CANINE ADIPONECTIN ELISA KIT
96-Well Plate (Cat. # EZCADP63K)

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CANINE ADIPONECTIN ELISA KIT
96 -Well Plate (Cat. # EZCADP63K)

I. INTENDED USE

This Canine Adiponectin (ACRP30) ELISA kit is used for the non-radioactive quantification of Canine Adiponectin in serum, plasma, tissue/cell lysate or cell culture supernatant samples. This kit has significant cross-reactivity to human, and rat Adiponectin. One kit is sufficient to measure 38 unknown samples in duplicate. *This kit is for research purpose only.*

II. PRINCIPLES OF PROCEDURE

This assay is a sandwich ELISA based, sequentially, on: 1) concurrent capture of canine Adiponectin molecules from samples to the wells of a microtiter plate coated with a pre-titered amount of anti-adiponectin monoclonal antibodies, and binding of a secondary biotinylated anti-adiponectin polyclonal antibody to captured canine adiponectin, 2) washing away of unbound materials from samples, 3) binding of conjugated horseradish peroxidase to the immobilized biotinylated antibodies, 4) washing away of free enzyme conjugates, and 5) quantification of immobilized antibody-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, corrected for the absorbance at 590nm. Since the increase in absorbance is directly proportional to the amount of captured canine Adiponectin in the unknown sample, the concentration can be calculated from the reference curve generated in the same assay with reference standards of known concentrations of canine Adiponectin.

III. REAGENTS SUPPLIED

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

A. Canine Adiponectin ELISA Plate
Coated with a Monoclonal Anti-Adiponectin Capture Antibody
Quantity: 1 strip plate
Preparation: Ready to Use
Note: Unused strips should be resealed in the foil pouch with the dessicant provided and stored at 2-8 °C.

B. Adhesive Plate Sealer
Quantity: 2 sheets
Preparation: Ready to Use

III. REAGENTS SUPPLIED (continued)

C. 10X Concentrate HRP Wash Buffer
10X concentrate of 50mM Tris Buffered Saline containing Tween-20.
Quantity: Two bottles containing 50 ml each
Preparation: Dilute 1:10 with distilled or deionized water prior to use.

D. Canine Adiponectin Calibrator (Standard)
Canine Adiponectin Calibrator, lyophilized.
Quantity: 0.5ml upon hydration
Preparation: Contents Lyophilized. Reconstitute well with 0.5 ml distilled or deionized water.

E. Quality Controls 1 and 2
One vial each, lyophilized, containing adiponectin in assay buffer at two different levels
Quantity: 0.5ml/vial upon hydration
Preparation: Reconstitute each control with 0.5 ml distilled or deionized water.

F. Assay Buffer
0.05M PBS pH 7.4 containing 0.025M EDTA, 0.08% Sodium Azide, 1% BSA
Quantity: 2 bottles containing 40 ml each
Preparation: Ready to use

G. Canine Adiponectin Detection Antibody
Pre-titered Biotinylated Anti-Canine Adiponectin Polyclonal Antibody
Quantity: 12 ml
Preparation: Ready to Use

H. Enzyme Solution
Pre-titered Streptavidin-Horseradish Peroxidase Conjugate in Buffer
Quantity: 12 ml
Preparation: Ready to Use

I. Substrate (Light sensitive, avoid unnecessary exposure to light)
3, 3′, 5, 5′-tetramethylbenzidine (TMB) in Assay Buffer
Quantity: 12 ml
Preparation: Ready to Use.

J. Stop Solution (Caution: Corrosive Solution)
0.3 M HCl
Quantity: 12 ml
Preparation: Ready to Use

IV. STORAGE AND STABILITY

Prior to use, all components in the kit can be stored up to 2 weeks at 2-8°C. For longer storage (> 2 weeks), freeze diluted Wash Buffer, Assay Buffer, and reconstituted Standards and Controls at ≤ –20°C. Minimize repeated freeze/ thaw cycles of the Adiponectin Standards and Quality Controls. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

V. REAGENT PRECAUTION

A. Sodium Azide

Sodium azide has been added to certain reagents as a preservative. Although the concentrations are low, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

B. 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.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and Pipette Tips: 10 µl - 20 µl or 20 µl - 100 µl
2. Multi-Channel Pipettes and Pipette Tips: 5 ~ 50 µl and 50 ~ 300 µl
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. Deionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm
7. Orbital Microtiter Plate Shaker
8. Absorbent Paper or Cloth

VII. SAMPLE COLLECTION AND STORAGE

1. To prepare serum samples, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Let blood clot at room temperature for 30 min.

