Quantikine® ELISA

Canine CCL2/MCP-1 Immunoassay

Catalog Number CACP00

For the quantitative determination of canine Monocyte Chemotactic Protein 1 (MCP-1) 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

Monocyte chemotactic protein 1 (MCP-1) is a member of the CC chemokine family and is designated CC chemokine ligand 2 (CCL2) (1, 2). Based on its protein sequence homology, gene structure, and chromosomal location, MCP-1 is a member of the MCP/Eotaxin subgroup within the CC chemokine family (3). MCP-1 is a pleiotropic cytokine with multiple pro-inflammatory activities (3-6). It is a potent chemoattractant for monocytes, basophils, NK cells, and T cells. It induces the release of effectors, including histamine, leukotriene C4, and enzymes and upregulates the expression of various adhesion molecules and cytokines from target cells. It stimulates the development of Th2 responses from Th0 cells. In neurons, MCP-1 signaling contributes to sensory neuron excitation and neuropathic pain (7).

Canine MCP-1 is produced as a 101 amino acid (aa) precursor with a 23 aa signal peptide. The 78 aa mature canine MCP-1 is secreted as a 8.7 kDa protein that contains two intrachain disulfide bonds but no potential N-linked glycosylation sites (8). In solution, MCP-1 exists in equilibrium between monomers and dimers (9, 10). Heterodimer formation has been observed between CCL2 and CCL8, CCL13, or CCL11 (11). Similarly to many chemokine family members, MCP-1 binds to specific glycosaminoglycans (GAGs) of the extracellular matrix and on cell surfaces (12, 13). The MCP-1 GAG binding site overlaps its receptor binding site, both of which are localized in the N-terminal half of the protein (14). Immobilization on GAGs can influence the localization, concentration, stability and presentation of chemokines. Upon binding to GAGs, MCP-1 oligomerizes to form tetramers and octamers (12, 13). The oligomeric state required for chemokine functions is controversial (12). In the case of MCP-1, GAG-binding appears to be essential for *in vivo* MCP-1 functions (5, 12). N-terminally or C-terminally truncated forms of human MCP-1 have been reported (15, 16). The N-terminal truncation is mediated by MMP-1 or MMP-3 and is associated with reduced MCP-1 activity (17). It is predicted that proteolytic processing can also modulate canine MCP-1 activity. A broad spectrum of cell types and tissues, including tumor cells, has been shown to express MCP-1 (3). Canine MCP-1 shares 80%, 79%, 77%, and 73% aa sequence identity with human, equine, porcine, and bovine MCP-1, respectively.

MCP-1 binds and signals through CC chemokine receptor 2 (CCR2), a seven transmembrane G-protein coupled receptor that also responds to CCL8 (MCP-2), CCL7 (MCP-3), CCL13 (MCP-4) and CCL16 (HCC-4) (18, 19). CCR2 is present on various hematopoietic cells including monocytes, basophils, immature dendritic cells, and memory T cells. It is also constitutively expressed on cells in the CNS including neurons, activated astrocytes, microglia and neural progenitor cells (7). As is the case for a number of chemokine receptors, the N-terminus of CCR2 is tyrosine sulfated (18, 19). Receptor activation initiates signaling cascades that lead to multiple biological activities (3). Besides CCR2, MCP-1 also binds a number of non-signaling chemokine receptors including D6, DARC, and viral chemokine receptors (20-22). These receptors may have a role in limiting inflammatory responses by sequestering MCP-1.

The Quantikine Canine CCL2/MCP-1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure canine MCP-1 in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant canine MCP-1 and antibodies raised against the recombinant protein. Results obtained for naturally occurring canine MCP-1 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 natural canine MCP-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for canine MCP-1 has been pre-coated onto a microplate. Standards, Control and samples are pipetted into the wells and any canine MCP-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for canine MCP-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of canine MCP-1 bound in the initial step. The sample values are then read from the standard curve.

