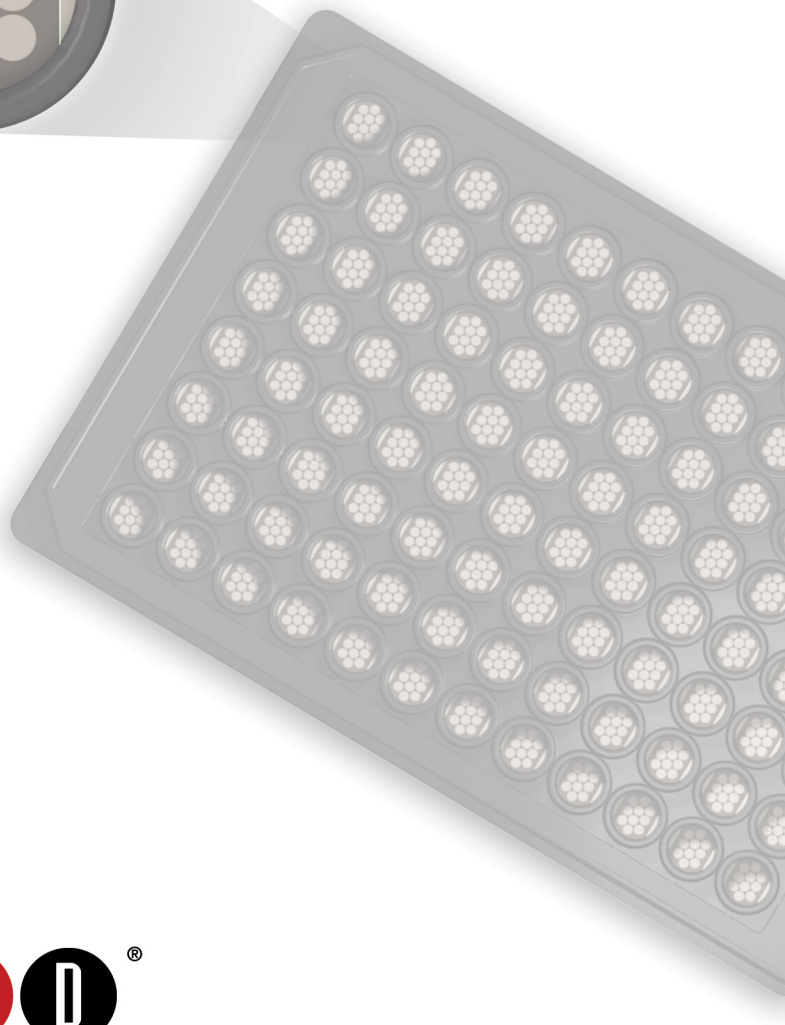
MSD® MULTI-SPOT Assay System

Chemokine Panel 1 (NHP) Kits

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	K15055D	K15055G
Individual Assay Kits		
NHP MIP-1β	K156NRD	K156NRG
NHP Eotaxin-3	K156NUD	K156NUG
NHP TARC	K156NTD	K156NTG
NHP IP-10	K156NVD	K156NVG
NHP MIP-1α	K156NQD	K156NQG
NHP IL-8	K156RAD	K156RAG
NHP MCP-1	K156NND	K156NNG
NHP MDC	K156NPD	K156NPG
NHP MCP-4	K156NOD	K156NOG

MSD Cytokine Assays

Chemokine Panel 1 (NHP) Kits

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

For use with non-human primate (NHP) 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.

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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[1] 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.[2] 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.[3] Chemokines function by activating specific G protein-coupled receptors resulting in the migration of inflammatory and non-inflammatory cells.[4] The pro-inflammatory chemokines are responsible for the migration of immune cells to the infection site,[5] while the homeostatic chemokines are responsible for recruiting cells for tissue maintenance and development.[6] Chemokines are associated with a number of diseases.[7,8] 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 (NHP) contains nine assays specifically validated for measuring chemokines in two species of non-human primates (NHP): rhesus (Macaca mulatta) and cynomolgus (M. fascicularis) monkeys. Validation involved measuring both stimulated and endogenous levels of chemokines from NHP specimens. To verify that the assays can measure immune responses, stimulated NHP peripheral blood monocytes were used to detect and measure chemokine production. To further validate the ability of the assays to reproducibly quantify physiologic levels of NHP chemokines, endogenous levels were measured in serum, plasma, and urine from normal rhesus and cynomolgus monkeys.

The Chemokine Panel 1 (NHP) employs anti-human detection and capture antibodies that react with rhesus and cynomolgus monkeys. These assays may be suitable for primate species in addition to *M. mulatta* and *M. fascicularis* since human chemokines are broadly homologous with chemokines from NHPs.[9] The Chemokine Panel 1 (NHP) Kit consists of seven CC chemokine assays (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 (NHP) 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 MDC and MCP-4 assays are provided on 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) for 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 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]

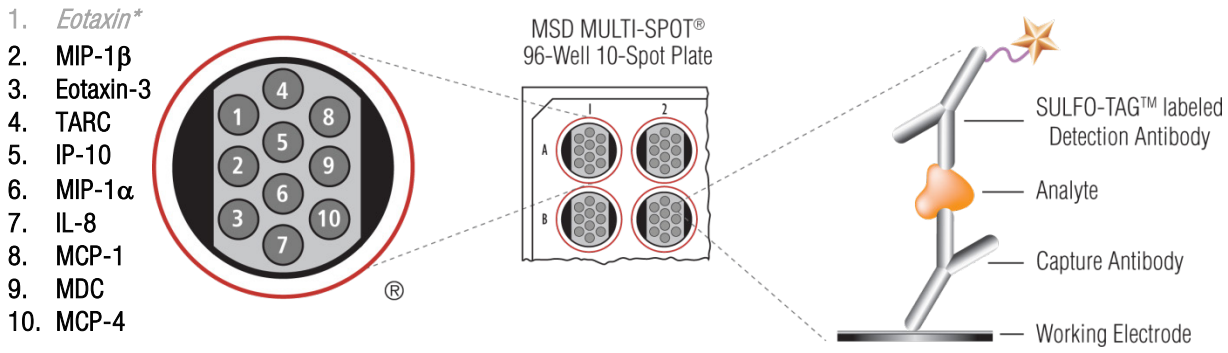

Figure 1. Multiplex plate spot diagram showing the placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

