User Guide

MILLIPLEX® Canine Cytokine Magnetic Bead Panel

96-Well Plate Assay

CCYTOMAG-90K
CCYTMG-90K-PX13
CCYTMAG90KPX13BK

For research use only. Not for use in diagnostic procedures.

Introduction

"Cytokine" is a general term used for a diverse group of soluble proteins and peptides, which act as regulators under both normal and pathological conditions to modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment. Cytokines differ from hormones in that they act on a wider spectrum of target cells and also, they are not produced by specialized cells which are organized in specialized glands. This group of proteins includes lymphokines, interferons, colony stimulating factors and chemokines.

Cytokine and chemokine research plays a significant role in achieving a deeper understanding of disease states such as allergic reactions, IBD, sepsis, and cancer.

The MILLIPLEX® portfolio offers the broadest selection of analytes across a wide range of disease states and species. Once the analytes of interest have been identified, you can rely on the quality that we build into each kit to produce results you can trust. In addition to the assay characteristics listed in the protocol, other performance criteria evaluated during the verification process include cross-reactivity, dilution linearity, kit stability, and sample behavior (for example, detectability and stability).

Each MILLIPLEX® panel and kit includes:

- Quality controls (QCs) provided to qualify assay performance
- Comparison of standard (calibrator) and QC lots to a reference lot to ensure lot-to-lot consistency
- Optimized serum matrix to mimic native analyte environment
- Detection antibody cocktails designed to yield consistent analyte profiles within panel

In addition, each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The MILLIPLEX® Canine Cytokine Magnetic Bead Panel thus enables you to focus on the therapeutic potential of cytokines. Coupled with the Luminex® xMAP® platform in a magnetic bead format, you receive the advantage of ideal speed and sensitivity, allowing quantitative multiplex detection of dozens of analytes simultaneously, which can dramatically improve productivity.

MILLIPLEX® Canine Cytokine Magnetic Bead Panel is the most versatile system available for Cytokine research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically verify and build the most comprehensive library available for protein detection and quantitation.

MILLIPLEX® products offer you:

- Select a premixed kit (13-plex).
- The ability to choose any combination of analytes from our panel of 13 analytes to design a custom kit that better meets your needs.
- A convenient "all-in-one" box format that gives you the assurance that you will have all the necessary reagents you need to run your assay.

The MILLIPLEX® Canine Cytokine Panel is to be used for the simultaneous quantification of any or all of the following analytes in canine tissue/cell lysate and culture supernatant samples and serum or plasma samples: GM-CSF, IFNγ, KC-like, IP-10, IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, MCP-1, and TNF-α.

For research use only. Not for use in diagnostic procedures.
Please read entire protocol before use.
It is important to use same assay incubation conditions throughout your study.

Principle

MILLIPLEX® products are based on the Luminex® xMAP® technology-one of the fastest growing and most respected multiplex technologies offering applications throughout the life-sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded magnetic bead (MagPlex®-C) and non-magnetic bead (MicroPlex®) microspheres.

- Luminex® products use proprietary techniques to internally color-code microspheres with multiple fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500-5.6 µm non-magnetic or 80-6.45 µm magnetic polystyrene microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- The following Luminex® instruments can be used to acquire and analyze data using two detection methods:
    - The Luminex® analyzers Luminex® 200™, FLEXMAP 3D®, and xMAP® INTELLIFLEX are flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex® analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified, and the result of its bioassay is quantified based on fluorescent reporter signals. We combine the streamlined data acquisition power of Luminex® xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex® instruments.
- xMAP® INTELLIFLEX runs on INTELLIFLEX software for instrument control, run setup and generating high quality data with flexible output options. Data can be exported in xPONENT® style CSV files for compatibility with many existing analytical applications, or in the new, customizable INTELLIFLEX file format. The INTELLIFLEX file format is intended for flexibility and simplicity, allowing the user The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. Open-architecture xMAP® technology enables multiplexing of many types of bioassays reducing time, labor and costs over traditional methods.

