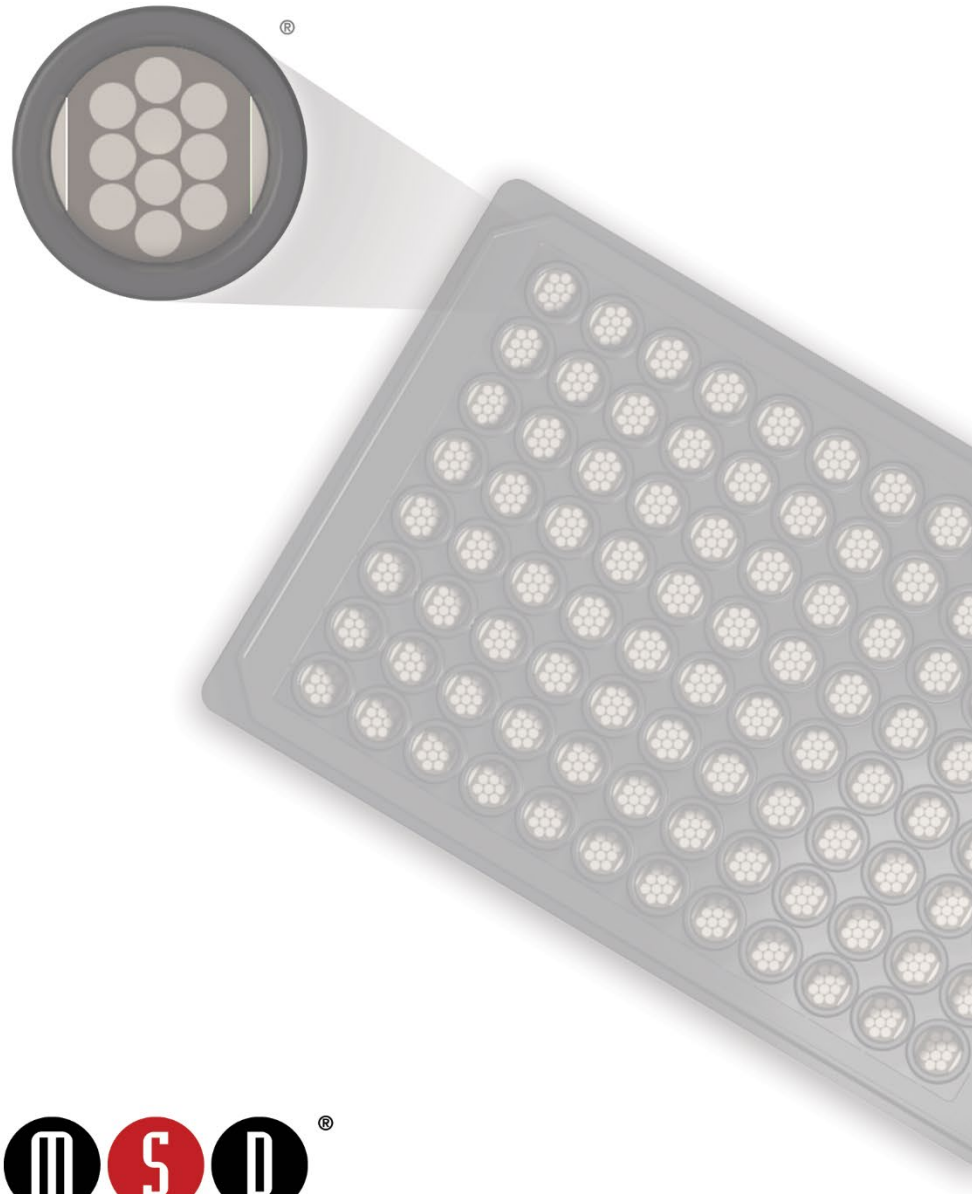
MSD® MULTI-SPOT Assay System

Chemokine Panel 1 Gen. B Kits

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

V-PLEX®



MSD®

MSD Cytokine Assays

Chemokine Panel 1 (human) Gen. B Kits

Chemokine Panel 1 (NHP) Gen. B Kits

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

For use with human serum, plasma, cerebral spinal fluid (CSF), urine, and cell culture supernatants, as well as non-human primate (NHP) 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 following MSD's Quality Management System. They offer exceptional sensitivity, simple protocols, reproducible results, and lot-to-lot consistency. In addition to the analytical validation, the 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 on 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 of 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 migrating immune cells to the infection site,[4] while the homeostatic chemokines recruit cells for tissue maintenance and development.[5] Chemokines are associated with several diseases.[6,7] Due to their association with a wide spectrum of diseases, these biomarkers are the subjects of drug discovery projects and basic research. The Chemokine Panel 1 (human) and (NHP) Gen. B kits consist of eight CC chemokine assays (Eotaxin, MIP-1β, Eotaxin-3, TARC, MIP-1α, MCP-1, MDC, MCP-4) and one CXC chemokine assays (IP-10). The Chemokine Panel 1 (NHP) Gen. B employs anti-human detection and capture antibodies that react with rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) monkeys. These assays may be suitable for primate species in addition to *Macaca mulatta* and *Macaca fascicularis* since human chemokines are broadly homologous with chemokines from NHPs.[9] Chemokine Panel 1 Gen. B kits are validated for sample types across human and NHP species, and assays are available as species-specific Chemokine Panels and Singleplex individual kits.

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 Gen. B kits 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, 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® GOLD Read Buffer B 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]

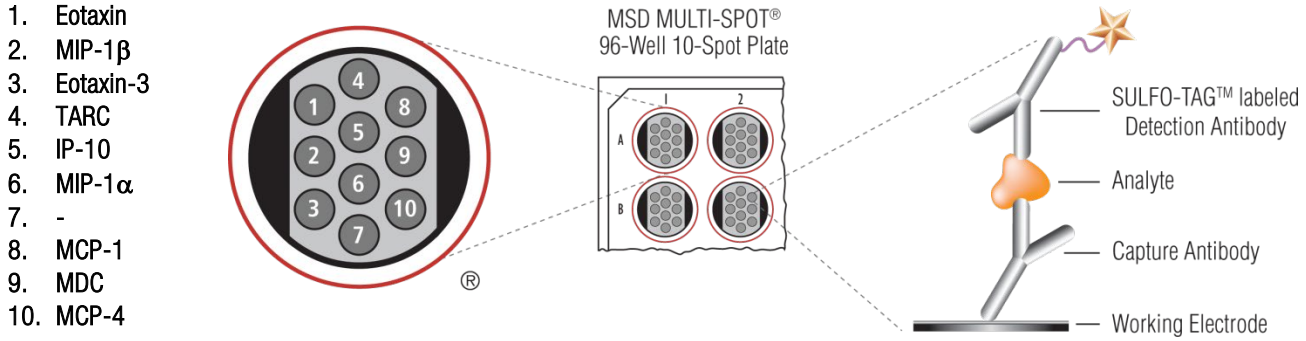

Figure 1. Multiplex plate spot diagram showing the placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

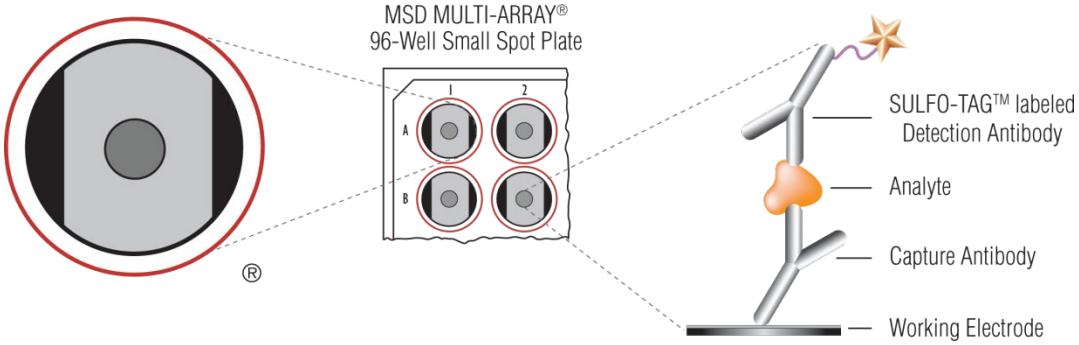

Figure 2. Small Spot plate diagram showing the placement of analyte capture antibodies.

Kit Components

Chemokine Panel 1 (human) and (NHP) Gen. B assays are available as multiplex, individual, and 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 a complete list of kits.