**Not cross-reactive with non-human primate samples*

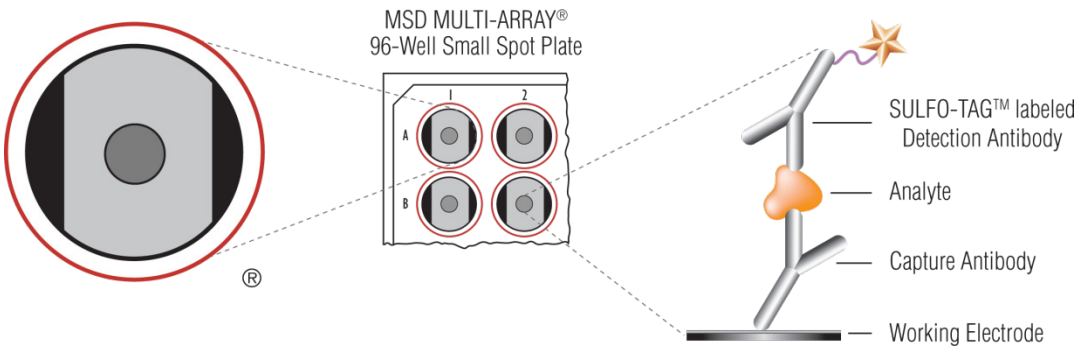

Figure 2. Small Spot plate diagram showing the placement of analyte capture antibodies.

Kit Components

Chemokine Panel 1 (NHP) assays are available as a 9-spot multiplex kit, as individual assay kits, or as custom V-PLEX kits with subsets of assays selected from the full panel. The assays use human recombinant chemokine calibrator sequences that are highly homologous to the NHP chemokines. 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	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.

Dash (—) = not applicable
RT = room temperature

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-primate 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 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 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 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 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 1,000 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 highest 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 the highest calibrator 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 (Calibrator 1) is stable for one day at 2-8 ºC. It may also be stored frozen at ≤-70 ºC 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.

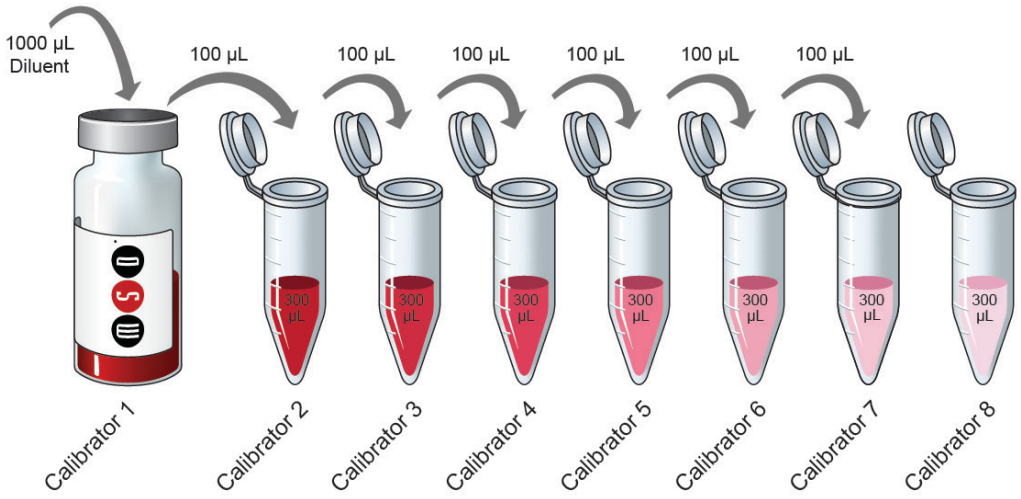

Figure 3. Dilution schema for preparation of Calibrator Standards.

Sample Collection and Handling

Below are general guidelines for NHP sample collection, storage, and handling. If possible, use published guidelines.43-45 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 ºC 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 NHP 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 (NHP) 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 (NHP) 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 2,460 µL of Diluent 3:

- ❑ 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.

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 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 the 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 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. Before 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 is 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 the 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 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.

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 (NHP) 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 nine detection antibodies. The calibration curves were comparable. Calibration curves for each lot are presented in the lot-specific COA.

