User Guide

Canine C-Peptide ELISA

96-Well Plate

EZCCP-47K
EZCCP47BK

Intended Use

This Canine C-Peptide ELISA kit is used for the non-radioactive quantification of C-peptide in canine serum and plasma. One kit is sufficient to measure 39 unknown samples in duplicate.

This kit is for Research Use Only. Not for use in Diagnostic Procedures.

Principles of Assay

This assay is a competitive ELISA based, sequentially, on:

- Capture of canine C-peptide molecules from samples/standard by a pre-titered limited amount of anti-canine C-peptide serum whose IgG fraction can be immobilized on the wells of a microtiter plate
- Subsequent competition of binding to the immobilized anti-canine C-peptide IgG by a pre-titered biotinylated canine C-peptide
- Washing of unbound materials from samples
- Binding of streptavidin-horseradish peroxidase conjugate to the immobilized biotinylated canine C-peptide
- Washing of excess free enzyme conjugates
- Quantification of immobilized enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3’,5,5’-tetramethylbenzidine

The enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm–590 nm after acidification of formed products. Since the decrease in absorbance is directly proportional to the amount of captured canine C-peptide in the unknown sample because of the fixed limited amount of anti-canine C-peptide IgG, the concentration can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of canine C-peptide.

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.
Microtiter Plate with 2 plate sealers **Note:** Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2–8 °C	-	1 plate 2 sealers	EP83
Canine C-Peptide Standard	Lyophilized	1 vial	E8047-K
Canine C-Peptide Quality Controls 1 and 2	Lyophilized	1 vial each	E6047-K
Matrix Solution	Lyophilized	1 vial	EMTX-1
Assay Buffer	25 mL	1 vial	EAB-GLU
10X Wash Buffer	50 mL	2 bottles	EWB-HRP
Canine C-Peptide Antibody	3 mL	1 bottle	E1047-P
Biotinylated Canine C-Peptide	3 mL	1 bottle	E1047-D
Enzyme Solution	12 mL	1 bottle	EHRP-5
Substrate Solution	12 mL	1 bottle	ESS-TMB2
Stop Solution	12 mL	1 bottle	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

Sodium Azide

Sodium azide or Proclin™ has been added to some reagents as a preservative. Although the concentrations are low, Sodium azide and Proclin™ 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.

Hydrochloric Acid

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

Note: See Full Labels of Hazardous components on next page.

Symbol Definitions

Ingredient	Cat. No.	Full Label	
Canine C-Peptide ELISA Standard	E8047-K		**Warning:** Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.
Canine C-Peptide Quality Control 1 and 2	E6047-K		**Warning:** Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.
Matrix Solution	EMTX-1	No symbol required	**Warning:** Toxic to aquatic life with long lasting effects. Avoid release to the environment.
Stop Solution	ET-TMB		**Warning:** May be corrosive to metals.
10X HRP Wash Buffer Concentrate	EWB-HRP		**Warning:** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.

Materials Required (Not Provided)

- Multi-channel Pipettes and pipette tips: 5-50 µL and 50-300 µL
- Pipettes and pipette tips: 10 µL-20 µL or 20 µL-100 µL
- Reagent Reservoirs
- Polypropylene Microfuge Tubes
- Vortex Mixer
- De-ionized water
- Microtiter Plate Reader capable of reading absorbency at 450 nm
- Orbital Microtiter Plate Shaker
- Absorbent Paper or Cloth

Sample Collection and Storage

Preparation of Serum Samples

- Whole blood is directly drawn into a Vacutainer® serum tube that contains no anti-coagulant. Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000 x *g*.
- Use freshly prepared serum or aliquot and store samples at ≤ −20 °C.
- Avoid multiple (> 2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.

Preparation of Plasma Samples

- Whole blood is directly drawn into a Vacutainer® EDTA-plasma tube. Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000 x *g*.
- Use freshly prepared plasma or aliquot and store samples at ≤ −20 °C.
- Avoid multiple (> 2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.