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.
- 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.

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
Canine MCP-1 Microplate	893129	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for canine MCP-1.	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.*
Canine MCP-1 Conjugate	893130	12 mL of a polyclonal antibody against canine MCP-1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Canine MCP-1 Standard	893131	5 ng of recombinant canine MCP-1 in a buffered protein base with preservatives; lyophilized.	
Canine MCP-1 Control	893132	Recombinant canine MCP-1 in a buffered protein base with preservatives; lyophilized. The concentration range of recombinant canine MCP-1 after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.	
Assay Diluent RD1W	895038	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-12	895214	21 mL of animal serum with preservatives.	
Wash Buffer Concentrate	895003	21 mL 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	895174	23 mL of diluted hydrochloric 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.
- 500 mL graduated cylinder.
- Test tubes for dilution of standards and samples.

PRECAUTIONS

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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 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 20 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.*

SAMPLE PREPARATION

Cell culture supernate samples may require dilution.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

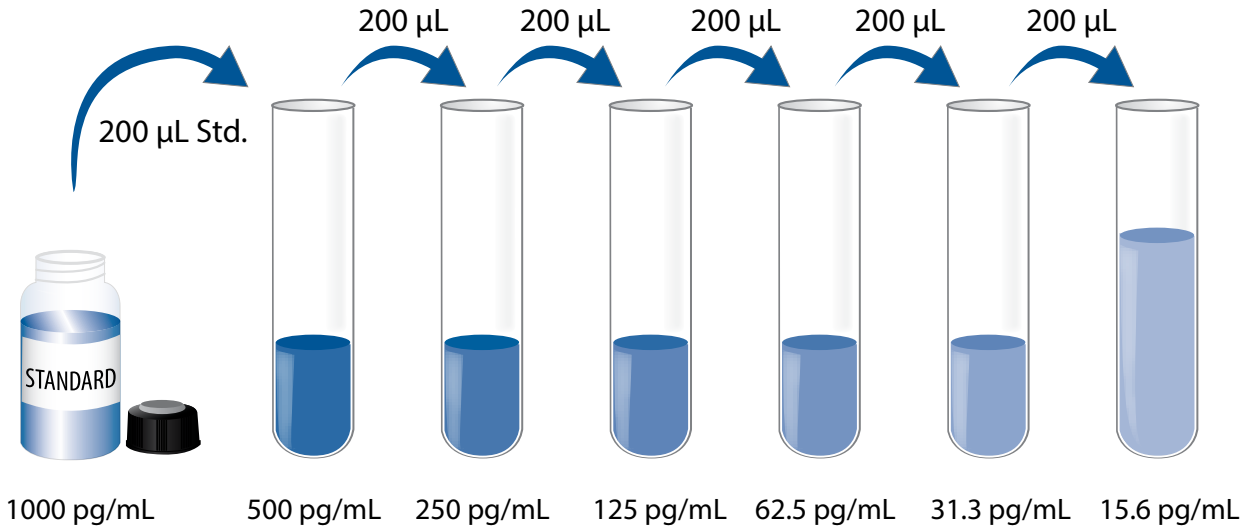
Canine MCP-1 Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 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. 100 µL of the resultant mixture is required per well.

Canine MCP-1 Standard - Reconstitute the Canine MCP-1 Standard with 5.0 mL of Calibrator Diluent RD6-12. Do not substitute other diluents. This reconstitution produces a stock solution of 1000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD6-12 into each tube. Use the standard stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Canine MCP-1 Standard (1000 pg/mL) serves as the high standard. Calibrator Diluent RD6-12 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

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

1. Prepare all reagents, standard dilutions, Control, 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. Add 50 µL of Assay Diluent RD1W to each well.
4. Add 50 µL of Standard, Control, or sample* per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process four times for a total of five washes. 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.
6. Add 100 µL of Canine MCP-1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. 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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