Reagents Supplied With All Kits

Table 1. Reagents that are supplied with V-PLEX and V-PLEX Plus Chemokine Panel 1 Gen. B Kits

Reagent	Storage	Catalog No.	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Chemokine Panel 1 (human) Gen. B Calibrator Blend	2–8 °C	C0705-2	1 vial	1 vial	5 vials	25 vials	Lyophilized kit calibrator
Diluent 57‡	≤-10 °C	R50BZ-1	10 mL	1 bottle	—	—	Diluent for samples and calibrator
		R50BZ-2	50 mL	—	1 bottle	5 bottles	
Diluent 3‡	≤-10 °C	R50AP-1	8 mL	1 bottle	—	—	Diluent for detection antibody
		R50AP-2	40 mL	—	1 bottle	5 bottles	
MSD GOLD™ Read Buffer B	RT	R60AM-1	18 mL	1 bottle	—	—	Buffer to catalyze the electro-chemiluminescence reaction
		R60AM-2	90 mL	—	1 bottle	5 bottles	

‡Diluent 57, 10 mL and Diluent 3, 8 mL are provied as Diluent Assembly 33 (Catalog No. R50CG-1) whereas Diluent 57, 50 mL and Diluent 3, 40 mL are provied as Diluent Assembly 34 (Catalog No. R50CH-1).
RT = room temperature
Dash (—) = not applicable

V-PLEX Plus Kits: Additional Components

Table 2. Additional components that are supplied with V-PLEX Plus Chemokine Panel 1 Gen. B Kits

Reagents	Storage	Catalog No.	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Chemokine Panel 1 (human) Gen. B Control 1*	2–8 °C	—	1 vial	1 vial	5 vials	25 vials	Multi-analyte controls
Chemokine Panel 1 (human) Gen. B Control 2*	2–8 °C		1 vial	1 vial	5 vials	25 vials	
Chemokine Panel 1 (human) Gen. B Control 3*	2–8 °C		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 plate wash buffer solution
Plate Seals	—	—	—	3	15	75	Adhesive seals for sealing plates during incubations

*Controls are provided as components in the Chemokine Panel 1 (human) Gen. B Control Pack (Catalog No. C4705-1, 5-plate size pack). Refer to the control pack insert for more details.
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			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Chemokine Panel 1 (human) Gen. B SECTOR Plate	2–8 °C	N05705A-1	10-spot	1	5	25	96-well plate, foil sealed, with desiccant
Human MIP-1β Gen. B SECTOR Plate	2–8 °C	L45ANHA-1	Small Spot	1	5	25	
Human Eotaxin-3 Gen. B SECTOR Plate	2–8 °C	L45ANJA-1	Small Spot	1	5	25	
Human TARC Gen. B SECTOR Plate	2–8 °C	L45ANKA-1	Small Spot	1	5	25	
Human IP-10 Gen. B SECTOR Plate Plate	2–8 °C	L45ANLA-1	Small Spot	1	5	25	
Human MIP-1α Gen. B SECTOR Plate	2–8 °C	L45ANMA-1	Small Spot	1	5	25	
Human MCP-1 Gen. B SECTOR Plate	2–8 °C	L45ANPA-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			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Human Eotaxin Antibody	2–8 °C	D21ANG-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D21ANG-3	375 µL	—	1	5	
Human MIP-1β Antibody	2–8 °C	D21ANH-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D21ANH-3	375 µL	—	1	5	
Human Eotaxin-3 Antibody	2–8 °C	D21ANJ-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D21ANJ-3	375 µL	—	1	5	
Human TARC Antibody	2–8 °C	D21ANK-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D21ANK-3	375 µL	—	1	5	
Human IP-10 Antibody	2–8 °C	D21ANL-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D21ANL-3	375 µL	—	1	5	
Human MIP-1α Antibody	2–8 °C	D21ANM-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D21ANM-3	375 µL	—	1	5	
Human MCP-1 Antibody	2–8 °C	D21ANP-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D21ANP-3	375 µL	—	1	5	
Human MDC Antibody	2–8 °C	D21ANQ-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D21ANQ-3	375 µL	—	1	5	
Human MCP-4 Antibody	2–8 °C	D21ANR-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D21ANR-3	375 µL	—	1	5	

Dash (—) = not applicable

Additional Materials and Equipment

- ❑ Appropriately sized tubes for reagent preparation
- ❑ Polypropylene microcentrifuge tubes for preparing dilutions
- ❑ Liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL/well into a 96-well microtiter plate
- ❑ Plate washing equipment: automated plate washer or multichannel pipette
- ❑ Microtiter plate shaker (rotary) capable of shaking at 500–1,000 rpm
- ❑ 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) Gen. B Control Pack, available for separate purchase from MSD, catalog no. C4705-1 (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 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 22–25 °C water bath before use. Thaw other reagents on wet ice and use them immediately.
- 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 need not 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 the 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.
- 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 seal before reading the plate.
- Read buffer should be at room temperature (20-26 ºC) when adding it to the plate.
- Do not shake the plate after adding the read buffer.
- Keep time intervals consistent between the addition of the read buffer and reading the plate to improve inter-plate precision. It is recommended that an MSD instrument be prepared to read a plate before adding the read buffer. Unless otherwise directed, read the plate as soon as possible after adding the read buffer.
- If the sample results are above the top of the calibration curve, dilute the samples and repeat the assay.

Reagent Preparation and Protocol

Bring all reagents to room temperature.

Important: Upon the first thaw, aliquot Diluent 57 and Diluent 3 into suitable volumes before refreezing.

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.

For one plate, combine:

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

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 57. (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 Dlluent 57 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 Dlluent 57 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 Dlluent 57. Mix well by vortexing. Repeat 4-fold serial dilutions 5 additional times to generate 7 calibrators.
3) Use Diluent 57 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.

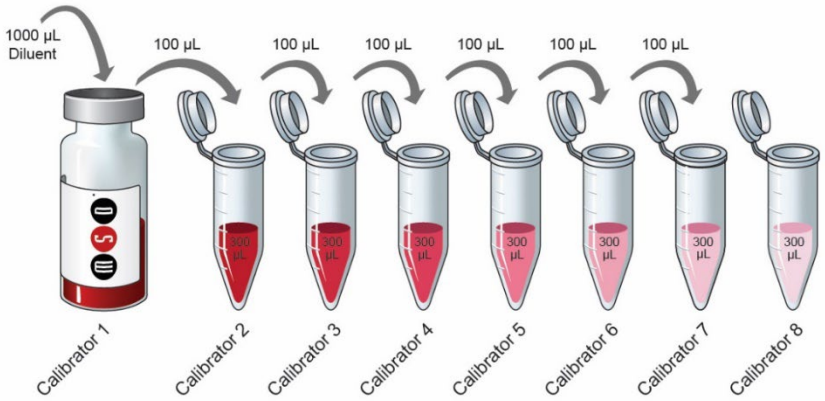

Figure 3. Dilution schema for preparation of Calibrator Standards.

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 ≤-70 °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 57. For human serum, plasma, and urine samples and NHP serum and plasma, MSD recommends a minimum 4-fold dilution. For example, when running samples in duplicate, add 50 µL of sample to 150 µL of Diluent 57. We recommend running at least two replicates per sample. When running unreplicated samples, add 25 µL of sample to 75 µL of Diluent 57. 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 diluents 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) Gen. B Control Pack, catalog no. C4705-1. (Controls are included only in V-PLEX Plus kits.)

Reconstitute the lyophilized controls in 250 µL of Diluent 57. 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 57. Vortex briefly using short pulses. For the lot-specific concentration of each analyte in the control, refer to the Chemokine Panel 1 (human) Gen. B Control Pack COA for analyte levels. Reconstituted controls must be stored frozen. They are stable through three freeze-thaw cycles.

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. Prewash plates before use as recommended in the assay protocol.

❖ 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.

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 Human Eotaxin Antibody
- ❑ 60 µL of SULFO-TAG Human MIP-1β Antibody
- ❑ 60 µL of SULFO-TAG Human Eotaxin-3 Antibody
- ❑ 60 µL of SULFO-TAG Human TARC Antibody
- ❑ 60 µL of SULFO-TAG Human IP-10 Antibody
- ❑ 60 µL of SULFO-TAG Human MIP-1α Antibody
- ❑ 60 µL of SULFO-TAG Human MCP-1 Antibody
- ❑ 60 µL of SULFO-TAG Human MDC Antibody
- ❑ 60 µL of SULFO-TAG Human MCP-4 Antibody

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.

❖ PROTOCOL 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.

Prepare MSD GOLD Read Buffer B

MSD provides MSD GOLD Read Buffer B ready for use. Do not dilute.

Note: Unlike Read Buffer T, which is provided at 4X concentration, MSD GOLD Read Buffer B is provided at the working concentration for the assay. Diluting MSD GOLD Read Buffer B may compromise assay results.

❖ STEP 3: Wash and Read

- ❑ Wash the plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 150 µL of MSD GOLD Read Buffer B to each well. Analyze the plate on an MSD instrument.

Summary 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 MSD GOLD Read Buffer B to each well. 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 similar to the recommended protocol (data not shown).

Validation

V-PLEX products are validated according to fit-for-purpose principles[8] 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 assessing all validation lots.

➢ Accuracy and Precision

Accuracy and precision are evaluated by measuring calibrators and controls across multiple runs and lots. For most assays, the results of control measurements fall within 20% of the expected concentration for each run (25% for Eotaxin-3 and IP-10). Precision is reported as the coefficient of variation (CV). Intra-run CVs and inter-run CVs are below 20%. Rigorous management of inter-lot reagent consistency and calibrator production results in typical inter-lot CVs below 20%. 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 20%.

➢ Matrix Effects and Samples

Matrix effects from serum, plasma, urine, and cell culture media are measured for 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. Depending on the samples, some assays may benefit from lower or higher dilution factors.

- **Specificity**

  The specificity of both capture and detection antibodies is measured during assay development. Antibody specificity is assessed by 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 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 the specific signal is greater than 50,000 counts.

- **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 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 60 months from manufacture.

