User Guide

Canine Leptin ELISA Kit

96-Well Plate

EZCL-31K

Intended Use

This kit is used for the non-radioactive quantification of Canine leptin in serum, plasma and other biological media. One kit is sufficient to measure 38 unknown samples in duplicate. **This kit is for Research Use Only. Not for Use in Diagnostic Procedures.**

Principles of Assay

This assay is a direct Sandwich ELISA based, sequentially, on:

- Capture of Canine leptin by a polyclonal Goat anti-Canine leptin antibody immobilized on a 96-well microtiter plate,
- Wash away unbound materials,
- Binding of a biotinylated monoclonal antibody to the captured Canine leptin,
- Wash away unbound materials,
- Binding of streptavidin-horseradish peroxidase to the immobilized biotinylated antibodies,
- Wash away free enzyme conjugates, and
- Quantification of bound streptavidin-horseradish peroxidase with the substrate 3,3’,5,5’-tetramethylbenzidine.

The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm-590 nm after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured Canine leptin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of Canine leptin.

Reagents Supplied

Each kit is sufficient to run one 96-well plate and contains the following reagents:

Note: Store all reagents at 2-8 °C

Reagents Supplied	Volume	Quantity	Cat. No.
Canine Leptin ELISA Plate **Note:** Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8 °C.	---------	1 plate	EP31
Adhesive Plate Sealer	---------	2 sheets	---------
Canine Leptin ELISA Standard Purified Recombinant Canine Leptin	0.5 mL	1 vial	E8031-K
Canine Leptin Quality Controls 1 and 2	0.5 mL	1 vials	E6031-K
Assay Buffer 0.05 M PBS, pH 7.4, containing 0.025 M EDTA, Sodium Azide, 1% BSA and 0.05% Triton™ X-100.	10 mL	1 vial	EABTR
10X HRP Wash Buffer Concentrate	50 mL	2 bottles	EWB-HRP
Canine Leptin Detection Antibody	11 mL	1 vial	E1031
Enzyme Solution	12 mL	1 vial	EHRP-5
Substrate Solution 3,3’5,5’-tetramethylbenzidine in buffer.	12 mL	1 bottle	ESS-TMB
Stop Solution (**Caution: Corrosive Solution**) 0.3 M HCl	12 mL	1 vial	ET-TMB

Storage and Stability

Recommended storage for kit components is 2-8 °C.

All components are shipped and stored at 2-8 °C. Reconstituted standards and controls can be frozen for future use but repeated freeze/thaw cycles should be avoided. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

Reagent Precautions

Hydrochloric Acid

Hydrochloric acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eyes. Do not swallow or ingest.

Sodium Azide

Sodium azide has been added to some reagents as a preservative. Although the concentrations are low, Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Ingredient	Cat. No.	Full Label	
Canine Leptin Detection Antibody	E1031		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
Canine Leptin ELISA Quality Controls 1 & 2	E6031-K		**Danger.** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention.
Canine Leptin ELISA Standard	E8031-K		**Danger.** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/attention.
Stop Solution	ET-TMB		**Danger.** May be corrosive to metals. Causes severe skin burns and eye damage. Wear protective gloves/ protective clothing/ eye protection/ face protection. IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. IF exposed or concerned: immediately call a POISON CENTER or doctor/ physician.
10X HRP Wash Buffer Concentrate for ELISA	EWB-HRP		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.

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Materials Required (Not Provided)

- Multi-channel Pipettes and pipette tips: 50 µL-300 µL
- Pipettes and pipette tips: 10 µL-200 µL
- Buffer and Reagent Reservoirs
- Vortex Mixer
- Deionized water
- Microtiter Plate Reader capable of reading absorbency at 450 nm
- Orbital Microtiter Plate Shaker
- Absorbent Paper or Cloth

Sample Collection and Storage

1. To prepare serum, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Let blood clot at room temperature for 30 minutes.
2. Promptly centrifuge the clotted blood at 2000 to 3000 x *g* for 15 minutes at 4 ± 2 °C.
3. Transfer and store serum samples in separate tubes. Date and identify each sample.
4. Avoid multiple (>5) freeze/thaw cycles.
5. To prepare plasma samples, whole blood should be collected into centrifuge tubes containing enough K_3EDTA to achieve a final concentration of 1.735 mg/mL and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.
6. If heparin is to be used as an anti-coagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.
7. Avoid using samples with gross hemolysis or lipemia.

