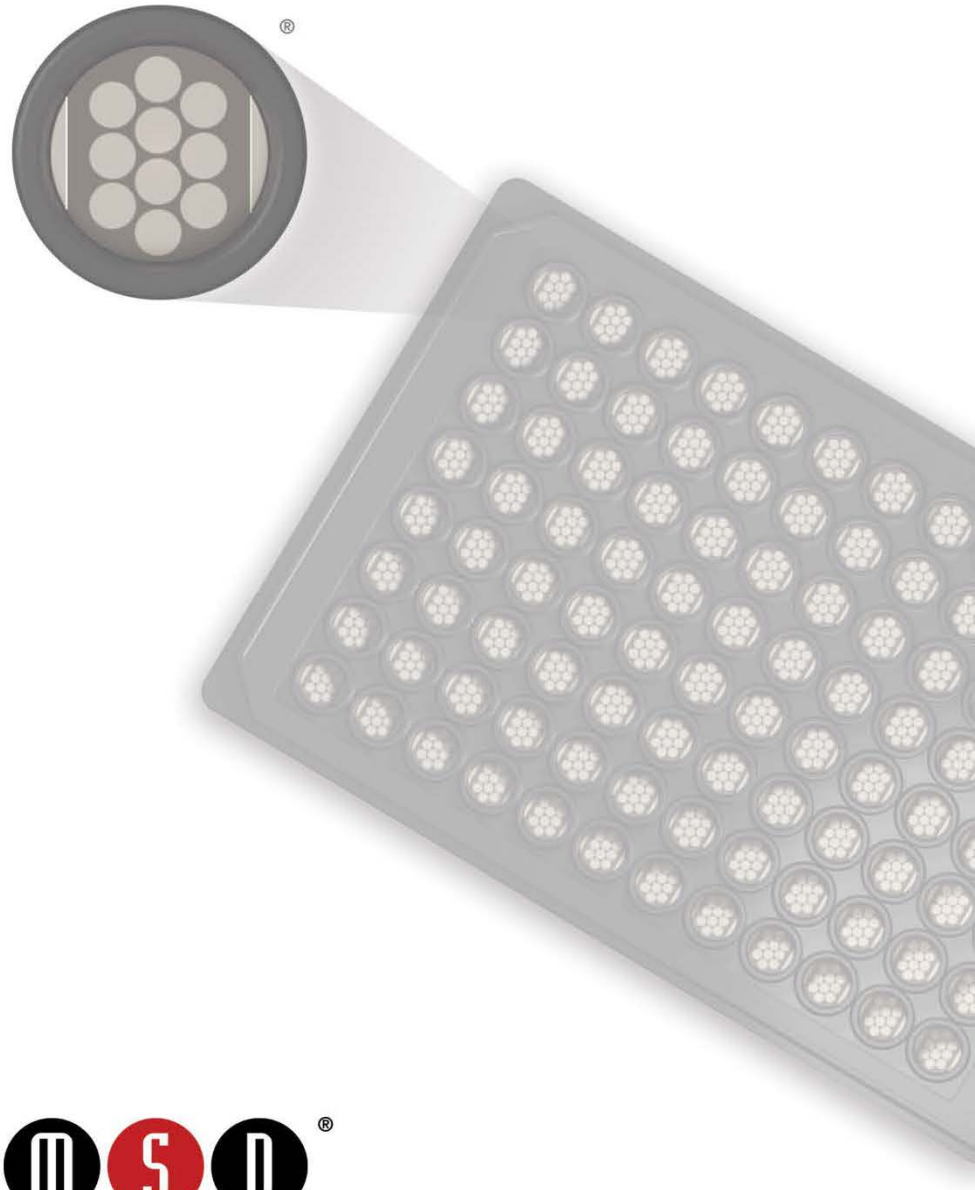
MSD® MULTI-SPOT Assay System

Proinflammatory Panel 1 (NHP) Kits

IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10

V-PLEX®

SECTOR™ Assay Kits
QuickPlex Ultra™ Assay Kits



MSD®

MSD Cytokine Assays

Proinflammatory Panel 1 (NHP) Kits

IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10

For use with non-human primate (NHP) cell culture supernatants, serum, plasma, and urine.

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

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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Table of Contents

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Introduction

MSD offers V-PLEX® assays for customers who require unsurpassed performance and quality. V-PLEX products are developed under rigorous design control and fully validated according to fit-for-purpose principles[1] in accordance with MSD's Quality Management System. They offer exceptional sensitivity, simple protocols, reproducible results, and lot-to-lot consistency. In addition to the analytical validation, robustness of the assay protocol is assessed during development along with the stability and robustness of the assay components and kits. V-PLEX assays are available in both single-assay and multiplex formats.

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

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

The Proinflammatory Panel 1 (NHP) employs human detection and capture antibodies that react with rhesus and cynomolgus monkeys. These assays may be suitable for primate species in addition to M. mulatta and M. fascicularis since human cytokines are broadly homologous with cytokines from NHPs.[2]

Proinflammatory Panel 1 (NHP) 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 (NHP) measures biomarkers that are implicated in a number of disorders, including rheumatoid arthritis,[3] Alzheimer's disease,[4] asthma,[5] atherosclerosis,[6] allergies,[7] systemic lupus erythematosus,[8] obesity,[9] cancer,[10] depression,[11] multiple sclerosis,[12] diabetes,[13] psoriasis,[14] and Crohn's disease.[15] As a result of their association with such a wide spectrum of disease, these biomarkers are the subjects of drug discovery projects and basic research. The biomarkers constituting the panel are described below.

Interferon gamma (IFN-**γ**), also known as immune interferon, is a glycosylated, 19.3 kDa pro-inflammatory cytokine. It exists as a non-covalently linked homodimer. IFN-γ dimers bind to the IFN-γ R1 (receptor 1), which is then triggered to bind the IFN-γ R2 (receptor 2) to form a functional receptor–ligand complex consisting of two receptor subunits. IFN-γ is produced by lymphocytes and is a potent activator of macrophages. It is involved in numerous pathways and is associated with a number of disorders including Huntington's disease[16] and hepatitis C.[17]

Interleukin-1beta (IL-1β), also known as IL-1F2, is a 30.7 kDa pro-inflammatory cytokine that is produced by activated macrophages. It stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity. There are multiple receptors of IL-1β. Along with IL-1α, IL-1β binds directly to IL-1 RI, which is then associated with IL-1 R accessory protein (IL-1RAcP) to form a high-affinity receptor complex for signal transduction. IL-1 RII has high affinity for IL-1β, but it is a negative regulator of IL-1β activity. IL-1 receptor antagonist (IL-1ra) interacts with IL-1 RI to prevent binding of IL-1α and IL-1β. IL-1β is involved in a number of biological activities ranging from aging[18] to wound healing.[19] Along with IFN-γ, IL-6, and TNF-α, IL-1β is a pyrogenic cytokine that induces the production of prostaglandins, the major mediators of fever induction.[20]

Interleukin-2 (IL-2), also known as T-cell growth factor (TCGF), is a glycosylated 17.6 kDa protein produced by T-cells. It is a crucial regulator of the immune system through T-cell proliferation and other activities. The receptor for this four α-helix bundle cytokine consists of three subunits: an α subunit that is specific for IL-2; a β subunit that is a component of the IL-15 receptor; and a common gamma chain (γc/IL-2 Rγ) that is shared with the receptors for IL-4, IL-7, IL-9, IL-15, and IL-21 but does not independently interact with IL-2.[21] IL-2 production levels are associated with HIV infection[22] and entamoebiasis.[23]

Interleukin-6 (IL-6)—also known as B-cell stimulatory factor 2 (BSF-2), CTL differentiation factor (CDF), hybridoma growth factor, and Interferon beta-2 (IFN-β2)—is a 23.7 kDa cytokine with two disulfide bonds that is secreted mainly by T cells and macrophages. It is involved in numerous biological processes including inflammation, aging, cell growth, apoptosis, and bone remodeling. It is released from muscle cells during exercise in response to muscle contraction. IL-6 induces an acute phase response[24] and plays an essential role in differentiating B cells into immunoglobulin-secreting cells. The receptor for IL-6 consists of a ligand-binding subunit (IL-6R) and a signal-transducing subunit (gp130) that is also a component of other protein receptors. IL-6 binding to IL-6R triggers the binding of the IL-6-receptor complex to gp130 and the homodimerization of gp130. IL-6 is involved in osteoporosis,[25] pulmonary fibrosis,[26] liver cirrhosis,[27] ischemia,[28] and berylliosis[29] among other disorders.