Storage Conditions Upon Receipt

- Recommended storage for kit components is 2-8 °C.
- For long-term storage, freeze reconstituted standards and controls at ≤ −20 °C. Avoid multiple (> 2) freeze/thaw cycles.
- **DO NOT FREEZE** Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.

Reagents Supplied

Store all reagents at 2-8 °C.

Reagents	Volume	Quantity	Cat. No.
Canine Cytokine Standard	Lyophilized	1 vial	LCC-8090
Canine Cytokine Quality Controls 1 and 2	Lyophilized	2 vials	LCC-6090
Serum Matrix (for serum/plasma samples only)	Lyophilized	1 vial	LMC-SD
Set of one 96-Well Plate with 2 sealers	-	1 set	-
Assay Buffer	30 mL	1 bottle	L-AB
10X Wash Buffer*	60 mL	1 bottle	L-WB
Canine Cytokine Detection Antibodies	3.2 mL	1 bottle	LCC-1090
Streptavidin-Phycoerythrin	3.2 mL	1 bottle	L-SAPE3
Mixing Bottle (not provided with premixed panel)	-	1 bottle	-

*Contains 0.05% ProClin™

Canine Cytokine Antibody-Immobilized Premixed Magnetic Beads:

	Volume		Quantity
Premixed 13-plex Beads	3.5 mL	CCYPMX13-MAG	1 bottle

Included Canine Cytokine Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel.

Canine Cytokine Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex® Bead Region	Customizable 13 Analytes (50X Concentration, 90 µL) Available	Cat. No.	13-Plex Premixed Beads
Anti-GM-CSF Bead	14	✔	CGMCSF-MAG	✔
Anti-IFN-γ Bead	25	✔	CIFNG-MAG	✔
Anti-KC-like Bead	61	✔	MKC-MAG	✔
Anti-IP-10 Bead	57	✔	MIP10-MAG	✔
Anti-IL-2 Bead	29	✔	CIL2-MAG	✔
Anti-IL-6	35	✔	CIL6-MAG	✔
Anti-IL-7	36	✔	MIL7-MAG	✔
Anti-IL-8	48	✔	CIL8-MAG	✔
Anti-IL-10	63	✔	CIL10-MAG	✔
Anti-IL-15	54	✔	MIL15-MAG	✔
Anti-IL-18	67	✔	CIL18-MAG	✔
Anti-MCP-1	74	✔	CMCP1-MAG	✔
Anti-TNFα	76	✔	CTNFA-MAG	✔

Materials Required (Not included)

Reagents

MAGPIX® Drive Fluid PLUS (40-50030), xMAP® Sheath Fluid PLUS (40-50021), or xMAP® Sheath Concentrate PLUS (40-50023)

Instrumentation/Materials

- Adjustable Pipettes with Tips capable of delivering 25 µL to 1000 µL
- Multichannel Pipettes capable of delivering 5 µL to 50 µL, or 25 µL to 200 µL
- Reagent Reservoirs
- Polypropylene Microfuge Tubes
- Rubber Bands
- Aluminum Foil
- Absorbent Pads
- Laboratory Vortex Mixer
- Sonicator (Branson Ultrasonic Cleaner Model No. B200 or equivalent)
- Titer Plate Shaker (VWR® Microplate Shaker 12620-926 or equivalent)
- Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® instrument with xPONENT® software, or xMAP® INTELLIFLEX instrument with INTELLIFLEX software by Luminex® Corporation
- Automatic Plate Washer for magnetic beads (BioTek® 405 LS and 405 TS, 40-094, 40-095, 40-096, 40-097 or equivalent) or Handheld Magnetic Separation Block (40-285 or equivalent).

Note: If a plate washer or handheld magnetic separation block for magnetic beads is not available, one can use a microtiter filter plate (MX-PLATE) to run the assay using a Vacuum Filtration Unit (Vacuum Manifold, MSVMHTS00 or equivalent with Vacuum Pump, WP6111560 or equivalent).

Safety Precautions

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- 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.

