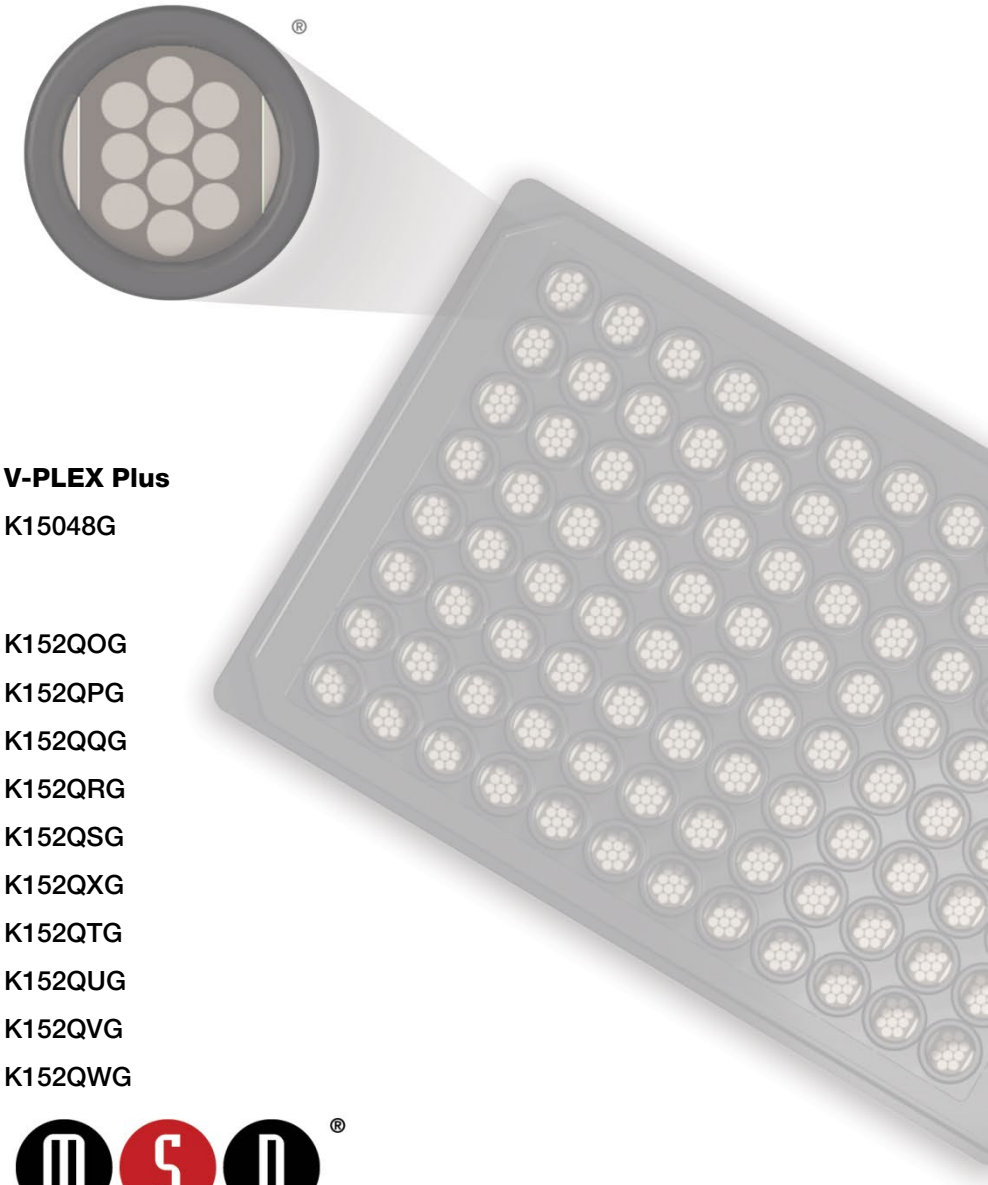
MSD® MULTI-SPOT Assay System

Proinflammatory Panel 1 (mouse) Kits

IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, TNF-α

V-PLEX®



	V-PLEX®	V-PLEX Plus
Multiplex Kits	K15048D	K15048G
Individual Assay Kits		
Mouse IFN-γ	K152QOD	K152QOG
Mouse IL-1β	K152QPD	K152QPG
Mouse IL-2	K152QQD	K152QQG
Mouse IL-4	K152QRD	K152QRG
Mouse IL-5	K152QSD	K152QSG
Mouse IL-6	K152QXD	K152QXG
Mouse KC/GRO	K152QTD	K152QTG
Mouse IL-10	K152QUD	K152QUG
Mouse IL-12p70	K152QVD	K152QVG
Mouse TNF-α	K152QWD	K152QWG

MSD Cytokine Assays

Proinflammatory Panel 1 (mouse) Kits

IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, TNF-α

For use with 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.

MESO SCALE DISCOVERY®
A division of Meso Scale Diagnostics, LLC.
1601 Research Blvd.
Rockville, MD 20850 USA
www.mesoscale.com

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Contact Information

MSD Customer Service

Phone: 1-240-314-2795
Fax: 1-301-990-2776
Email: CustomerService@mesoscale.com

MSD Scientific Support

Phone: 1-240-314-2798
Fax: 1-240-632-2219 attn: Scientific Support
Email: ScientificSupport@mesoscale.com

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] in accordance with MSD's Quality Management System. They offer exceptional sensitivity, simple protocols, reproducible results, and lot-to-lot consistency. In addition to the analytical validation, the robustness of the assay protocol is assessed during development along with the stability and robustness of the assay components and kits. V-PLEX assays are available in both single-assay and multiplex formats.

The V-PLEX assay menu is organized by panels. Grouping the assays into panels by species, analytical compatibility, clinical range, and expected use ensures optimal and consistent performance from each assay while still providing the benefits and efficiencies of multiplexing. V-PLEX panels are provided in MSD's MULTI-SPOT® 96-well plate format. The composition of each panel and the location of each assay (i.e., its spot within the well) are maintained from lot to lot. Most individual V-PLEX assays are provided on MSD's single-spot, 96-well plates. The remaining are provided on the multiplex panel plate.

The Proinflammatory Panel 1 (mouse) measures ten cytokines that are important in inflammation response 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 under-expression may indicate a shift in biological equilibrium. This panel also includes assays for many of the Th1/Th2 pathway biomarkers. The Proinflammatory Panel 1 (mouse) measures biomarkers that are associated with a number of disorders, including rheumatoid arthritis,[1] Alzheimer's disease,[2] asthma,[3] atherosclerosis,[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 Proinflammatory Panel 1 (mouse) kit are: **a)** interferon gamma (IFN-γ), **b)** interleukin-1 beta (IL-1β), **c)** interleukin-2 (IL-2), **d)** interleukin-4 (IL-4), **e)** interleukin-5 (IL-5), **f)** interleukin-6 (IL-6), **g)** KC/GRO, **h)** interleukin-10 (IL-10), **i)** interleukin-12p70 (IL-12p70), and **j)** tumor necrosis factor alpha (TNF-α).

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 Proinflammatory Panel 1 (mouse) are sandwich immunoassays. MSD provides a plate pre-coated with capture antibodies on independent and well-defined spots, as shown in the layouts below. Multiplex assays and the individual IL-4, IL-5, KC/GRO, IL-10, and IL-12p70 assays are provided on 10-spot MULTI-SPOT plates (Figure 1); the individual IFN-γ, IL-1β, IL-2, IL-6, and TNF-α 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.[14]

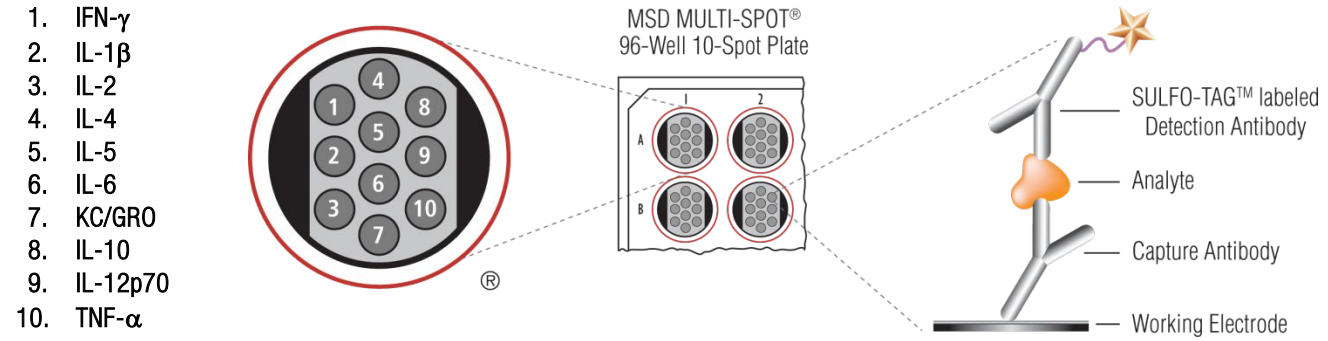


Figure 1. Multiplex plate spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

