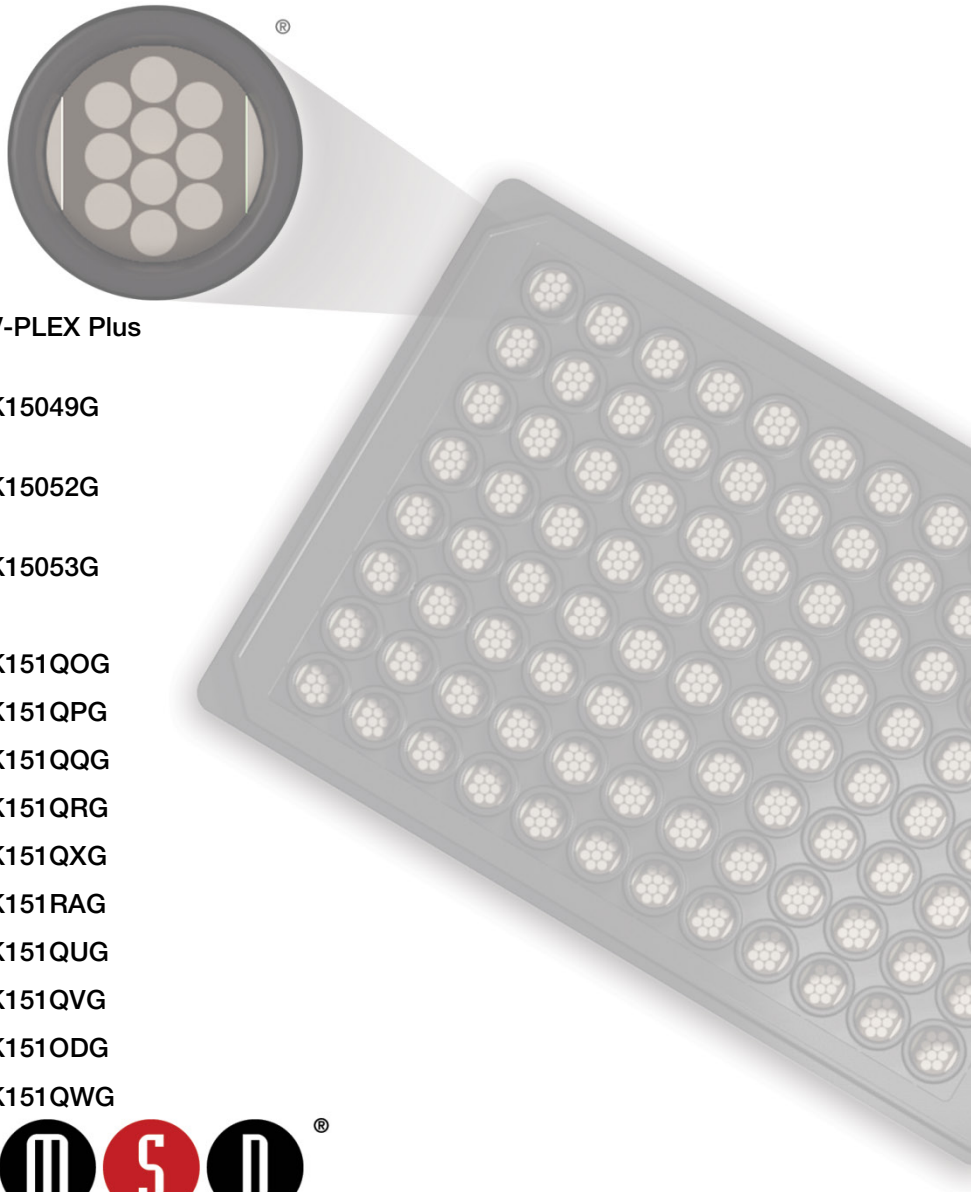
MSD® MULTI-SPOT Assay System

Proinflammatory Panel 1 (human) Kits

IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α

V-PLEX®



	V-PLEX®	V-PLEX Plus
Proinflammatory Panel 1 (human)	K15049D	K15049G
Human Proinflammatory Panel I (4-Plex)	K15052D	K15052G
Human Proinflammatory Panel II (4-Plex)	K15053D	K15053G
Individual Assay Kits		
Human IFN-γ	K151QOD	K151QOG
Human IL-1β	K151QPD	K151QPG
Human IL-2	K151QQD	K151QQG
Human IL-4	K151QRD	K151QRG
Human IL-6	K151QXD	K151QXG
Human IL-8	K151RAD	K151RAG
Human IL-10	K151QUD	K151QUG
Human IL-12p70	K151QVD	K151QVG
Human IL-13	K151ODD	K151ODG
Human TNF-α	K151QWD	K151QWG

MSD®

MSD Cytokine Assays

Proinflammatory Panel 1 (human) Kits

IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α

Human Proinflammatory Panel I (4-Plex)

IFN-γ, IL-1β, IL-6, TNF-α

Human Proinflammatory Panel II (4-Plex)

IL-1β, IL-6, IL-8, TNF-α

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

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

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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Introduction

MSD offers V-PLEX assays for customers who require unsurpassed performance and quality. V-PLEX products are developed under rigorous design control and are fully validated according to fit-for-purpose[39] principles 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. 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 (human) measures ten cytokines that are important in inflammatory responses and immune system regulation as well as many 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 (human) measures biomarkers that are implicated in 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 panel are described below.

Human 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[14] and hepatitis C.[15]

Human interleukin-1 beta (IL-1β), also known as IL-1F2, is a 30.7 kDa pro-inflammatory cytokine that is produced by activated macrophages. IL-1β 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. Together with IL-1α, IL-1β binds directly to IL-1 R1, which is then associated with IL-1 R accessory protein (IL-1RAcP) to form a high-affinity receptor complex for signal transduction. IL-1 R2 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 R1 to prevent binding of IL-1α and IL-1β. IL-1β is involved in a number of biological activities ranging from aging[16] to wound healing.[17] 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.[18]

Human interleukin-2 (IL-2), also known as T-cell growth factor (TCGF), is a glycosylated 17.6 kDa protein produced by T-cells. IL-2 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.[19] IL-2 production levels are associated with HIV infection[20] and entamoebiasis.[21]

Human interleukin-4 (IL-4), also known as B-cell stimulatory factor 1 (BSF-1) and lymphocyte stimulatory factor 1, is a glycosylated 17.5 kDa protein with three intra-chain disulfide bonds. It is produced by Th2 cells and participates in activation of B-cells and other cell types. It also stimulates DNA synthesis and enhances the expression of IgE and IgG1.[22] IL-4 can bind to two different receptor complexes. One complex is a heterodimer of IL-4 Rα and the common gamma chain complex. This complex is found on T cells. The second receptor complex consists of IL-4 Rα and IL-13 Rα1 and it is found on other cell types such as B cells. IL-4 suppresses the function of Th1 cells and macrophages as well as inhibits IFN-γ and IL-12 production. It is associated with severe asthma[23] among other disorders.

Human 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.