   Promptly centrifuge the clotted blood at 2,000 to 3,000g for 15 minutes at 4 ± 2°C.

   Transfer and store serum samples in separate tubes. Date and identify each sample.

   Use freshly prepared serum or aliquot and store samples at ≤ −20°C for later use. For long-term storage, keep at -70°C. Avoid freeze/thaw cycles.

   To prepare plasma samples, whole blood should be collected in a centrifuge tube containing an appropriate anti-coagulant and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.

2. If heparin is to be used as an anticoagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.

3. Avoid using samples with gross hemolysis or lipemia.

VIII. SAMPLE AND REAGENT PREPARATION

Sample Preparation:

1. Allow all reagents to come to room temperature.

2. Dilute serum or plasma samples 1:2,000 in Assay Buffer. Cell extract and culture media dilutions will vary depending on experimental conditions. A dilution factor of 1:2,000 can be completed in following example.

   a. Prepare dilution (A) by adding 10 µl canine sample to 190 µl Assay Buffer and vortex.

   b. Prepare dilution (B) by adding 10 µl of dilution (A) to 990 µl of Assay Buffer and vortex. Dilution B (1:2,000) is the final dilution used in the assay procedure.

VIII. SAMPLE AND REAGENT PREPARATION (continued)

Reagent Preparation:

3. Canine Adiponectin Standard (Calibrator) Preparation

a. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the canine adiponectin standard with 0.5ml distilled or deionized water to give a concentration described on the analysis sheet. Invert and mix gently, allow the vial to set for 5 minutes, mix again. Transfer the reconstituted standard to a polypropylene tube labeled Tube 1. This will be the highest standard concentration point.

b. Label six polypropylene tubes 2, 3, 4, 5, 6, and 7. Add 0.4 ml Assay Buffer to tubes 2 through 7. Prepare a serial dilution by transferring 0.2 ml of the reconstituted standard (Tube 1) to Tube 2, mix well, and transfer 0.2ml of Tube 2 to Tube 3, mix well and transfer 0.2ml of Tube 3 to Tube 4, mix well and transfer 0.2ml of Tube 4 to Tube 5, mix well and transfer 0.2ml of Tube 5 to Tube 6, mix well and transfer 0.2ml of Tube 6 to Tube 7 and mix well.

Note: Do not use a Repeater pipette. Change tips for every dilution. Pre-wet pipetting tips with the standards before pipetting. The unused portion of reconstituted standard can be stored at ≤-20°C and re-used at a later date. Avoid multiple freeze/thaw cycles.

Tube #	Volume of Deionized Water to Add	Volume of Standard to Add	Standard Concentration
1	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
2	0.4 ml	0.2 ml of reconstituted standard (Tube 1)	X/3
3	0.4 ml	0.2 ml of Tube 2	X/9
4	0.4 ml	0.2 ml of Tube 3	X/27
5	0.4 ml	0.2 ml of Tube 4	X/81
6	0.4 ml	0.2 ml of Tube 5	X/243
7	0.4 ml	0.2 ml of Tube 6	X/729

VIII. SAMPLE AND REAGENT PREPARATION (continued)

4. Canine Adiponectin Quality Control 1 and 2 Preparation

a. Use care in opening the lyophilized Quality Control vials. Using an Eppendorf or equivalent pipette, reconstitute each of the Canine Adiponectin Quality Control 1 and Quality Control 2 vials with 0.5 ml of distilled or deionized water. Invert gently to mix, let set for 5 minutes, mix well. Transfer rehydrated controls to an appropriately labeled polypropylene microfuge tube or equivalent for futher use.

IX. ASSAY PROCEDURE

Pre-warm all reagents to room temperature prior to 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 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 and wash 3 times with 300µl of diluted 1X Wash Buffer. Decant Wash Buffer from all wells by inverting the plate and tapping firmly 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, use a gentle wash program for all washing steps described in this protocol.

3. Add 50 µl Assay Buffer to all wells.

4. Add an additional 50 µl Assay Buffer to the duplicate blank wells.

5. Add 50 µl Canine Adiponectin Standard in duplicate to the appropriate wells standards, pipetting in ascending order of concentration. Add in duplicate 50 µl QC1 and QC2 to the appropriate wells for Quality Controls. Add sequentially 50 µl of diluted (1:2000 for serum or plasma) samples in duplicate to the remaining wells for samples.