Reagent Preparation

Canine Leptin Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Canine Leptin Standard with 0.5 mL distilled or deionized water to give a concentration described in the analysis sheet. Invert and mix gently, let sit for 5 minutes then vortex gently.

2. Label six tubes 1, 2, 3, 4, 5, and 6. Add 0.25 mL Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 0.25 mL of the reconstituted standard to Tube 1, mix well and transfer 0.25 mL of Tube 1 to Tube 2, mix well and transfer 0.25 mL of Tube 2 to Tube 3, mix well and transfer 0.25 mL of Tube 3 to Tube 4, mix well and transfer 0.25 mL of Tube 4 to Tube 5, mix well and transfer 0.25 mL of Tube 5 to Tube 6 and mix well.

   Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of standard should be stored at ≤ −20 °C. Avoid multiple freeze/thaw cycles.

Volume of Deionized Water to Add	Volume of Standard to Add	Standard Concentration (ng/mL)
0.5 mL	0	X (refer to analysis sheet for exact concentration)

Tube #	Volume of Assay Buffer to Add	Volume of Standard to Add	Standard Concentration (ng/mL)
Tube 1	0.25 mL	0.25 mL of reconstituted standard	X/2
Tube 2	0.25 mL	0.25 mL of Tube 1	X/4
Tube 3	0.25 mL	0.25 mL of Tube 2	X/8
Tube 4	0.25 mL	0.25 mL of Tube 3	X/16
Tube 5	0.25 mL	0.25 mL of Tube 4	X/32
Tube 6	0.25 mL	0.25 mL of Tube 5	X/64

Canine Leptin Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the Canine Leptin Quality Control 1 and Quality Control 2 with 0.5 mL distilled or deionized water into the vials. Invert and mix gently, let sit for 5 minutes then mix well.

Canine Leptin ELISA Assay Procedure

Warm all reagents to room temperature before setting up the assay.

1. Dilute the 10X Wash Buffer concentrate 10-fold by mixing the entire content of each bottle of Wash Buffer with 450 mL deionized or distilled water (dilute both bottles with 900 mL deionized water).
2. Remove required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8 °C. Assemble strips in an empty plate holder and wash each well 3 times with 300 µL of 1X Wash Buffer per wash. Decant wash buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.
3. Add 80 µL Assay Buffer into all wells.
4. Add in duplicate 20 µL Assay Buffer to blank wells. (Refer to Assay Procedure for suggested well orientations.)
5. Add in duplicate 20 µL Canine Leptin Standards in order of ascending concentration to the appropriate wells. Add in duplicate 20 µL QC1 and 20 µL QC2 to the appropriate wells. Add sequentially 20 µL of samples in duplicate to the remaining wells**. For best results all additions should be completed within one hour**.
6. Cover the plate with plate sealer and incubate at room temperature for 2 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.
7. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.
8. Wash wells 3 times with 1X Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.
9. Add 100 µL Detection Antibody to each well. Cover the plate with sealer and incubate at room temperature for 1 hour on the microtiter plate shaker.
10. Remove sealer and decant solution from the plate. Tap as before to remove residual solutions in the wells.
11. Wash wells 3 times with 1X Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

12. Add 100 µL Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.

13. Remove sealer, decant solution from the plate, and tap plate to remove the residual fluid.

14. Wash wells 6 times with 1X Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

15. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for 5-20 minutes. Blue color should be formed in wells of Leptin standards with intensity proportional to increasing concentrations of Leptin.

    Note: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time. One can monitor color development using 370 nm filter, if available on the spectrophotometer. When the absorbance is between 1.2 and 1.8 at 370 nm, the stop solution can be added to terminate the color development.

16. Remove sealer and add 100 µL of Stop Solution (**Caution: Corrosive solution**) and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Wipe the bottom of the microtiter plate to remove any residue prior to reading on plate reader. Read absorbance at 450nm and 590nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference in absorbance units. The absorbance of highest canine leptin standard should be approximately 2.2-2.8, or not to exceed the capability of the plate reader used.