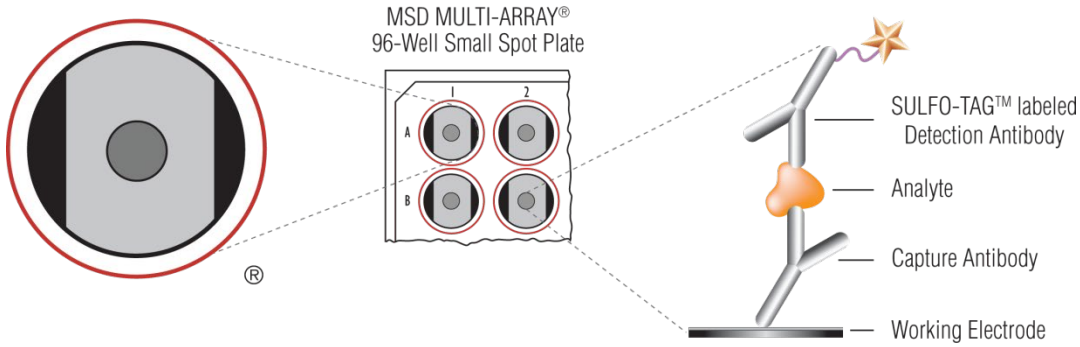


Figure 2. Small Spot plate diagram showing placement of analyte capture antibodies.

Kit Components

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

See the **Catalog Numbers** section for complete kits.

Reagents Supplied With All Kits

Table 1. Reagents that are supplied with V-PLEX and V-PLEX Plus Kits

Reagent	Storage	Catalog No.	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Proinflammatory Panel 1 (mouse) Calibrator Blend	2–8 °C	C0048-2	1 vial	1 vial	5 vials	25 vials	Ten recombinant mouse proteins in diluent, buffered and lyophilized. Individual analyte concentration is provided in the lot-specific certificate of analysis (COA).
Diluent 41	≤-10 °C	R50AH-1	10 mL	1 bottle			Diluent for samples and calibrator; contains protein, blockers, and preservatives.
		R50AH-2	50 mL		1 bottle	5 bottles	
Diluent 45	≤-10 °C	R50AI-1	5 mL	1 bottle			Diluent for detection antibody; contains protein, blockers, and preservatives.
		R50AI-2	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	
Proinflammatory Panel 1 (mouse) Control 1*	2–8 °C	C4048-1	1 vial	1 vial	5 vials	25 vials	Multi-analyte controls in mouse serum, buffered, lyophilized, and spiked with recombinant mouse analytes. The concentration of the controls is provided in the lot-specific COA.
Proinflammatory Panel 1 (mouse) Control 2*	2–8 °C	C4048-1	1 vial	1 vial	5 vials	25 vials	
Proinflammatory Panel 1 (mouse) Control 3*	2–8 °C	C4048-1	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 Proinflammatory Panel 1 (mouse) Control Pack

Kit-Specific Components

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

Plates	Storage	Part No.	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Proinflammatory Panel 1 (mouse) Plate	2–8 °C	N05048A-1	10-spot	1	5	25	96-well plate, foil sealed, with desiccant.
Mouse IFN-γ Plate	2–8 °C	L452QOA-1	Small Spot	1	5	25	
Mouse IL-1β Plate	2–8 °C	L452QPA-1	Small Spot	1	5	25	
Mouse IL-2 Plate	2–8 °C	L452QQA-1	Small Spot	1	5	25	
Mouse IL-6 Plate	2–8 °C	L452QXA-1	Small Spot	1	5	25	
Mouse TNF-α Plate	2–8 °C	L452QWA-1	Small Spot	1	5	25	

Table 4. *Individual detection antibodies for each assay are supplied with specific kits*

SULFO-TAG Detection Antibody	Storage	Catalog No.	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Anti-ms IFN-γ Antibody (50X)	2–8 °C	D22QO-2	75 µL	1			SULFO-TAG conjugated antibody.
		D22QO-3	375 µL		1	5	
Anti-ms IL-1β Antibody (50X)	2–8 °C	D22QP-2	75 µL	1			SULFO-TAG conjugated antibody.
		D22QP-3	375 µL		1	5	
Anti-ms IL-2 Antibody (50X)	2–8 °C	D22QQ-2	75 µL	1			SULFO-TAG conjugated antibody.
		D22QQ-3	375 µL		1	5	
Anti-ms IL-4 Antibody (50X)	2–8 °C	D22QR-2	75 µL	1			SULFO-TAG conjugated antibody.
		D22QR-3	375 µL		1	5	
Anti-ms IL-5 Antibody (50X)	2–8 °C	D22QS-2	75 µL	1			SULFO-TAG conjugated antibody.
		D22QS-3	375 µL		1	5	
Anti-ms IL-6 Antibody (50X)	2–8 °C	D22QX-2	75 µL	1			SULFO-TAG conjugated antibody.
		D22QX-3	375 µL		1	5	
Anti-ms KC/GRO Antibody (50X)	2–8 °C	D22QT-2	75 µL	1			SULFO-TAG conjugated antibody.
		D22QT-3	375 µL		1	5	
Anti-ms IL-10 Antibody (50X)	2–8 °C	D22QU-2	75 µL	1			SULFO-TAG conjugated antibody.
		D22QU-3	375 µL		1	5	
Anti-ms IL-12p70 Antibody (50X)	2–8 °C	D22QV-2	75 µL	1			SULFO-TAG conjugated antibody.
		D22QV-3	375 µL		1	5	
Anti-ms TNF-α Antibody (50X)	2–8 °C	D22QW-2	75 µL	1			SULFO-TAG conjugated antibody.
		D22QW-3	375 µL		1	5	

Additional Materials and Equipment

- ❑ Appropriately sized tubes for reagent preparation
- ❑ Polypropylene microcentrifuge tubes for preparing dilutions
- ❑ Liquid handling equipment for the desired throughput, capable of dispensing 10 to 150 µL/well into a 96-well microtiter plate
- ❑ Plate washing equipment: automated plate washer or multichannel pipette
- ❑ Microtiter plate shaker (rotary) capable of shaking at 500-1,000 rpm
- ❑ Phosphate-buffered saline (PBS) plus 0.05% Tween-20 for plate washing or MSD Wash Buffer catalog no. R61AA-1 (included in V-PLEX Plus kit)
- ❑ Adhesive plate seals (3 per plate included in V-PLEX Plus kits)
- ❑ Deionized water
- ❑ Vortex mixer

Optional Materials and Equipment

- ❑ Proinflammatory Panel 1 (mouse) Control Pack, available for separate purchase from MSD, catalog no. C4048-1 (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 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.
- Gently tap the plate on a paper towel to remove residual fluid after washing.
- If an incubation step needs to be extended, avoid letting the plate dry out by keeping sample or detection antibody solution in the plate.
- Remove plate seals prior to 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 inter-plate 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, separate Diluent 41 and Diluent 45 into suitable volumes before refreezing.

Prepare Calibrator Dilutions

MSD supplies a multi-analyte lyophilized calibrator that yields the recommended highest calibrator concentration when reconstituted in 1,000 µL of Diluent 41. (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 41 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 41 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 41. Mix well by vortexing. Repeat 4-fold serial dilutions 5 additional times to generate 7 calibrators.
3) Use Diluent 41 as the zero calibrator.

Note: Reconstituted calibrator (Calibrator 1) is stable for 30 days at 2–8 °C. 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.

