MESO SCALE DISCOVERY

MULTI-SPOT Assay System

Canine ProInflammatory Panel 3 Assay Ultra-Sensitive Kit

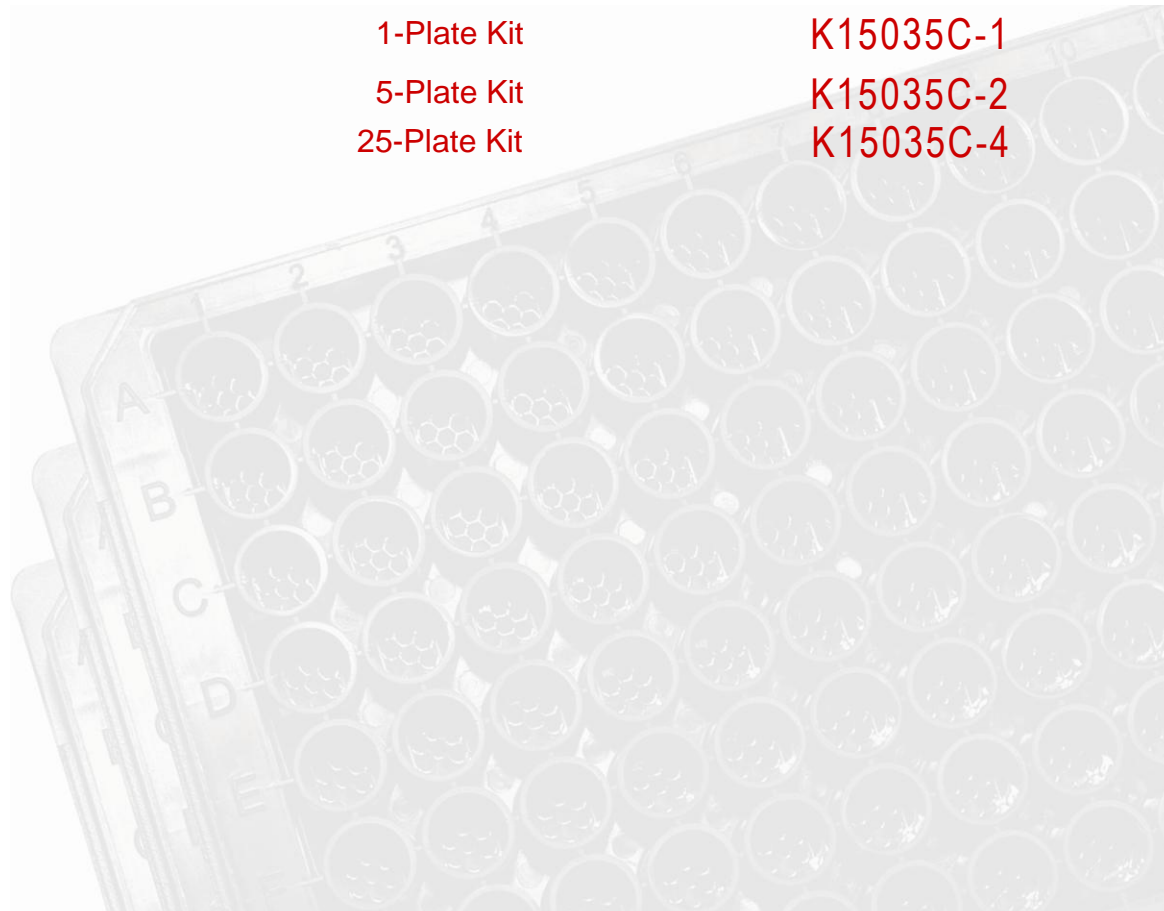|	
1-Plate Kit	K15035C-1
5-Plate Kit	K15035C-2
25-Plate Kit	K15035C-4

MESO SCALE DISCOVERY MESO SCALE DISCOVERY MESO SCALE DISCOVERY MESO SCALE DISCOVERY MESO SCALE DISCOVERY MESO SCALE DISCOVERY