Human 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. It 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]

Human 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]

Human interleukin-12p70 (IL-12p70) is a disulfide-linked heterodimer consisting of IL-12a (p35 subunit) and IL-12b (p40 subunit) that is mainly produced by macrophages and T lymphocytes. The IL-12a subunit also dimerizes with Ebi3 to form IL-35, and the IL-12b subunit dimerizes with IL-23a to form IL-23. IL-12, IL-23, IL-27, and IL-35 are all heterodimeric proteins and members of the IL-12 cytokine family. The IL-12 receptor consists of two chains, IL-12 Rβ1 and IL-12 Rβ2. Binding of IL-12 to the receptor complex induces phosphorylation of tyrosine kinase 2 (TYK2) and Janus kinase 2 (JAK2). IL-12p70 activates T cells and natural killer cells that stimulate the production of IFN-γ. It is involved in a number of disorders including peritonitis[32] and chronic toxoplasmosis.[33]

Human interleukin-13 (IL-13) is a 15.8 kDa, glycosylated monomeric cytokine with two internal disulfide bonds. It is secreted by a variety of immune cells. IL-13 is involved in a number of biological processes, such as positive regulation of B-cell proliferation, macrophage activation, immunoglobulin production, protein secretion, and phosphorylation of Stat6 protein. IL-13 initially interacts with IL-13 Rα1 to form a low-affinity complex. The formation of this complex triggers association with IL-4 Rα to form a high-affinity complex that also functions as the type 2 IL-4 receptor complex. IL-13 also binds with high affinity to IL-13 Rα2, which is expressed on the cell surface, intracellularly, and in soluble form. It is involved in a number of disorders including allergic rhinitis,[34] inflammatory bowel disease, and colorectal cancer. [35]

Human tumor necrosis factor alpha (TNF-α), also known as tumor necrosis factor ligand superfamily member 2 (TNFSF2) and cachectin, is a 25.6 kDa cytokine. TNF is a transmembrane protein that oligomerizes intracellularly to form a non-covalent homotrimer. The membrane-bound soluble portion of the homotrimer is cleaved by TACE/ADAM17 to form TNF-α. The homotrimer binds to the receptors TNF RI and TNF RII, both of which are also expressed as homotrimers. TNF-α is mainly secreted by macrophages and can induce apoptosis in some tumor cell lines. It stimulates IL-1, which induces cachexia and causes fever. The intracellular form of TNF induces IL-12 production in dendritic cells. It can induce sepsis[36] and inflammation and can inhibit tumorigenesis[37] and viral replication.[38]

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 (human) 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 and individual IL-4 and IL-13 assays are provided on 10-spot MULTI-SPOT plates (Figure 1); individual IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, 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™) 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.[39]

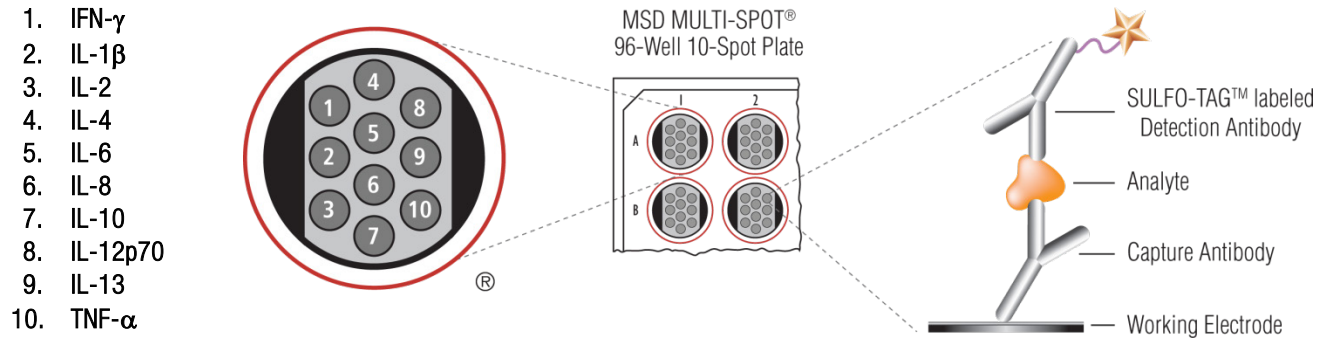

Figure 1. Multiplex plate spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

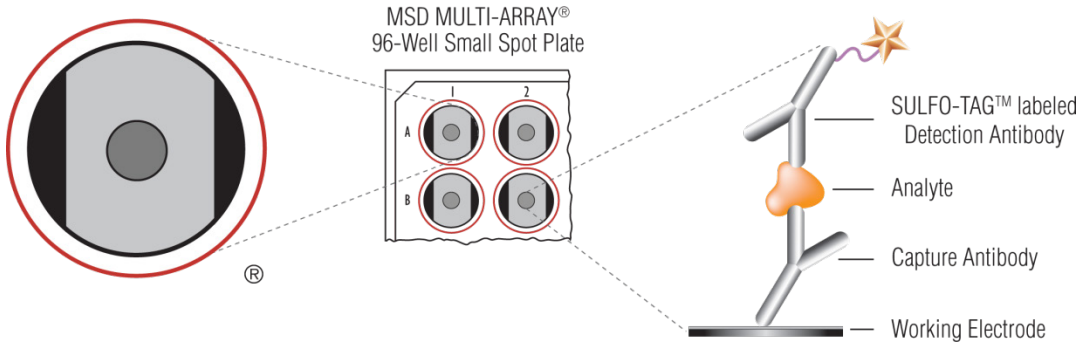

Figure 2. Small Spot plate diagram showing placement of analyte capture antibodies.

Kit Components

Proinflammatory Panel 1 (human) 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 #	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	Ten 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 protein, 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	
Read Buffer T (4X)	RT	R92TC-3	50 mL	1 bottle	1 bottle	5 bottles	Buffer to catalyze the electro-chemiluminescence reaction.

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-human matrix, buffered, lyophilized, and spiked with recombinant human analytes. The concentration of the controls is provided in the lot-specific COA.
Proinflammatory Panel 1 (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			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Proinflammatory Panel 1 (human) Plate	2–8 °C	N05049A-1	10-spot	1	5	25	96-well plate, foil sealed, with desiccant.
Human IFN-γ Plate	2–8 °C	L451QOA-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-4 Plate	2–8 °C	N05049A-1	10-spot	1	5	25	
Human IL-6 Plate	2–8 °C	L451QXA-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	
Human IL-12p70 Plate	2–8 °C	L451QVA-1	Small Spot	1	5	25	
Human IL-13 Plate	2–8 °C	N05049A-1	10-spot	1	5	25	
Human TNF-α Plate	2–8 °C	L451QWA-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			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Anti-hu IFN-γ Antibody (50X)	2–8 °C	D21QO-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21QO-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-4 Antibody (50X)	2–8 °C	D21QR-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21QR-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	
Anti-hu IL-12p70 Antibody (50X)	2–8 °C	D21QV-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21QV-3	375 µL		1	5	
Anti-hu IL-13 Antibody (50X)	2–8 °C	D21OD-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21OD-3	375 µL		1	5	
Anti-hu TNF-α Antibody (50X)	2–8 °C	D21BH-2	75 µL	1			SULFO-TAG conjugated antibody.
		D21BH-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 kit)
- ❑ Adhesive plate seals (3 per plate included in V-PLEX Plus kits)
- ❑ Deionized water
- ❑ Vortex mixer

Optional Materials and Equipment

- ❑ Proinflammatory Panel 1 (human) Control Pack, available for separate purchase from MSD, catalog # C4049-1 (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 safety data sheet (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, 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 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 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 such a 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. You can also find a copy of the COA at www.mesoscale.com.