Interleukin-8 (IL-8)—also known as C-X-C motif chemokine 8 (CXCL8), granulocyte chemotactic protein 1 (GCP-1), monocyte-derived neutrophil chemotactic factor (MDNCF), monocyte-derived neutrophil-activating peptide (MONAP), neutrophil-activating protein 1 (NAP-1), protein 3-10C, and T-cell chemotactic factor—is an 11.1 kDa CXC chemokine. It is expressed by several cell types as a response to inflammation. It attracts neutrophils, basophils, and T-cells but not monocytes and is also involved in neutrophil activation. There are several N-terminal processed forms of this protein with varying activity resulting from proteolytic cleavage upon secretion. This proinflammatory chemokine is associated with numerous disorders including acute pancreatitis.[30]

Interleukin-10 (IL-10), also known as cytokine synthesis inhibitory factor (CSIF), is a 20.5 kDa, glycosylated homodimeric cytokine with two disulfide bonds. It is produced by a variety of cell lines such as T-cells, macrophages, and mast cells. The homodimer binds to two IL-10 Rα subunits resulting in recruitment of two IL-10 Rβ chains to initiate the IL-10–mediated signal cascades. IL-10 Rβ is also associated with receptors of IL-22, IL-26, IL-28, and IL-29. IL-10 inhibits the synthesis of numerous cytokines (including IFN-γ, IL-2, IL-3, TNF-α, TNF-β, and GM-CSF) that suppress Th1 proinflammatory responses and promote phagocytic uptake. IL-10 has been shown to prevent liver necrosis during parasitic infection in mice.[31]

Principle of the Assay

MESO SCALE DISCOVERY® 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 (NHP) are sandwich immunoassays. MSD provides a plate pre-coated with capture antibodies on independent and well-defined spots, as shown in the layout below. Multiplex assays are provided on MULTI-SPOT plates (Figure 1); individual 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™) over the course of one or more incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. The user adds an MSD buffer that creates the appropriate chemical environment for electrochemiluminescence (ECL) and loads the plate into an MSD instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light (which is proportional to the amount of analyte present in the sample) and provides a quantitative measure of each analyte in the sample. V-PLEX assay kits have been validated according to the principles outlined in "Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement" by J. W. Lee, et al.[1]

1. IFN-γ
2. IL-1β
3. IL-2
4. *IL-4**
5. IL-6
6. IL-8
7. IL-10
8. *IL-12p70**
9. *IL-13**
10. *TNF-α**

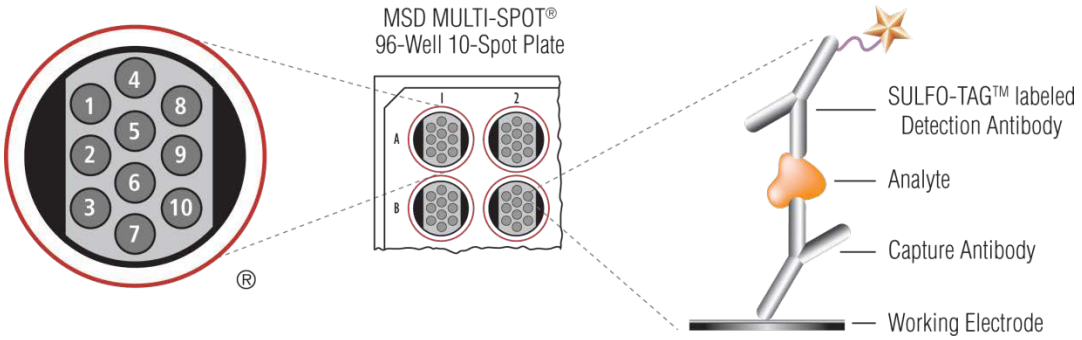

***Figure 1**. Multiplex plate spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.*

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

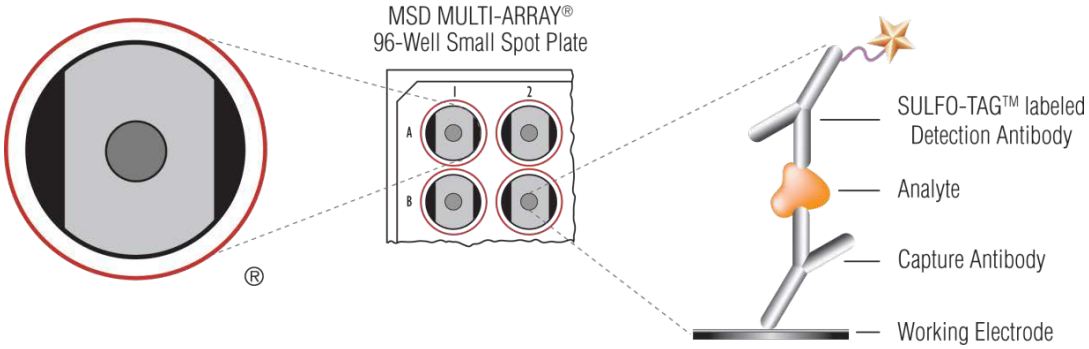

***Figure 2**. Small Spot plate diagram showing placement of analyte capture antibodies.*

Materials and Equipment

Kit Components

Proinflammatory Panel 1 (NHP) assays are available as a 6-spot multiplex kit, as individual assay kits, or as custom V-PLEX kits with subsets of assays selected from the full panel. The assays use human recombinant cytokine calibrator sequences that are highly homologous to the non-human primate cytokines. 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 #	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Proinflammatory Panel 1 (human) Calibrator Blend	2–8°C	C0049-2	1 vial	1 vial	5 vials	25 vials	Recombinant human proteins in diluent, buffered and lyophilized. Individual analyte concentration is provided in the lot-specific certificate of analysis (COA).
Diluent 2	≤-10°C	R51BB-4	8 mL	1 bottle	-	-	Diluent for samples and calibrator; contains serum, blockers, and preservatives.
		R51BB-3	40 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	
MSD GOLD™ Read Buffer B	RT	R60AM-1	18 mL	1 bottle	-	-	Buffer to catalyze the electro-Chemiluminescence reaction on **QuickPlex Ultra™** plates.
		R60AM-2	90 mL	-	1 bottle	5 bottles	
Read Buffer T (4X)	RT	R92TC-3	50 mL	1 bottle	1 bottle	5 bottles	Buffer to catalyze the electro-Chemiluminescence reaction on **SECTOR™** plates.

V-PLEX Plus Kits: Additional Components

Table 2. Additional components that are supplied with V-PLEX Plus Kits

Reagents	Storage	Catalog #	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Proinflammatory Panel 1 (human) Control 1*	2–8°C	C4049-1	1 vial	1 vial	5 vials	25 vials	Multi-analyte controls in a non-primate matrix, buffered, lyophilized, and spiked with recombinant human analytes. The concentration of the controls is provided in the lot-specific COA.
Proinflammatory Panel 2 (human) Control 2*	2–8°C	C4049-1	1 vial	1 vial	5 vials	25 vials	
Proinflammatory Panel 1 (human) Control 3*	2–8°C	C4049-1	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 (human) Control Pack*