M S D ®

MSD MULTI-SPOT Assays

Ultra-Sensitive Kit

Canine ProInflammatory Panel 3 Assay

IL-2, IL-6, IL-8, TNF-α

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

MESO SCALE DISCOVERY®

A division of Meso Scale Diagnostics, LLC.
9238 Gaither Road
Gaithersburg, MD 20877   USA
www.mesoscale.com

Table of Contents

Ordering Information

MSD Customer Service

Phone: 1-301-947-2085
Fax: 1-301-990-2776
Email: CustomerService@mesoscale.com

MSD Scientific Support

Phone: 1-301-947-2025
Fax: 1-240-632-2219 attn: Scientific Support
Email: ScientificSupport@mesoscale.com

MESO SCALE DISCOVERY'S MULTI-ARRAY® Technology is a multiplex immunoassay system that enables the measurement of biomarkers utilizing the next generation of electrochemiluminescent detection. In an MSD® assay, specific Capture Antibodies for the analytes are coated in arrays in each well of a 96-well carbon electrode plate surface. The detection system uses patented SULFO-TAG™ labels, which emit light upon electrochemical stimulation initiated at the electrode surfaces of the MULTI-ARRAY and MULTI-SPOT® plates. The electrical stimulation is decoupled from the output signal, which is light, to generate assays with minimal background. MSD labels can be conveniently conjugated to biological molecules, are stable and are non-radioactive. Additionally, only labels near the electrode surface are detected, enabling non-washed assays.

One of the advantages of MSD assays is the minimal sample volume required as compared to a traditional ELISA, which is also limited by its inability to measure more than a single analyte. With an MSD assay, ten different biomarkers can be analyzed simultaneously using as little as 10-25 µL of sample. These assays have high sensitivity, up to five logs of linear dynamic range, and excellent performance in complex biological matrices. Combined, these advantages enable the measurement of native levels of biomarkers in normal and diseased samples without multiple dilutions. Further, the simple and rapid protocols of MSD assays provide a powerful tool to generate reproducible and reliable results. The MSD product line offers a diverse menu of assay kits for profiling biomarkers, cell signaling pathways, and other applications, as well as a variety of plates and reagents for assay development.

Introduction

Inflammatory processes are involved in many physiological events, from fighting infection and wound healing to a multitude of disease states including autoimmune disorders. Cytokines and chemokines are soluble factors that mediate both acute and chronic inflammatory response.

Interleukin (IL)-2 is produced primarily by activated CD4^{+} T cells. IL-2 is an important regulator of proliferation and maintenance of several T- and NK-cell subsets. The presence of IL-2 has also been demonstrated to play an important role in the long-term survival of activated helper T cells (Th) and CD8^{+} cytotoxic T cells (Tc).

IL-6 is a proinflammatory cytokine secreted by monocytes, macrophages and certain non-lymphoid cell types in response to tissue damage or infection. It plays a role in the acute phase response, the regulation of fever, and the generation of plasma B cells. IL-6 has been recently shown to act in concert with TGF-β to induce the differentiation of IL-17 producing helper T cells from naïve progenitors.

IL-8 also known as CLXL8, is a chemokine responsible for the attraction of neutrophils to vascular endothelium and extravasation into inflamed tissues. It is produced primarily by activated macrophages in response to toll-like receptor agonists and certain bacterial pathogens.

Tumor necrosis factor-α (TNF-α) plays a key role in the acute phase reaction and systemic inflammation. TNF-α is primarily produced by activated macrophages, but it is also secreted by a variety of other cell types under pathogenic conditions. Upon receptor binding, it has been shown to trigger diverse cell signaling pathways including apoptosis, proliferation, differentiation, chemoattraction, hypothalamic regulation, and cytokine production. TNF-α can also contribute to tumorigenesis and viral replication.

