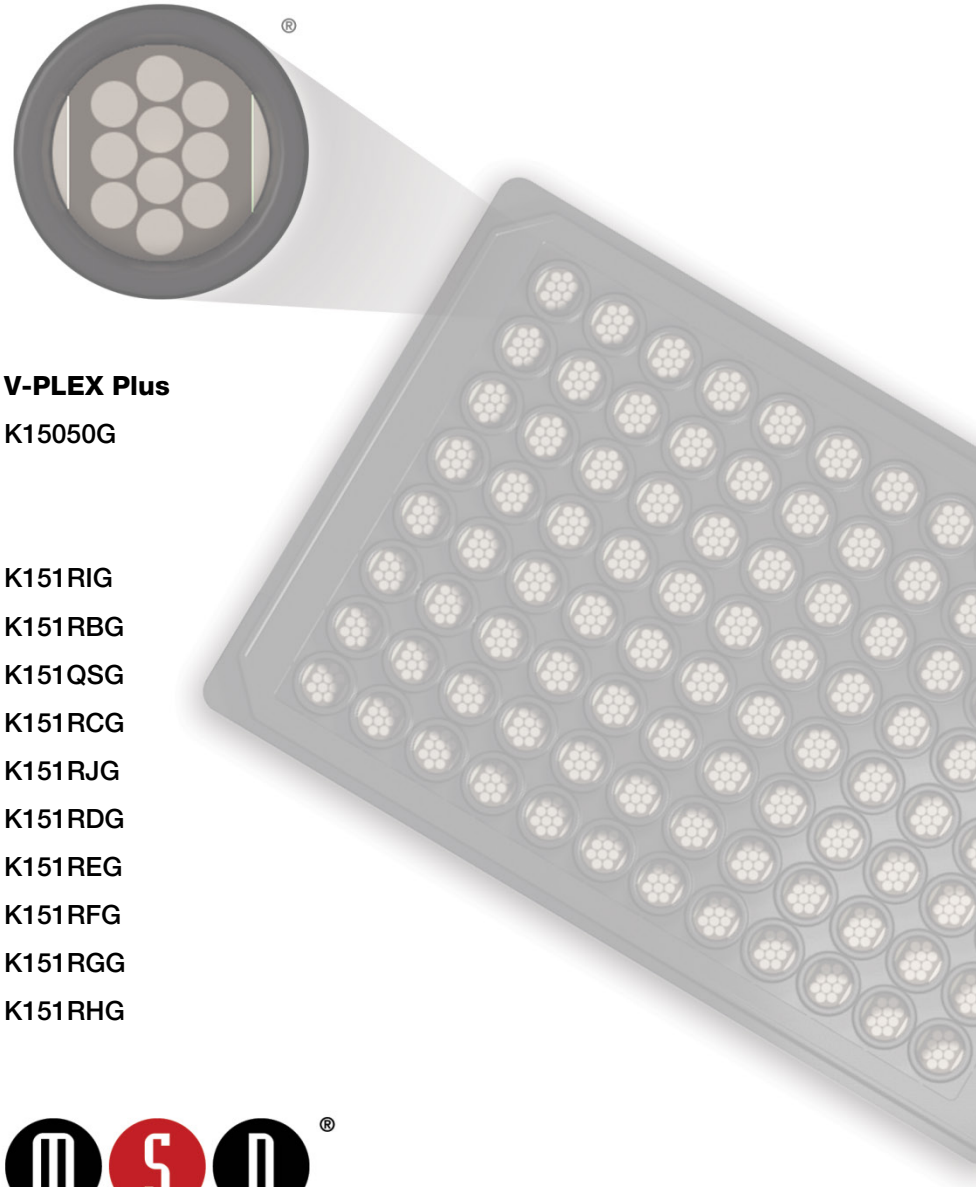
MSD® MULTI-SPOT Assay System

Cytokine Panel 1 (human) Kits

GM-CSF, IL-1α, 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	K15050D	K15050G
Individual Assay Kits		
Human GM-CSF	K151RID	K151RIG
Human IL-1α	K151RBD	K151RBG
Human IL-5	K151QSD	K151QSG
Human IL-7	K151RCD	K151RCG
Human IL-12/IL-23p40	K151RJD	K151RJG
Human IL-15	K151RDD	K151RDG
Human IL-16	K151RED	K151REG
Human IL-17A	K151RFD	K151RFG
Human TNF-β	K151RGD	K151RGG
Human VEGF-A	K151RHD	K151RHG

MSD®

MSD Cytokine Assays

Cytokine Panel 1 (human) Kits

GM-CSF, IL-1α, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF-A

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

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

MESO SCALE DISCOVERY®
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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[14] 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 singleplex 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 plates.

The Cytokine Panel 1 (human) measures 10 cytokines that are important in inflammation responses and immune system regulation as well as numerous other biological processes. These 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 (human) measures biomarkers that are associated with several disorders, including rheumatoid arthritis,[1] Alzheimer's disease,[2] asthma,[3] various autoimmune diseases,[4] allergies,[5] systemic lupus erythematosus,[6] obesity,[7] cancer,[8] depression,[9] multiple sclerosis,[10] diabetes,[11] psoriasis,[12] and Crohn's disease.[13] 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 (human) kits are: **a)** granulocyte-macrophage colony stimulating factor (GM-CSF), **b)** interleukin-1alpha (IL-1α) or IL-1F1, **c)** interleukin-5 (IL-5), **d)** interleukin-7 (IL-7), **e)** interleukin-12/interleukin 23 p40 subunit (IL-12/IL-23p40), **f)** interleukin-15 (IL-15), **g)** interleukin-16 (IL-16), **h)** interleukin-17A (IL-17A), **i)** tumor necrosis factor-beta (TNF-β), and **j)** 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 (human) 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-1α, IL-5, IL-12/IL-23p40, IL-15, IL-16, 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 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.[14]