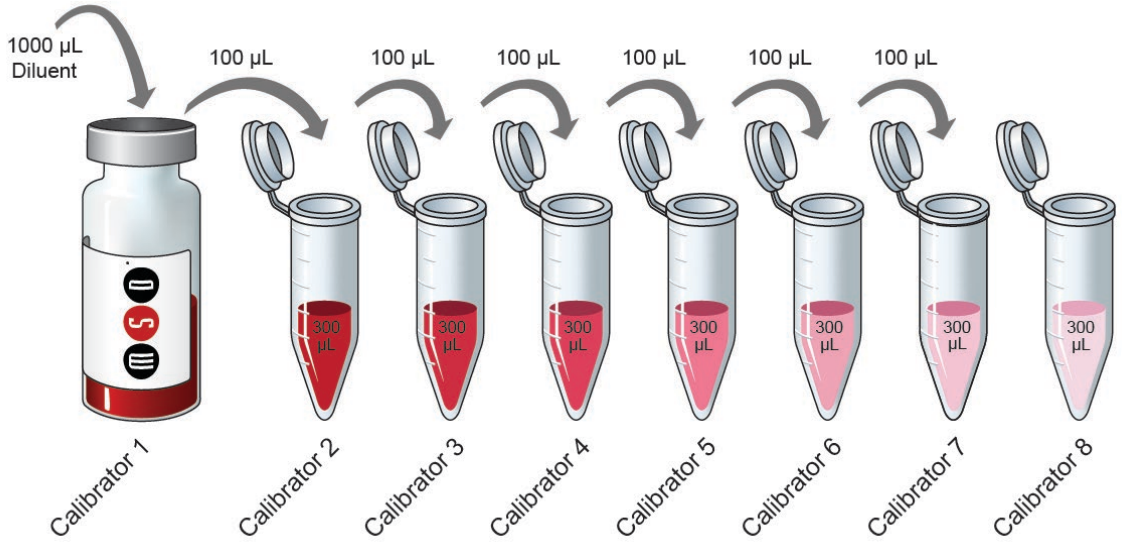


***Figure 3**. Dilution schema for preparation of Calibrator Standards*

Sample Collection and Handling

Below are general guidelines for mouse sample collection, storage, and handling. If possible, use published guidelines.[15,16] Evaluate sample stability under the selected method as needed.

- Serum and plasma. When preparing serum, allow samples to clot for 2 hours at room temperature, then centrifuge for 20 minutes at 2,000g prior to 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 2,000g for 3 minutes to remove particulates before sample preparation.

Dilute Samples

Dilute samples with Diluent 41. For mouse serum, plasma, and urine samples, MSD recommends a minimum 2-fold dilution. For example, to dilute 2-fold, add 60 µL of sample to 60 µL of Diluent 41. 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 duplicates. Additional diluent can be purchased at www.mesoscale.com.

Prepare Controls

Three levels of multi-analyte lyophilized controls are available for separate purchase from MSD in the Proinflammatory Panel 1 (mouse) Control Pack, catalog no. C4048-1. (Controls are included only in V-PLEX Plus kits.)

Reconstitute the lyophilized controls in 250 µL of Diluent 41. Do not invert or vortex the vials. Wait for a minimum of 15–30 minutes before diluting controls 2-fold in Diluent 41. Vortex briefly using short pulses. Refer to the Proinflammatory Panel 1 (mouse) Control Pack product insert for analyte levels. Once reconstituted in 250 µL of Diluent 41, the controls are stable for seven days at 2-8 ºC.

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.

10-plex Proinflammatory Panel 1 (mouse) kit

For one plate, combine the following detection antibodies and add to 2,400 µL of Diluent 45:

- ❑ 60 µL of SULFO-TAG Anti-ms IFN-γ Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IL-1β Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IL-2 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IL-4 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IL-5 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IL-6 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms KC/GRO Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IL-10 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms IL-12p70 Antibody
- ❑ 60 µL of SULFO-TAG Anti-ms TNF-α Antibody

Custom multiplex kits

For one plate, combine 60 µL of each supplied detection antibody, then add Diluent 45 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 45.

Prepare Wash Buffer

MSD provides 100 mL of Wash Buffer as a 20X stock solution in the V-PLEX Plus kit. Dilute the stock solution to 1X before use. PBS + 0.05% Tween-20 can be used instead.

For one plate, combine:

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

Prepare Read Buffer T

MSD provides Read Buffer T as a 4X stock solution. The working solution is 2X.

For one plate, combine:

- ❑ 10 mL of Read Buffer T (4X)
- ❑ 10 mL of deionized water

You may keep excess diluted Read Buffer T in a tightly sealed container at room temperature for up to one month.

Prepare MSD Plate

MSD plates are pre-coated with capture antibodies (Figure 1) and exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies. Plates may be used as delivered; no additional preparation is required.

Assay Protocol

Note: Follow **Reagent Preparation** before beginning this assay protocol.

STEP 1: Wash and Add Sample

- ❑ Wash the plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 50 µL of prepared samples, calibrators, or controls per well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.

Note: Washing the plate before sample addition is an optional step that may provide greater uniformity of results for certain assays. Analytical parameters, including limits of quantification, recovery of controls, and sample quantification, are not affected by washing the plate before sample addition.

STEP 2: Wash and Add Detection Antibody Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 25 µL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.

STEP 3: Wash and Read

- ❑ Wash the plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 150 µL of 2X Read Buffer T to each well. 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 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). Intra-run CVs are typically below 7%, and inter-run CVs are typically below 15%. Rigorous management of inter-lot reagent consistency and calibrator production results in typical inter-lot CVs below 10%. Validation lots are compared using controls and at least 40 samples in various sample matrices. Samples are well correlated with an inter-lot bias typically below 10%.

- ### Matrix Effects and Samples

  Matrix effects from serum, plasma, urine, and cell culture media are measured as part of development and validation. Dilution linearity and spike recovery studies are performed on individual samples rather than pooled samples to assess the variability of results due to matrix effects. The sample dilution suggested in the protocol gives an appropriate dilution factor for all assays in the multiplex. Some assays may benefit from lower or higher dilution factors, depending on the samples and application (data are provided in this product insert). In addition to the matrices listed above, blood, PBMCs,

and/or cell lines that have been stimulated to generate elevated levels of analytes are tested. Results confirm the measurement of native proteins at concentrations that are often higher than those found in individual native samples.

- **Specificity**

  The specificity of both capture and detection antibodies is measured during assay development. Antibody specificity is assessed by first running each assay using the multiplex plate with assay-specific detection antibody and assay-specific calibrator. These results are compared to the assay's performance when the plate is run 1) with the multi-analyte calibrator and assay-specific detection antibodies and 2) with assay-specific calibrator and all detection antibodies. For each validation lot and product release, assay specificity is measured using a multi-analyte calibrator and individual detection antibodies. The calibrator concentration used for specificity testing is chosen to ensure that the specific signal is greater than 50,000 counts.

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

- **Assay Robustness and Stability**

  The robustness of the assay protocol is assessed by examining the boundaries of the selected incubation times and evaluating the stability of assay components during the experiment and the stability of reconstituted lyophilized components during storage. For example, the stability of reconstituted calibrator is assessed in real-time over 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.

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 Proinflammatory Panel 1 (mouse) were collected over six months of testing by five operators (63 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.

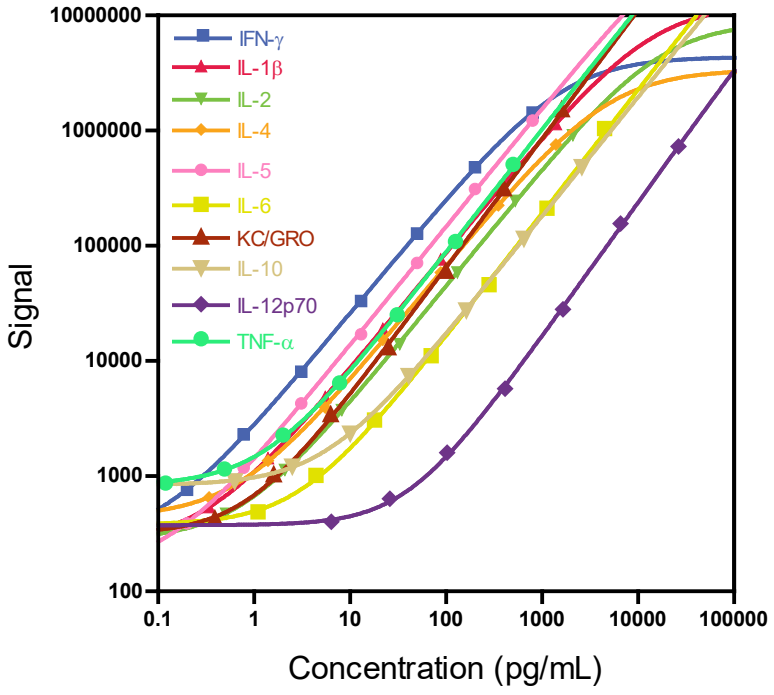


Figure 4. Typical calibration curves for the Proinflammatory Panel 1 (mouse) assay

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 63 runs.

