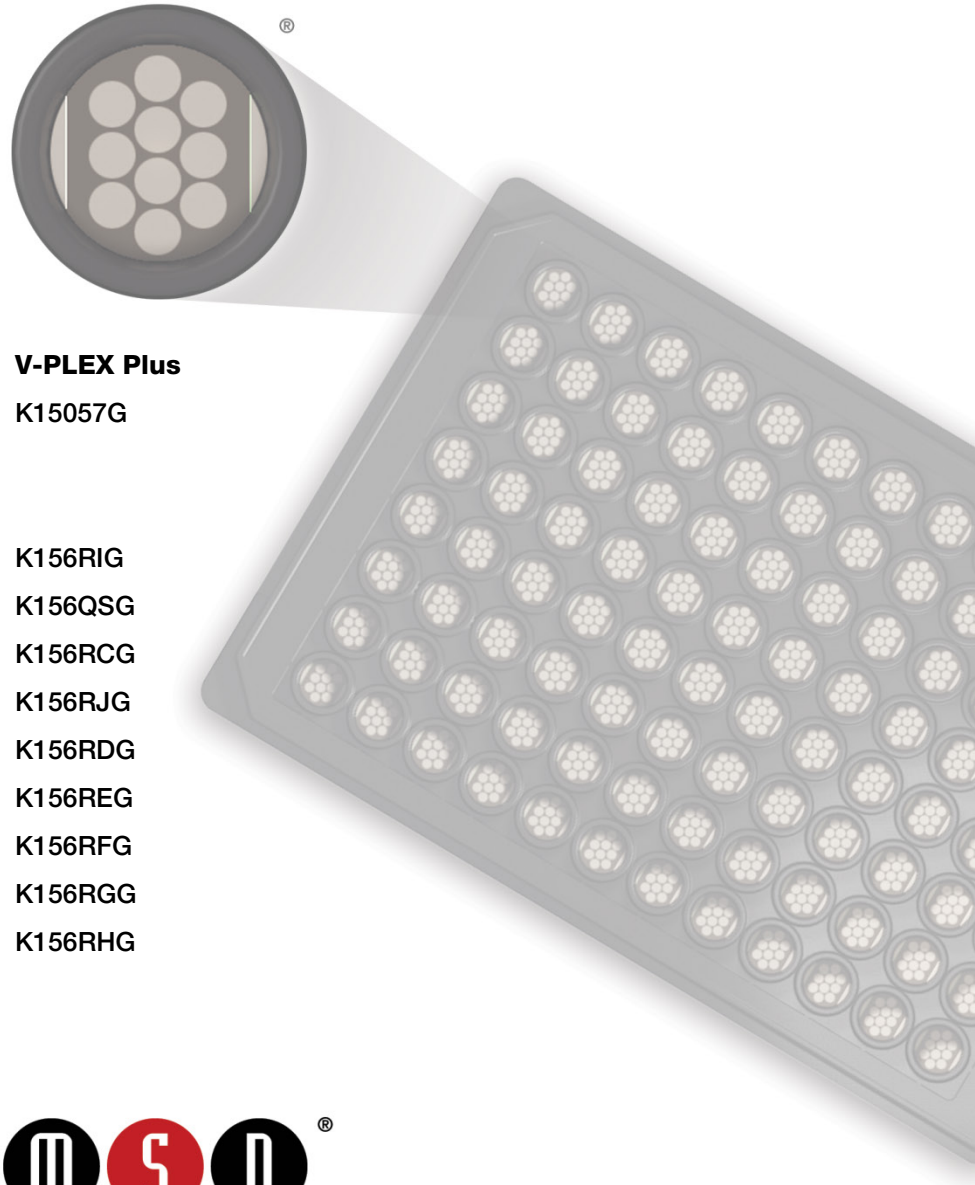
MSD® MULTI-SPOT Assay System

Cytokine Panel 1 (NHP) Kits

GM-CSF, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF-A

V-PLEX®



	V-PLEX®	V-PLEX Plus
Multiplex Kits	K15057D	K15057G
Individual Assay Kits		
NHP GM-CSF	K156RID	K156RIG
NHP IL-5	K156QSD	K156QSG
NHP IL-7	K156RCD	K156RCG
NHP IL-12/IL-23p40	K156RJD	K156RJG
NHP IL-15	K156RDD	K156RDG
NHP IL-16	K156RED	K156REG
NHP IL-17A	K156RFD	K156RFG
NHP TNF-β	K156RGD	K156RGG
NHP VEGF-A	K156RHD	K156RHG

MSD Cytokine Assays

Cytokine Panel 1 (NHP) Kits

GM-CSF, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF-A

For use with nonhuman 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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Table of Contents

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Introduction

MSD offers V-PLEX assays for customers who require unsurpassed performance and quality. V-PLEX products are developed under rigorous design control and are fully validated according to fit-for-purpose principles[1] 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, 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 multiplex panel plates.

The Cytokine Panel 1 (NHP) contains nine assays specifically validated for measuring cytokines in two species of nonhuman primates (NHP): rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) monkeys. Validation involved measuring both stimulated and endogenous levels of cytokines from NHP specimens. To verify that the assays can measure immune responses, stimulated NHP peripheral blood monocytes were used to detect and measure cytokine production. To further validate the ability of the assays to reproducibly quantify physiologic levels of NHP cytokines, endogenous levels were measured in serum, plasma, and urine from normal rhesus and cynomolgus monkeys.

The Cytokine Panel 1 (NHP) employs anti-human detection and capture antibodies that react with rhesus and cynomolgus monkeys. These assays may be suitable for multiple NHP species in addition to *M. mulatta* and *M. fascicularis* because human cytokines are broadly similar to cytokines from NHPs.[2]

Cytokine Panel 1 (NHP) assays can detect secreted biomarkers in a variety of tissues and body fluids where over- or underexpression may indicate a shift in biological equilibriums. The Cytokine Panel 1 (NHP) measures biomarkers that are associated with many disorders, including rheumatoid arthritis,[3] Alzheimer's disease,[4] asthma,[5] various autoimmune diseases,[6] allergies,[7] systemic lupus erythematosus,[8] obesity,[9] cancer,[10] depression,[11] multiple sclerosis,[12] diabetes,[13] psoriasis,[14] and Crohn's disease.[15] As a result of their association with such a wide spectrum of disease, these biomarkers are the subjects of drug discovery projects, diagnostics development, and basic research. The biomarkers constituting the Cytokine Panel 1 (NHP) kits are: **a)** granulocyte-macrophage colony stimulating factor (GM-CSF), **b)** interleukin-5 (IL-5), **c)** interleukin-7 (IL-7), **d)** interleukin-12/interleukin 23 p40 subunit (IL-12/IL-23p40), **e)** interleukin-15 (IL-15), **f)** interleukin-16 (IL-16), **g)** interleukin-17A (IL-17A), h) tumor necrosis factor-beta (TNF-β), and i) vascular endothelial growth factor (VEGF).