6. Cover plate with sealer and incubate at room temperature for 2.0 hours with shaking on a microtiter plate shaker set at approximately 400 to 500 rpm.

7. Remove the plate sealer and decant solutions from each well. Tap as before to remove residual solutions from the plate.

8. Wash plate 3 times with 300 µl 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer.

IX. ASSAY PROCEDURE (continued)

9. Add 100 µl Detection Antibody to all wells. Cover the plate with a plate sealer and incubate at room temperature for 1.0 hour with shaking on a microtiter plate shaker set at approximately 400 to 500 rpm.

10. Remove the plate sealer and decant solutions from each well. Tap as before to remove residual solutions from the plate.

11. Wash plate 3 times with 300 µl 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer.

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

12. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid.

14. Wash plate 3 times with 300µl 1X Wash Buffer. 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 with the same setting for 5 - 20 minutes. Blue color should be formed in wells of Adiponectin standards with intensity proportional to increasing concentrations of Adiponectin.

    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 a 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 Stop Solution, [CAUTION: CORROSIVE SOLUTION] shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Read absorbance at 450 nm and 590nm 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 highest Adiponectin standard should be approximately 2.6-2.8 or not to exceed the capability of the plate reader used.

Assay Procedure for Canine Adiponectin ELISA Kit (Cat. # EZCADP63K)

	Step 1	Step 2	Step 3-4	Step 5	Step 6-8	Step 9	Step 10-11	Step 12	Step 13-14	Step 15	Step 15	Step 16	Step 16
Well #	Dilute each bottle of 10X Wash Buffer with 450ml Deionized Water.	Wash plate 3X with 300 µl Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels	Assay Buffer	Standards/ Controls/ Samples	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µl Wash Buffer	Detection Ab	Seal, Agitate, Incubate 1.0 hours at Room Temperature. Wash 3X with 300 µl Wash Buffer	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature . Wash 3X with 300 µl Wash Buffer	Substrate	Seal, Agitate, Incubate approximately 5-20 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, A2			100 µl	______		100 µl		100 µl		100 µl		100 µl	
A3, A4			50 µl	50 µl of Tube 7		↓		↓		↓		↓	
A5, A6			50 µl	50 µl of Tube 6									
A7, A8			50 µl	50 µl of Tube 5									
A9, A10			50 µ l	50 µl of Tube 4									
A11, A12			50 µl	50 µl of Tube 3									
B1, B2			50 µl	50 µl of Tube 2									
B3, B4			50 µl	50 µl of Tube 1									
B5, B6			50 µl	50 µl of QC 1									
B7, B8			50 µl	50 µl of QC 2									
B9, B10			50 µl	50 µl of Sample									
B11, B12			50 µl	50 µl of Sample									
C1, C2 ↓			50 µl	50 µl of Sample									

X. MICROTITER PLATE ARRANGEMENT

Canine Adiponectin ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 4	QC 1	Etc.								
B	Blank	Tube 4	QC 1	Etc.								
C	Tube 7	Tube 3	QC 2									
D	Tube 7	Tube 3	QC 2									
E	Tube 6	Tube 2	Sample									
F	Tube 6	Tube 2	Sample									
G	Tube 5	Tube 1	Sample									
H	Tube 5	Tube 1	Sample									

XI. 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, or cubic spline functions. Alternatively, sample concentrations may be obtained using linear models. Final results should be multiplied by the dilution factor used for samples.

