Mercodia
Developing diagnostics

Mercodia Canine Insulin ELISA

Directions for Use

10-1203-01
REAGENTS FOR 96 DETERMINATIONS

EXPLANATION OF SYMBOLS USED ON LABELS

Σ Σ = **96**	Reagents for 96 determinations
	Expiry date
	Store between 2-8°C
LOT	Lot No.

INTENDED USE

Mercodia Canine Insulin ELISA provides a method for the quantitative determination of insulin in canine serum and plasma.

SUMMARY AND EXPLANATION OF THE TEST

Insulin is the principal hormone responsible for the control of glucose metabolism. It is synthesised in the ß-cells of the islets of Langerhans as the precursor, proinsulin, which is processed to form C-peptide and insulin. Both are secreted in equimolar amounts into the portal circulation. The mature insulin molecule comprises two polypeptide chains, the A chain and the B chain. The two chains are linked together by two inter-chain disulphide bridges. There is also an intra-chain disulphide bridge in the A chain.

Secretion of insulin is mainly controlled by plasma glucose concentration, and the hormone has a number of important metabolic actions. Its principal function is to control the uptake and utilisation of glucose in peripheral tissues via the glucose transporter. This and other hypoglycaemic activities, such as the inhibition of hepatic gluconeogenesis and glycogenolysis are counteracted by the hyperglycaemic hormones including glucagon, epinephrine (adrenaline), growth hormone and cortisol.

Diabetes mellitus is one of the most common endocrine disorders in dogs, with clinical features resembling the human type 1 diabetes, such as polydipsia, polyuria and weight loss (1-2). Diabetes is diagnosed in dogs with persistant hyperglycaemia (>9 mmol/L) and glucosuria. Diabetes typically occurs in dogs between 5 to 12 years of age. Furthermore, certain breeds of dog appear to be predisposed to diabetes and female dogs are more likely to develop diabetes (3).

PRINCIPLE OF THE PROCEDURE

Mercodia Canine Insulin 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. During incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microplate. After a simple washing step that removes unbound enzyme labelled antibody, the bound conjugate is detected by reaction with 3,3´-5,5´-tetramethylbenzidine (TMB). The reaction is stopped by the addition of acid, giving a colorimetric endpoint that can be read spectrophotometrically.

WARNINGS AND PRECAUTIONS

- The content of this kit and their residues must not be allowed to come into contact with ruminating animal or swine.
- The Stop Solution in this kit contains 0.5 M H_2SO_4. Follow routine precautions for handling hazardous chemicals.
- All samples should be handled as capable of transmitting infections.

MATERIAL REQUIRED BUT NOT PROVIDED
- Pipettes for 25, 50, 100, 200 and 1000 µL (repeat pipettes preferred for addition of enzyme conjugate 1X solution, Substrate TMB and Stop Solution).
- Beakers and cylinders for reagent preparation.
- Redistilled water.
- Microplate reader (450 nm filter).
- Plate shaker (The recommended velocity is 700-900 cycles per minute, orbital movement).
- Microplate washing device.

REAGENTS
Each Mercodia Canine Insulin ELISA (12-1203-01) kit contains reagents for 96 wells, sufficient for 42 samples and one calibrator curve in duplicate. For larger series of assays, use pooled reagents from packages bearing identical lot numbers. 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 Porcine insulin Color coded yellow Concentration stated on vial label	5 vials	1000 µL	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	Preparation, see below
Enzyme Conjugate Buffer Color coded blue	1 vial	13 mL	Ready for use
Wash Buffer 21X Storage after dilution: 2-8°C for 8 weeks	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_2SO_4	1 vial	7 mL	Ready for Use

Preparation of enzyme conjugate 1X solution

Prepare the needed volume of enzyme conjugate 1X solution by dilution of Enzyme Conjugate 11X, (1+10) in Enzyme Conjugate Buffer according to the table below. Mix gently.

When preparing enzyme conjugate 1X solution for the whole plate or if the reagents are to be used within 2 weeks, pour all of the Enzyme Conjugate Buffer into the Enzyme Conjugate 11X vial.

Number of strips	Enzyme Conjugate 11X	Enzyme Conjugate Buffer
12 strips	1 vial	1 vial
8 strips	700 µL	7.0 mL
4 strips	400 µL	4.0 mL

Storage after dilution: 2-8°C for 2 weeks.

SPECIMEN COLLECTION AND HANDLING

Serum

Collect blood by venipuncture, allow to clot and separate the serum by centrifugation. 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.

Plasma

Collect blood by venipuncture into tubes containing heparin, citrate or EDTA as anticoagulant, and separate the plasma fraction. 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

No dilution is normally required for serum or plasma. All samples containing canine insulin above the highest calibrator should be diluted with Calibrator 0 or with Mercodia Diabetes Sample Buffer (10-1195-01).

TEST PROCEDURE

All reagents and samples must be brought to room temperature before use. Perform each determination in duplicate for calibrators and samples. Prepare a calibrator curve for each assay run.

1. Prepare enzyme conjugate 1X solution (according to the table on previous page) and wash buffer 1X solution.
2. Prepare sufficient microplate wells to accommodate Calibrators and samples in duplicate.
3. Pipette 25 µL each of Calibrators and samples into appropriate wells.
4. Add 100 µL of enzyme conjugate 1X solution into each well.
5. Incubate on a plate shaker (700-900 rpm) for 2 hours at room temperature (18-25°C).
6. Wash 6 times with 700 µL wash buffer 1X solution per well using an automatic plate washer with overflow-wash function, after final wash, invert and tap the plate firmly against absorbent paper. Do not include soak step in washing procedure.
   Or manually,
   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.
7. Add 200 µL Substrate TMB into each well.
   Incubate for 15 minutes at room temperature (18-25°C).
8. Add 50 µL Stop Solution to each well.
   Place the plate on the shaker for approximately 5 seconds to ensure mixing.
9. Read optical density at 450 nm and calculate results.
   Read within 30 minutes.