Principle of the Assay

MSD cytokine assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The assays in the Cytokine Panel 1 (NHP) are sandwich immunoassays. MSD provides a plate precoated with capture antibodies on independent and well-defined spots, as shown in the layouts below. Multiplex assays and the individual IL-7, IL-16, and TNF-β assays are provided on 10-spot MULTI-SPOT plates (Figure 1); the individual GM-CSF, IL-5, IL-12/IL-23p40, IL-15, IL-17A, and VEGF-A assays are provided on Small Spot plates (Figure 2). The user adds the sample and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) throughout one or more incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. The user adds an MSD buffer that creates the appropriate chemical environment for electrochemiluminescence (ECL) and loads the plate into an MSD® instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light (which is proportional to the amount of analyte present in the sample) and provides a quantitative measure of each analyte in the sample. V-PLEX assay kits have been validated according to the principles outlined in "Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement" by J. W. Lee, et al.[1]

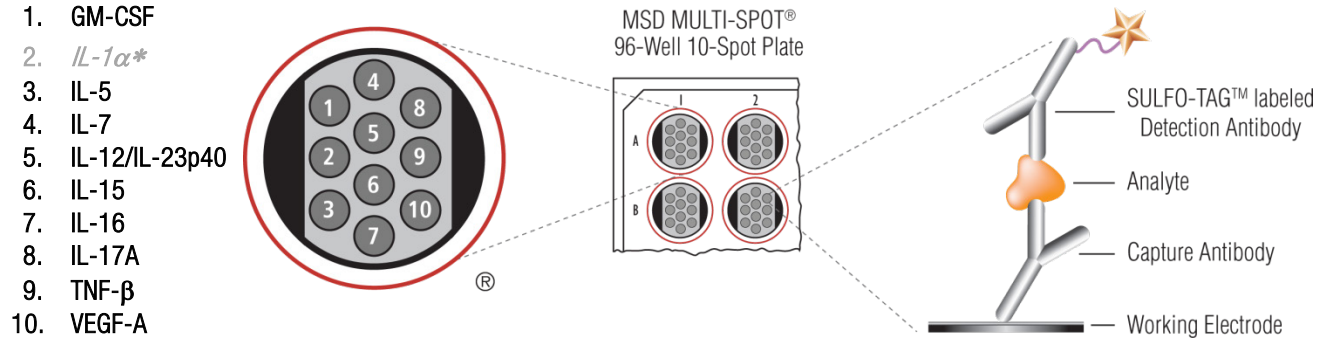

Figure 1. Multiplex plate spot diagram showing the placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

**Not cross-reactive with NHP samples*

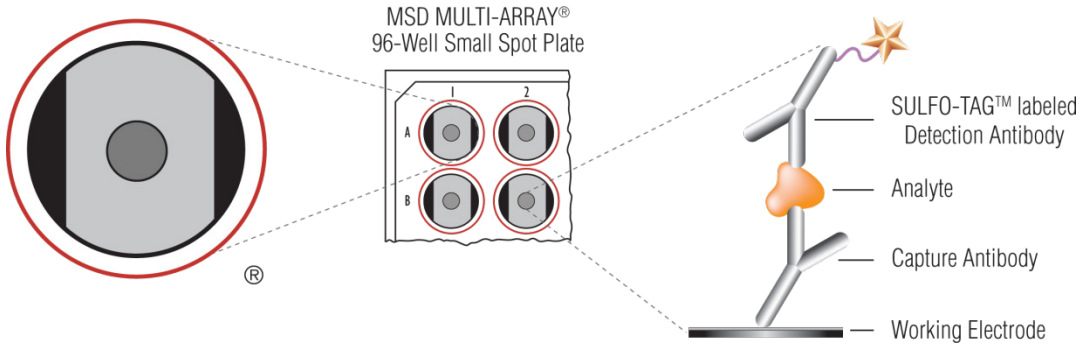

Figure 2. Small Spot plate diagram showing the placement of analyte capture antibodies.

Kit Components

Cytokine 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 cytokine calibrator sequences that are highly homologous to the NHP cytokines. V-PLEX Plus kits include additional items (controls, wash buffer, and plate seals). See below for details.

See the **Catalog Numbers** section (below) 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	
Cytokine Panel 1 (human) Calibrator Blend	2–8 °C	C0050-2	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-chemiluminescent reaction.

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	
Cytokine Panel 1 (human) Control 1*	2–8 °C	C4050-1	1 vial	1 vial	5 vials	25 vials	Multianalyte controls in an NHP matrix, buffered, lyophilized, and spiked with recombinant human analytes. The concentration of the controls is provided in the lot-specific COA.
Cytokine Panel 1 (human) Control 2*	2–8 °C	C4050-1	1 vial	1 vial	5 vials	25 vials	
Cytokine Panel 1 (human) Control 3*	2–8 °C	C4050-1	1 vial	1 vial	5 vials	25 vials	
Wash Buffer (20X)	RT	R61AA-1	100 mL	1 bottle	1 bottle	5 bottles	20-fold concentrated phosphate buffered solution with surfactant.
Plate Seals	-	-	-	3	15	75	Adhesive seals for sealing plates during incubations.

*Provided as components in the Cytokine Panel 1 (human) Control Pack.

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	
Cytokine Panel 1 (human) Plate	2–8 °C	N05050A-1	10-spot	1	5	25	96-well plate, foil sealed, with desiccant.
Human GM-CSF Plate	2–8 °C	L451RIA-1	Small Spot	1	5	25	
Human IL-5 Plate	2–8 °C	L451QSA-1	Small Spot	1	5	25	
Human IL-12/IL-23p40 Plate	2–8 °C	L451RJA-1	Small Spot	1	5	25	
Human IL-15 Plate	2–8 °C	L451RDA-1	Small Spot	1	5	25	
Human IL-17A Plate	2–8 °C	L451RFA-1	Small Spot	1	5	25	
Human VEGF-A Plate	2–8 °C	L451RHA-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	
Anti-hu GM-CSF Antibody (50X)	2–8 °C	D21RI-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21RI-3	375 µL		1	5	
Anti-hu IL-5 Antibody (50X)	2–8 °C	D21QS-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21QS-3	375 µL		1	5	
Anti-hu IL-7 Antibody (50X)	2–8 °C	D21RC-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21RC-3	375 µL		1	5	
Anti-hu IL-12/IL-23p40 Antibody (50X)	2–8 °C	D21RJ-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21RJ-3	375 µL		1	5	
Anti-hu IL-15 Antibody (50X)	2–8 °C	D21RD-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21RD-3	375 µL		1	5	
Anti-hu IL-16 Antibody (50X)	2–8 °C	D21RE-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21RE-3	375 µL		1	5	
Anti-hu IL-17A Antibody (50X)	2–8 °C	D21RF-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21RF-3	375 µL		1	5	
Anti-hu TNF-β Antibody (50X)	2–8 °C	D21LW-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21LW-3	375 µL		1	5	
Anti-hu VEGF-A Antibody (50X)	2–8 °C	D21RH-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21RH-3	375 µL		1	5	

Additional Materials and Equipment (not supplied)

- ❑ 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

- ❑ Cytokine Panel 1 (human) Control Pack, available for separate purchase from MSD, catalog no. C4050-1 (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

- Do not mix or substitute reagents from different sources or different kit lots. Lot information is provided in the lot-specific COA.
- Assay incubation steps should be performed between 20-26 °C to achieve the most consistent signals between runs.
- Bring frozen diluent to room temperature in a 24 °C water bath. Thaw other reagents on wet ice and use as directed without delay.
- Prepare calibrators, samples, and controls in polypropylene microcentrifuge tubes; use a fresh pipette tip for each dilution; vortex after each dilution before proceeding.
- Do not touch the pipette tip on the bottom of the wells when pipetting into the MSD plate.
- Avoid prolonged exposure of detection antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be shielded from light except for direct sunlight.
- Avoid bubbles in wells at all pipetting steps. Bubbles may lead to variable results; bubbles introduced when adding Read Buffer T may interfere with signal detection.
- Use reverse pipetting when necessary to avoid the introduction of bubbles. For empty wells, pipette to the bottom corner.
- Plate shaking should be vigorous, with a rotary motion between 500 and 1,000 rpm. Binding reactions may reach equilibrium sooner if you use shaking at the middle of this range (~700 rpm) or above.
- When using an automated plate washer, rotate the plate 180 degrees between wash steps to improve assay precision.
- Washing plates with a high volume of wash buffer, 3 times with 300 µL/well, may provide improvement in assay precision for certain assays without impacting the analytical parameters, including LOQs, control recovery, and sample quantification.
- 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.
- Make sure that Read Buffer T is at room temperature when added to a plate.
- Do not shake the plate after adding Read Buffer T.
- To improve interplate precision, keep time intervals consistent between adding Read Buffer T and reading the plate. Unless otherwise directed, read the plate as soon as possible after adding Read Buffer T.
- If assay results are above the top of the calibration curve, dilute the samples and repeat the assay.
- When running a partial plate, seal the unused sectors to avoid contaminating unused wells. Remove all seals before reading. Partially used plates may be sealed and stored up to 30 days at 2-8 °C in the original foil pouch with desiccant. You may adjust volumes proportionally when preparing reagents.