Representative data from the validation studies are presented in the following sections. The stock concentration of calibrator, measured limits of quantification, and specificity data for each lot can be found in the lot-specific COA 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® and Methodical Mind 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 Gen. B kits were collected over three months of testing by multiple operators (93 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. 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. Appendix D and E provide sample concordance data between the assays in the V-PLEX Chemokine Panel 1 and V-PLEX Chemokine Panel 1 Gen. B kits.

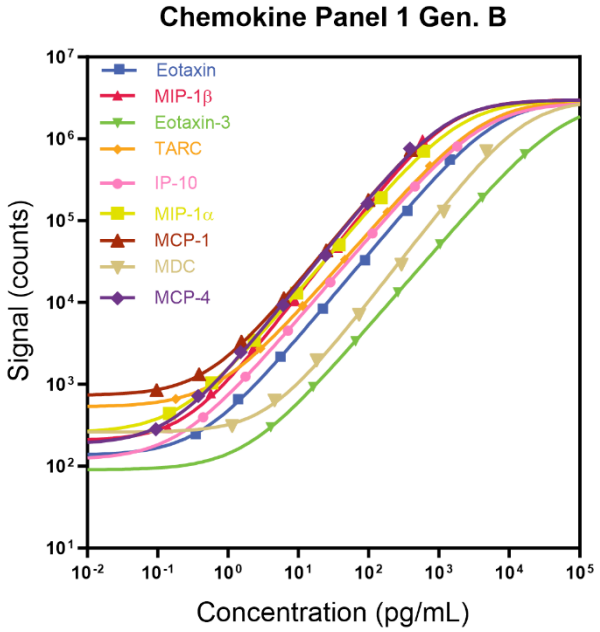

Figure 4. Typical calibration curves for assays in the V-PLEX Chemokine Panel 1 Gen. B Kits.

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 93 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 (25% for 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.

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 included with each kit and available at www.mesoscale.com.

Table 5. LLOD, LLOQ, and ULOQ for each analyte in the Chemokine Panel 1 Gen. B Kits

	Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
Eotaxin	0.20	0.13–0.61	2.14	910
MIP-1β	0.09	0.04–0.25	1.20	390
Eotaxin-3	1.44	0.89–2.21	11.1	11,100
TARC	0.11	0.06–1.28	2.14	481
IP-10	0.12	0.06–0.60	1.29	1,240
MIP-1α	0.05	0.03–0.16	0.357	403
MCP-1	0.16	0.03–2.60	1.45	247
MDC	1.00	0.59–19.50	6.43	2,930
MCP-4	0.05	0.03–0.50	0.518	234

Precision

Precision was evaluated using the Chemokine Panel 1 (human) Gen. B Controls 1, 2, and 3. Analyte levels were measured by a minimum of three replicates on 48 runs over multiple days. The results are shown below. While a typical specification for precision is a concentration CV of less than 20% for controls on both intra- and inter-day runs, for this panel, the data shows all assays are below 15%, most are below 10%.

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 12 runs, averaged across three kit lots.

Inter-lot %CV is the variability of controls across three kit lots (48 runs).

Table 6. Intra-run and inter-run %CVs for each analyte in the Chemokine Panel 1 Gen. B Kits

	Control	Average Conc. (pg/mL)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
Eotaxin	Control 1	1,174	3.9	6.1	7.6
	Control 2	178	2.6	4.1	5.8
	Control 3	29	3.6	5.8	6.8
MIP-1β	Control 1	495	2.6	4.0	6.6
	Control 2	109	2.7	3.6	3.8
	Control 3	25	2.9	4.6	4.4
Eotaxin-3	Control 1	12,758	4.7	6.0	10.5
	Control 2	1,245	4.2	6.6	8.0
	Control 3	127	3.8	10.3	7.1
TARC	Control 1	592	3.9	6.9	9.8
	Control 2	93	3.4	6.8	10.1
	Control 3	16	3.4	8.4	11.9
IP-10	Control 1	1,618	3.1	7.7	10.0
	Control 2	175	2.6	7.0	10.4
	Control 3	19	2.6	8.4	11.3
MIP-1α	Control 1	518	2.2	4.1	7.9
	Control 2	46	2.4	4.3	6.8
	Control 3	5	2.5	7.8	7.6
MCP-1	Control 1	316	2.7	5.3	7.4
	Control 2	47	2.4	6.3	6.5
	Control 3	7	3.3	6.3	6.9
MDC	Control 1	3,723	3.4	5.6	8.3
	Control 2	528	3.2	4.5	6.1
	Control 3	86	3.2	7.1	7.3
MCP-4	Control 1	308	4.9	7.7	9.3
	Control 2	41	6.4	11.3	10.6
	Control 3	7	6.0	7.1	9.1

Tested Samples (Human)

Normal Samples

Normal human serum, EDTA plasma, heparin plasma, citrate plasma, urine, and CSF 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. The median and range are calculated from samples with concentrations at or above the LLOD. The percentage detected is the percentage of samples with concentrations at or above the LLOD.

Table 7. Normal human samples tested in the Chemokine Panel 1 (human) Gen. B Kit

Sample Type	Statistic	Eotaxin	MIP-1β	Eotaxin-3	TARC	IP-10	MIP-1α	MCP-1	MDC	MCP-4
Serum (N=10)	Median (pg/mL)	158	63.9	16.8	343	127	6.47	209	752	132
	Range (pg/mL)	27.9–486	19.7–496	12.2–32.5	174–1,230	68.0–428	3.70–27.6	127–357	509–2,630	48.2–191
	% Detected	100	100	90	100	100	100	100	100	100
EDTA Plasma (N=10)	Median (pg/mL)	140	37.9	21.1	209	219	4.74	69.5	846	64.5
	Range (pg/mL)	47.2–256	15.2–486	8.03–34.3	75.7–410	118–853	2.66–25.6	51.5–109	585–2,300	30.9–102
	% Detected	100	100	100	100	100	100	100	100	100
Heparin Plasma (N=10)	Median (pg/mL)	684	34.7	125	96.3	156	4.02	124	482	248
	Range (pg/mL)	9.40–1,230	16.8–59.0	6.23–351	5.28–173	1.44–625	0.31–10.5	1.49–177	13.9–1,640	6.00–389
	% Detected	100	100	90	100	100	100	100	100	100
Citrate Plasma (N=10)	Median (pg/mL)	121	29.1	14.3	82.1	190	3.63	71.5	674	43
	Range (pg/mL)	59.3–229	12.8–493	7.56–22.1	30.9–140	98.0–770	2.35–25.1	56.0–98.3	483–2,310	27.0–67.7
	% Detected	100	100	100	100	100	100	100	100	100
Urine (N=10)	Median (pg/mL)	6.4	4.51	ND	1.94	17.4	0.53	205	4.89	7.86
	Range (pg/mL)	3.21–15.9	0.25–58.1	NA	1.94–1.94	1.76–41.1	0.22–1.62	6.55–598	3.59–10.3	0.31–38.6
	% Detected	90	100	0	20	90	80	100	80	100
CSF (N=10)	Median (pg/mL)	2.65	8.44	9.85	2.7	255	2.6	436	6.85	0.5
	Range (pg/mL)	0.96–8.15	3.86–20.5	9.32–10.38	2.03–112	47.8–9,990	0.64–14.1	115–891	3.72–42.3	0.22–5.25
	% Detected	100	100	20	90	100	100	100	80	100

ND = not detectable; NA = not available

Dilution Linearity (Human)

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

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

Table 8. Analyte percent recovery at various dilutions in each sample type in the Chemokine Panel 1 (human) Gen. B Kit

		Eotaxin		MIP-1β		Eotaxin-3		TARC		IP-10	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=6)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	8	111	103–122	101	100–103	96	92–105	103	91–121	111	107–118
	16	109	95–122	96	94–99	89	87–94	111	95–157	108	100–122
	32	111	96–126	95	93–99	83	82–87	112	87–187	104	94–121
EDTA Plasma (N=6)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	8	102	84–117	101	95–104	98	94–105	96	80–106	112	105–120
	16	105	87–117	97	89–101	95	90–108	99	81–114	117	109–123
	32	102	90–108	95	90–99	89	86–95	101	78–145	117	106–125
EDTA Plasma (N=6)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	8	98	93–103	104	91–144	101	96–105	100	85–118	104	100–111
	16	95	81–104	109	85–201	99	91–104	103	88–126	98	83–115
	32	93	84–99	120	77–270	96	88–104	100	87–119	93	78–110
Heparin Plasma (N=6)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	8	95	91–100	88	82–91	91	91–92	91	84–98	107	106–109
	16	92	88–95	83	80–85	83	80–87	91	83–114	105	100–111
	32	92	85–100	80	78–84	79	74–82	90	76–126	106	101–114
Citrate Plasma (N=6)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	8	95	82–107	94	89–101	104	95–113	105	92–124	97	86–122
	16	93	85–100	89	86–90	104	83–120	114	87–144	92	83–110
	32	91	82–100	87	81–90	101	81–117	107	75–142	91	81–114
Urine (N=6)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	8	107	102–111	94	91–99	101	97–106	100	95–106	117	113–119
	16	112	108–115	90	87–94	99	92–106	93	88–102	122	117–127
	32	109	100–116	86	80–94	95	85–106	91	79–112	122	118–126
CSF (N=6)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	8	99	90–110	96	86–101	97	91–105	96	87–108	113	95–125
	16	111	93–127	91	85–96	97	88–106	105	91–118	126	109–137
	32	111	96–128	91	87–94	95	88–103	96	83–111	133	111–149
Cell Culture Media (N=6)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	8	111	103–122	101	100–103	96	92–105	103	91–121	111	107–118
	16	109	95–122	96	94–99	89	87–94	111	95–157	108	100–122
	32	111	96–126	95	93–99	83	82–87	112	87–187	104	94–121