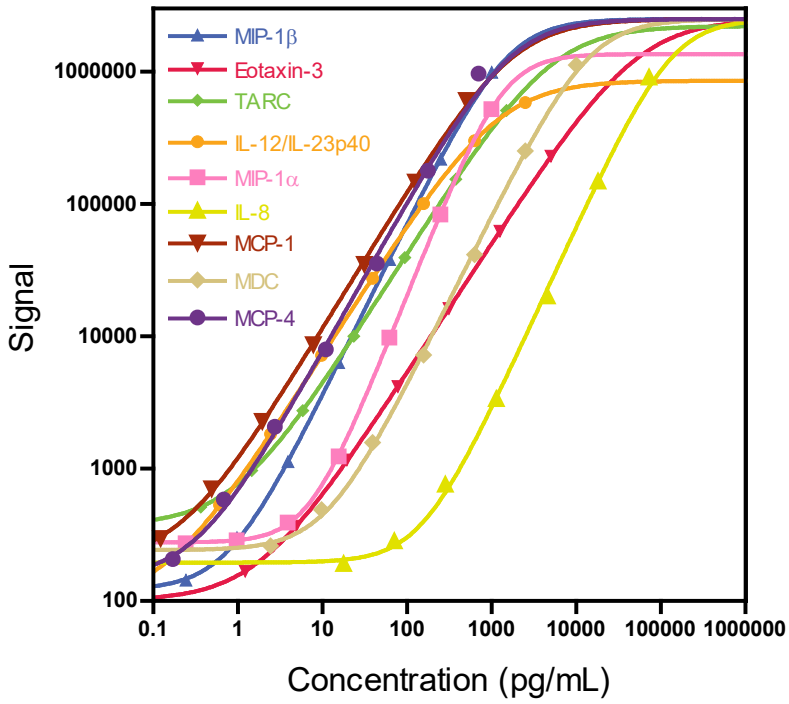

Figure 4. Typical calibration curves for the Chemokine Panel 1 (NHP) assay.

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, an 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 (NHP) Kit

	Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
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 (NHP) 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-primate 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 interday 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 (NHP) Kit

	Control	Average Conc. (pg/mL)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
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

*Due to 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, commercially available serum, EDTA plasma, and urine from rhesus and cynomolgus monkeys 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.

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

Rhesus Monkey

Table 7. Analyte percent recovery at various dilutions in serum, EDTA plasma, urine, and cell culture supernatant rhesus monkey samples

		MIP-1β		Eotaxin-3		TARC		IP-10		MIP-1α	
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=5)	2	97	88–114	100	84–144	104	92–122	146	110–233	107	85–135
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	100	95–108	99	92–108	96	88–107	87	81–95	101	93–106
	16	100	91–109	101	91–111	101	90–117	87	80–93	101	85–109
	32	98	81–115	99	82–116	95	76–112	88	80–99	104	84–118
	64	103	88–127	121	105–146	104	83–128	96	85–112	111	90–130
EDTA Plasma (N=5)	2	90	86–96	107	94–121	99	90–106	148	89–258	104	98–110
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	102	97–112	73	63–82	94	83–108	76	65–90	98	92–106
	16	100	90–119	78	69–96	94	80–109	70	58–90	96	83–111
	32	101	93–121	83	73–107	88	74–107	70	58–94	101	87–118
	64	101	92–121	94	81–121	94	81–110	76	64–101	105	89–122
Urine (N=5)	2	117	103–154	109	98–136	126	110–170	144	107–208	120	107–155
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	94	91–96	95	91–105	90	84–93	84	79–89	94	91–99
	16	91	88–97	96	90–110	91	86–93	82	75–90	88	84–95
	32	89	86–91	99	92–113	85	78–94	81	75–89	91	89–94
	64	89	85–93	118	109–129	90	82–96	87	78–94	91	87–94
Cell Culture Supernatant (N=6)	2	110	98–116	93	88–97	93	84–101	179	127–256	120	104–128
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	95	91–100	100	96–102	91	87–98	73	55–84	90	85–94
	16	94	90–99	108	101–114	87	80–94	68	60–88	85	78–94
	32	91	87–99	112	105–118	82	75–89	63	52–77	80	72–89
	64	95	89–102	126	116–133	90	76–99	68	57–81	81	73–95

N/A = not applicable or not available

Table 7. continued

		IL-8		MCP-1		MDC		MCP-4*	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=5)	2	113	96–132	96	83–117	109	98–131	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	100	N/A
	8	87	80–101	99	89–109	96	87–101	111	94–127
	16	78	63–83	102	98–108	88	82–102	115	94–135
	32	80	66–91	100	88–116	82	75–99	116	85–146
	64	86	75–93	109	93–137	80	66–98	N/A	N/A
EDTA Plasma (N=5)	2	111	87–134	98	89–108	104	90–116	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	100	N/A
	8	88	81–100	93	81–112	95	89–105	100	92–104
	16	81	76–89	91	77–115	84	78–88	107	90–118
	32	84	74–96	99	87–122	81	74–87	104	85–115
	64	97	86–109	109	88–149	80	74–86	N/A	N/A
Urine (N=5)	2	135	111–188	110	94–135	155	133–202	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	N/A	N/A
	8	84	79–92	90	82–96	83	81–87	114	108–117
	16	78	70–84	94	82–105	71	65–75	123	113–128
	32	78	70–82	93	81–117	69	66–75	128	117–136
	64	94	82–108	95	86–108	66	60–74	N/A	N/A
Cell Culture Supernatant (N=6)	2	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
	8	96	89–113	95	89–98	75	73–76	106	N/A
	16	89	83–95	94	89–101	63	61–65	116	N/A
	32	93	82–97	88	81–91	56	54–59	N/A	N/A
	64	107	95–121	98	90–103	55	52–57	N/A	N/A

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

Cynomolgus Monkey

Table 8. Analyte percent recovery at various dilutions in serum, EDTA plasma, urine, and cell culture supernatant cynomolgus monkey samples

