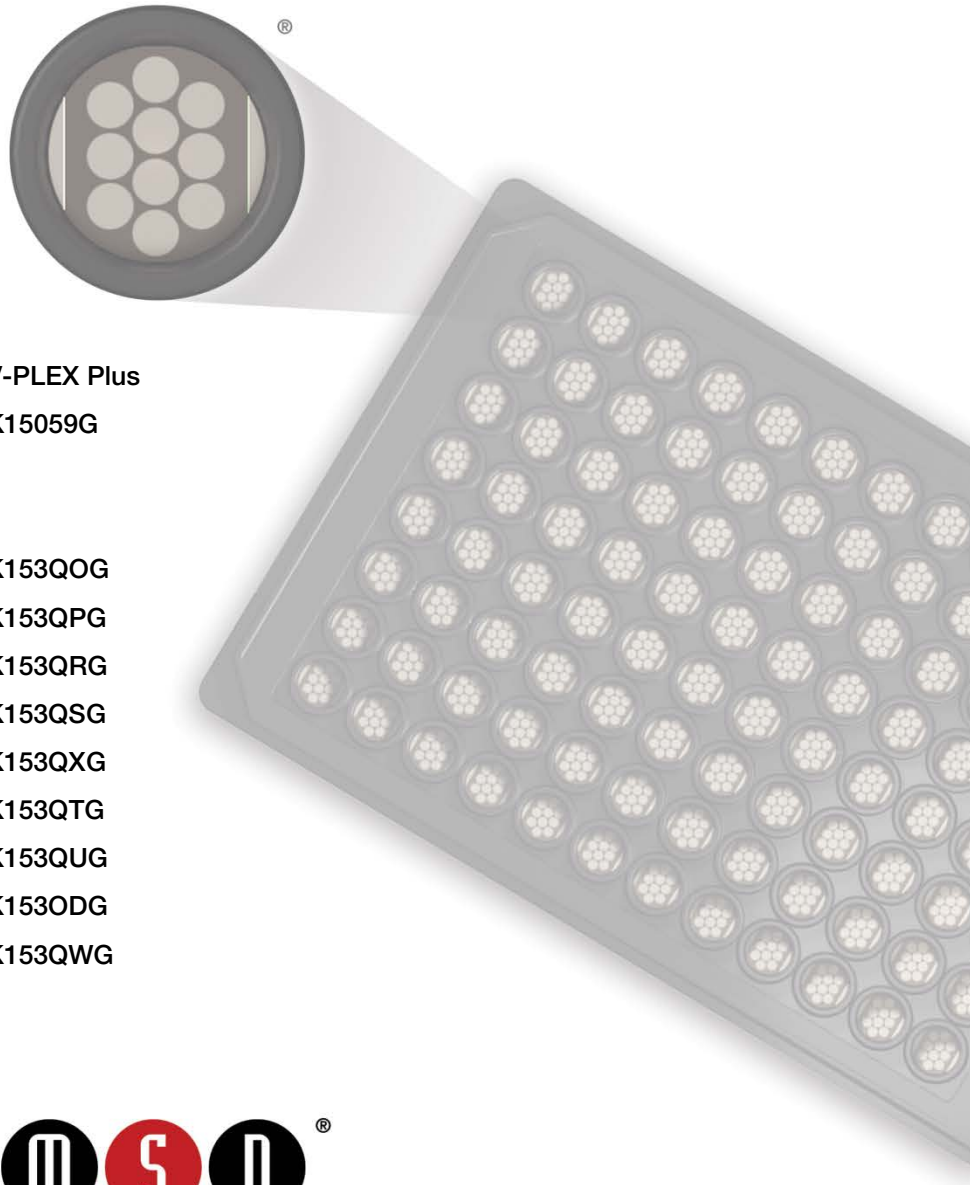
MSD® MULTI-SPOT Assay System

Proinflammatory Panel 2 (rat) Kits

IFN-γ, IL-1β, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, TNF-α

V-PLEX®



	V-PLEX®	V-PLEX Plus
Multiplex Kits	K15059D	K15059G
Individual Assay Kits		
Rat IFN-γ	K153QOD	K153QOG
Rat IL-1β	K153QPD	K153QPG
Rat IL-4	K153QRD	K153QRG
Rat IL-5	K153QSD	K153QSG
Rat IL-6	K153QXD	K153QXG
Rat KC/GRO	K153QTD	K153QTG
Rat IL-10	K153QUD	K153QUG
Rat IL-13	K153ODD	K153ODG
Rat TNF-α	K153QWD	K153QWG