The ULOQ is the highest concentration at which the CV of the calculated concentration is <25% and the recovery of each analyte is within 75% to 125% of the known value.

The LLOQ is the lowest concentration at which the CV of the calculated concentration is <25% and the recovery of each analyte is within 75% to 125% 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 Proinflammatory Panel 1 (mouse) Kit

	Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
IFN-γ	0.04	0.01–0.14	0.390	570
IL-1β	0.11	0.05–0.23	2.04	1,030
IL-2	0.22	0.07–1.40	1.03	1,570
IL-4	0.11	0.07–0.18	0.818	1,060
IL-5	0.06	0.04–0.07	0.302	590
IL-6	0.61	0.06–1.38	7.61	3,140
KC/GRO	0.24	0.13–0.71	3.29	1,230
IL-10	0.94	0.42–2.22	7.26	2,030
IL-12p70	9.95	2.92–25.3	179	20,600
TNF-α	0.13	0.05–3.01	0.980	403

Precision

Controls were made by spiking calibrator into mouse serum at three levels within the quantitative range of the assay. Analyte levels were measured by five operators using a minimum of three replicates on 49 runs over five months. Results are shown below. While a typical specification for precision is a concentration CV of less than 25% for controls on both intra- and inter-day runs, for this panel, the data shows most assays are below 15%.

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

Inter-run %CV is the variability of controls across 49 runs.

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

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

	Control	Average Conc. (pg/mL)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
IFN-γ	Control 1	740	4.4	10.9	4.7
	Control 2	56.1	2.2	10.3	6.6
	Control 3	5.03	2.4	11.7	7.4
IL-1β	Control 1	1,412	2.9	8.7	2.9
	Control 2	105	2.0	10.3	5.3
	Control 3	9.31	2.3	12.5	7.5
IL-2	Control 1	2,504	2.5	8.4	6.0
	Control 2	187	2.6	11.1	9.0
	Control 3	14.8	2.9	11.9	6.4
IL-4	Control 1	701	2.5	8.2	4.8
	Control 2	74.0	2.4	9.3	6.1
	Control 3	10.7	3.1	12.5	9.6
IL-5	Control 1	832	2.7	12.2	6.3
	Control 2	53.2	2.2	11.4	8.3
	Control 3	2.88	3.1	20.0	4.9
IL-6	Control 1	5,031	2.3	10.6	4.8
	Control 2	542	2.6	10.4	1.1
	Control 3	61.5	2.6	11.1	5.3
KC/GRO	Control 1	1,922	2.2	10.1	1.8
	Control 2	237	2.1	9.9	6.8
	Control 3	25.5	2.7	10.8	9.7
IL-10	Control 1	2,730	4.1	8.3	6.3
	Control 2	619	3.9	10.8	7.1
	Control 3	137	4.2	11.8	6.1
IL-12p70	Control 1	32,794	2.2	10.6	3.1
	Control 2	4,494	1.8	12.1	5.4
	Control 3	643	2.3	12.6	6.2
TNF-α	Control 1	380	2.8	12.6	6.0
	Control 2	103	2.1	8.5	3.6
	Control 3	28.5	2.5	13.6	8.7

Dilution Linearity

To assess linearity, normal mouse 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

		IFN-γ		IL-1β		IL-2		IL-4		IL-5	
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=8)	4	107	94–115	108	99–122	106	98–115	144	128–154	106	92–129
	8	99	86–107	96	82–102	100	88–110	156	136–169	101	85–124
	16	98	86–108	98	88–105	94	82–110	170	145–182	99	84–126
	32	95	82–101	99	87–109	96	78–110	171	139–190	94	83–116
	64	98	82–109	100	88–109	97	83–111	184	144–204	100	86–124
EDTA Plasma (N=8)	4	101	90–112	103	98–109	98	92–106	123	114–129	100	90–107
	8	100	80–115	101	86–111	94	84–104	134	127–149	96	79–110
	16	106	78–155	100	85–112	90	78–105	147	138–167	95	80–114
	32	103	79–124	100	83–115	93	82–108	149	133–177	99	79–120
	64	114	80–167	104	79–125	94	79–109	160	143–196	105	82–133
Heparin Plasma (N=8)	4	105	82–122	106	100–112	101	94–112	134	124–139	100	89–113
	8	98	81–123	102	97–109	95	90–104	147	136–152	95	87–105
	16	107	91–171	100	93–111	92	83–107	162	153–179	93	85–110
	32	103	86–161	100	92–111	95	81–109	159	129–174	93	78–121
	64	106	87–147	101	94–115	93	78–108	174	154–194	98	81–131
Citrate Plasma (N=8)	4	99	83–109	103	95–109	102	94–111	120	105–131	97	94–106
	8	91	79–100	95	85–102	94	88–103	125	112–137	87	76–97
	16	88	71–103	92	83–101	90	85–99	133	112–149	83	70–102
	32	83	71–96	89	75–104	87	74–97	129	105–147	83	66–102
	64	86	74–101	91	73–108	87	74–98	134	111–155	83	58–107
Urine (N=6)	4	99	93–104	104	93–110	105	97–115	122	118–128	98	84–114
	8	96	87–104	102	93–111	103	95–119	136	129–143	97	88–115
	16	96	89–101	99	81–107	100	94–118	149	145–153	91	75–106
	32	94	88–100	101	94–112	103	95–124	151	146–157	93	82–106
	64	97	86–103	102	86–112	103	91–122	166	160–169	95	81–105
Cell Culture Supernatant (N=4)	4	103	100–105	105	101–108	96	93–100	112	108–118	96	94–98
	8	98	96–99	102	99–107	92	89–95	112	108–115	93	92–97
	16	100	96–104	102	99–108	88	87–90	115	112–122	92	86–96
	32	95	94–97	101	97–106	92	91–93	112	109–115	92	85–99
	64	100	96–105	104	101–107	93	87–96	119	111–125	95	89–100

Table 7 continued

		IL-6		KC/GRO		IL-10		IL-12p70		TNF-α	
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=8)	4	98	87–109	118	105–154	120	105–142	98	84–112	111	100–124
	8	90	72–102	110	96–146	117	102–135	87	73–100	108	99–120
	16	90	74–100	106	95–136	119	105–136	81	65–94	109	97–123
	32	94	73–112	111	98–145	117	101–143	78	62–90	117	105–142
	64	98	74–117	118	103–147	126	107–159	81	61–93	123	107–156
EDTA Plasma (N=8)	4	97	83–95	91	83–95	103	99–117	93	87–101	99	90–110
	8	91	72–93	85	72–93	101	90–118	85	78–94	100	88–111
	16	88	64–90	82	64–90	102	94–123	81	75–89	98	84–112
	32	91	68–106	87	68–106	99	85–121	81	68–92	102	85–124
	64	95	72–112	90	72–112	102	88–127	86	69–102	108	89–136
Heparin Plasma (N=8)	4	98	76–111	93	76–111	111	103–127	87	77–95	102	95–111
	8	96	68–115	90	68–115	107	94–122	77	66–89	100	89–109
	16	97	65–118	86	65–118	109	95–124	75	66–84	100	88–109
	32	101	65–122	88	65–122	105	90–129	74	62–87	105	89–118
	64	104	64–125	88	64–125	111	92–131	78	63–90	107	91–119
Citrate Plasma (N=8)	4	91	86–99	90	77–99	99	86–106	96	86–105	99	92–106
	8	83	72–97	79	64–94	89	75–99	84	68–96	94	88–97
	16	79	67–94	73	59–86	87	75–101	79	52–94	91	85–95
	32	80	65–99	71	53–91	83	71–98	76	46–98	92	89–97
	64	82	60–102	74	54–91	88	76–103	79	44–103	93	87–98
Urine (N=6)	4	99	95–104	85	78–95	103	87–114	108	96–122	98	91–107
	8	94	83–106	70	59–79	98	84–109	107	87–133	96	84–112
	16	93	81–101	64	53–75	94	74–111	106	90–132	97	85–116
	32	96	85–105	63	49–77	91	82–106	106	91–140	102	89–122
	64	101	92–108	64	50–78	95	85–108	113	101–148	107	94–130
Cell Culture Supernatant (N=4)	4	93	91–94	76	72–80	93	87–100	94	92–98	93	92–94
	8	90	88–92	69	63–72	84	79–92	87	85–88	89	87–93
	16	88	83–91	65	60–69	84	77–94	83	79–88	89	84–93
	32	93	88–97	67	62–72	81	74–90	81	78–87	93	88–96
	64	96	90–102	70	66–76	85	77–101	85	79–93	96	92–100

Note: For the IL-4 assay, dilution linearity performance may be improved by normalizing each dilution point to the 4-fold dilution, rather than to the 2-fold dilution.

