Chemokine Panel 1 (human) Kits

Eotaxin, MIP-1β, Eotaxin-3, TARC, IP-10, MIP-1α, IL-8, MCP-1, MDC, MCP-4

V-PLEX®

	V-PLEX®	V-PLEX Plus
Multiplex Kits	K15047D	K15047G
Individual Assay Kits		
Human Eotaxin	K151NSD	K151NSG
Human MIP-1β	K151NRD	K151NRG
Human Eotaxin-3	K151NUD	K151NUG
Human TARC	K151NTD	K151NTG
Human IP-10	K151NVD	K151NVG
Human MIP-1α	K151NQD	K151NQG
Human IL-8	K151RAD	K151RAG
Human MCP-1	K151NND	K151NNG
Human MDC	K151NPD	K151NPG
Human MCP-4	K151NOD	K151NOG

MSD®

MSD Cytokine Assays

Chemokine Panel 1 (human) Kits

Eotaxin, MIP-1β, Eotaxin-3, TARC, IP-10, MIP-1α, IL-8, MCP-1, MDC, MCP-4

For use with cell culture supernatants, serum, plasma, cerebral spinal fluid, 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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Table of Contents

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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 principles[8] 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.

Chemokines are small chemotactic cytokines with molecular weights around 8–10 kDa that are capable of inducing directed chemotaxis. Four cysteine residues in conserved locations result in a compact 3-dimensional structure.[1] Based on the spacing of the first two cysteine residues, chemokines are divided into four families: CC chemokines, CXC chemokines, C chemokines, and CX3C chemokines, where C represents cysteine and X represents any other amino acid.[2] Chemokines function by activating specific G protein-coupled receptors resulting in the migration of inflammatory and non-inflammatory cells.[3] The pro-inflammatory chemokines are responsible for the migration of immune cells to the infection site,[4] while the homeostatic chemokines are responsible for recruiting cells for tissue maintenance and development.[5] Chemokines are associated with a number of diseases.[6,7] As a result of their association with a wide spectrum of diseases, these biomarkers are the subjects of drug discovery projects, diagnostics development, and basic research. The Chemokine Panel 1 (human) Kit consists of eight CC chemokine assays (Eotaxin, MIP-1β, Eotaxin-3, TARC, MIP-1α, MCP-1, MDC, MCP-4) and two CXC chemokine assays (IP-10 and IL-8).

Note: The detection antibody used in the human MCP-4 assay has been replaced with a new version of the antibody, resulting in improved performance and greater consistency than the previous generation.

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 Chemokine Panel 1 (human) are sandwich immunoassays. MSD provides a plate pre-coated with capture antibodies on independent and well-defined spots, as shown in the layouts below. Multiplex assays and the individual Eotaxin, MDC, and MCP-4 assays are provided on 10-spot MULTI-SPOT plates (Figure 1); the individual MIP-1β, Eotaxin-3, TARC, IP-10, MIP-1α, IL-8, and MCP-1 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 GOLD™ SULFO-TAG) throughout 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.[8]

1. Eotaxin
2. MIP-1β
3. Eotaxin-3
4. TARC
5. IP-10
6. MIP-1α
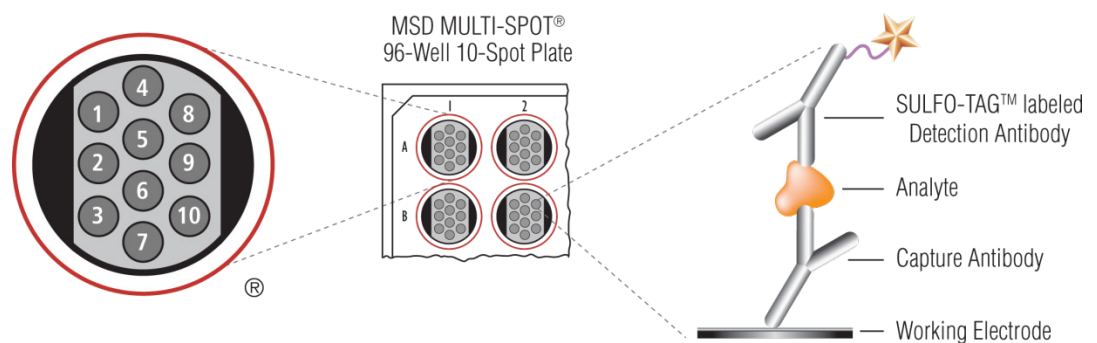7. IL-8
8. MCP-1
9. MDC
10. MCP-4

Figure 1. Multiplex plate spot diagram showing the 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.
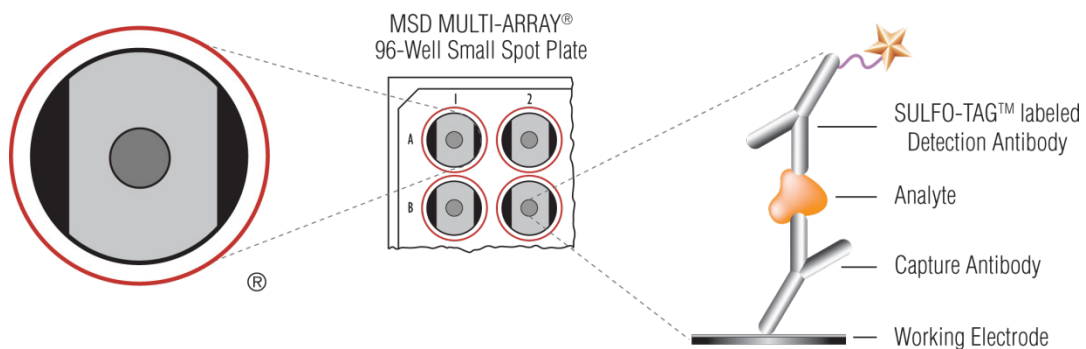

Figure 2. Small Spot plate diagram showing the placement of analyte capture antibodies.

Kit Components

Chemokine Panel 1 (human) assays are available as a 10-spot multiplex kit, 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 No. | Size | Quantity Supplied | | | Description |
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Chemokine Panel 1 (human) Calibrator Blend	2–8 °C	C0047-2	1 vial	1 vial	5 vials	25 vials	Ten recombinant human proteins in diluent, buffered and lyophilized. Individual analyte concentration is provided in the lot-specific certificate of analysis (COA).
Diluent 43	≤-10 °C	R50AG-1	10 mL	1 bottle	—	—	Diluent for samples and calibrator; contains protein, blockers, and preservatives.
		R50AG-2	50 mL	—	1 bottle	5 bottles	
Diluent 3	≤-10 °C	R51BA-4	5 mL	1 bottle	—	—	Diluent for detection antibody; contains protein, blockers, and preservatives.
		R51BA-5	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 electro-chemiluminescence reaction.

RT = room temperature
Dash (—) = not applicable or not available

V-PLEX Plus Kits: Additional Components

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

| Reagents | Storage | Catalog No. | Size | Quantity Supplied | | | Description |
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Chemokine Panel 1 (human) Control 1*	2–8 °C	C4047-1	1 vial	1 vial	5 vials	25 vials	Multi-analyte controls in a non-human matrix, buffered, lyophilized, and spiked with recombinant human analytes. The concentration of the controls is provided in the lot-specific COA.
Chemokine Panel 1 (human) Control 2*	2–8 °C	C4047-1	1 vial	1 vial	5 vials	25 vials	
Chemokine Panel 1 (human) Control 3*	2–8 °C	C4047-1	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 Chemokine Panel 1 (human) Control Pack
RT = room temperature
Dash (—) = not available or not applicable