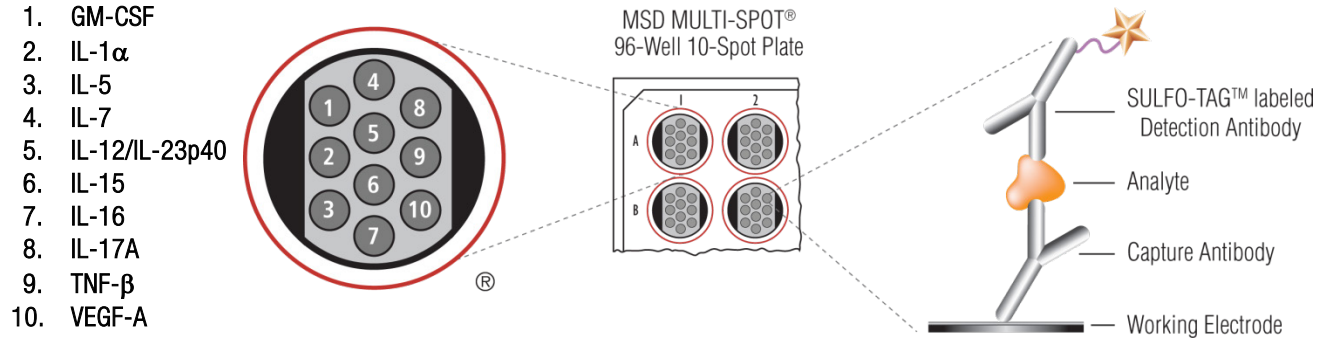

Figure 1. Multiplex plate spot diagram showing the placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

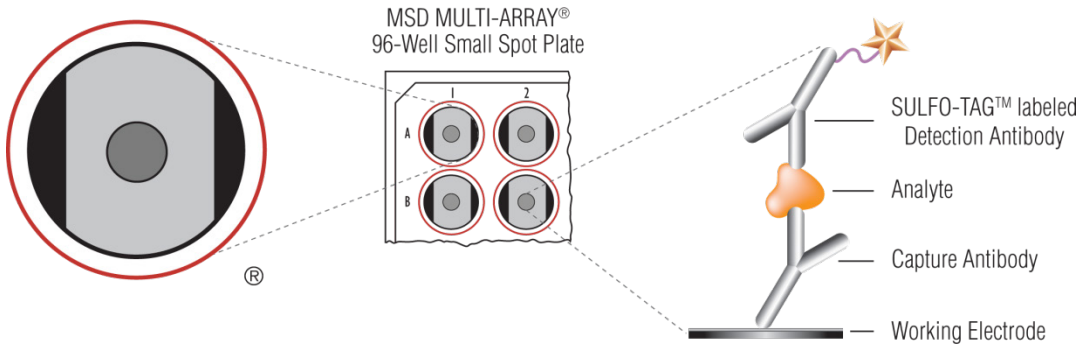

Figure 2. Small Spot plate diagram showing placement of analyte capture antibodies.

Kit Components

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

See the **Catalog Numbers** section (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 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Cytokine Panel 1 (human) Calibrator Blend	2–8 °C	C0050-2	1 vial	1 vial	5 vials	25 vials	Ten recombinant human proteins in diluent, buffered and lyophilized. Individual analyte concentration is provided in the lot-specific certificate of analysis (COA).
Diluent 43	≤–10 °C	R50AG-1	10 mL	1 bottle	-	-	Diluent for samples and calibrator; contains protein, blockers, and preservatives.
		R50AG-2	50 mL	-	1 bottle	5 bottles	
Diluent 3	≤–10 °C	R51BA-4	5 mL	1 bottle	-	-	Diluent for detection antibody; contains protein, blockers, and preservatives.
		R51BA-5	25 mL	-	1 bottle	5 bottles	
Read Buffer T (4X)	RT	R92TC-3	50 mL	1 bottle	1 bottle	5 bottles	Buffer to catalyze the electro-chemiluminescent reaction.

Dash (-) = not applicable

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 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Cytokine Panel 1 (human) Control 1*	2–8 °C	C4050-1	1 vial	1 vial	5 vials	25 vials	Multianalyte controls in a nonhuman 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-1α Plate	2–8 °C	L451RBA-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-16 Plate							
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-1α Antibody (50X)	2–8 °C	D21RB-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21RB-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	

Dash (-) = not applicable

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 the www.mesoscale.com® website.

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 may interfere with signal detection.
- 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.
- Use reverse pipetting when necessary to avoid the introduction of bubbles. For empty wells, pipette to the bottom corner.
- 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 1,000 µL of Diluent 43. (For individual assays that do not saturate at the highest calibrator concentration, the calibration curve can be extended by creating a more concentrated highest calibrator. In such a case, follow the steps below using 250 µL instead of 1000 µL of Diluent 43 when reconstituting the lyophilized calibrator.

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

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.

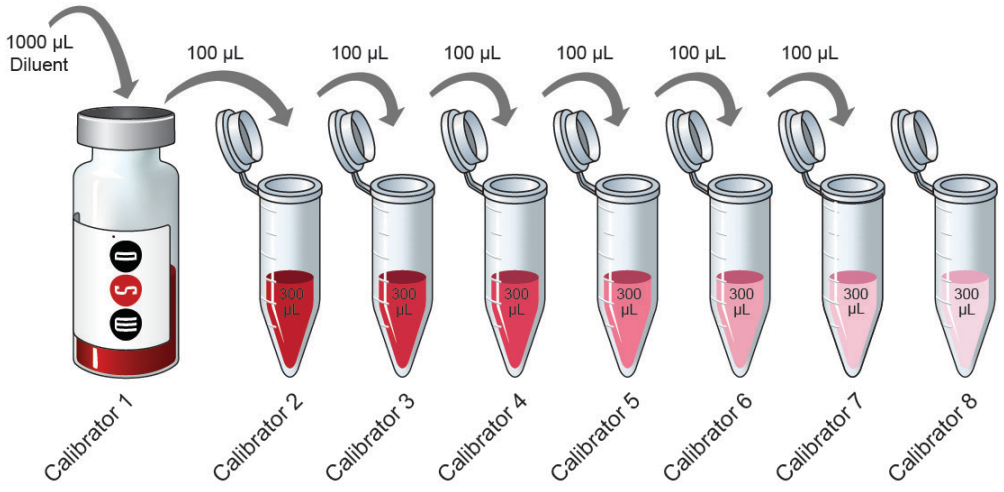

***Figure 3**. Dilution schema for preparation of Calibrator Standards.*

Sample Collection and Handling

Below are general guidelines for human sample collection, storage, and handling. If possible, use published guidelines.[15-18] 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 °C until needed. Repeated freeze-thaw of samples is not recommended. After thawing, centrifuge samples at 2,000 × *g* for 3 minutes to remove particulates before sample preparation.

Dilute Samples

Dilute samples with Diluent 43. For human serum, plasma, and urine samples, MSD recommends a minimum 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 three freeze-thaw cycles.

Prepare Detection Antibody Solution

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

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

- ❑ 60 µL of SULFO-TAG Anti-hu GM-CSF Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-1α 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 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[14] 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 the assessment of all validation lots.