Reagent Preparation

Bring all reagents to room temperature.

Important: Upon 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 multianalyte lyophilized calibrator that yields the recommended highest calibrator concentration when reconstituted in 1000 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. In that case, 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:

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 °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.

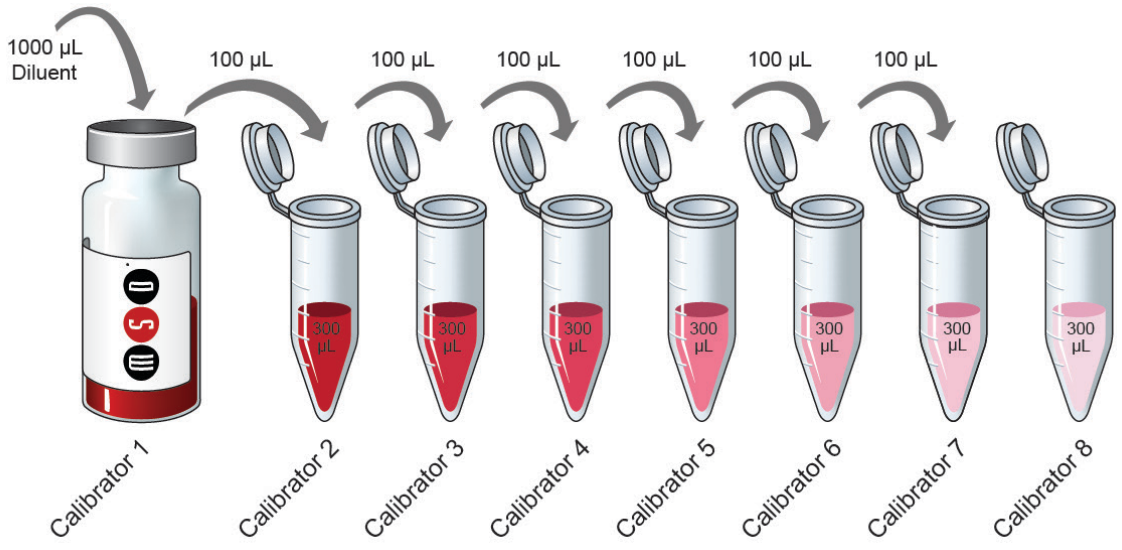

***Figure 3**. Dilution schema for preparation of Calibrator Standards.*

Sample Collection and Handling

Below are general guidelines for NHP sample collection, storage, and handling. If possible, use published guidelines.[16-19] 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 2,000 × *g* 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 ≤–10 ºC until needed. Repeated freezing and thawing of samples is not recommended. After thawing, centrifuge samples at 2,000× g for 3 minutes removing particulates before sample preparation.

Dilute Samples

Dilute samples with Diluent 43. For serum, plasma, and urine samples, MSD recommends a minimum 2-fold dilution. For example, when running samples in duplicate, add 60 µL of sample to 60 µL of Diluent 43. We recommend running at least two replicates per sample. When running single replicates of samples, add 40 µL of sample to 40 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 for running samples in duplicate. Additional diluent can be purchased at www.mesoscale.com.

Prepare Controls

Three levels of multianalyte lyophilized controls are available for separate purchase from MSD in the Cytokine Panel 1 (human) Control Pack, catalog no. C4050-1. (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 before diluting controls 2-fold in Diluent 43. Vortex briefly using short pulses. Refer to the Cytokine Panel 1 (human) Control Pack product insert for analyte levels. Reconstituted controls must be stored frozen. They are stable through 3 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.

9-plex Cytokine Panel 1 (NHP) kit

For one plate, combine the following detection antibodies and add to 2,460 µL of Diluent 3:

- ❑ 60 µL of SULFO-TAG Anti-hu GM-CSF Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-5 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-7 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-12/IL-23p40 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-15 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-16 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-17A Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu TNF-β Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu VEGF-A 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.

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 V-PLEX plates are precoated 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** (above) 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. Analyze the plate on an MSD instrument. Incubation in Read Buffer T is not required before reading the plate.

Alternate Protocols

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

- **Alternate Protocol 1, Extended Sample Incubation:** Incubating samples overnight at 2-8 ºC may improve sensitivity for some assays. See **Appendix A** for specific assays that may benefit from this alternate protocol.
- **Alternate Protocol 2, Reduced Wash:** For tissue culture samples, you may simplify the protocol by eliminating one of the wash steps. After incubating diluted sample, calibrator, or control, add detection antibody solution to the plate without decanting or washing the plate. See **Appendix A** for assay performance using this protocol.
- **Alternate Protocol 3, Dilute-in-Plate:** To limit sample handling, you may dilute samples and controls in the plate. For 2-fold dilution, add 25 µL of assay diluent to each sample/control well, and then add 25 µL of neat control or sample. Calibrators should not be diluted in the plate; add 50 µL of each calibrator directly into empty wells. Tests conducted according to this alternate protocol produced results that were similar to the recommended protocol (data not shown).

Validation

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 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 assessment of all validation lots.

- ### Accuracy and Precision

  Accuracy and precision are evaluated by measuring calibrators and matrix-based validation samples or controls across multiple runs and multiple lots. For most assays, the results of control measurements fall within 20% of the expected concentration for each run. Precision is reported as the coefficient of variation (CV). Intrarun CVs are typically below 7%, and interrun CVs are typically below 15%. Rigorous management of interlot reagent consistency and calibrator production results in typical interlot CVs of less than 10%. Validation lots are compared using controls and at least 40 samples in various sample matrices. Samples are well correlated with an interlot 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 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 stimulated to generate elevated levels of analytes are tested. Results confirm the measurements 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 multianalyte calibrator and assay-specific detection antibodies and 2) with assay-specific calibrator and all detection antibodies. For each validation lot for product release, assay specificity is measured using a multianalyte calibrator and individual detection antibodies. The calibrator concentration used for specificity testing is chosen to ensure that the specific signal is greater than 50,000 counts.

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

- **Assay Robustness and Stability**

  The robustness of the assay protocol is assessed by examining the boundaries of the selected incubation times and evaluating the stability of assay components during the experiment and the stability of reconstituted lyophilized components during storage. For example, the stability of reconstituted calibrator is assessed in real-time over 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. The calibration curve and measured limits of detection for each lot can be found in the lot-specific COA that is included with each kit and available for download at www.mesoscale.com.