NA = not available

Table 8. continued

		MIP-1α		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=6)	4	100	NA	100	NA	100	NA	100	NA
	8	94	85–98	99	88–111	103	100–109	108	106–112
	16	92	88–96	99	95–113	102	100–107	112	108–116
	32	92	87–96	98	92–110	102	94–114	114	102–122
EDTA Plasma (N=6)	4	100	NA	100	NA	100	NA	100	NA
	8	97	92–99	102	98–106	100	88–108	107	98–114
	16	96	89–103	104	92–113	103	90–119	114	101–128
	32	95	88–100	105	95–114	104	94–116	114	101–124
EDTA Plasma (N=6)	4	100	NA	100	NA	100	NA	100	NA
	8	93	88–107	100	96–106	99	91–106	102	97–107
	16	88	82–102	99	92–105	100	87–110	99	89–106
	32	84	79–98	97	90–105	102	86–112	100	84–107
Heparin Plasma (N=6)	4	100	NA	100	NA	100	NA	100	NA
	8	90	87–92	93	88–95	94	91–99	104	102–108
	16	83	82–85	89	87–92	94	90–100	106	101–109
	32	78	75–81	86	83–88	96	89–106	111	103–116
Citrate Plasma (N=6)	4	100	NA	100	NA	100	NA	100	NA
	8	91	87–95	98	94–103	98	90–107	90	78–111
	16	86	84–88	98	89–109	100	95–103	87	76–103
	32	83	78–88	93	86–98	99	98–102	86	73–102
Urine (N=6)	4	100	NA	100	NA	100	NA	100	NA
	8	95	93–98	99	94–102	106	101–109	107	104–110
	16	91	88–95	96	88–103	110	105–112	110	106–112
	32	90	87–92	96	89–103	112	102–119	107	103–112
CSF (N=6)	4	100	NA	100	NA	100	NA	100	NA
	8	92	90–96	95	87–100	101	96–104	105	99–109
	16	91	87–95	97	88–102	112	107–120	113	105–120
	32	88	84–92	94	87–99	116	108–122	118	110–125
Cell Culture Media (N=6)	4	100	NA	100	NA	100	NA	100	NA
	8	94	85–98	99	88–111	103	100–109	108	106–112
	16	92	88–96	99	95–113	102	100–107	112	108–116
	32	92	87–96	98	92–110	102	94–114	114	102–122

NA = not available

Spike Recovery (Human)

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, urine, and CSF) were obtained from a commercial source. These samples and cell culture media were spiked with calibrators at three levels (high, mid, and low) and then diluted 4-fold. The average percent recovery for each sample type is reported along with %CV and % recovery range.

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

Table 9. Spike recovery measurements of different sample types in the Chemokine Panel 1 (human) Gen. B Kit

	Serum (N=6)			EDTA Plasma (N=6)			Heparin Plasma (N=6)			Citrate Plasma (N=6)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
Eotaxin	92	6.7	83–106	97	6.6	87–107	104	4.5	99–108	94	6.6	86–108
MIP-1β	100	3.4	94–107	100	10.3	74–113	87	12.4	67–100	99	7.3	86–108
Eotaxin-3	117	7.0	101–132	115	5.9	102–124	113	6.1	96–125	119	4.2	111–128
TARC	130	11.2	111–148	111	18.3	65–127	102	15.1	81–125	104	18.9	60–121
IP-10	91	9.6	75–106	76	8.6	62–83	95	15.4	65–111	81	9.9	68–92
MIP-1α	117	4.2	110–127	115	5.9	103–126	109	11.9	80–122	116	3.1	110–124
MCP-1	100	5.0	96–106	72	12.6	63–88	82	10.3	70–92	74	15.4	66–91
MDC	103	7.2	88–113	105	6.9	90–119	93	17.5	76–135	97	5.8	85–104
MCP-4	83	3.1	79–87	82	4.0	77–87	87	4.4	84–89	78	5.4	73–87

	Urine (N=6)			CSF (N=6)			Cell Culture Media (N=3)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
Eotaxin	120	7.9	98–136	100	5.6	92–110	84	8.5	72–99
MIP-1β	114	3.9	105–120	101	6.2	90–111	79	9.8	73–101
Eotaxin-3	89	8.9	72–102	86	14.0	60–98	79	7.9	71–93
TARC	100	18.2	68–136	122	10.6	103–159	100	9.3	89–119
IP-10	109	14.8	82–133	75	8.5	67–83	69	11.4	53–85
MIP-1α	130	5.3	115–141	118	3.9	110–126	88	10.9	79–111
MCP-1	113	11.7	96–129	101	NA	NA	85	8.0	77–98
MDC	102	8.3	84–115	95	6.7	84–104	85	9.0	74–98
MCP-4	123	12.1	97–142	94	5.3	86–103	73	8.7	66–87

NA = not available

Tested Samples (NHP)

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. The median and range are calculated from samples with concentrations at or above the LLOD. The percentage detected is the percentage of samples with concentrations at or above the LLOD.

Rhesus Monkey

Table 10. Rhesus monkey samples tested in the Chemokine Panel 1 (NHP) Gen. B Kit

Sample Type	Statistic	Eotaxin	MIP-1β	Eotaxin-3	TARC	IP-10	MIP-1α	MCP-1	MDC	MCP-4
Serum (N=5)	Median (pg/mL)	217	118	ND	1.09	472	11.2	180	238	21
	Range (pg/mL)	38.1–233	78.9–167	NA	NA	279–920	7.33–15.3	147–327	108–421	5.10–33.7
	% Detected	100	100	0	20	100	100	100	100	100
EDTA Plasma (N=5)	Median (pg/mL)	47.7	77.6	ND	1.7	492	1.48	55.5	164	8.08
	Range (pg/mL)	30.7–121	27.9–94.3	NA	1.45–1.94	366–603	1.20–2.57	35.1–87.3	128–313	5.81–18.3
	% Detected	100	100	0	40	100	100	100	100	100
Urine (N=5)	Median (pg/mL)	ND	ND	ND	ND	ND	ND	3.44	ND	ND
	Range (pg/mL)	NA	NA	NA	NA	NA	NA	2.49–4.40	NA	NA
	% Detected	0	0	0	0	0	0	40	0	0

ND = not detectable; NA = not available

Cynomolgus Monkey

Table 11. Cynomolgus monkey samples tested in the Chemokine Panel 1 (NHP) Gen. B Kit

Sample Type	Statistic	Eotaxin	MIP-1β	Eotaxin-3	TARC	IP-10	MIP-1α	MCP-1	MDC	MCP-4
Serum (N=5)	Median (pg/mL)	142	154	5.12	4.4	935	14.6	354	200	6.19
	Range (pg/mL)	69.3–221	109–188	NA	1.80–6.47	665–3,160	10.7–21.1	255–470	91.2–345	2.26–16.6
	% Detected	100	100	20	80	100	100	100	100	100
EDTA Plasma (N=5)	Median (pg/mL)	25.1	82.8	ND	2.1	914	1.08	70.9	294	4.03
	Range (pg/mL)	18.5–58.3	44.6–209	NA	1.64–3.86	427–1,760	0.76–2.69	55.4–102	253–962	1.98–6.66
	% Detected	100	100	0	100	100	100	100	100	100
Urine (N=5)	Median (pg/mL)	ND	ND	ND	ND	ND	ND	5.97	ND	0.13
	Range (pg/mL)	NA	NA	NA	NA	NA	NA	1.18–10.8	NA	NA
	% Detected	0	0	0	0	0	0	40	0	20

ND = not detectable; NA = not available

Dilution Linearity (NHP)

To assess linearity, commercially available serum, EDTA plasma, and urine from rhesus and cynomolgus monkeys were spiked with recombinant calibrators and diluted 4-fold, 8-fold, 16-fold, and 32-fold before testing. Percent recovery at each dilution level was normalized to the dilution-adjusted, 4-fold concentration. The average percent recovery shown below is based on samples within the quantitative range of the assay.