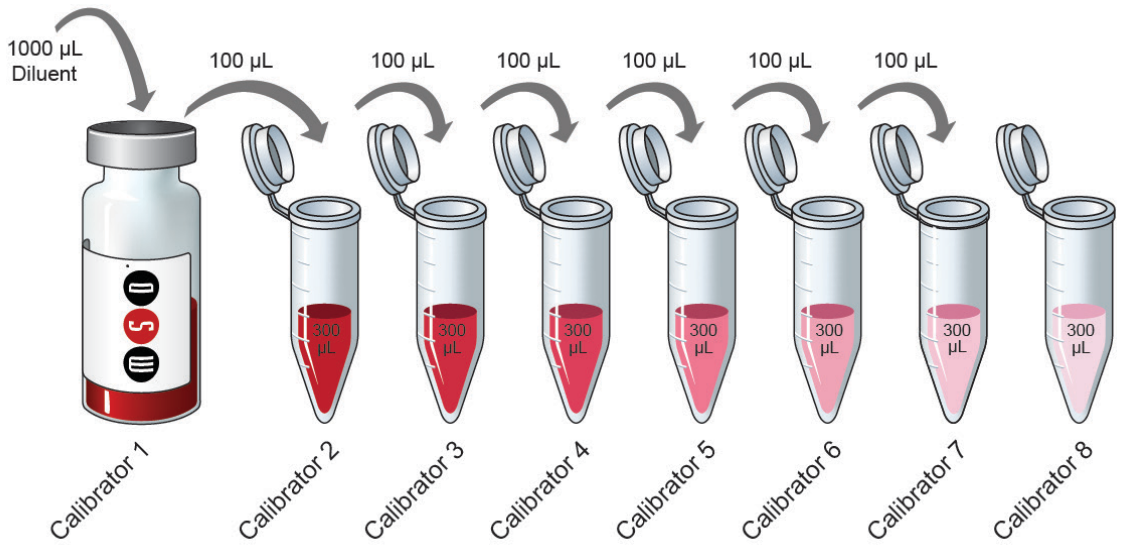

***Figure 3**. Dilution schema for preparation of Calibrator Standards*

Sample Collection and Handling

Below are general guidelines for human sample collection, storage, and handling. If possible, use published guidelines.[40-44] 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 ºC 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 human 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. 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. 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 (human) 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.

10-plex Proinflammatory Panel 1 (human) kit

For one plate, combine the following detection antibodies and add to 2,400 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-4 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
- ❑ 60 µL of SULFO-TAG Anti-hu IL-12p70 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu IL-13 Antibody
- ❑ 60 µL of SULFO-TAG Anti-hu TNF-α Antibody

Custom multiplex kits

For one plate, combine 60 µL of each supplied detection antibody, then add Diluent 3 to bring the final volume to 3,000 µL.

Individual assay kits

For one plate, add 60 µL of the supplied detection antibody to 2,940 µL of Diluent 3.

Prepare Wash Buffer

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

For one plate, combine:

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

Prepare Read Buffer T

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

For one plate, combine:

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

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

Prepare MSD Plate

MSD 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 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[39] and MSD design control procedures. V-PLEX assay components go through an extensive critical reagents program to ensure that the reagents are controlled and well characterized. Prior to the release of each V-PLEX panel, at least three independent kit lots are produced. Using results from multiple runs (typically greater than 50) and multiple operators, these lots are used to establish production specifications for sensitivity, specificity, accuracy, and precision. During validation, each individual assay is analytically validated as a singleplex and is also independently evaluated as a multiplex component by running the full multiplex plate using only the single detection antibody for that assay. These results are compared with the results from the multiplex panel when using all detection antibodies. This demonstrates that each assay is specific and independent, allowing them to be multiplexed in any combination. The COA provided with each kit outlines the kit release specifications for sensitivity, specificity, accuracy, and precision.

- ### Dynamic Range

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

- ### Sensitivity

  The lower limit of detection (LLOD) is a calculated concentration corresponding to the average signal 2.5 standard deviations above the background (zero calibrator). The LLOD is calculated using results from multiple plates for each lot, and the median and range of calculated LLODs for a representative kit lot are reported in this product insert. The upper limit of quantification (ULOQ) and lower limit of quantification (LLOQ) are established for each lot by measuring multiple levels near the expected LLOQ and ULOQ levels. The final LLOQ and ULOQ specifications for the product are established after 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 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 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 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 (human) 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 ten detection antibodies. The calibration curves were comparable. Calibration curves for each lot are presented in the lot-specific COA.

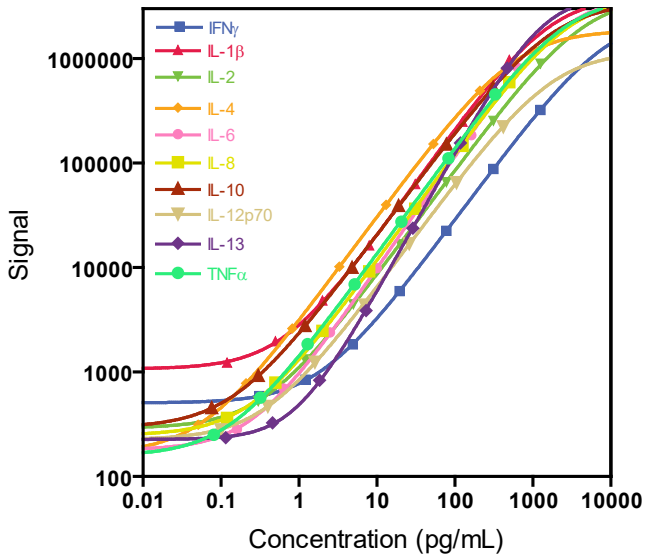

Figure 4. Typical calibration curves for the Proinflammatory Panel 1 (human) assay

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 (human) 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-4	0.02	0.01–0.03	0.218	158
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
IL-12p70	0.11	0.02–0.89	1.22	315
IL-13	0.24	0.03–0.73	4.21	353
TNF-α	0.04	0.01–0.13	0.690	248