Kit-Specific Components

Table 3. Components that are supplied with specific kits

Plates	Storage	Part #	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Proinflammatory Panel 1 (human) Plate	2–8°C	N05049A-1	10-spot	1	5	25	96-well plate, foil sealed, with desiccant.
Proinflammatory Panel 1 (human) QuickPlex Ultra Plate	2–8°C	N0B049A-1	10-spot	1	5	25	
Human IFN-γ Plate	2–8°C	L451QOA-1	Small Spot	1	5	25	
Human IFN-γ QuickPlex Ultra Plate	2–8°C	L4B1QOA-1	Small Spot	1	5	25	
Human IL-1β Plate	2–8°C	L451QPA-1	Small Spot	1	5	25	
Human IL-2 Plate	2–8°C	L451QQA-1	Small Spot	1	5	25	
Human IL-2 QuickPlex Ultra Plate	2–8°C	L4B1QQA-1	Small Spot	1	5	25	
Human IL-6 Plate	2–8°C	L451QXA-1	Small Spot	1	5	25	
Human IL-6 QuickPlex Ultra Plate	2–8°C	L4B1QXA-1	Small Spot	1	5	25	
Human IL-8 Plate	2–8°C	L451RAA-1	Small Spot	1	5	25	
Human IL-10 Plate	2–8°C	L451QUA-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 #	Size	Quantity Supplied 1-Plate Kit	5-Plate Kit	25-Plate Kit	Description
Anti-hu IFN-γ Antibody (50X)	2–8°C	D21Q0-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21Q0-3	375 µL	-	1	5	
Anti-hu IL-1β Antibody (50X)	2–8°C	D21AG-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21AG-3	375 µL	-	1	5	
Anti-hu IL-2 Antibody (50X)	2–8°C	D21QQ-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21QQ-3	375 µL	-	1	5	
Anti-hu IL-6 Antibody (50X)	2–8°C	D21AK-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21AK-3	375 µL	-	1	5	
Anti-hu IL-8 Antibody (50X)	2–8°C	D21AN-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21AN-3	375 µL	-	1	5	
Anti-hu IL-10 Antibody (50X)	2–8°C	D21QU-2	75 µL	1	-	-	SULFO-TAG conjugated antibody.
		D21QU-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 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 # R61AA-1 (included in V-PLEX Plus kits)
- ❑ Adhesive plate seals (3 per plate included in V-PLEX Plus kits)
- ❑ Deionized water
- ❑ Vortex mixer

Optional Materials and Equipment

- ❑ Proinflammatory Panel 1 (NHP) Control Pack, available for separate purchase from MSD, catalog # C4049-1 (included in V-PLEX Plus kits)
- ❑ 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 safety data sheet (SDS), which can be obtained from MSD Customer Service or at www.mesoscale.com.

Protocol

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.
- 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 when 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, leave the sample or detection antibody solution in the plate to keep the plate from drying out.
- Remove the plate seals prior to reading the plate.
- Make sure that read buffer is at room temperature when added to a plate.
- Do not shake the plate after adding read buffer.
- To improve inter-plate precision, keep time intervals consistent between adding read buffer and reading the plate. Unless otherwise directed, read the plate as soon as possible after adding read buffer.
- 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. Consider adjusting volumes proportionally when preparing reagents.

Reagent Preparation

Bring all reagents to room temperature.

Important: Upon first thaw, aliquot Diluent 2 and Diluent 3 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 2. 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 2 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 2 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 2. Mix well by vortexing. Repeat 4-fold serial dilutions 5 additional times to generate 7 calibrators.
3) Use Diluent 2 as the zero calibrator.

Note: Reconstituted calibrator (Calibrator 1) is not stable when stored at 2-8°C; however, it may 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. A copy of the COA is also available at www.mesoscale.com.

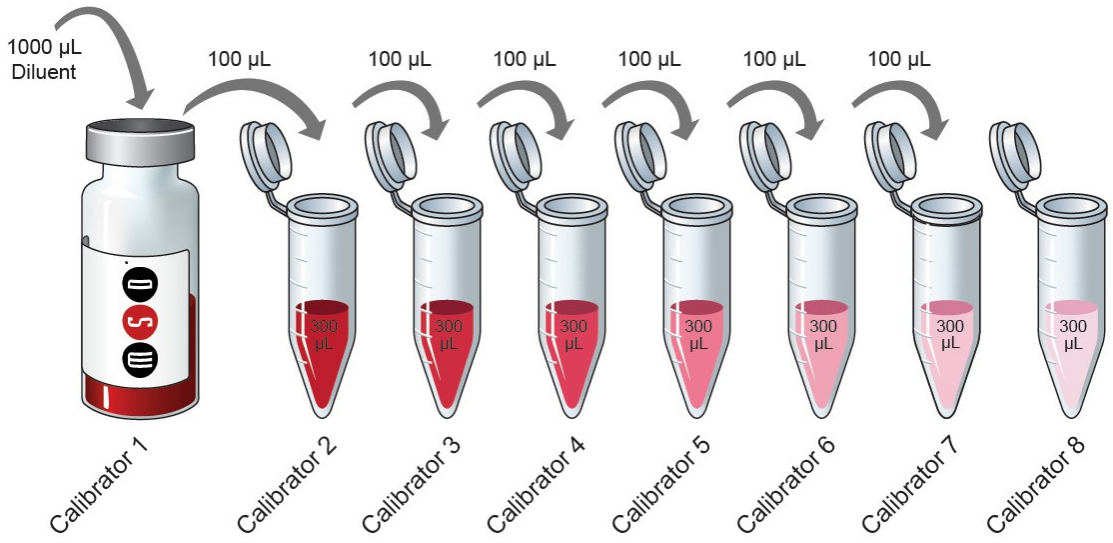

Figure 3. Dilution schema for preparation of Calibrator Standards

Sample Collection and Handling

Below are general guidelines for NHP sample collection, storage, and handling. If possible, use published guidelines.[32-34] 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, centrifugation may not be necessary.
- Other samples. Use immediately or freeze.

Freeze all samples in suitably-sized aliquots; they may be stored at ≤-10ºC until needed. Repeated freeze–thaw of samples is not recommended. After thawing, centrifuge samples at 2,000g for 3 minutes to remove particulates prior to sample preparation.

Dilute Samples

Dilute samples with Diluent 2. For 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 2. Optionally, conserve sample volume by using a higher dilution.

Tissue culture supernatants may require additional dilution based on stimulation and analyte concentrations in the sample. 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 (NHP) Control Pack, catalog # C4049-1. (Controls are included only in V-PLEX Plus kits.)

Reconstitute the lyophilized controls in 250 µL of Diluent 2. Do not invert or vortex the vials. Wait for a minimum of 15-30 minutes before diluting controls 2-fold in Diluent 2. Vortex briefly using short pulses. After use, remaining diluted control material should be discarded.

Once reconstituted, controls are stable for 10 days at 2-8°C. For long-term storage, reconstituted controls must be stored at

≤-70°C and are stable through three freeze–thaw cycles. Refer to the Proinflammatory Panel 1 (human) Control Pack product insert for analyte levels.

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 prior to use.

6-plex Proinflammatory Panel 1 (NHP) kit

For one plate, combine the following detection antibodies and add to 2,640 µL of Diluent 3:

- ❑ 60 µL of SULFO-TAG Anti-hu IFN-γ Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-1β Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-2 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-6 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-8 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-10 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. The working solution is 1X. 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: SECTOR Plates Only

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

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

MSD GOLD Read Buffer B: QuickPlex Ultra Plates Only

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

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

Prepare MSD Plate

MSD V-PLEX 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 prior to 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 prior to 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 Read Buffer to each well. Analyze the plate on an MSD instrument. Incubation in Read Buffer 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 2-8ºC 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).

Assay Characteristics

Validation

V-PLEX products are validated following fit-for-purpose principles[1] and MSD design control procedures. V-PLEX assay components go through an extensive critical reagents program to ensure that the reagents are controlled and well characterized. Prior to the release of each V-PLEX panel, at least three independent kit lots are produced. Using results from multiple runs (typically greater than 50) and multiple operators, these lots are used to establish production specifications for sensitivity, specificity, accuracy, and precision. During validation, each individual assay is analytically validated on single-spot plates. Each assay is also independently evaluated as a multiplex component by running the full multiplex plate using only the single detection antibody for that assay. These results are compared with the results from the multiplex panel when using all detection antibodies. This demonstrates that each assay is specific and independent, allowing them to be multiplexed in any combination. The COA provided with each kit outlines the kit release specifications for sensitivity, specificity, accuracy, and precision.

