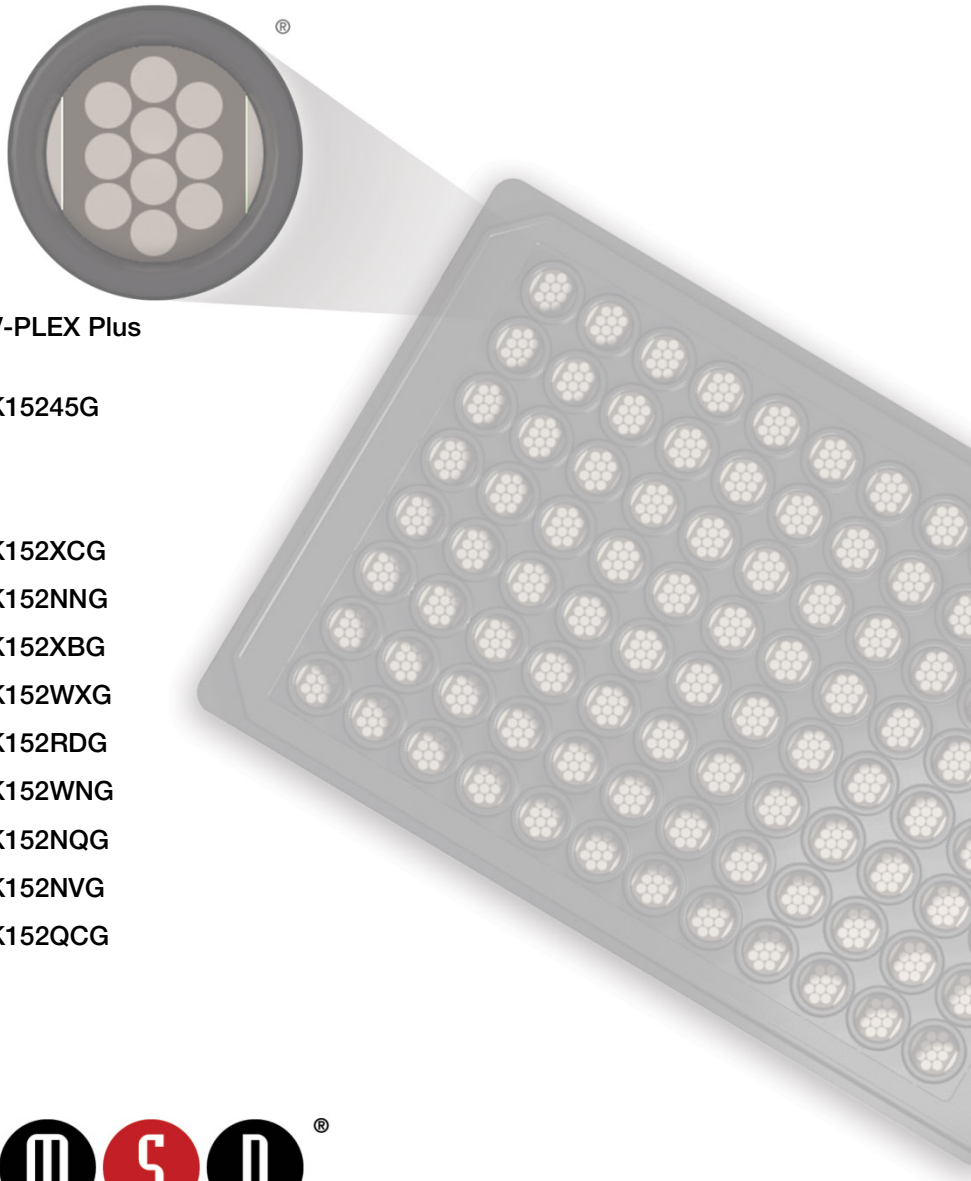
MSD® MULTI-SPOT Assay System

Cytokine Panel 1 (mouse) Kits

IL-9, MCP-1, IL-33, IL-27p28/IL-30, IL-15, IL-17A/F, MIP-1α, IP-10, MIP-2

V-PLEX®



	V-PLEX®	V-PLEX Plus
Cytokine Panel 1 (mouse)	K15245D	K15245G
Individual Assay Kits		
Mouse IL-9	K152XCD	K152XCG
Mouse MCP-1	K152NND	K152NNG
Mouse IL-33	K152XBD	K152XBG
Mouse IL-27p28/IL-30	K152WXD	K152WXG
Mouse IL-15	K152RDD	K152RDG
Mouse IL-17A/F	K152WND	K152WNG
Mouse MIP-1α	K152NQD	K152NQG
Mouse IP-10	K152NVD	K152NVG
Mouse MIP-2	K152QCD	K152QCG

MSD Cytokine Assays

Cytokine Panel 1 (mouse) Kits

IL-9, MCP-1, IL-33, IL-27p28/IL-30, IL-15, IL-17A/F, MIP-1α, IP-10, MIP-2

For use with cell culture supernatants, serum, plasma, and urine.

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®
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Introduction

MSD offers V-PLEX assays for customers who require unsurpassed performance and quality. V-PLEX products are developed under rigorous design control and are fully validated according to fit-for-purpose[1] principles in accordance with MSD's Quality Management System. They offer exceptional sensitivity, simple protocols, reproducible results, and lot-to-lot consistency. In addition to the analytical validation, robustness of the assay protocol is assessed during development along with the stability and robustness of the assay components and kits. V-PLEX assays are available in both single-assay and multiplex formats.

The V-PLEX assay menu is organized by panels. Grouping the assays into panels by species, analytical compatibility, clinical range, and expected use, ensures optimal and consistent performance from each assay while still providing the benefits and efficiencies of multiplexing. V-PLEX panels are provided in MSD's MULTI-SPOT® 96-well plate format. The composition of each panel and the location of each assay (i.e., its spot within the well) are maintained from lot to lot. Most individual V-PLEX assays are provided on MSD's single-spot, 96-well plates. The remaining are provided on the multiplex panel plate.

The Cytokine Panel 1 (mouse) measures nine cytokines that are important in a number of key inflammatory pathways. The biomarkers constituting the panel are described below.

Mouse Interleukin-9 (IL-9 also known as P40) is a pleiotropic cytokine first recognized produced by both TH2 cells and TH17 T cells.[2] Naïve T cells treated with TGF-β and IL-4 differentiate into the TH9 subtype.[3,4] IL-9 binds to its cognate receptor IL-9R, also known as CD129, which activates the JAK/STAT network to exert effects on both myeloid and lymphoid cells.[2] It is associated with allergic inflammation, immune response to parasite infection, and TH17 mediated immunity.[2,4,5]

Mouse Monocyte Chemoattractant Protein 1 (MCP-1, also known as CCL2) is chemotactic for monocytes and basophils and is a key regulator for infiltration of tissues by monocytes. MCP-1 is produced by a variety of cell types, especially after oxidative stress or stimulation by cytokines. It both induces angiogenesis and plays a role in tumor progression while also augmenting monocyte anti-tumor activity.[6,7] MCP-1 naturally occurs as either a monomer or homodimer and binds to CCR2A and CCR2B, splice variants of the CCR2 receptor.[5,8,] Elevated MCP-1 expression is associated with chronic systemic and intestinal inflammation in colon cancer models, obesity, and heart disease.[9-11]

Mouse Interleukin-33 (IL-33) is a member of the IL-1 cytokine family, and is structurally similar to IL-1β and IL-18.[12] It is synthesized as a 31 kDa precursor protein that undergoes cleavage by caspase-1 to generate an 18 kDa protein.[12] By binding to either its soluble or membrane-bound receptors, the stimulatory effect of IL-33 on several immune cell types is regulated.[5] IL-33 induces mast cells, lymphocytes, basophils, and eosinophils to produce proinflammatory and TH2 cytokines was well as chemokines.[13] It has been shown to play a role in chronic inflammation in asthma and autoimmune and cardiovascular disease.[13,14]

Mouse Interleukin-27 p28 subunit, also known as IL-30 (IL-27p28/IL-30) is a monomer that associates with the soluble receptor EBI3 (Epstein-Barr virus induced gene 3) to form IL-27.[15,16] p28 is related to CLC (cardiotrophin-like cytokine) and can dimerize with CLF (cytokine like factor 1).[17] Both heterodimers (p28-EBI3, p28-CLF) are secreted by dendritic cells and macrophages. p28-EBI3 and p28-CLF act on NK cells, mast cells, monocytes, and cytotoxic T cells through the IL-27Rα receptor, which is composed of WSX-1 and gp130, as well as through IL-6R and gp130.[5,16,17] p28 expression is upregulated by inflammatory stimuli; the resulting biological effects depend on which binding partner is expressed and which receptor is engaged.