MSD®

MSD Cytokine Assays

Proinflammatory Panel 2 (rat) Kits

IFN-γ, IL-1β, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, TNF-α

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

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

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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Table of Contents

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Introduction

MSD offers V-PLEX assays for customers who require unsurpassed performance and quality. V-PLEX products are developed under rigorous design control and are fully validated according to fit-for-purpose principles[35] 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 10-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 2 (rat) measures nine cytokines that are important in inflammation, immune system regulation and numerous other biological processes. These assays can detect secreted biomarkers in a variety of tissues and body fluids where over- or under-expression may indicate a shift in biological equilibrium. This panel also includes assays for many of the Th1/Th2 pathway biomarkers. The Proinflammatory Panel 2 (rat) measures biomarkers that are implicated in a number of disorders including rheumatoid arthritis,[1] Alzheimer's disease,[2] asthma,[3] atherosclerosis,[4] allergy,[5] systemic lupus erythematosus,[6] obesity,[7] cancer,[8] depression,[9] multiple sclerosis,[10] diabetes,[11] psoriasis[12] and Crohn's disease.[13] Because of their association with such a wide spectrum of disease these biomarkers are the focus of drug discovery efforts, diagnostics development, and basic research. The biomarkers constituting the panel are described below.

Rat interferon gamma (IFN-γ) is a glycosylated 17.9 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]

Rat interleukin-1beta (IL-1β), also known as IL-1F2, is a 17 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. It 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]

Rat interleukin-4 (IL-4), also known as B-cell stimulatory factor 1 (BSF-1) and lymphocyte stimulatory factor 1, is a glycosylated 16.2 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.[19] IL-4 decreases the production of Th1 cells, macrophages, IFN-γ, and IL-12. It is associated with severe asthma[20] among other disorders.

Rat interleukin-5 (IL-5), also known as B-cell growth factor II (BCGF-II) and T-cell replacing factor (TRF), is a glycosylated homodimer with two disulfide bonds and a monomeric molecular weight around 15.2 kDa. It is mainly produced by eosinophils and Th2 cells, and its primary function is to induce terminal differentiation of late-developing B-cells into immunoglobulin-secreting cells. The IL-5 receptor consists of α and βc subunits with IL-5 initially binding to the α subunit with low affinity and then associating with the βc subunit homodimer to yield a high-affinity interaction. IL-5 is associated with eosinophilia[21] and other disorders.

Rat interleukin-6 (IL-6) is a 24.4 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[22] 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,[23] pulmonary fibrosis,[24] liver cirrhosis,[25] ischemia,[26] and berylliosis[27] among other disorders.

Rat KC/GRO - also known as CXCL1, GRO-α, neutrophil-activating protein 3 (NAP-3), and melanoma growth stimulating activity alpha (MGSA-α) - is a 10.2 kDa CXC chemokine. KC/GRO is produced by fibroblasts induced by platelet-derived growth factor and expressed in macrophages and endothelial cells. It produces a biological signal by binding to its receptor, CXCR2. During inflammation, it is involved in neutrophil activation and shows hematopoietic activity. Upon secretion from bone marrow stromal cells through proteolytic cleavage, the N-terminal processed form of KC (5-72) shows highly enhanced hematopoietic activity. This chemokine and its receptor are responsible for neutrophil chemotaxis in epidemic keratoconjunctivitis.[28]

Rat interleukin-10 (IL-10), also known as cytokine synthesis inhibitory factor (CSIF), is a 20.4 kDa, glycosylated homodimeric cytokine with two disulfide bonds. 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.[29]

Rat interleukin-13 (IL-13) is a 14.1 kDa glycosylated immunoregulatory cytokine with two intra-molecular disulfide bonds forming a bundled four α-helix configuration. It is secreted by a variety of immune cells. IL-13 is involved in a number of biological processes, including 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 intracellularly as a soluble protein as well as on the cell surface. It is involved in a number of disorders including allergic rhinitis,[30] inflammatory bowel disease, and colorectal cancer.[31]