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

Rhesus Monkey

Table 12. Analyte percent recovery at various dilutions in different rhesus monkey sample types in the Chemokine Panel 1 (NHP) Gen. B Kit

		Eotaxin		MIP-1β		Eotaxin-3		TARC		IP-10	
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=3)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	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
EDTA Plasma (N=3)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	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
Urine (N=3)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	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

NA = not available

		MIP-1α		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=3)	4	100	NA	100	NA	100	NA	100	NA
	8	100	95–108	99	92–108	96	88–107	87	81–95
	16	100	91–109	101	91–111	101	90–117	87	80–93
	32	98	81–115	99	82–116	95	76–112	88	80–99
EDTA Plasma (N=3)	4	100	NA	100	NA	100	NA	100	NA
	8	102	97–112	73	63–82	94	83–108	76	65–90
	16	100	90–119	78	69–96	94	80–109	70	58–90
	32	101	93–121	83	73–107	88	74–107	70	58–94
Urine (N=3)	4	100	NA	100	NA	100	NA	100	NA
	8	94	91–96	95	91–105	90	84–93	84	79–89
	16	91	88–97	96	90–110	91	86–93	82	75–90
	32	89	86–91	99	92–113	85	78–94	81	75–89

NA = not available

Cynomolgus Monkey

Table 13. Analyte percent recovery at various dilutions in different cynomolgus monkey sample types in the Chemokine Panel 1 (NHP) Gen. B Kit

		Eotaxin		MIP-1β		Eotaxin-3		TARC		IP-10	
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=3)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	8	93	90-97	94	92-96	96	94-98	96	94-99	107	105-109
	16	91	87-95	93	90-95	91	90-92	100	96-107	103	101-106
	32	91	86-99	89	86-92	88	85-93	103	98-113	102	101-105
EDTA Plasma (N=3)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	8	102	100-105	97	93-103	95	93-96	94	91-100	114	113-115
	16	105	100-110	99	94-109	90	89-91	94	88-100	116	114-120
	32	104	100-109	99	86-110	86	84-88	90	89-94	116	114-119
Urine (N=3)	4	100	NA	100	NA	100	NA	100	NA	100	NA
	8	105	103-108	94	92-96	94	93-96	96	95-97	119	116-121
	16	110	104-114	92	91-92	94	92-95	97	94-101	129	127-132
	32	107	104-109	92	87-98	93	92-94	88	84-92	132	122-138

NA = not available

		MIP-1α		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=3)	4	100	NA	100	NA	100	NA	100	NA
	8	99	91-106	100	95-106	104	103-106	104	102-107
	16	102	90-117	101	97-103	111	110-112	108	102-117
	32	99	85-116	100	97-102	118	117-120	112	104-123
EDTA Plasma (N=3)	4	100	NA	100	NA	100	NA	100	NA
	8	95	94-97	100	96-107	102	100-103	115	109-124
	16	95	93-97	103	98-111	106	104-109	125	118-136
	32	91	88-92	99	93-109	106	104-111	131	122-146
Urine (N=3)	4	100	NA	100	NA	100	NA	100	NA
	8	91	90-92	101	99-103	103	101-105	110	108-111
	16	91	89-92	99	94-103	110	109-111	117	116-118
	32	87	86-89	103	97-110	114	111-115	120	117-123

NA = not available

Spike Recovery (NHP)

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 were spiked with calibrators at three levels (high, mid, and low) and then diluted 4-fold. The average percent recovery for each sample type is reported along with %CV and % recovery range.

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

Rhesus Monkey

Table 14. Spike recovery measurements of different rhesus monkey sample types in the Chemokine Panel 1 (NHP) Gen. B Kit

	Serum (N=3)			EDTA Plasma (N=3)			Urine (N=3)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
Eotaxin	103	17.6	78–125	116	36.7	55–154	65	17.8	46–80
MIP-1β	97	9.1	87–108	165	24.3	124–204	67	19.0	50–90
Eotaxin-3	92	22.5	52–112	97	61.3	43–218	71	16.2	54–86
TARC	77	25.4	46–104	98	66.1	44–224	80	18.1	61–102
IP-10	85	4.5	82–89	102	NA	NA	48	19.3	33–58
MIP-1α	87	22.6	49–105	103	63.4	47–246	78	20.7	56–101
MCP-1	88	NA	NA	106	27.3	85–139	70	17.8	52–86
MDC	75	26.9	43–95	95	42.5	54–147	68	21.5	46–89
MCP-4	80	15.7	57–92	89	50.5	44–148	60	19.2	42–74

NA = not available

Cynomolgus Monkey

Table 15. Spike and Recovery measurements of different cynomolgus monkey sample types in the Chemokine Panel 1 (NHP) Gen. B Kit

	Serum (N=3)			EDTA Plasma (N=3)			Urine (N=3)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
Eotaxin	92	16.0	76–107	98	68.0	40–227	84	17.0	62–100
MIP-1β	97	1.9	95–98	159	59.0	99–267	89	14.0	66–106
Eotaxin-3	86	21.0	51–108	107	69.0	53–288	89	16.0	65–103
TARC	79	22.0	49–100	99	76.0	37–286	104	12.0	82–116
IP-10	NA•	NA	NA	124	NA	NA	64	14.0	53–80
MIP-1α	79	24.0	59–110	104	70.0	48–284	98	13.0	76–115
MCP-1	NA•	NA	NA	110	52.0	72–175	86	16.0	70–104
MDC	74	15.0	60–93	135	59.0	82–225	87	16.0	73–110
MCP-4	72	17.0	54–88	77	73.0	32–198	78	15.0	65–93

*Cynomolgus serum samples had high level of endogenous IP-10 and MCP-1. Spiking with the calibrator resulted in a sample concentration outside of the quantifiable range.
NA = not available

Specificity

To assess specificity, each assay in the panel was tested individually. Nonspecific binding was less than 1% for all assays in the kit. Non-specificity reported in the COA for this panel is measured using blended calibrators and individual detection antibodies.

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

Stability

The reconstituted calibrator, controls, and diluents were tested for freeze-thaw stability. Results (not shown) demonstrated that the reconstituted calibrator, controls, Diluent 57, and Diluent 3 can go through three freeze-thaw cycles without significantly affecting the assay's performance. Once reconstituted, the multi-analyte calibrator is stable for one day at 2–8 °C. The validation study includes a real-time stability study with scheduled performance evaluations of complete kits for up to 60 months from 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 16. 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	0.000106
MCP-1	92/794	0.000636

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 17. 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
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 18. Antibody source species

	Source Species		
Analyte	MSD Capture Antibody	MSD Detection Antibody	Antibody Pair Generation
Eotaxin	Mouse Monoclonal	Mouse Monoclonal	C
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	C
MCP-1	Mouse Monoclonal	Mouse Monoclonal	B
MDC	Mouse Monoclonal	Mouse Monoclonal	B
MCP-4	Mouse Monoclonal	Mouse Monoclonal	C

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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).

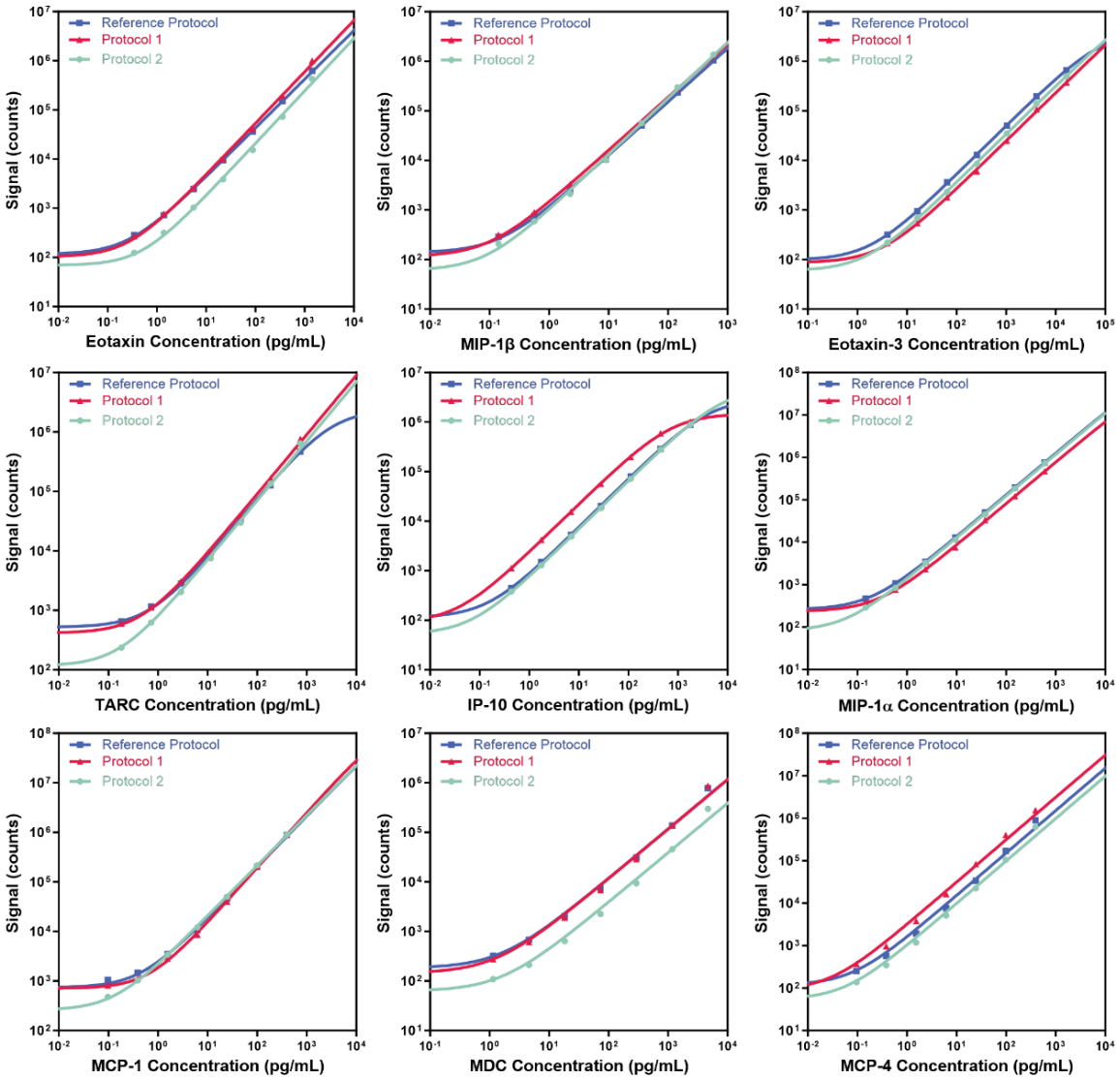

Table 19. Relative sensitivity when using alternate protocols

	LLOD Comparison (pg/mL)		
Assay	Reference Protocol	Protocol 1	Protocol 2
Eotaxin	0.170	0.191	0.513
MIP-1β	0.088	0.068	0.110
Eotaxin-3	1.353	2.605	1.887
TARC	0.079	0.120	0.128
IP-10	0.095	0.030	0.099
MIP-1α	0.050	0.082	0.054
MCP-1	0.112	0.530	0.075
MDC	0.917	1.045	3.093
MCP-4	0.085	0.039	0.125