- **Accuracy and Precision**

  Accuracy and precision are evaluated by measuring calibrators and matrix-based validation samples or controls across multiple runs and multiple lots. For most assays, the results of control measurements fall within 20% of the expected concentration for each run. 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 below 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 the variability of results due to matrix effects. The sample dilution suggested in the protocol gives an appropriate dilution factor for all assays in the multiplex. Some assays may benefit from lower or higher dilution factors, depending on the samples and application (data are provided in this product insert). In addition to the matrices listed above, blood, PBMCs, and/or cell lines that have been stimulated to generate elevated levels of analytes are tested. Results confirm the measurement of native proteins at concentrations that are often higher than those found in individual native samples.

- **Specificity**

  The specificity of both capture and detection antibodies is measured during assay development. Antibody specificity is assessed by first running each assay using the multiplex plate with assay-specific detection antibody and assay-specific calibrator. These results are compared to the assay's performance when the plate is run 1) with the multianalyte 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 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 30 days. Assay component (calibrator, antibody, control) stability was assessed through freeze-thaw testing and accelerated stability studies. The validation program includes a real-time stability study with scheduled performance evaluations of complete kits for up to 54 months from the date of manufacture.

Representative data from the validation studies are presented in the following sections. 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 (human) 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 ten detection antibodies. The calibration curves were comparable. Calibration curves for each lot are presented in the lot-specific COA.

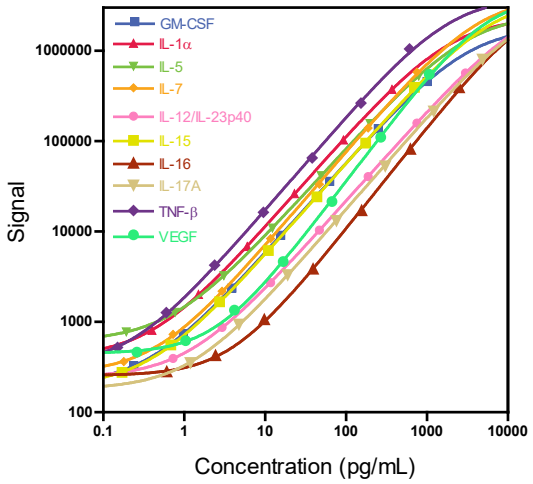

Figure 4. *Typical calibration curves for the Cytokine Panel 1 (human) assay.*

Note: Outliers are defined as wells with signals that are more than 2-fold away from the expected mean signal value. The percentage of outliers observed in V-PLEX assays is typically <0.1% of the wells in the sample set. A slightly elevated percentage of outliers (approximately 1%) is observed for the IL-1α 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 (human) 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-1α	0.09	0.05–2.40	2.85	278
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 a nonhuman 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 show most assays are below 15% for this panel.

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 (human) 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-1α	Control 1	134	3.6	5.5	7.4
	Control 2	36.3	3.5	5.6	6.1
	Control 3	8.09	4.1	8.0	6.4
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, normal human serum, EDTA plasma, heparin plasma, citrate plasma, and urine from a commercial source as well as cell culture supernatants were spiked with recombinant calibrators and diluted 2-fold, 4-fold, 8-fold, 16-fold, 32-fold, and 64-fold before testing. Percent recovery at each dilution level was normalized to the dilution-adjusted, 2-fold concentration. The average percent recovery shown below is based on samples within the quantitative range of the assay.

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

Table 7. Analyte percent recovery at various dilutions in each sample type

		GM-CSF		IL-1α		IL-5		IL-7		IL-12/IL-23p40	
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=11)	4	108	91–136	118	98–170	108	89–169	96	84–120	101	90–128
	8	98	77–141	120	65–220	101	76–134	85	65–104	91	65–114
	16	94	68–145	138	59–320	94	74–129	82	64–107	90	66–114
	32	89	64–144	175	63–621	99	73–129	78	66–108	87	71–107
	64	92	66–143	209	75–834	99	76–125	82	71–122	89	78–119
EDTA Plasma (N=11)	4	100	89–122	103	85–137	102	93–116	91	86–95	98	85–110
	8	91	80–123	101	78–182	98	82–124	82	75–91	91	72–106
	16	87	74–119	100	66–202	90	69–114	78	70–88	87	68–102
	32	80	66–103	104	62–247	88	66–115	72	62–86	81	62–97
	64	81	68–106	114	65–276	86	65–107	76	62–84	83	61–99
Heparin Plasma (N=11)	4	102	88–135	105	88–139	106	87–123	98	84–106	107	91–133
	8	93	78–142	102	74–181	103	86–127	91	73–99	101	82–131
	16	93	72–152	109	63–258	96	77–127	89	72–99	103	80–145
	32	89	72–144	114	59–294	97	73–124	87	65–100	99	73–130
	64	93	74–159	134	66–422	96	65–122	93	66–110	103	78–144
Citrate Plasma (N=10)	4	97	95–99	120	92–156	98	89–114	92	88–100	102	95–112
	8	85	81–88	129	88–209	92	78–115	82	73–94	96	77–113
	16	81	72–86	139	86–253	86	73–112	79	70–93	97	82–116
	32	74	65–80	140	84–266	82	71–109	74	66–86	90	72–113
	64	75	69–83	145	83–320	78	68–111	75	68–82	93	74–110
Urine (N=5)	4	101	95–109	109	101–123	99	88–114	102	98–109	101	96–104
	8	101	93–110	114	101–126	110	88–133	100	95–104	99	94–105
	16	100	89–112	110	96–126	109	81–138	102	94–108	97	91–104
	32	98	86–112	112	96–130	105	80–123	97	90–103	95	89–101
	64	104	89–122	114	94–130	105	77–125	103	95–108	100	90–110
Cell Culture Supernatant (N=6)	4	93	86–98	110	95–124	94	89–98	88	85–91	103	94–109
	8	91	86–98	109	96–137	89	87–92	89	83–93	95	90–101
	16	89	83–101	101	89–116	83	80–85	86	82–91	93	86–100
	32	88	83–95	105	96–122	83	81–85	89	82–97	90	87–94
	64	91	84–99	104	88–120	80	78–83	92	86–100	94	90–100