Precision

Controls were made by spiking calibrator into non-human 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 below. While a typical specification for precision is a concentration CV of less than 20% for controls on both intra- and inter-day runs, for this panel, the data shows 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 (human) 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-4	Control 1	73.3	5.6	9.6	9.8
	Control 2	19.6	5.4	9.6	9.4
	Control 3	5.19	6.4	9.9	9.2
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
IL-12p70	Control 1	142	4.9	7.3	6.0
	Control 2	38.6	4.3	7.6	6.3
	Control 3	9.59	6.1	10.2	4.9
IL-13	Control 1	157	3.6	5.5	3.4
	Control 2	34.6	3.2	5.0	3.6
	Control 3	8.09	3.8	7.9	4.7
TNF-α	Control 1	75.5	2.7	6.1	7.2
	Control 2	19.2	2.4	7.4	6.3
	Control 3	4.45	3.4	10.1	6.2

Dilution Linearity

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

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

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

		IFN-γ		IL-1β		IL-2		IL-4		IL-6	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=11)	4	105	96–109	106	100–118	91	78–121	106	93–128	105	95–113
	8	101	91–112	103	92–129	91	71–158	103	87–133	106	96–124
	16	100	92–119	102	85–121	94	63–196	107	94–139	104	89–117
	32	98	87–120	105	85–136	107	63–283	103	88–135	104	93–118
	64	102	88–125	110	88–143	120	63–402	108	88–142	110	95–127
EDTA Plasma (N=11)	4	108	101–124	108	100–115	92	81–121	110	96–129	104	99–115
	8	107	93–131	106	94–119	91	75–157	108	86–140	106	97–121
	16	108	89–135	107	85–125	96	69–210	111	80–153	106	90–132
	32	103	79–135	107	81–128	105	66–282	105	71–140	105	88–133
	64	109	80–141	112	84–136	116	65–412	109	76–152	113	93–144
Heparin Plasma (N=11)	4	106	97–116	109	100–123	94	76–122	107	92–128	108	100–130
	8	101	90–110	108	99–118	96	70–161	104	84–142	105	94–121
	16	102	89–112	106	93–122	102	65–206	108	82–151	106	84–121
	32	98	84–112	108	98–124	110	61–277	105	81–149	104	81–121
	64	101	83–124	109	93–137	125	54–435	110	81–157	108	89–130
Citrate Plasma (N=9)	4	102	95–107	100	92–105	79	61–116	103	91–109	107	92–169
	8	97	87–104	99	94–107	74	50–146	99	89–110	112	83–264
	16	94	85–105	96	89–109	71	46–174	98	86–115	127	85–416
	32	89	80–104	94	80–113	72	46–191	95	82–124	136	78–550
	64	91	81–106	94	84–113	73	45–207	99	85–129	156	79–702
Urine (N=5)	4	99	97–100	99	91–106	90	82–102	92	77–98	115	100–127
	8	97	96–98	103	91–118	86	77–101	90	78–98	117	98–135
	16	96	89–103	105	93–119	82	70–97	89	73–124	115	92–134
	32	93	89–100	111	93–128	84	70–97	90	72–107	122	97–144
	64	99	94–108	113	95–125	83	70–100	96	76–119	125	96–143
Cell Culture Supernatant (N=5)	4	102	95–105	100	95–105	87	85–88	101	96–108	97	80–107
	8	97	92–103	96	90–104	83	78–88	100	94–107	93	84–101
	16	96	89–105	89	83–97	77	73–81	102	94–114	87	79–95
	32	88	82–94	86	81–99	75	71–77	95	87–103	84	74–92
	64	102	95–105	100	95–105	87	85–88	101	96–108	84	75–96

Table 7 continued

		IL-8		IL-10		IL-12p70		IL-13		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=11)	4	97	89–103	102	93–108	104	99–117	88	79–103	98	88–107
	8	93	86–104	102	91–114	102	90–111	79	70–101	95	89–111
	16	88	78–100	97	89–113	104	91–119	73	63–101	90	82–110
	32	92	79–106	102	90–123	105	93–118	74	62–108	94	85–115
	64	95	79–110	104	89–124	110	94–131	79	65–114	95	86–119
EDTA Plasma (N=11)	4	97	94–104	106	100–116	106	94–126	90	84–102	97	93–103
	8	92	86–99	106	93–120	107	95–133	83	74–111	94	87–103
	16	90	74–104	103	82–119	108	92–142	77	62–117	89	77–103
	32	90	71–102	107	81–131	108	87–152	76	53–117	92	77–104
	64	95	72–108	106	81–132	114	88–154	81	59–127	94	76–107
Heparin Plasma (N=11)	4	99	92–104	101	91–108	105	96–115	93	83–109	99	89–105
	8	94	83–101	101	86–111	102	94–116	84	72–114	96	81–101
	16	92	83–100	97	81–111	106	93–131	79	63–111	93	78–102
	32	94	80–102	100	83–112	103	93–126	77	60–114	94	78–97
	64	96	82–108	99	81–118	109	92–155	82	62–126	95	77–105
Citrate Plasma (N=9)	4	97	93–107	97	94–100	101	90–115	87	81–95	95	90–101
	8	89	83–97	94	87–108	96	84–111	76	67–88	89	80–106
	16	85	74–95	88	74–105	94	78–109	66	57–78	83	70–108
	32	86	72–97	90	75–114	91	73–111	65	57–79	84	71–107
	64	86	73–99	88	71–111	95	74–118	67	60–88	84	71–108
Urine (N=5)	4	96	89–103	98	96–102	99	95–108	97	87–111	89	86–92
	8	98	92–104	97	93–101	96	91–101	92	77–113	87	85–91
	16	97	85–108	94	88–110	93	83–113	84	70–101	84	79–95
	32	102	94–111	96	89–103	93	83–105	80	67–98	86	80–92
	64	102	91–114	97	90–109	98	79–120	76	64–91	88	84–97
Cell Culture Supernatant (N=5)	4	96	90–98	101	99–104	88	77–95	89	84–98	86	80–91
	8	92	89–98	100	95–106	86	81–89	79	74–83	79	74–85
	16	85	80–92	94	87–100	84	81–89	70	65–73	72	63–81
	32	86	82–95	94	87–101	79	70–85	67	64–71	73	67–80
	64	84	78–91	90	83–98	81	74–88	68	65–73	71	61–78

Spike Recovery

Spike recovery measurements of different sample types throughout the quantitative range of the assays were evaluated. Multiple individual human samples (serum, EDTA plasma, heparin plasma, citrate plasma, and urine) were obtained from a commercial source. These samples, along with cell culture supernatants, were spiked with calibrators at 3 levels (high, mid, and low) then diluted 2-fold. The average % recovery for each sample type is reported along with %CV and % recovery range.