Analysis of Results

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

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

Typical Data

Data from the Cytokine Panel 1 (NHP) were collected over four months of testing by four operators (34 runs in total). Calibration curve accuracy and precision were assessed for three kit lots. Representative data from one lot are presented below. (Data from individual assays are presented in **Appendix B**.) The multiplex panel was tested with individual detection antibodies to demonstrate that the assays are independent of one another. **Appendix C** compares results for each assay in the kit when the panel is run using the individual detection antibody versus all nine detection antibodies. The calibration curves were comparable. Calibration curves for each lot are presented in the lot-specific COA.

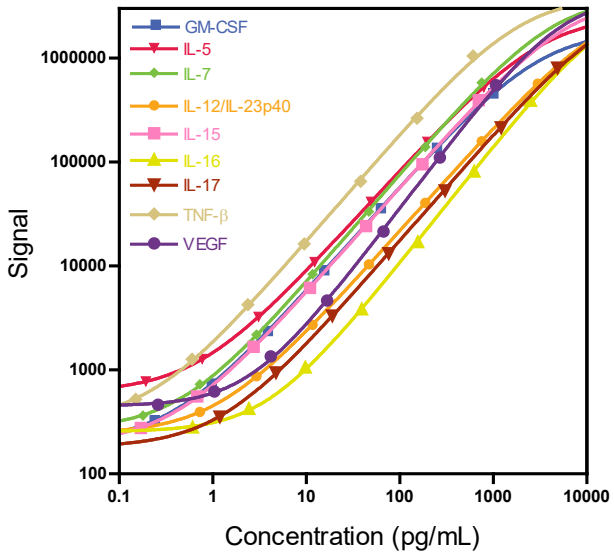

Figure 4. *Typical calibration curves for the Cytokine Panel 1 (NHP) assay.*

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.

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

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

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

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

	Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
GM-CSF	0.16	0.08-0.19	0.842	750
IL-5	0.14	0.04-0.46	4.41	562
IL-7	0.12	0.08-0.17	0.851	563
IL-12/IL-23p40	0.33	0.25-0.42	1.32	2,250
IL-15	0.15	0.09-0.25	0.774	525
IL-16	2.83	0.88–9.33	19.1	1,870
IL-17A	0.31	0.19-0.55	3.19	3,650
TNF-β	0.08	0.04-0.17	0.465	458
VEGF-A	1.12	0.55–6.06	7.70	562

Precision

Controls were made by spiking calibrator into an NHP 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 29 runs over a month. Results are shown below. While a typical specification for precision is a concentration CV of less than 20% for controls on both intra- and interday runs, for this panel, the data shows most assays are below 15%.

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

Interrun %CV is the variability of controls across 29 runs.

Interlot %CV is the variability of controls across two kit lots.

Table 6. *Intrarun and Interrun %CVs for each analyte in the Cytokine Panel 1 (NHP) Kit*

	Control	Average Conc. (pg/mL)	Average Intrarun %CV	Interrun %CV	Interlot %CV
GM-CSF	Control 1	267	3.2	5.5	0.8
	Control 2	70.2	4.4	9.1	2.3
	Control 3	17.4	4.1	9.6	1.6
IL-5	Control 1	239	6.8	8.5	4.7
	Control 2	61.6	5.3	10.8	6.1
	Control 3	14.1	6.3	13.0	7.0
IL-7	Control 1	254	3.7	6.4	0.6
	Control 2	67.5	4.0	9.1	0.6
	Control 3	16.5	5.1	10.0	0.9
IL-12/IL-23p40	Control 1	935	3.2	6.5	2.9
	Control 2	249	3.7	6.0	2.8
	Control 3	60.4	3.2	7.2	2.1
IL-15	Control 1	195	3.4	5.9	3.6
	Control 2	52.8	4.0	7.0	4.7
	Control 3	13.2	3.8	8.3	5.4
IL-16	Control 1	935	3.4	6.1	1.7
	Control 2	230	3.5	7.0	0.9
	Control 3	59.1	4.4	9.9	1.6
IL-17A	Control 1	1282	5.1	8.3	6.8
	Control 2	362	4.7	10.6	8.8
	Control 3	69.5	5.4	12.1	7.5
TNF-β	Control 1	166	3.0	5.9	0
	Control 2	44.0	2.9	6.1	0.5
	Control 3	10.2	2.7	8.2	0.7
VEGF-A	Control 1	294	2.3	16.4	6.7
	Control 2	78.2	2.4	16.7	7.8
	Control 3	18.8	3.4	17.8	7.2

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, 2-fold concentration. The average percent recovery shown below is based on samples within the quantitative range of the assay.

$$\%\ recovery = \frac{measured\ concentration}{expected\ concentration} \times 100$$

Rhesus Monkey

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

		GM-CSF		IL-5		IL-7		IL-12/IL-23p40		IL-15	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=5)	4	112	99–122	101	95–112	96	87–105	116	94–142	84	73–95
	8	109	82–121	104	84–113	100	72–111	131	106–186	83	75–92
	16	112	95–123	102	88–114	94	77–116	142	96–245	81	75–89
	32	110	98–117	105	93–118	95	76–112	147	90–272	87	78–97
	64	112	94–125	104	84–121	97	76–119	158	91–303	88	75–100
EDTA Plasma (N=5)	4	108	97–116	106	102–112	111	101–121	104	95–119	81	78–84
	8	99	93–103	100	93–108	101	94–116	104	93–132	80	73–84
	16	103	99–110	108	86–131	102	82–121	108	89–167	82	75–97
	32	97	91–103	105	93–114	93	84–102	106	90–159	84	79–90
	64	107	93–121	107	87–134	103	93–116	117	94–182	90	82–104
Urine (N=5)	4	100	96–103	91	85–107	89	81–94	98	94–107	79	75–84
	8	90	86–92	85	80–97	82	79–87	91	87–98	79	77–80
	16	89	86–101	82	67–95	84	81–88	92	85–102	73	68–80
	32	81	69–95	78	68–84	79	75–85	88	80–96	77	71–83
	64	87	82–93	82	78–86	83	75–99	93	86–104	79	74–87
Cell Culture Supernatant (N=6)	4	93	86–98	94	89–98	88	85–91	103	94–109	80	78–81
	8	91	86–98	89	87–92	89	83–93	95	90–101	80	76–85
	16	89	83–101	83	80–85	86	82–91	93	86–100	82	77–87
	32	88	83–95	83	81–85	89	82–97	90	87–94	84	77–90
	64	91	84–99	80	78–83	92	86–100	94	90–100	90	86–95

Table 7 continued

		IL-16		IL-17A		TNF-β		VEGF-A	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=5)	4	98	86–104	105	91–124	123	93–142	105	90–121
	8	94	81–104	109	80–129	137	92–173	108	87–134
	16	90	77–102	106	85–121	141	84–182	108	88–146
	32	93	86–104	105	77–126	150	83–203	120	94–163
	64	91	79–107	108	80–125	155	82–213	127	95–181
EDTA Plasma (N=5)	4	95	80–105	133	109–150	130	121–156	95	61–111
	8	91	84–96	143	108–169	145	130–189	86	70–97
	16	92	79–99	164	95–212	157	132–210	100	91–111
	32	98	92–106	166	105–239	169	140–234	116	96–129
	64	95	89–107	189	110–309	183	146–256	126	106–155
Urine (N=5)	4	98	92–108	95	86–102	90	86–95	90	85–94
	8	91	87–97	83	79–91	82	80–88	84	77–88
	16	91	84–100	87	79–91	77	75–82	79	69–84
	32	90	86–96	75	71–79	76	73–81	81	73–87
	64	96	91–104	81	74–93	78	72–86	84	79–92
Cell Culture Supernatant (N=6)	4	101	95–110	89	83–91	87	86–89	89	87–90
	8	99	95–103	81	74–89	84	82–86	79	76–82
	16	96	91–114	79	75–84	81	79–83	75	70–80
	32	107	97–129	79	73–84	81	79–83	76	72–82
	64	112	102–140	84	76–89	82	77–85	77	72–82