		MIP-1β		Eotaxin-3		TARC		IP-10		MIP-1α	
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=5)	2	98	89–104	118	92–138	112	90–164	204	66–473	105	102–109
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	100	95–104	92	87–97	100	94–109	81	62–116	101	99–109
	16	104	88–121	107	95–126	114	96–160	82	57–126	104	96–130
	32	100	85–120	122	105–151	118	86–210	81	56–133	111	99–142
	64	101	84–124	136	112–169	137	96–274	89	59–147	119	106–156
EDTA Plasma (N=5)	2	94	78–103	135	110–184	94	81–116	170	105–250	104	86–113
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	102	101–105	97	90–106	96	90–101	73	55–87	97	96–99
	16	101	97–109	101	86–117	97	88–104	68	48–80	93	90–99
	32	101	92–115	111	86–135	93	84–103	66	48–77	95	90–107
	64	101	89–120	125	90–160	99	87–111	69	48–82	104	87–128
Urine (N=5)	2	112	107–116	105	94–113	120	107–151	138	123–149	121	115–127
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	92	88–99	93	88–96	88	83–103	81	74–85	95	89–102
	16	90	87–96	94	91–97	86	83–92	79	72–82	91	88–96
	32	88	84–95	98	93–102	82	72–93	75	72–80	92	87–98
	64	88	81–93	115	107–123	81	74–91	82	76–89	92	87–98
Cell Culture Supernatant (N=6)	2	110	98–116	93	88–97	93	84–101	179	127–256	120	104–128
	4	100	N/A	100	N/A	100	N/A	100	N/A	100	N/A
	8	95	91–100	100	96–102	91	87–98	73	55–84	90	85–94
	16	94	90–99	108	101–114	87	80–94	68	60–88	85	78–94
	32	91	87–99	112	105–118	82	75–89	63	52–77	80	72–89
	64	95	89–102	126	116–133	90	76–99	68	57–81	81	73–95

N/A = not applicable or not available

Table 8. continued

Sample Type	Fold Dilution	IL-8		MCP-1		MDC		MCP-4*	
		Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=5)	2	112	104–126	96	93–100	109	94–128	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	100	N/A
	8	86	83–91	94	89–100	91	86–96	119	110–127
	16	79	76–83	102	86–115	85	73–96	129	112–138
	32	78	76–80	94	82–109	80	69–89	138	120–149
	64	86	81–91	101	83–131	81	67–93	N/A	N/A
EDTA Plasma (N=5)	2	108	86–132	99	80–110	109	95–135	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	100	N/A
	8	86	82–89	84	78–91	95	90–107	94	92–95
	16	77	72–82	86	75–98	86	77–101	90	88–92
	32	78	71–85	83	69–98	83	70–101	89	84–92
	64	94	72–125	92	73–110	83	73–108	145	118–192
Urine (N=5)	2	137	118–174	105	101–109	137	125–150	N/A	N/A
	4	100	N/A	100	N/A	100	N/A	100	N/A
	8	85	77–98	90	84–98	81	76–85	113	109–117
	16	76	69–85	88	82–103	72	66–76	114	110–118
	32	77	73–80	83	81–86	66	60–70	107	88–117
	64	84	72–88	87	78–100	65	61–73	N/A	N/A
Cell Culture Supernatant (N=6)	2	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
	8	96	89–113	95	89–98	75	73–76	106	N/A
	16	89	83–95	94	89–101	63	61–65	116	N/A
	32	93	82–97	88	81–91	56	54–59	N/A	N/A
	64	107	95–121	98	90–103	55	52–57	N/A	N/A

N/A = not applicable or not available

*MCP-4 was tested using N=3 for serum, EDTA plasma, and urine, and N=1 for cell culture supernatant

Spike Recovery

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

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

Rhesus Monkey

Table 9. Spike and Recovery measurements of different sample types from rhesus monkeys

	Serum (N=5)			EDTA Plasma (N=5)			Urine (N=5)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
MIP-1β	92	3.8	88–97	88	3.8	85–92	82	3.7	79–87
Eotaxin-3	99	25.5	68–135	110	7.7	99–120	102	11.6	85–118
TARC	81	5.5	75–87	85	13.2	74–98	86	6.8	79–92
IP-10	89	6.0	83–94	100	4.4	95–106	85	2.8	82–88
MIP-1α	84	5.3	78–89	80	8.7	73–91	89	4.0	86–95
IL-8	65	8.8	55–69	58	7.3	54–64	71	11.4	61–82
MCP-1	94	9.5	83–102	77	10.3	67–87	90	9.4	78–100
MDC	93	5.4	84–96	95	5.2	88–101	109	4.1	102–113
MCP-4*	84	10.1	97–126	84	17.2	61–97	113	22.4	88–136

*MCP-4 was tested using N=3 for serum, EDTA plasma, and uine

	Cell Culture Supernatants (N=6)		
	Average % Recovery	%CV	% Recovery Range
MIP-1β	107	6.8	90–122
Eotaxin-3	95	5.8	84–108
TARC	121	5.6	110–135
IP-10	147	35.2	97–267
MIP-1α	116	5.7	100–127
IL-8	97	10.0	71–109
MCP-1	104	4.9	96–119
MDC	140	7.5	122–159
MCP-4*	71	N/A	N/A

*MCP-4 was tested using N=1 for cell culture supernatant

Cynomolgus Monkey

Table 10. Spike and Recovery measurements of different sample types from cynomolgus monkeys

	Serum (N=5)			EDTA Plasma (N=5)			Urine (N=5)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
MIP-1β	91	4.2	87–95	83	4.9	77–88	82	1.0	81–83
Eotaxin-3	86	15.1	70–104	82	30.6	51–112	92	8.2	81–99
TARC	62	43.6	14–76	76	11.8	62–86	86	6.5	82–95
IP-10	90	18.0	63–105	100	5.5	94–106	85	1.5	83–86
MIP-1α	85	9.5	76–93	85	4.0	81–88	90	2.0	87–92
IL-8	62	11.3	55–69	57	11.7	50–65	70	10.9	59–79
MCP-1	94	17.1	77–113	86	10.1	75–98	93	8.4	87–106
MDC	90	1.9	89–92	92	4.9	86–97	103	4.4	96–107
MCP-4*	106	36.2	54–182	99	4.8	93–104	85	3.3	82–90

*MCP-4 was tested using N=3 for serum, EDTA plasma, and uine

	Cell Culture Supernatants (N=6)		
	Average % Recovery	%CV	% Recovery Range
MIP-1β	107	6.8	90–122
Eotaxin-3	95	5.8	84–108
TARC	121	5.6	110–135
IP-10	147	35.2	97–267
MIP-1α	116	5.7	100–127
IL-8	97	10.0	71–109
MCP-1	104	4.9	96–119
MDC	140	7.5	122–159
MCP-4*	71	N/A	N/A

*MCP-4 was tested using 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 ºC. Partially used MSD plates may be sealed and stored up to 30 days at 2-8 ºC 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.