Mouse Interleukin-15 (IL-15) is a glycosylated 18.1 kDa protein with two disulfide bonds and has structural similarity to IL-2.[18] It stimulates the proliferation of T-lymphocytes and NK cells and plays important roles in both innate and adaptive immunity.[18] IL-15 supports naive and memory CD8+ T cells maintenance.[19] The IL-15 receptor complex consists of IL-2Rβ, IL-2Rγ, and IL-15Rα.[5] The IL-15Rα receptor, both in a soluble and membrane-bound form, binds IL-15 with high affinity.[18]

Mouse Interleukin-17A/F heterodimer (IL-17A/F) is a cross-linked heterodimer composed of IL-17A (CTLA8; Gene ID: 16171) and IL-17F (CANDF6, ML1; Gene ID: 257630) proteins that is secreted by IL-23 activated Th17 cells.[20,21] Activity is mediated by binding its receptor, a heterodimer of IL-17RA and IL-17RC expressed on multiple cell types including fibroblasts, which results in the activation of the Erk1/2 and NF-κB pathways.[22] IL-17A/F signal responses have similar but intermediate potency between IL-17A/A and IL-17F/F homodimers. Elevated expression is associated with corticosteroid-resistant 'neutrophilic' asthma, bacterial infections, and autoimmune diseases. [14,23,24]

Mouse Macrophage Inflammatory Protein 1α (MIP-1α, also known as CCL3) is a chemokine with inflammatory and chemotactic properties.[25] It attracts T cells, B cells, monocytes, and eosinophils. MIP-1α binds to CCR1, CCR4, and CCR5, and like MIP-1β, it is one of the major HIV suppressive factors produced by CD8+ T cells.[25] MIP-1α also induces mast cell degranulation and NK cell activation. Elevated MIP-1α levels are associated with fungal infection and lung irritation.[26,27]

Mouse Interferon Gamma-Induced Protein 10 (IP-10, also known as interferon gamma induced protein or CXCL10) is produced by monocytes and is chemotactic to monocytes, NK cells, and T lymphocytes. As its name suggests, IP-10 production is induced by IFN-γ. It has antimicrobial, antitumor, and antiparasitic effects *in vivo*.[28-30] IP-10 shares the CXCR3 receptor that is expressed on activated T cells with the related MIG (CXCL9) and I-TAC (CXCL11) chemokines.[31] However, antibody neutralization studies demonstrate that their signaling is non-redundant.[30]

Mouse Macrophage Inflammatory Protein 2 (MIP-2, also known as CXCL2) is structurally and functionally related to CXCL1/GRO-α/Gm1960, CXCL3/KC, and CXCL8/IL-8.[31] MIP-2 is secreted by monocytes and macrophages and is chemotactic for granulocytes including neutrophils.[8,32] Its receptor (CXCR2) is homologous to human IL-8R. Elevated levels of MIP-2 are detected after microbial infection and lung irritation.[5,28,29,33]

Principle of the Assay

MSD cytokine assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The assays in the Cytokine Panel 1 (mouse) are sandwich immunoassays. MSD provides a plate pre-coated with capture antibodies on independent and well-defined spots, as shown in the layout below. Multiplex assays and the individual IL-9, IL-33, IL-27p28/IL-30, IL-15, IL-17A/F, MIP-1α, and MIP-2 assays are provided on 10-spot MULTI-SPOT plates (Figure 1); the individual MCP-1 and IP-10 assays are provided on Small Spot plates (Figure 2). The user adds the sample and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) 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 detection antibodies by the bound analytes completes the sandwich. The user adds an MSD buffer that creates the appropriate chemical environment for electrochemiluminescence (ECL) and loads the plate into an MSD instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light (which is proportional to the amount of analyte present in the sample) and provides a quantitative measure of each analyte in the sample. V-PLEX assay kits have been validated according to the principles outlined in "Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement" by J. W. Lee, et al.[1]

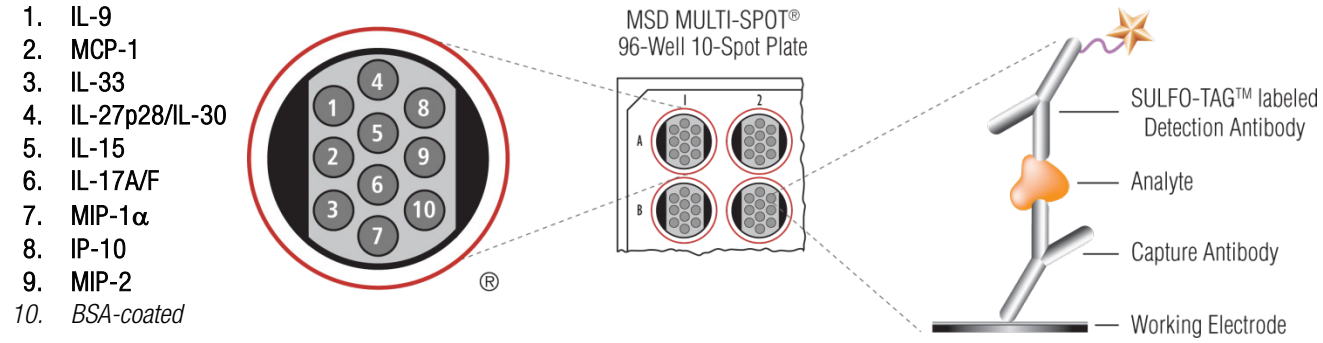


Figure 1. Multiplex plate spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

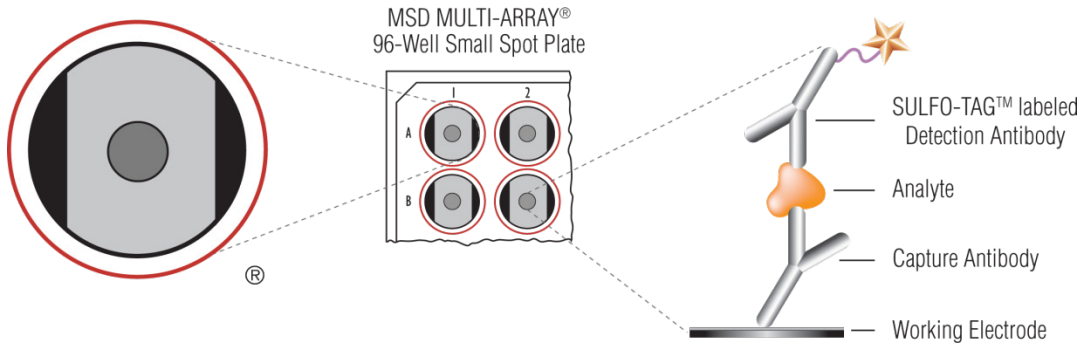


Figure 2. Small Spot plate diagram showing placement of analyte capture antibodies.

Kit Components

Cytokine Panel 1 (mouse) assays are available as a multiplex kit on 10-Spot plate, as individual assay kits, or as custom V-PLEX kits with subsets of assays selected from the full panel. V-PLEX Plus kits include additional items (controls, wash buffer, and plate seals). See below for details.

See the **Catalog Numbers** section for complete kits.

Reagents Supplied With All Kits

Table 1. Reagents that are supplied with V-PLEX and V-PLEX Plus Kits

Reagent	Storage	Catalog #	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Cytokine Panel 1 (mouse) Calibrator Blend	2–8 °C	C0245-2	1 vial	1 vial	5 vials	25 vials	Recombinant mouse proteins in diluent, buffered and lyophilized. Individual analyte concentration is provided in the lot-specific certificate of analysis (COA).
Diluent 41	≤-10 °C	R50AH-1	10 mL	1 bottle			Diluent for samples and calibrator; contains protein, blockers, and preservatives.
		R50AH-2	50 mL		1 bottle	5 bottles	
Diluent 45	≤-10 °C	R50AI-1	5 mL	1 bottle			Diluent for detection antibody; contains protein, blockers, and preservatives.
		R50AI-2	25 mL		1 bottle	5 bottles	
Read Buffer T (4X)	RT	R92TC-3	50 mL	1 bottle	1 bottle	5 bottles	Buffer to catalyze the ECL reaction.

V-PLEX Plus Kits: Additional Components

Table 2. Additional components that are supplied with V-PLEX Plus Kits

Reagents	Storage	Catalog #	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Cytokine Panel 1 (mouse) Control 1*	2–8 °C	C4245-1	1 vial	1 vial	5 vials	25 vials	Recombinant mouse proteins in diluent, buffered and lyophilized. The concentration of the controls is provided in the lot-specific COA.
Cytokine Panel 1 (mouse) Control 2*	2–8 °C	C4245-1	1 vial	1 vial	5 vials	25 vials	
Cytokine Panel 1 (mouse) Control 3*	2–8 °C	C4245-1	1 vial	1 vial	5 vials	25 vials	
Wash Buffer (20X)	RT	R61AA-1	100 mL	1 bottle	1 bottle	5 bottles	20-fold concentrated phosphate buffered solution with surfactant.
Plate Seals	-	-	-	3	15	75	Adhesive seals for sealing plates during incubations.

*Provided as components in the Cytokine Panel 1 (mouse) Control Pack (catalog # C4245-1)

Kit-Specific Components

Table 3. Components that are supplied with specific kits

Plates	Storage	Part #	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Cytokine Panel 1 (mouse) SECTOR® Plate	2–8 °C	N05245A-1	10-spot	1	5	25	96-well plate, foil sealed, with desiccant.
Mouse MCP-1 SECTOR Plate	2–8 °C	L450NNA-1	Small Spot	1	5	25	
Mouse IP-10 SECTOR Plate	2–8 °C	L452NVA-1	Small Spot	1	5	25	

Table 4. Individual detection antibodies for each assay are supplied with specific kits

SULFO-TAG Detection Antibody	Storage	Catalog #	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Anti-ms IL-9 Antibody (50X)	2–8 °C	D22XC-2	75 µL	1			SULFO-TAG conjugated antibody
		D22XC-3	375 µL		1	5	
Anti-ms MCP-1 Antibody (50X)	2–8 °C	D20NN-2	75 µL	1			SULFO-TAG conjugated antibody
		D20NN-3	375 µL		1	5	
Anti-ms IL-33 Antibody (50X)	2–8 °C	D22XB-2	75 µL	1			SULFO-TAG conjugated antibody
		D22XB-3	375 µL		1	5	
Anti-ms IL-27p28/IL-30 Antibody (50X)	2–8 °C	D22WX-2	75 µL	1			SULFO-TAG conjugated antibody
		D22WX-3	375 µL		1	5	
Anti-ms IL-15 Antibody (50X)	2–8 °C	D22RD-2	75 µL	1			SULFO-TAG conjugated antibody
		D22RD-3	375 µL		1	5	
Anti-ms IL-17A/F Antibody (50X)	2–8 °C	D22WN-2	75 µL	1			SULFO-TAG conjugated antibody
		D22WN-3	375 µL		1	5	
Anti-ms MIP-1α Antibody (50X)	2–8 °C	D22NQ-2	75 µL	1			SULFO-TAG conjugated antibody
		D22NQ-3	375 µL		1	5	
Anti-ms IP-10 Antibody (50X)	2–8 °C	D22NV-2	75 µL	1			SULFO-TAG conjugated antibody
		D22NV-3	375 µL		1	5	
Anti-ms MIP-2 Antibody (50X)	2–8 °C	D22QC-2	75 µL	1			SULFO-TAG conjugated antibody
		D22QC-3	375 µL		1	5	

