pmol/L)	% Detectable	Range (pmol/L)
EDTA plasma (n=5)	96.4	80	ND-145

ND=Non-detectable

Porcine Samples	Mean of Detectable (pmol/L)	% Detectable	Range (pmol/L)
Serum (n=10)	22.6	90	ND-34.3
EDTA plasma (n=5)	27.1	80	ND-40.8
Heparin plasma (n=5)	30.4	80	ND-40.9

ND=Non-detectable

Cell Culture Supernates - Four cell culture supernate samples were tested. No detectable levels were observed.

SPECIFICITY

This assay recognizes recombinant human Insulin and natural human, canine, and porcine Insulin.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant Insulin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

IGF-I
IGF-II
INSL3
Insulin C-Peptide
Insulin R
Relaxin-1
Relaxin-2
Relaxin-3

Recombinant human Proinsulin cross-reacts approximately 0.25% in this assay.

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