Appendix B

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

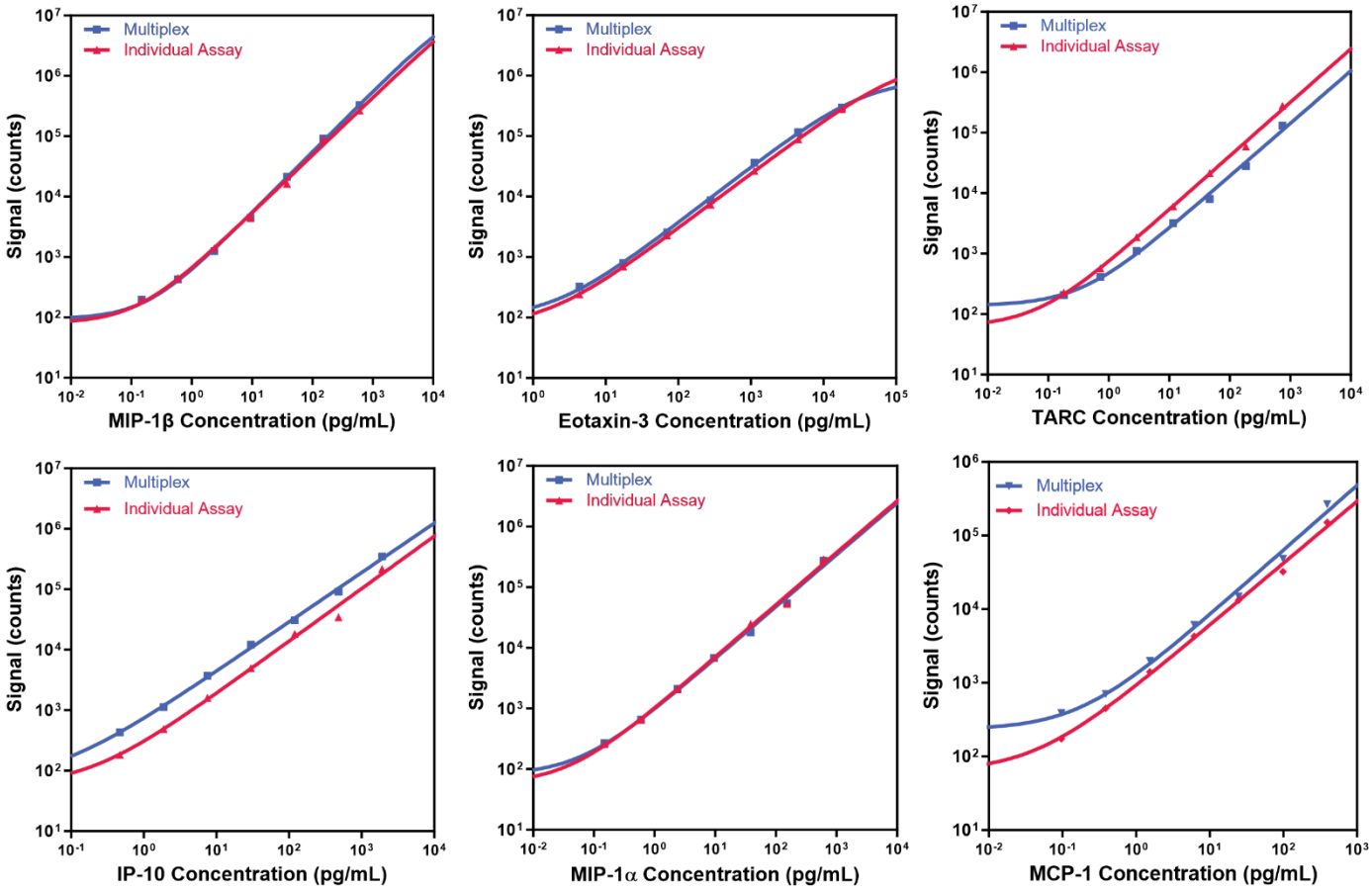

Table 20. Assay performance for individual and 9-plex assays

	LLOD (pg/mL)	
Assay	Individual	9-plex
MIP-1β	0.038	0.146
Eotaxin-3	0.506	1.561
TARC	0.023	0.302
IP-10	0.062	0.069
MIP-1α	0.013	0.052
MCP-1	0.014	0.261

In general, assays in the single spot format yielded a lower overall signal compared to the 9-plex format. The spots on single-spot plates have a larger binding surface than those on multiplex plates, but the same amount of calibrator was used for each test; therefore, the bound calibrator was spread over a larger surface area reducing the average signal.

Note: Assay performance for Eotaxin, MCP-4, and MDC is not included since the individual assays are provided on a multiplex plate.

Appendix C

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

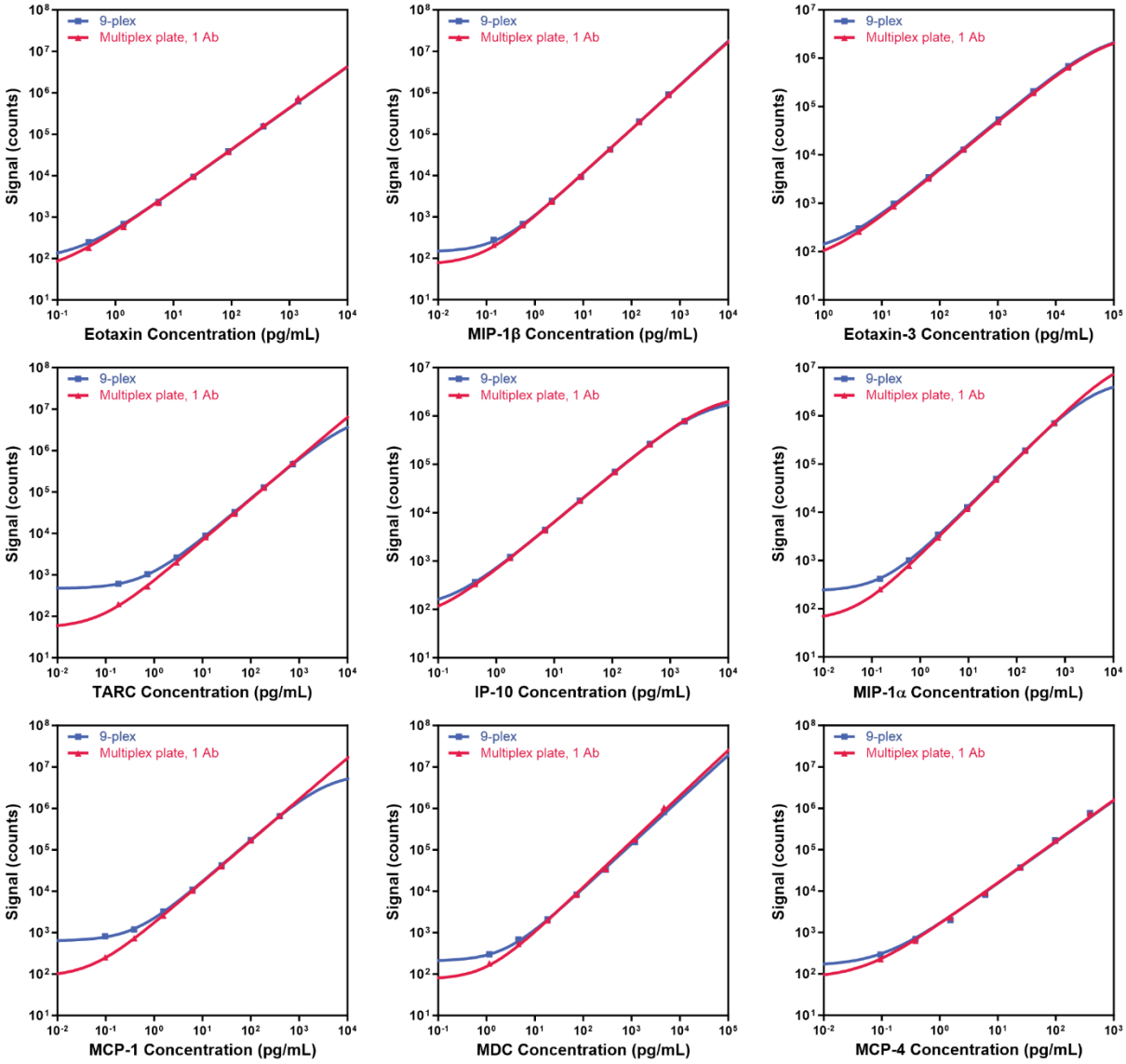

Table 21. LLODs for detection of a single antibody vs. blended antibodies

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	Multiplex plate, 1 Ab	9-plex
Eotaxin	0.213	0.183
MIP-1β	0.088	0.093
Eotaxin-3	1.461	1.417
TARC	0.109	0.136
IP-10	0.119	0.123
MIP-1α	0.059	0.056
MCP-1	0.046	0.062
MDC	0.967	0.923
MCP-4	0.058	0.097

Appendix D

Sample concordance was performed using serum, plasma, urine, and CSF samples between assays in the V-PLEX Chemokine Panel 1 (human) and V-PLEX Chemokine Panel 1 (human) Gen. B kits. Concordance was evaluated using slope (Deming regression) and r^2 (Pearson correlation).