Additional Materials and Equipment

- ❑ Appropriately sized tubes for reagent preparation
- ❑ Polypropylene microcentrifuge tubes for preparing dilutions
- ❑ Liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL/well into a 96-well microtiter plate
- ❑ Plate washing equipment: automated plate washer or multichannel pipette
- ❑ Microtiter plate shaker (rotary) capable of shaking at 500-1,000 rpm
- ❑ Phosphate-buffered saline (PBS) plus 0.05% Tween-20 for plate washing or MSD Wash Buffer catalog # R61AA-1 (included in V-PLEX Plus kit)
- ❑ Adhesive plate seals (3 per plate included in V-PLEX Plus kits)
- ❑ Deionized water
- ❑ Vortex mixer

Optional Materials and Equipment

- ❑ Cytokine Panel 1 (mouse) Control Pack, available for separate purchase from MSD, catalog # C4245-1 (included in V-PLEX Plus kit)
- ❑ Centrifuge for sample preparation
- ❑ De-crimping tool for opening calibrator and control vials

Safety

Use safe laboratory practices and wear gloves, safety glasses, and lab coats when handling kit components. Handle and dispose of all hazardous samples properly in accordance with local, state, and federal guidelines.

Additional product-specific safety information is available in the safety data sheet (SDS), which can be obtained from MSD Customer Service or at www.mesoscale.com.

Best Practices

- Do not mix or substitute reagents from different sources or different kit lots. Lot information is provided in the lot-specific COA.
- Assay incubation steps should be performed between 20-26 °C to achieve the most consistent signals between runs.
- Bring frozen diluent to room temperature in a 24 °C water bath. Thaw other reagents on wet ice and use as directed without delay.
- Prepare calibrators, samples, and controls in polypropylene microcentrifuge tubes; use a fresh pipette tip for each dilution; vortex after each dilution before proceeding.
- Do not touch the pipette tip on the bottom of the wells when pipetting into the MSD plate.
- Avoid prolonged exposure of detection antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be shielded from light except for direct sunlight.
- Avoid bubbles in wells at all pipetting steps. Bubbles may lead to variable results; bubbles introduced when adding Read Buffer T may interfere with signal detection.
- Use reverse pipetting when necessary to avoid introduction of bubbles. For empty wells, pipette to the bottom corner.
- Plate shaking should be vigorous, with a rotary motion between 500 and 1,000 rpm. Binding reactions may reach equilibrium sooner if you use shaking at the middle of this range (~700 rpm) or above.
- When using an automated plate washer, rotate the plate 180 degrees between wash steps to improve assay precision.
- Gently tap the plate on a paper towel to remove residual fluid after washing.
- If an incubation step needs to be extended, leave the sample or detection antibody solution in the plate to keep the plate from drying out.
- Remove the plate seals prior to reading the plate.
- Make sure that Read Buffer T is at room temperature when added to a plate.
- Do not shake the plate after adding Read Buffer T.
- To improve inter-plate precision, keep time intervals consistent between adding Read Buffer T and reading the plate. Unless otherwise directed, read the plate as soon as possible after adding Read Buffer T.
- If assay results are above the top of the calibration curve, dilute the samples and repeat the assay.
- When running a partial plate, seal the unused sectors to avoid contaminating unused wells. Remove all seals before reading. Partially used plates may be sealed and stored up to 30 days at 2-8 °C in the original foil pouch with desiccant. You may adjust volumes proportionally when preparing reagents.

Reagent Preparation

Bring all reagents to room temperature.

Important: Upon first thaw, aliquot Diluent 41 and Diluent 45 into suitable volumes before refreezing.

Prepare Calibrator Dilutions

MSD supplies a multi-analyte lyophilized calibrator that yields the recommended highest calibrator concentration when reconstituted in 1,000 µL of Diluent 41. (For individual assays that do not saturate at the highest calibrator concentration, the calibration curve can be extended by creating a more concentrated highest calibrator. In this case, follow the steps below using 250 µL instead of 1,000 µL of Diluent 41 when reconstituting the lyophilized calibrator.) Keep reconstituted calibrator and calibrator solutions on wet ice until use.

To prepare 7 calibrator solutions plus a zero calibrator for up to 4 replicates:

1) Prepare the highest calibrator (Calibrator 1) by adding 1,000 µL of Diluent 41 to the lyophilized calibrator vial. After reconstituting, invert at least 3 times (do not vortex). Let the reconstituted solution equilibrate at room temperature for **30-45 minutes** and then vortex briefly using short pulses.

   Note: It is critical that the reconstituted calibrator equilibrates at room temperature for 30-45 minutes prior to first use. Reconstituted calibrator is stable when stored at 2–8 °C up to 30 days. It may also be stored frozen at ≤-70˚C in suitable aliquots and subjected to up to 3 freeze–thaw cycles.

2) Prepare the next calibrator by transferring 100 µL of the highest calibrator to 300 µL of Diluent 41. Mix well by vortexing. Repeat 4-fold serial dilutions 5 additional times to generate 7 calibrators.

3) Use Diluent 41 as the zero calibrator.

Note: For the lot-specific concentration of each calibrator in the blend, refer to the COA supplied with the kit. You can also find a copy of the COA at www.mesoscale.com.

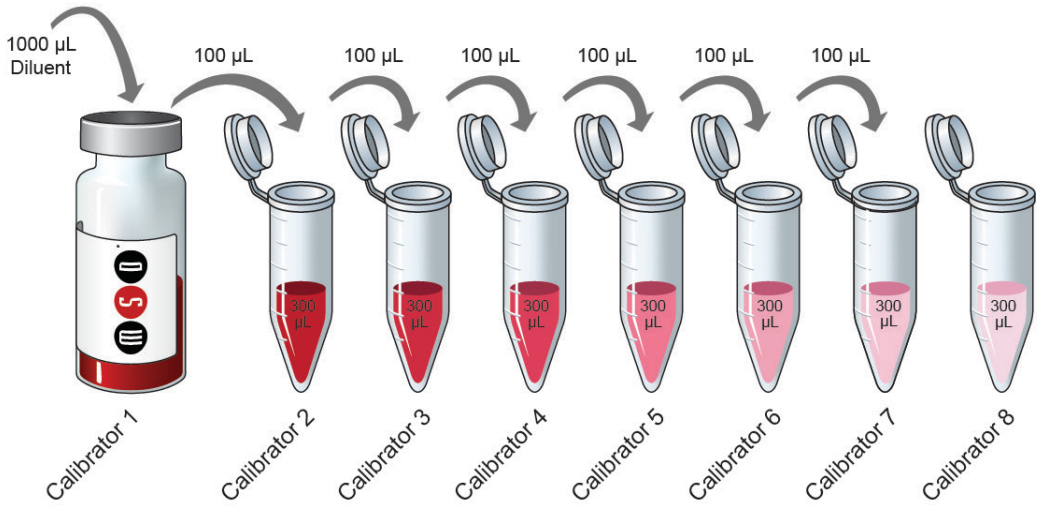


***Figure 3**. Dilution schema for preparation of Calibrator Standards.*

Sample Collection and Handling

Below are general guidelines for mouse sample collection, storage, and handling. If possible, use published guidelines.[34,35] Evaluate sample stability under the selected method as needed.

- Serum and plasma. When preparing serum, allow samples to clot for 2 hours at room temperature, then centrifuge for 20 minutes at 2,000g prior to using or freezing. If no particulates are visible, you may not need to centrifuge.
- Other samples. Use immediately or freeze.

Freeze all samples in suitably-sized aliquots; they may be stored at ≤-10 ºC until needed. Repeated freeze-thaw of samples is not recommended. After thawing, centrifuge samples at 2,000g for 3 minutes to remove particulates prior to sample preparation.

Dilute Samples

Dilute samples with Diluent 41. For mouse serum, plasma, and urine samples, MSD recommends a minimum 4-fold dilution. For example, to dilute 4-fold, add 30 µL of sample to 90 µL of Diluent 41. You may conserve sample volume by using a higher dilution.

Tissue culture supernatants may require additional dilution based on stimulation and analyte concentrations in the sample. Additional diluent can be purchased at www.mesoscale.com.

Prepare Controls

Three levels of multi-analyte lyophilized controls are available for separate purchase from MSD in the Cytokine Panel 1 (mouse) Control Pack, catalog # C4245-1 (controls are included only in V-PLEX Plus kits).

Reconstitute the lyophilized controls in 250 µL of Diluent 41. After reconstituting, invert at least 3 times (do not vortex). Wait a minimum of 30-45 minutes at room temperature and then vortex briefly using short pulses. Dilute the controls 4-fold in Diluent 41. Add diluted control solutions directly to the MSD Cytokine Panel 1 (mouse) plate, and assay as unknown samples.

Reconstituted controls can be stored at 2-8 °C for up 5 days. Controls can also be stored frozen at ≤-70 °C and are stable through three freeze-thaws.

Prepare Detection Antibody Solution

MSD provides each detection antibody separately as a 50X stock solution. The working solution is 1X. Prepare the detection antibody solution immediately prior to use.