Rat tumor necrosis factor alpha (TNF-α), also known as tumor necrosis factor ligand superfamily member 2 (TNFSF2) and cachectin, is a 25.8 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 produced by many cell types including 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[32] and inflammation and can inhibit tumorigenesis[33] and viral replication.[34]

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 2 (rat) are sandwich immunoassays. MSD provides a plate pre-coated with capture antibodies on independent and well-defined spots, as shown in the layouts below. Multiplex assays are provided on 10-spot 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 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.[35]

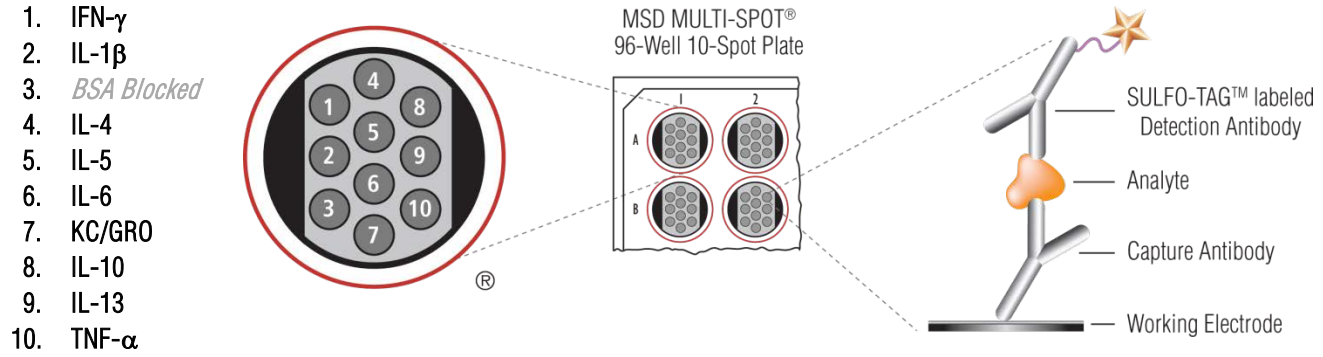

Figure 1. Multiplex plate spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

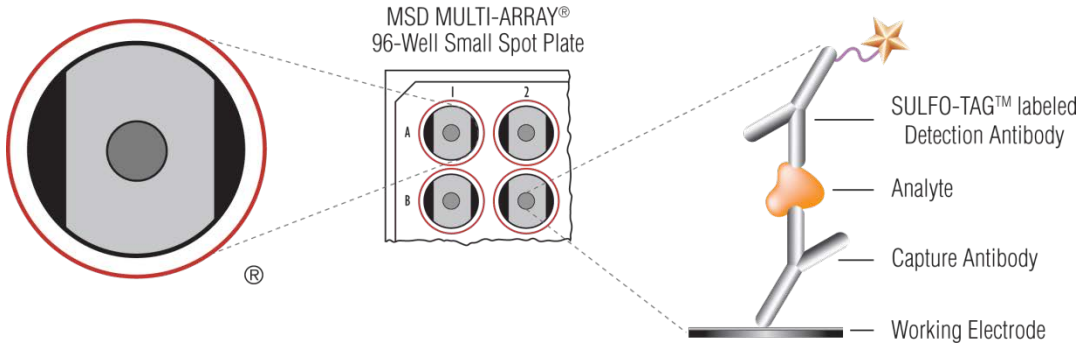

Figure 2. Small Spot plate diagram showing placement of analyte capture antibodies.