- Dynamic Range

  Calibration curve concentrations for each assay are optimized for a maximum dynamic range while maintaining enough calibration points near the bottom of the curve to ensure a proper fit for accurate quantification of samples that require high sensitivity.

- Sensitivity

  The lower limit of detection (LLOD) is a calculated concentration corresponding to the average signal 2.5 standard deviations above the background (zero calibrator). The LLOD is calculated using results from multiple plates for each lot, and the median and range of calculated LLODs for a representative kit lot are reported in this product insert. The upper limit of quantification (ULOQ) and lower limit of quantification (LLOQ) are established for each lot by measuring multiple levels near the expected LLOQ and ULOQ levels. The final LLOQ and ULOQ specifications for the product are established after assessment of all validation lots.

- Accuracy and Precision

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

- Specificity

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

  In addition to measuring the specificity of antibodies to analytes in the multiplex kit, specificity and interference from other related markers are tested during development. This includes 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 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 backfitting 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 (NHP) were collected over five months of testing by five operators (38 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 six detection antibodies. The calibration curves were comparable. Calibration curves for each lot are presented in the lot-specific COA.

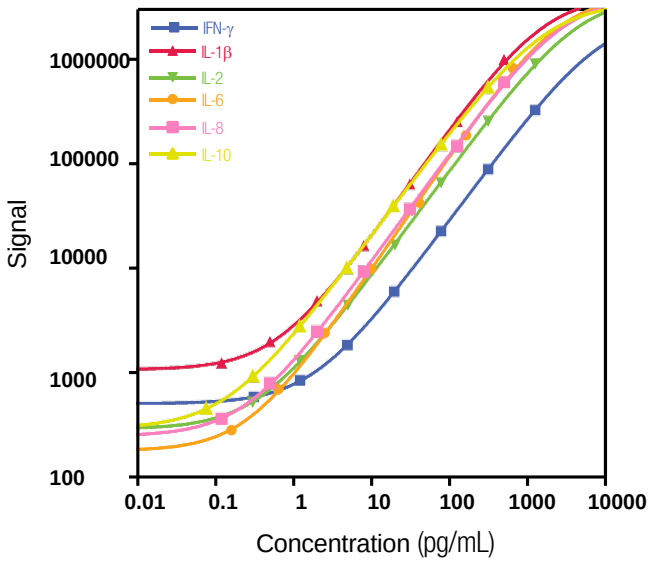

Figure 4. Typical calibration curves for the Proinflammatory Panel 1 (NHP) assay

Sensitivity

The LLOD is a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator). The LLOD shown below was calculated based on 38 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 Proinflammatory Panel 1 (NHP) Kit

	Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
IFN-γ	0.37	0.21–0.62	1.76	938
IL-1β	0.05	0.01–0.17	0.646	375
IL-2	0.09	0.01–0.29	0.890	938
IL-6	0.06	0.05–0.09	0.633	488
IL-8	0.07	0.03–0.14	0.591	375
IL-10	0.04	0.02–0.08	0.298	233

Precision

Controls were made by spiking calibrator into non-primate matrix at three levels within the quantitative range of the assay. Analyte levels were measured by five operators using a minimum of three replicates on 49 runs over five months. Results are shown in Table 6. While a typical specification for precision is a concentration CV of less than 20% for controls on both intra- and inter-day runs, for this panel, the data shows most assays are below 10%.

Average intra-run %CV is the average %CV of the control replicates within an individual run. Inter-run %CV is the variability of controls across 34 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 (NHP) Kit

	Control	Average Conc. (pg/mL)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
IFN-γ	Control 1	447	3.6	9.2	7.2
	Control 2	108	3.8	8.2	6.5
	Control 3	37.8	5.0	7.3	5.0
IL-1β	Control 1	152	3.3	5.5	1.7
	Control 2	41.0	4.0	6.1	3.2
	Control 3	11.2	4.1	7.7	5.8
IL-2	Control 1	315	4.4	6.2	4.9
	Control 2	82.5	5.8	8.2	4.8
	Control 3	20.7	5.0	10.5	4.5
IL-6	Control 1	239	3.6	5.2	4.2
	Control 2	61.9	3.9	6.8	5.1
	Control 3	18.4	4.5	7.3	5.5
IL-8	Control 1	166	2.7	5.0	5.6
	Control 2	44.9	3.6	7.1	3.2
	Control 3	12.5	3.0	7.1	6.0
IL-10	Control 1	107	2.6	5.7	6.2
	Control 2	27.3	3.1	7.9	5.6
	Control 3	7.18	3.7	10.1	4.8

Dilution Linearity

To assess linearity, commercially available serum, EDTA plasma, and urine from rhesus and cynomolgus monkeys as well as cell culture supernatants were spiked with recombinant calibrators and diluted 2-fold, 4-fold, 8-fold, 16-fold, 32-fold, and 64-fold before testing. Percent recovery at each dilution level was normalized to the dilution-adjusted, 2-fold concentration. The average percent recovery shown below is based on samples within the quantitative range of the assay.

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

Rhesus Monkey

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

		IFN-γ		IL-1β		IL-2	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=5)	4	116	94–157	148	125–170	98	83–115
	8	111	89–152	176	148–205	95	81–104
	16	113	85–164	185	147–213	92	75–108
	32	108	83–155	202	147–251	92	77–109
	64	116	88–168	211	149–263	93	77–114
EDTA Plasma (N=5)	4	119	91–179	135	120–145	96	88–103
	8	126	86–232	153	131–161	94	82–109
	16	128	84–241	160	137–174	93	87–110
	32	122	81–227	168	147–184	95	85–113
	64	131	84–250	179	150–195	96	87–116
Urine (N=5)	4	99	96–101	96	94–98	82	79–85
	8	93	87–95	94	89–97	73	70–76
	16	90	87–91	87	85–88	66	63–68
	32	86	83–90	89	86–92	66	64–69
	64	90	86–93	88	83–93	64	61–68
Cell Culture Supernatant (N=5)	4	102	95–105	100	95–105	87	85–88
	8	97	92–103	96	90–104	83	78–88
	16	96	89–105	89	83–97	77	73–81
	32	88	82–94	86	81–99	75	71–77
	64	102	95–105	100	95–105	87	85–88

		IL-6		IL-8		IL-10	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=5)	4	110	91–143	101	89–124	108	94–129
	8	110	88–130	99	88–113	108	94–121
	16	113	83–138	95	79–111	105	89–120
	32	113	86–146	101	85–124	108	90–127
	64	117	85–149	99	82–125	107	92–131
EDTA Plasma (N=5)	4	112	95–146	97	83–104	102	88–107
	8	129	92–209	97	84–108	101	86–114
	16	139	93–240	93	81–110	99	85–118
	32	151	92–266	98	88–113	101	84–121
	64	165	97–296	99	86–116	100	83–124
Urine (N=5)	4	99	88–103	93	91–95	98	92–102
	8	91	81–99	88	85–91	96	91–100
	16	82	79–87	83	78–90	93	85–97
	32	82	77–87	86	83–89	93	86–98
	64	84	76–92	86	81–91	91	83–99
Cell Culture Supernatant (N=5)	4	97	80–107	96	90–98	101	99–104
	8	93	84–101	92	89–98	100	95–106
	16	87	79–95	85	80–92	94	87–100
	32	84	74–92	86	82–95	94	87–101
	64	84	75–96	84	78–91	90	83–98

Cynomolgus Monkey

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

		IFN-γ		IL-1β		IL-2	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=5)	4	110	103–118	143	136–157	114	96–152
	8	107	95–119	162	142–199	137	98–234
	16	105	91–118	171	139–220	149	97–277
	32	97	84–113	175	136–238	160	96–311
	64	103	86–125	183	141–259	165	101–320
EDTA Plasma (N=5)	4	109	104–111	164	147–180	99	89–105
	8	101	94–109	213	172–253	99	91–103
	16	99	95–107	238	189–291	101	95–111
	32	94	89–110	260	196–315	108	96–125
	64	100	90–120	281	205–356	113	96–136
Urine (N=5)	4	99	96–101	102	95–107	78	76–81
	8	91	88–93	99	96–106	74	69–78
	16	90	83–93	97	87–106	73	68–80
	32	84	80–89	95	88–104	77	72–84
	64	88	84–93	98	91–111	82	76–88
Cell Culture Supernatant (N=5)	4	102	95–105	100	95–105	87	85–88
	8	97	92–103	96	90–104	83	78–88
	16	96	89–105	89	83–97	77	73–81
	32	88	82–94	86	81–99	75	71–77
	64	102	95–105	100	95–105	87	85–88