Symbol Definitions

Ingredient	Cat. No	Label	
Canine Cytokine Detection Antibody	LCC-1090		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Canine Cytokine Quality Controls 1 and 2	LCC-6090	 	**Danger.** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/attention.
Canine Cytokine Standard	LCC-8090	 	**Danger.** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/attention.
Serum Matrix	LMC-SD	No Symbol Required	Harmful to aquatic life with long lasting effects. Avoid release to the environment.
Streptavidin-Phycoerythrin	L-SAPE3		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
10X Wash Buffer	L-WB		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.

Technical Guidelines

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set up.

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing the beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25 °C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at ≤ −20 °C for 1 month and at ≤ −80 °C for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8 °C for up to one month.
- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8 °C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.

- When reading the assay on the Luminex® 200™ instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended filter plates using 3 alignment discs. When reading the assay on the MAGPIX® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended filter plates using 2 alignment discs. When reading the assay on the FLEXMAP 3D® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 1 alignment disc.
- For the FLEXMAP 3D® instrument, when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid PLUS in each well and 75 µL should be aspirated.
- For the xMAP® INTELLIFLEX instrument, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells. If samples are diluted in Assay Buffer, use the Assay Buffer as matrix.
- For serum/plasma samples that require further dilution use the Serum Matrix provided in the kit for further dilution.
- For cell/tissue homogenate, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strong denaturing detergents, and has an ionic strength close to physiological concentration. Avoid debris, lipids, and cell/tissue aggregates. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

Sample Collection and Storage

Preparation of Serum Samples

- Allow the blood to clot at room temperature for at least 30 minutes before centrifugation for 10 minutes at 1000 x g. Remove serum and assay immediately 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.
- Serum samples should be diluted 1:3 in the Assay Buffer provided in the kit (for example, adding 20 µL of serum to 40 µL Assay Buffer). When further dilution beyond 1:3 is required, use Serum Matrix as the diluent.

Preparation of Plasma Samples

- Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000 x g within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at ≤ −20 °C.
- Avoid multiple (> 2) freeze/thaw cycles.
- Plasma samples should be diluted 1:3 in the Assay Buffer provided in the kit (for example, adding 20 µL of serum to 40 µL Assay Buffer). When further dilution beyond 1:3 is required, use Serum Matrix as the diluent.
- 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 Tissue Culture Supernatant

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ −20 °C.
- Avoid multiple (> 2) freeze/thaw cycles.
- Tissue culture supernatant may require a dilution with an appropriate control medium prior to assay. Tissue/cell extracts should be done in neutral buffers containing reagents and conditions that do not interfere with assay performance. Excess concentrations of detergent, salt, denaturants, high or low pH, etc. will negatively affect the assay. Organic solvents should be avoided. The tissue/cell extract samples should be free of particles such as cells or tissue debris.

Note:

- A maximum of 25 µL per well of serum or plasma can be used. Tissue culture or other media may also 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 anticoagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

Preparation of Reagents for Immunoassay

Preparation of Antibody-Immobilized Beads

- If premixed beads are used, sonicate the premixed bead vial 30 seconds and then vortex for 1 minute before use.

  Note: Due to the composition of magnetic beads, you may notice a slight color in the bead solution. This does not affect the performance of the beads or the kit.

- For individual vials of beads, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 60 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Assay Buffer. Vortex the mixed beads well. Unused portions may be stored at 2-8 °C for up to one month.

  Note: Due to the composition of magnetic beads, you may notice a slight color in the bead solution. This does not affect the performance of the beads or the kit.

Example 1: When using 13 cytokine antibody-immobilized beads, add 60 µL from each of the 13 bead sets to the Mixing Bottle. Then add 2.22 mL Assay Buffer.

Example 2: When using 5 cytokine antibody-immobilized beads, add 60 µL from each of the 5 bead sets to the Mixing Bottle. Then add 2.7 mL Assay Buffer.

Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water. Invert the vial several times to mix and vortex. Allow the vial to sit for 5-10 minutes. Transfer the reconstituted Quality Control 1 and Quality Control 2 into two polypropylene microfuge tubes and set in an ice bath. These should be added to plate within 1 hour of reconstitution. Unused portion may be stored at ≤ −20 °C for up to one month.

Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 60 mL of 10X Wash Buffer with 540 mL deionized water. Store unused portion at 2-8 °C for up to one month.

Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 1.0 mL deionized water to the bottle containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Then add 2.0 mL of Assay Buffer to the bottle and mix well. Leftover reconstituted Serum Matrix should be stored at ≤ −20 °C for up to one month.

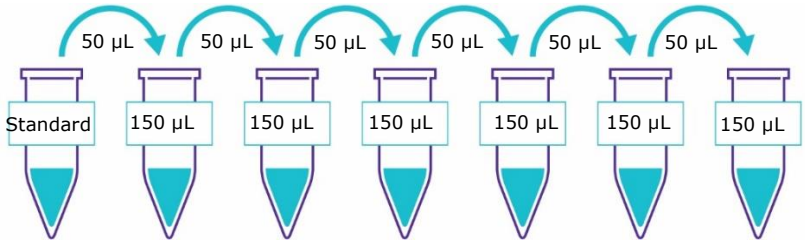
Preparation of Canine Cytokine Panel Standard

1. Prior to use, reconstitute the Canine Cytokine Panel Standard with 250 µL deionized water. Invert the vial several times to mix and vortex for 5-10 seconds. Allow the vial to sit for 5-10 minutes and vortex. Transfer the reconstituted standard to a polypropylene microfuge tube. This will be used as "standard 7"; This reconstituted standard and the serially diluted standards in the following steps should be set in an ice bath, during the assay procedure. These need to be added to the plate within 1 hour of preparation. The unused portion of the "Standard 7" may be stored at ≤ –20 °C for up to one month.

2. Label six polypropylene microfuge tubes Standard 6, Standard 5, Standard 4, Standard 3, Standard 2, and Standard 1, and add 150 µL of Assay Buffer to each of the six tubes. Perform 4-fold serial dilutions by adding 50 µL of the Standard 7 to the Standard 6 tube, mix well and transfer 50 µL of the Standard 6 to the Standard 5 tube, mix well and transfer 50 µL of the Standard 5 to the Standard 4 tube, mix well and transfer 50 µL of the Standard 4 to the Standard 3 tube, mix well and transfer 50 µL of the Standard 3 to the Standard 2 tube, mix well and transfer 50 µL of the Standard 2 to the Standard 1 tube, and mix Standard 1 well. The Standard 0 (Background) will be Assay Buffer.

Standard Tube No.	**Add Deionized Water (µL)**	**Add Standard (volume)**
Standard 7 (reconstituted standard)	250	0
Standard Tube No.	**Add Assay Buffer (µL)**	**Add Standard (volume)**
Standard 6	150	50 µL of Standard 7
Standard 5	150	50 µL of Standard 6
Standard 4	150	50 µL of Standard 5
Standard 3	150	50 µL of Standard 4
Standard 2	150	50 µL of Standard 3
Standard 1	150	50 µL of Standard 2

Preparation of Standards



Reconstituted Standard 7	Standard 6	Standard 5	Standard 4	Standard 3	Standard 2	Standard 1

After serial dilutions, the tubes should have the following concentrations for constructing standard curves.

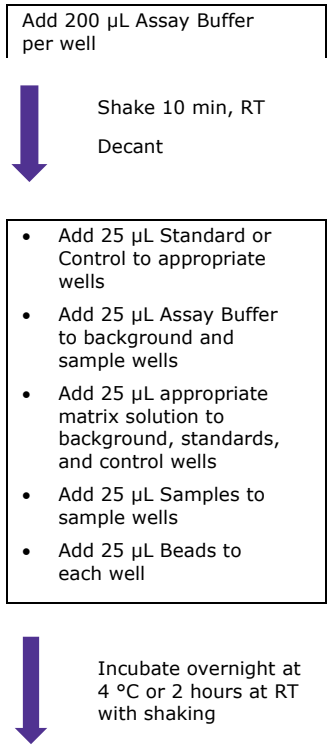
Standard Tube No.	IFN-γ (pg/mL)	All Other Analytes (pg/mL)
1	2.44	12.2
2	9.77	48.8
3	39.1	195
4	156	781
5	625	3,125
6	2,500	12,500
7	10,000	50,000

Immunoassay Procedure

- Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.
- Allow all reagents to warm to room temperature (20-25 °C) before use in the assay.
- Diagram the placement of Standards [0 (Background), 1, 2, 3, 4, 5, 6, 7], Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration.

  Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
- If using a filter plate, set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Add 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25 °C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of each Standard or Control into the appropriate wells. Add 25 µL Assay Buffer for Standard 0 (Background).
4. Add 25 µL of Assay Buffer to the sample wells.
5. Add 25 µL of appropriate matrix solution to the background, standards, and control wells. When assaying serum or plasma, use the Serum Matrix provided in the kit. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
6. Add 25 µL of Sample into the appropriate wells.
7. Vortex Mixing Bottle and add 25 µL of the Mixed Beads to each well. (**Note**: During addition of Beads, shake bead bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-18 hr) at 4 °C or 2 hours at room temperature (20-25 °C). An overnight incubation may improve assay sensitivity for some analytes.



9. Gently remove well contents and wash plate 2 times following instructions listed in the Plate Washing section.

10. Add 25 µL of Detection Antibodies into each well. (**Note:** Allow the Detection Antibodies to warm to room temperature prior to addition.)

11. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25 °C). **DO NOT ASPIRATE AFTER INCUBATION.**

12. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.

13. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25 °C).

14. Gently remove well contents and wash plate 2 times following instructions listed in the Plate Washing section.

15. Add 150 µL of Sheath Fluid PLUS (or Drive Fluid PLUS if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes at room temperature.

16. Run plate on Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® instrument with xPONENT® software or xMAP® INTELLIFLEX instrument with INTELLIFLEX software.

17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. (**Note:** If samples are diluted, multiply the result by the dilution factor.)

Remove well contents and wash 2X with 200 µL Wash Buffer

Add 25 µL Detection Antibodies per well

Incubate for 1 hour at RT

Do Not Aspirate

Add 25 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

Remove well contents and wash 2X with 200 µL Wash Buffer

Add 150 µL Sheath Fluid PLUS or Drive Fluid PLUS per well

Read on Luminex® instrument (100 µL, 50 beads per bead set)

Plate Washing

Solid Plate

If using a solid plate, use either a handheld magnet or magnetic plate washer.

- Handheld magnet (40-285)
  Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.
- Magnetic plate washer (40-094, 40-095, 40-096 and 40-097)
  Please refer to specific automatic plate washer manual for appropriate equipment settings. Please note that after the final aspiration, there will be approximately 25 µL of residual wash buffer in each well. This is expected when using the BioTek® plate washer and this volume does not need to be aspirated from the plate.

If using an automatic plate washer other than BioTek® 405 LS or 405 TS, please refer to the manufacturer's recommendations for programming instructions.

Filter Plate (MX-PLATE)

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration after each wash. Repeat wash steps as recommended in the Assay Procedure.

Equipment Settings

Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® instruments with xPONENT® software and xMAP® INTELLIFLEX instrument with INTELLIFLEX software:

These specifications are for the above listed instruments and software. Luminex® instruments with other software (for example, MasterPlex®, StarStation, LiquiChip, Bio-Plex® Manager™, LABScan™100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex® magnetic beads.

For magnetic bead assays, each instrument must be calibrated and performance verified with the indicated calibration and verification kits.

Instrument	Calibration Kit	Verification Kit
Luminex® 200™ and HTS	xPONENT® 3.1 compatible Calibration Kit (LX2R-CAL-K25)	Performance Verification Kit (LX2R-PVER-K25)
FLEXMAP 3D®	FLEXMAP 3D® Calibrator Kit (F3D-CAL-K25)	FLEXMAP 3D® Performance Verification Kit (F3D-PVER-K25)
xMAP® INTELLIFLEX	xMAP® INTELLIFLEX Calibration Kit (IFX-CAL-K20)	xMAP® INTELLIFLEX Performance Verification Kit (IFX-PVER-K20)
MAGPIX®	MAGPIX® Calibration Kit (Cat, No. MPX-CAL-K25)	MAGPIX® Performance Verification Kit (MPX-PVER-K25)

Note: When setting up a Protocol using the xPONENT® software, you must select MagPlex® as the Bead Type in the Acquisition settings. These assays cannot be run on any instruments using Luminex® IS 2.3 or Luminex® 1.7 software.