Table 7 continued

		IL-15		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	Average % Recovery	% Recovery Range
Serum (N=11)	4	90	79–115	95	86–103	104	74–128	106	86–143	106	91–121
	8	85	69–94	88	72–99	95	58–108	100	76–116	95	77–113
	16	80	62–94	86	73–101	90	64–95	98	72–115	96	70–122
	32	83	73–98	91	77–101	87	72–96	99	69–120	118	74–170
	64	83	70–101	98	83–122	88	77–100	97	71–116	145	82–213
EDTA Plasma (N=11)	4	85	77–93	93	81–104	101	92–111	100	93–108	92	74–107
	8	82	75–94	79	68–93	96	86–108	94	83–108	83	69–96
	16	77	69–82	74	58–88	93	79–111	85	74–104	77	65–90
	32	74	62–83	77	60–100	86	68–106	80	68–92	84	68–101
	64	73	63–81	80	60–106	87	67–102	79	66–95	95	71–121
Heparin Plasma (N=11)	4	83	72–93	97	79–115	102	83–110	102	90–114	107	85–115
	8	78	64–87	89	64–102	95	84–108	99	83–115	94	80–110
	16	75	64–84	87	57–107	94	82–104	96	82–114	82	65–99
	32	74	58–89	93	58–120	87	76–97	94	77–111	96	57–130
	64	75	61–86	99	63–123	89	75–109	94	77–114	139	54–234
Citrate Plasma (N=10)	4	85	76–93	92	86–99	102	93–128	117	106–143	96	87–108
	8	79	70–90	81	76–91	94	81–143	122	102–159	89	77–102
	16	77	68–88	75	67–85	93	75–158	120	94–167	89	69–114
	32	75	67–87	76	67–91	89	70–156	113	82–169	102	71–137
	64	72	64–81	78	70–93	90	73–152	108	81–153	116	76–148
Urine (N=5)	4	84	80–90	123	106–144	116	100–162	93	90–95	90	89–94
	8	82	77–92	130	104–161	112	95–166	89	85–91	84	82–87
	16	81	74–92	131	99–173	112	90–169	84	80–88	79	75–83
	32	85	77–92	135	101–173	107	86–163	86	81–89	81	79–86
	64	86	77–98	140	99–192	114	87–178	87	81–91	85	82–90
Cell Culture Supernatant (N=6)	4	80	78–81	101	95–110	89	83–91	87	86–89	89	87–90
	8	80	76–85	99	95–103	81	74–89	84	82–86	79	76–82
	16	82	77–87	96	91–114	79	75–84	81	79–83	75	70–80
	32	84	77–90	107	97–129	79	73–84	81	79–83	76	72–82
	64	90	86–95	112	102–140	84	76–89	82	77–85	77	72–82

Spike Recovery

Spike recovery measurements of different sample types throughout the quantitative range of the assays were evaluated. Multiple individual human samples (serum, EDTA plasma, heparin plasma, citrate plasma, and urine) were obtained from a commercial source. These samples, along with cell culture supernatants, were spiked with calibrators at three levels (high, mid, and low) then diluted 2-fold. The average % recovery for each sample type is reported along with %CV and % recovery range.

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

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

	Citrate Plasma (N=11)			Heparin Plasma (N=11)			EDTA Plasma (N=11)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
GM-CSF	91	13.0	65–108	96	15.8	60–128	92	17.9	51–127
IL-1α	72	27.4	18–107	86	23.7	17–112	69	32.2	11–100
IL-5	86	14.8	54–110	86	19.5	52–120	84	15.5	55–112
IL-7	100	11.2	68–117	104	8.7	91–130	86	15.2	72–126
IL-12/IL-23p40	92	8.1	78–107	94	10.8	79–130	88	13.3	71–127
IL-15	106	13.8	76–138	110	11.1	92–143	106	17.0	78–150
IL-16	91	9.9	73–110	94	8.3	82–116	91	9.1	78–114
IL-17A	99	14.6	77–132	98	11.0	80–120	97	15.0	60–145
TNF-β	85	12.5	63–108	92	11.3	66–110	89	19.4	64–128
VEGF-A	73	27.0	37–104	89	10.1	74–115	63	39.0	32–118

	Serum (N=10)			Urine (N=5)			Cell Culture Supernatants (N=6)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
GM-CSF	108	7.5	96–132	122	7.8	106–138	99	6.1	89–114
IL-1α	64	41.6	22–101	100	9.4	90–115	93	5.4	83–105
IL-5	100	14.0	75–126	109	5.9	99–125	109	10.4	92–130
IL-7	110	8.6	88–130	114	8.9	97–135	86	5.4	77–93
IL-12/IL-23p40	91	9.8	71–117	125	7.4	102–140	120	7.5	103–143
IL-15	124	18.7	87–173	122	13.9	98–152	78	11.6	68–95
IL-16	98	10.7	83–124	74	16.9	48–96	92	13.9	78–131
IL-17A	97	17.7	49–117	135	11.1	105–171	121	6.1	107–133
TNF-β	69	20.5	42–90	137	6.1	123–152	118	6.8	107–132
VEGF-A	77	25.4	35–121	110	12.7	88–134	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 3 freeze-thaw cycles without significantly affecting the performance of the assay. Once reconstituted, the multianalyte calibrator is stable for one day at 2–8 °C. Partially used MSD plates may be sealed and stored up to 30 days at 2–8 °C in the original foil pouch with desiccant. Results from control measurements changed by ≤30% after partially used plates were stored for 30 days. The validation study includes a real-time stability study with scheduled performance evaluations of complete kits for up to 54 months from the date of manufacture.

Calibration

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

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

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

Analyte	NIBSC/WHO Catalog Number	NIBSC (IU/mL): MSD (pg/mL)
GM-CSF	88/646	0.0079
IL-1α	86/632	0.099
IL-5	90/586	0.0078
IL-7	90/530	0.099
IL-15	95/554	0.024
IL-17A	01/420	0.0039
TNF-β	87/640	0.14
VEGF-A	02/286	0.0015

Tested Samples

Normal Samples

Normal human serum, EDTA plasma, heparin plasma, citrate plasma, and urine samples from a commercial source 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.

Table 10. *Normal human samples tested in the Cytokine Panel 1 (human) Kit*

Sample Type	Statistic	GM-CSF	IL-1α	IL-5	IL-7	IL-12/IL-23p40	IL-15	IL-16	IL-17A	TNF-β	VEGF-A
Serum (N=20)	Median (pg/mL)	0.183	1.18	0.283	0.919	53.3	1.29	59.9	0.927	0.15	9.62
	Range (pg/mL)	0.18–0.18	0.29–62.1	0.11–0.62	0.37–2.78	13.1–159	0.56–3.01	24–137	0.28–4.87	–	1.79–187
	% Detected	5	55	30	95	100	100	100	40	5	70
EDTA Plasma (N=20)	Median (pg/mL)	0.83	3.15	0.20	1.88	62.5	1.28	93.7	0.59	0.12	54.5
	Range (pg/mL)	0.17–1.49	0.74–99.2	0.08–0.54	0.37–39.9	15.1–395	0.98–3.05	51.3–973	0.22–8.51	–	17.7–347
	% Detected	10	100	45	100	100	100	100	55	5	100
Heparin Plasma (N=20)	Median (pg/mL)	ND	0.87	0.17	4.14	45.2	1.16	100	0.55	0.12	64.4
	Range (pg/mL)	ND	0.29–57.5	0.08–0.56	0.20–38.8	9.02–148	0.77–2.26	34.9–1109	0.28–1.59	–	4.95–484
	% Detected	0	50	40	95	95	95	95	30	5	85
Citrate Plasma (N=10)	Median (pg/mL)	ND	ND	0.28	0.92	48.3	1.13	58.4	0.37	0.13	15.8
	Range (pg/mL)	ND	ND	0.11–0.62	0.37–2.00	13.1–133	0.88–2.13	32.5–95.4	0.28–4.87	0.11–0.15	4.10–187
	% Detected	0	0	40	90	90	90	90	60	20	70
Urine (N=5)	Median (pg/mL)	1.03	2.33	ND	0.67	14.0	0.61	46.8	3.05	ND	49.5
	Range (pg/mL)	0.25–1.82	0.21–3.07	ND	0.19–1.44	0.54–27.4	0.13–1.96	–	0.91–5.18	ND	37.3–85.3
	% Detected	40	80	0	60	40	80	20	40	0	80