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

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

	Citrate Plasma (N=11)			Heparin Plasma (N=11)			EDTA Plasma (N=11)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
IFN-γ	102	7.0	91–121	101	9.3	85–123	102	11.7	78–127
IL-1β	94	13.9	66–117	96	11.0	78–118	97	15.6	64–125
IL-2	105	38.8	34–158	115	21.8	56–149	107	35.2	41–181
IL-4	94	12.9	70–121	94	10.2	74–116	98	11.8	81–128
IL-6	88	33.1	8–120	87	20.2	39–112	92	14.3	72–122
IL-8	104	8.3	85–119	103	9.8	87–124	105	8.6	81–122
IL-10	105	8.4	93–126	105	8.9	90–124	103	9.1	80–127
IL-12p70	100	14.4	71–132	102	11.7	80–126	100	10.8	80–122
IL-13	122	9.3	103–149	114	12.0	81–138	119	13.1	87–157
TNF-α	111	7.7	95–131	110	8.1	96–131	110	8.0	88–132

	Serum (N=10)			Urine (N=5)			Cell Culture Supernatants (N=6)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
IFN-γ	103	9.6	85–126	101	6.1	91–111	102	11.0	86–123
IL-1β	93	9.6	81–112	97	7.5	83–108	108	12.3	87–113
IL-2	104	34.8	23–155	120	18.3	86–163	131	14.0	106–165
IL-4	96	10.0	82–115	99	7.8	87–112	92	13.3	70–115
IL-6	86	14.7	59–119	95	9.3	74–106	109	13.2	86–139
IL-8	100	9.3	87–118	102	7.5	84–114	111	11.6	84–138
IL-10	105	9.7	83–129	97	7.9	79–108	100	12.6	75–123
IL-12p70	100	15.7	79–158	106	9.0	89–121	114	12.2	93–137
IL-13	117	19.1	74–205	119	8.1	98–132	126	13.3	95–169
TNF-α	107	15.8	79–166	104	12.3	83–120	123	11.6	99–154

Specificity

To assess specificity, each assay in the panel was tested individually. Nonspecific binding was also evaluated with additional recombinant human analytes (Abeta 38, Abeta 40, Abeta 42, c-Kit, CTACK, CRP, EGF, Eotaxin, Eotaxin-2, Eotaxin-3, EPO, FGF (basic), Fractalkine, G-CSF, 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 controls must be stored frozen at ≤-70ºC; reconstituted controls can be stored up to ten days at 2–8ºC. 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 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	NIBSC (IU/mL): MSD (pg/mL)
IFN-γ	87/586	0.0023
IL-1β	86/680	0.22
IL-2	86/504	0.013
IL-4	88/656	0.032
IL-6	89/548	0.24
IL-8	89/520	0.0012
IL-10	93/722	0.010
IL-12p70	95/544	0.025
IL-13	94/622	0.0029
TNF-α	88/786	0.106

Tested Samples

Normal Samples

Normal human serum, EDTA plasma, heparin plasma, citrate plasma, and urine samples from a commercial source were diluted 2-fold and tested. Results for each sample set are displayed below. Concentrations are corrected for sample dilution. Median and range are calculated from samples with concentrations at or above the LLOD. Percent Detected is the percentage of samples with concentrations at or above the LLOD.

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

Sample Type	Statistic	IFN-γ	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-13	TNF-α
Serum (N=27)	Median (pg/mL)	3.77	0.16	0.52	ND	0.47	9.61	0.20	0.29	1.65	0.36
	Range (pg/mL)	0.64–14.4	0.11–24.3	0.22–2.68	NA	0.16–27.2	1.48–1720	0.06–3.08	0.26–0.38	0.60–2.78	0.10–1.75
	% Detected	96	22	33	0	37	100	52	11	11	70
EDTA Plasma (N=27)	Median (pg/mL)	3.80	0.20	0.38	0.05	0.29	0.52	0.21	0.38	1.34	0.74
	Range (pg/mL)	0.46–22.8	0.11–0.94	0.20–3.69	0.04–0.06	0.12–0.99	0.14–20.4	0.09–2.71	0.29–0.51	–	0.31–2.32
	% Detected	78	37	30	7	37	81	48	11	4	81
Heparin Plasma (N=27)	Median (pg/mL)	2.88	0.66	2.73	ND	0.30	60.1	0.16	ND	0.88	0.46
	Range (pg/mL)	0.58–7.90	0.21–11.0	1.23–3.48	NA	0.12–3.11	1.93–2630	0.06–2.64	NA	0.51–2.95	0.17–1.48
	% Detected	96	41	11	0	33	100	52	0	15	100
Citrate Plasma (N=20)	Median (pg/mL)	3.28	0.18	0.39	ND	0.21	2.85	0.21	0.32	ND	0.48
	Range (pg/mL)	1.44–34.9	0.13–0.36	0.26–1.46	NA	0.12–0.47	0.19–112	0.09–2.21	–	NA	0.19–3.17
	% Detected	100	15	15	0	40	100	65	5	0	100
Urine (N=15)	Median (pg/mL)	0.77	0.99	0.36	0.10	0.30	31.6	0.11	0.37	0.96	0.15
	Range (pg/mL)	0.45–1.36	0.11–75.7	0.14–0.93	0.06–0.13	0.19–2.11	0.30–1080	0.08–0.15	0.31–0.43	0.56–4.95	0.13–0.17
	% Detected	60	100	27	20	67	100	27	13	33	33