9-plex Cytokine Panel 1 (mouse) kit

For one plate, combine the following detection antibodies, then add 2,460 µL of Diluent 45:

- ❑ 60 µL of SULFO-TAG Anti-ms IL-9 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms MCP-1 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IL-33 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IL-27p28/IL-30 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IL-15 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IL-17A/F Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms MIP-1α Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IP-10 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms MIP-2 Antibody

Custom multiplex kits

For one plate, combine 60 µL of each supplied detection antibody, then add Diluent 45 to bring the final volume to 3,000 µL.

Individual assay kits

For one plate, add 60 µL of the supplied detection antibody to 2,940 µL of Diluent 45.

Prepare Wash Buffer

MSD provides 100 mL of Wash Buffer as a 20X stock solution in the V-PLEX Plus kit. Dilute the stock solution to 1X before use. PBS + 0.05% Tween-20 can be used instead.

For one plate, combine:

- ❑ 15 mL of MSD Wash Buffer (20X)
- ❑ 285 mL of deionized water

Prepare Read Buffer

MSD provides Read Buffer T as a 4X stock solution. The working solution is 2X.

For one plate, combine:

- ❑ 10 mL of Read Buffer T (4X)
- ❑ 10 mL of deionized water

You may keep excess diluted read buffer in a tightly sealed container at room temperature for up to one month.

Prepare MSD Plate

MSD V-PLEX plates are pre-coated with capture antibodies (Figure 1) and exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies. Pre-wash plates before use as recommended in the assay protocol.

Protocol

Note: Follow **Reagent Preparation** before beginning this assay protocol.

STEP 1: Wash and Add Sample

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of prepared samples, calibrators or controls per well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.

Note: Washing the plate prior to sample addition is an optional step that may provide greater uniformity of results for certain assays. Analytical parameters, including limits of quantification, recovery of controls, and sample quantification, are not affected by washing the plate prior to sample addition.

STEP 2: Wash and Add Detection Antibody Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 25 µL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.

STEP 3: Wash and Read

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 150 µL of 2X Read Buffer T to each well. Analyze the plate on an MSD instrument. Incubation in Read Buffer T is not required before reading the plate.

Alternate Protocols

The suggestions below may be useful as alternate protocols; however, not all were tested using multiple kit lots.

- **Alternate Protocol 1, Extended Sample Incubation:** Incubating samples overnight at 2–8 ºC may improve sensitivity for some assays. See **Appendix A** for specific assays that may benefit from this alternate protocol.
- **Alternate Protocol 2, Reduced Wash:** For tissue culture samples, you may simplify the protocol by eliminating one of the wash steps. After incubating diluted sample, calibrator, or control, add detection antibody solution to the plate without decanting or washing the plate. See **Appendix A** for assay performance using this protocol.
- **Alternate Protocol 3, Dilute-in-Plate:** To limit sample handling, you may dilute samples and controls in the plate. For 4-fold dilution, add 37.5 µL of assay diluent to each sample/control well, and then add 12.5 µL of neat control or sample. Calibrators should not be diluted in the plate; add 50 µL of each calibrator directly into empty wells. Tests conducted according to this alternate protocol produced results that were similar to the recommended protocol (data not shown).

Validation

MSD's V-PLEX products are validated following fit-for-purpose principles[1] and MSD design control procedures. V-PLEX assay components go through an extensive critical reagents program to ensure that the reagents are controlled and well characterized. Prior to the release of each V-PLEX panel, at least three independent kit lots are produced. Using results from multiple runs (typically greater than 50) and multiple operators, these lots are used to establish production specifications for sensitivity, specificity, accuracy, and precision. During validation, each individual assay is analytically validated as a singleplex and is also independently evaluated as a multiplex component by running the full multiplex plate using only the single detection antibody for that assay. These results are compared with the results from the multiplex panel when using all detection antibodies. This demonstrates that each assay is specific and independent, allowing them to be multiplexed in any combination. The COA provided with each kit outlines the kit release specifications for sensitivity, specificity, accuracy, and precision.

- **Development**

  Calibration curve concentrations for each assay are optimized for a maximum dynamic range while maintaining enough calibration points near the bottom of the curve to ensure a proper fit for accurate quantification of samples that require high sensitivity.

- **Sensitivity**

  The lower limit of detection (LLOD) is a calculated concentration corresponding to the average signal 2.5 standard deviations above the background (zero calibrator). The LLOD is calculated using results from multiple plates for each lot, and the median and range of calculated LLODs for a representative kit lot are reported in the product insert. The upper limit of quantification (ULOQ) and lower limit of quantification (LLOQ) are established for each lot by measuring multiple levels near the expected LLOQ and ULOQ levels. The final LLOQ and ULOQ specifications for the product are established after assessment of all validation lots.

- **Accuracy and Precision**

  Accuracy and precision are evaluated by measuring calibrators and matrix-based validation samples or controls across multiple runs and multiple lots. For most assays, the results of control measurements fall within 20% of the expected concentration for each run. Precision is reported as the coefficient of variation (CV). Intra-run CVs are typically below 6%, and inter-run CVs are typically below 15%. Rigorous management of inter-lot reagent consistency and calibrator production results in typical inter-lot CVs below 15%. Validation lots are compared using controls and at least 35 samples in various sample matrices. Samples are well correlated with an inter-lot bias typically below 15%.

- **Matrix Effects and Samples**

  Matrix effects from serum, plasma, urine, and cell culture media are measured as part of development and validation. Dilution linearity and spike recovery studies are performed on individual samples to assess variability of results due to matrix effects. The sample dilution suggested in the protocol gives an appropriate dilution factor for all assays in the multiplex. Some assays may benefit from lower or higher dilution factors, depending on the samples and application (data are provided in this product insert). In addition to the matrices listed above, blood, PBMCs, and/or cell lines that have

been stimulated to generate elevated levels of analytes are tested. Results confirm measurement of native proteins at concentrations that are often higher than those found in individual native samples.

- **Specificity**

  The specificity of both capture and detection antibodies is measured during assay development. Antibody specificity is assessed by first running each assay using the multiplex plate with assay-specific detection antibody and assay-specific calibrator. These results are compared to the assay's performance when the plate is run 1) with the multi-analyte calibrator and assay-specific detection antibodies, and 2) with assay-specific calibrator and all detection antibodies. For each validation lot and for product release, assay specificity is measured using a multi-analyte calibrator and individual detection antibodies. The calibrator concentration used for specificity testing is chosen to ensure that the specific signal is greater than 50,000 counts.

  In addition to measuring the specificity of antibodies to analytes in the multiplex kit, specificity and interference from other related markers are tested during development. This includes evaluation of selected related proteins and receptors or binding partners.

- **Assay Robustness and Stability**

  The robustness of the assay protocol is assessed by examining the boundaries of the selected incubation times and evaluating the stability of assay components during the experiment and the stability of reconstituted lyophilized components during storage. For example, the stability of reconstituted calibrator is assessed in real time over a 30-day period. Assay component (calibrator, antibody, control) stability was assessed through freeze–thaw testing and accelerated stability studies. The validation program includes a real-time stability study with scheduled performance evaluations of complete kits for up to 54 months from date of manufacture.

Representative data from the development studies are presented in the following sections. The calibration curve and measured limits of detection for each lot can be found in the lot-specific COA that is included with each kit and available for download at www.mesoscale.com.

Analysis of Results

The calibration curves used to calculate analyte concentrations were established by fitting the signals from the calibrators to a 4-parameter logistic (or sigmoidal dose-response) model with a $1/Y^2$ weighting. The weighting function provides a better fit of data over a wide dynamic range, particularly at the low end of the calibration curve. Analyte concentrations were determined from the ECL signals by back-fitting to the calibration curve. These assays have a wide dynamic range (4 logs), which allows accurate quantification of samples without the need for multiple dilutions or repeated testing. The calculations to establish calibration curves and determine concentrations were carried out using the MSD DISCOVERY WORKBENCH® analysis software.

Best quantification of unknown samples will be achieved by generating a calibration curve for each plate using a minimum of two replicates at each calibrator level.

Typical Data

Data from the Cytokine Panel 1 (mouse) were collected over one month of testing by five operators (78 runs in total). Calibration curve accuracy and precision were assessed for three kit lots. Representative data from one lot are presented below. Data from individual assays are presented in Appendix B. The multiplex panel was tested with individual detection antibodies to demonstrate that the assays are independent of one another. Appendix C compares results for each assay in the kit when the panel is run using the individual detection antibody versus all 9 detection antibodies. The calibration curves were comparable. Calibration curves for each lot are presented in the lot-specific COA.

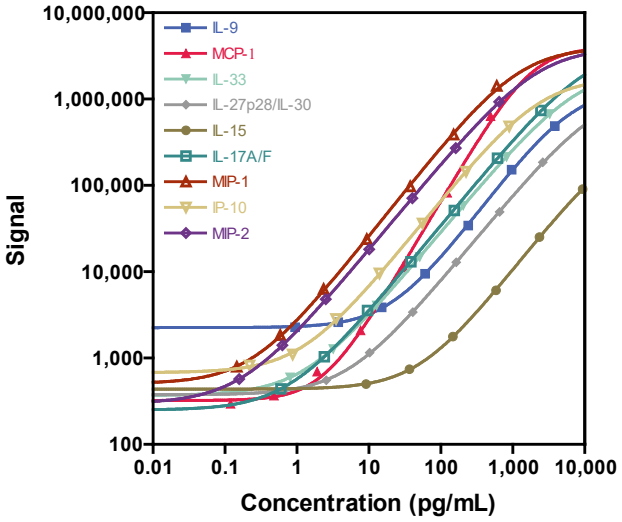


***Figure 4**. Typical calibration curves for the Cytokine Panel 1 (mouse) assays.*

Sensitivity

The LLOD is a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator). The LLOD shown below was calculated based on 12 runs.