		IL-6		IL-8		IL-10	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=5)	4	134	104–244	99	95–102	102	101–107
	8	144	101–295	98	90–106	102	97–113
	16	149	99–316	95	85–103	98	91–109
	32	150	95–332	97	85–115	98	90–119
	64	166	97–369	99	85–121	97	86–122
EDTA Plasma (N=5)	4	113	100–125	101	96–105	103	99–110
	8	112	100–124	101	96–109	102	97–112
	16	109	99–121	97	92–107	97	90–113
	32	110	96–131	102	91–121	100	94–121
	64	116	100–140	103	94–125	101	91–127
Urine (N=5)	4	101	98–105	97	95–100	98	96–100
	8	93	89–95	95	93–99	101	95–103
	16	88	78–96	94	89–102	101	95–109
	32	83	74–92	98	92–103	108	101–113
	64	87	81–94	98	92–104	112	105–119
Cell Culture Supernatant (N=5)	4	97	80–107	96	90–98	101	99–104
	8	93	84–101	92	89–98	100	95–106
	16	87	79–95	85	80–92	94	87–100
	32	84	74–92	86	82–95	94	87–101
	64	84	75–96	84	78–91	90	83–98

Spike Recovery

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

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

Rhesus Monkey

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

	Serum (N=5)			EDTA Plasma (N=5)			Urine (N=5)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
IFN-γ	75	16.9	53–83	70	34.5	28–90	83	4.1	79–87
IL-1β	49	15.8	35–55	52	8.5	45–56	100	4.1	96–105
IL-2	98	7.0	90–108	92	9.4	84–106	127	3.7	121–134
IL-6	73	10.4	65–84	58	38.2	27–87	92	3.3	88–95
IL-8	103	9.3	96–120	95	6.9	87–105	99	3.2	94–103
IL-10	98	15.7	82–122	95	7.4	88–105	93	4.1	88–97

	Cell Culture Supernatants (N=6)		
	Average % Recovery	%CV	% Recovery Range
IFN-γ	102	11.0	86–123
IL-1β	108	12.3	87–113
IL-2	131	14.0	106–165
IL-6	92	13.3	70–115
IL-8	109	13.2	86–139
IL-10	111	11.6	84–138

Cynomolgus Monkey

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

	Serum (N=5)			EDTA Plasma (N=5)			Urine (N=5)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
IFN-γ	75	11.4	63–86	83	6.3	74–87	88	3.8	85–93
IL-1β	53	7.0	48–57	36	20.7	26–44	100	4.7	96–108
IL-2	56	40.4	17–72	74	25.2	52–101	127	7.2	117–142
IL-6	56	29.7	30–72	71	12.7	56–80	97	5.2	90–104
IL-8	89	8.0	80–100	86	4.3	82–91	102	6.1	94–111
IL-10	88	9.9	78–101	89	2.6	86–91	93	7.7	85–105

	Cell Culture Supernatants (N=6)		
	Average % Recovery	%CV	% Recovery Range
IFN-γ	102	11.0	86–123
IL-1β	108	12.3	87–113
IL-2	131	14.0	106–165
IL-6	92	13.3	70–115
IL-8	109	13.2	86–139
IL-10	111	11.6	84–138

Specificity

To assess specificity, each assay in the panel was tested individually. Nonspecific binding was also evaluated with additional recombinant human analytes (Aβ 38, Aβ 40, Aβ 42, c-Kit, CTACK, CRP, EGF, Eotaxin, Eotaxin-2, Eotaxin-3, EPO, FGF (basic), Fractalkine, G-CSF, GM-CSF, HGF, I-309, ICAM-1, ICAM-3, IFN-α2a, IL-1α, IL-5, IL-6R, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, IL-17B, IL-17D, IL-18, IP-10, I-TAC, MCP-1, MCP-2, MCP-4, M-CSF, MDC, MIF, MIG, MIP-1α, MIP-3α, MIP-1β, MIP-4, MIP-5, MMP-1, MMP-2, MMP-3, MMP-9, MMP-10, NT-proBNP, RANTES, SAA, Thrombomodulin, Tie, TARC, TNF-β,

TNF-RI, TNF-RII, TPO, VCAM-1, VEGF-A, VEGF-C, VEGF-D, and VEGF-RI). Nonspecific binding was less than 0.6% for all assays in the kit.

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

Stability

The reconstituted calibrator, reconstituted controls, and diluents were tested for freeze–thaw stability. Results (not shown) demonstrated that reconstituted calibrator, reconstituted controls, and diluents can go through three freeze–thaw cycles without significantly affecting the performance of the assay. Reconstituted calibrator and diluents must be stored frozen at -70ºC; reconstituted controls can be stored up to 10 days at 2-8ºC or frozen at ≤-70ºC for long-term storage. Seal and store partially used MSD plates for 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 date of manufacture.

Calibration

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

Tested Samples

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

Rhesus Monkey

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

Sample Type	Statistic	IFN-γ	IL-1β	IL-2	IL-6	IL-8	IL-10
Serum (N=30)	Median (pg/mL)	1.18	1.00	0.30	0.98	2.16	0.22
	Range (pg/mL)	0.45–133	0.12–32.3	0.20–23.5	0.12–9.15	0.73–67.0	0.06–28.3
	% Detected	83	30	67	87	100	60
EDTA Plasma (N=30)	Median (pg/mL)	2.11	0.39	0.29	0.86	0.38	0.19
	Range (pg/mL)	0.47–10.9	0.08–2.17	0.20–1.91	0.22–11.0	0.10–2.40	0.07–1.01
	% Detected	73	40	47	93	73	60
Urine (N=30)	Median (pg/mL)	ND	ND	ND	ND	0.31	0.08
	Range (pg/mL)	ND	ND	ND	ND	0.31	0.08
	% Detected	0	0	0	0	3	3

ND = Non-detectable

Cynomolgus Monkey

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

Sample Type	Statistic	IFN-γ	IL-1β	IL-2	IL-6	IL-8	IL-10
Serum (N=30)	Median (pg/mL)	1.18	0.22	0.42	0.98	0.41	0.12
	Range (pg/mL)	0.42–4.43	0.09–5.39	0.20–3.27	0.14–9.25	0.09–4.87	0.06–0.26
	% Detected	77	77	70	93	100	70
EDTA Plasma (N=30)	Median (pg/mL)	0.71	0.33	0.34	0.93	0.13	0.15
	Range (pg/mL)	0.43–130	0.12–20.2	0.19–2.07	0.24–2.09	0.08–45.7	0.07–17.3
	% Detected	73	23	93	100	80	40
Urine (N=30)	Median (pg/mL)	0.62	0.10	ND	0.16	0.16	0.09
	Range (pg/mL)	0.41–1.01	0.08–0.15	ND	0.14–0.65	0.08–0.20	0.08–0.14
	% Detected	30	37	0	17	20	13

ND = Non-detectable

Stimulated Samples

Peripheral blood mononuclear cells (PBMC) from rhesus or cynomolgus monkeys were incubated at 37°C either unstimulated or with stimulant (lipopolysaccharide [LPS], phytohaemagglutinin [PHA], pokeweed mitogen [PWM], or concanavalin A [Con A]). Changes in cytokine production were assessed at 6, 24, and 48 hours. Specifically, we assessed changes occurring either spontaneously during cell culture or induced by stimulants.[46] The following tables summarize the maximum changes observed in cytokine production. When analytes were initially undetectable, calculations for fold increase were based on the assay's LLOD. At each time point, fold changes were calculated by normalizing the stimulated levels to cytokine levels from untreated controls rather than background levels from unconditioned cell culture media.

