

# Quantikine<sup>®</sup> ELISA

## Canine IL-6 Immunoassay

Catalog Number CA6000

For the quantitative determination of canine Interleukin 6 (IL-6) 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

Interleukin 6 (IL-6) is a pleiotropic,  $\alpha$ -helical, 22-28 kDa phosphorylated and variably glycosylated cytokine that plays important roles in the acute phase reaction, inflammation, hematopoiesis, bone metabolism, and cancer progression (1-5). Mature canine IL-6 is 187 amino acids (aa) in length and shares 58%, 37%, 39%, and 76% aa sequence identity with human, mouse, rat, and feline IL-6, respectively. Cells known to express IL-6 include CD8<sup>+</sup> T cells, fibroblasts, synoviocytes, adipocytes, osteoblasts, megakaryocytes, endothelial cells (under the influence of endothelins), sympathetic neurons, cerebral cortex neurons, adrenal medulla chromaffin cells, retinal pigment cells, mast cells, keratinocytes, Langerhans cells, fetal and adult astrocytes, neutrophils, monocytes, eosinophils, colonic epithelial cells, B1 B cells and pancreatic islet beta cells (2, 6-28). IL-6 production is generally correlated with cell activation and is normally kept in control by glucocorticoids, catecholamines, and secondary sex steroids (2). Normal human circulating IL-6 is in the 1 pg/mL range, with slight elevations during the menstrual cycle, modest elevations in certain cancers, and large elevations after surgery (29-33).

IL-6 induces signaling through a cell surface heterodimeric receptor complex composed of a ligand binding subunit (IL-6 R alpha) and a signal transducing subunit (gp130). IL-6 binds to IL-6 R $\alpha$ , triggering IL-6 R $\alpha$  association with gp130 and gp130 dimerization (34). gp130 is also a component of the receptors for CLC, CNTF, CT-1, IL-11, IL-27, LIF, and OSM (35). Soluble forms of IL-6 R $\alpha$  are generated by both alternative splicing and proteolytic cleavage (5). In a mechanism known as trans-signaling, complexes of soluble IL-6 and IL-6 R $\alpha$  elicit responses from gp130-expressing cells that lack cell surface IL-6 R $\alpha$  (5). Trans-signaling enables a wider range of cell types to respond to IL-6, as the expression of gp130 is ubiquitous, while that of IL-6 R $\alpha$  is predominantly restricted to hepatocytes, monocytes, and resting lymphocytes (2, 5). Soluble splice forms of gp130 block trans-signaling from IL-6/IL-6 R $\alpha$  but not from other cytokines that use gp130 as a co-receptor (5, 36).

IL-6, along with TNF- $\alpha$  and IL-1, drives the acute inflammatory response. IL-6 is almost solely responsible for fever and the acute phase response in the liver, and it is important in the transition from acute inflammation to either acquired immunity or chronic inflammatory disease (1-5). When dysregulated, it contributes to chronic inflammation in conditions such as obesity, insulin resistance, inflammatory bowel disease, arthritis, and sepsis (2, 5). IL-6 modulates bone resorption and is a major effector of inflammatory joint destruction in rheumatoid arthritis through its promotion of Th17 cell development and activity (1). It contributes to atherosclerotic plaque development and destabilization as well as the development of inflammation-associated carcinogenesis (1, 2). IL-6 can also function as an anti-inflammatory molecule, as in skeletal muscle where it is secreted in response to exercise (2). In addition, it enhances hematopoietic stem cell proliferation and the differentiation of memory B cells and plasma cells (37).

The Quantikine Canine IL-6 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure canine IL-6 in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant canine IL-6 and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained using natural canine IL-6 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 naturally occurring canine IL-6.

## PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for canine IL-6 has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any canine IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for canine IL-6 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 IL-6 bound in the initial step. The sample values are then read off 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 the 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.
- For best results, pipette reagents and samples into the center of each well.
- 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
<b>Canine IL-6 Microplate</b>	893125	96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for canine IL-6.	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.*  May be stored for up to 1 month at 2-8 °C.*
<b>Canine IL-6 Conjugate</b>	893126	21 mL of a polyclonal antibody specific for canine IL-6 conjugated to horseradish peroxidase with preservatives.	
<b>Canine IL-6 Standard</b>	893127	10 ng of recombinant canine IL-6 in a buffered protein base with preservatives; lyophilized.	
<b>Canine IL-6 Control</b>	893128	1 vial of recombinant canine IL-6 in a buffered protein base with preservatives; lyophilized. The assay value of the Control should be within the range specified on the label.	
<b>Assay Diluent RD1-75</b>	895811	11 mL of a buffered protein base with preservatives. <i>May contain a precipitate. Mix well before and during use.</i>	
<b>Calibrator Diluent RD5T</b>	895175	21 mL of a buffered protein base with preservatives.	
<b>Wash Buffer Concentrate</b>	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. <i>May turn yellow over time.</i>	
<b>Color Reagent A</b>	895000	12 mL of stabilized hydrogen peroxide.	
<b>Color Reagent B</b>	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
<b>Stop Solution</b>	895174	23 mL of diluted hydrochloric acid.	
<b>Plate Sealers</b>	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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of  $500 \pm 50$  rpm.
- Test tubes for dilution of standards.

## PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin<sup>®</sup> 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  $\leq -20$  °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  $\leq -20$  °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  $\leq -20$  °C. Avoid repeated freeze-thaw cycles.

**Note:** Citrate plasma has not been validated for use in this assay.

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## REAGENT PREPARATION

**Bring all reagents to room temperature before use.**

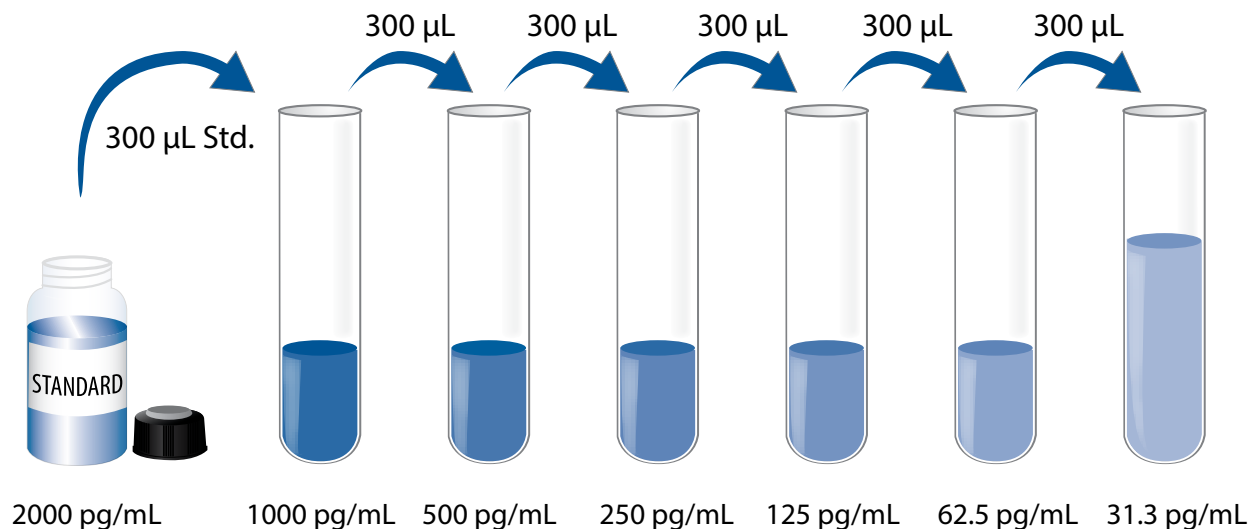
**Canine IL-6 Control** - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

**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. 120  $\mu$ L of the resultant mixture is required per well.

**Canine IL-6 Standard** - Reconstitute the Canine IL-6 Standard with 5.0 mL of Calibrator Diluent RD5T. Do not substitute other diluents. This reconstitution produces a stock solution of 2000 pg/mL. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 300  $\mu$ L of Calibrator Diluent RD5T into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The undiluted canine IL-6 Standard serves as the high standard (2000 pg/mL). Calibrator Diluent RD5T 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 samples, standards, and control 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  $\mu\text{L}$  of Assay Diluent RD1-75 to each well.
4. Add 100  $\mu\text{L}$  of Standard, Control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at  $500 \pm 50$  rpm.
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  $\mu\text{L}$ ) 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 200  $\mu\text{L}$  of Canine IL-6 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 120  $\mu\text{L}$  of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 120  $\mu\text{L}$  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.