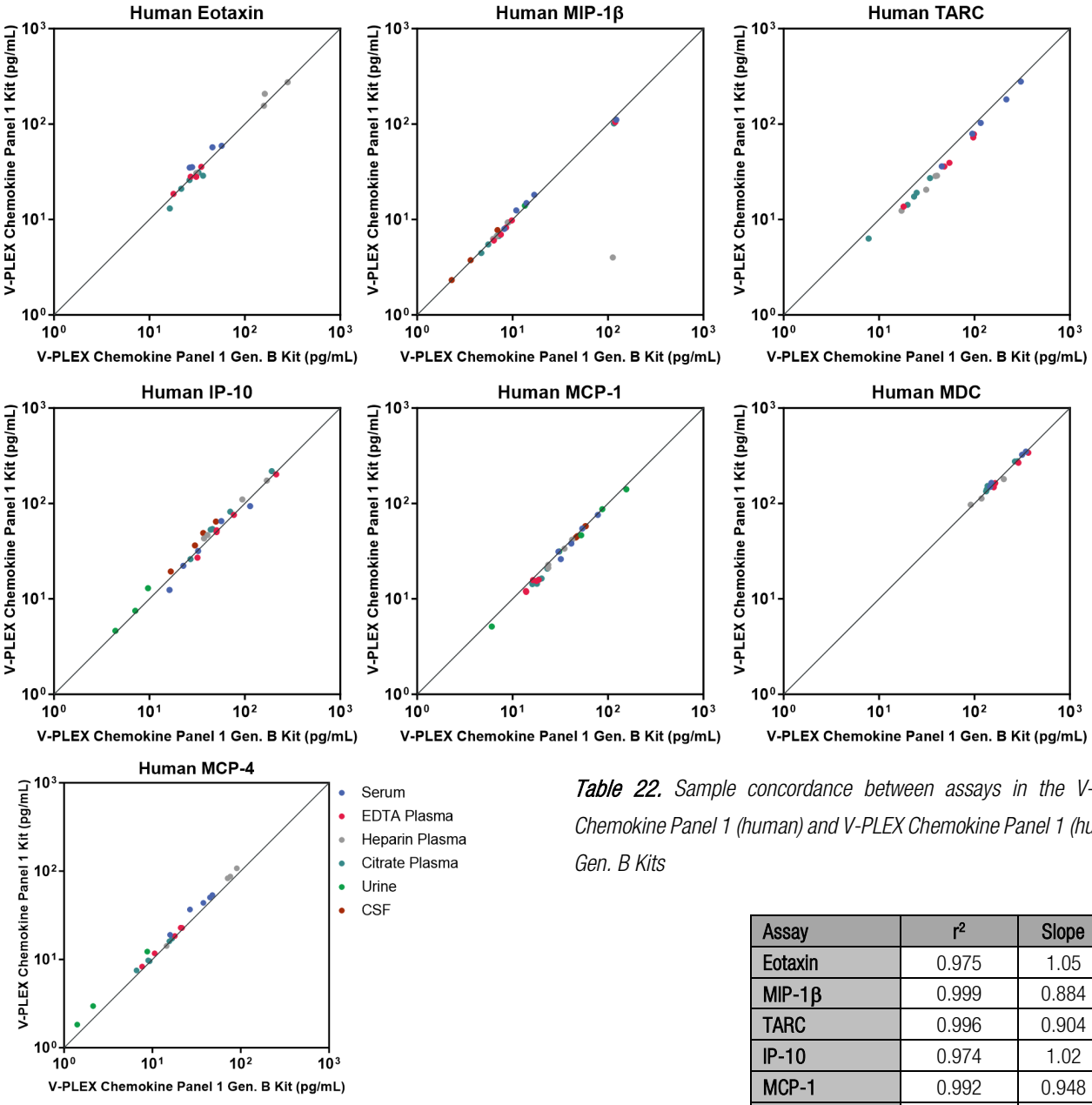

Table 22. Sample concordance between assays in the V-PLEX Chemokine Panel 1 (human) and V-PLEX Chemokine Panel 1 (human) Gen. B Kits

Assay	r^2	Slope
Eotaxin	0.975	1.05
MIP-1β	0.999	0.884
TARC	0.996	0.904
IP-10	0.974	1.02
MCP-1	0.992	0.948
MDC	0.981	0.955
MCP-4	0.996	1.17

Due to low quantification of Eotaxin-3 and MIP-1α in the V-PLEX Chemokine Panel 1 (human) kit, concordance was not calculated.

Appendix E

Sample concordance was performed using serum, plasma and urine samples between assays in the V-PLEX Chemokine Panel 1 (NHP) and V-PLEX Chemokine Panel 1 (NHP) Gen. B kits. Concordance was evaluated using slope (Deming regression) and r^2 (Pearson correlation).

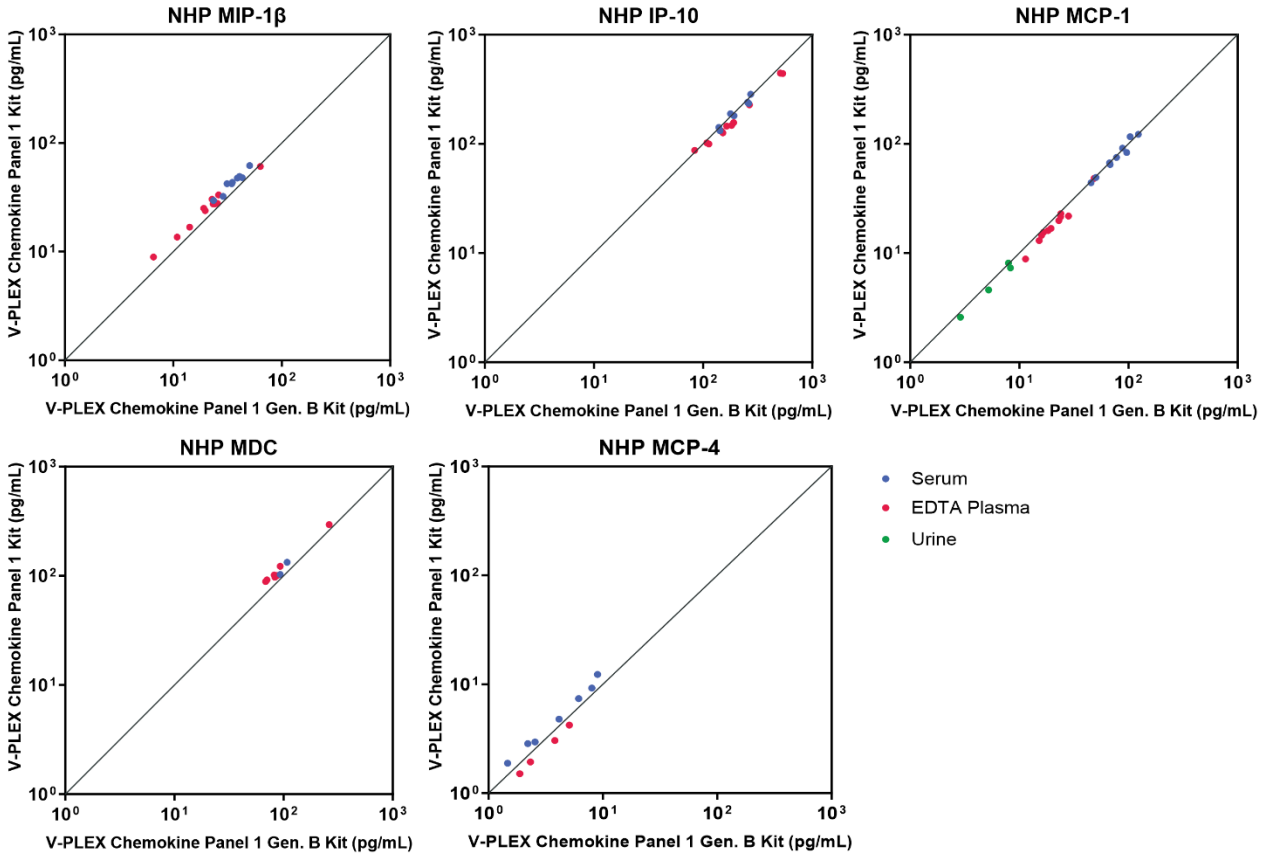

Table 23. Sample concordance between assays in the V-PLEX Chemokine Panel 1 (NHP) and V-PLEX Chemokine Panel 1 (NHP) Gen. B Kits

Assay	r^2	Slope
MIP-1β	0.947	1.06
IP-10	0.977	0.839
MCP-1	0.987	1.03
MDC	0.991	1.08
MCP-4	0.938	1.37

Due to the low detection of Eotaxin-3, TARC, and MIP-1α in the V-PLEX Chemokine Panel 1 (NHP) kit, concordance was not calculated. Eotaxin assay didn't cross-react with NHP samples in the V-PLEX Chemokine Panel 1 (NHP) kit.