The ULOQ is the highest concentration at which the CV of the calculated concentration is <20% and the recovery of each analyte is within 80% to 120% of the known value.

The LLOQ is the lowest concentration at which the CV of the calculated concentration is <20% and the recovery of each analyte is within 80% to 120% of the known value.

The quantitative range of the assay lies between the LLOQ and ULOQ.

The LLOQ and ULOQ are verified for each kit lot and the results are provided in the lot-specific COA that is included with each kit and available at www.mesoscale.com.

Table 5. LLOD, LLOQ, and ULOQ for each analyte in the Cytokine Panel 1 (mouse) Kit

	Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
IL-9	3.84	0.895 - 13.0	21.9	2,600
MCP-1	0.672	0.405 - 1.17	4.42	325
IL-33	0.364	0.222 - 0.879	1.85	1,950
IL-27p28/IL-30	1.39	0.656 - 4.40	5.91	6,500
IL-15	16.0	8.00 - 39.0	43.2	26,000
IL-17A/F	0.231	0.106 - 0.443	1.39	1,620
MIP-1α	0.081	0.027 - 0.194	0.380	390
IP-10	0.328	0.067 - 3.13	2.15	650
MIP-2	0.053	0.030 - 0.354	0.580	423

Precision

Blended controls were made by spiking recombinant mouse proteins in buffered diluent at three levels within the quantitative range of each assay, followed by lyophilization. Analyte levels were measured by 5 operators using a minimum of 9 replicates on 4 runs over one month. Results are shown below. While a typical specification for precision is a concentration CV of less than 20% for controls on both intra- and inter-day runs, for this panel, the data show most assays are below 15%.

Average intra-run %CV is the average %CV of the control replicates within an individual run.

Inter-run %CV is the variability of controls across 71 runs.

Inter-lot %CV is the variability of controls across 4 kit lots.

Table 6. Intra-run and Inter-run %CVs for each analyte in the Cytokine Panel 1 (mouse) Kit

	Control	Average Conc. (pg/mL)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
IL-9	Control 1	3,474	3.2	6.0	6.3
	Control 2	846	2.8	7.1	7.1
	Control 3	200	5.2	12.6	15.0
MCP-1	Control 1	444	3.8	7.2	7.7
	Control 2	146	4.3	8.1	8.6
	Control 3	52.0	5.1	10.7	10.7
IL-33	Control 1	2,290	3.6	10.1	10.5
	Control 2	273	3.8	11.4	11.2
	Control 3	34.9	3.8	13.6	13.3
IL-27p28/ IL-30	Control 1	8,466	3.9	8.8	9.0
	Control 2	1,153	3.1	8.3	8.3
	Control 3	173	4.0	10.1	10.3
IL-15	Control 1	27,692	5.1	12.6	13.4
	Control 2	4,085	4.6	11.0	11.4
	Control 3	618	5.5	10.9	11.4
IL-17A/F	Control 1	2,132	5.2	7.9	8.1
	Control 2	192	4.7	7.9	8.4
	Control 3	16.6	5.6	9.8	9.6
MIP-1α	Control 1	558	3.2	5.4	7.7
	Control 2	47.5	4.5	7.8	8.3
	Control 3	4.02	4.8	10.7	10.6
IP-10	Control 1	848	4.2	9.8	10.4
	Control 2	215	4.0	9.6	10.2
	Control 3	54.8	4.9	11.0	11.9
MIP-2	Control 1	568	2.7	5.3	5.5
	Control 2	65.9	2.7	7.3	7.5
	Control 3	9.71	3.7	9.4	9.6

Dilution Linearity

To assess linearity, normal mouse serum, EDTA plasma, heparin plasma, citrate plasma, and urine from a commercial source as well as cell culture supernatants were spiked with recombinant calibrators and diluted 4-fold, 8-fold, 16-fold, and 32-fold before testing. Percent recovery at each dilution level was normalized to the dilution-adjusted 4-fold concentration. The average percent recovery shown below is based on samples within the quantitative range of the assay.

$$\%\ Recovery = \frac{measured\ concentration}{expected\ concentration} * 100$$

Table 7. Analyte percent recovery at various dilutions in serum, EDTA plasma, heparin plasma, citrate plasma, and urine samples

		IL-9		MCP-1		IL-33		IL-27p28/IL-30		IL-15	
Sample Type	Fold Dilution	Average % Recovery	%Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=6)	8	104	99 - 107	90	85 - 96	100	98 - 104	120	116 - 127	131	124 - 135
	16	100	96 - 104	91	85 - 100	102	100 - 105	122	115 - 129	152	140 - 166
	32	100	90 - 107	94	83 - 103	90	78 - 97	118	98 - 133	157	137 - 171
EDTA Plasma (N=6)	8	109	101 - 118	94	90 - 103	101	97 - 108	120	117 - 127	116	108 - 121
	16	104	93 - 114	86	79 - 94	100	93 - 106	119	113 - 130	125	105 - 137
	32	105	98 - 111	89	81 - 94	94	91 - 100	122	116 - 142	130	117 - 141
Citrate Plasma (N=6)	8	105	102 - 108	92	90 - 93	99	97 - 102	108	106 - 110	116	115 - 118
	16	104	99 - 108	91	87 - 94	96	92 - 97	103	97 - 109	125	118 - 132
	32	106	99 - 116	97	88 - 102	94	86 - 99	98	93 - 102	136	122 - 144
Heparin Plasma (N=6)	8	108	102 - 113	91	87 - 96	105	101 - 110	120	115 - 125	126	122 - 132
	16	110	101 - 116	92	86 - 97	110	106 - 114	129	123 - 136	150	141 - 156
	32	121	109 - 132	103	95 - 112	113	111 - 115	136	119 - 147	170	164 - 178
Urine (N=5)	8	102	96 - 106	95	88 - 109	93	89 - 97	105	99 - 123	89	82 - 106
	16	102	95 - 109	94	89 - 114	83	79 - 90	96	87 - 118	75	66 - 91
	32	103	98 - 108	95	87 - 109	71	62 - 82	88	80 - 109	66	59 - 79
Cell Culture Media (N=6)	8	103	100 - 104	90	85 - 92	90	87 - 94	99	96 - 103	91	86 - 95
	16	107	103 - 112	87	82 - 90	86	80 - 92	96	93 - 98	88	83 - 97
	32	110	102 - 119	96	89 - 105	82	78 - 85	95	89 - 100	86	80 - 92

Table 7 continued.

		IL-17A/F		MIP-1α		IP-10		MIP-2	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=6)	8	98	77 - 105	100	98 - 102	97	94 - 102	102	98 - 107
	16	99	95 - 107	97	95 - 100	93	88 - 96	101	97 - 107
	32	87	77 - 100	89	81 - 97	86	74 - 94	89	79 - 102
EDTA Plasma (N=6)	8	104	101 - 107	106	101 - 115	112	107 - 120	104	100 - 109
	16	98	88 - 104	102	96 - 111	115	103 - 124	102	96 - 109
	32	93	86 - 101	99	92 - 104	119	111 - 134	100	95 - 105
Heparin Plasma (N=6)	8	99	93 - 102	101	97 - 107	98	96 - 101	102	99 - 106
	16	94	92 - 96	98	94 - 102	93	91 - 96	100	97 - 102
	32	88	84 - 92	93	90 - 99	91	87 - 94	94	90 - 99
Citrate Plasma (N=6)	8	104	96 - 109	105	102 - 109	109	106 - 120	105	102 - 110
	16	103	96 - 109	105	99 - 109	106	102 - 112	103	97 - 109
	32	105	95 - 112	105	98 - 115	106	97 - 113	105	97 - 112
Urine (N=5)	8	96	91 - 100	98	96 - 99	116	94 - 144	101	97 - 104
	16	91	85 - 95	94	90 - 97	118	79 - 182	97	89 - 102
	32	79	77 - 81	87	83 - 92	108	71 - 175	89	86 - 94
Cell Culture Media (N=6)	8	99	96 - 104	99	95 - 102	100	94 - 108	99	93 - 103
	16	97	92 - 105	98	95 - 102	99	88 - 114	100	98 - 105
	32	95	88 - 103	96	94 - 97	93	83 - 105	97	94 - 102

Spike Recovery

Spike recovery measurements were evaluated for different sample types throughout the quantitative range of the assays. Multiple individual mouse samples (serum, EDTA plasma, heparin plasma, citrate plasma, and urine) were obtained from a commercial source. These samples, along with cell culture supernatants, were spiked with calibrators at three levels (high, mid, and low) then diluted 4-fold. The average % recovery for each sample type is reported along with the % recovery range.

$$\%\ Recovery = \frac{measured\ concentration}{expected\ concentration} * 100$$

Table 8. Analyte percent recovery at various dilutions in serum, EDTA plasma, heparin plasma, citrate plasma, and urine samples

	Serum (N=6)		EDTA Plasma (N=6)		Cell Culture Media (N=6)	
	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
IL-9	87	39 - 106	82	33 - 134	94	39 - 141
MCP-1	94	88 - 104	111	103 - 117	125	118 - 131
IL-33	64	48 - 84	65	51 - 72	110	98 - 121
IL-27p28/ IL-30	61	52 - 76	61	39 - 69	98	87 - 106
IL-15	55	48 - 70	62	49 - 69	115	103 - 127
IL-17A/F	91	80 - 104	85	71 - 94	105	95 - 111
MIP-1α	96	84 - 108	88	69 - 93	106	98 - 117
IP-10	106	98 - 112	76	71 - 82	88	75 - 99
MIP-2	97	88 - 106	86	69 - 97	104	97 - 111