Cynomolgus Monkey

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

		GM-CSF		IL-5		IL-7		IL-12/IL-23p40		IL-15	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=5)	4	111	104–117	106	97–114	102	93–108	108	91–133	79	75–84
	8	109	97–120	120	105–138	97	87–103	102	82–136	79	70–87
	16	110	93–124	127	106–142	98	84–106	106	81–150	78	69–93
	32	103	89–119	128	114–157	91	79–100	104	80–153	83	68–102
	64	114	94–131	127	102–156	99	89–106	113	91–169	86	74–104
EDTA Plasma (N=5)	4	106	97–112	106	88–132	100	92–105	98	85–107	76	67–80
	8	102	95–109	114	90–145	96	91–101	90	84–97	72	65–78
	16	105	92–118	115	96–141	99	91–104	97	81–120	73	58–81
	32	101	88–119	113	81–144	93	85–103	96	78–120	78	63–88
	64	105	90–123	121	84–156	99	91–110	101	85–118	76	65–88
Urine (N=5)	4	102	94–106	101	94–104	97	89–100	94	92–98	76	73–80
	8	97	91–108	104	97–114	94	85–104	92	86–111	79	76–83
	16	98	89–112	95	69–109	96	85–111	92	84–114	74	73–79
	32	90	85–106	94	83–102	90	83–106	87	78–104	78	76–81
	64	97	89–108	97	91–112	99	91–115	93	84–110	77	72–81
Cell Culture Supernatant (N=6)	4	93	86–98	94	89–98	88	85–91	103	94–109	80	78–81
	8	91	86–98	89	87–92	89	83–93	95	90–101	80	76–85
	16	89	83–101	83	80–85	86	82–91	93	86–100	82	77–87
	32	88	83–95	83	81–85	89	82–97	90	87–94	84	77–90
	64	91	84–99	80	78–83	92	86–100	94	90–100	90	86–95

Table 8 continued

		IL-16		IL-17A		TNF-β		VEGF-A	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=5)	4	86	54–106	124	108–142	133	96–176	95	34–118
	8	85	56–106	127	114–154	159	101–260	117	45–155
	16	92	77–104	131	117–156	180	103–337	150	67–239
	32	100	80–111	125	111–143	194	103–375	196	84–372
	64	110	99–121	133	117–179	211	113–422	225	102–451
EDTA Plasma (N=5)	4	73	59–85	123	98–139	120	109–133	93	44–150
	8	74	62–88	130	106–148	135	110–147	120	56–271
	16	85	73–92	140	110–160	145	115–159	173	76–498
	32	94	86–102	135	104–157	157	117–184	240	85–785
	64	105	93–118	138	112–163	164	119–193	282	92–960
Urine (N=5)	4	102	97–111	99	89–108	88	86–90	90	87–93
	8	109	101–125	94	80–110	83	80–92	88	81–102
	16	109	102–128	92	83–105	79	76–89	82	76–97
	32	111	100–126	84	72–101	77	74–83	82	74–94
	64	114	100–126	94	82–111	79	74–85	86	77–98
Cell Culture Supernatant (N=6)	4	101	95–110	89	83–91	87	86–89	89	87–90
	8	99	95–103	81	74–89	84	82–86	79	76–82
	16	96	91–114	79	75–84	81	79–83	75	70–80
	32	107	97–129	79	73–84	81	79–83	76	72–82
	64	112	102–140	84	76–89	82	77–85	77	72–82

Spike Recovery

Spike and recovery measurements of different sample types across 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 2-fold. The average % recovery for each sample type is reported along with %CV and % recovery range.

$$\%\, recovery = \frac{measured\ concentration}{expected\ concentration} \times 100$$

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
GM-CSF	81	6.4	76–87	83	9.1	76–91	100	7.6	89–108
IL-5	85	8.4	77–93	73	20.8	47–86	89	13.1	80–109
IL-7	92	5.3	88–100	84	10.2	75–96	107	5.4	97–112
IL-12/IL-23p40	82	19.1	56–98	94	9.6	80–102	112	7.3	100–119
IL-15	104	4.8	98–110	97	5.5	91–102	99	6.1	91–108
IL-16	88	8.1	76–93	92	6.3	83–99	78	7.9	70–87
IL-17A	95	11.2	82–111	53	39.9	28–84	112	2.6	107–114
TNF-β	65	36.0	42–105	55	19.0	38–62	115	4.4	108–122
VEGF-A	100	19.6	69–118	99	3.4	96–105	129	2.9	125–134

	Cell Culture Supernatants (N=6)		
	Average % Recovery	%CV	% Recovery Range
GM-CSF	99	6.1	89–114
IL-5	109	10.4	92–130
IL-7	86	5.4	77–93
IL-12/IL-23p40	120	7.5	103–143
IL-15	78	11.6	68–95
IL-16	92	13.9	78–131
IL-17A	121	6.1	107–133
TNF-β	118	6.8	107–132
VEGF-A	108	22.6	85–151

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
GM-CSF	79	11.8	67–92	81	14.6	63–91	94	9.0	85–107
IL-5	71	12.1	57–80	77	7.8	71–84	89	10.7	77–101
IL-7	102	7.5	93–112	90	5.6	82–94	96	10.7	81–109
IL-12/IL-23p40	100	12.8	78–110	99	2.8	96–103	107	4.0	103–113
IL-15	93	13.0	72–100	103	6.4	97–113	102	5.5	95–109
IL-16	81	3.6	77–85	81	8.9	69–87	73	6.8	67–78
IL-17A	67	16.8	51–79	68	6.5	63–74	101	7.5	93–112
TNF-β	48	33.4	20–60	59	25.4	38–75	110	2.4	107–113
VEGF-A	62	55.5	15–95	107	14.7	84–127	122	2.6	117–126

	Cell Culture Supernatants (N=6)		
	Average % Recovery	%CV	% Recovery Range
GM-CSF	99	6.1	89–114
IL-5	109	10.4	92–130
IL-7	86	5.4	77–93
IL-12/IL-23p40	120	7.5	103–143
IL-15	78	11.6	68–95
IL-16	92	13.9	78–131
IL-17A	121	6.1	107–133
TNF-β	118	6.8	107–132
VEGF-A	108	22.6	85–151

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, Eotaxin-2, Eotaxin-3, EPO, FGF (basic), Fractalkine, G-CSF, HGF, I-309, ICAM-1, ICAM-3, IFN-α2a, IL-1β, IL-2, IL-4, IL-6, IL-6R, IL-10, IL-12p70, IL-13, IL-17B, IL-17D, IL-18, INF-γ, IP-10, I-TAC, MCP-1, MCP-2, MCP-4, M-CSF, MDC, MIF, MIG, MIP-1α, MIP-3α, MIP-4, MIP-5, MMP-1, MMP-2, MMP-3, MMP-9, MMP-10, NT-proBNP, RANTES, SAA, Thrombomodulin, TARC, Tie, TNF-α, TNF-RI, TNF-RII, TPO, VCAM-1, VEGF-A, VEGF-C, VEGF-D, and VEGF-RI). Nonspecific binding was less than 0.5% for all assays in the kit.

$$\%\, nonspecificity = \frac{nonspecific\ signal}{specific\ signal} \times 100$$

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 multianalyte 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 °C in the original foil pouch with desiccant. Results from control measurements changed by ≤30% after partially used plates were stored for 30 days. The validation study includes a real-time stability study with scheduled performance evaluations of complete kits for up to 54 months from the date of manufacture.