Rhesus Monkey

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

+++ > 100-fold

++ > 10-fold

\+ > 2-fold

– no significant response

	ConA	LPS	PHA	PWM	Spontaneous
IFN-γ	+++	+	+++	++	+
IL-1β	+	+	+	+	–
IL-2	++	+	++	+	+
IL-6	+	–	+	+	+
IL-8	+	+	++	+	+
IL-10	+	+	+	+	+

Cynomolgus Monkey

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

+++ > 100-fold

++ > 10-fold

\+ > 2-fold

– no significant response

	ConA	LPS	PHA	PWM	Spontaneous
IFN-γ	+++	–	+++	+++	–
IL-1β	–	+	+	+	–
IL-2	++	+	++	++	+
IL-6	+	–	+	+	–
IL-8	+	+	++	+	+
IL-10	+	+	–	+	++

Assay Components

Calibrators

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

Table 15. Recombinant human proteins used in the Calibrators

Calibrator	Expression System
IFN-γ	E. coli
IL-1β	E. coli
IL-2	E. coli
IL-6	E. coli
IL-8	E. coli
IL-10	Insect cell line

Antibodies

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

Table 16. Antibody source species

	Source Species		
Analyte	MSD Capture Antibody	MSD Detection Antibody	Assay Generation
IFN-γ	Mouse Monoclonal	Mouse Monoclonal	C
IL-1β	Mouse Monoclonal	Goat Polyclonal	C
IL-2	Recombinant Monoclonal	Recombinant Monoclonal	B
IL-6	Mouse Monoclonal	Goat Polyclonal	C
IL-8	Mouse Monoclonal	Goat Polyclonal	B
IL-10	Mouse Monoclonal	Mouse Monoclonal	B

References

1. Lee JW, et al. Fit-for-purpose method development and validation for successful biomarker measurement. Pharm Res. 2006;23:312-28.
2. Villinger F, et al. Comparative sequence analysis of cytokine genes from human and nonhuman primates. J Immunol. 1995;155:3946- 3954.
3. 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.
4. Holmes C, et al. Proinflammatory cytokines, sickness behavior, and Alzheimer disease. Neurology. 2011;77:212-8.
5. Desai D, et al. Cytokines and cytokine-specific therapy in asthma. Ad Clin Chem. 2012;57:57-97.
6. Gui T, et al. Diverse roles of macrophages in atherosclerosis: from inflammatory biology to biomarker discovery. Mediators Inflamm. 2012;693083.
7. Islam SA, et al. T cell homing to epithelial barriers in allergic disease. Nat Med. 2012;18:705-15.
8. Su DL, et al. Roles of pro- and anti-inflammatory cytokines in the pathogenesis of SLE. J Biomed Biotechnol. 2012;347141.
9. Lukens JR, et al. Inflammasome activation in obesity-related inflammatory disease and autoimmunity. Discov Med. 2011;12:65-74.
10. Laoui D, et al. Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. Int J Dev Biol. 2011;55:861-7.
11. Hallberg L, et al. Exercise-induced release of cytokines in patients with major depressive disorder. J Affect Disord. 2010;126:262-7.
12. 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.
13. Svensson J, et al. Few differences in cytokines between patients newly diagnosed with type 1 diabetes and their healthy siblings. Hum Immunol. 2012;73:1116-26.
14. Yehuda H, et al. Isothiocyanates inhibit psoriasis-related proinflammatory factors in human skin. Inflamm Res. 2012;61:735-42.
15. 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 Colitis. 2012;S1873-9946:00393-5
16. Kwan W, et al. Bone marrow transplantation confers modest benefits in mouse models of Huntington's disease. J Neurosci. 2012;32:133-42.
17. Crotta S, et al. Hepatitis C virions subvert natural killer cell activation to generate a cytokine environment permissive for infection. J Hepatol. 2010;52:183-90.
18. Liu X, et al. Age-dependent neuroinflammatory responses and deficits in long-term potentiation in the hippocampus during systemic inflammation. Neuroscience. 2012;216:133-42.
19. Moon MH, et al. Sphingosine-1-phosphate inhibits interleukin-1b-induced inflammation in human articular chondrocytes. Int J Mol Med. 2012;30:1451-8.
20. Mihai G, et al. Circulating cytokines as mediators of fever. Clin Infect Dis. 2000:s178-84.
21. Liao W, et al. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. Curr Opin Immunol. 2011;23:598-604.
22. Aberg JA. Aging, inflammation, and HIV infection. Top Antivir Med. 2012;20:101-5.
23. Sharma M, et al. Enhanced pro-inflammatory chemokine/cytokine response triggered by pathogenic Entamoeba histolytica : basis of invasive disease. Parasitology. 2005;131:783-96.
24. Deng B, et al. Cytokine and chemokine levels in patients with severe fever with thrombocytopenia syndrome virus. PLoS One. 2012;7:e41365.
25. Zupan J, et al. The relationship between osteoclastogenic and anti-osteoclastogenic pro-inflammatory cytokines differs in human osteoporotic and osteoarthritic bone tissues. J Biomed Sci. 2012;19:28.
26. O'Donoghue RJ, et al. Genetic partitioning of interleukin-6 signalling in mice dissociates Stat3 from Smad3-mediated lung fibrosis. EMBO Mol Med. 2012;4:939-51.
27. Goral V, et al. The relation between pathogenesis of liver cirrhosis, hepatic encephalopathy and serum cytokine levels: what is the role of tumor necrosis factor α Hepatogastoenterology. 2011;58:943-8.
28. Smith PD, et al. The evolution of chemokine release supports a bimodal mechanism of spinal cord ischemia and reperfusion injury. Circulation. 2012;126:S110-7.

29. Tinkle SS, et al. Beryllium-stimulated release of tumor necrosis factor-alpha, interleukin-6, and their soluble receptors in chronic beryllium disease. Am J Respir Crit Care Med. 1997;156:1884-91.

30. Aoun E, et al. Diagnostic accuracy of interleukin-6 and interleukin-8 in predicting severe acute pancreatitis: a meta-analysis. Pancreatology. 2009;9:777-85.

31. Bliss SK, et al. IL-10 prevents liver necrosis during murine infection with Trichinella spiralis. J Immunol. 2003; 6:3142-7.

32. Bowen RA, et al. Impact of blood collection devices on clinical chemistry assays. Clin Biochem. 2010;43:4-25.

33. Zhou H, et al. Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney. 2006;69:1471-6.

34. Thomas CE, et al. Urine collection and processing for protein biomarker discovery and quantification. Cancer Epidemiol Biomarkers & Prevention. 2010;19:953-9.

35. Godoy-Ramirez K, et al. Optimum culture conditions for specific and nonspecific activation of whole blood and PBMC for intracellular cytokine assessment by flow cytometry. J Immunol Methods. 2004;292:1-15.