Kit-Specific Components

Table 3. *Components that are supplied with specific kits*

Plates	Storage	Part No.	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Chemokine Panel 1 (human) Plate	2–8 °C	N05047A-1	10-spot	1	5	25	96-well plate, foil sealed, with desiccant.
Human MIP-1β Plate	2–8 °C	L451NRA-1	Small Spot	1	5	25	
Human Eotaxin-3 Plate	2–8 °C	L451NUA-1	Small Spot	1	5	25	
Human TARC Plate	2–8 °C	L451NTA-1	Small Spot	1	5	25	
Human IP-10 Plate	2–8 °C	L451NVA-1	Small Spot	1	5	25	
Human MIP-1α Plate	2–8 °C	L451NQA-1	Small Spot	1	5	25	
Human IL-8 Plate	2–8 °C	L451RAA-1	Small Spot	1	5	25	
Human MCP-1 Plate	2–8 °C	L451NNA-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 No.	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Anti-hu Eotaxin Antibody (50X)	2–8 °C	D21NS-2	75 µL	1	—	—	SULFO-TAG™ conjugated antibody.
		D21NS-3	375 µL	—	1	5	
Anti-hu MIP-1β Antibody (50X)	2–8 °C	D21NR-2	75 µL	1	—	—	SULFO-TAG conjugated antibody.
		D21NR-3	375 µL	—	1	5	
Anti-hu Eotaxin-3 Antibody (50X)	2–8 °C	D21NU-2	75 µL	1	—	—	SULFO-TAG conjugated antibody.
		D21NU-3	375 µL	—	1	5	
Anti-hu TARC Antibody (50X)	2–8 °C	D21NT-2	75 µL	1	—	—	SULFO-TAG conjugated antibody.
		D21NT-3	375 µL	—	1	5	
Anti-hu IP-10 Antibody (50X)	2–8 °C	D21NV-2	75 µL	1	—	—	SULFO-TAG conjugated antibody.
		D21NV-3	375 µL	—	1	5	
Anti-hu MIP-1α Antibody (50X)	2–8 °C	D21NQ-2	75 µL	1	—	—	SULFO-TAG conjugated antibody.
		D21NQ-3	375 µL	—	1	5	
Anti-hu IL-8 (HA) Antibody (50X)*	2–8 °C	D21RO-2	75 µL	1	—	—	SULFO-TAG conjugated antibody.
		D21RO-3	375 µL	—	1	5	
Anti-hu IL-8 Antibody (50X)*	2–8 °C	D21AN-2	75 µL	1	—	—	SULFO-TAG conjugated antibody.
		D21AN-3	375 µL	—	1	5	
Anti-hu MCP-1 Antibody (50X)	2–8 °C	D21NN-2	75 µL	1	—	—	SULFO-TAG conjugated antibody.
		D21NN-3	375 µL	—	1	5	
Anti-hu MDC Antibody (50X)	2–8 °C	D21NP-2	75 µL	1	—	—	SULFO-TAG conjugated antibody.
		D21NP-3	375 µL	—	1	5	
Anti-hu MCP-4 Antibody (50X)	2–8 °C	D21AJE-2	75 µL	1	—	—	SULFO-TAG conjugated antibody.
		D21AJE-3	375 µL	—	1	5	

*Two detection antibodies for IL-8 are provided. The anti-hu IL-8 (HA) antibody (D21RO-2 or D21RO-3) has been validated and is recommended when high IL-8 levels are anticipated. Data reported in the product insert were obtained using the anti-hu IL-8 (HA) antibody. The anti-hu IL-8 antibody (D21AN-2 or D21AN-3) may be used in place of the anti-hu IL-8 (HA) antibody to reduce the lower limit of quantitation (LLOQ) (i.e., to increase sensitivity). When anti-hu IL-8 antibody is used, verification of performance to specific applications is recommended.
Dash (—) = not available or not applicable

Additional Materials and Equipment

- ❑ Appropriately sized tubes for reagent preparation

- ❑ Polypropylene microcentrifuge tubes for preparing dilutions

- ❑ Liquid handling equipment for the 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 no. 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

- ❑ Chemokine Panel 1 (human) Control Pack, available for separate purchase from MSD, catalog no. C4047-1 (included in V-PLEX Plus kit)

- ❑ Centrifuge for sample preparation

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 applicable safety data sheet(s) (SDS), which can be obtained from MSD Customer Service or at www.mesoscale.com®.

Best Practices

- Mixing or substituting reagents from different sources or different kit lots is not recommended. 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 diluents to room temperature in a 20–26 °C water bath prior to use. If a controlled water bath is not available, thaw at room temperature. Diluents may also be thawed overnight at 2-8 °C. Thaw other reagents on wet ice and use it as directed without delay.

- Prepare calibrators, samples, and controls in polypropylene microcentrifuge tubes. Use a fresh pipette tip for each dilution and mix by vortexing after each dilution.

- 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 during all pipetting steps as they may lead to variable results. Bubbles introduced when adding read buffer may interfere with signal detection.

- Plate shaking should be vigorous with a rotary motion between 500–1,000 rpm. Binding reactions may reach equilibrium sooner if shaken in the middle of this range (~700 rpm) or above.

- Use reverse pipetting when necessary to avoid the introduction of bubbles. For empty wells, pipette gently to the bottom corner. Do not touch the pipette tip to the bottom of the wells when pipetting into the MSD plate.

- 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 before reading the plate.

- Read buffer should be at room temperature (20-26 ºC) before adding it to the plate.

- Do not shake the plate after adding read buffer.

- Keep time intervals consistent between addition of read buffer and reading the plate to improve inter-plate precision. It is recommended that a MSD instrument be prepared to read a plate before adding Read Buffer. Unless otherwise directed, read the plate as soon as possible after adding read buffer.

- If the sample results are above the top of the calibration curve, dilute the samples, and repeat the assay.

- The anti-hu IL-8 detection antibody (D21AN-2 or D21AN-3) may be substituted for the recommended anti-hu IL-8 (HA) detection antibody when a reduced lower limit of quantitation (LLOQ) is desired. Since the IL-8 assay was validated using the anti-hu IL-8 (HA) antibody, when anti-hu IL-8 detection antibody is used, verification of assay performance for specific applications is recommended.

Reagent Preparation

Bring all reagents to room temperature.

Important: Upon the first thaw, aliquot Diluent 43 and Diluent 3 into suitable volumes before refreezing. After thawing Diluent 43, you may see precipitate in the solution. Mix or vortex the diluent and proceed with the assay. Any remaining precipitate will not compromise assay performance.

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 43. (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. Follow the steps below using 250 µL instead of 1000 µL of Diluent 43 when reconstituting the lyophilized calibrator.

To prepare 7 calibrator solutions plus a zero calibrator for up to 4 replicates (Figure 3):

1) Prepare the most concentrated calibrator (Calibrator 1) by adding 1,000 µL of Diluent 43 to the lyophilized calibrator vial. After reconstituting, invert at least 3 times (do not vortex). Let the reconstituted solution equilibrate at room temperature for 15-30 minutes and then vortex briefly using short pulses.

2) Prepare the next calibrator by transferring 100 µL of Calibrator 1 to 300 µL of Diluent 43. Mix well by vortexing. Repeat 4-fold serial dilutions 5 additional times to generate 7 calibrators.

3) Use Diluent 43 as the zero calibrator.

Note: Reconstituted calibrator is stable for one day at 2–8 °C. It may also be stored frozen at ≤-70 °C and is stable through three freeze-thaw cycles. 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.
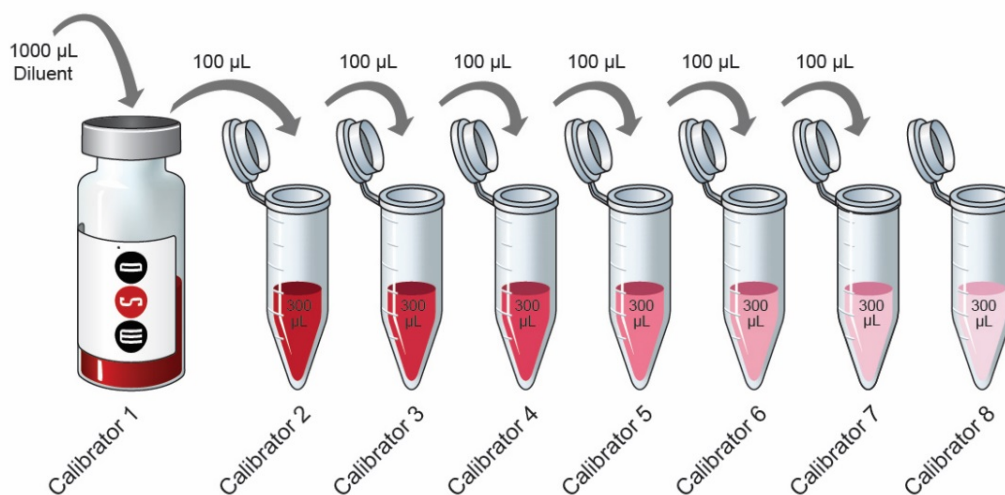

Figure 3. Dilution schema for preparation of Calibrator Standards.

Sample Collection and Handling

Below are general guidelines for human sample collection, storage, and handling. If possible, use published guidelines.[9-13] 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 before 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 ≤-20 °C until needed. Repeated freeze-thaw of samples is not recommended. After thawing, centrifuge samples at 2,000g for 3 minutes to remove particulates before sample preparation.