Principle of the Assay

MSD assays provide a rapid and convenient method for measuring the levels of protein targets within a single small-volume sample. The assays are available in both singleplex and multiplex formats. In a singleplex assay, an antibody for a specific protein target is coated on one electrode (or "spot") per well. In a multiplex assay, an array of capture antibodies against different targets is patterned on distinct spots in the same well. The Canine ProInflammatory Panel 3 Assay detects IL-2, IL-6, IL-8, and TNF-α in a sandwich immunoassay format (Figure 1). MSD provides a plate that has been pre-coated with capture antibody on spatially distinct spots – antibodies for IL-2, IL-6, IL-8, and TNF-α. The user adds the sample and a solution containing the labeled detection antibodies— anti-IL-2, anti-IL-6, anti-IL-8, and anti-TNF-α labeled with an electrochemiluminescent compound, MSD SULFO-TAG label—over the course of one or more incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the labeled detection antibodies by bound analytes completes the sandwich. The user adds an MSD read buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD SECTOR® instrument for analysis. Inside the SECTOR instrument, a voltage applied to the plate electrodes causes the labels bound to the electrode surface to emit light. The instrument measures intensity of emitted light to afford a quantitative measure of IL-2, IL-6, IL-8, and TNF-α present in the sample.
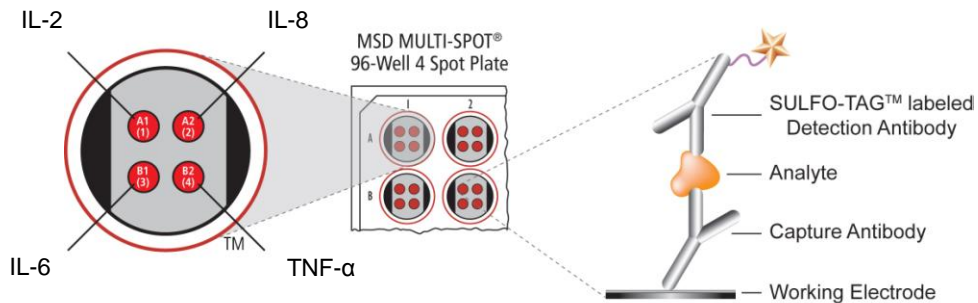

Figure 1. *Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing records.*

IV Reagents Supplied

Product Description	Storage	Quantity per Kit		
		K15035C-1	K15035C-2	K15035C-4
MULTI-SPOT 96-well 4 Spot Canine ProInflammatory Panel 3 Plate N45035A-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG Anti-canine IL-2 Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-canine IL-6 Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-canine IL-8 Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-canine TNF-α Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Canine ProInflammatory Panel 3 Calibrator Blend (100X)	≤-70°C	1 vial (15 µL)	5 vials (15 µL ea)	25 vials (15 µL ea)
Blocker D-M (2%)	≤-10°C	1 vial (0.2 mL)	1 vial (0.9 mL)	5 vials (0.9 mL ea)
Blocker D-G (10%)	≤-10°C	1 vial (0.05 mL)	1 vial (0.2 mL)	1 vial (1 mL)
Diluent 2 R51BB-4 (8 mL) R51BB-3 (40 mL)	≤-10°C	1 bottle (8 mL)	1 bottle (40 mL)	5 bottles (40 mL ea)
Diluent 100 R50AA-4 (50 mL) R50AA-2 (200 mL)	2–8°C	1 bottle (50 mL)	1 bottle (50 mL)	1 bottle (200 mL)
Read Buffer T (4X) R92TC-3 (50 mL) R92TC-2 (200 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	2 bottles (200 mL ea)

V Required Materials and Equipment - not supplied

- Deionized water for diluting concentrated buffers
- 50 mL tubes for reagent preparation
- 15 mL tubes for reagent preparation
- Microcentrifuge tubes for preparing serial dilutions
- Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing
- Appropriate liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL into a 96-well microtiter plate
- Plate washing equipment: automated plate washer or multichannel pipette
- Adhesive plate seals
- Microtiter plate shaker

---

[1] Some SULFO-TAG labeled detection antibodies may be light-sensitive, so they should be stored in the dark.

VI Safety

safety

Safe laboratory practices and personal protective equipment such as gloves, safety glasses, and lab coats should be used at all times during the handling of all kit components. All hazardous samples should be handled and disposed of properly, in accordance with local, state, and federal guidelines.