Calibration

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

Tested Samples

Commercially available normal serum, EDTA plasma, and urine samples from rhesus and cynomolgus monkeys were diluted 2-fold and tested. Results for each sample set are displayed below. Concentrations are corrected for sample dilution. Median and range are calculated from samples with concentrations at or above the LLOD. Percent detected is the percentage of samples with concentrations at or above the LLOD.

Rhesus Monkey

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

Sample Type	Statistic	GM-CSF	IL-5	IL-7	IL-12/IL-23p40	IL-15	IL-16	IL-17A	TNF-β	VEGF-A
Serum (N=30)	Median (pg/mL)	1.34	0.96	80.1	173	12.0	53.6	3.01	0.16	4.24
	Range (pg/mL)	0.34–18.1	0.45–17.8	17.5–151	27.2–465	5.76–18.3	7.82–137	1.55–61.0	0.10–0.18	2.43–18.7
	% Detected	43	63	100	100	100	93	77	20	53
EDTA Plasma (N=30)	Median (pg/mL)	0.55	0.69	9.14	306	11.0	134	2.85	0.16	8.80
	Range (pg/mL)	0.28–9.84	0.45–9.64	0.60–39.4	24.4–659	4.74–19.7	30.2–446	1.62–18.4	0.10–0.32	3.66–65.4
	% Detected	80	57	100	100	100	100	83	50	100
Urine (N=30)	Median (pg/mL)	0.32	0.47	ND	ND	0.52	ND	1.78	ND	8.22
	Range (pg/mL)	0.29–0.34	0.45–0.57	ND	ND	0.35–1.02	ND	1.64–1.91	ND	3.82–17.3
	% Detected	17	20	0	0	47	0	7	0	83

ND = Nondetectable

Cynomolgus Monkey

Table 12. *Cynomolgus monkey samples tested in the Cytokine Panel 1 (NHP) Kit*

Sample Type	Statistic	GM-CSF	IL-5	IL-7	IL-12/IL-23p40	IL-15	IL-16	IL-17A	TNF-β	VEGF-A
Serum (N=30)	Median (pg/mL)	0.42	1.47	107	189	5.45	15.9	2.67	0.17	9.76
	Range (pg/mL)	0.33–1.37	1.06–4.76	5.78–304	1.82–607	2.76–13.5	6.18–57.4	1.63–7.28	0.10–0.23	2.31–36.8
	% Detected	23	17	100	100	100	87	37	23	70
EDTA Plasma (N=30)	Median (pg/mL)	4.91	1.23	2.12	140	5.15	14.4	2.09	0.11	7.58
	Range (pg/mL)	0.36–7.92	0.61–9.26	0.49–62.7	38.8–482	3.09–10.6	6.74–54.1	1.67–14.4	0.11–0.11	2.59–61.2
	% Detected	20	33	100	100	100	97	33	7	97
Urine (N=30)	Median (pg/mL)	ND	ND	0.47	ND	0.40	ND	ND	ND	23.0
	Range (pg/mL)	ND	ND	0.47	ND	0.36–0.95	ND	ND	ND	7.08–80.2
	% Detected	0	0	3	0	30	0	0	0	100

ND = Nondetectable

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.[20] 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 Cytokine Panel 1 (NHP) Kit*

	ConA	LPS	PHA	PWM	Spontaneous
GM-CSF	+	+	+	++	–
IL-5	–	–	+	–	–
IL-7	–	–	–	–	–
IL-12/IL-23p40	–	+	–	+	–
IL-15	–	–	–	+	++
IL-16	–	+	+	–	++
IL-17A	+++	+	+++	+++	+
TNF-β	+++	+	++	+++	–
VEGF-A	–	+	+	+	–

+++ > 100-fold
++ > 10-fold
+ > 2-fold
– no significant response

Cynomolgus Monkey

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

	ConA	LPS	PHA	PWM	Spontaneous
GM-CSF	+++	+	+++	+++	–
IL-5	++	–	+++	++	–
IL-7	–	–	+	+	–
IL-12/IL-23p40	+	–	–	+	–
IL-15	–	–	–	–	+
IL-16	–	+	+	+	–
IL-17A	++	+	++	++	–
TNF-β	+++	+	+++	+++	–
VEGF-A	+	+	+	+	++

+++ > 100-fold
++ > 10-fold
+ > 2-fold
– no significant response

Assay Components

Calibrators

Cytokine calibrators are recombinant proteins encoding human sequences, which are highly homologous to cytokines in NHPs. The assay calibrator blend uses the following recombinant human proteins:

Table 15. Recombinant human proteins used in the Calibrators

Calibrator	Expression System
GM-CSF	*E. coli*
IL-5	Insect cell line
IL-7	*E. coli*
IL-12/IL-23p40	Insect cell line
IL-15	*E. coli*
IL-16	*E. coli*
IL-17A	*E. coli*
TNF-β	*E. coli*
VEGF-A	Insect cell line

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
GM-CSF	Mouse Monoclonal	Rat Monoclonal	A
IL-5	Mouse Monoclonal	Rat Monoclonal	B
IL-7	Mouse Monoclonal	Goat Polyclonal	A
IL-12/IL-23p40	Mouse Monoclonal	Mouse Monoclonal	C
IL-15	Mouse Monoclonal	Mouse Monoclonal	A
IL-16	Mouse Monoclonal	Goat Polyclonal	A
IL-17A	Mouse Monoclonal	Goat Polyclonal	A
TNF-β	Mouse Monoclonal	Mouse Monoclonal	A
VEGF-A	Mouse Monoclonal	Mouse Monoclonal	C