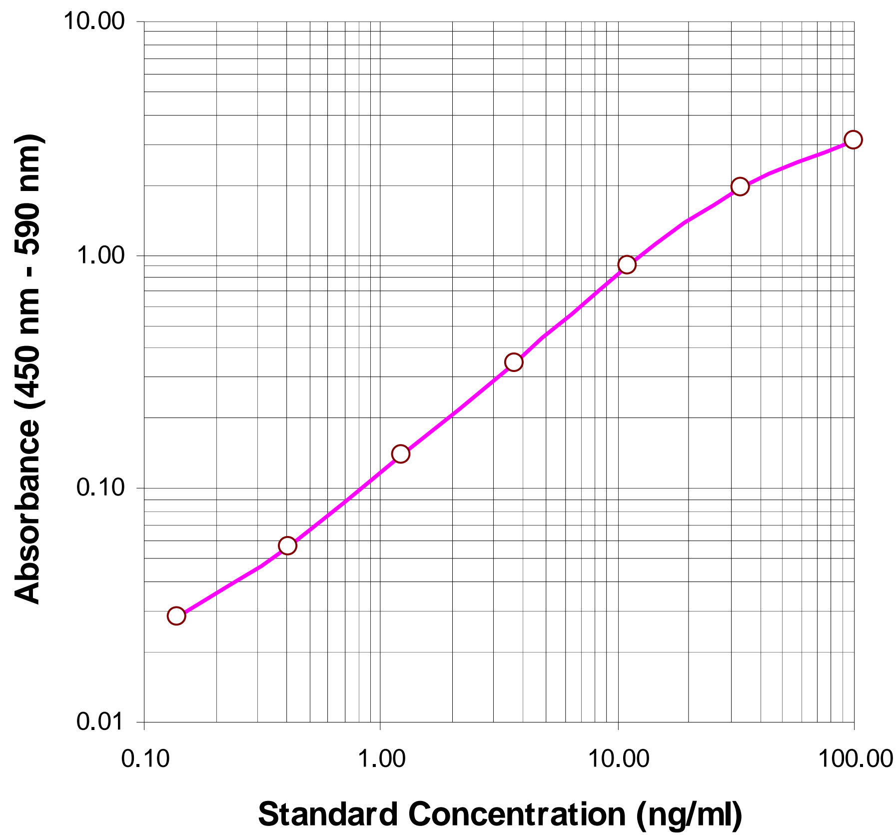
Note: When sample volume differs from 50 µl, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (i.e., if 25 µl of sample is used, then calculated data must be multiplied by 2).

XII. INTERPRETATION

A. Acceptance Criteria

1. The assay will be considered acceptable when all Quality Control values fall within the calculated Quality Control Range. If any QC's fall outside the control range, review results with a 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 approximately 0.05 ng/ml Canine Adiponectin.
4. The approximate dynamic range of this assay is 0.137 - 100 ng/ml Canine Adiponectin in a 50 µl sample size. Any results greater than 100 ng/ml in a 50 µl sample should be further diluted using the assay buffer provided in the kit, and the assay repeated until the results fall within range.

XIII. STANDARD CURVE



Typical Standard Curve, not to be used to calculate results. Users need to establish a standard curve each time the assay is run.

XIV. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Canine Adiponectin that can be detected by this assay is 0.048ng/ml when using a 50 µl sample size.

B. Specificity

The antibody pair used in this assay cross reacts with rat and human adiponectin. No significant signal was detected with tested using 14 canine cytokines, 29 human cytokines, 22 mouse cytokines and 24 rat cytokines. See Table below for details.

Analyte	Max. Conc.	Cross Reactivity	Analyte	Max. Conc.	Cross Reactivity
Human Adiponectin	200ng/ml	~33%	IL-7	10000pg/ml	n.d.
Mouse Adiponectin	50ng/ml	n.d.	TGF-α	10000pg/ml	n.d.
Rat Adiponectin	–	100%	Fractalkine	10000pg/ml	n.d.
Canine Cytokines:			IL-8	10000pg/ml	n.d.
GM-CSF	50000pg/ml	n.d.	IL-10	10000pg/ml	n.d.
IFNy	50000pg/ml	n.d.	IL-12p70	10000pg/ml	n.d.
KC	50000pg/ml	n.d.	IL-13	10000pg/ml	n.d.
IP-10	50000pg/ml	n.d.	IL-15	10000pg/ml	n.d.
IL-2	50000pg/ml	n.d.	IL-17	10000pg/ml	n.d.
IL-4	50000pg/ml	n.d.	IL-1-α	10000pg/ml	n.d.
IL-6	50000pg/ml	n.d.	IFNy	10000pg/ml	n.d.
IL-7	50000pg/ml	n.d.	G-CSF	10000pg/ml	n.d.
IL-8	50000pg/ml	n.d.	GM-CSF	10000pg/ml	n.d.
IL-10	50000pg/ml	n.d.	TNF-α	10000pg/ml	n.d.
IL-15	50000pg/ml	n.d.	Eotaxin	10000pg/ml	n.d.
IL-18	50000pg/ml	n.d.	MCP-1	10000pg/ml	n.d.
MCP-1	50000pg/ml	n.d.	sCD40L	10000pg/ml	n.d.
TNF-α	50000pg/ml	n.d.	IL-12p40	10000pg/ml	n.d.
Human Cytokine:			MIP-1α	10000pg/ml	n.d.
IL-1ß	10000pg/ml	n.d.	MIP-1ß	10000pg/ml	n.d.
IL-2	10000pg/ml	n.d.	IP-10	10000pg/ml	n.d.
IL-1ra	10000pg/ml	n.d.	VEGF	10000pg/ml	n.d.
IL-4	10000pg/ml	n.d.			
IL-5	10000pg/ml	n.d.			
EGF	10000pg/ml	n.d.			
IL-6	10000pg/ml	n.d.			