Spike Recovery

Spike recovery measurements of different sample types throughout the quantitative range of the assays were evaluated. Multiple individual mice 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 Proinflammatory Panel 1 (mouse) Kit

	Citrate Plasma (N=8)			Heparin Plasma (N=7)			EDTA Plasma (N=7)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
IFN-γ	109	9.8	92–131	99	11.8	69–114	103	18.8	84–143
IL-1β	102	10.8	85–123	99	8.1	87–117	105	11.9	90–135
IL-2	99	6.2	90–111	97	4.9	90–104	106	4.0	99–117
IL-4	66	6.7	55–75	58	6.4	48–67	67	7.9	58–81
IL-5	101	9.1	81–115	99	10.6	79–116	102	10.9	83–126
IL-6	99	10.6	78–115	97	11.7	80–116	105	17.1	84–143
KC/GRO	108	9.4	91–127	100	10.3	89–129	98	8.6	82–113
IL-10	90	6.2	77–100	92	8.8	73–111	101	8.9	88–130
IL-12p70	121	20.5	85–193	117	13.2	91–143	131	17.1	111–194
TNF-α	95	4.6	87–106	91	5.0	82–99	95	6.4	88–113

	Serum (N=8)			Urine (N=5)			Cell Culture Supernatants (N=4)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
IFN-γ	115	11.3	91–137	104	8.9	82–105	108	5.2	96–112
IL-1β	105	10.3	81–121	91	6.8	80–101	103	4.6	93–108
IL-2	106	6.4	94–117	91	8.4	85–119	106	3.5	98–111
IL-4	71	12.4	53–86	60	12.0	46–71	101	5.1	91–105
IL-5	108	7.7	92–122	96	10.0	68–106	105	6.9	93–117
IL-6	107	15.3	72–131	88	11.7	116–164	109	2.9	103–114
KC/GRO	84	6.2	74–91	135	9.7	84–106	128	6.6	122–141
IL-10	88	7.8	73–99	96	6.3	79–124	121	7.5	100–128
IL-12p70	124	7.7	107–144	94	12.6	63–95	117	4.5	112–125
TNF-α	91	6.9	75–101	78	11.6	82–105	114	4.1	105–121

Specificity

To assess specificity, each assay in the panel was tested individually. Nonspecific binding was also evaluated with additional recombinant mouse analytes (IL-13, IL-17, GM-CSF, MCP-1, MIP-3α, RANTES, TNF-RI, TNF-RII, and VEGF). Nonspecific binding was less than 0.5% for all assays in the kit.

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

To evaluate the TNF-α assay for interference by its receptors, the Proinflammatory Panel 1 (mouse) was run with TNF-α-containing serum to which 10 ng/mL of TNF-RI or 15 ng/mL of TNF-RII was added. Assay interference was not observed.

Stability

The reconstituted calibrator, reconstituted controls, and diluents were tested for freeze–thaw stability. Results (not shown) demonstrated that reconstituted calibrator, reconstituted controls, Diluent 41, and Diluent 45 can go through five freeze–thaw cycles without significantly affecting the performance of the assay. Once reconstituted, the multi-analyte calibrator is stable for 30 days at 2–8 °C. Partially used MSD plates may be sealed and stored up to 30 days at 2–8 °C in the original foil pouch with desiccant. Results from control measurements changed by ≤30% after partially used plates were stored for 30 days. The validation study includes a real-time stability study with scheduled performance evaluations of complete kits for up to 54 months from the date of manufacture.

Calibration

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

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

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

Analyte	NIBSC/WHO Catalog Number	Concentration Ratio (MSD Reference: NIBSC)
IL-1β	96/668	1.18
IL-2	93/566	1.0
IL-4	91/656	0.89
IL-6	93/730	1.0
TNF-α	88/532	1.0

Tested Samples

Normal Samples

Normal mouse 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 mouse samples tested in the Proinflammatory Panel 1 (mouse) Kit

Sample Type	Statistic	IFN-γ	IL-1β	IL-2	IL-4	IL-5	IL-6	KC/GRO	IL-10	IL-12p70	TNF-α
Serum (N=16)	Median (pg/mL)	0.95	2.27	1.02	0.43	2.72	21.6	48.3	11.0	81.0	12.0
	Range (pg/mL)	0.34–28.7	1.13–3.95	0.55–3.98	0.23–1.10	0.58–6.52	5.28–111	28.7–102	5.71–45.4	64.8–97.1	8.23–34.4
	% Detected	100	100	100	94	100	100	100	100	13	100
EDTA Plasma (N=15)	Median (pg/mL)	41.2	0.86	3.86	0.63	2.59	117	70.5	56.5	69.3	38.5
	Range (pg/mL)	18.6–262	0.46–2.40	2.60– 5.89	0.48–0.70	1.50–2.88	11.0–185	54.2–96.9	31.5–74.7	50.2–171	21.3–47.0
	% Detected	100	87	100	60	100	100	100	100	73	100
Heparin Plasma (N=15)	Median (pg/mL)	262	1.62	4.63	0.75	4.01	175	269	76.4	85.6	65.3
	Range (pg/mL)	156–352	0.61–2.25	3.35–7.36	0.42–1.49	2.26–5.72	28.8–355	220–369	63.7–105	38.0–152	35.0–76.7
	% Detected	100	87	100	60	100	100	100	100	53	100
Citrate Plasma (N=16)	Median (pg/mL)	7.04	1.01	3.09	0.73	3.37	41.9	65.3	30.7	71.2	42.8
	Range (pg/mL)	0.31–122	0.45–2.02	0.65–5.03	0.39–1.47	1.72–8.24	6.84–74.2	34.9–172	5.30–68.2	50.4–107	5.45–58.8
	% Detected	100	100	94	100	100	100	100	100	94	100
Urine (N=10)	Median (pg/mL)	0.32	0.57	0.50	0.95	ND	ND	2.31	1.36	102	0.63
	Range (pg/mL)	0.09–0.66	0.35–1.34	0.49–0.65	0.33–1.31	ND	ND	1.91–2.84	0.98–1.53	67.3–125	0.48–3.90
	% Detected	70	60	30	90	0	0	100	40	90	80

ND = Non-detectable.
% Detected = % of samples with concentrations at or above the LLOD

Stimulated Samples

Freshly collected, normal, pooled, mouse whole blood was incubated at 37ºC for different time periods either with lipopolysaccharide (LPS) or with peptidoglycan (PG) and zymosan (ZY) as shown below; plasma was isolated at the end of incubations. The dilution-adjusted concentrations (pg/mL) for each stimulation model are displayed below. Assays that showed a significant difference in analyte level with prolonged stimulation are identified with an asterisk.

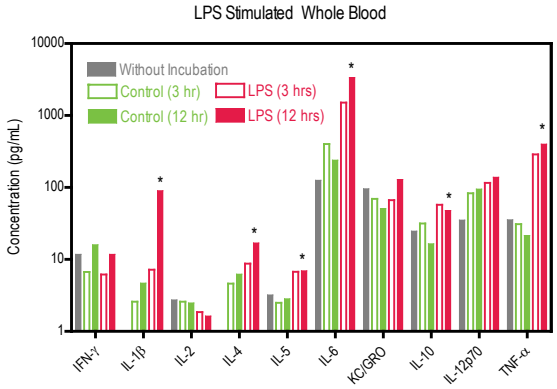


Figure 6. Normal mouse whole blood stimulated with LPS

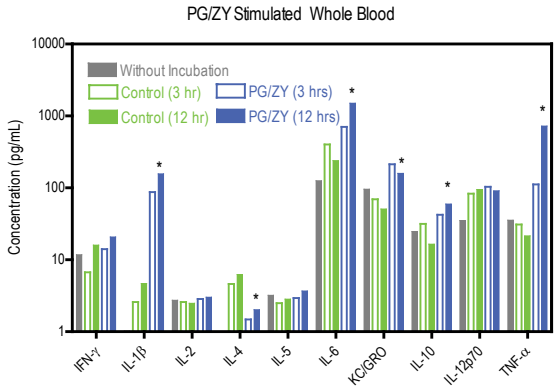


Figure 7. Normal mouse whole blood stimulated with PG/ZY

A mouse monocyte macrophage cell line (J774A.1) was stimulated for 4 hours with LPS or pokeweed mitogen (PWM). The lysate was collected and tested. The concentrations were normalized for 50 µg of lysate per well. Analyte levels for IFN-γ, IL-4, IL-5, and IL-12p70 were non-detectable. Measurements that were above saturation levels are identified with an arrow.