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

INTERNAL QUALITY CONTROL

Commercial controls such as Mercodia Animal Insulin Control (10-1221-01) and/or internal serum pools with low, intermediate and high canine insulin concentrations should routinely be assayed as samples, and results charted from day to day. It is good laboratory practice to record the following data for each assay: kit lot number, preparation dates of kit components, OD values for the blank, Calibrators and controls.

CALCULATION OF RESULTS
Computerized calculation
The concentration of canine insulin is obtained by computerized data reduction of the absorbance for the Calibrators, except Calibrator 0, versus the concentration using cubic spline regression.

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

Example of results

Wells	Identity	A_{450}	Mean conc. µg/L
1A–B	Calibrator 0	0.054/0.053	
1C–D	Calibrator 1*	0.090/0.086	
1E–F	Calibrator 2*	0.135/0.133	
1G–H	Calibrator 3*	0.319/0.329	
2A–B	Calibrator 4*	0.907/0.880	
2C–D	Calibrator 5*	2.405/2.304	
2E–F	Sample 1	0.184/0.185	0.079
2G–H	Sample 2	0.571/0.560	0.290
3A–B	Sample 3	1.597/1.543	0.977

*Concentration stated on vial label.

Example of calibrator curve
A typical calibrator curve is shown here. Do not use this curve to determine actual assay results.
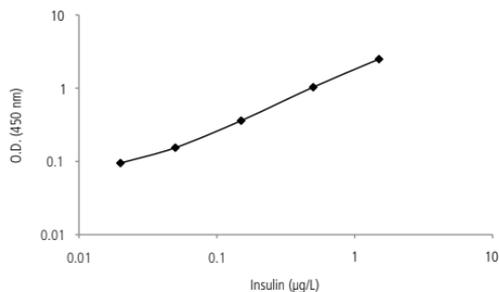
LIMITATIONS OF THE PROCEDURE

Grossly lipemic, icteric or hemolyzed samples do not interfere in the assay. Insulin is, however, degraded over time in hemolyzed samples. The degradation could give falsely low values and contributes to higher inter assay variation.

EXPECTED VALUES

Good practice dictates that each laboratory establishes its own expected range of values.

PERFORMANCE CHARACTERISTICS
Detection limit

Detection limit is defined as the Capability of Detection according to ISO11843-Part 1. Capability of Detection should be seen as part of a method validation, rather than the lowest concentration that can be measured.

The detection limit is 0.01 (µg/L) determined with the methodology described in ISO11843-Part 4.

Concentration of samples with absorbance below Calibrator 1 should not be calculated, instead expressed as less or equal to ≤ the concentration indicated on the vial for Calibrator 1.

Recovery

Recovery upon addition is 103-124 % (mean 111 %).
Recovery upon dilution is 75-102 % (mean 90 %).

Hook effect

Samples with a concentration of up to 1000 µg/L can be measured without giving falsely low results.

Precision

Each sample was analyzed in 4 replicates on 13 different occasions.

| Sample | Mean value µg/L | Coefficient of variation | | |
		within assay %	between assay %	total assay %
1	0.057	4.5	4.1	4.6
2	0.128	2.9	6.2	6.3
3	0.205	2.1	3.6	3.8

Specificity

The following cross reaction have been found:

Porcine C-peptide	< 0.001 %
Porcine Insulin	100 %
Porcine Proinsulin	< 0.2 %
Human C-peptide	< 0.01 %
Human Insulin	28 %
Human Proinsulin	< 0.1 %
Humalog	< 0.0000006 %
Lantus	5.4 %
Levemir	< 0.0000002 %
NovoRapid	0.7 %

CALIBRATION

Mercodia Canine Insulin ELISA is calibrated against an in-house reference preparation of porcine insulin.

WARRANTY

The performance data presented here was obtained using the procedure indicated. Any change or modification in the procedure not recommended by Mercodia AB may affect the results, in which event Mercodia AB disclaims all warranties expressed, implied or statutory, including the implied warranty of merchantability and fitness for use.

Mercodia AB and its authorized distributors, in such event, shall not be liable for damages indirect of consequential.

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2. Kim SP, Ellmerer M, Van Citters GW, Bergman RN (2003) Primacy of Hepatic Insulin Resistance in the Development of the Metabolic Syndrome Induced by an Isocaloric Moderate-Fat Diet in the Dog. *Diabetes* 52:2543-2460

3. Catchpole B, Ristic JM, Fleeman LM, Davison LJ (2005) Canine diabetes mellitus: can old dogs teach us new tricks? *Diabetologia* 48:1948-1956

SUMMARY OF PROTOCOL SHEET
Mercodia Canine Insulin ELISA

Add Calibrators and samples	25 µL
Add enzyme conjugate 1X solution	100 µL
Incubate	2 hours at 18-25°C on a plate shaker
Wash plate with wash buffer 1X solution	6 times
Add Substrate TMB	200 µL
Incubate	15 minutes at 18-25°C
Add Stop Solution	50 µL Shake for 5 seconds to ensure mixing
Measure A_{450}	Evaluate results