Dilute Samples

Dilute samples with Diluent 43. For human serum, plasma, and urine samples, MSD recommends a minimum 4-fold dilution. For example, when running samples in duplicate, add 50 μL of sample to 150 μL of Diluent 43. We recommend running at least two replicates per sample. When running unreplicated samples, add 25 μL of sample to 75 μL of Diluent 43. 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. The kit includes diluent sufficient enough for running samples in duplicates. 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 Chemokine Panel 1 (human) Control Pack, catalog no. C4047-1. (Controls are included only in V-PLEX Plus kits.)

Reconstitute the lyophilized controls in 250 μL of Diluent 43. Do not invert or vortex the vials. Wait for a minimum of 15–30 minutes at room temperature before diluting controls 4-fold in Diluent 43. Vortex briefly using short pulses. Refer to the Chemokine Panel 1 (human) Control Pack product insert for analyte levels. Reconstituted controls must be stored frozen. They are stable through three freeze-thaw cycles.

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 before use.

For one plate, combine the following detection antibodies and add to 2,400 μL of Diluent 3:

- ☐ 60 μL of SULFO-TAG Anti-hu Eotaxin Antibody
- ☐ 60 μL of SULFO-TAG Anti-hu MIP-1β Antibody
- ☐ 60 μL of SULFO-TAG Anti-hu Eotaxin-3 Antibody
- ☐ 60 μL of SULFO-TAG Anti-hu TARC Antibody
- ☐ 60 μL of SULFO-TAG Anti-hu IP-10 Antibody
- ☐ 60 μL of SULFO-TAG Anti-hu MIP-1α Antibody
- ☐ 60 μL of SULFO-TAG Anti-hu IL-8 (HA) Antibody*
- ☐ 60 μL of SULFO-TAG Anti-hu MCP-1 Antibody
- ☐ 60 μL of SULFO-TAG Anti-hu MDC Antibody
- ☐ 60 μL of SULFO-TAG Anti-hu MCP-4 Antibody

*For each assay, please select either the recommended anti-hu IL-8 (HA) antibody or the alternative anti-hu IL-8 antibody. Do not combine the anti-IL-8 antibodies. Data and specifications reported in this product insert were obtained using the anti-hu IL-8 (HA) antibody. If high sensitivity is desired, MSD recommends that testing be done to verify the suitability of the anti-hu IL-8 for specific applications.

Spot the Difference®

Custom multiplex kits

For one plate, combine 60 μL of each supplied detection antibody, then add Diluent 3 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 3.

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 T

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 T in a tightly sealed container at room temperature for up to one month.

Prepare MSD Plate

MSD 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. Plates may be used as delivered; no additional preparation is required.

Assay 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 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 before 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 before sample addition.

STEP 2: Wash and Add Detection Antibody Solution

❑ Wash the plate 3 times with at least 150 µL/well of 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 Wash Buffer.

❑ Add 150 µL of 2X Read Buffer T to each well and incubate at room temperature for 10 minutes. Analyze the plate on an MSD instrument.

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 °C 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).

- **Alternative Protocol 4, Higher Sensitivity for IL-8:** To achieve higher sensitivity for IL-8, the recommended anti-hu IL-8 (HA) detection antibody may be replaced with the anti-hu IL-8 detection antibody, which is boxed separately. The use of the alternative anti-hu IL-8 antibody, however, was not tested during the validation process.

Validation

V-PLEX products are validated according to fit-for-purpose principles[53] 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.

> ➢ **Dynamic Range**
>
> 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 this 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 the 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 (25% for Eotaxin-3). Precision is reported as the coefficient of variation (CV). Intra-run CVs are typically below 7%, 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 10%. Validation lots are compared using controls and at least 40 samples in various sample matrices. Samples are well correlated with an inter-lot bias typically below 10%.

> ➢ **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 rather than pooled samples to assess the 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 the 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 the 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 30 days. 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 the date of manufacture.

Representative data from the validation studies are presented in the following sections. All data were obtained using the recommended anti-hu IL-8 (HA) antibody. The use of the alternative anti-hu IL-8 antibody was not tested during the validation process. 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.

The 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 Chemokine Panel 1 (human) were collected over four months of testing by five operators (34 runs in total). Calibration curve accuracy and precision were assessed for three kit lots. Representative data from one lot are presented below (Figure 4). 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 ten detection antibodies. The calibration curves were comparable. Calibration curves for each lot are presented in the lot-specific COA.
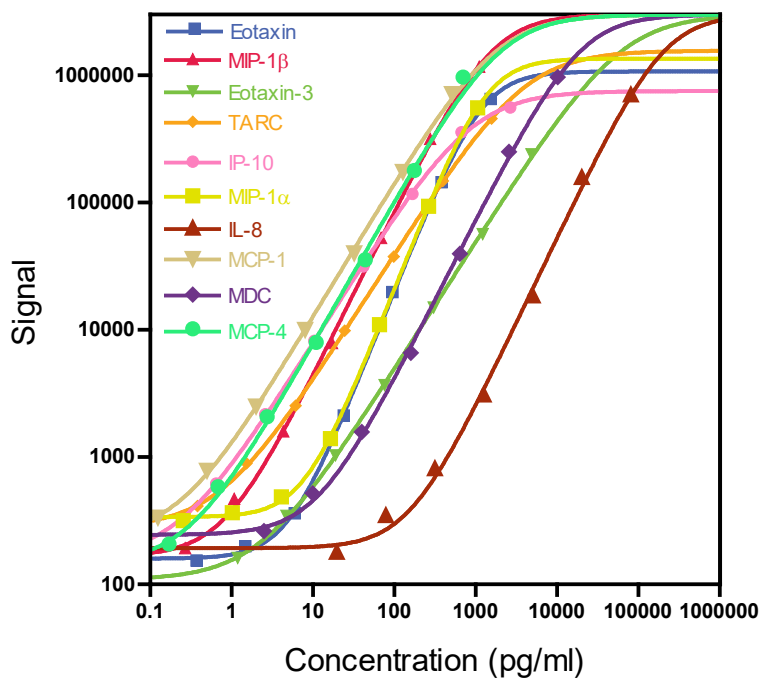
Figure 4. *Typical calibration curves for the Chemokine Panel 1 (human) assay.*

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 34 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 (75% to 125% for IL-8*, TARC, and MDC).

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 (75% to 125% for IL-8*).

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 Chemokine Panel 1 (human) Kit

	Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
Eotaxin	3.26	2.41–5.12	12.3	1,120
MIP-1β	0.17	0.08–0.32	1.88	520
Eotaxin-3	1.77	1.29–4.13	10.2	3,750
TARC	0.22	0.17–0.54	3.32	1,120
IP-10	0.37	0.22–0.72	1.37	500
MIP-1α	3.02	2.28–4.01	13.8	743
IL-8*	95.6	35.2–238	713	43,400
MCP-1	0.09	0.06–0.31	1.09	375
MDC	1.22	1.14–1.26	88.3	3,700
MCP-4	0.18	0.15–0.20	1.49	472

*Due to IL-8's high abundance in some sample types, Chemokine Panel 1 (human) uses a low-sensitivity IL-8 assay, which exhibits more variability compared to the highly sensitive assays in this panel. Assay sensitivity may be increased by replacing the recommended anti-hu IL-8 (HA) detection antibody (D21RO-2 or D21RO-3) with the high anti-hu IL-8 detection antibody (D21AN-2 or D21AN-3). However, use of the anti-hu IL-8 detection antibody was not validated.

Precision

Controls were made by spiking calibrator into a non-human matrix at three levels within the quantitative range of the assay. Analyte levels were measured by five operators using a minimum of three replicates on 49 runs over five months. The results are shown below. While a typical specification for precision is a concentration CV of less than 25% for controls on both intra- and inter-day runs, for this panel, the data shows 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 25 runs.