Note:

- A maximum of 20 µL per well of neat serum or plasma can be used.
- All samples must be stored in polypropylene tubes. DO NOT STORE SAMPLES IN GLASS.
- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anti-coagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

Reagent Preparation

Canine C-Peptide Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the canine C-peptide Standard with 0.5 mL distilled or de-ionized water. Invert and mix gently, let sit for 5 minutes then mix well.

2. Label 5 polypropylene microfuge tubes as 1, 2, 3, 4, and 5. Add 100 µL Assay Buffer to each of the tubes. Prepare serial dilutions by adding 100 µL of the reconstituted standard to Tube 5, mix well and transfer 100 µL of Tube 5 to Tube 4, mix well and transfer 100 µL of Tube 4 to Tube 3, mix well and transfer 100 µL of Tube 3 to Tube 2, mix well and transfer 100 µL of the Tube 2 to Tube 1, mix well. The 0 ng/mL Canine C-Peptide standard (Background) will be Assay Buffer.

Note: Change tip for every dilution. Wet tip with standard before dispensing. Unused portions of reconstituted standard should be stored in small aliquots at ≤ −20 °C. Avoid multiple freeze/thaw cycles.

Tube #	Volume of Deionized Water to Add	Volume of Standard to Add	Standard Stock Concentration
Reconstituted standard/Tube 6	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)
5	100 µL	100 µL of reconstituted standard	X/2
4	100 µL	100 µL of Tube 5	X/4
3	100 µL	100 µL of Tube 4	X/8
2	100 µL	100 µL of Tube 3	X/16
1	100 µL	100 µL of Tube 2	X/32

Canine C-Peptide Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Reconstitute each Canine C-Peptide Quality Control 1 and Quality Control 2 with 0.5 mL distilled or de-ionized water and gently invert to ensure complete hydration. Unused portions of the reconstituted Quality Controls should be stored in small aliquots at ≤ −20 °C. Avoid further freeze/thaw cycles.

Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 50 mL of 10X Wash Buffer with 450 mL deionized water. Store unused portion at 2-8 °C for up to one month.

Preparation of Matrix Solution

Add 1 mL distilled or de-ionized water to the bottle containing lyophilized Matrix Solution. Mix well. Allow at least 5 minutes for complete reconstitution. Any unused portion of the reconstituted Matrix Solution should be stored at ≤ −20 °C for up to one month.

Canine C-Peptide ELISA Assay Procedure

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

1. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8 °C. Assemble the strips in an empty plate holder. Add 300 µL diluted Wash Buffer to each well of the plate. Decant Wash Buffer and remove the residual volume by inverting the plate and tapping it smartly onto absorbent towels several times. Repeat wash procedure 2 additional 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.
2. Add 30 µL Assay Buffer to B_0 wells, 10 µL to Standard/Quality Control wells and 30 µL to sample wells.
3. Add 20 µL of appropriate Matrix Solution to B_0, Standards and Quality Control wells (refer to Microtiter Plate Arrangement section for suggested sample order placement).
4. Add 20 µL Standards and Quality Controls to the appropriate wells.
5. Add 20 µL of sample to the appropriate wells.
6. Add 25 µL Canine C-Peptide Antibody to all wells.
7. Cover the plate with plate sealer and incubate at room temperature for 3 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.
8. Remove plate sealer and add 25 µL Biotinylated Canine C-Peptide to all wells. Re-cover plate with sealer and incubate at room temperature for 1 hour on an orbital microtiter plate shaker set to rotate at a moderate speed, about 400 to 500 rpm.
9. Remove plate sealer and decant reagents from the plate. Tap as before to remove residual volume in well. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.
10. Add 100 µL Enzyme Solution to each well. Cover plate with sealer and incubate at room temperature for 30 minutes on an orbital microtiter plate shaker set to rotate at a moderate speed, about 400 to 500 rpm.
11. Remove sealer, decant reagents from the plate and tap plate to remove the residual volume. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

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

    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.