Kit Components

Proinflammatory Panel 2 (rat) assays are available as a 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 (rat) Calibrator Blend	2–8°C	C0044-2	1 vial	1 vial	5 vials	25 vials	Nine recombinant rat proteins in diluent, buffered and lyophilized. Individual analyte concentration is provided in the lot-specific certificate of analysis (COA).
Blocker H	RT	R93BI-1	20 mL	1 bottle			Reagent required to block coated plates prior to adding calibrator, controls, or samples.
		R93BI-2	100 mL		1 bottle	5 bottles	
Diluent 42	≤-10°C	R50AK-1	10 mL	1 bottle			Diluent for samples and calibrator; contains serum, blockers, and preservatives.
		R50AK-2	50 mL		1 bottle	5 bottles	
Diluent 40	≤-10°C	R50AJ-1	5 mL	1 bottle			Diluent for detection antibody; contains protein, blockers, and preservatives.
		R50AJ-2	25 mL		1 bottle	5 bottles	
Read Buffer T (4X)	RT	R92TC-3	50 mL	1 bottle	1 bottle	5 bottles	Buffer to catalyze the electro-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 (rat) Control 1*	2–8°C	C4044-1	1 vial	1 vial	5 vials	25 vials	Multi-analyte controls in rat EDTA plasma, buffered, lyophilized, and spiked with recombinant rat analytes. The concentration of the controls is provided in the lot-specific COA.
Proinflammatory Panel 1 (rat) Control 2*	2–8°C	C4044-1	1 vial	1 vial	5 vials	25 vials	
Proinflammatory Panel 1 (rat) Control 3*	2–8°C	C4044-1	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 (rat) 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 2 (rat) Plate	2–8°C	N05059A-1	10-spot	1	5	25	96-well plate, foil sealed, with desiccant.
Rat IFN-γ Plate	2–8°C	L453QOA-1	Small Spot	1	5	25	
Rat IL-1β Plate	2–8°C	L453QPA-1	Small Spot	1	5	25	
Rat IL-4 Plate	2–8°C	L453QRA-1	Small Spot	1	5	25	
Rat IL-5 Plate	2–8°C	L453QSA-1	Small Spot	1	5	25	
Rat IL-6 Plate	2–8°C	L453QXA-1	Small Spot	1	5	25	
Rat KC/GRO Plate	2–8°C	L453QTA-1	Small Spot	1	5	25	
Rat IL-10 Plate	2–8°C	L453QUA-1	Small Spot	1	5	25	
Rat IL-13 Plate	2–8°C	L453ODA-1	Small Spot	1	5	25	
Rat TNF-α Plate	2–8°C	L453QWA-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-rat IFN-γ Antibody (50X)	2–8°C	D23QO-2	75 µL	1			SULFO-TAG–conjugated antibody
		D23QO-3	375 µL		1	5	
Anti-rat IL-1β Antibody (50X)	2–8°C	D23QP-2	75 µL	1			SULFO-TAG–conjugated antibody
		D23QP-3	375 µL		1	5	
Anti-rat IL-4 Antibody (50X)	2–8°C	D23QR-2	75 µL	1			SULFO-TAG–conjugated antibody.
		D23QR-3	375 µL		1	5	
Anti-rat IL-5 Antibody (50X)	2–8°C	D23QS-2	75 µL	1			SULFO-TAG–conjugated antibody.
		D23QS-3	375 µL		1	5	
Anti-rat IL-6 Antibody (50X)	2–8°C	D23QX-2	75 µL	1			SULFO-TAG–conjugated antibody.
		D23QX-3	375 µL		1	5	
Anti-rat KC/GRO Antibody (50X)	2–8°C	D23QT-2	75 µL	1			SULFO-TAG–conjugated antibody.
		D23QT-3	375 µL		1	5	
Anti-rat IL-10 Antibody (50X)	2–8°C	D23QU-2	75 µL	1			SULFO-TAG–conjugated antibody.
		D23QU-3	375 µL		1	5	
Anti-rat IL-13 Antibody (50X)	2–8°C	D23OD-2	75 µL	1			SULFO-TAG–conjugated antibody.
		D23OD-3	375 µL		1	5	
Anti-rat TNF-α Antibody (50X)	2–8°C	D23QW-2	75 µL	1			SULFO-TAG–conjugated antibody.
		D23QW-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

Optional Materials and Equipment

- ❑ Proinflammatory Panel 1 (rat) Control Pack, available for separate purchase from MSD, catalog # C4044-1 (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.
- Shaking should be vigorous with a rotary motion between 500 and 1,000 rpm. Binding reactions may reach equilibrium sooner if you use shaking at the middle of this range (~700 rpm) or above.
- When using an automated plate washer, rotate the plate 180 degrees between wash steps to improve assay precision.
- Gently tap the plate on a paper towel to remove residual fluid after washing.
- If an incubation step needs to be extended, avoid letting the plate dry out by keeping sample or detection antibody solution in the plate.
- Remove plate seals prior to reading the plate.
- Make sure that Read Buffer T is at room temperature when added to a plate.
- Do not shake the plate after adding Read Buffer T.
- To improve inter-plate precision, keep time intervals consistent between adding Read Buffer T and reading the plate. Unless otherwise directed, read the plate as soon as possible after adding Read Buffer T.
- If assay results are above the top of the calibration curve, dilute samples and repeat the assay.
- We do not recommend attempting to use a partial plate when running this panel.