Additional Information

Appendix A: Alternate Protocols

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

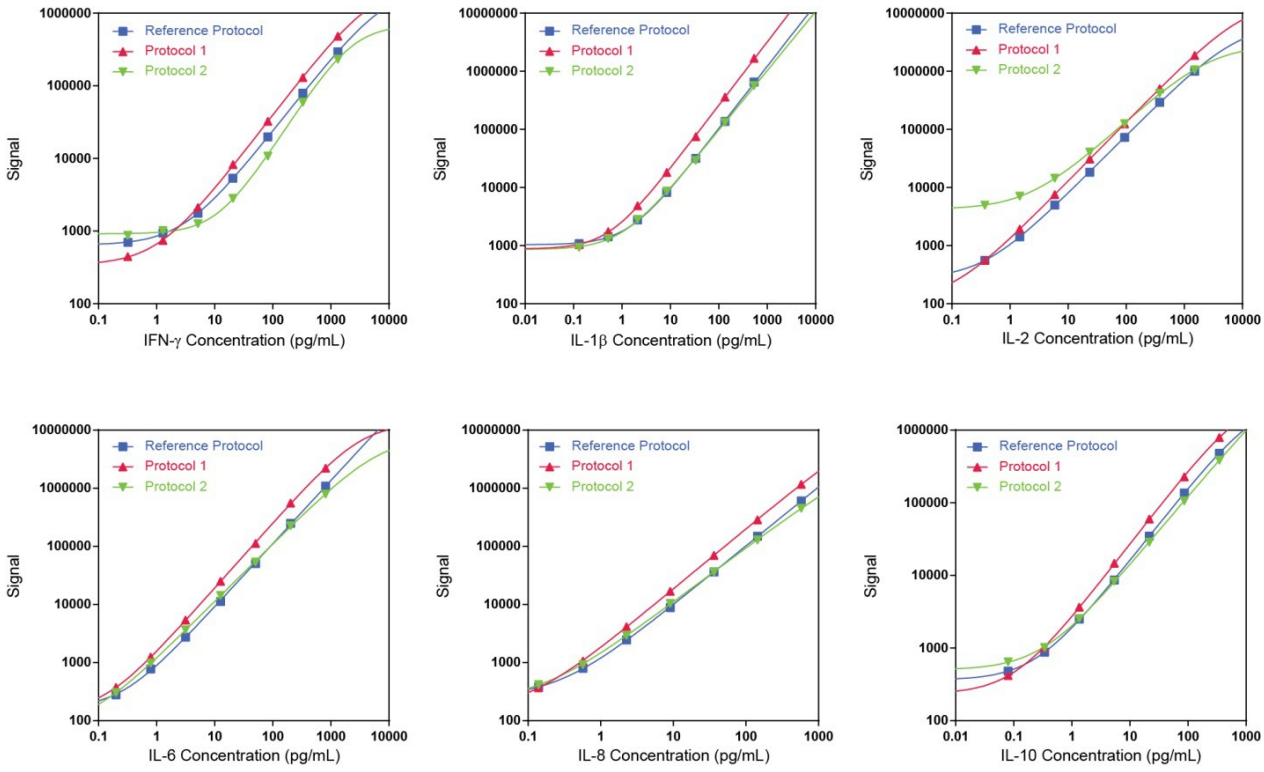

Table 17. Relative sensitivity for each assay when using alternative protocols

	LLOD Comparison (pg/mL)		
Assay	Reference Protocol	Protocol 1	Protocol 2
IFN-γ	0.37	0.25	4.84
IL-1β	0.05	0.09	0.20
IL-2	0.09	0.16	0.20
IL-6	0.06	0.07	0.07
IL-8	0.07	0.05	0.05
IL-10	0.04	0.03	0.09

Appendix B: Single Spot vs Multiplex Plate

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

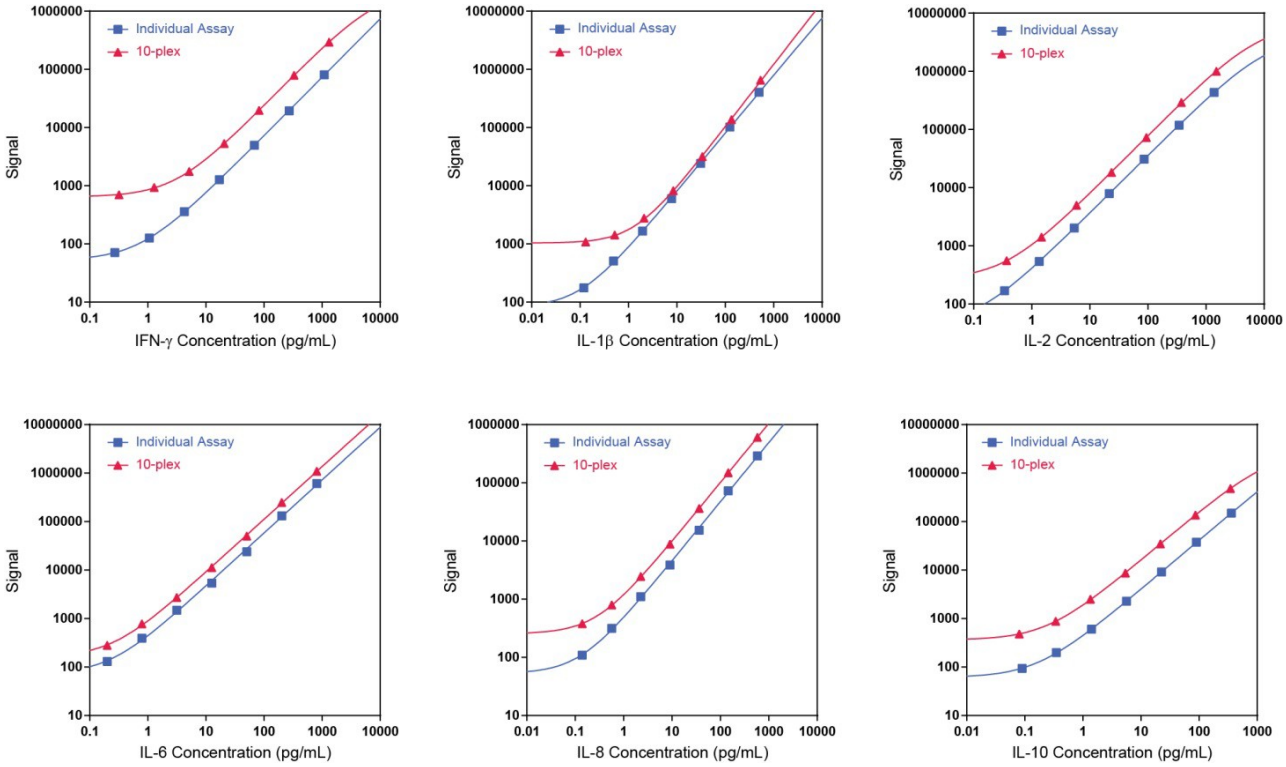

Table 18. Assay performance for individual and 10-plex assays

	LLOD (pg/mL)	
Assay	Individual	10-plex
IFN-γ	0.35	0.37
IL-1β	0.03	0.05
IL-2	0.07	0.09
IL-6	0.08	0.06
IL-8	0.07	0.07
IL-10	0.07	0.04

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.