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

Stimulated Samples

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

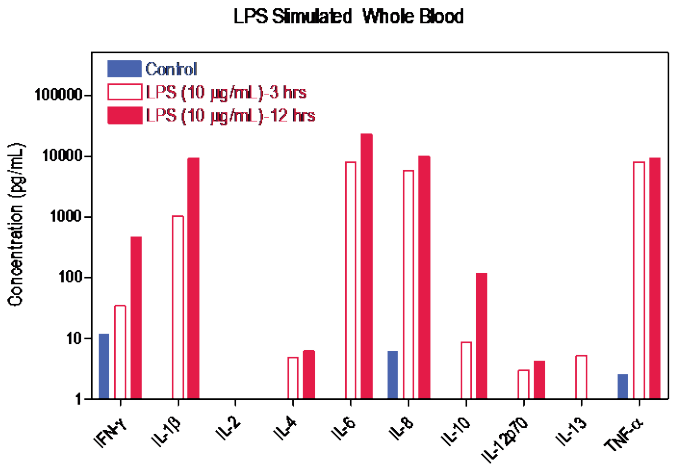

Figure 5. Normal human whole blood stimulated with LPS

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

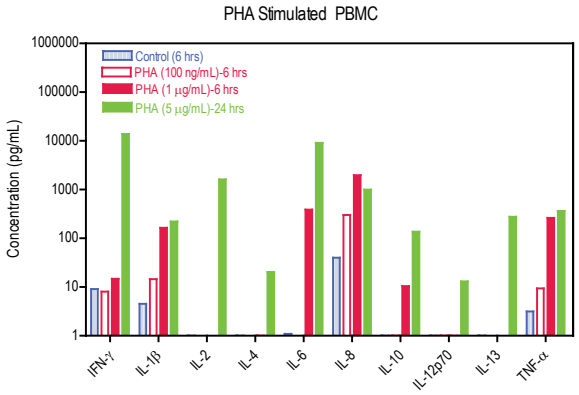

Figure 6. *Enriched whole blood treated with PHA*

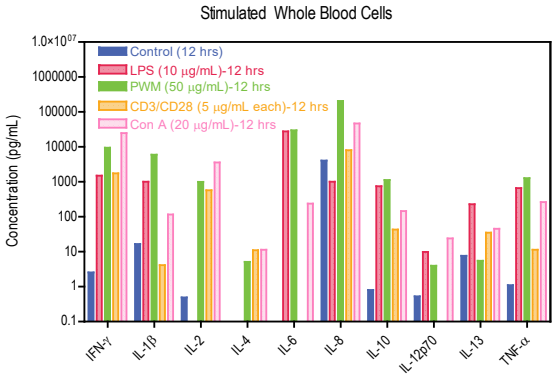

Figure 7. *Enriched whole blood treated with LPS, PWM, Con A, and CD3/CD28*

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

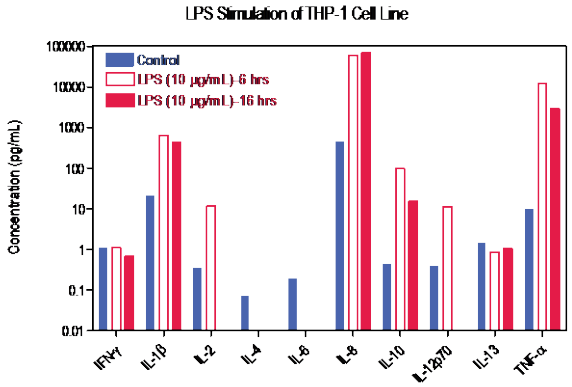

Figure 8. *THP-1 cell line stimulated for different time periods with LPS*

Assay Components

Calibrators

The assay calibrator blend uses the following recombinant human proteins:

Table 11. Recombinant human proteins used in the Calibrators

Calibrator	Expression System
IFN-γ	*E. coli*
IL-1β	*E. coli*
IL-2	*E. coli*
IL-4	*E. coli*
IL-6	*E. coli*
IL-8	*E. coli*
IL-10	Insect cell line
IL-12p70	Insect cell line
IL-13	*E. coli*
TNF-α	*E. coli*

Antibodies

Table 12. 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	Mouse Monoclonal	Mouse Monoclonal	B
IL-4	Mouse Monoclonal	Mouse Monoclonal	B
IL-6	Mouse Monoclonal	Goat Polyclonal	C
IL-8	Mouse Monoclonal	Goat Polyclonal	B
IL-10	Mouse Monoclonal	Mouse Monoclonal	B
IL-12p70	Mouse Monoclonal	Mouse Monoclonal	B
IL-13	Rat Monoclonal	Mouse Monoclonal	B
TNF-α	Mouse Monoclonal	Goat Polyclonal	B

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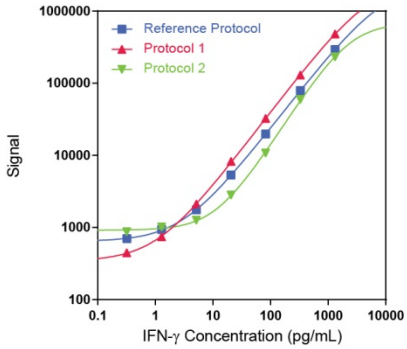
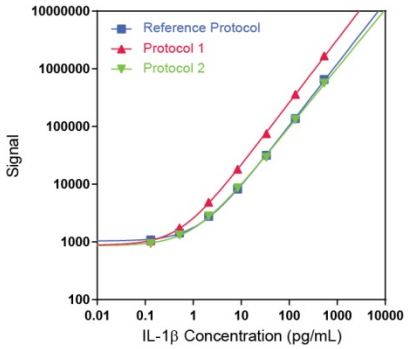
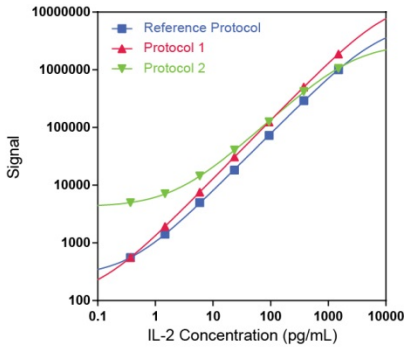
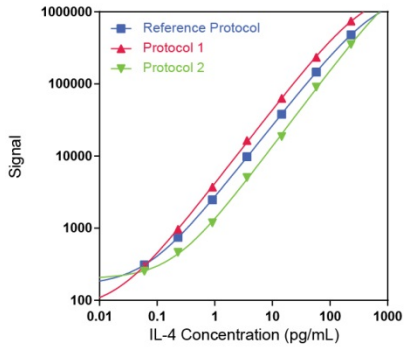
Appendix A

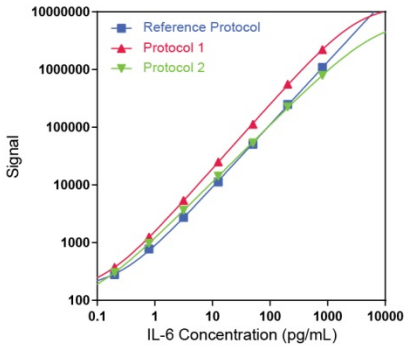
Calibration curves below illustrate the relative sensitivity for each assay under **Alternate Protocols**: Reference Protocol (2-hour sample incubation/2 wash steps, blue curve), Alternate Protocol 1 (overnight sample incubation, red curve), and Alternate Protocol 2 (tissue culture: single wash, green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