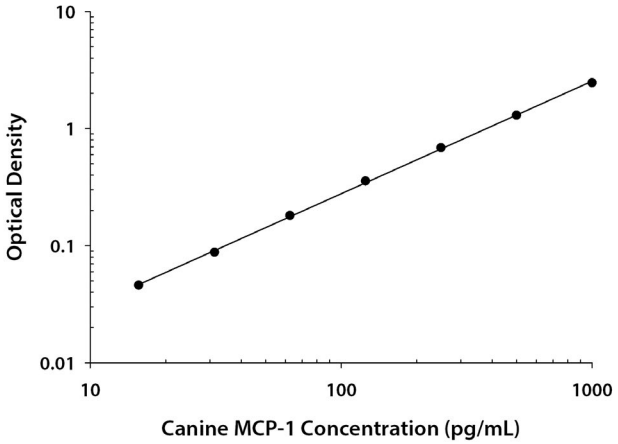
Average the duplicate readings for each standard, Control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log 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 a log/log graph. The data may be linearized by plotting the log of the canine MCP-1 concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the 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.



(pg/mL)	O.D.	Average	Corrected
0	0.040 0.041	0.041	—
15.6	0.087 0.087	0.087	0.046
31.3	0.127 0.131	0.129	0.088
62.5	0.219 0.225	0.222	0.181
125	0.390 0.405	0.398	0.357
250	0.712 0.741	0.727	0.686
500	1.320 1.362	1.341	1.300
1000	2.478 2.526	2.502	2.461

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 forty-three separate assays to assess inter-assay precision.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	43	43	43
Mean (pg/mL)	37.1	144	632	42.7	133	655
Standard deviation	2.2	5.8	22	5.4	7.5	30
CV (%)	5.9	4.0	3.5	12.6	5.6	4.6

RECOVERY

The recovery of canine MCP-1 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n=4)	112	106-121%
Serum (n=4)	93	84-102%
EDTA plasma (n=4)	110	102-116%
Heparin plasma (n=4)	103	96-112%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of canine MCP-1 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	94	100	99	102
	Range (%)	90-96	98-104	96-100	96-104
1:4	Average % of Expected	88	95	96	99
	Range (%)	85-92	89-106	89-105	97-104
1:8	Average % of Expected	88	93	93	96
	Range (%)	87-89	83-105	86-97	89-101
1:16	Average % of Expected	91	89	93	88
	Range (%)	83-96	83-97	89-101	81-94

*Sample were diluted prior to assay.

SENSITIVITY

Thirty-seven assays were evaluated and the minimum detectable dose (MDD) of canine MCP-1 ranged from 1.2-10.8 pg/mL. The mean MDD was 3.4 pg/mL.

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 *E. coli*-expressed recombinant canine MCP-1 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma -Samples were evaluated for detectable levels of canine MCP-1 in this assay.

Sample	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=14)	374	170-561	112
EDTA plasma (n=17)	100	49-151	29
Heparin plasma (n=13)	134	67-192	36

Cell Culture Supernates:
Canine peripheral blood cells (2 x 10^5 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum and stimulated with 10 µg/mL Concanavilin A and 10 µM β-mercaptoethanol. An aliquot of the cell culture supernate was removed, assayed for canine MCP-1, and measured 30,588 pg/mL.

MDCK canine kidney epithelial cells (1 x 10^5 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum and stimulated with 10 µg/mL PHA and 10 ng/mL PMA for 3 days. An aliquot of the cell culture supernate was removed, assayed for canine MCP-1, and measured 3857 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant canine MCP-1.

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 canine MCP-1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant canine:

GM-CSF
IFN-γ
IL-2
IL-4
IL-5
IL-6
IL-8
IL-10
IL-12/IL-23 p40
KGF
SCF
TNF-α
VEGF

Recombinant mouse:

Eotaxin
JE/MCP-1

Recombinant human:

Eotaxin
MCP-1
MCP-2
MCP-3
MCP-4

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