VII Reagent Preparation

reagent preparation

Bring all reagents to room temperature and thaw the Calibrator stock on ice.

> **Important:** Upon first thaw, separate Diluent 2 into aliquots appropriate to the size of your assay needs. These diluents can go through up to three freeze-thaw cycles without significantly affecting the performance of the assay.

Prepare Calibrator and Control Solutions

MSD recommends the preparation of an 8-point standard curve consisting of at least 2 replicates of each point. Each well requires 25 µL of Calibrator. For the assay, MSD recommends 4-fold serial dilution steps and Diluent 2 alone for the 8th point:

Standard	IL-2 (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	TNF-α (pg/mL)	Dilution Factor
100X Stock	2000000	1000000	1000000	500000	
STD-01	20000	10000	10000	5000	100
STD-02	5000	2500	2500	1250	4
STD-03	1250	625	625	313	4
STD-04	313	156	156	78	4
STD-05	78	39	39	20	4
STD-06	20	9.8	9.8	4.9	4
STD-07	4.9	2.4	2.4	1.2	4
STD-08	0	0	0	0	n/a

To prepare this 8-point standard curve for up to 4 replicates:

1) Prepare the highest Calibrator point (STD-01) by transferring 10 µL of the Canine ProInflammatory Panel 3 Calibrator Blend to 990 µL Diluent 2.
2) Prepare the next Calibrator by transferring 50 µL of the diluted Calibrator to 150 µL of Diluent 2. Repeat 4-fold serial dilutions 5 additional times to generate 7 Calibrators.
3) The recommended 8th Standard is Diluent 2 (i.e. zero Calibrator).

Notes:

a. Alternatively, Calibrators can be prepared in the sample matrix or diluent of choice to verify acceptable performance in these matrices. In general, the presence of some protein (for example, 1% BSA) in the sample matrix is helpful for preventing loss of analyte by adsorption onto the sides of tubes, pipette tips, and other surfaces. If your sample matrix is serum-free tissue culture media, then the addition of 10% FBS or 1% BSA is recommended.

b. The standard curve can be modified as necessary to meet specific assay requirements.

17838-v1-2010Dec   Page 8

Dilution of Samples

Serum and Plasma

All solid material should be removed by centrifugation. Plasma prepared in heparin tubes commonly displays additional clotting following the thawing of the sample. Remove any additional clotted material by centrifugation. Avoid multiple freeze/thaw cycles for serum and plasma samples. Normal serum or plasma samples may not require a dilution prior to being used in the MSD Canine ProInflammatory Panel 3 Assay. Serum or plasma with high levels of these analytes may require 2-fold dilution in Diluent 2.

Tissue Culture

Tissue culture supernatant samples may not require dilution prior to being used in the MSD Canine ProInflammatory Panel 3 Assay. If using serum-free medium, the presence of carrier protein (e.g., 1% BSA) in the solution is helpful to prevent loss of analyte to the labware. Samples from experimental conditions with extremely high levels of cytokines may require a dilution.

Other Matrices

Information on preparing samples in other matrices, including sputum, CSF, and tissue homogenates can be obtained by contacting MSD Scientific Support at 1-301-947-2025 or ScientificSupport@mesoscale.com.

Prepare Detection Antibody Solution

The Detection Antibodies are provided as a 50X stock solution. The working Detection Antibody Solution should contain 1X as final concentration of each antibody and 0.1% of each blocker.

In a 15 mL tube combine (per plate):

- ❑ 60 µL of 50X SULFO-TAG Anti-canine IL-2 Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-canine IL-6 Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-canine IL-8 Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-canine TNF-α Antibody
- ❑ 30 µL of Blocker D-G (10%)
- ❑ 150 µL of Blocker D-M (2%)
- ❑ 2580 µL of Diluent 100

Prepare Read Buffer

The Read Buffer should be diluted 2-fold in deionized water to make a final concentration of 2X Read Buffer T. Add 10 mL of 4X Read Buffer T to 10 mL of deionized water for each plate.

Prepare MSD Plate

This plate has been pre-coated with antibody for the analyte shown in Figure 1. The plate can be used as delivered; no additional preparation (e.g., pre-wetting) is required. The plate has also been exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies.