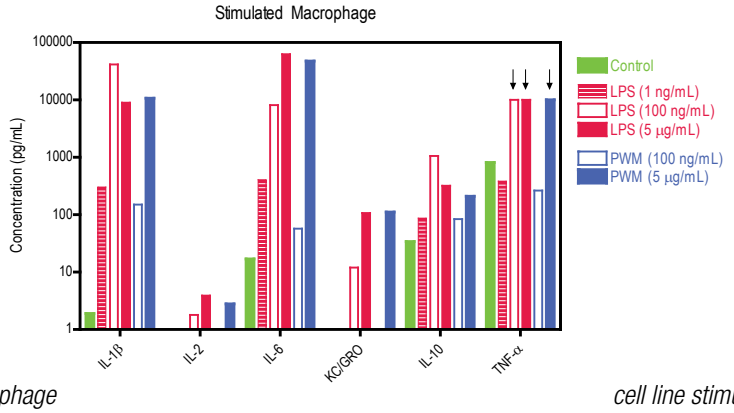


Figure 8. J774A.1 mouse macrophage cell line stimulated with LPS or PWM

Assay Components

Calibrators

The assay calibrator blend uses the following recombinant mouse proteins:

Table 11. Recombinant mouse proteins used in the Calibrators

Calibrator	Expression System
IFN-γ	*E. coli*
IL-1β	*E. coli*
IL-2	*E. coli*
IL-4	*E. coli*
IL-5	Insect cell line
IL-6	*E. coli*
KC/GRO	*E. coli*
IL-10	*E. coli*
IL-12p70	Insect cell line
TNF-α	*E. coli*

Antibodies

Table 12. Antibody source species

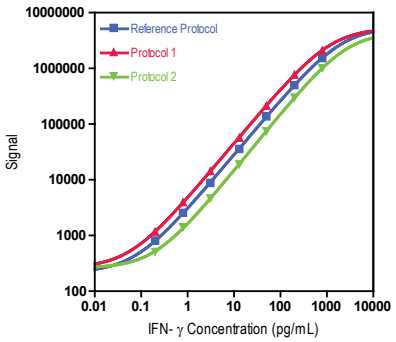
	Source Species		
Analyte	MSD Capture Antibody	MSD Detection Antibody	Assay Generation
IFN-γ	Rat Monoclonal	Rat Monoclonal	A
IL-1β	Mouse Monoclonal	Goat Polyclonal	A
IL-2	Rat Monoclonal	Rat Monoclonal	A
IL-4	Rat Monoclonal	Rat Monoclonal	A
IL-5	Rat Monoclonal	Rat Monoclonal	A
IL-6	Rat Monoclonal	Goat Polyclonal	B
KC/GRO	Rat Monoclonal	Goat Polyclonal	A
IL-10	Rat Monoclonal	Goat Polyclonal	A
IL-12p70	Rat Monoclonal	Rat Monoclonal	A
TNF-α	Hamster Monoclonal	Goat Polyclonal	B

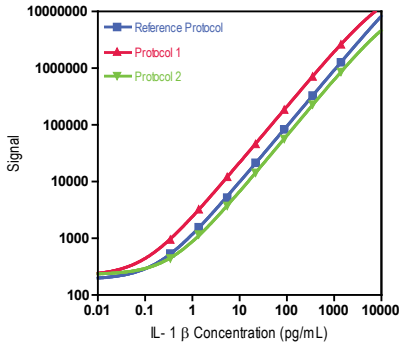
References

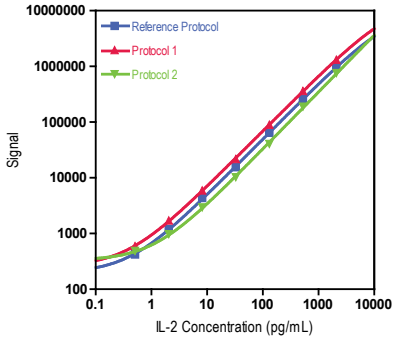
1. Kause ML, et al. Assessing immune function by profiling cytokine release from stimulated blood leukocytes and the risk of infection in rheumatoid arthritis. Clin Immunol. 2011;141:67-72.
2. Holmes C, et al. Proinflammatory cytokines, sickness behavior, and Alzheimer disease. Neurology. 2011;77:212-8.
3. Desai D, et al. Cytokines and cytokine-specific therapy in asthma. Ad Clin Chem. 2012;57:57-97.
4. Gui T, et al. Diverse roles of macrophages in atherosclerosis: from inflammatory biology to biomarker discovery. Mediators Inflam. 2012;693083.
5. Islam SA, et al. T cell homing to epithelial barriers in allergic disease. Nat Med. 2012;18:705-15.
6. Su DL, et al. Roles of pro- and anti-inflammatory cytokines in the pathogenesis of SLE. J Biomed Biotechnol. 2012;347141.
7. Lukens JR, et al. Inflammasome activation in obesity-related inflammatory disease and autoimmunity. Discov Med. 2011;12:65-74.
8. Laoui D, et al. Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. Intern J Dev Bio. 2011;55:861-7.
9. Hallberg L, et al. Exercise-induced release of cytokines in patients with major depressive disorder. J Affect Disord. 2010;126:262-7.
10. Oreja-Guevara C, et al. TH1/TH2 Cytokine profile in relapsing-remitting multiple sclerosis patients treated with Glatiramer acetate or Natalizumab. BMC Neurol. 2012;12:95.
11. Svensson J, et al. Few differences in cytokines between patients newly diagnosed with type 1 diabetes and their healthy siblings. Hum Immunol. 2012 Nov;73:1116-26
12. Yehuda H, et al. Isothiocyanates inhibit psoriasis-related proinflammatory factors in human skin. Inflamm Res. 2012;61:735-42.
13. Gologan S, et al. Inflammatory gene expression profiles in Crohn's disease and ulcerative colitis: A comparative analysis using a reverse transcriptase multiplex ligation-dependent probe amplification protocol. J Crohns. Epub Sept 26, 2012. (j.crohns.2012.08.015).
14. Lee JW, et al. Fit-for-purpose method development and validation for successful biomarker measurement. Pharm Res. 2006;23:312-28.
15. Hem A, et al. Saphenous vein puncture for blood sampling of the mouse, rat, hamster, gerbil, ferret and mink. Lab Anim. 1998;32:364-8.
16. Removal of blood from laboratory animals and birds: First report of the BVA/FRAME/RSPCA/UFAW joint working group on refinement. Lab Anim. 1993;27:1-22.

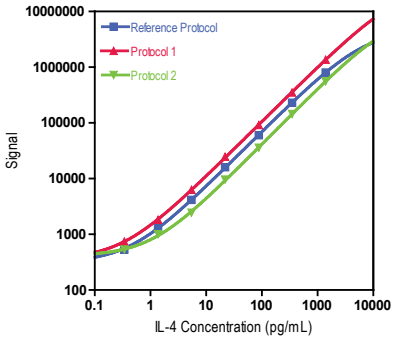
Appendix A

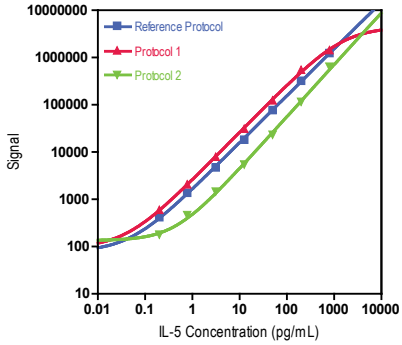
Calibration curves below illustrate the relative sensitivity of each assay under **Alternate Protocols**: Reference Protocol (2-hour sample incubation/2 wash steps, blue curve), Alternate Protocol 1 (overnight sample incubation, red curve), and Alternate Protocol 2 (tissue culture: single wash, green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