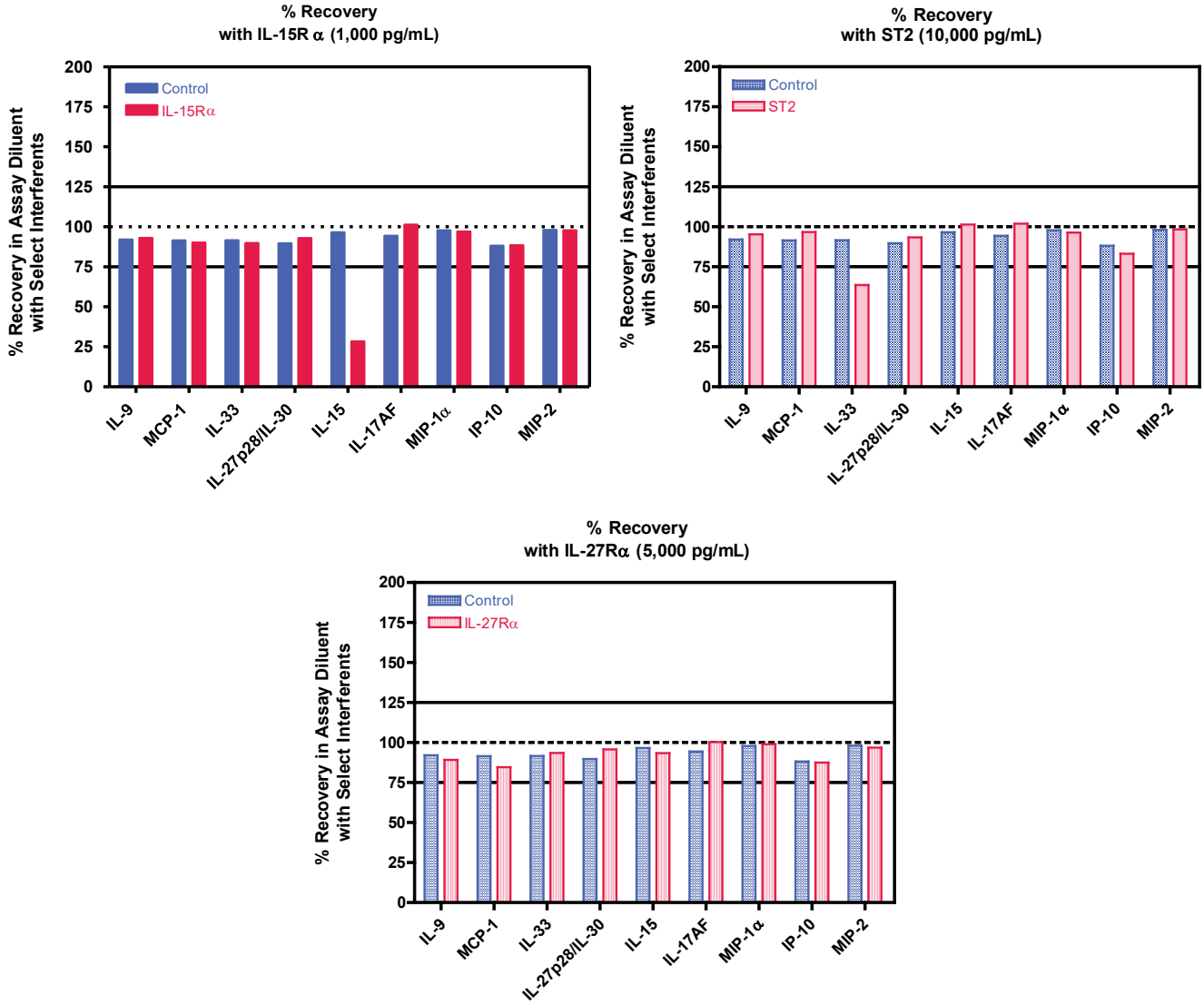
	Heparin Plasma (N=6)		Citrate Plasma (N=6)		Urine (N=5)	
	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
IL-9	105	57 - 139	98	64 - 114	109	73 - 146
MCP-1	110	104 - 120	111	104 - 115	109	90 - 124
IL-33	88	75 - 119	73	47 - 85	139	128 - 154
IL-27p28/ IL-30	76	64 - 99	57	53 - 64	88	71 - 100
IL-15	70	60 - 105	59	48 - 72	125	97 - 153
IL-17A/F	104	90 - 134	92	79 - 102	102	93 - 113
MIP-1α	110	98 - 149	95	88 - 105	96	90 - 104
IP-10	105	92 - 125	106	90 - 126	72	33 - 127
MIP-2	111	100 - 133	100	93 - 107	98	90 - 111

Specificity

To assess specificity, each assay antibody set in the panel was tested individually. Nonspecific binding was also evaluated with additional recombinant mouse analytes (IFN-γ, IL-10, IL-12p70, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-31, IL-21, IL-22, IL-17A, IL-17C, IL-17E/IL-25, IL-17F, IL-16, IL-12/IL-23p40, KC/GRO, MIP-3α, and TNF-α). Nonspecific binding was less than 0.5% for all assays in the kit. The IL-27p28/IL-30 assay measures both the heterodimer p28-EBI3 and the p28 subunit.

To evaluate interference from soluble receptors present in normal samples, IL-15, IL-33, and IL-27p28/IL-30 assays were examined in the presence of 1,000 pg/mL IL-15Rα, 10,000 pg/mL ST2, and 5,000 pg/mL IL-27Rα, respectively. Cross-reactivity was less than 0.5% for all assays, but the presence of IL-15Rα and ST2 did affect quantitation of their respective analytes.

Figure 5. Analyte percent recovery in assay diluent with select interferents.



Stability

The reconstituted calibrator, reconstituted controls, and diluents were tested for freeze–thaw stability. Results (not shown) demonstrated that reconstituted calibrator and reconstituted controls can be stored at 2-8 °C or frozen at ≤-70ºC. Refrigerated calibrator and controls should be used within 30 and 5 days, respectively. Frozen reconstituted calibrator and reconstituted controls can go through three freeze–thaw cycles without significantly affecting the performance of the assay. Diluent must be stored frozen and can go through one freeze-thaw cycle without affecting performance. Partially used MSD plates can be stored up to 30 days at 2–8 °C in the original foil pouch with desiccant. Results from control measurements changed by ≤30% after partially used plates were stored for 30 days. The validation study includes a real-time stability study with scheduled performance evaluations of complete kits for up to 54 months from date of manufacture.

Tested Samples

Normal Samples

Normal mouse serum, EDTA plasma, heparin plasma, citrate plasma, and urine samples from a commercial source were diluted 4-fold and tested. Results for each sample set are displayed below. Concentrations are corrected for sample dilution. Median and range are calculated from samples with concentrations at or above the LLOD. Percent Detected is the percentage of samples with concentrations at or above the LLOD.