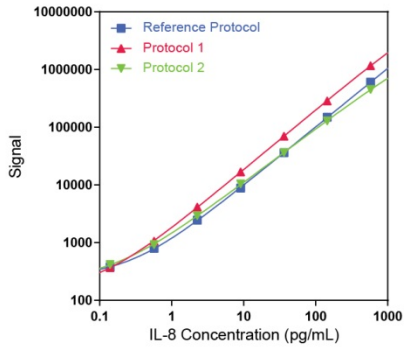


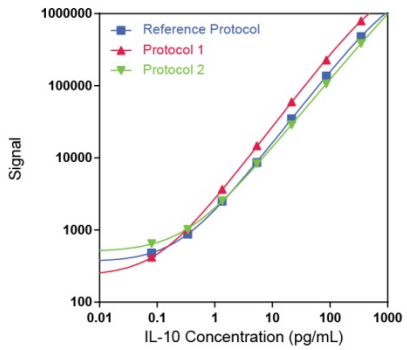


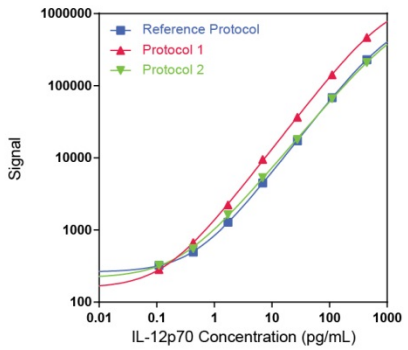


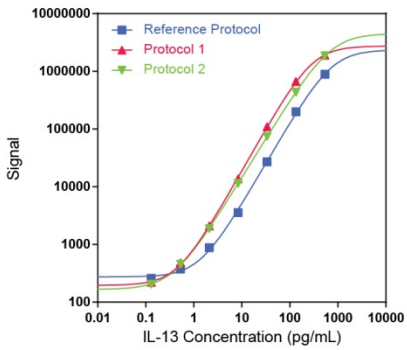












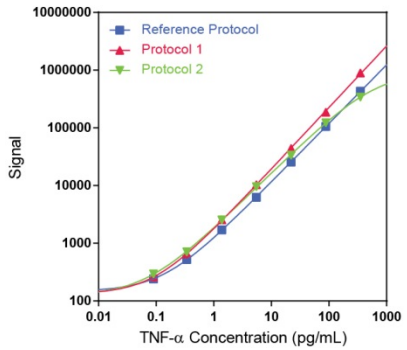


Table 13. Relative sensitivity when using alternate protocols

	LLOD Comparison (pg/mL)		
	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-4	0.02	0.02	0.08
IL-6	0.06	0.07	0.07
IL-8	0.07	0.05	0.05
IL-10	0.04	0.03	0.09
IL-12p70	0.11	0.07	0.12
IL-13	0.24	0.22	0.19
TNF-α	0.04	0.05	0.06

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).

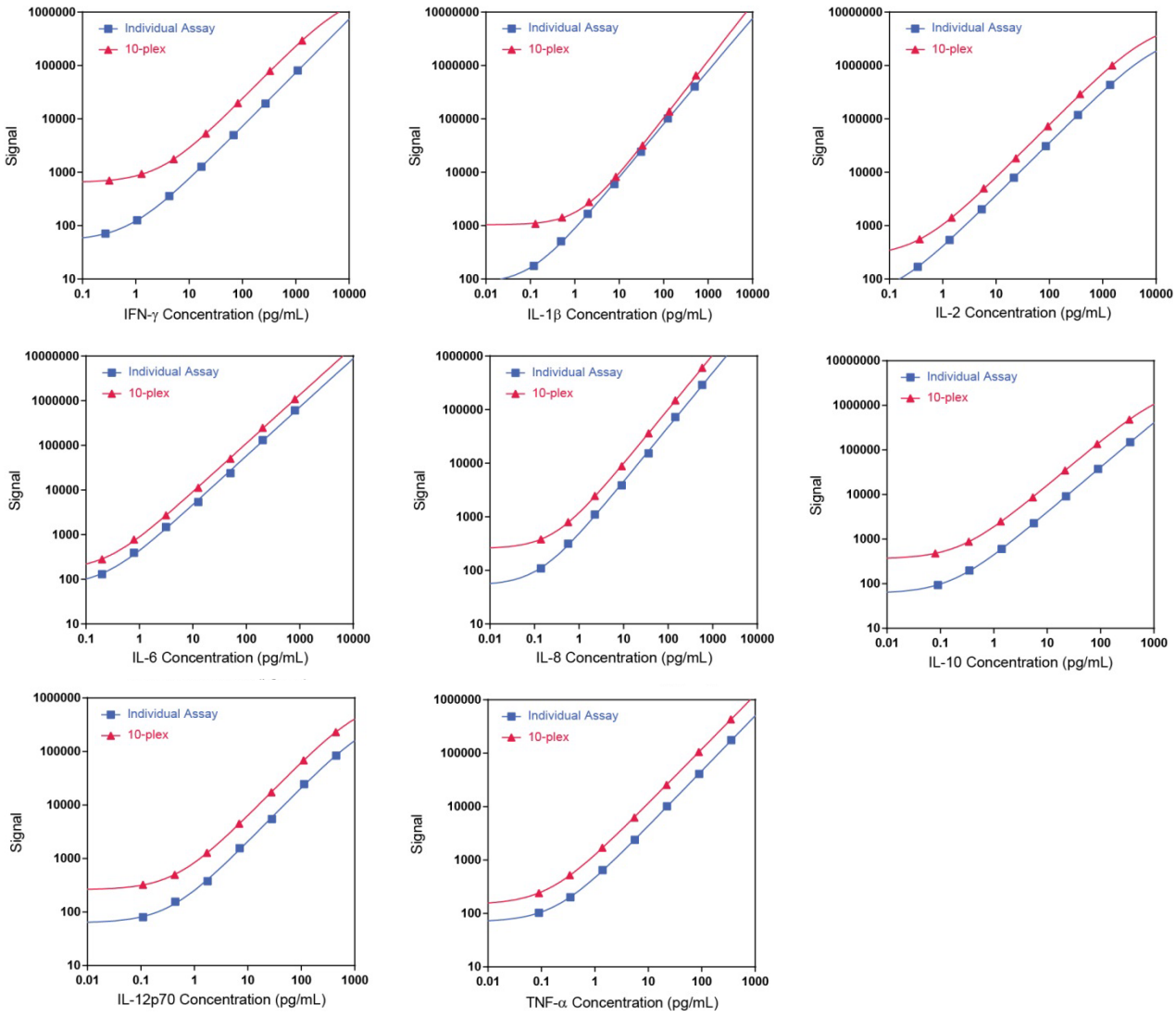


Table 14. Assay performance for individual and 10-plex assays

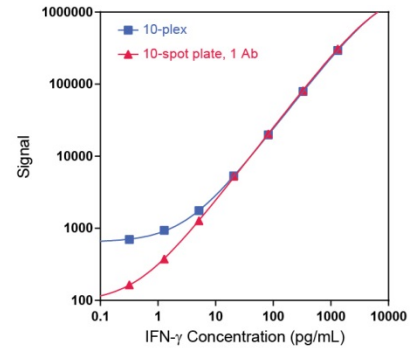
	LLOD (pg/mL)	
Assay	Individual	10-plex
IFN-γ	0.35	0.37
IL-1β	0.03	0.05
IL-2	0.07	0.09
IL-6	0.08	0.06
IL-8	0.07	0.07
IL-10	0.07	0.04
IL-12p70	0.14	0.11
TNF-α	0.06	0.04

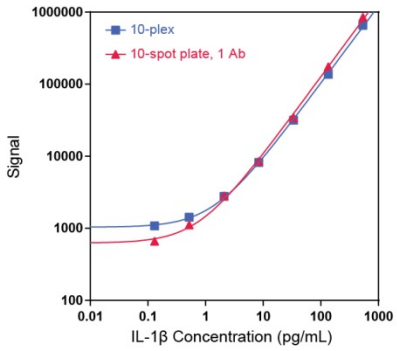
In general, assays in the single spot format yielded a lower overall signal compared to the 10-plex format. The spots on single-spot plates have a larger binding surface than those on multiplex plates, but the same amount of calibrator was used for each test; therefore, the bound calibrator was spread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

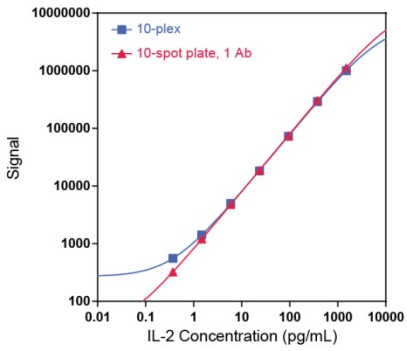
Note: Assay performance for IL-4 and IL-13 is not included since the individual assays are run on multiplex plates.