ND = nondetectable
% detected = % of samples with concentrations at or above the LLOD
Dash (-) = not applicable

Stimulated Samples

Freshly collected, normal, pooled, human whole blood was incubated at 37 °C for different periods with lipopolysaccharide (LPS) as shown below; plasma was isolated at the end of incubations. The dilution-adjusted concentrations (pg/mL) for each stimulation model are displayed below. All analytes assayed showed a significant difference in analyte level with prolonged stimulation.

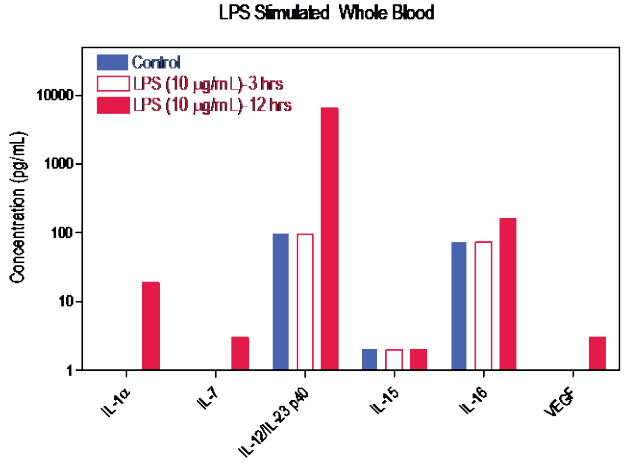

Figure 5*. Normal human whole blood stimulated with LPS.*

Normal human whole blood was enriched for leukocytes and platelets and was treated with LPS, phytohaemagglutinin (PHA), pokeweed mitogen (PWM), or concanavalin A (Con A), and costimulated with CD3 and CD28 antibodies. The samples were collected and tested. The dilution-adjusted concentrations (pg/mL) for each stimulation model are displayed below. In most cases, assays showed a significant difference in analyte level with prolonged stimulation.

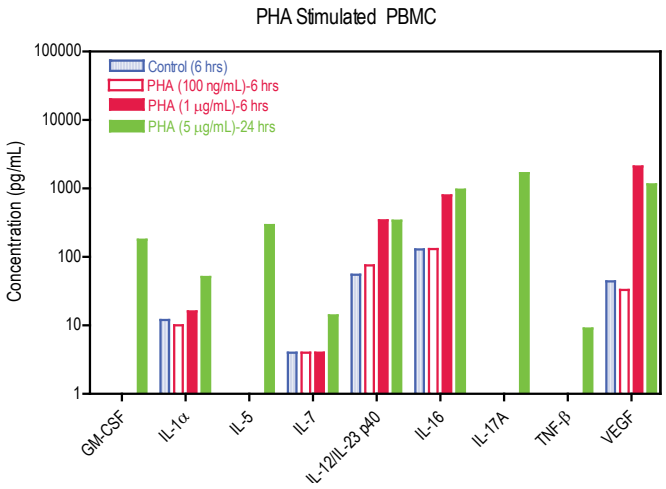

Figure 6. Enriched whole blood treated with PHA

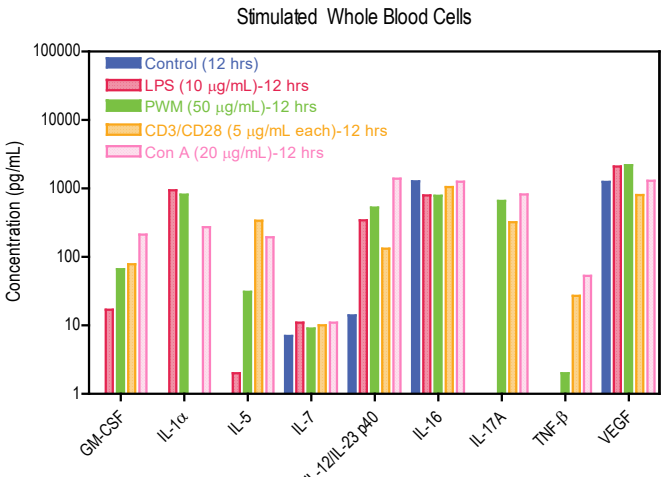

Figure 7. Enriched whole blood treated with LPS, PWM, Con A, and CD3/CD28.

A human acute monocyte leukemia cell line (THP-1) was stimulated for different periods with LPS (10 µg/mL) as shown below. Supernatants were then isolated and tested. The dilution-adjusted concentrations (pg/mL) for each stimulation model are displayed below. All assays showed a significant difference in analyte level with prolonged stimulation.

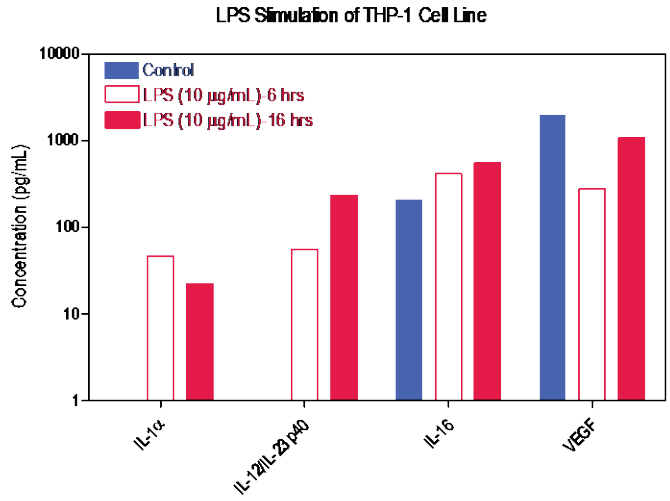

Figure 8. THP-1 cell line stimulated for different times with LPS

Assay Components

Calibrators

The assay calibrator blend uses the following recombinant human proteins:

Table 11. Recombinant human proteins used in the Calibrators

Calibrator	Expression System
GM-CSF	*E. coli*
IL-1α	*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

Table 12. Antibody source species

	Source Species		
Analyte	MSD Capture Antibody	MSD Detection Antibody	Assay Generation
GM-CSF	Mouse Monoclonal	Rat Monoclonal	A
IL-1α	Mouse Monoclonal	Goat Polyclonal	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).