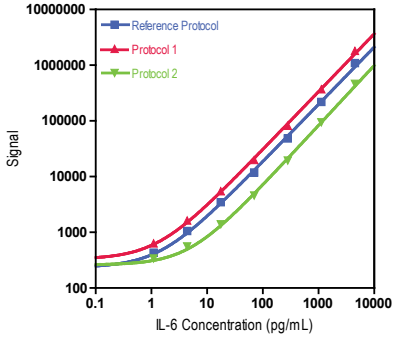


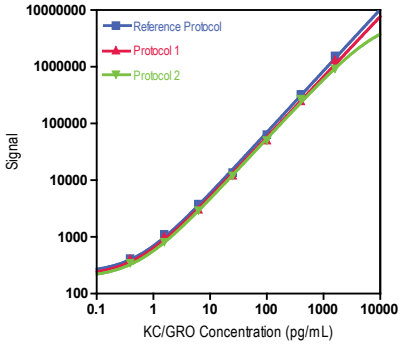


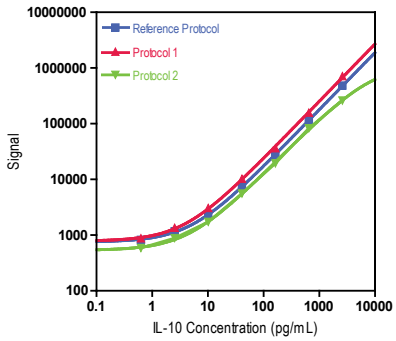


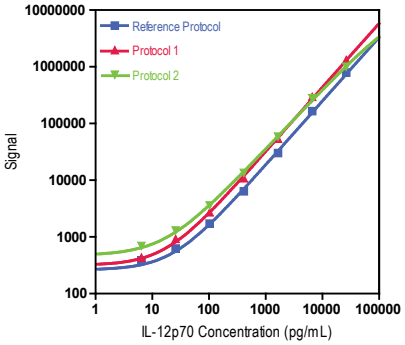












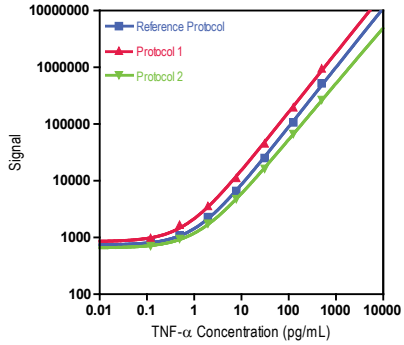


Table 13. Relative sensitivity when using alternate protocols

	LLOD Comparison (pg/mL)		
	Reference Protocol	Protocol 1	Protocol 2
IFN-γ	0.04	0.02	0.07
Il-1β	0.11	0.05	0.12
IL-2	0.22	0.11	0.35
IL-4	0.11	0.08	0.26
IL-5	0.06	0.03	0.25
IL-6	0.61	0.62	2.88
KC/GRO	0.24	0.29	0.51
IL-10	0.94	0.39	0.64
IL-12p70	9.95	4.83	2.92
TNF-α	0.13	0.13	0.40

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).

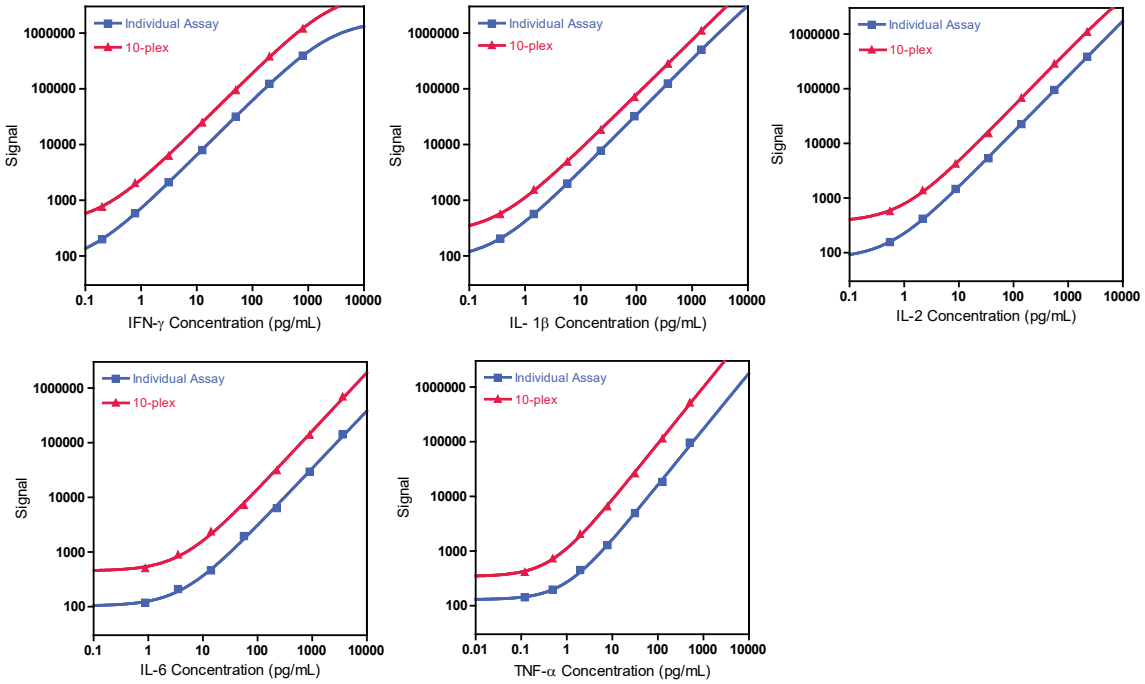


Table 14. Assay performance for individual and 10-plex assays

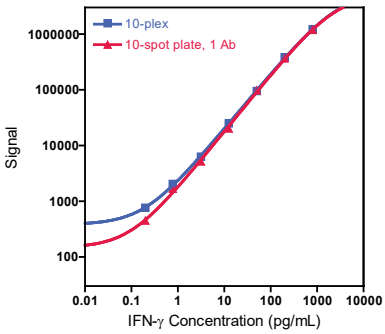
In general, assays in the single spot format yielded a lower overall signal compared to the 10-plex format. The spots on single-spot plates have a larger binding surface than those on multiplex plates, but the same amount of calibrator was used for each test; therefore, the bound calibrator was spread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

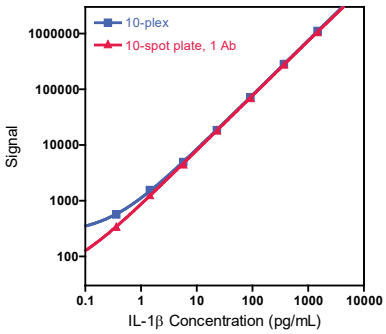
Note: Assay performance for IL-4, IL-5, KC/GRO, IL-10, and IL-12p70 are not included since the individual assays are run on multiplex plates.

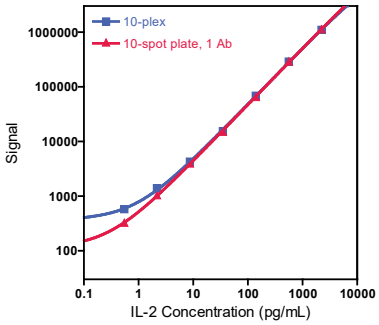
	LLOD (pg/mL)	
Assay	Individual	10-plex
IFN-γ	0.05	0.04
IL-1β	0.09	0.11
IL-2	0.29	0.22
IL-6	1.24	0.61
TNF-α	0.18	0.13

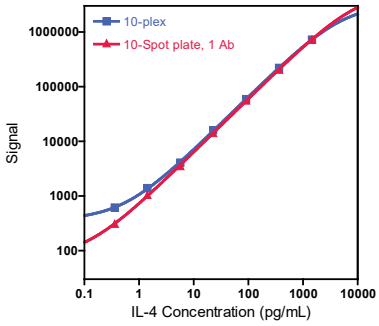
Appendix C

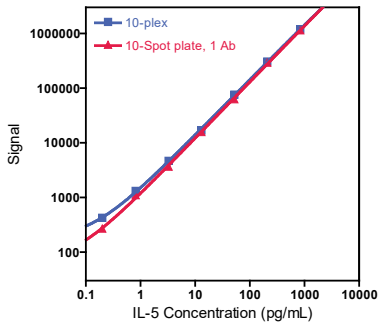
The calibration curves below compare results for each assay in the panel when the assays were run on the 10-spot plate using all detection antibodies (blue curve) vs. running each assay using a single, assay-specific detection antibody (red curve).