VIII Assay Protocol

assay protocol

1. **Addition of Diluent 2:** Dispense 25 µL of Diluent 2 into each well. Seal the plate with an adhesive plate seal and incubate for 30 min with vigorous shaking (300–1000 rpm) at room temperature.

2. **Addition of the Sample or Calibrator:** Dispense 25 µL of sample or Calibrator into separate wells of the MSD plate. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.

   Shaking a 96-well MSD plate typically accelerates capture at the working electrode.

3. **Wash and Addition of the Detection Antibody Solution:** Wash the plate 3X with PBS-T. Dispense 25 µL of the 1X Detection Antibody Solution into each well of the MSD plate. Seal the plate and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.

4. **Wash and Read:** Wash the plate 3X with PBS-T. Add 150 µL of 2X Read Buffer T to each well of the MSD plate. Analyze the plate on the SECTOR Imager. Plates may be read immediately after the addition of Read Buffer.

   Bubbles in the fluid will interfere with reliable reading of plate. Use reverse pipetting techniques to insure bubbles are not created when dispensing the Read Buffer.

IX Analysis of Results

analysis of results

The Calibrators should be run in duplicate to generate a standard curve. The standard curve is modeled using least squares fitting algorithms so that signals from samples with known levels of the analyte of interest can be used to calculate the concentration of analyte in the sample. The assays have a wide dynamic range (3–4 logs) which allows accurate quantitation in many samples without the need for dilution. The MSD Discovery Workbench® analysis software utilizes a 4-parameter logistic model (or sigmoidal dose-response) and includes a $1/Y^2$ weighting function. The weighting function is important because it provides a better fit of data over a wide dynamic range, particularly at the low end of the standard curve.

Typical Standard Curve

typical standard curve

The following standard curve is an example of the dynamic range of the assay. The actual signals may vary and a standard curve should be run for each set of samples and on each plate for the best quantitation of unknown samples.
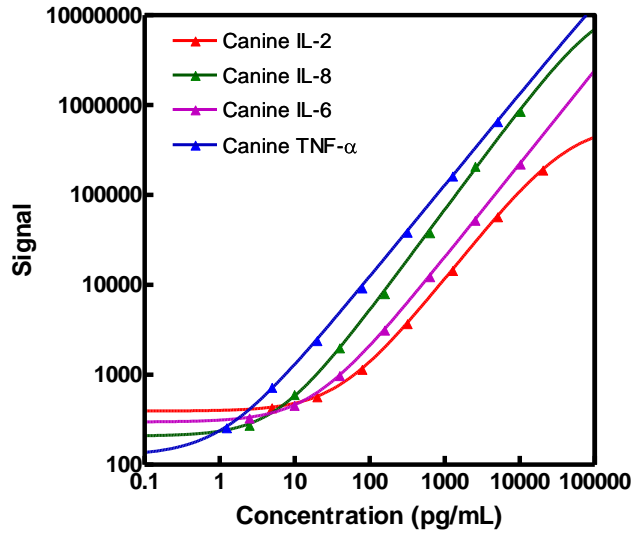
IL-2		
Conc. (pg/mL)	Average Signal	%CV
0	364	5.6
4.9	435	4.2
20	575	3.3
78	1162	3.5
313	3740	6.0
1250	14670	2.2
5000	57711	7.4
20000	190798	5.1

IL-6		
Conc. (pg/mL)	Average Signal	%CV
0	282	6.4
2.4	334	4.0
9.8	462	2.9
39	989	2.3
156	3169	3.1
625	12475	4.7
2500	52827	5.8
10000	222700	3.1

IL-8		
Conc. (pg/mL)	Average Signal	%CV
0	178	14.0
2.4	277	7.0
9.8	608	5.0
39	2016	3.8
156	8091	6.4
625	38505	3.3
2500	210357	4.3
10000	863178	6.7

TNF-α		
Conc. (pg/mL)	Average Signal	%CV
0	135	6.8
1.2	260	8.5
4.9	735	11.6
20	2430	3.5
78	9346	2.0
313	38915	3.0
1250	163861	1.8
5000	664033	2.3

XI Sensitivity

The lower limit of detection (LLOD) is the calculated concentration of the signal that is 2.5 standard deviations over the zero calibrator. The values below represent the average LLOD over multiple kit lots.