Table 9. Normal mouse samples tested in the Cytokine Panel 1 (mouse) Kit

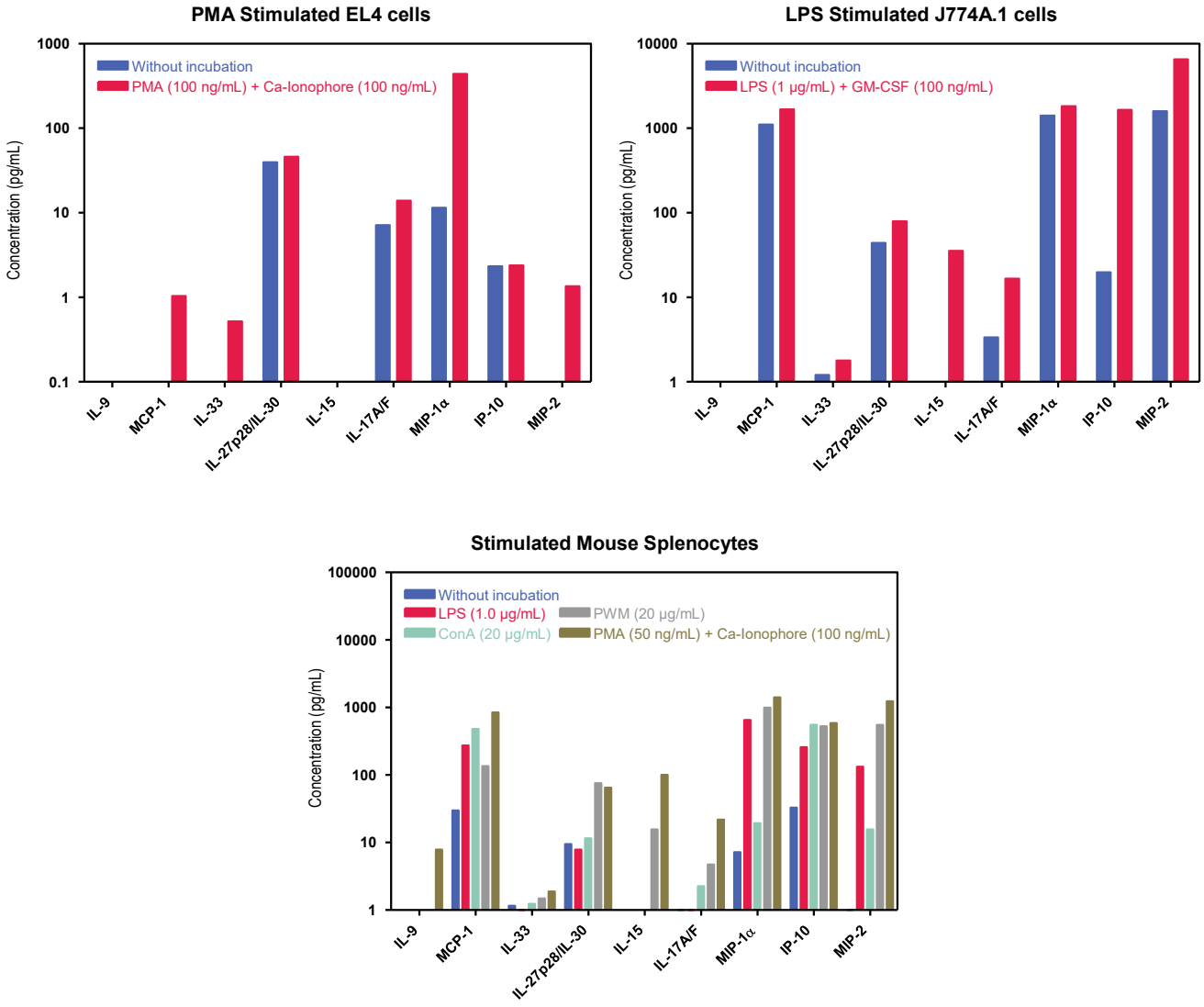
Sample Type	Statistic	IL-9	MCP-1	IL-33	IL27p28/IL-30	IL-15	IL-17A/F	MIP-1α	IP-10	MIP-2
Serum (N=10)	Median (pg/mL)	ND	7.71	0.168	2.48	3.58	0.133	0.387	26.0	2.52
	Range (pg/mL)	ND - 7.65	4.39 - 8.62	ND - 11.3	0.062 - 5.61	ND - 19.8	0.013 - 0.300	0.187- 1.23	14.8 - 34.2	1.31 - 2.95
	% Detected	10	100	40	70	20	30	100	100	100
EDTA Plasma (N=9)	Median (pg/mL)	ND	15.3	0.164	2.68	9.85	0.207	0.376	51.5	3.39
	Range (pg/mL)	ND	10.9 - 23.5	ND - 0.366	0.787 - 5.21	4.35 - 25.4	0.076 - 0.299	0.329 - 0.472	40.8 - 70.1	1.90 - 18.7
	% Detected	0	100	20	80	60	40	100	100	100
Heparin Plasma (N=10)	Median (pg/mL)	ND	8.58	0.286	3.43	14.7	0.259	0.634	52.4	1.16
	Range (pg/mL)	ND - 1.15	7.64 - 11.6	0.065 - 0.393	2.07 - 4.76	10.2 - 31.6	0.140 - 0.339	0.344 - 1.16	41.1 - 72.5	0.882 - 1.73
	% Detected	0	100	60	100	100	60	100	100	100
Citrate Plasma (N=10)	Median (pg/mL)	ND	7.82	0.119	1.08	11.4	0.135	0.199	31.6	1.17
	Range (pg/mL)	ND	6.69 - 9.98	ND - 0.280	0.362 - 1.86	2.93 - 20.6	0.026 - 0.217	0.151 - 0.243	17.1 - 39.2	0.767 - 1.26
	% Detected	0	100	10	10	90	0	100	100	100
Urine (N=5)	Median (pg/mL)	ND	1.81	0.344	ND	19.3	0.081	1.88	0.730	2.50
	Range (pg/mL)	ND	0.750 - 6.01	0.016 - 1.54	ND - 0.279	1.37 - 29.7	0.028 - 0.101	0.750 - 2.06	0.500 - 4.73	1.70 - 4.90
	% Detected	0	100	60	0	80	0	100	100	100
Cell Culture Supernatant (N=9)	Median (pg/mL)	1.95	273	1.21	43.9	5.52	4.71	652	257	132
	Range (pg/mL)	ND - 7.85	0.244 – 1,667	0.219 - 1.88	7.84 - 79.2	ND - 101	0.244 - 21.8	7.18 – 1,821	2.33 - 1652	0.099 – 6,543
	% Detected	11	89	89	100	33	100	100	100	89

% Detected = Percentage of samples with concentrations at or above the LLOD
ND = below LLOD

Stimulated Samples

Mouse model cell lines and mouse splenocytes were incubated at 37 °C with various stimulating compounds, including Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), Concanavalin A (ConA), and Pokeweed mitogen (PMW). The dilution-adjusted concentrations (pg/mL) for each stimulation model and its unstimulated control are displayed below.

Figure 6. Effect of cell stimulation on cytokine production as measured in the Cytokine Panel 1 (ms) Kit.



Assay Components

Calibrators

The assay calibrator blend uses the following recombinant mouse proteins:

Table 10. Recombinant mouse proteins used in the Calibrators

Calibrator	Expression System
IL-9	Insect cell line
MCP-1	*E. coli*
IL-33	*E. coli*
IL-27p28/IL-30	Mouse cell line
IL-15	*E. coli*
IL-17A/F	*E. coli*
MIP-1α	*E. coli*
IP-10	*E. coli*
MIP-2	*E. coli*

Antibodies

Table 11. Antibody source species

	Source Species		
Analyte	MSD Capture Antibody	MSD Detection Antibody	Assay Generation
IL-9	Mouse Monoclonal	Goat Polyclonal	A
MCP-1	Rat Monoclonal	Goat Polyclonal	A
IL-33	Goat Polyclonal	Goat Polyclonal	A
IL-27p28/IL-30	Goat Polyclonal	Goat Polyclonal	A
IL-15	Goat Polyclonal	Goat Polyclonal	A
IL-17A/F	Rat Monoclonal	Rat Monoclonal	A
MIP-1α	Goat Polyclonal	Goat Polyclonal	A
IP-10	Goat Polyclonal	Goat Polyclonal	A
MIP-2	Goat Polyclonal	Goat Polyclonal	A

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Appendix A

Calibration curves below illustrate the relative sensitivity for each assay under **Alternate Protocols**: Reference Protocol (2-hour sample incubation/2 wash steps, blue curve), in-well dilution (in-well sample dilution, red curve), O/N incubation (overnight sample incubation at 4 °C with shaking, green curve), and tissue culture protocol (tissue culture: single wash, gray curve).

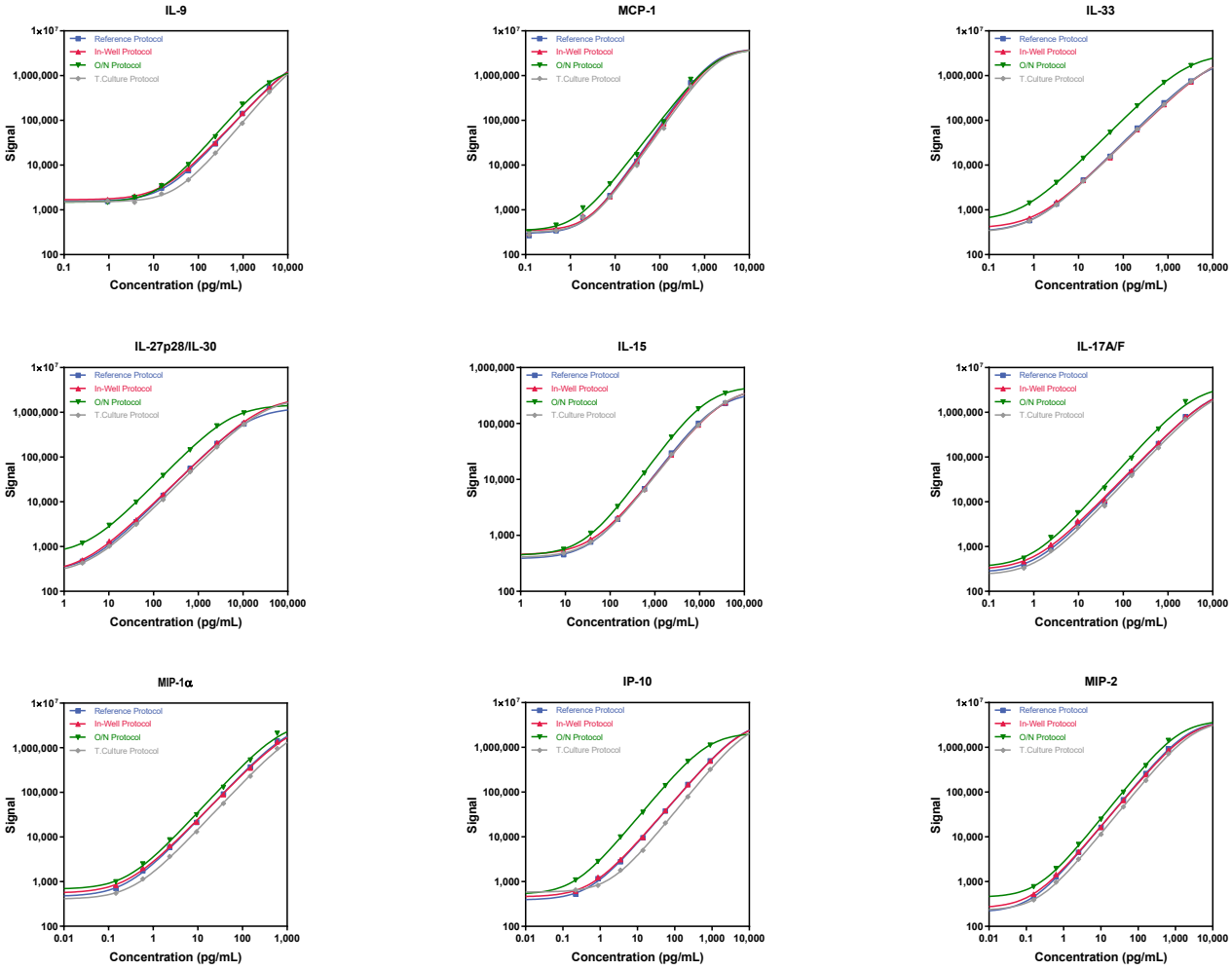

Table 12. Relative sensitivity when using alternate protocols

	LLOD Comparison (pg/mL)			
	Reference Protocol	In-Well Protocol	O/N Protocol	T. Culture Protocol
IL-9	9.03	4.00	3.91	15.8
MCP-1	0.756	0.751	0.442	0.772
IL-33	0.226	0.185	0.067	0.373
IL-27p28/IL-30	1.45	2.06	0.582	1.05
IL-15	8.09	6.52	20.6	10.9
IL-17A/F	0.265	0.203	0.231	0.338
MIP-1α	0.031	0.026	0.022	0.059
IP-10	0.270	0.152	0.186	6.63
MIP-2	0.044	0.034	0.430	0.059

Appendix B

The calibration curves below compare assay performance when the assay is run as an individual assay on a single spot plate (blue curve) vs. on the multiplex plate (red curve).