Tested Samples

Commercially available normal serum, EDTA plasma, and urine samples from rhesus and cynomolgus monkeys 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.

Rhesus Monkey

Table 11. Rhesus monkey samples tested in the Chemokine Panel 1 (NHP) Kit

Sample Type	Statistic	MIP-1β	Eotaxin-3	TARC	IP-10	MIP-1α	IL-8	MCP-1	MDC	MCP-4*
Serum (N=30)	Median (pg/mL)	90.0	13.0	3.60	164	24.6	700	248	115	9.30
	Range (pg/mL)	35.8–146	13.0	1.03–43.0	59.4–397	15.7–122	395–8484	97.9–760	8.81–443	5.80–34.3
	% Detected	100	3	100	100	97	50	100	100	100
EDTA Plasma (N=30)	Median (pg/mL)	75.4	7.57	2.80	461	17.1	623	85.9	311	6.6
	Range (pg/mL)	46.3–184	7.57–7.57	1.29–11.8	195–4204	13.0–27.5	406–1213	54.5–274	96.1–1127	2.70–85.1
	% Detected	100	3	100	100	80	50	100	100	80
Urine (N=30)	Median (pg/mL)	ND	ND	1.23	ND	ND	392	24.9	ND	ND
	Range (pg/mL)	ND	ND	1.23	ND	ND	392	3.11–138	ND	ND
	% Detected	0	0	3	0	0	3	100	0	0

% detected = % of samples with concentrations at or above the LLOD
ND = Not detectable
*MCP-4 was tested using N=5 for serum, EDTA plasma, and urine

Cynomolgus Monkey

Table 12. Rhesus monkey samples tested in the Chemokine Panel 1 (NHP) Kit

Sample Type	Statistic	MIP-1β	Eotaxin-3	TARC	IP-10	MIP-1α	IL-8	MCP-1	MDC	MCP-4*
Serum (N=30)	Median (pg/mL)	165	ND	6.01	426	156	ND	504	356	14.7
	Range (pg/mL)	86.0–1172	ND	1.44–46.2	65.4–3764	13.4–1170	ND	96.5–1614	125–1398	9.60–47.5
	% Detected	100	0	93	100	100	0	100	97	100
EDTA Plasma (N=30)	Median (pg/mL)	85.3	ND	3.28	397	24.2	628	117	365	3.9
	Range (pg/mL)	14.4–178	ND	1.24–14.0	161–1458	14.0–75.1	486–1378	55.0–230	165–647	2.60–42.8
	% Detected	100	0	100	100	90	10	100	100	100
Urine (N=30)	Median (pg/mL)	1.72	ND	1.09	2.26	ND	521	63.3	7.22	ND
	Range (pg/mL)	1.60–1.79	ND	0.93–1.50	1.74–12.2	ND	404–893	7.31–416	5.11–17.4	ND
	% Detected	10	0	33	60	0	67	100	40	0

% detected = % of samples with concentrations at or above the LLOD
ND = not detectable
*MCP-4 was tested using N=5 for serum, EDTA plasma, and urine

Stimulated Samples

Peripheral blood mononuclear cells (PBMC) from rhesus or cynomolgus monkeys were incubated at 37 °C either unstimulated or with stimulant [lipopolysaccharide (LPS), phytohaemagglutinin (PHA), pokeweed mitogen (PWM), or concanavalin A (Con A)]. Changes in cytokine production were assessed at 6, 24, and 48 hours. Specifically, we assessed changes occurring either spontaneously during cell culture or induced by stimulants.[10] The following tables summarize the maximum changes observed in cytokine production. When analytes were initially undetectable, calculations for fold increase were based on the assay's LLOD. At each time point, fold changes were calculated by normalizing the stimulated levels to cytokine levels from untreated controls rather than background levels from unconditioned cell culture media.

Rhesus Monkey

Table 13. Effect of stimulated rhesus monkey samples on cytokine production in the Chemokine Panel 1 (NHP) Kit

Assays*	ConA	LPS	PHA	PWM	Spontaneous
MIP-1β	–	–	+	+	+
Eotaxin-3	–	+	–	+	–
TARC	++	–	+	+	–
IP-10	++	+	+	+	+
MIP-1α	–	+	–	–	+
IL-8	+	+	+	++	++
MCP-1	+	+	+	+	+
MDC	–	+	+	–	+

*Data not available for MCP-4 assay

++ > 10-fold
+ > 2-fold
– No significant response

Cynomolgus Monkey

Table 14. Effect of stimulated cynomolgus monkey samples on cytokine production in the Chemokine Panel 1 (NHP) Kit

	ConA	LPS	PHA	PWM	Spontaneous
MIP-1β	+	+	+	++	++
Eotaxin-3	–	++	–	–	–
TARC	+	–	–	–	–
IP-10	+	+	+	+	+
MIP-1α	+	+	++	++	++
IL-8	+	+	+	++	+
MCP-1	+	+	+	+	–
MDC	+	+	+	+	+

*Data not available for MCP-4 assay

++ > 10-fold
+ > 2-fold
- No significant response

Assay Components

Calibrators

Chemokine calibrators are recombinant proteins encoding human sequences, which are highly homologous to chemokines in non-human primates. The assay calibrator blend uses the following recombinant human proteins:

Table 15. Recombinant human proteins used in the Calibrators

Calibrator	Expression System
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

Cross-reactivity to homologs in rhesus and cynomolgus monkeys has been verified for all antibodies in this kit.