Reagent Preparation

Bring all reagents to room temperature.

Important: Upon first thaw, aliquot Diluent 42 and Diluent 40 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 42. (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 42 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 42 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 42. Mix well by vortexing. Repeat 4-fold serial dilutions 5 additional times to generate 7 calibrators.
3) Use Diluent 42 as the zero calibrator.

Note: Reconstituted calibrator (Calibrator 1) is not stable when stored at 2-8°C; however, the material may be frozen at ≤-70°C. It 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.

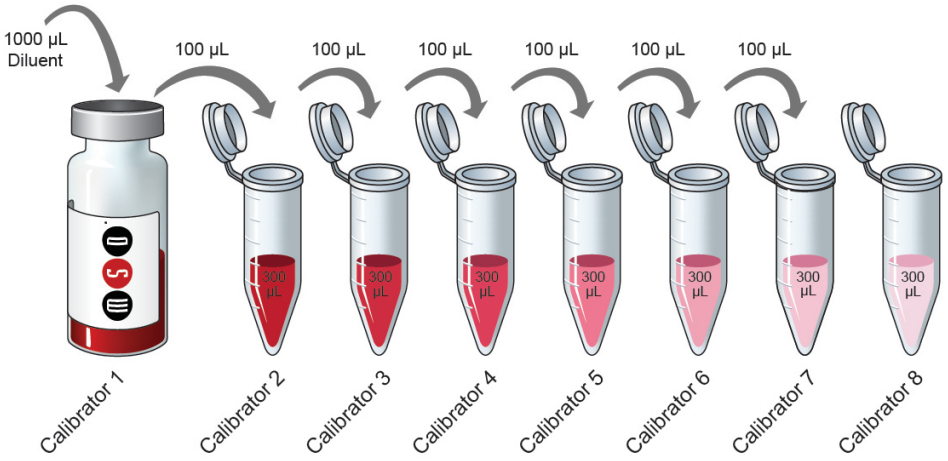

***Figure 3**. Dilution schema for preparation of Calibrator Standards*

Sample Collection and Handling

Below are general guidelines for rat sample collection, storage, and handling. If possible, use published guidelines.[36,37] 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 ≤-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 42. For rat serum, plasma, and urine samples, MSD recommends a minimum 4-fold dilution. For example, when running samples in duplicate, add 50 µL of sample to 150 µL of Diluent 42. We recommend running at least two replicates per sample. When running unreplicated samples use 25 µL of sample to 75 µL of Diluent 42. 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 2 (rat) Control Pack, catalog # C4044-1. (Controls are included only in V-PLEX Plus kits.)

Reconstitute the lyophilized controls in 250 µL of Diluent 42. Do not invert or vortex the vials. Wait for a minimum of 15-30 minutes before diluting controls 4-fold in Diluent 42. Vortex briefly using short pulses. Refer to the Proinflammatory Panel 1 (rat) Control Pack product insert for analyte levels. Controls are a one-time use product and are not stable when frozen or stored at 2-8°C.

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.

9-plex Proinflammatory Panel 2 (rat) kit

For one plate, combine the following detection antibodies and add to 2,460 µL of Diluent 40:

- ❑ 60 µL of SULFO-TAG Anti-rat IFN-γ Antibody
- ❑ 60 µL of SULFO-TAG Anti-rat IL-1β Antibody
- ❑ 60 µL of SULFO-TAG Anti-rat IL-4 Antibody
- ❑ 60 µL of SULFO-TAG Anti-rat IL-5 Antibody
- ❑ 60 µL of SULFO-TAG Anti-rat IL-6 Antibody
- ❑ 60 µL of SULFO-TAG Anti-rat KC/GRO Antibody
- ❑ 60 µL of SULFO-TAG Anti-rat IL-10 Antibody
- ❑ 60 µL of SULFO-TAG Anti-rat IL-13 Antibody
- ❑ 60 µL of SULFO-TAG Anti-rat TNF-α Antibody

Custom multiplex kits

For one plate, combine 60 µL of each supplied detection antibody, then add Diluent 40 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 40.