## CALCULATION OF RESULTS

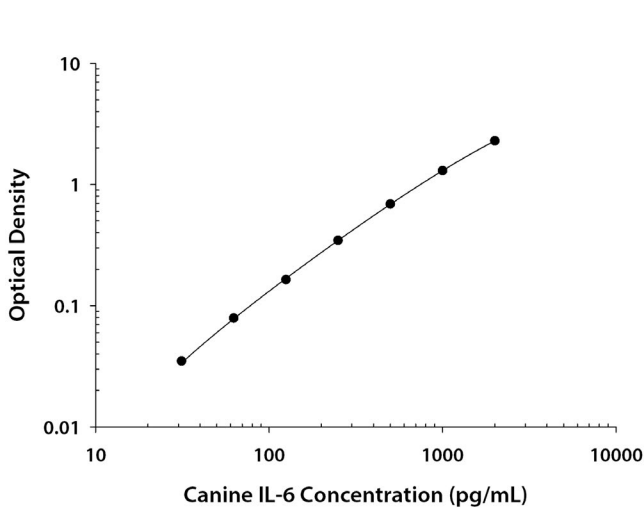
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) 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 the graph. The data may be linearized by plotting the log of the canine IL-6 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If 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	0.046 0.056	0.051	—
31.3	0.083 0.088	0.086	0.035
62.5	0.129 0.131	0.130	0.079
125	0.212 0.217	0.215	0.164
250	0.392 0.401	0.397	0.346
500	0.737 0.745	0.741	0.690
1000	1.331 1.373	1.352	1.301
2000	2.326 2.360	2.343	2.292

## 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 separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of kit components.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	67	65	66
Mean (pg/mL)	92.6	256	685	90.7	262	666
Standard deviation	3.1	8.3	15.3	6.6	15.5	30.2
CV (%)	3.3	3.2	2.2	7.3	5.9	4.5

## RECOVERY

The recovery of canine IL-6 spiked to three levels throughout the range of the assay in various matrices was evaluated

Sample Type	Average % Recovery	Range
Cell culture supernates (n=6)	94	87-103%
Serum (n=4)	93	84-102%
EDTA plasma (n=4)	97	91-103%
Heparin plasma (n=4)	92	83-100%

## LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with various concentrations of canine IL-6 in each matrix were diluted with Calibrator Diluent and assayed.

		Cell culture supernates (n=7)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	102	105	100	107
	Range (%)	93-106	97-110	96-105	102-112
1:4	Average % of Expected	102	109	98	108
	Range (%)	92-106	102-113	93-100	105-111
1:8	Average % of Expected	101	108	95	106
	Range (%)	86-108	101-111	90-98	104-110
1:16	Average % of Expected	96	100	90	103
	Range (%)	84-113	91-108	86-100	99-111

## SENSITIVITY

Eighteen assays were evaluated and the minimum detectable dose (MDD) of canine IL-6 ranged from 1.5-11.8 pg/mL. The mean MDD was 6.1 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 IL-6 produced at R&D Systems.

## SAMPLE VALUES

**Serum/Plasma** - Twelve individual canine serum, eleven EDTA plasma, and thirteen heparin plasma samples were evaluated for detectable levels of canine IL-6 in this assay. One serum sample measured 162 pg/mL, two EDTA plasma samples measured 32 and 107 pg/mL, and two heparin plasma samples measured 68 and 148 pg/mL. All other serum and plasma samples tested measured below the lowest standard, 31.3 pg/mL.

**Cell Culture Supernates** - Canine peripheral blood cells ( $1 \times 10^6$  cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum and stimulated with 1  $\mu$ g/mL LPS and 1  $\mu$ M histamine for 48 hours. An aliquot of the cell culture supernate was removed, assayed for canine IL-6, and measured 620 pg/mL.

## SPECIFICITY

This assay recognizes natural and recombinant canine IL-6.

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 IL-6 control were assayed for interference. No significant cross-reactivity or interference was observed.

### Recombinant canine:

IFN- $\gamma$   
IL-2  
IL-4  
IL-5  
IL-8  
IL-10  
IL-12 p40  
IL-18

### Recombinant human:

IL-6  
IL-6 R  
IL-6 R/gp130

### Recombinant mouse:

IL-6  
IL-6 R

### Other recombinants:

rat IL-6  
cotton rat IL-6

Recombinant feline IL-6 cross-reacts approximately 7.9% in this assay.

Recombinant porcine IL-6 cross-reacts approximately 1.7% in this assay.

Recombinant equine IL-6 cross-reacts approximately 0.7% in this assay.

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