Assay Procedure for Canine Leptin ELISA Kit

	Step 1	Step 2	Step 3-4	Step 5	Step 6-8	Step 9	Step 9-11	Step 12	Step 12-14	Step 15	Step 15	Step 16	Step 16
Well #			Assay Buffer	Standards/ QCs/Samples		Detection Antibody		Enzyme Solution		Substrate		Stop Solution	
A1, B1	Dilute each bottle of 10X with 450 mL Deionized Water.	Wash plate 3X with 300 µL Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels.	100 µL	--	Seal, Agitate, and Incubate 2 hours at Room Temperature. Wash 3X with 300 µL Wash Buffer	100 µL	Seal, Agitate, Incubate 1 hour at Room Temperature. Wash 3X with 300 µL Wash Buffer	100 µL	Seal, Agitate, and Incubate 30 minutes at Room Temperature. Wash 6X with 300 µL Wash Buffer	100 µL	Seal, Agitate, Incubate 5-20 minutes at Room Temperature.	100 µL	Read Absorbance at 450 nm and 590 nm.
C1, D1			80 µL	20 µL of X/64 Standard									
E1, F1				20 µL of X/32 Standard									
G1, H1				20 µL of X/16 Standard									
A2, B2				20 µL of X/8 Standard									
C2, D2				20 µL of X/4 Standard									
E2, F2				20 µL of X/2 Standard									
G2, H2				20 µL of X/1 Standard									
A3, B3				20 µL of QC 1									
C3, D3				20 µL of QC 2									
E3, F3				20 µL of Sample									
G3, H3				20 µL of Sample									
G4, H4 ↓				20 µL of Sample									

Microtiter Plate Arrangement

Canine Leptin ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	X/8 Standard	QC 1	Etc.								
B	Blank	X/8 Standard	QC 1									
C	X/64 Standard	X/4 Standard	QC 2									
D	X/64 Standard	X/4 Standard	QC 2									
E	X/32 Standard	X/2 Standard	Sample 1									
F	X/32 Standard	X/2 Standard	Sample 1									
G	X/16 Standard	X Standard	Sample 2									
H	X/16 Standard	X Standard	Sample 2									

Calculations

The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function.

Note: When sample volumes assayed differ from 20 µL (In normal assay), an appropriate mathematical adjustment must be made to accommodate for the dilution factor (for example, if 10 µL of sample is used, then calculated data must be multiplied by 2).

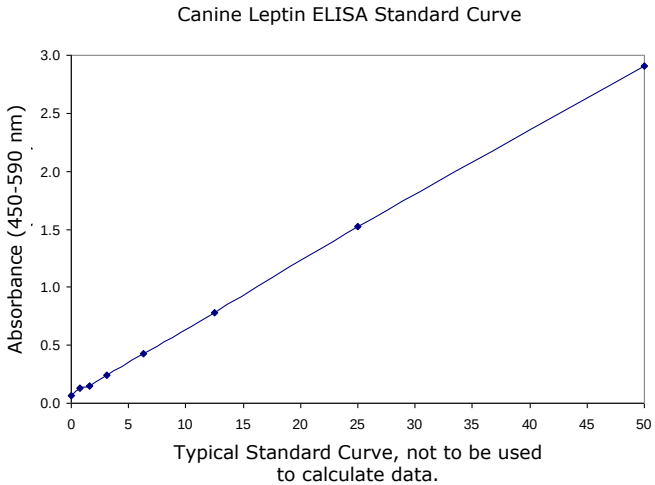
Interpretation

1. The assay should be rejected if one of the two QCs falls outside of 2 standard deviations of the applicable mean. See the supervisor.
2. If the difference between duplicate results of a sample is > 15% CV, repeat the sample.
3. The limit of sensitivity of this assay is 0.21 ng/mL Canine leptin (20 µL sample size).
4. The appropriate range of this assay is 0.78 to 50 ng/mL Canine leptin (20 µL sample size). Any result greater than 50 ng/mL in a 20 µL sample assayed should be diluted and repeated using assay buffer as diluent until it falls within range.

Normal Range

Normal range: Leptin levels are directly correlated with degree of adiposity.