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

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

	Control	Average Conc. (pg/mL)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
Eotaxin	Control 1	1,084	6.7	6.0	6.4
	Control 2	286	3.4	1.9	8.5
	Control 3	65	6.9	4.9	9.4
MIP-1β	Control 1	700	12.8	8.1	11.9
	Control 2	170	4.3	2.4	10.0
	Control 3	35	6.8	3.2	10.6
Eotaxin-3	Control 1	3,796	10.7	8.8	10.8
	Control 2	958	7.1	3.8	12.9
	Control 3	221	8.2	5.0	12.0
TARC	Control 1	993	8.4	6.3	9.9
	Control 2	257	6.9	3.0	9.8
	Control 3	59	7.9	4.6	10.3
IP-10	Control 1	1,436	10.3	8.9	10.1
	Control 2	348	6.8	3.4	9.4
	Control 3	86	10.9	5.3	12.4
MIP-1α	Control 1	749	8.7	6.2	9.0
	Control 2	181	4.8	1.7	10.4
	Control 3	47	6.3	3.2	10.0
IL-8*	Control 1	152,116	6.7	8.6	7.9
	Control 2	53,673	11.5	3.1	10.8
MCP-1	Control 1	403	11.2	8.9	10.5
	Control 2	95	6.2	3.6	10.4
	Control 3	24	6.6	5.0	10.4
MDC	Control 1	6,498	9.5	6.9	11.0
	Control 2	1,338	4.4	2.4	10.8
	Control 3	326	7.3	3.3	11.7
MCP-4	Control 1	713	4.7	4.9	5.3
	Control 2	226	7.0	5.9	5.6
	Control 3	66	8.0	5.4	5.4

*Because of IL-8's high abundance in some sample types, this panel uses a low-sensitivity IL-8 assay; therefore, only two controls are provided.

Dilution Linearity

To assess linearity, normal human 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 2-fold, 4-fold, 8-fold, 16-fold, 32-fold, and 64-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 each sample type*

Sample Type	Fold Dilution	Eotaxin		MIP-1β		Eotaxin-3		TARC		IP-10	
		Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=12)	2	88	73–106	97	55–117	113	88–134	92	80–108	118	107–130
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	104	100–112	105	91–150	93	86–106	94	89–102	89	80–95
	16	106	95–124	113	87–192	90	74–105	94	83–108	84	76–93
	32	110	85–145	118	87–226	96	73–119	92	83–111	81	71–90
	64	111	81–146	120	81–245	100	73–128	98	81–126	84	72–95
EDTA Plasma (N=12)	2	91	84–102	93	59–107	131	95–163	94	79–109	117	104–149
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	104	86–115	108	99–143	78	61–96	95	85–106	89	81–96
	16	106	92–119	114	96–177	72	59–93	95	78–112	86	73–99
	32	105	87–135	123	96–207	73	60–103	88	72–127	86	71–100
	64	105	85–151	122	91–220	77	64–115	92	76–131	91	75–106
Heparin Plasma (N=12)	2	89	75–119	94	61–109	112	97–143	75	61–103	112	98–124
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	108	101–119	107	96–138	89	78–100	110	95–121	89	80–98
	16	120	80–135	111	93–173	89	71–104	118	67–135	86	75–97
	32	135	86–157	117	95–197	92	60–125	120	80–139	84	69–101
	64	145	87–170	113	90–202	101	66–138	126	80–170	95	75–110
Citrate Plasma (N=10)	2	95	85–104	106	99–117	122	111–137	97	89–141	131	98–169
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	102	98–105	99	91–105	86	74–98	85	60–102	88	72–97
	16	102	96–110	95	89–101	78	66–87	78	48–97	82	68–95
	32	99	88–110	95	86–106	79	64–90	72	44–86	79	62–89
	64	98	83–118	93	82–110	87	71–100	74	43–89	84	65–101
Urine (N=5)	2	96	78–116	88	63–107	106	97–119	92	78–110	93	75–102
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	106	102–110	105	101–109	97	92–102	94	87–108	97	93–106
	16	114	108–116	109	103–114	94	87–99	92	85–110	97	87–109
Cell Culture Supernatant (N=6)	2	134	117–141	110	98–116	93	88–97	93	84–101	179	127–256
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	95	91–100	95	91–100	100	96–102	91	87–98	73	55–84
	16	95	88–99	94	90–99	108	101–114	87	80–94	68	60–88
	32	95	89–101	91	87–99	112	105–118	82	75–89	63	52–77
	64	99	88–107	95	89–102	126	116–133	90	76–99	68	57–81

NA = not available or not applicable

Table 7. continued

Sample Type	Fold Dilution	MIP-1α Average % Recovery	MIP-1α % Recovery Range	IL-8 Average % Recovery	IL-8 % Recovery Range	MCP-1 Average % Recovery	MCP-1 % Recovery Range	MDC Average % Recovery	MDC % Recovery Range	MCP-4* Average % Recovery	MCP-4* % Recovery Range
Serum (N=12)	2	105	91–115	91	82–104	92	81–97	109	101–121	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	97	89–104	102	85–108	98	92–107	89	81–96	97	94–101
	16	93	81–106	98	77–112	94	86–100	79	66–87	95	86–100
	32	93	78–118	112	88–135	92	82–102	72	57–83	N/A	N/A
	64	92	71–119	128	97–162	98	82–116	69	48–81	N/A	N/A
EDTA Plasma (N=12)	2	103	73–112	93	84–108	99	93–107	106	91–118	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	99	90–111	90	79–100	92	83–97	93	83–101	112	109–114
	16	97	88–113	87	75–100	88	78–96	84	69–94	115	112–117
	32	98	86–116	98	83–109	88	77–98	79	65–89	N/A	N/A
	64	97	80–119	112	94–127	95	83–108	76	64–87	N/A	N/A
Heparin Plasma (N=12)	2	107	101–116	97	87–123	96	82–106	106	98–118	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	95	90–100	91	76–113	96	87–105	90	78–99	103	103–104
	16	92	80–103	82	66–99	92	81–106	78	62–89	110	109–111
	32	90	76–107	88	65–116	90	79–105	73	57–83	N/A	N/A
	64	88	71–109	100	75–140	94	76–112	70	57–82	N/A	N/A
Citrate Plasma (N=10)	2	109	102–115	98	90–109	99	90–105	105	77–130	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	94	88–105	88	79–100	91	83–101	88	69–94	117	110–122
	16	87	78–108	79	73–90	87	82–95	74	57–85	125	113–135
	32	83	74–111	87	79–102	83	74–89	68	55–75	N/A	N/A
	64	79	70–108	97	87–114	86	78–94	64	52–69	N/A	N/A
Urine (N=5)	2	111	104–115	117	107–126	95	91–101	92	71–112	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	96	95–99	87	78–94	98	95–102	86	61–102	75	73–78
	16	92	89–97	77	68–85	96	91–103	75	54–89	66	64–70
Cell Culture Supernatant (N=6)	2	120	104–128	89	84–94	98	93–108	191	158–211	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	90	85–94	96	89–113	95	89–98	75	73–76	106	N/A
	16	85	78–94	89	83–95	94	89–101	63	61–65	116	N/A
	32	80	72–89	93	82–97	88	81–91	56	54–59	N/A	N/A
	64	81	73–95	107	95–121	98	90–103	55	52–57	N/A	N/A

N/A = not available or not applicable
*MCP-4 was tested using N=3 for serum, EDTA plasma, heparin plasma, citrate plasma and urine, and N=1 for cell culture supernatant

Spike Recovery

Spike recovery measurements of different sample types across the quantitative range of the assays were evaluated. Multiple individual human 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 %CV and % recovery range.

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

Table 8. Spike and Recovery measurements of different sample types in the Chemokine Panel 1 (human) Kit

	Citrate Plasma (N=10)			Heparin Plasma (N=11)			EDTA Plasma (N=11)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
Eotaxin	98	11.2	75–119	93	19.0	64–126	105	8.0	95–129
MIP-1β	106	6.7	92–121	100	16.6	50–117	103	15.1	56–120
Eotaxin-3	109	9.7	93–130	113	15.9	85–157	129	9.2	102–150
TARC	112	20.9	94–226	94	23.1	44–140	102	12.1	69–127
IP-10	105	11.1	87–139	107	11.3	77–138	111	8.7	91–134
MIP-1α	109	15.8	65–141	104	12.8	77–130	109	9.8	85–125
IL-8	98	8.7	78–115	88	8.7	71–102	97	9.9	74–115
MCP-1	93	9.0	81–110	103	10.1	80–130	88	7.7	72–102
MDC	111	10.8	93–151	108	8.2	92–130	109	8.9	91–128
MCP-4*	51	8.0	48–56	63	5.0	57–62	60	5.0	57–62

*MCP-4 was tested using N=3 for citrate plasma, heparin plasma and EDTA plasma

	Serum (N=11)			Urine (N=5)			Cell Culture Supernatants (N=6)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
Eotaxin	105	13.8	79–135	106	12.1	75–125	124	8.2	104–145
MIP-1β	103	17.5	47–127	107	8.4	89–121	107	6.8	90–122
Eotaxin-3	136	20.3	78–164	102	9.3	89–119	95	5.8	84–108
TARC	102	16.9	71–153	119	17.8	83–162	121	5.6	110–135
IP-10	111	10.3	79–131	99	15.8	55–124	147	35.2	97–267
MIP-1α	109	10.1	88–125	117	6.6	104–129	116	5.7	100–127
IL-8	97	7.1	76–110	108	4.9	101–119	97	10.0	71–109
MCP-1	88	6.8	77–109	104	7.9	90–117	104	4.9	96–119
MDC	109	12.7	86–129	126	10.9	96–140	140	7.5	122–159
MCP-4*	92	19	82–112	123	11.0	108–133	71	N/A	N/A