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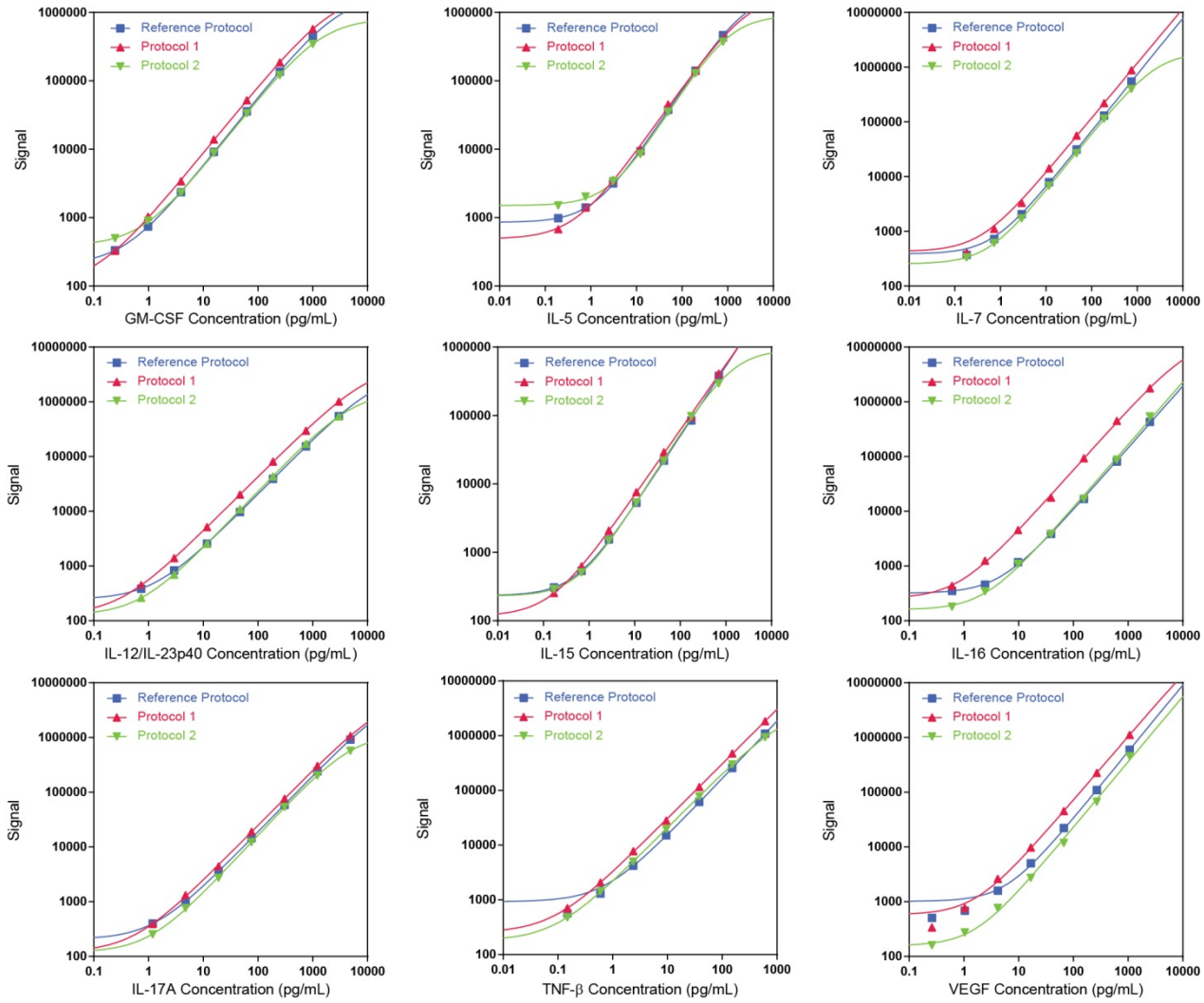
Appendix A

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).



	LLOD Comparison (pg/mL)		
Assay	Reference Protocol	Protocol 1	Protocol 2
GM-CSF	0.16	0.08	0.18
IL-5	0.14	0.16	0.93
IL-7	0.12	0.10	0.31
IL-12/IL-23p40	0.33	0.17	0.39
IL-15	0.15	0.17	0.25
IL-16	2.83	0.22	1.12
IL-17A	0.31	0.33	1.13
TNF-β	0.08	0.02	0.04
VEGF-A	1.12	0.18	0.73

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).

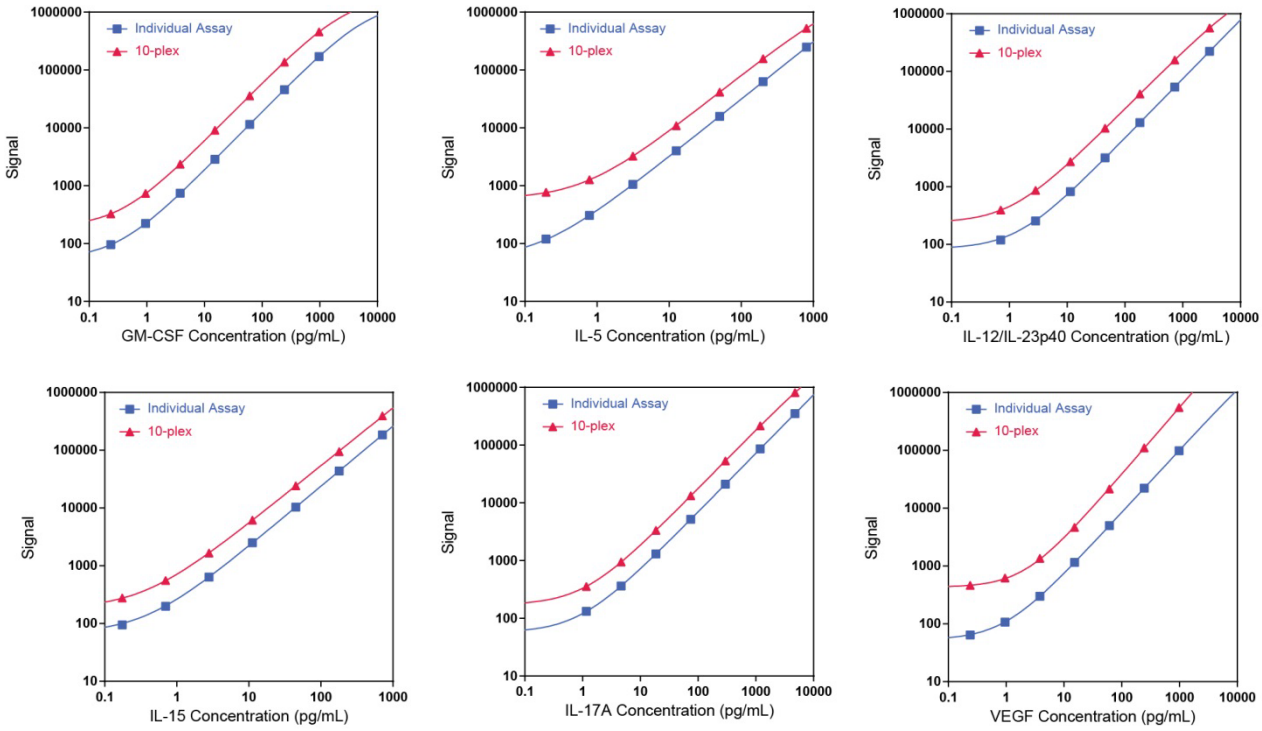


Table 18. Assay performance for individual and 10-plex assays

	LLOD (pg/mL)	
Assay	Individual	10–plex
GM-CSF	0.13	0.16
IL-5	0.08	0.14
IL-12/IL-23p40	0.39	0.33
IL-15	0.11	0.15
IL-17A	0.74	0.31
VEGF-A	1.12	1.12

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

Note: Assay performance for IL-7, IL-16 and TNF-β are not included since the individual assays are run on multiplex plates.

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).