n.d. = not detectable

XIV. ASSAY CHARACTERISTICS (continued)

C. Precision

Intra- and Inter-Assay Variation

Sample No.	Mean Adiponectin Levels (ng/ml)	Intra-Assay CV (%)
1	1.58	4.21
2	13.39	2.86

Sample No.	Mean Adiponectin Levels (ng/ml)	Inter-Assay CV (%)
1	1.73	8.97
2	13.47	0.46

The assay variations of Millipore Canine Adiponectin ELISA kits were studied on two Canine samples at two levels on the Adiponectin std curve. The mean intra-assay variation was calculated from eight determinations of the indicated samples. The mean inter-assay variations of each sample was calculated from results of four separate assays with duplicate samples in each assay.

XIV. ASSAY CHARACTERISTICS (continued)

D. Recovery

Spike & Recovery of Canine Adiponectin in Serum

Sample No.	Adiponectin Spiked (ng/ml)	Expected (ng/ml)	Observed (ng/ml)	% of Recovery
1	0	4.23	4.23	100.0
	3.71	7.94	7.84	98.7
	11.1	15.33	15.22	99.3
	33.3	37.53	36.97	98.5
2	0	3.41	3.41	100.0
	3.71	7.12	6.79	95.4
	11.1	14.51	14.55	100.3
	33.3	36.71	35.77	97.4
3	0	3.44	3.44	100.0
	3.71	7.15	7.10	99.3
	11.1	14.54	14.61	100.5
	33.3	36.74	35.39	96.3

Varying concentrations of Canine Adiponectin was spiked to three separate Canine serum samples. The % recovery was determined as follows: Observed Adiponectin concentrations/expected Adiponectin concentrations x 100%.

XIV. ASSAY CHARACTERISTICS (continued)

E. Linearity of Dilution

Effect of Serum Dilution

Sample No.	Volume Sampled (µl)	Expected (ng/ml)	Observed (ng/ml)	% Of Expected
1	50	51.93	51.93	100.0
	25	25.97	24.63	94.8
	12.5	12.98	11.89	91.6
	6.25	6.49	6.39	98.5
2	50	47.48	47.48	100.0
	25	23.74	21.94	92.4
	12.5	11.87	11.08	93.3
	6.25	5.94	5.65	95.1
3	50	31.79	31.79	100.0
	25	15.90	14.78	93.0
	12.5	7.95	7.01	88.2
	6.25	3.97	3.91	98.5

Three Canine serum samples with the indicated sample volumes were assayed in three separate experiments. Required amounts of assay buffer were added to compensate for lost volumes below 50 µl. The resulting dilution factors of 1.0, 2.0, 4.0, and 8.0 representing 50 µl, 25 µl, 12.5 µl, and 6.25 µl sample volumes assayed, respectively, were applied in the calculation of observed Adiponectin concentrations. % expected = observed/expected x 100%.

XV. QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert included in the kit or can be located at the Millipore website www.millipore.com.

XVI. TROUBLESHOOTING GUIDE

1. 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.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. 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.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure all reagents and samples are added to the bottom of each well.
6. 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.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. Do not let the absorbency reading of the highest standard reach 3.0 units or higher after acidification.
9. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with HRP Wash Buffer or 3) overexposure to light after substrate has been added.

XVII. REPLACEMENT REAGENTS

Reagents	Cat. #
Canine Adiponectin ELISA Plate	EP63
10X HRP Wash Buffer Concentrate (50 ml)	EWB-HRP
Canine Adiponectin Standards	E8063-K
Quality Controls 1 and 2	E6363-K
Assay Buffer	AB-P
Canine Adiponectin Detection Antibody	E1063
Enzyme Solution	EHRP
Substrate	ESS-TMB2
Stop Solution	ET-TMB

XVIII. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

TELEPHONE ORDERS:
Toll Free US (866) 441-8400
(636) 441-8400
FAX ORDERS: (636) 441-8050
MAIL ORDERS: MILLIPORE
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers, place an order or to obtain additional information about Millipore products, please refer to the MILLIPORE website located at www.millipore.com.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to human or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.