N/A = not available or not applicable
*MCP-4 was tested using N=3 for serum and urine, and N=1 for cell culture supernatant

Specificity

To assess specificity, each assay in the panel was tested individually. Nonspecific binding was also evaluated with additional recombinant human analytes (Abeta 38, Abeta 40, Abeta 42, c-Kit, CTACK, CRP, EGF, eotaxin-2, EPO, FGF (basic), Fractalkine, G-CSF, GM-CSF, HGF, I-309, ICAM-1, ICAM-3, IFN-α2a, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-6R, IL-7, IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-17B, IL-17D, IL-18, IFN-γ, I-TAC, MCP-2, M-CSF, MIF, MIG, MIP-3α, MIP-4, MIP-5, MMP-1, MMP-2, MMP-3, MMP-9, MMP-10, NT-proBNP, RANTES, SAA, Thrombomodulin, Tie, TNF-α, TNF-β, TNF-RI, TNF-RII, TPO, VCAM-1, VEGF-A, VEGF-C, VEGF-D, and VEGF-RI). Nonspecific binding was less than 0.8% for all assays in the kit. Since chemokines are heavily charged, non-specific binding of detection antibodies to calibrators through very weak interactions is observed and the level of non-specificity can vary from run to run. However, this interaction does not interfere with assay performance because non-specific antibody does not out-compete specific antibody when binding to the calibrator (data not shown). Non-specificity reported in the COA for this panel is measured using individual calibrators and blended detection antibodies.

$$\% \, Nonspecificity = \frac{nonspecific \, signal}{specific \, signal} * 100$$

Stability

The reconstituted calibrator, reconstituted controls, and diluents were tested for freeze-thaw stability. Results (not shown) demonstrated that reconstituted calibrator, reconstituted controls, Diluent 43, and Diluent 3 can go through three freeze-thaw cycles without significantly affecting the performance of the assay. Once reconstituted, the multi-analyte calibrator is stable for one day at 2–8 °C. Partially used MSD plates may be sealed and 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 the date of manufacture.

Calibration

All the assays in the panel are calibrated against a reference calibrator generated at MSD.

MSD reference calibrators for the following analytes were evaluated against the NIBSC/WHO International Standards; the ratios of International Units of biological activity per mL (IU/mL) of NIBSC standard relative to pg/mL of MSD calibrator are shown in the table below. To convert MSD concentrations to biological activity relative to the WHO International Standard, multiply the MSD concentration by the ratio provided.

Table 9. Ratios of International Units (IU/mL) relative to MSD Calibrators (pg/mL)

Analyte	NIBSC/WHO Catalog Number	NIBSC (IU/mL): MSD (pg/mL)
MIP-1α	92/518	0.00022
MCP-1	92/794	0.0018

Tested Samples

Normal Samples

Normal human serum, EDTA plasma, heparin plasma, citrate plasma, and urine samples from a commercial source were diluted 4-fold and tested. The 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 10. Normal human samples tested in the Chemokine Panel 1 (human) Kit

Sample Type	Statistic	Eotaxin	MIP-1β	Eotaxin-3	TARC	IP-10	MIP-1α	IL-8	MCP-1	MDC	MCP-4*
Serum (N=27)	Median (pg/mL)	55.9	53.1	8.18	29.1	80.9	37.0	575	118	1350	75.3
	Range (pg/mL)	19.0–145	7.29–95.2	7.63–8.73	5.39–70.0	28.5–237	12–202	488–665	75.7–205	606–3249	61.1–304
	% Detected	96	100	7	100	100	33	22	100	100	100
EDTA Plasma (N=27)	Median (pg/mL)	162	70.4	17.9	127	229	84.5	1047	87.5	1408	65.2
	Range (pg/mL)	37.2–795	7.99–153	6.71–115	13.4–373	102–676	11.3–651	347–2478	42.3–185	869–2144	29.5–136
	% Detected	100	100	85	100	100	37	37	100	100	100
Heparin Plasma (N=27)	Median (pg/mL)	409	130	42.4	175	208	171	1114	158	1135	110
	Range (pg/mL)	22.3–1522	9.53–301	6.40–147	5.97–957	90.7–625	12.3–2231	340–3281	68.9–319	589–1963	60.7–332
	% Detected	100	100	96	100	100	85	22	100	100	100
Citrate Plasma (N=20)	Median (pg/mL)	182	51.9	13.2	60.9	115	16.4	1031	138	1010	50.5
	Range (pg/mL)	71.9–288	18.5–123	9.03–18.5	25.3–131	35.6–373	10.2–30.4	328–1869	76.1–242	576–1364	32.3–65.9
	% Detected	100	100	90	100	100	20	20	100	100	100
Urine (N=5)	Median (pg/mL)	21.1	10.0	9.22	3.53	24.0	15.5	511	247	32.2	15.7
	Range (pg/mL)	12.9–35.5	2.31–27.7	7.13–13.5	1.40–6.97	0.45–160	11.6–17.9	296–870	1.95–1173	19.8–44.4	7.9–103
	% Detected	73	100	60	67	80	60	20	100	47	100

% detected = % of samples with concentrations at or above the LLOD
*MCP-4 was tested using N=7 for serum, EDTA plasma, heparin plasma, citrate plasma and urine, and N=1 for cell culture supernatant

Stimulated Samples

Freshly collected, normal, pooled human whole blood was incubated at 37 °C for different time periods with lipopolysaccharide (LPS) as shown below; plasma was isolated at the end of incubation (Figure 5). Additionally, a human acute monocyle leukemia cell line (THP-1) was stimulated for different time periods with LPS (10 ug/mL) as show below; supernatants were then isolated and tested (Figure 6). The dilution-adjusted concentrations (pg/mL) for each stimulation model are displayed below. All assays showed a significant difference in analyte level with prolonged stimulation.
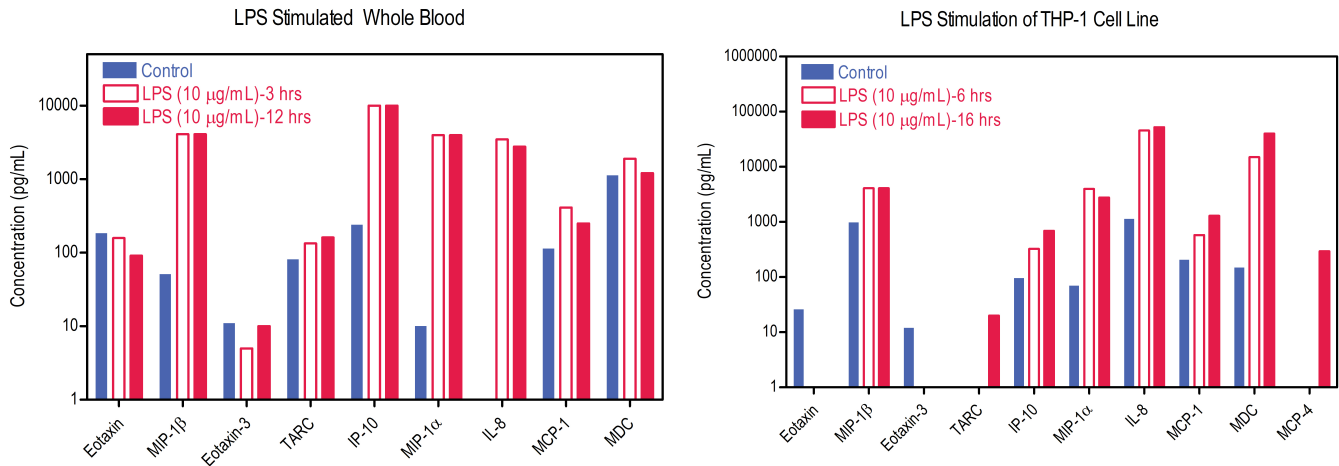

Figure 5. Normal human whole blood and THP-1 cell line stimulated with LPS.