	IL-2	IL-6	IL-8	TNF-α
LLOD (pg/mL)	7.6	2.4	1.3	0.17

XII Spike Recovery

Beagle and Mongrel serum and plasma samples were spiked with Calibrator at multiple values throughout the range of the assay. Each spike was done in ≥ 3 replicates.
% Recovery = measured / expected x 100

IL-2	Spike Conc. (pg/mL)	Measured Conc. (pg/mL)	Measured Conc. % CV	% Recovery
Beagle Serum	0	2.8	6.1	
	125	134	3.5	105
	500	526	1.1	105
	2000	2029	0.7	101
Beagle Plasma	0	17	1.5	
	125	131	4.4	92
	500	468	0.9	90
	2000	1795	0.5	89
Mongrel Serum	0	2.7	3.9	
	125	136	4.0	107
	500	516	0.8	103
	2000	2011	1.7	100
Mongrel Plasma	0	5.8	0.4	
	125	123	0.6	94
	500	512	2.8	101
	2000	1952	2.6	97

IL-6	Spike Conc. (pg/mL)	Measured Conc. (pg/mL)	Measured Conc. % CV	% Recovery
Beagle Serum	0	0.05	3.4	
	63	61	0.6	97
	250	229	6.5	92
	1000	920	1.3	92
Beagle Plasma	0	4.0	6.6	
	63	62	5.4	93
	250	229	0.7	90
	1000	912	0.9	91
Mongrel Serum	0	79	2.3	
	63	126	2.5	89
	250	268	3.2	82
	1000	815	0.8	76
Mongrel Plasma	0	29	6.4	
	63	80	3.5	87
	250	267	0.8	96
	1000	930	2.0	90

IL-8	Spike Conc. (pg/mL)	Measured Conc. (pg/mL)	Measured Conc. % CV	% Recovery
Beagle Serum	0	0.3	12.7	
	63	60	3.9	95
	250	251	1.0	100
	1000	1127	2.6	113
Beagle Plasma	0	1945	0.1	
	63	2048	4.7	102
	250	2319	0.3	106
	1000	2994	6.4	102
Mongrel Serum	0	17	0.8	
	63	66	0.1	82
	250	217	0.4	81
	1000	892	4.3	88
Mongrel Plasma	0	434	2.1	
	63	482	2.6	97
	250	688	1.1	101
	1000	1356	0.6	95

TNF-α	Spike Conc. (pg/mL)	Measured Conc. (pg/mL)	Measured Conc. % CV	% Recovery
Beagle Serum	0	0	8.6	
	31	29.4	0.1	94
	125	115	0.8	92
	500	540	1.2	108
Beagle Plasma	0	0.02	22.0	
	31	29	3.6	91
	125	112	4.7	89
	500	497	0.2	99
Mongrel Serum	0	0.04	14.3	
	31	29	1.1	94
	125	110	0.7	88
	500	487	1.5	97
Mongrel Plasma	0	1.1	4.4	
	31	29	8.3	89
	125	114	1.7	91
	500	504	4.1	101

XIII Linearity

linearity

Dilution linearity was tested by spiking calibrators in different matrices including beagle serum, beagle plasma, mongrel serum and mongrel plasma and conducting 2-fold dilutions.

The concentrations shown below have been corrected for dilution (concentration = measured x dilution factor). Percent recovery is calculated as the measured concentration divided by the concentration measured from the previous dilution (expected).