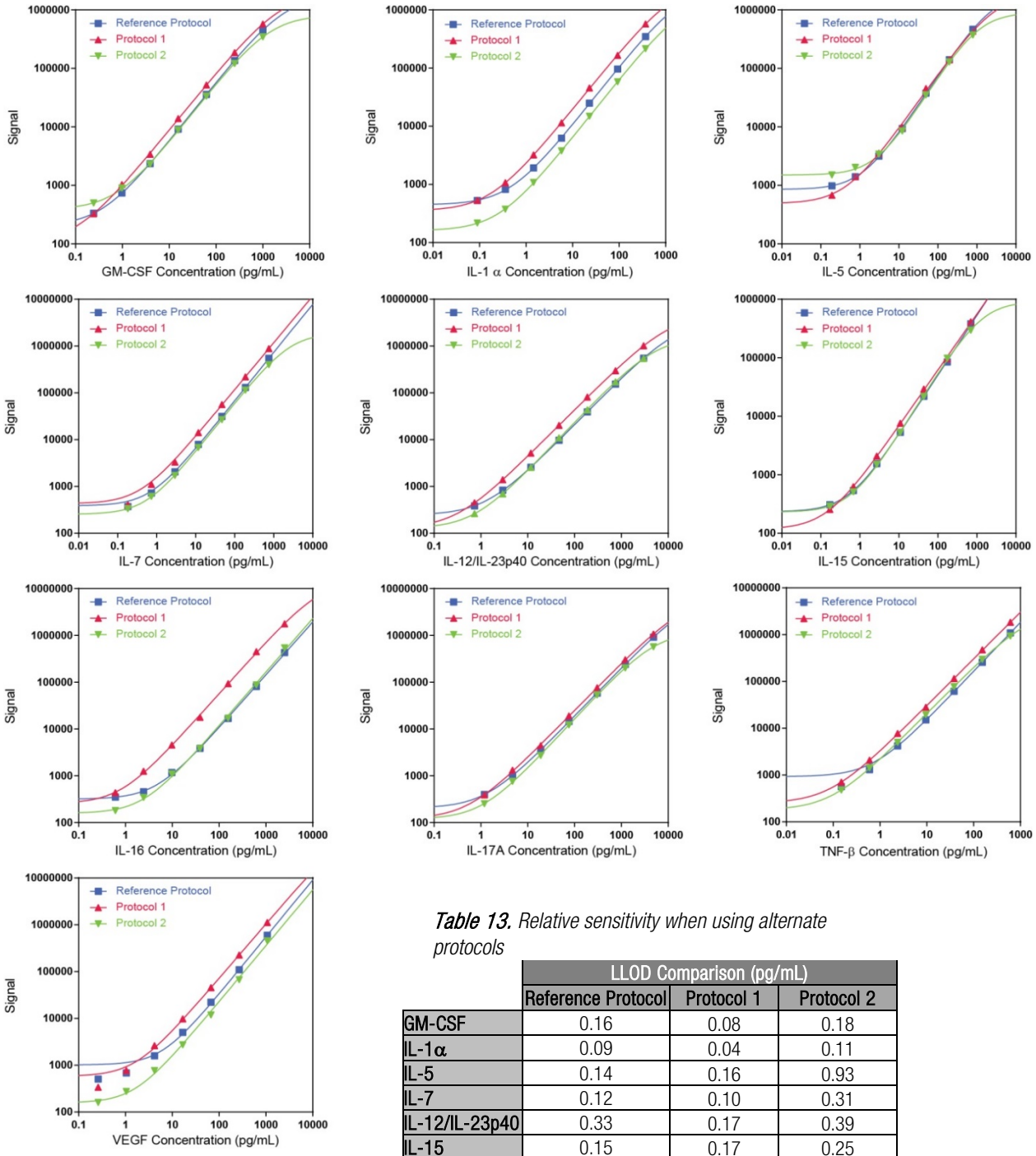


Table 13. Relative sensitivity when using alternate protocols

	LLOD Comparison (pg/mL)		
	Reference Protocol	Protocol 1	Protocol 2
GM-CSF	0.16	0.08	0.18
IL-1α	0.09	0.04	0.11
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 on a single spot plate (blue curve) vs. on the multiplex plate (red curve).