Normal human whole blood was enriched for leukocytes and platelets and was treated with LPS, phytohaemagglutinin (PHA), pokeweed mitogen (PWM), or concanavalin A (Con A), and co-stimulated with CD3 and CD28 antibodies. The dilution-adjusted concentrations (pg/mL) for each stimulation model are displayed below (Figures 6 and 7).
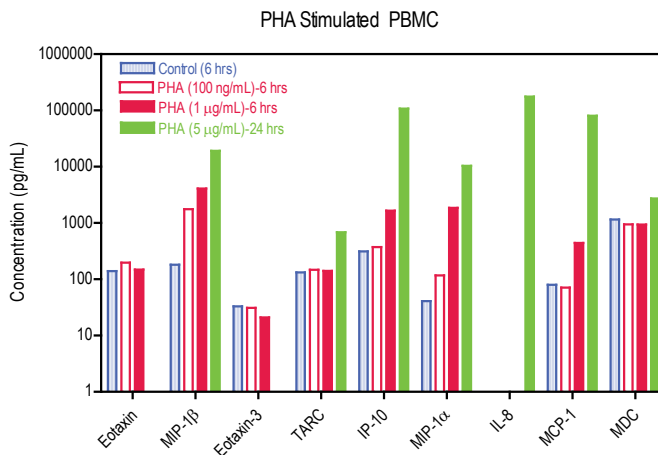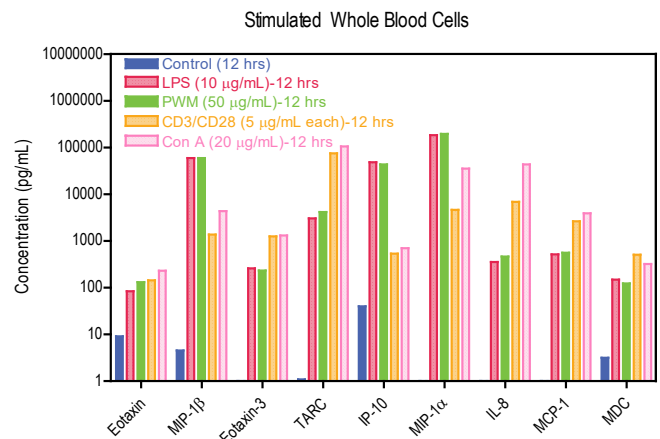

Figure 6. Enriched whole blood treated with PHA.

Figure 7. Enriched whole blood treated with LPS, PWM, ConA, and CD3/CD28.

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Assay Components

Calibrators

The assay calibrator blend uses the following recombinant human proteins:

Table 11. *Recombinant human proteins used in the Calibrators*

Calibrator	Expression System
Eotaxin	E. coli
MIP-1β	E. coli
Eotaxin-3	E. coli
TARC	E. coli
IP-10	E. coli
MIP-1α	E. coli
IL-8	E. coli
MCP-1	E. coli
MDC	E. coli
MCP-4	E. coli

Antibodies

Table 12. *Antibody source species*

Analyte	Source Species		Assay Generation
	MSD Capture Antibody	MSD Detection Antibody	
Eotaxin	Mouse Monoclonal	Mouse Monoclonal	B
MIP-1β	Mouse Monoclonal	Mouse Monoclonal	B
Eotaxin-3	Mouse Monoclonal	Mouse Monoclonal	B
TARC	Mouse Monoclonal	Mouse Monoclonal	B
IP-10	Mouse Monoclonal	Mouse Monoclonal	B
MIP-1α	Mouse Monoclonal	Mouse Monoclonal	B
IL-8	Mouse Monoclonal	Goat Polyclonal	B
MCP-1	Mouse Monoclonal	Mouse Monoclonal	B
MDC	Mouse Monoclonal	Mouse Monoclonal	B
MCP-4	Mouse Monoclonal	Mouse Monoclonal	C

References

1. Laing KJ, et al. Chemokines. Dev Comp Immunol. 2004;28:443-60.
2. Rollins B. Chemokines. Blood. 1997;90:909-28.
3. Onuffer JJ, et al. Chemokines, chemokine receptors and small-molecule antagonists: recent developments. Trends Pharmacol Sci. 2002;23:459-67.
4. Yoshie O, Role of chemokines in trafficking of lymphocytes and dendritic cells. Int. J Hematol 2000;72:399-407.
5. Zlotnik A, et al. The chemokine superfamily revisited. Immunity. 2012;36:705-16.
6. Gerard C, et al. Chemokines and disease. Nat Immunol. 2001;2:108-15.
7. Gangur V, et al. Chemokines in health and disease. Veterinary Immunology and Immunotherapy. 2002;86:127-136.
8. Lee JW, et al. Fit-for-purpose method development and validation for successful biomarker measurement. Pharm Res. 2006;23:312-28.
9. Bowen RA, et al. Impact of blood collection devices on clinical chemistry assays. Clin Biochem. 2010;43:4-25.
10. Zhou H, et al. Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney. 2006;69:1471-6.
11. Thomas CE, et al. Urine collection and processing for protein biomarker discovery and quantification. Cancer Epidemiol Biomarkers & Prevention. 2010:953-9.
12. Schoonenboom NS, et al. Effects of processing and storage conditions on amyloid beta (1-42) and tau concentrations in cerebrospinal fluid: implications for use in clinical practice. Clin Chem. 2005;51:189-95.
13. Girgrah N, et al. Purification and characterization of the P-80 glycoprotein from human brain. Biochem J. 1988;256:351-6.

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Appendix A

The calibration curves below illustrate the relative sensitivity of each assay under **Alternate Protocols**: Reference Protocol (2-hour sample incubation/2 wash steps, blue curve), Alternate Protocol 1 (overnight sample incubation, red curve), and Alternate Protocol 2 (tissue culture: single wash, green curve).
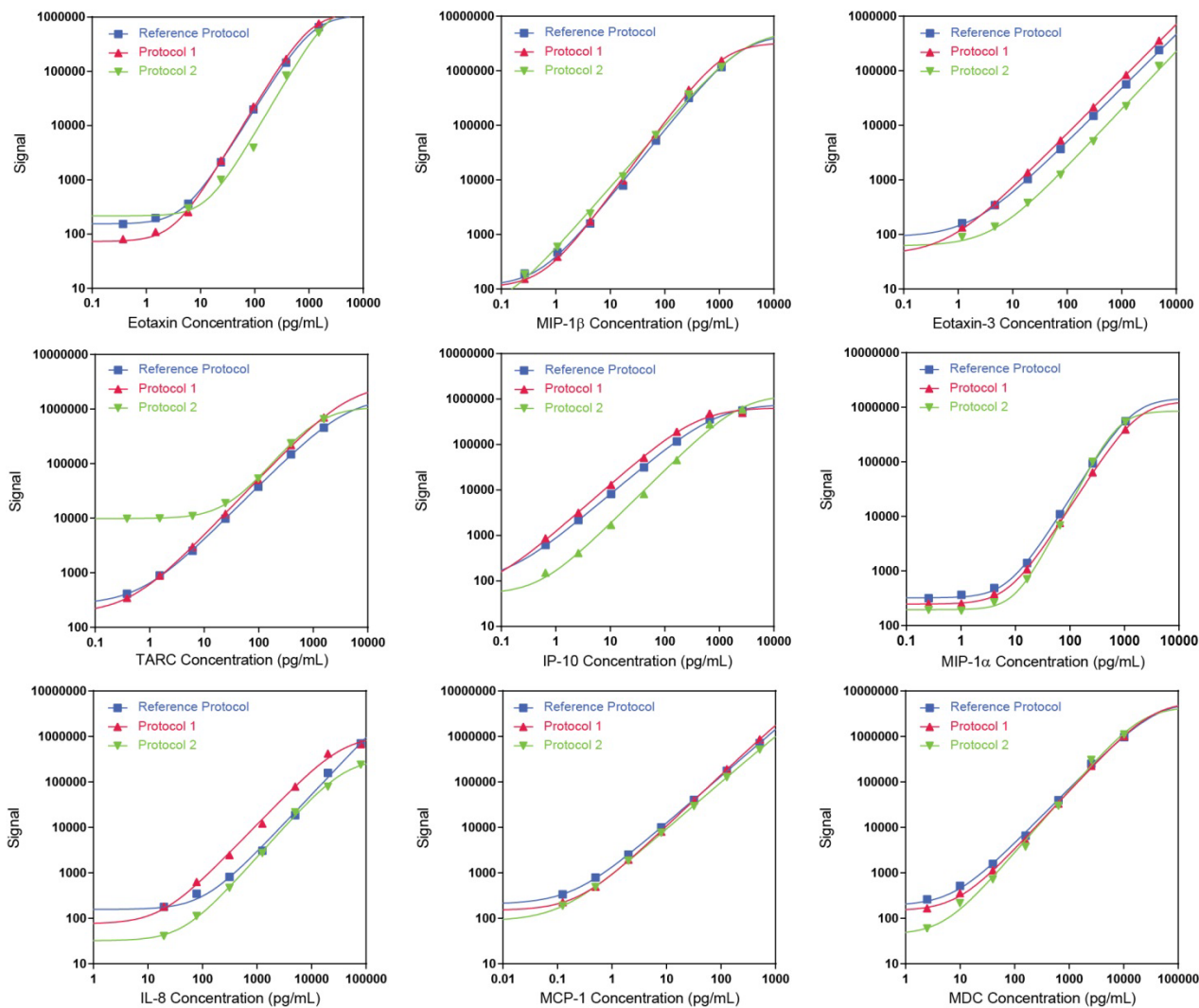