Summary Protocol

Chemokine Panel 1 (human) Gen. B Kits

Chemokine Panel 1 (NHP) Gen. B Kits

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol before performing the Chemokine Panel 1 Gen. B assays.

Sample and Reagent Preparation

- ❑ Bring all reagents to room temperature.
- ❑ Prepare calibration solutions in Diluent 57 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 57 before adding to the plate.
- ❑ Prepare combined detection antibody solution by diluting each 50X detection antibody 50-fold in Diluent 3.

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 MSD GOLD Read Buffer B.
- ❑ Analyze plate on the MSD instrument.

Catalog Numbers

Table 24. Catalog numbers for V-PLEX and V-PLEX Plus Chemokine Panel 1 Gen. B multiplex, individual assays, and multi-panel 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) Gen. B	K15705D-1	K15705D-2	K15705D-4	K15705G-1	K15705G-2	K15705G-4
Chemokine Panel 1 (NHP) Gen. B	K15712D-1	K15712D-2	K15712D-4	K15712G-1	K15712G-2	K15712G-4
Individual Assay Kits						
Human Eotaxin Gen. B	K151ANGD-1	K151ANGD-2	K151ANGD-4	K151ANGG-1	K151ANGG-2	K151ANGG-4
Human MIP-1β Gen. B	K151ANHD-1	K151ANHD-2	K151ANHD-4	K151ANHG-1	K151ANHG-2	K151ANHG-4
Human Eotaxin-3 Gen. B	K151ANJD-1	K151ANJD-2	K151ANJD-4	K151ANJG-1	K151ANJG-2	K151ANJG-4
Human TARC Gen. B	K151ANKD-1	K151ANKD-2	K151ANKD-4	K151ANKG-1	K151ANKG-2	K151ANKG-4
Human IP-10 Gen. B	K151ANLD-1	K151ANLD-2	K151ANLD-4	K151ANLG-1	K151ANLG-2	K151ANLG-4
Human MIP-1α Gen. B	K151ANMD-1	K151ANMD-2	K151ANMD-4	K151ANMG-1	K151ANMG-2	K151ANMG-4
Human MCP-1 Gen. B	K151ANPD-1	K151ANPD-2	K151ANPD-4	K151ANPG-1	K151ANPG-2	K151ANPG-4
Human MDC Gen. B	K151ANQD-1	K151ANQD-2	K151ANQD-4	K151ANQG-1	K151ANQG-2	K151ANQG-4
Human MCP-4 Gen. B	K151ANRD-1	K151ANRD-2	K151ANRD-4	K151ANRG-1	K151ANRG-2	K151ANRG-4
NHP Eotaxin Gen. B	K156ANGD-1	K156ANGD-2	K156ANGD-4	K156ANGG-1	K156ANGG-2	K156ANGG-4
NHP MIP-1β Gen. B	K156ANHD-1	K156ANHD-2	K156ANHD-4	K156ANHG-1	K156ANHG-2	K156ANHG-4
NHP Eotaxin-3 Gen. B	K156ANJD-1	K156ANJD-2	K156ANJD-4	K156ANJG-1	K156ANJG-2	K156ANJG-4
NHP TARC Gen. B	K156ANKD-1	K156ANKD-2	K156ANKD-4	K156ANKG-1	K156ANKG-2	K156ANKG-4
NHP IP-10 Gen. B	K156ANLD-1	K156ANLD-2	K156ANLD-4	K156ANLG-1	K156ANLG-2	K156ANLG-4
NHP MIP-1α Gen. B	K156ANMD-1	K156ANMD-2	K156ANMD-4	K156ANMG-1	K156ANMG-2	K156ANMG-4
NHP MCP-1 Gen. B	K156ANPD-1	K156ANPD-2	K156ANPD-4	K156ANPG-1	K156ANPG-2	K156ANPG-4
NHP MDC Gen. B	K156ANQD-1	K156ANQD-2	K156ANQD-4	K156ANQG-1	K156ANQG-2	K156ANQG-4
NHP MCP-4 Gen. B	K156ANRD-1	K156ANRD-2	K156ANRD-4	K156ANRG-1	K156ANRG-2	K156ANRG-4
Multi-Panel Kits						
Human Cytokine 29-Plex	K15054D-1	K15054D-2	K15054D-4	K15054G-1	K15054G-2	K15054G-4
Human Cytokine 35-Plex	K15089D-1	K15089D-2	K15089D-4	K15089G-1	K15089G-2	K15089G-4
Human Cytokine 43-Plex	K15249D-1	K15249D-2	K15249D-4	K15249G-1	K15249G-2	K15249G-4
Human Biomarker 39-Plex	K15209D-1	K15209D-2	K15209D-4	K15209G-1	K15209G-2	K15209G-4
Human Biomarker 45-Plex	K15088D-1	K15088D-2	K15088D-4	K15088G-1	K15088G-2	K15088G-4
Human Biomarker 53-Plex	K15248D-1	K15248D-2	K15248D-4	K15248G-1	K15248G-2	K15248G-4
NHP Cytokine 24-Plex	K15058D-1	K15058D-2	K15058D-4	K15058G-1	K15058G-2	K15058G-4
Neuroinflammation Panel 1 Human	K15210D-1	K15210D-2	K15210D-4	K15210G-1	K15210G-2	K15210G-4

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

Plate Diagram

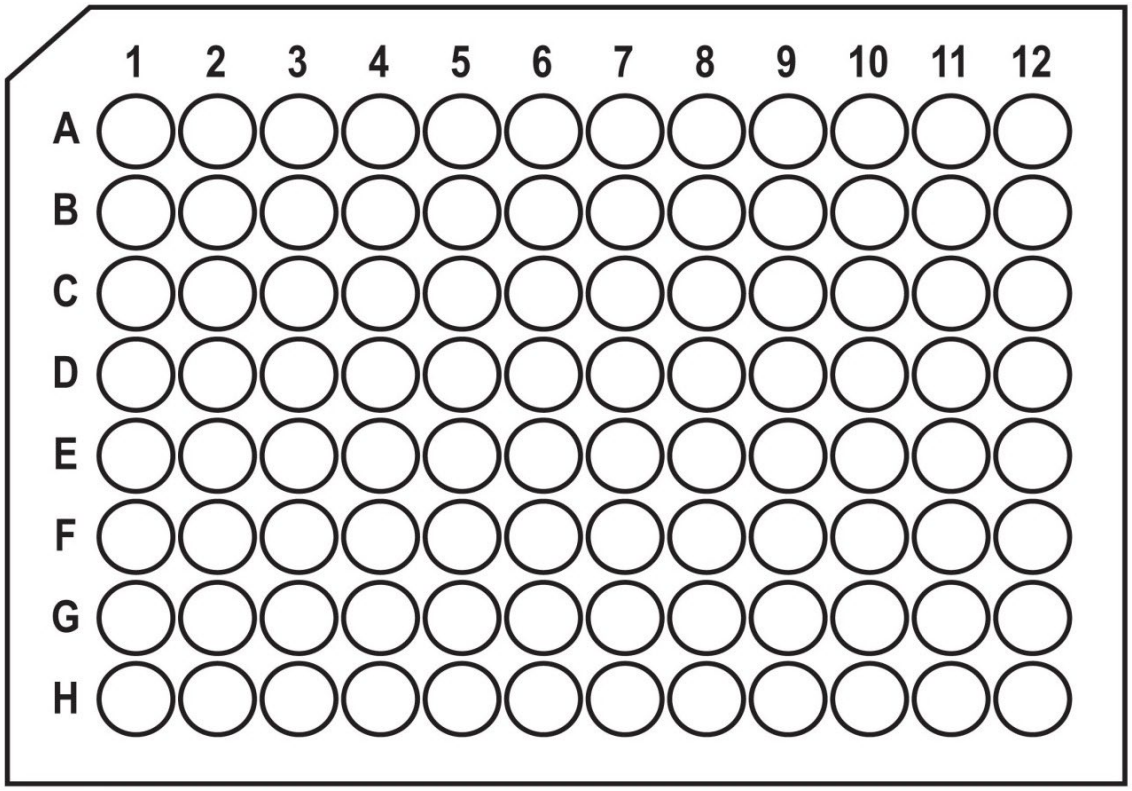

Figure 5. Plate diagram.

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		Sample-06		Sample-14		Sample-22		Sample-30	
B	CAL-02		Control 2		Sample-07		Sample-15		Sample-23		Sample-31	
C	CAL-03		Control 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 (V-PLEX Plus kits) is measured in duplicate in side-by-side wells.*