Table 16. Antibody source species

	Source Species		
Analyte	MSD Capture Antibody	MSD Detection Antibody	Assay Generation
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

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10. Wang G, et al. Genetic polymorphism in chemokine CCL22 and susceptibility to Helicobacter pylori infection-related gastric carcinoma. Cancer. 2009;115:2430-7.

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).

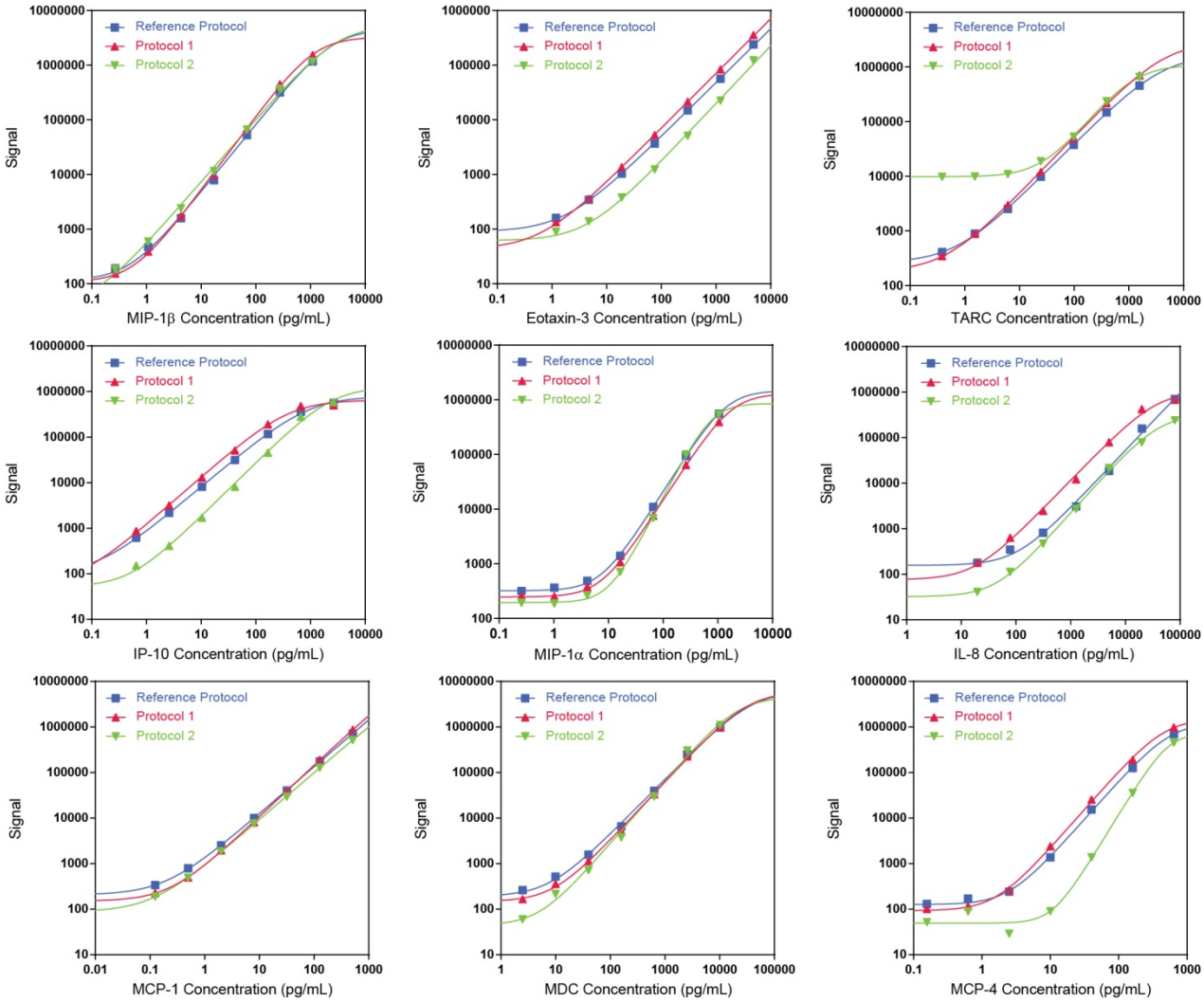

Table 17. Relative sensitivity when using alternative protocols

	LLOD Comparison (pg/mL)		
Assay*	**Reference Protocol**	**Protocol 1**	**Protocol 2**
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