Table 13. Relative sensitivity when using alternate protocols

	LLOD Comparison (pg/mL)		
Assay*	Reference Protocol	Protocol 1	Protocol 2
Eotaxin	3.26	3.16	20.4
MIP-1β	0.17	0.45	0.19
Eotaxin-3	1.77	1.08	5.58
TARC	0.22	0.19	5.81
IP-10	0.37	0.07	0.70
MIP-1α	3.02	3.43	6.11
IL-8	95.6	18.1	85.8
MCP-1	0.09	0.12	0.10
MDC	1.22	8.85	7.94

*Data not available for MCP-4 assay

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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).
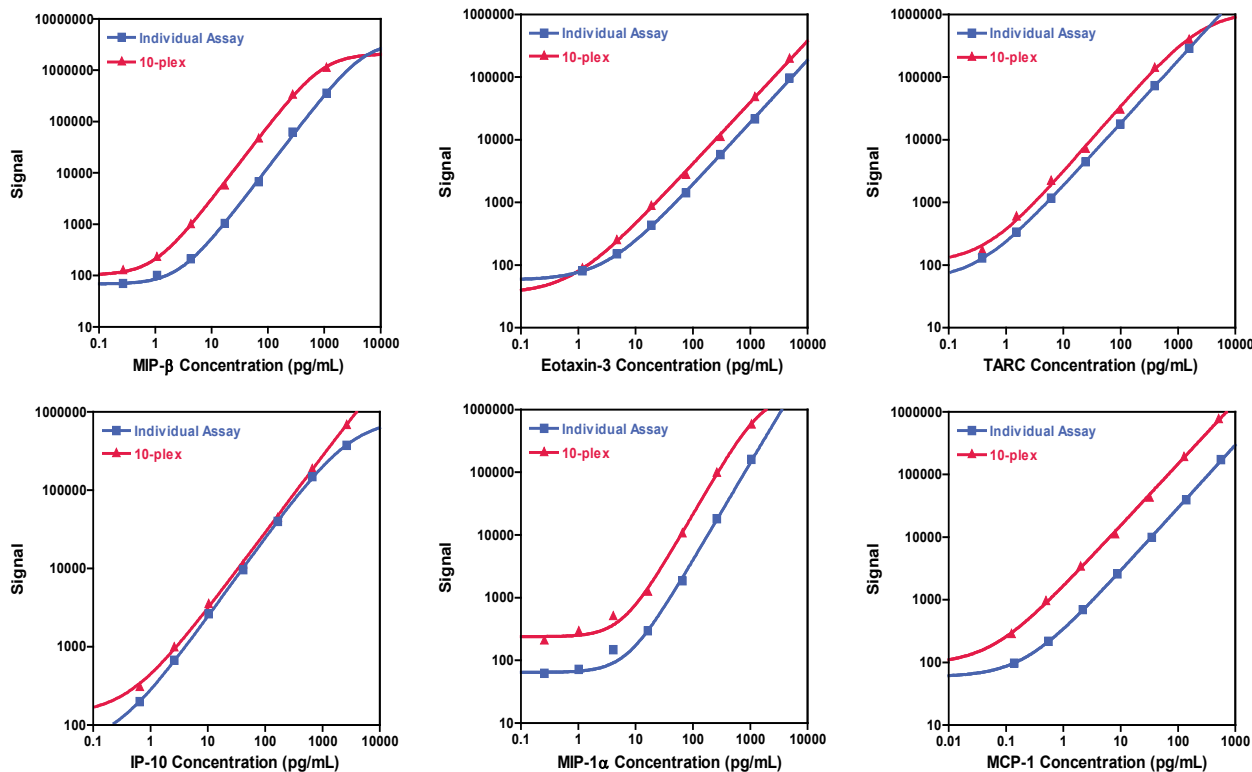

Table 14. Assay performance for individual and 10-plex assays

	LLOD (pg/mL)	
Assay	Individual*	10-plex
MIP-1β	1.29	0.17
Eotaxin-3	1.30	1.77
TARC	0.15	0.22
IP-10	0.11	0.37
MIP-1α	4.72	3.02
MCP-1	0.08	0.09

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.

*Due to its higher sensitivity, the IL-8 assay from the Proinflammatory Panel 1 (human) is the IL-8 assay provided on single spot plates. Data are shown in the Proinflammatory Panel 1 (human) product insert.

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 detection antibodies (blue curve) vs. running each assay using a single, assay-specific detection antibody (red curve).
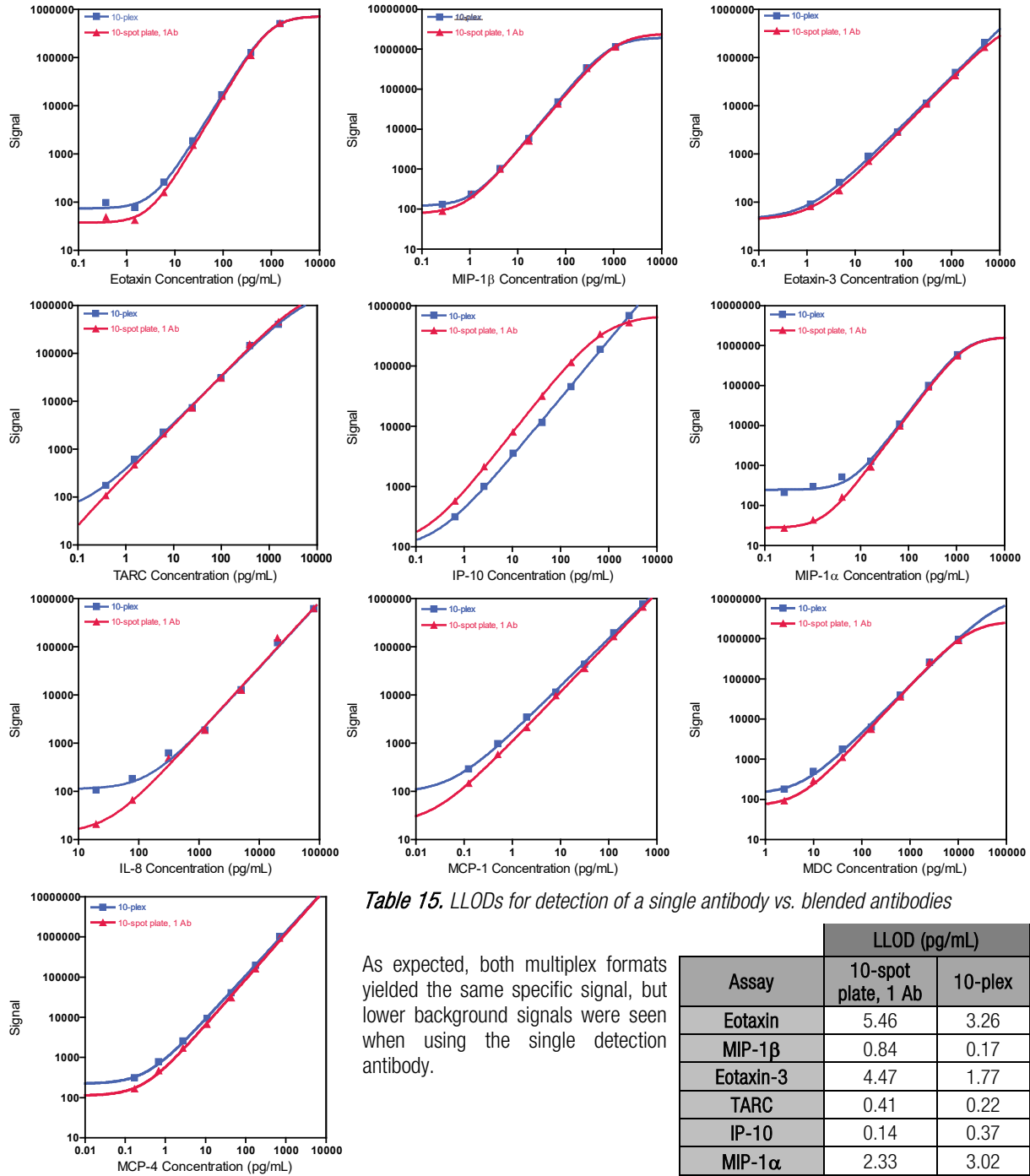

Table 15. *LLODs for detection of a single antibody vs. blended antibodies*

As expected, both multiplex formats yielded the same specific signal, but lower background signals were seen when using the single detection antibody.