13. Remove sealer and add 100 µL Stop Solution (**Caution:** Corrosive Solution) and gently 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 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference of absorbance units. The absorbance of the lowest Canine C-Peptide standard should be approximately 2.0-3.0, or not to exceed the capability of the plate reader used.

    Note: When sample volumes assayed differ from 20 µL, 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). When sample volume assayed is less than 20 µL, compensate for the volume deficit with the Matrix Solution provided.

Assay Procedure for Rat/Mouse Insulin ELISA Kit

Option A: For Samples with Significant Serum Matrix Effect

Well #	Step 1	Step 2	Step 3	Step 4-5	Step 6	Step 7	Step 8	Step 9	Step 10	Step 11	Step 12-13		
		Assay Buffer	Matrix Solution	Standards/ QCs/Samples	Canine C-Peptide Antibody		Biotinylated Canine C-Peptide		Enzyme Solution		Substrate Solution		Stop Solution
A1, B1	Wash plate 3X with 300 µL Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels.	30 µL	20 µL	-	25 µL	Seal, Agitate, Incubate 3 hours at Room Temperature.	25 µL	Seal, Agitate, Incubate 1 hour at Room Temperature. Wash 3X with 300 µL Wash Buffer.	100 µL	Seal, Agitate, Incubate 30 mins at Room Temp. Wash 3X 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		10 µL	20 µL	20 µL of Tube 1									
E1, F1		10 µL	20 µL	20 µL of Tube 2									
G1, H1		10 µL	20 µL	20 µL of Tube 3									
A2, B2		10 µL	20 µL	20 µL of Tube 4									
C2, D2		10 µL	20 µL	20 µL of Tube 5									
E2, F2		10 µL	20 µL	20 µL of Tube 6									
G2, H2		10 µL	20 µL	20 µL of QC 1									
A3, B3		10 µL	20 µL	20 µL of QC 2									
C3, D3		30 µL	-	20 µL of Sample									
E3, F3		30 µL	-	20 µL of Sample									
G3, H3 Etc.		30 µL	-	20 µL of Sample									

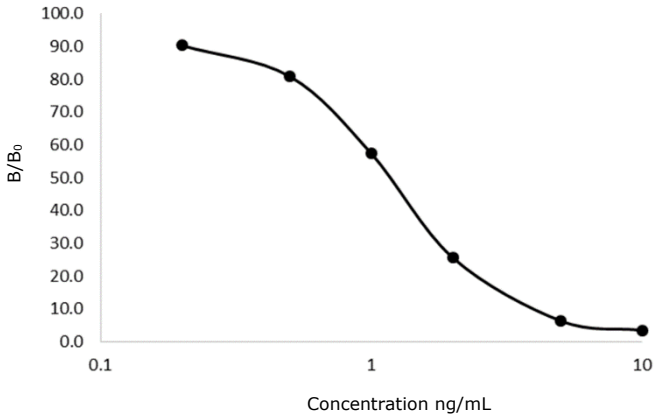
Microtiter Plate Arrangement

Canine C-Peptide ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	B_0	Tube 4	QC2									
B	B_0	Tube 4	QC2									
C	Tube 1	Tube 5	Sample 1									
D	Tube 1	Tube 5	Sample 1									
E	Tube 2	Reconstituted Standard	Sample 2									
F	Tube 2	Reconstituted Standard	Sample 2									
G	Tube 3	QC1	Sample 3									
H	Tube 3	QC1	Sample 3									

Graph of Typical Reference Curve

Canine C-Peptide Standard Curve



Typical Standard Curve, not to be used to calculate data.

Assay Characteristics

Sensitivity

The Minimum Detectable Concentration (MinDC) of Canine C-Peptide is 0.24 ng/mL. It is calculated by using MILLIPLEX® Analyst 5.1. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions. This reported value is the mean plus 2 standard deviations of the MinDC of multiple assays (n= 8).