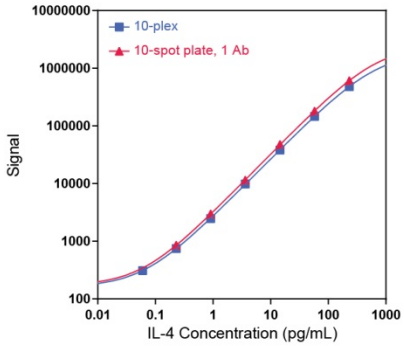
Appendix C

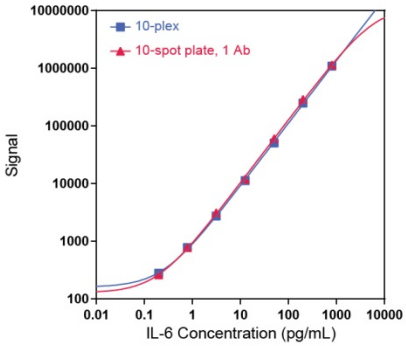
The calibration curves below compare results for each assay in the panel when the assays were run on the 10-spot plate using all detection antibodies (blue curve) vs. running each assay using a single, assay-specific detection antibody (red curve).

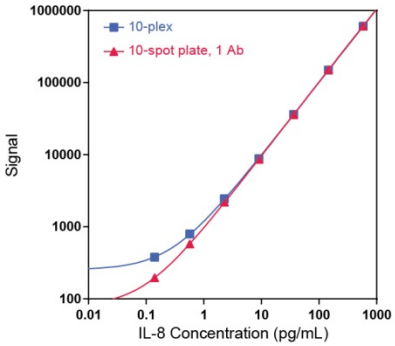


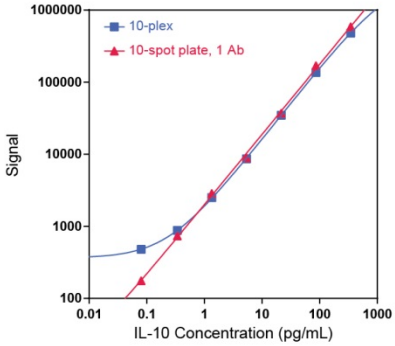


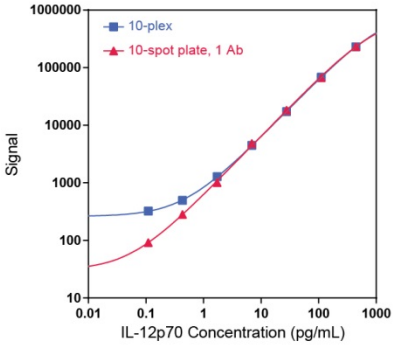


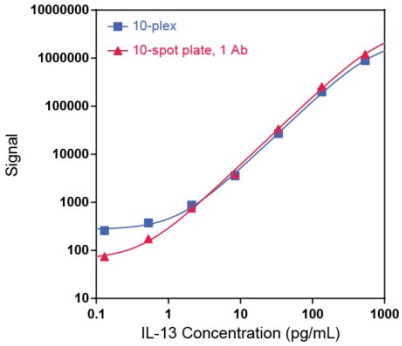












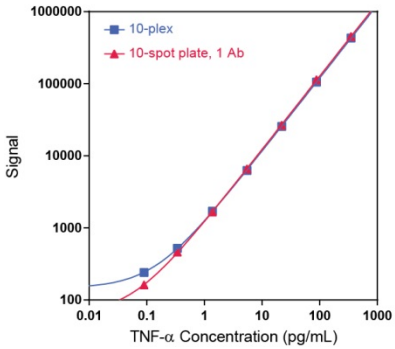


Table 15. LLODs for detection of a single Ab vs. blended Abs

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	0.40	0.37
IL-1β	0.13	0.05
IL-2	0.11	0.09
IL-4	0.06	0.02
IL-6	0.11	0.06
IL-8	0.08	0.07
IL-10	0.04	0.04
IL-12p70	0.15	0.11
IL-13	0.44	0.24
TNF-α	0.09	0.04

Summary Protocol

Proinflammatory Panel 1 (human) Kits

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the proinflammatory panel 1 (human) assays.

Sample and Reagent Preparation

- ❑ Bring all reagents to room temperature.
- ❑ Prepare calibration solutions in Diluent 2 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 2 before adding to the plate.
- ❑ Prepare combined detection antibody solution by diluting each 50X detection antibody 50-fold in Diluent 3.
- ❑ Prepare 2X Read Buffer T by diluting 4X Read Buffer T 2-fold with deionized water.

STEP 1: Wash* and Add Sample

- ❑ Wash 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 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 16. *Catalog numbers for V-PLEX and V-PLEX Plus proinflammatory (human) multiplex and single assay kits*

Kit Name	V-PLEX			V-PLEX Plus*		
	1-Plate Kit	5-Plate kit	25-Plate Kit	1-Plate Kit	5-Plate Kit	25-Plate Kit
Multiplex Kits						
Proinflammatory Panel 1 (human)	K15049D-1	K15049D-2	K15049D-4	K15049G-1	K15049G-2	K15049G-4
Human Proinflammatory Panel I (4-Plex)	K15052D-1	K15052D-2	K15052D-4	K15052G-1	K15052G-2	K15052G-4
Human Proinflammatory Panel II (4-Plex)	K15053D-1	K15053D-2	K15053D-4	K15053G-1	K15053G-2	K15053G-4
Individual Assay Kits						
Human IFN-γ	K151QOD-1	K151QOD-2	K151QOD-4	K151QOG-1	K151QOG-2	K151QOG-4
Human IL-1β	K151QPD-1	K151QPD-2	K151QPD-4	K151QPG-1	K151QPG-2	K151QPG-4
Human IL-2	K151QQD-1	K151QQD-2	K151QQD-4	K151QQG-1	K151QQG-2	K151QQG-4
Human IL-4	K151QRD-1	K151QRD-2	K151QRD-4	K151QRG-1	K151QRG-2	K151QRG-4
Human IL-6	K151QXD-1	K151QXD-2	K151QXD-4	K151QXG-1	K151QXG-2	K151QXG-4
Human IL-8	K151RAD-1	K151RAD-2	K151RAD-4	K151RAG-1	K151RAG-2	K151RAG-4
Human IL-10	K151QUD-1	K151QUD-2	K151QUD-4	K151QUG-1	K151QUG-2	K151QUG-4
Human IL-12p70	K151QVD-1	K151QVD-2	K151QVD-4	K151QVG-1	K151QVG-2	K151QVG-4
Human IL-13	K1510DD-1	K1510DD-2	K1510DD-4	K1510DG-1	K1510DG-2	K1510DG-4
Human TNF-α	K151QWD-1	K151QWD-2	K151QWD-4	K151QWG-1	K151QWG-2	K151QWG-4

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

Plate Diagram