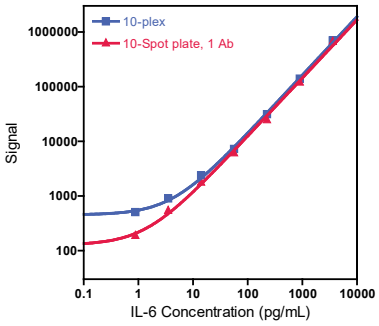


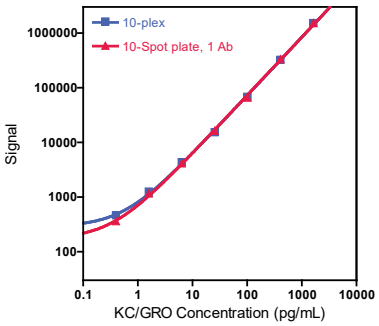


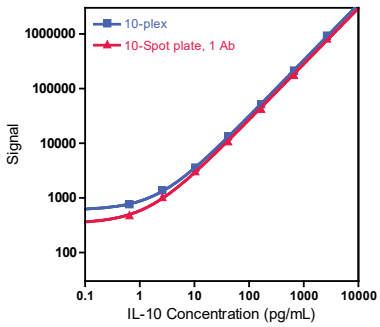


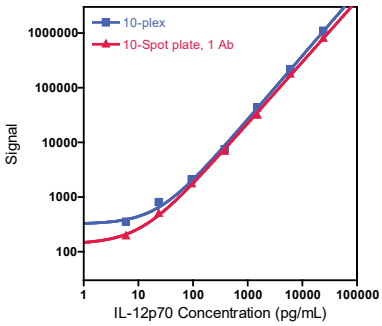












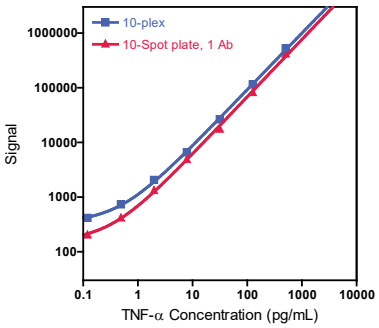


Table 15. LLODs for detection of a single Ab vs. blended Abs

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

	LLOD (pg/mL)	
Assay	10-spot plate, 1 Ab	10-plex
IFN-γ	0.04	0.04
IL-1β	0.10	0.11
IL-2	0.28	0.22
IL-4	0.11	0.11
IL-5	0.05	0.06
IL-6	0.73	0.61
KC/GRO	0.13	0.24
IL-10	0.30	0.94
IL-12p70	5.23	9.95
TNF-α	0.14	0.13

Summary Protocol

Proinflammatory Panel 1 (mouse) Kits

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol before performing the Proinflammatory Panel 1 (mouse) assays.

Sample and Reagent Preparation

- ❑ Bring all reagents to room temperature.
- ❑ Prepare calibration solutions in Diluent 41 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 41 before adding to the plate.
- ❑ Prepare combined detection antibody solution by diluting each 50X detection antibody 50-fold in Diluent 45.
- ❑ Prepare 2X Read Buffer T by diluting 4X Read Buffer T 2-fold with deionized water.

STEP 1: Wash* and Add Sample

- ❑ Wash plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 50 µL/well of sample (calibrators, controls, or unknowns).
- ❑ Incubate at room temperature with shaking for 2 hours.

STEP 2: Wash and Add Detection Antibody Solution

- ❑ Wash plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 25 µL/well of 1X detection antibody solution.
- ❑ Incubate at room temperature with shaking for 2 hours.

STEP 3: Wash and Read Plate

- ❑ Wash plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 150 µL/well of 2X Read Buffer T.
- ❑ Analyze the plate on the MSD instrument.

*Note: Washing the plate before sample addition is an optional step that may provide greater uniformity of results for certain assays. Analytical parameters, including limits of quantification, recovery of controls, and sample quantification, are not affected by washing the plate before sample addition.

Catalog Numbers

Table 16. *Catalog numbers for V-PLEX and V-PLEX Plus* Proinflammatory (mouse) 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						
Proinflammatory Panel 1 (mouse)	K15048D-1	K15048D-2	K15048D-4	K15048G-1	K15048G-2	K15048G-4
Single Assay Kits						
Mouse IFN-γ	K152QOD-1	K152QOD-2	K152QOD-4	K152QOG-1	K152QOG-2	K152QOG-4
Mouse IL-1β	K152QPD-1	K152QPD-2	K152QPD-4	K152QPG-1	K152QPG-2	K152QPG-4
Mouse IL-2	K152QQD-1	K152QQD-2	K152QQD-4	K152QQG-1	K152QQG-2	K152QQG-4
Mouse IL-4	K152QRD-1	K152QRD-2	K152QRD-4	K152QRG-1	K152QRG-2	K152QRG-4
Mouse IL-5	K152QSD-1	K152QSD-2	K152QSD-4	K152QSG-1	K152QSG-2	K152QSG-4
Mouse IL-6	K152QXD-1	K152QXD-2	K152QXD-4	K152QXG-1	K152QXG-2	K152QXG-4
Mouse KC/GRO	K152QTD-1	K152QTD-2	K152QTD-4	K152QTG-1	K152QTG-2	K152QTG-4
Mouse IL-10	K152QUD-1	K152QUD-2	K152QUD-4	K152QUG-1	K152QUG-2	K152QUG-4
Mouse IL-12p70	K152QVD-1	K152QVD-2	K152QVD-4	K152QVG-1	K152QVG-2	K152QVG-4
Mouse TNF-α	K152QWD-1	K152QWD-2	K152QWD-4	K152QWG-1	K152QWG-2	K152QWG-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 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

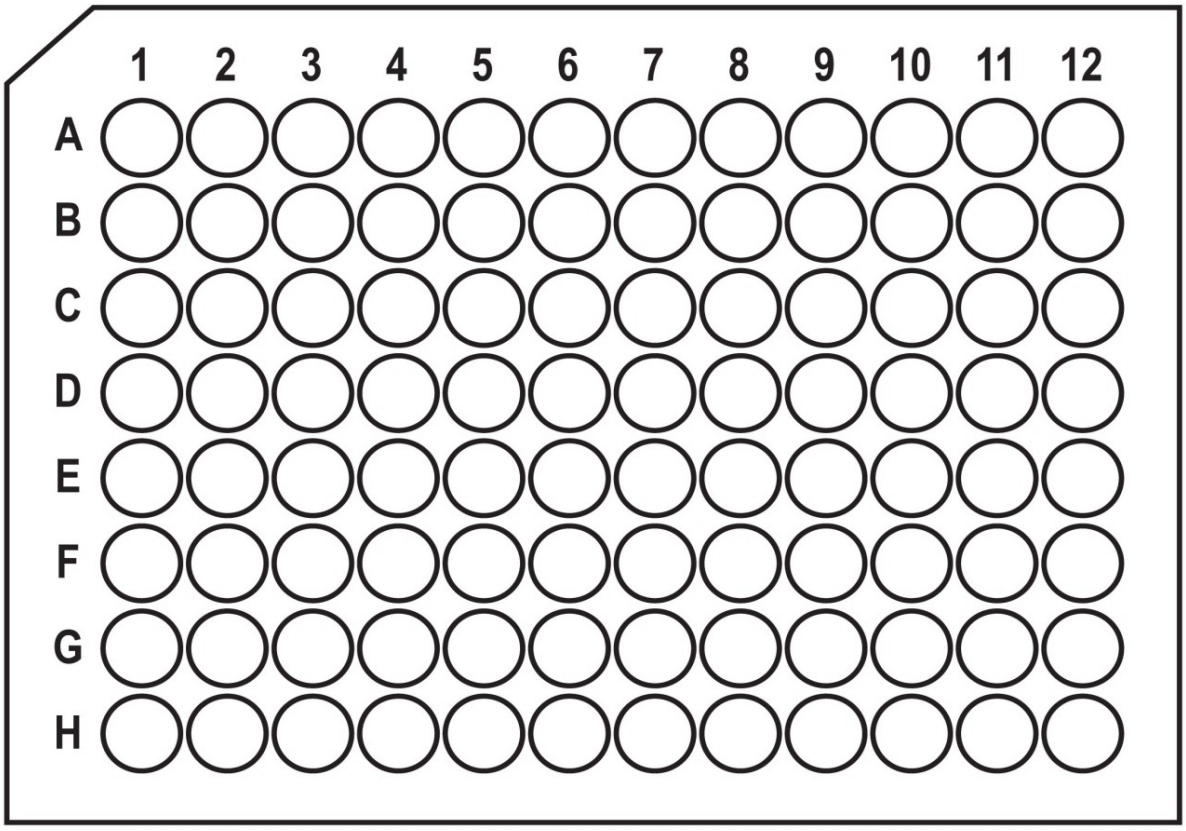


Figure 10. Plate diagram.