Appendix C: 10-spot Plate vs Single Antibody

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

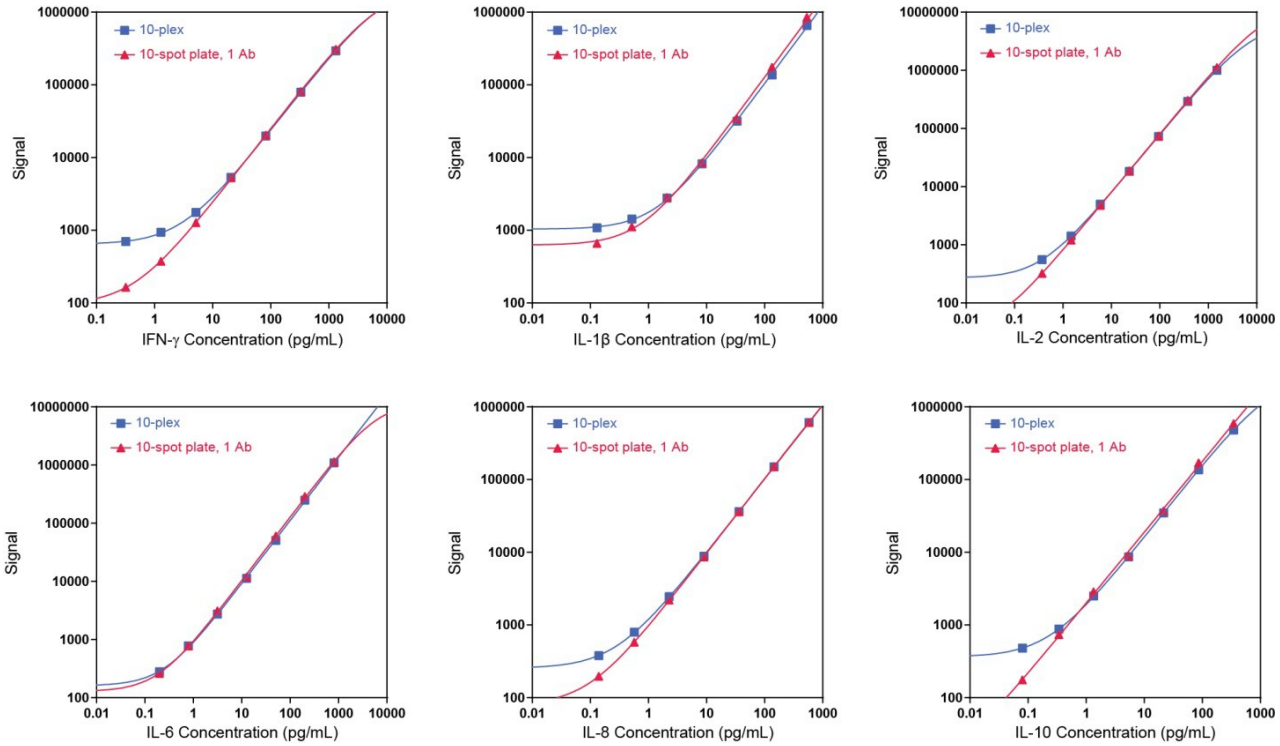

Table 19. LLODs for detection of a single antibody vs. blended antibodies

	LLOD (pg/mL)	
Assay	10-spot plate, 1 Ab	10-plex
IFN-γ	0.40	0.37
IL-1β	0.13	0.05
IL-2	0.11	0.09
IL-6	0.11	0.06
IL-8	0.08	0.07
IL-10	0.04	0.04

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

Summary Protocol

Proinflammatory Panel 1 (NHP) Kits

MSD provides this summary protocol for your convenience. Read the entire detailed protocol prior to performing the Proinflammatory Panel 1 (NHP) assays.

Sample and Reagent Preparation

- ❑ Bring all reagents to room temperature.
- ❑ Prepare calibration solutions in Diluent 2 using the supplied calibrator:
  - o Reconstitute the lyophilized calibrator blend.
  - o Invert 3 times, equilibrate 15-30 minutes at room temperature.
  - o Vortex briefly using short pulses.
  - o Perform a series of 4-fold dilution steps and prepare a zero calibrator.
- ❑ Dilute samples and controls 2-fold in Diluent 2 before adding to the plate.
- ❑ Prepare combined detection antibody solution by diluting each 50X detection antibody 50-fold in Diluent 3.
- ❑ SECTOR Plates Only: Prepare 2X Read Buffer T by diluting 4X Read Buffer T 2-fold with deionized water.
- ❑ QuickPlex Plates Only: MSD provides MSD GOLD Read Buffer B ready for use. Do not dilute.

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

*Note: Washing the plate prior to 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 prior to sample addition.

Catalog Numbers

Table 20. Catalog numbers for V-PLEX and V-PLEX Plus Proinflammatory Panel 1 (NHP) multiplex and single assay kits

Kit Name	V-PLEX			V-PLEX Plus*		
	1-Plate Kit	5-Plate kit	25-Plate Kit	1-Plate Kit	5-Plate Kit	25-Plate Kit
Multiplex Kits						
Proinflammatory Panel 1 (NHP)	K15056D-1	K15056D-2	K15056D-4	K15056G-1	K15056G-2	K15056G-4
Proinflammatory Panel 1 QuickPlex Ultra (NHP)	K15056D-21	K15056D-22	K15056D-24	K15056G-21	K15056G-22	K15056G-24
Individual Assay Kits						
NHP IFN-γ	K156QOD-1	K156QOD-2	K156QOD-4	K156QOG-1	K156QOG-2	K156QOG-4
NHP IL-1β	K156QPD-1	K156QPD-2	K156QPD-4	K156QPG-1	K156QPG-2	K156QPG-4
NHP IL-2	K156QQD-1	K156QQD-2	K156QQD-4	K156QQG-1	K156QQG-2	K156QQG-4
NHP IL-6	K156QXD-1	K156QXD-2	K156QXD-4	K156QXG-1	K156QXG-2	K156QXG-4
NHP IL-8	K156RAD-1	K156RAD-2	K156RAD-4	K156RAG-1	K156RAG-2	K156RAG-4
NHP IL-10	K156QUD-1	K156QUD-2	K156QUD-4	K156QUG-1	K156QUG-2	K156QUG-4
Individual QuickPlex® Ultra Assay Kits						
NHP IFN-γ	K156QOD-21	K156QOD-22	K156QOD-24	K156QOG-21	K156QOG-22	K156QOG-24
NHP IL-1β	K156QPD-21	K156QPD-22	K156QPD-24	K156QPG-21	K156QPG-22	K156QPG-24
NHP IL-2	K156QQD-21	K156QQD-22	K156QQD-24	K156QQG-21	K156QQG-22	K156QQG-24
NHP IL-6	K156QXD-21	K156QXD-22	K156QXD-24	K156QXG-21	K156QXG-22	K156QXG-24
NHP IL-8	K156RAD-21	K156RAD-22	K156RAD-24	K156RAG-21	K156RAG-22	K156RAG-24
NHP IL-10	K156QUD-21	K156QUD-22	K156QUD-24	K156QUG-21	K156QUG-22	K156QUG-24

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

Plate Diagram

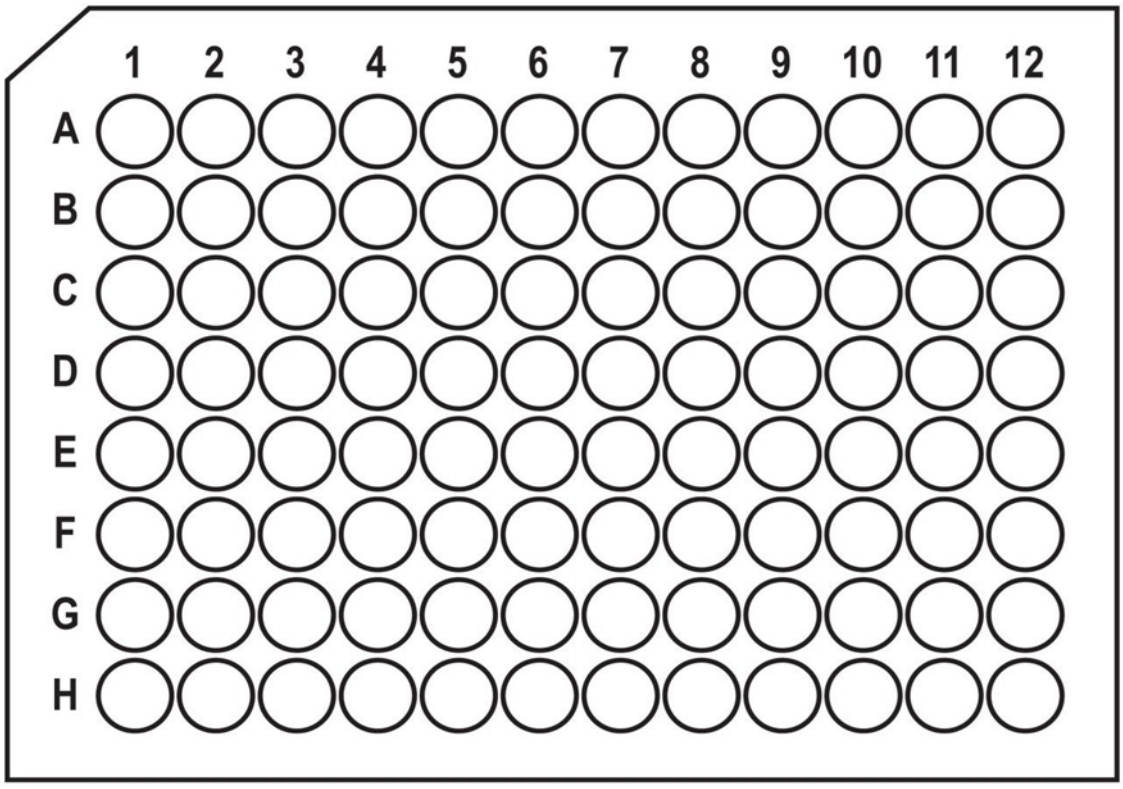

Figure 5. Plate Diagram

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