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

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. We do not recommend attempting to use a partial plate when running this panel.

Assay Protocol

Note: Follow **Reagent Preparation** before beginning this assay protocol**.** We do not recommend attempting to use a partial plate when running this panel.

STEP 1: Add Blocker H

- ❑ Add 150 µL of Blocker H per well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 1 hour.

STEP 2: 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.

STEP 3: 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 4: 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 2-8ºC 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 reducing the number of 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 4-fold dilution, add 37.5 µL of assay diluent to each sample/control well, and then add 12.5 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[35] 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.

- **Development**

  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 assay-specific 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 2 (rat) were collected over two months of testing by three operators (42 runs in total). Calibration curve accuracy and precision were assessed for three kit lots. Representative data from one lot are presented below. Data from individual assays are presented in **Appendix B**. The multiplex panel was tested with individual detection antibodies to demonstrate that the assays are independent of one another. **Appendix C** compares results for each assay in the kit when the panel is run using the individual detection antibody versus all nine detection antibodies. The calibration curves were comparable. Calibration curves for each lot are presented in the lot-specific COA.

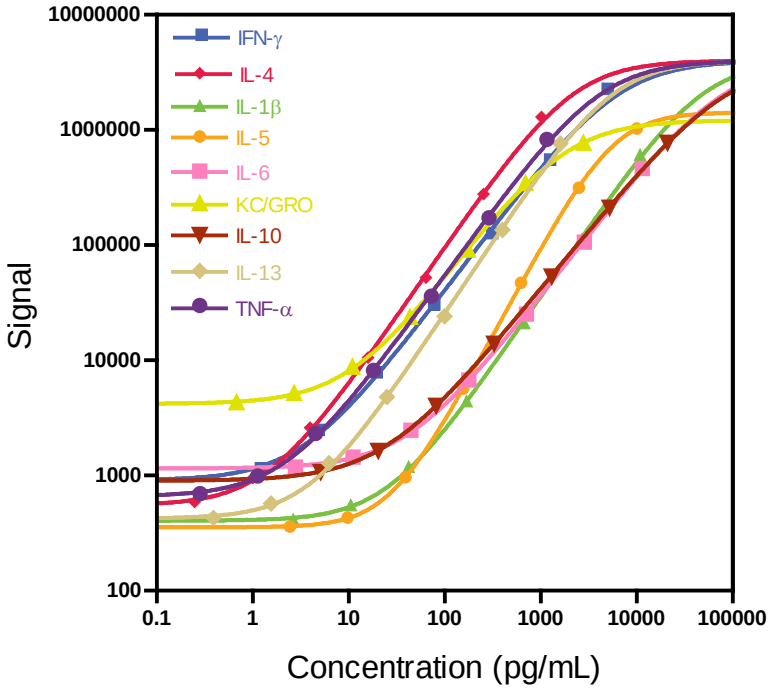

Figure 4. *Typical calibration curves for the Proinflammatory Panel 1 (rat) assay*

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 42 runs.

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

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

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

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

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

	Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
IFN-γ	0.65	0.14–3.50	39.7	3,750
IL-1β	6.92	3.40–27.6	102	8,100
IL-4	0.69	0.20–1.28	8.00	723
IL-5	14.1	5.73–63.0	82.0	3,000
IL-6	13.8	0.67–37.9	96.9	8,550
KC/GRO	1.04	0.26–2.86	21.7	728
IL-10	16.4	1.53–70.6	163	15,700
IL-13	1.97	0.95–21.7	12.5	1,080
TNF-α	0.72	0.26–2.04	9.10	793

Precision

Controls were made by spiking calibrator into rat EDTA plasma at two levels within the quantitative range of the assay. Analyte levels were measured by five operators using a minimum of two replicates on 41 runs over four months. Results are shown below. While a typical specification for precision is a concentration CV of less than 25% for controls on both intra- and inter-day runs, for this panel, the data shows most assays are below 15%.