% Recovery = (measured x dilution factor) / expected x 100

Sample	Fold Dilution	IL-2 Conc. (pg/mL)	IL-2 Conc. % CV	IL-2 % Recovery	IL-6 Conc. (pg/mL)	IL-6 Conc. % CV	IL-6 % Recovery
Beagle Serum	1	2068	1.5		1128	0.9	
	2	2139	2.4	103	1010	2.2	90
	4	2108	2.4	99	1028	0.6	102
	8	2024	0.2	96	998	0.8	97
Beagle Plasma	1	2123	3.1		1205	0.3	
	2	2061	5.4	97	1023	3.1	85
	4	2141	1.2	104	996	3.0	97
	8	2103	3.6	98	967	0.4	97
Mongrel Serum	1	2176	2.0		1152	0.4	
	2	2179	2.7	100	1003	3.2	87
	4	2124	1.5	97	990	0.0	99
	8	2031	3.4	96	964	1.6	97
Mongrel Plasma	1	2201	0.2		1163	0.3	
	2	2240	5.2	102	1007	2.3	87
	4	2219	3.9	99	915	5.1	91
	8	2191	2.6	99	927	5.5	101

Sample	Fold Dilution	IL-8 Conc. (pg/mL)	IL-8 Conc. % CV	IL-8 % Recovery	TNFα Conc. (pg/mL)	TNFα Conc. % CV	TNFα % Recovery
Beagle Serum	1	1161	1.0		640	0.1	
	2	994	2.5	86	492	0.5	77
	4	947	1.1	95	461	2.0	94
	8	960	2.4	101	452	2.9	98
Beagle Plasma	1	1145	3.1		657	0.4	
	2	1021	3.7	89	507	0.2	77
	4	949	5.0	93	477	2.0	94
	8	942	4.9	99	464	0.1	97
Mongrel Serum	1	1106	1.9		624	1.1	
	2	986	3.0	89	498	0.5	80
	4	961	0.9	97	467	2.5	94
	8	975	0.9	101	445	0.1	95
Mongrel Plasma	1	1132	6.6		635	2.4	
	2	983	3.2	87	487	1.1	77
	4	920	0.1	94	455	0.8	93
	8	932	1.7	101	454	2.7	100

XIV Samples

s a m p l e s

The average endogenous levels of canine cytokines detected in beagle and mongrel serum and plasma are shown below.

		IL-2 (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	TNF-α (pg/mL)
Beagle	**Serum**	<LLOD	6.8	2055	<LLOD
	Plasma	19	111	16	<LLOD
Mongrel	**Serum**	<LLOD	33	458	<LLOD
	Plasma	3.2	12	963	<LLOD

.

MSD 96-well MULTI-SPOT Canine ProInflammatory Panel 3 Assay: Ultra-Sensitive Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the
MSD Canine ProInflammatory Panel 3 Assay.

Step 1 : Sample and Reagent Preparation

Bring all reagents to room temperature and thaw the Calibrator stock on ice.

If necessary, samples should be diluted in Diluent 2.

Prepare calibrator solutions and standard curve.

Use the 100X Calibrator stock to prepare an 8-point standard curve by diluting in Diluent 2.

> ***Note:*** *The standard curve can be modified as necessary to meet specific assay requirements.*

Prepare Detection Antibody Solution by diluting Detection Antibodies to 1X, Blocker D-M to 0.1% and Blocker D-G to 0.1% in 3.0 mL of Diluent 100 (per plate). Keep the Detection Antibody Solution in the dark.

Prepare 20 mL of 2X Read Buffer T by diluting 4X MSD Read Buffer T with deionized water.

SERUM OR PLASMA SAMPLES

Step 2: Add Diluent 2

Dispense 25 µL/well Diluent 2.

Incubate at room temperature with vigorous shaking (300-1000 rpm) for 30 minutes.

Step 3: Add Sample or Calibrator

Dispense 25 µL/well Calibrator or sample.

Incubate at room temperature with vigorous shaking (300-1000 rpm) for 2 hours.

Step 4: Wash and Add Detection Antibody Solution

Wash plate 3X with PBS-T.

Dispense 25 µL/well 1X Detection Antibody Solution.

Incubate at room temperature with vigorous shaking (300-1000 rpm) for 2 hours.

Step 5: Wash and Read Plate

Wash plate 3X with PBS-T.

Dispense 150 µL/well 2X Read Buffer T.

Analyze plate on SECTOR Imager instrument.