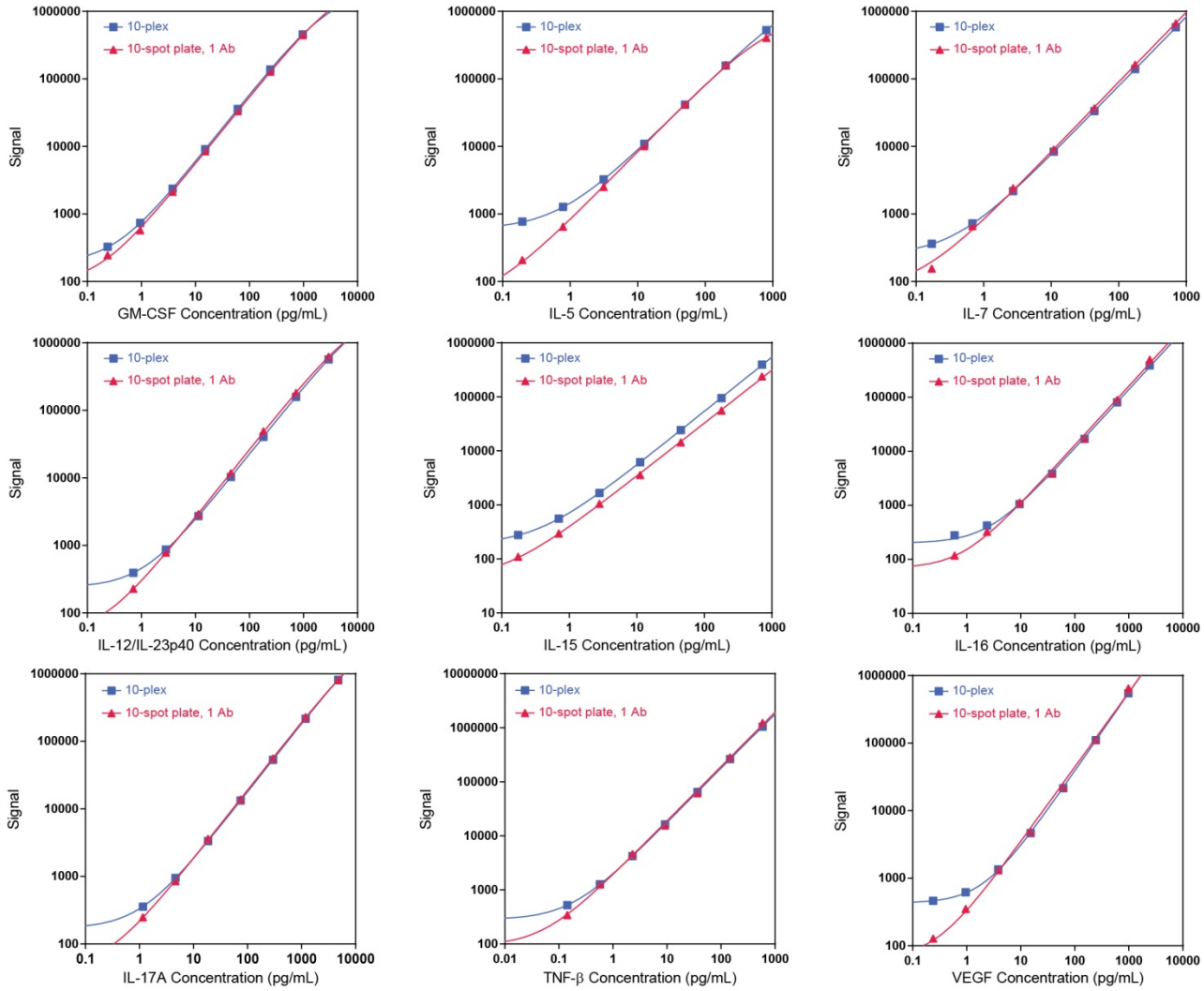


Table 19. LLODs for detection of a single antibody vs. blended antibodies

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

	LLOD (pg/mL)	
Assay	10–spot plate, 1 Ab	10–plex
GM-CSF	0.14	0.16
IL-5	0.10	0.14
IL-7	0.16	0.12
IL-12/IL-23p40	0.30	0.33
IL-15	0.35	0.15
IL-16	1.32	2.83
IL-17A	0.53	0.31
TNF-β	0.04	0.08
VEGF-A	0.32	1.12

Summary Protocol

Cytokine Panel 1 (NHP) Kits

MSD provides this summary protocol for your convenience.

Please read the entire detailed protocol before performing the cytokine 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 2–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 the 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 the 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 the plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 150 µL/well of 2X Read Buffer T.
- ❑ Analyze the plate on the MSD instrument.

*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 cytokine (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						
Cytokine Panel 1 (NHP)	K15057D–1	K15057D–2	K15057D–4	K15057G–1	K15057G–2	K15057G–4
Individual Assay Kits						
NHP GM-CSF	K156RID–1	K156RID–2	K156RID–4	K156RIG–1	K156RIG–2	K156RIG–4
NHP IL-5	K156QSD–1	K156QSD–2	K156QSD–4	K156QSG–1	K156QSG–2	K156QSG–4
NHP IL-7	K156RCD–1	K156RCD–2	K156RCD–4	K156RCG–1	K156RCG–2	K156RCG–4
NHP IL-12/IL-23p40	K156RJD–1	K156RJD–2	K156RJD–4	K156RJG–1	K156RJG–2	K156RJG–4
NHP IL-15	K156RDD–1	K156RDD–2	K156RDD–4	K156RDG–1	K156RDG–2	K156RDG–4
NHP IL-16	K156RED–1	K156RED–2	K156RED–4	K156REG–1	K156REG–2	K156REG–4
NHP IL-17A	K156RFD–1	K156RFD–2	K156RFD–4	K156RFG–1	K156RFG–2	K156RFG–4
NHP TNF-β	K156RGD–1	K156RGD–2	K156RGD–4	K156RGG–1	K156RGG–2	K156RGG–4
NHP VEGF-A	K156RHD–1	K156RHD–2	K156RHD–4	K156RHG–1	K156RHG–2	K156RHG–4

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

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 5. 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.

Plate Diagram

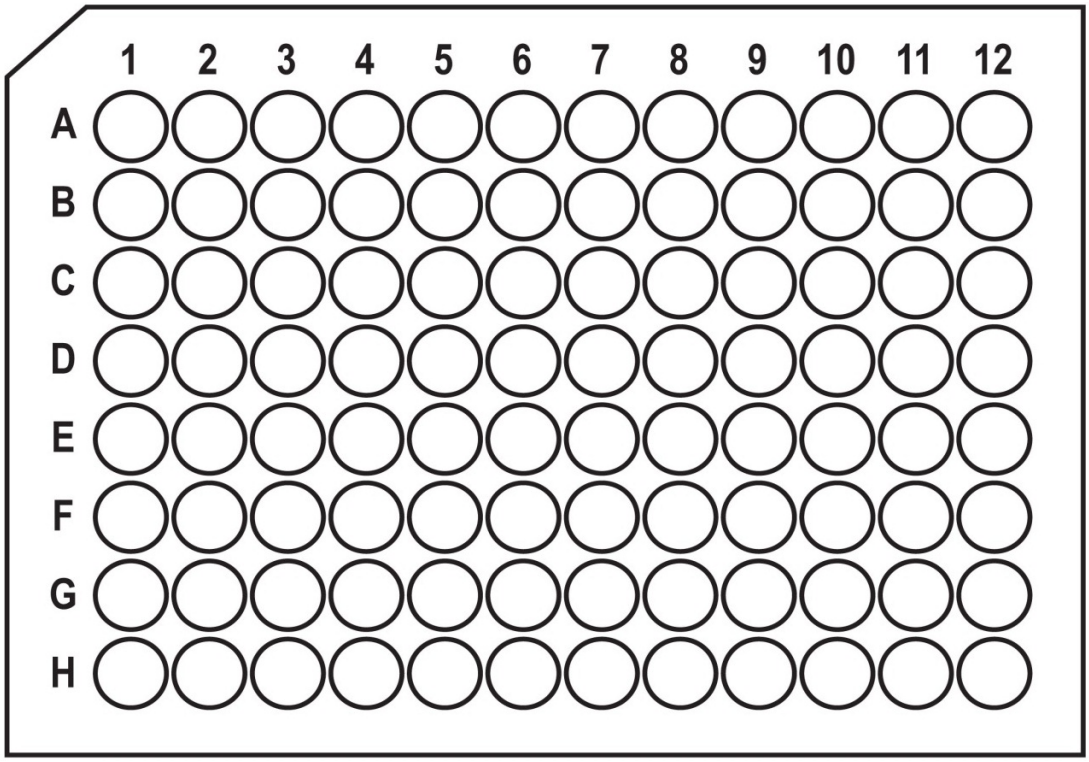


Figure 6. Plate diagram.