Specificity

Canine C-Peptide	100%
Human C-Peptide	0.8%
Porcine C-Peptide	n.d.
Rat C-Peptide 1	n.d.
Rat C-Peptide 2	n.d.
Human Proinsulin	n.d.
Porcine Proinsulin	n.d.
Bovine Proinsulin	n.d.
Human Insulin	n.d.
Glucagon	n.d.

n.d.: Not detectable

Precision

	Mean Canine-C Peptide Levels (ng/mL)	Intra-Assay
1	0.39	< 15
2	1.95	< 10

	Mean Canine-C Peptide Levels (ng/mL)	Inter-Assay
1	0.39	< 10
2	1.96	< 10

The assay variations of Canine C-Peptide ELISA kit were studied on two samples at two levels on the Canine C-Peptide standard curve. The mean intra-assay variation was calculated from results of eight determinations of the indicated samples. The mean inter-assay variations of each sample were calculated from results of 6 separate assays with duplicate samples in each assay.

Spike Recovery of Canine C-Peptide in Assay Samples

Sample	Canine C-Peptide Added (ng/mL)	Expected (ng/mL)	Observed (ng/mL)	Recovery
1	0.5	1.17	1.12	96
	1	1.67	1.54	92
	2	2.67	2.38	89
2	0.5	0.84	0.87	104
	1	1.34	1.26	94
	2	2.34	2.11	90
3	0.5	1.07	1.06	99
	1	1.57	1.47	94
	2	2.57	2.4	93
4	0.5	0.83	0.86	104
	1	1.33	1.31	98
	2	2.33	2.28	98
5	0.5	1.01	0.99	98
	1	1.51	1.41	93
	2	2.51	2.2	88
Average				95

Varying amounts of C-Peptide were added to individual canine serum and plasma samples and the resulting C-peptide content of each sample was assayed by the Canine C-Peptide ELISA. The recovery = [(observed C-peptide / (spiked C-peptide concentration + basal C-peptide level)] x 100%.

Linearity of Sample Dilution

Sample	Volume (µL)	Expected (ng/mL)	Observed (ng/mL)	Recovery
1	20	5.53	5.53	-
	10	2.77	2.84	103
	5	1.39	1.55	112
	2.5	0.69	0.79	114
2	20	4.84	4.84	-
	10	2.42	2.7	112
	5	1.21	1.43	118
	2.5	0.61	0.66	108
3	20	4.8	4.8	-
	10	2.4	2.63	110
	5	1.2	1.42	118
	2.5	0.6	0.62	103
4	20	4.95	4.95	-
	10	2.48	2.67	108
	5	1.24	1.42	115
	2.5	0.62	0.65	105
5	20	5.49	5.49	-
	10	2.75	2.78	101
	5	1.37	1.45	106
	2.5	0.68	0.65	96
Average				109

Five canine serum and plasma samples with the indicated sample volumes were assayed. Required amounts of serum matrix were added to compensate for lost volumes below 20 µL. The resulting dilution factors of neat, 2, 4 and 8 representing 20 µL, 10 µL, 5 µL and 2.5 µL sample volumes assayed, respectively, were applied in the calculation of observed Canine C-Peptide concentrations. % expected = (observed/expected) x 100%.

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.
- High absorbance in background or blank wells could be due to
    - cross well contamination by standard solution or sample
    - inadequate washing of wells with Wash Buffer
    - overexposure to light after substrate has been added.

Product Ordering

Products are available for online ordering at SigmaAldrich.com.

Replacement Reagents

Reagents	Cat. No.
ELISA Plate	EP83
10X HRP Wash Buffer Concentrate	EWB-HRP
Canine C-Peptide ELISA Standard	E8047-K
Canine C-Peptide Quality Control 1 and 2	E6047-K
Matrix Solution	EMTX-1
Assay Buffer	EAB-GLU
Canine C-Peptide Antibody	E1047-P
Biotinylated Canine C-Peptide	E1047-D
Enzyme Solution	EHRP-5
Substrate Solution	ESS-TMB2
Stop Solution	ET-TMB
10 pack of Canine C-Peptide ELISA Kits	EZCCP47BK

Notice

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