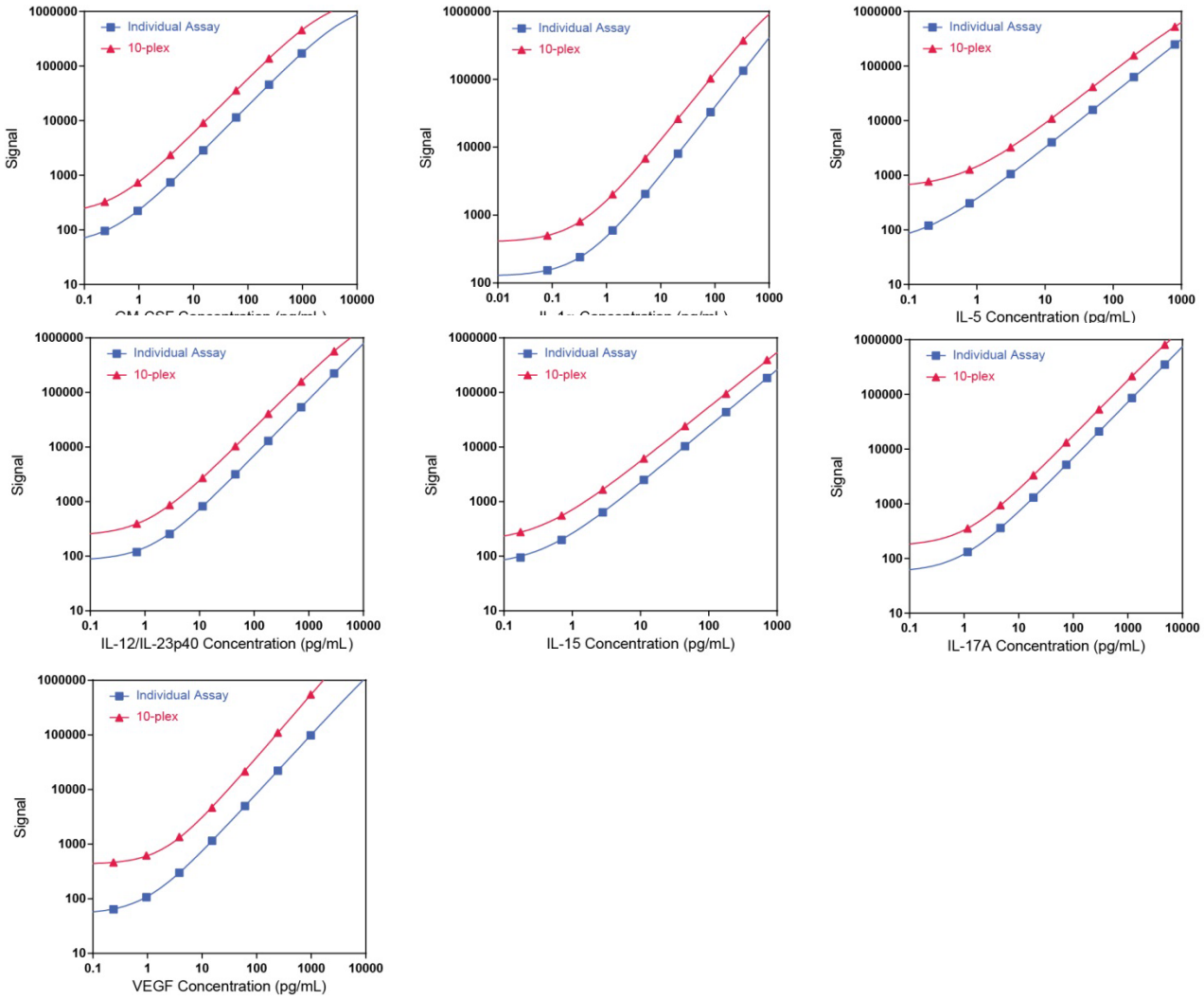


Table 14. *Assay performance for individual and 10-plex assays*

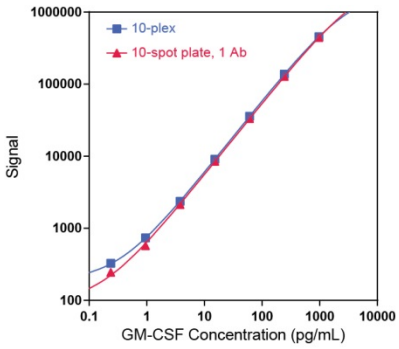
In general, assays in the single spot format yielded a lower overall signal compared to the 10-plex format. The spots on single-spot plates have a larger binding surface than those on multiplex plates, but the same amount of calibrator was used for each test; therefore, the bound calibrator was spread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

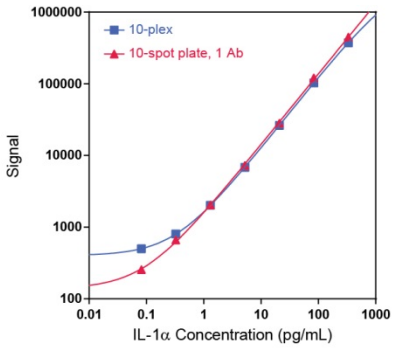
Note: Assay performance for IL-7, IL-16 and TNF-β are not included since the individual assays are run on multiplex plates.

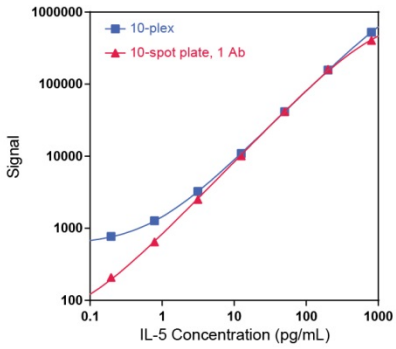
	LLOD (pg/mL)	
Assay	**Individual**	**10-plex**
GM-CSF	0.13	0.16
IL-1α	0.08	0.09
IL-5	0.08	0.14
IL-12p40	0.39	0.33
IL-15	0.11	0.15
IL-17	0.74	0.31
VEGF	1.12	1.12

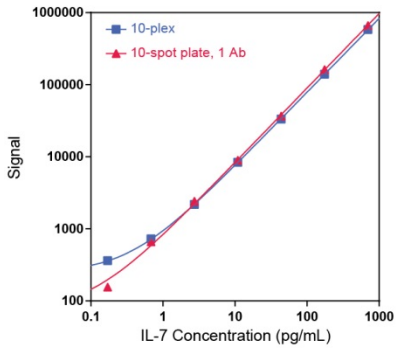
Appendix C

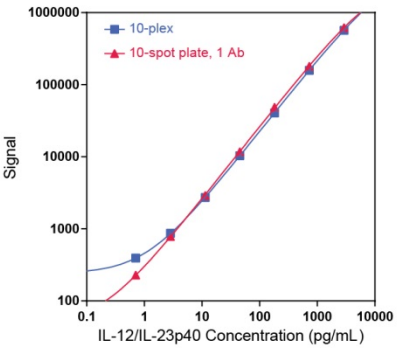
The calibration curves below compare results for each assay in the panel when the assays were run on the 10-spot plate using all detection antibodies (blue curve) vs. running each assay using a single, assay-specific detection antibody (red curve).