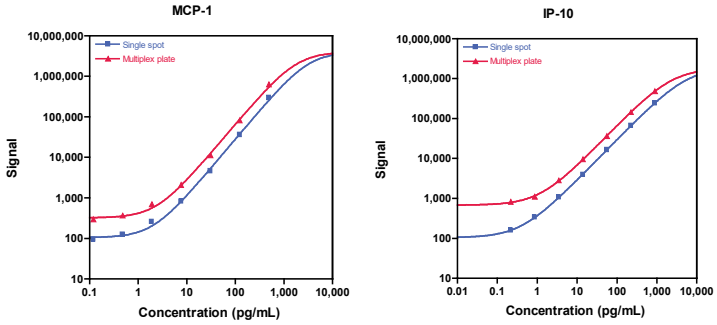

Table 13. Assay performance for individual and multiplex assays

	LLOD (pg/mL)	
Assay	Individual Assay	Multiplex
MCP-1	0.689	0.721
IP-10	0.097	2.099

In general, assays in the single spot format yielded a lower overall signal compared to the 10-plex format. The spots on single-spot plates have a larger binding surface than those on multiplex plates, but the same amount of calibrator was used for each test; therefore, the bound calibrator was spread over a larger surface area reducing the average signal.

Note: Assay performance for IL-9, IL-33, IL-27p28/IL-30, IL-15, IL-17A/F, MIP-1α, and MIP-2 is not included since the individual assay is run on a multiplex plate.

Appendix C

The calibration curves below compare results for each assay in the panel when the assays were run on the 10-spot plate using all 9 detection antibodies (red curve) vs. running each assay using a single, assay-specific detection antibody (blue curve).

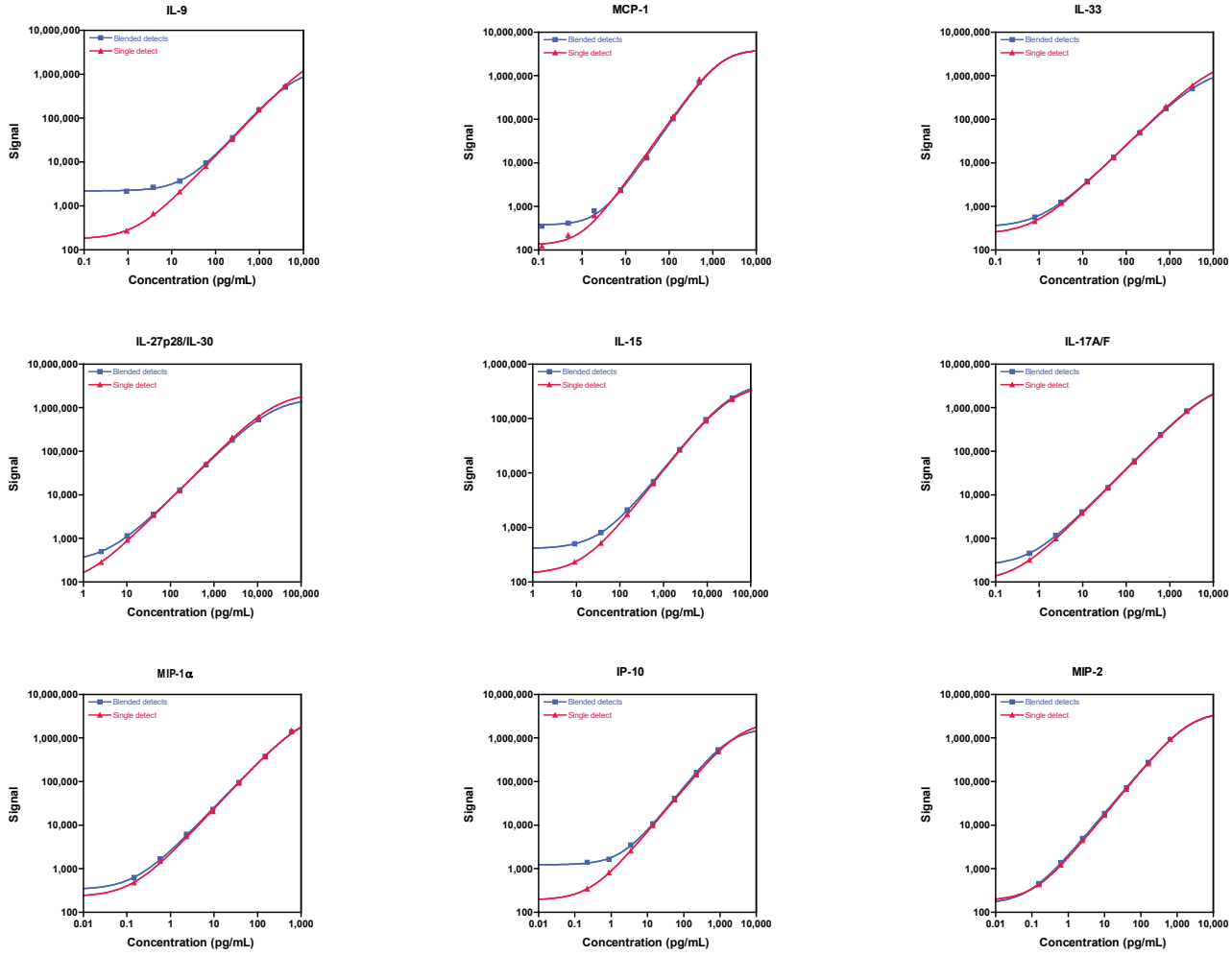

Table 14. LLODs for detection of a single antibody vs. blended antibodies

	LLOD (pg/mL)	
Assay	Blended Detect Ab	Single Detect Ab
IL-9	3.26	0.540
MCP-1	0.783	0.540
IL-33	0.374	0.223
IL-27p28/IL-30	4.22	0.942
IL-15	6.83	7.35
IL-17A/F	0.194	0.190
MIP-1α	0.032	0.033
IP-10	1.41	0.105
MIP-2	0.038	0.041

Summary Protocol

Cytokine Panel 1 (mouse) Kits

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the Cytokine Panel 1 (mouse) assays.

Sample and Reagent Preparation

- ❑ Bring all reagents to room temperature.
- ❑ Prepare calibration solutions in Diluent 41 using the supplied calibrator:
  - Reconstitute the lyophilized calibrator blend.
  - Invert 3 times, equilibrate 30-45 minutes at room temperature.
  - Vortex briefly using short pulses.
  - Perform a series of 4-fold dilution steps and prepare a zero calibrator.
- ❑ Dilute the samples and controls 4-fold in Diluent 41 before adding to the plate.
- ❑ Prepare combined detection antibody solution by diluting each 50X detection antibody 50-fold in Diluent 45.
- ❑ Prepare 2X Read Buffer T by diluting 4X Read Buffer T 2-fold with deionized water.

STEP 1: Wash* and Add Sample

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL/well of sample (calibrators, controls, or unknowns).
- ❑ Incubate at room temperature with shaking for 2 hours.

STEP 2: Wash and Add Detection Antibody Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 25 µL/well of 1X detection antibody solution.
- ❑ Incubate at room temperature with shaking for 2 hours.

STEP 3: Wash and Read Plate

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 150 µL/well of 2X Read Buffer T.
- ❑ Analyze the plate on the MSD instrument.

*Note: Washing the plate prior to sample addition is an optional step that may provide greater uniformity of results for certain assays. Analytical parameters including limits of quantification, recovery of controls, and sample quantification, are not affected by washing the plate prior to sample addition.

Catalog Numbers

Kit Name	V-PLEX			V-PLEX Plus*		
	1-Plate Kit	5-Plate kit	25-Plate Kit	1-Plate Kit	5-Plate Kit	25-Plate Kit
Multiplex Kits						
Cytokine Panel 1 (mouse)	K15245D-1	K15245D-2	K15245D-4	K15245G-1	K15245G-2	K15245G-4
Individual Assay Kits						
Mouse IL-9	K152XCD-1	K152XCD-2	K152XCD-4	K152XCG-1	K152XCG-2	K152XCG-4
Mouse MCP-1	K152NND-1	K152NND-2	K152NND-4	K152NNG-1	K152NNG-2	K152NNG-4
Mouse IL-3	K152XBD-1	K152XBD-2	K152XBD-4	K152XBG-1	K152XBG-2	K152XBG-4
Mouse IL27p28/IL-30	K152WXD-1	K152WXD-2	K152WXD-4	K152WXG-1	K152WXG-2	K152WXG-4
Mouse IL-15	K152RDD-1	K152RDD-2	K152RDD-4	K152RDG-1	K152RDG-2	K152RDG-4
Mouse IL-17A/F	K152WND-1	K152WND-2	K152WND-4	K152WNG-1	K152WNG-2	K152WNG-4
Mouse MIP-1α	K152NQD-1	K152NQD-2	K152NQD-4	K152NQG-1	K152NQG-2	K152NQG-4
Mouse IP-10	K152NVD-1	K152NVD-2	K152NVD-4	K152NVG-1	K152NVG-2	K152NVG-4
Mouse MIP-2	K152QCD-1	K152QCD-2	K152QCD-4	K152QCG-1	K152QCG-2	K152QCG-4

*V-PLEX Plus kits include controls, plate seals, and wash buffer. See **Kit Components** for details.

Plate Diagram