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

Inter-run %CV is the variability of controls across 41 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 (rat) Kit

	Control	Average Conc. (pg/mL)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
IFN-γ	Control 1	867	5.0	14.8	5.1
	Control 2	200	5.2	17.2	6.1
IL-1β	Control 1	3,642	2.7	13.6	9.2
	Control 2	959	2.7	18.6	16.4
IL-4	Control 1	779	2.8	10.9	8.1
	Control 2	166	2.6	13.7	4.5
IL-5	Control 1	6,531	3.7	9.9	6.7
	Control 2	1,257	3.8	12.7	9.5
IL-6	Control 1	7,187	2.6	10.7	4.5
	Control 2	2,195	3.2	12.9	4.5
KC/GRO	Control 1	1,995	3.2	6.5	4.8
	Control 2	691	2.0	7.9	4.1
IL-10	Control 1	15,214	3.6	10.7	4.9
	Control 2	3,893	3.6	11.5	3.4
IL-13	Control 1	966	2.9	10.5	5.5
	Control 2	122	3.0	14.6	2.2
TNF-α	Control 1	553	2.4	11.6	4.9
	Control 2	108	2.3	15.9	4.5

Dilution Linearity

To assess linearity, normal rat 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, 4-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-4		IL-5	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=6)	2	30	17–70	143	131–153	40	33–51	101	98–104
	4	100	–	100	–	100	–	100	–
	8	121	111–130	95	92–98	144	102–187	103	99–108
	16	132	118–155	102	99–105	152	100–197	104	99–109
	32	137	124–154	114	110–117	150	101–193	107	103–114
	64	153	132–173	135	128–140	160	103–212	107	95–118
EDTA Plasma (N=6)	2	70	66–75	111	105–120	108	105–111	111	93–124
	4	100	–	100	–	100	–	100	–
	8	122	108–126	122	111–135	84	69–91	100	95–114
	16	129	118–140	143	128–158	78	69–85	106	94–122
	32	137	119–156	161	138–180	77	66–84	104	93–128
	64	151	133–178	178	156–205	83	75–88	106	89–140
Heparin Plasma (N=6)	2	17	10–27	143	114–173	48	39–71	90	33–106
	4	100	–	100	–	100	–	100	–
	8	266	137–739	103	94–107	229	181–291	101	94–106
	16	323	142–1,010	113	100–120	331	237–621	106	96–114
	32	337	149–1,051	124	111–134	343	245–723	102	92–108
	64	363	168–1,114	141	129–149	383	262–816	109	97–122
Citrate Plasma (N=8)	2	49	29–73	172	157–206	73	66–80	107	103–110
	4	100	–	100	–	100	–	100	–
	8	143	113–176	85	82–91	170	115–224	106	103–113
	16	167	134–204	82	74–88	229	122–343	112	103–117
	32	177	141–227	91	79–102	233	120–360	116	110–122
	64	187	146–238	101	88–112	250	130–374	124	116–129
Urine (N=6)	2	99	88–107	57	37–78	73	57–89	62	52–77
	4	100	–	100	–	100	–	100	–
	8	94	89–97	139	93–197	117	99–133	128	107–149
	16	94	86–102	161	79–261	130	91–161	159	117–200
	32	92	81–103	150	67–268	129	87–172	154	110–204
	64	98	90–107	142	63–249	138	95–178	159	112–209
Cell Culture Supernatant (N=4)	2	111	106–119	173	162–194	104	102–108	147	136–156
	4	100	–	100	–	100	–	100	–
	8	95	91–109	72	68–77	96	95–102	97	87–106
	16	97	93–110	54	50–59	89	82–95	92	90–98
	32	94	87–106	53	47–58	87	85–97	92	88–104
	64	98	94–117	54	49–62	88	85–102	94	88–103