Appendix B

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

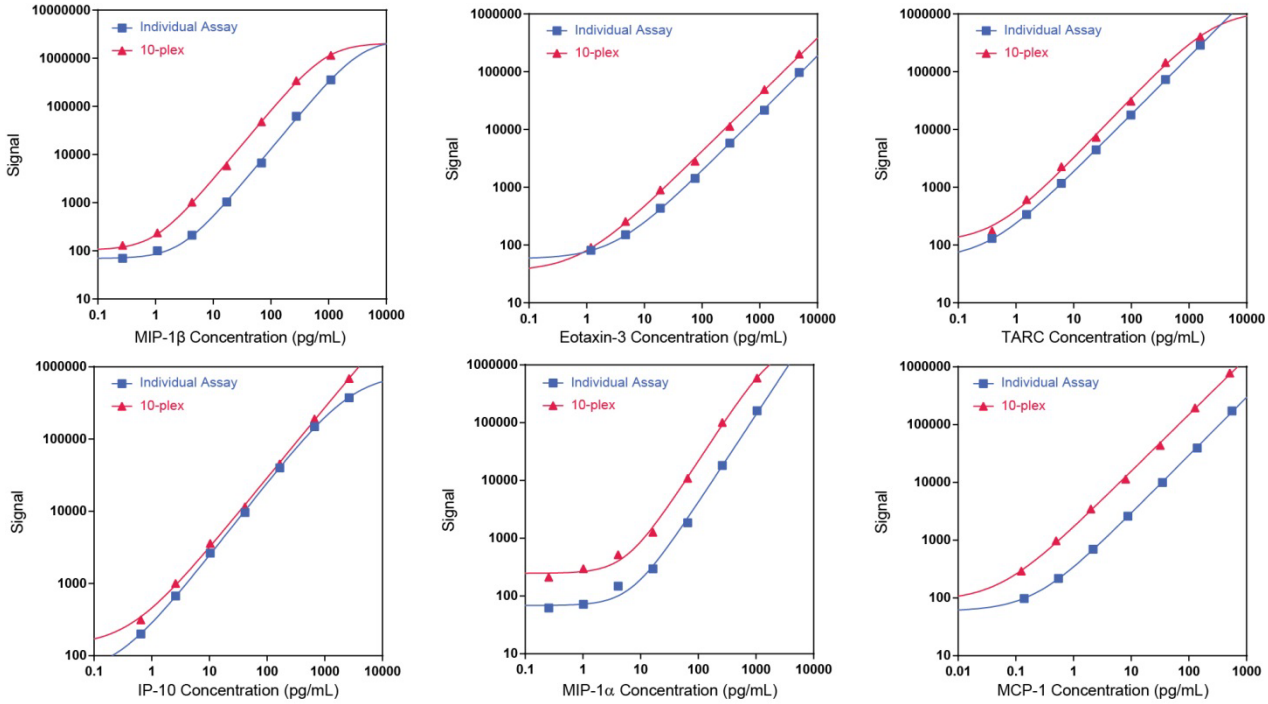

Table 18. Assay performance for individual and 10-plex assays

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 (NHP) is the IL-8 assay provided on single spot plates. Data are shown in the Proinflammatory Panel 1 (NHP) product insert.

Note: Assay performance for MCP-4 and MDC is not included since the individual assay is run on a multiplex plate.

	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

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).

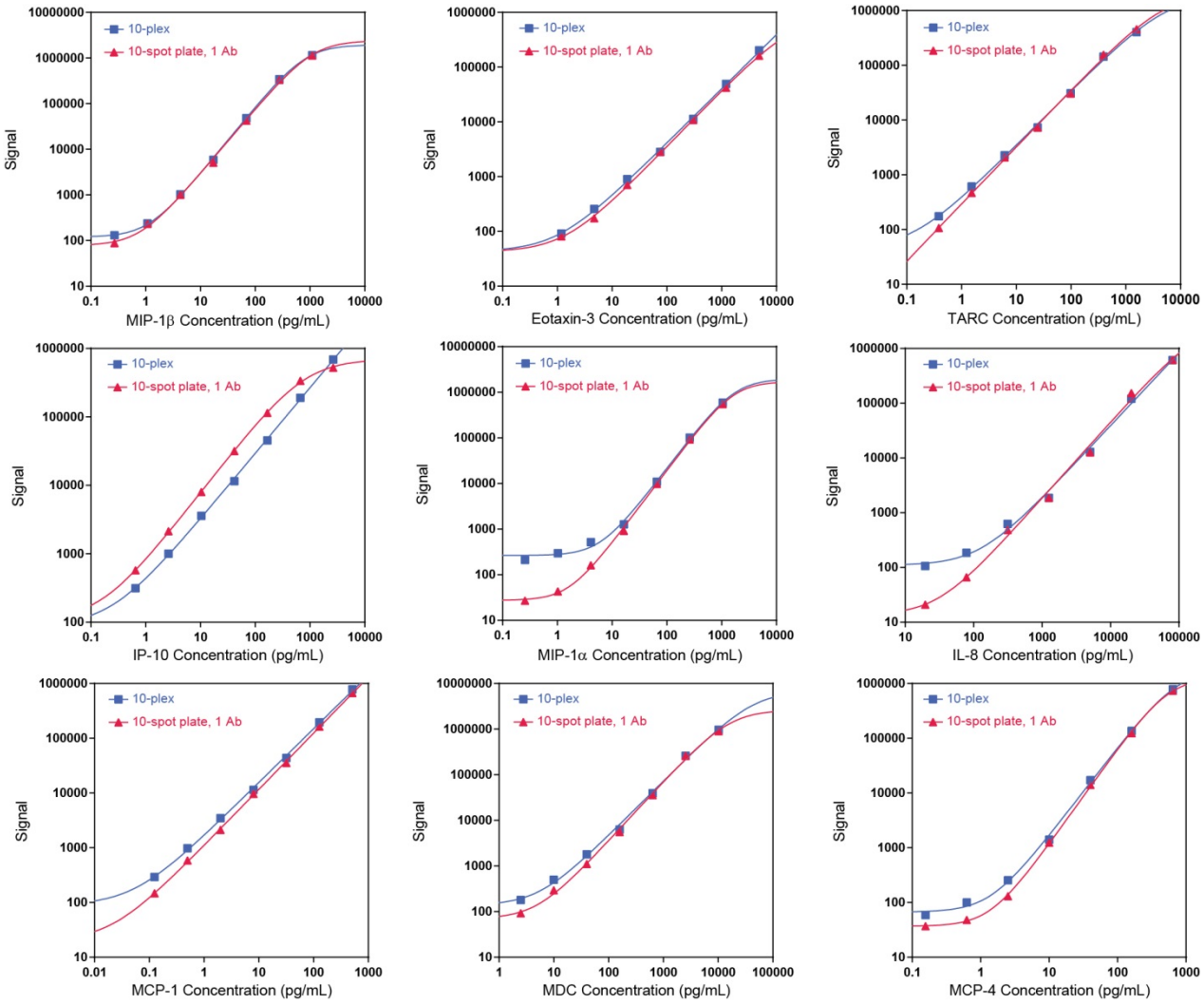

Table 19. LLODs for detection of a single antibody vs. blended antibodies

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
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	2.14	0.18