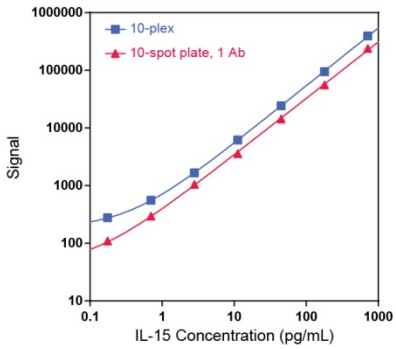


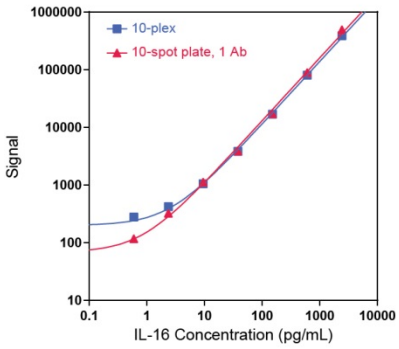


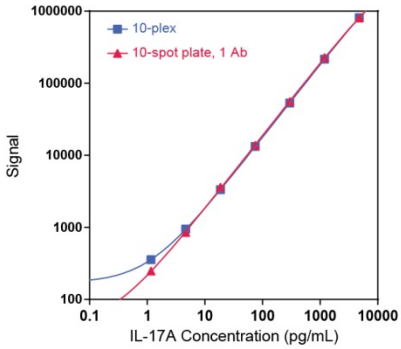


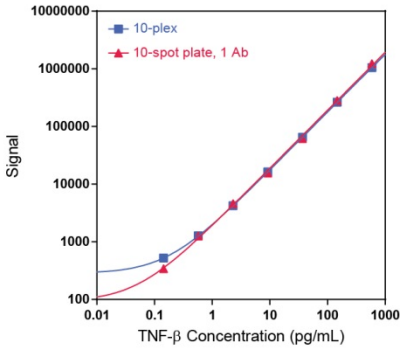












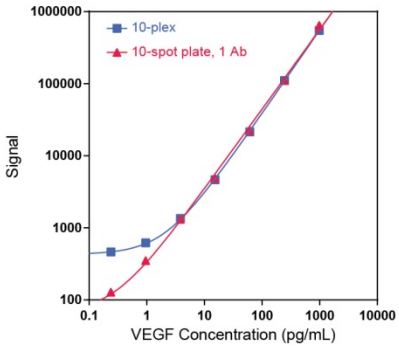


Table 15. LLODs for detection of a single antibody vs. blended antibodies

As expected, both multiplex formats yielded the same specific signal, but lower background signals were 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-1α	0.19	0.09
IL-5	0.10	0.14
IL-7	0.16	0.12
IL-12p40	0.30	0.33
IL-15	0.35	0.15
IL-16	1.32	2.83
IL-17	0.53	0.31
TNF-β	0.04	0.08
VEGF	0.32	1.12

Summary Protocol

Cytokine Panel 1 (human) Kits

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol before performing the cytokine panel 1 (human) 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 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 16. Catalog numbers for V-PLEX and V-PLEX Plus cytokine (human) multiplex and single assay kits

Kit Name	V-PLEX			V-PLEX Plus*		
	1-Plate Kit	5-Plate Kit	25-Plate Kit	1-Plate Kit	5-Plate Kit	25-Plate Kit
Multiplex Kits						
Cytokine Panel 1 (human)	K15050D-1	K15050D-2	K15050D-4	K15050G-1	K15050G-2	K15050G-4
Individual Assay Kits						
Human GM-CSF	K151RID-1	K151RID-2	K151RID-4	K151RIG-1	K151RIG-2	K151RIG-4
Human IL-1α	K151RBD-1	K151RBD-2	K151RBD-4	K151RBG-1	K151RBG-2	K151RBG-4
Human IL-5	K151QSD-1	K151QSD-2	K151QSD-4	K151QSG-1	K151QSG-2	K151QSG-4
Human IL-7	K151RCD-1	K151RCD-2	K151RCD-4	K151RCG-1	K151RCG-2	K151RCG-4
Human IL-12/IL-23p40	K151RJD-1	K151RJD-2	K151RJD-4	K151RJG-1	K151RJG-2	K151RJG-4
Human IL-15	K151RDD-1	K151RDD-2	K151RDD-4	K151RDG-1	K151RDG-2	K151RDG-4
Human IL-16	K151RED-1	K151RED-2	K151RED-4	K151REG-1	K151REG-2	K151REG-4
Human IL-17A	K151RFD-1	K151RFD-2	K151RFD-4	K151RFG-1	K151RFG-2	K151RFG-4
Human TNF-β	K151RGD-1	K151RGD-2	K151RGD-4	K151RGG-1	K151RGG-2	K151RGG-4
Human VEGF-A	K151RHD-1	K151RHD-2	K151RHD-4	K151RHG-1	K151RHG-2	K151RHG-4

*V-PLEX Plus kits include controls, plate seals, and wash buffer. See **Kit Components** (above) or 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 9. Sample plate layout that can be used for the assays. Each sample, calibrator, and control (Plus Kit) is measured in duplicate in side-by-side wells.

Plate Diagram

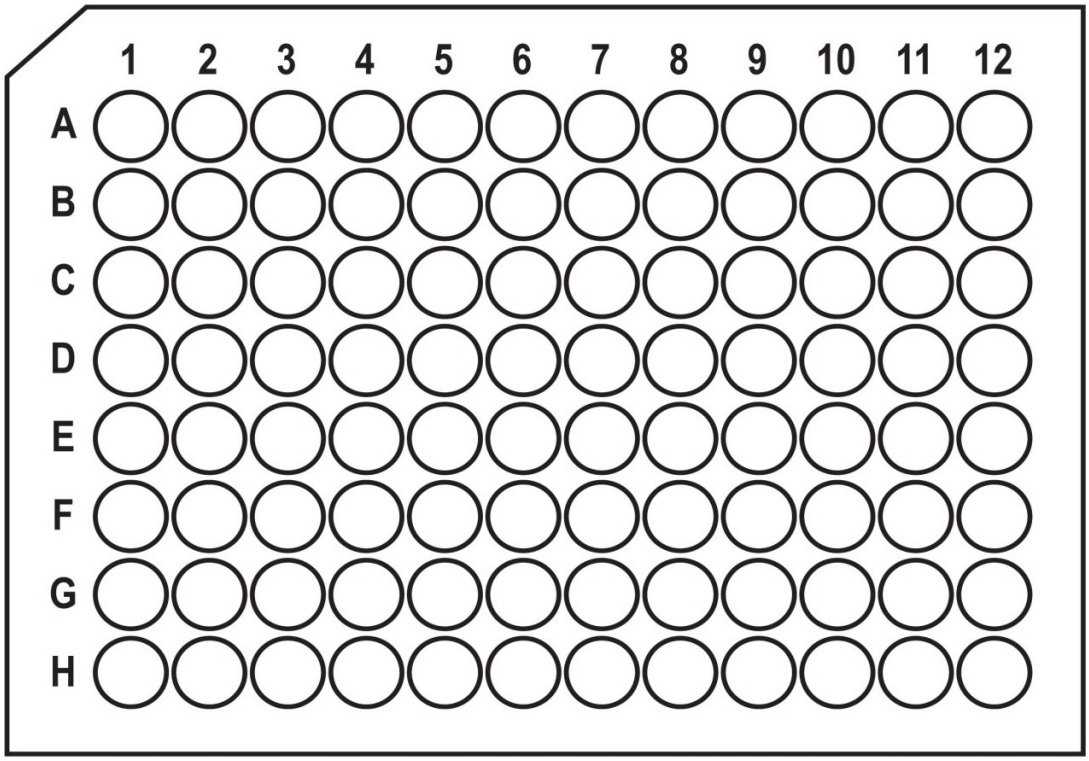


Figure 10. Plate diagram.