	LLOD (pg/mL)	
Assay	10-spot plate, 1 Ab	10-plex
Eotaxin	5.46	3.26
MIP-1β	0.84	0.17
Eotaxin-3	4.47	1.77
TARC	0.41	0.22
IP-10	0.14	0.37
MIP-1α	2.33	3.02
IL-8	74.1	95.6
MCP-1	0.13	0.09
MDC	4.32	1.22
MCP-4	0.17	0.18

Summary Protocol

<p style="text-align:center">Chemokine Panel 1 (human) Kits</p>

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol before performing the chemokine panel 1 (human) assays.

Sample and Reagent Preparation

❑ Bring all reagents to room temperature.

❑ Prepare calibration solutions in Diluent 43 using the supplied calibrator:

- o Reconstitute the lyophilized calibrator blend.
- o Invert 3 times, equilibrate 15-30 minutes at room temperature.
- o Vortex briefly using short pulses.
- o Perform a series of 4-fold dilution steps and prepare a zero calibrator.

❑ Dilute samples and controls 4-fold in Diluent 43 before adding to the plate.

❑ Prepare combined detection antibody solution by diluting each 50X detection antibody 50-fold in Diluent 3.

❑ Prepare 2X Read Buffer T by diluting 4X Read Buffer T 2-fold with deionized water.

STEP 1: Wash* and Add Sample

❑ Wash plate 3 times with at least 150 µL/well of 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 plate 3 times with at least 150 µL/well of 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 plate 3 times with at least 150 µL/well of Wash Buffer.

❑ Add 150 µL/well of 2X Read Buffer T and incubate at room temperature for 10 minutes.

❑ Analyze plate on the MSD instrument.

*Note: Washing the plate before 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 before sample addition.

Catalog Numbers

Table 16. Catalog numbers for V-PLEX and V-PLEX Plus chemokine (human) multiplex and single assay kits

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						
Chemokine Panel 1 (human)	K15047D-1	K15047D-2	K15047D-4	K15047G-1	K15047G-2	K15047G-4
Individual Assay Kits						
Human Eotaxin	K151NSD-1	K151NSD-2	K151NSD-4	K151NSG-1	K151NSG-2	K151NSG-4
Human MIP-1β	K151NRD-1	K151NRD-2	K151NRD-4	K151NRG-1	K151NRG-2	K151NRG-4
Human Eotaxin-3	K151NUD-1	K151NUD-2	K151NUD-4	K151NUG-1	K151NUG-2	K151NUG-4
Human TARC	K151NTD-1	K151NTD-2	K151NTD-4	K151NTG-1	K151NTG-2	K151NTG-4
Human IP-10	K151NVD-1	K151NVD-2	K151NVD-4	K151NVG-1	K151NVG-2	K151NVG-4
Human MIP-1α	K151NQD-1	K151NQD-2	K151NQD-4	K151NQG-1	K151NQG-2	K151NQG-4
Human IL-8	K151RAD-1	K151RAD-2	K151RAD-4	K151RAG-1	K151RAG-2	K151RAG-4
Human MCP-1	K151NND-1	K151NND-2	K151NND-4	K151NNG-1	K151NNG-2	K151NNG-4
Human MDC	K151NPD-1	K151NPD-2	K151NPD-4	K151NPG-1	K151NPG-2	K151NPG-4
Human MCP-4	K151NOD-1	K151NOD-2	K151NOD-4	K151NOG-1	K151NOG-2	K151NOG-4

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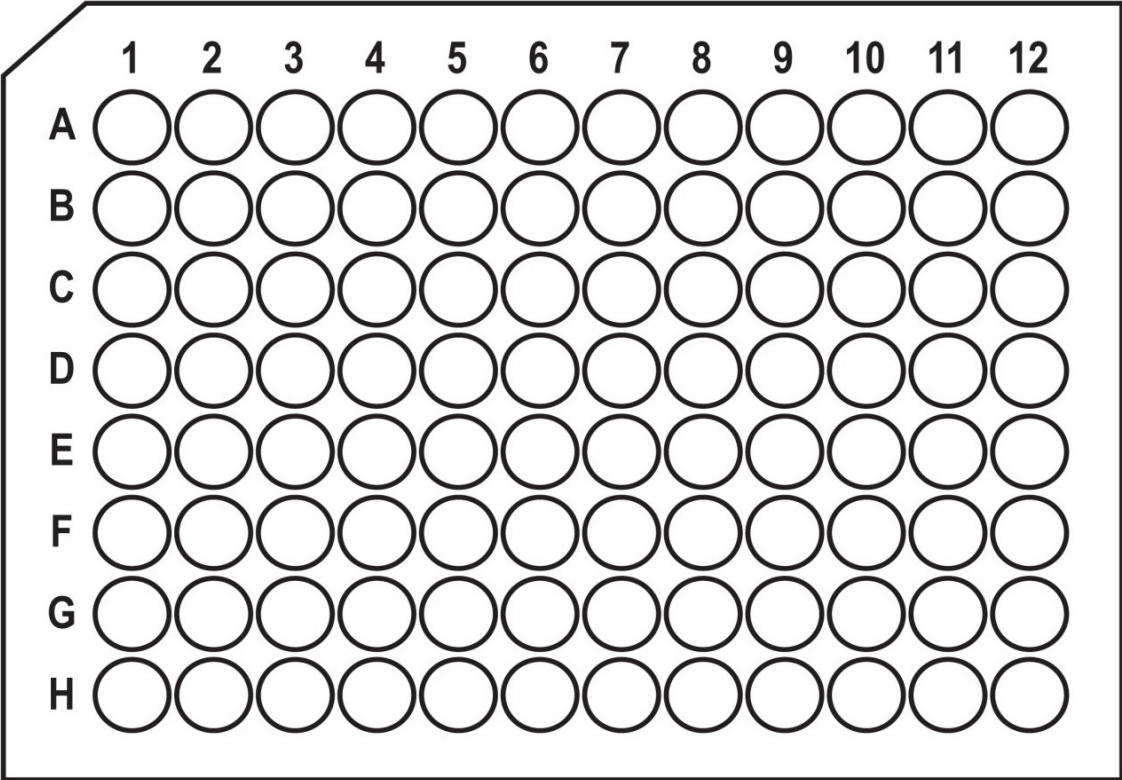
Plate Diagram

Figure 9. Plate diagram.

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	CAL-01		Sample-01		Sample-09		Sample-17		Sample-25		Sample-33	
B	CAL-02		Sample-02		Sample-10		Sample-18		Sample-26		Sample-34	
C	CAL-03		Sample-03		Sample-11		Sample-19		Sample-27		Sample-35	
D	CAL-04		Sample-04		Sample-12		Sample-20		Sample-28		Sample-36	
E	CAL-05		Sample-05		Sample-13		Sample-21		Sample-29		Sample-37	
F	CAL-06		Sample-06		Sample-14		Sample-22		Sample-30		Sample-38	
G	CAL-07		Sample-07		Sample-15		Sample-23		Sample-31		Sample-39	
H	CAL-08		Sample-08		Sample-16		Sample-24		Sample-32		Sample-40	

	1	2	3	4	5	6	7	8	9	10	11	12
A	CAL-01		Control 1.1		Sample-06		Sample-14		Sample-22		Sample-30	
B	CAL-02		Control 1.2		Sample-07		Sample-15		Sample-23		Sample-31	
C	CAL-03		Control 1.3		Sample-08		Sample-16		Sample-24		Sample-32	
D	CAL-04		Sample-01		Sample-09		Sample-17		Sample-25		Sample-33	
E	CAL-05		Sample-02		Sample-10		Sample-18		Sample-26		Sample-34	
F	CAL-06		Sample-03		Sample-11		Sample-19		Sample-27		Sample-35	
G	CAL-07		Sample-04		Sample-12		Sample-20		Sample-28		Sample-36	
H	CAL-08		Sample-05		Sample-13		Sample-21		Sample-29		Sample-37	

Figure 10. Sample plate layout that can be used for the assay. Each sample, calibrator, and control (Plus Kit) is measured in duplicate in side-by-side wells.