Summary Protocol

Chemokine Panel 1 (NHP) Kits

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

Sample and Reagent Preparation

- ❑ Bring all reagents to room temperature.
- ❑ Prepare calibration solutions in Diluent 43 using the supplied calibrator:
    - Reconstitute the lyophilized calibrator blend.
    - Invert 3 times, equilibrate 15-30 minutes at room temperature.
    - Vortex briefly using short pulses.
    - 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 a 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 the 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 20. Catalog numbers for V-PLEX and V-PLEX Plus chemokine (NHP) 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 (NHP)	K15055D-1	K15055D-2	K15055D-4	K15055G-1	K15055G-2	K15055G-4
Individual Assay Kits						
NHP MIP-1β	K156NRD-1	K156NRD-2	K156NRD-4	K156NRG-1	K156NRG-2	K156NRG-4
NHP Eotaxin-3	K156NUD-1	K156NUD-2	K156NUD-4	K156NUG-1	K156NUG-2	K156NUG-4
NHP TARC	K156NTD-1	K156NTD-2	K156NTD-4	K156NTG-1	K156NTG-2	K156NTG-4
NHP IP-10	K156NVD-1	K156NVD-2	K156NVD-4	K156NVG-1	K156NVG-2	K156NVG-4
NHP MIP-1α	K156NQD-1	K156NQD-2	K156NQD-4	K156NQG-1	K156NQG-2	K156NQG-4
NHP IL-8	K156RAD-1	K156RAD-2	K156RAD-4	K156RAG-1	K156RAG-2	K156RAG-4
NHP MCP-1	K156NND-1	K156NND-2	K156NND-4	K156NNG-1	K156NNG-2	K156NNG-4
NHP MDC	K156NPD-1	K156NPD-2	K156NPD-4	K156NPG-1	K156NPG-2	K156NPG-4
NHP MCP-4	K156NOD-1	K156NOD-2	K156NOD-4	K156NOG-1	K156NOG-2	K156NOG-4

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

Plate Diagram

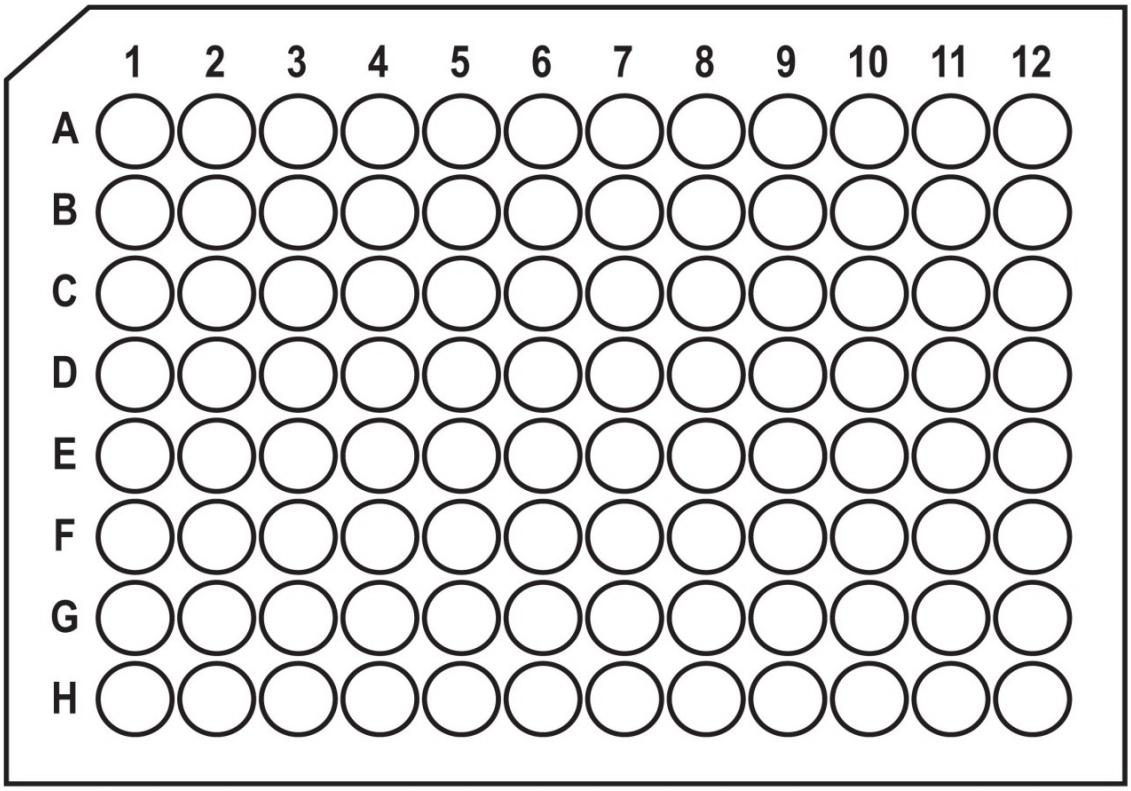

Figure 5. Plate diagram.

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 6. 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.