Canine leptin levels in “normal” dogs range from undetectable to 20 ng/mL.



Assay Characteristics

Sensitivity

The lowest level of Canine leptin used in this assay this is 0.21 ng/mL (20 µL sample size). Sensitivity for this assay is 0.4 ng/mL as determined by minimum detection limit using 5 parametric analyses from 8 different assays (mean ± 2 SD).

Specificity

The antibody pair used in this assay is specific to Canine Leptin and does not significantly cross-react to the following molecules/hormones tested:

Canine Leptin	100%
Rat Leptin 30 ng/mL	n.d.
Mouse Leptin 20 ng/mL	n.d.
Human Insulin 200 µU/mL	n.d.
Human Proinsulin 100 pM	n.d.
Glucagon 200 pg/mL	n.d.
GLP-1 100 pM	n.d.
Human Adiponectin 100 ng/mL	n.d.
PYY 3-36 1.28 ng/mL	n.d.
Mouse Visafatin 100 ng/mL	n.d.
Rat Obestatin 10 ng/mL	n.d.
IL-6 10 ng/mL	n.d.
TNF-ALPHA 10 ng/mL	n.d.
PAI-1 10 ng/mL	n.d.
Human Resistin 10 ng/mL	n.d.

n.d.: Not detectable

Precision

Intra- and Inter-Assay Variation

	Mean Leptin Levels (ng/mL)	Intra-Assay % CV	Inter-Assay % CV
1	3.1	5.9	6.7
2	20.8	2.3	5.5

The assay variations of Canine Leptin ELISA kits were studied on two samples with varying concentrations of exogenous leptin. Intra-assay variations were calculated from ten duplicate determinations from a single assay. Inter-assay variations were calculated from single determinations in duplicate from eight separate assays.

Spike Recovery of Canine Leptin in Assay Samples

Exogenous Canine Leptin	% Expected (n=3)
3.12 ng/mL	105.9 ± 1.7
6.25 ng/mL	103.5 ± 1.8
12.5 ng/mL	100.0 ± 7.6

Two serum and one plasma samples were spiked with different amounts of exogenous Canine Leptin. These spiked serum and plasma samples were assayed by Canine Leptin ELISA. Expected values are the basal levels plus the spiked amount (3.125, 6.25 and 12.5 ng/mL) of Canine Leptin. The % Expected is observed value divided by expected value X 100 (Mean ± SD).

Linearity of Sample Dilution

Dilution Factor	Endogenous (n=8) % Expected	Exogenous (n=2) % Expected
1/8	Not Done	93.5 ± 17.9
1/4	91.5 ± 21.1	93.5 ± 4.5
1/2	98.6 ± 7.2	84.5 ± 2.4
1/1	100.0 ± 0.0	100.0 ± 0.0

Eight plasma samples with relatively high endogenous Canine Leptin levels and two serum samples with low canine leptin levels after spiking with 12.5 ng/mL of exogenous Canine Leptin were diluted 1/8, 1/4, and 1/2 with assay buffer and then assayed by Canine Leptin ELISA. % Expected values (mean ± SD) are 1/8, 1/4, 1/2 and 1/1 of the 20 µL sample value.

Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert, or available at our website SigmaAldrich.com.

Troubleshooting

- To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
- Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
- Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
- Avoid cross contamination of any reagents or samples to be used in the assay.
- Make sure all reagents and samples are added to the bottom of each well.
- Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
- Remove any air bubbles formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
- Do not let the absorbency reading of the highest standard reach 2.0 units or higher before adding the stop solution.
- High signal in background or blank wells could be due to:
    - cross well contamination by standard solution or sample, or
    - inadequate washing of wells with HRP

Product Ordering

Products are available for online ordering at SigmaAldrich.com.

Replacement Reagents

Reagents	Cat. No.
Canine Leptin ELISA Plate	EP31
10X HRP Wash Buffer Concentrate	EWB-HRP
Canine Leptin ELISA Standard	E8031-K
Canine Leptin Quality Controls 1 and 2	E6031-K
Assay Buffer	EABTR
Canine Leptin ELISA Detection Antibody	E1031
Enzyme Solution	EHRP-5
Substrate Solution	ESS-TMB
Stop Solution	ET-TMB

Notice

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