The Luminex® probe height must be adjusted to the plate provided in the kit. Please use MAG-PLATE, if additional plates are required for this purpose.

Events	50, per bead
Sample Size	100 µL
Gate Settings	8,000 to 15,000
Time Out	60 seconds

Bead Set	13-Plex Premix Beads		Customizable 13-Plex Beads	
	GM-CSF	14	GM-CSF	14
	IFNγ	25	IFNγ	25
	IL-2	29	IL-2	29
	IL-6	35	IL-6	35
	IL-7	36	IL-7	36
	IL-8	48	IL-8	48
	IL-15	54	IL-15	54
	IP-10	57	IP-10	57
	KC-like	61	KC-like	61
	IL-10	63	IL-10	63
	IL-18	67	IL-18	67
	MCP-1	74	MCP-1	74
	TNF-A	76	TNF-A	76

Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at our website SigmaAldrich.com using the catalogue number as the keyword.

Assay Characteristics

Cross-Reactivity and Cross-Species Reactivity

There was no or negligible cross-reactivity among different analytes within the panel.

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) is calculated 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.

Analyte	MinDC (pg/mL) Average + 2SD
GM-CSF	9.2
IFN-γ	10.5
KC-like	5.3
IP-10	3.2
IL-2	3.5
IL-6	3.7
IL-7	7.5
IL-8	21.7
IL-10	8.5
IL-15	9.0
IL-18	5.8
MCP-1	21.0
TNFα	6.1

Precision

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across two different concentrations of analytes in a single assay. Inter-assay precision is generated from the mean of the %CV's across two different concentrations of analytes across 6 different assays.

Analyte	Intra-Assay (CV%)	Inter-Assay (CV%)
GM-CSF	< 5	< 15
IFN-γ	< 5	< 15
KC-like	< 5	< 15
IP-10	< 5	< 15
IL-2	< 5	< 17
IL-6	< 5	< 15
IL-7	< 5	< 15
IL-8	< 5	< 15
IL-10	< 5	< 15
IL-15	< 5	< 17
IL-18	< 5	< 15
MCP-1	< 5	< 15
TNFα	< 5	< 15

Accuracy

Spike Recovery: The data represent mean percent recovery of spiked standards ranging from low, medium, and high concentration in serum matrix samples (n=6).

Analyte	Recovery
GM-CSF	95%
IFN-γ	98%
KC-like	91%
IP-10	94%
IL-2	96%
IL-6	98%
IL-7	102%
IL-8	101%
IL-10	95%
IL-15	109%
IL-18	96%
MCP-1	91%
TNFα	98%

Troubleshooting

Problem	Probable Cause	Solution
Insufficient bead count	Plate washer aspirate height set too low	Adjust aspiration height according to manufacturers' instructions.
	Bead mix prepared inappropriately	Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting this into the plate.
	Samples cause interference due to particulate matter or viscosity	See above. Also sample probe may need to be cleaned with alcohol flushes, back flushes and washes; or if needed, probe should be removed and sonicated.
	Probe height not adjusted correctly	When reading the assay on the Luminex® 200™ instrument, adjust probe height to the kit solid plate or to the recommended filter plates using 3 alignment discs. When reading the assay on the MAGPIX® instrument, adjust probe height to the kit solid plate or to the recommended filter plates using 2 alignment discs. When reading the assay on the FLEXMAP 3D® instrument, adjust probe height to the kit solid plate using 1 alignment disc. For the FLEXMAP 3D® instrument, when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid PLUS in each well and 75 µL should be aspirated. When reading the assay on the xMAP® INTELLIFLEX instrument, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.

Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and pipetting with multichannel pipettes without touching reagent in plate.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for cross reacting components (for example, interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.
Beads not in region or gate	Luminex® instrument not calibrated correctly or recently	Calibrate Luminex® instrument based on manufacturer's instructions, at least once a week or if temperature has changed by > 3 °C.
	Gate settings not adjusted correctly	Some Luminex® instruments (for example, Bio-Plex®) require different gate settings than those described in the kit protocol. Use instrument default settings.
	Wrong bead regions in protocol template	Check kit protocol for correct bead regions or analyte selection.
	Incorrect sample type used	Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required.
	Instrument not washed or primed	Prime the Luminex® instrument 4 times to rid of air bubbles, wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid.
	Beads were exposed to light	Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.
Signal for whole plate is same as background	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue.
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low.

Low signal for standard curve	Detection Antibody may have been removed prior to adding Streptavidin-Phycoerythrin	May need to repeat assay if desired sensitivity not achieved.
	Incubations done at inappropriate temperatures, timings or agitation	Assay conditions need to be checked.
Signals too high, standard curves are saturated	Calibration target value set too high	With some Luminex® instruments (for example, Bio-Plex®) default target setting for RP1 calibrator is set at high PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with standard curve and samples	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	If below detectable levels, it may be possible to use higher sample volume. Check with tech support for appropriate protocol modifications.
	Samples contain analyte concentrations higher than highest standard point.	Samples may require dilution and reanalysis for just that particular analyte.
	Standard curve was saturated at higher end of curve.	See above.

High Variation in samples and/or standards	Multichannel pipette may not be calibrated	Calibrate pipettes.
	Plate washing was not uniform	Confirm all reagents are removed completely in all wash steps.
	Samples may have high particulate matter or other interfering substances	See above.
	Plate agitation was insufficient	Plate should be agitated during all incubation steps using an orbital plate shaker at a speed where beads are in constant motion without causing splashing.
	Cross-well contamination	Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipette tips that are used for reagent additions and that pipette tip does not touch reagent in plate.

FOR FILTER PLATES ONLY

Problem	Probable Cause	Solution
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay setup and use supernatant.
	High lipid concentration	After centrifugation, remove lipid layer and use supernatant.
Plate leaked	Vacuum pressure too high	Adjust vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (blocked) plate and continue.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate holder or raised edge so bottom of filter is not touching any surface.
	Insufficient blotting of filter plate bottom causing wicking	Blot the bottom of the filter plate well with absorbent towels after each wash step.
	Pipette touching plate filter during additions	Pipette to the side of plate.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
Sample too viscous	Sample too viscous	May need to dilute sample.

Product Ordering

Replacement Reagents	Cat. No.
Canine Cytokine Standard	LCC-8090
Canine Cytokine Quality Controls	LCC-6090
Serum Matrix	LMC-SD
Canine Cytokine Detection Antibodies	LCC-1090
Streptavidin-Phycoerythrin	L-SAPE3
Assay Buffer	L-AB
Set of two 96-Well Plate with 4 sealers	MAG-PLATE
10X Wash Buffer	L-WB
Canine Cytokine/Chemokine 13 Plex Premixed Magnetic Bead Panel-BULK PACKAGING	CCYTMAG90KPX 13BK

Antibody-Immobilized Magnetic Beads

Analyte	Bead No.	Cat.No.
GM-CSF	14	CGMCSF-MAG
IFN-γ	25	CIFNG-MAG
KC-like	61	MKC-MAG
IP-10	57	MIP10-MAG
IL-2	29	CIL2-MAG
IL-6	35	CIL6-MAG
IL-7	36	MIL7-MAG
IL-8	48	CIL8-MAG
IL-10	63	CIL10-MAG
IL-15	54	MIL15-MAG
IL-18	67	CIL18-MAG
MCP-1	74	CMCP1-MAG
TNFα	76	CTNFA-MAG

Well Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/mL Standard (Background)	Standard 4	QC-1 Control	Etc.								
B	0 pg/mL Standard (Background)	Standard 4	QC-1 Control									
C	Standard 1	Standard 5	QC-2 Control									
D	Standard 1	Standard 5	QC-2 Control									
E	Standard 2	Standard 6	Sample 1									
F	Standard 2	Standard 6	Sample 1									
G	Standard 3	Standard 7 Reconstituted	Sample 2									
H	Standard 3	Standard 7 Reconstituted	Sample 2									

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