Table 7 continued

		IL-6		KC/GRO		IL-10		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=6)	2	79	52–105	91	70–100	28	17–41	76	31–115	81	19–100
	4	100	–	100	–	100	–	100	–	100	–
	8	104	100–109	95	93–100	259	135–361	106	98–122	111	101–137
	16	102	93–110	92	88–101	319	137–465	108	96–132	113	99–155
	32	109	100–114	91	86–97	335	148–497	123	116–144	129	114–183
	64	110	102–116	93	86–101	340	143–509	137	120–156	136	120–192
EDTA Plasma (N=6)	2	115	113–118	103	98–110	109	103–114	113	110–116	94	91–98
	4	100	–	100	–	100	–	100	–	100	–
	8	95	89–100	93	90–98	86	60–95	98	95–103	105	100–114
	16	96	86–103	89	83–95	82	71–92	100	89–109	106	94–117
	32	98	83–110	88	81–97	92	87–96	109	94–116	112	95–123
	64	104	91–122	90	84–95	93	83–100	119	109–128	116	105–129
Heparin Plasma (N=6)	2	27	15–48	64	26–80	42	35–55	43	35–59	48	10–71
	4	100	–	100	–	100	–	100	–	100	–
	8	146	104–275	105	91–127	370	274–434	134	105–232	116	102–138
	16	149	97–281	103	87–131	758	518–1232	142	106–261	121	101–148
	32	153	99–283	103	83–130	917	620–1710	153	117–280	127	111–149
	64	160	115–279	107	90–132	984	672–1931	170	133–305	139	117–165
Citrate Plasma (N=5)	2	85	68–97	86	82–93	57	51–62	90	78–99	88	85–92
	4	100	–	100	–	100	–	100	–	100	–
	8	104	100–112	104	101–108	246	149–331	104	99–115	107	102–110
	16	101	98–105	101	95–106	451	174–683	104	99–117	108	102–110
	32	107	99–113	105	95–113	574	202–995	108	103–117	116	107–121
	64	108	101–115	114	105–124	570	179–984	114	110–123	123	114–128
Urine (N=6)	2	98	87–112	88	80–99	105	96–109	86	75–98	75	63–86
	4	100	–	100	–	100	–	100	–	100	–
	8	98	91–105	99	96–103	99	90–104	100	89–110	117	103–138
	16	98	82–109	102	92–109	94	87–107	104	88–113	137	102–162
	32	91	82–104	101	89–114	92	87–98	103	90–119	148	108–190
	64	91	83–102	106	93–120	88	81–95	110	80–133	159	116–194
Cell Culture Supernatant (N=6)	2	118	110–128	121	101–147	103	100–104	114	109–124	117	113–116
	4	100	–	100	–	100	–	100	–	100	–
	8	93	90–97	92	87–95	103	101–106	93	88–100	96	88–98
	16	89	82–96	88	77–89	101	100–108	87	83–90	90	81–94
	32	90	84–101	88	61–97	100	94–108	89	78–96	92	81–97
	64	91	84–102	94	67–105	104	103–114	95	93–100	95	83–101

Note: Some assays showed significant matrix effects, which can be minimized by higher sample dilution.

Spike Recovery

Spike recovery measurements of different sample types throughout the quantitative range of the assays were evaluated. Multiple individual rat 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 4-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 (rat) Kit

	Citrate Plasma (N=3)			Heparin Plasma (N=5)			EDTA Plasma (N=5)		
	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
IFN-γ	55	42.2	16–85	54	17.6	30–67	57	10.5	49–71
IL-1β	58	23.5	36–72	62	24.5	46–113	56	12.1	48–68
IL-4	46	37.2	16–68	51	30.7	20–69	100	7.7	85–110
IL-5	71	26.7	37–90	83	6.4	75–94	78	18.4	52–96
IL-6	92	25.3	45–111	95	10.5	74–112	106	13.4	84–131
KC/GRO	120	7.8	107–129	85	5.2	77–94	89	5.2	83–98
IL-10	22	55.8	6–37	24	45.9	8–54	97	14.8	78–133
IL-13	73	33.7	26–96	74	20.5	42–89	76	22.3	51–102
TNF-α	74	18.3	50–85	78	7.9	66–86	79	9.2	64–91

	Serum (N=5)			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	61	12.6	50–81	87	15.0	65–121	113	6.4	103–122
IL-1β	43	9.6	36–50	200	21.4	137–274	153	16.6	123–185
IL-4	85	13.2	69–104	87	13.0	62–100	127	8.6	111–133
IL-5	84	12.6	68–101	86	21.1	45–103	179	13.0	149–195
IL-6	109	9.1	92–126	132	16.9	111–207	124	6.6	110–131
KC/GRO	94	6.0	85–105	85	10.9	67–97	128	9.9	109–143
IL-10	54	21.7	39–78	99	15.1	87–148	138	14.2	107–157
IL-13	77	23.8	46–107	107	14.2	84–154	128	9.7	107–140
TNF-α	76	16.4	48–94	79	13.4	55–92	124	9.1	107–131

Specificity

To assess specificity, each assay in the panel was tested individually. Nonspecific binding was less than 0.5% 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 after the first thaw, Diluent 42, and Diluent 40 can go through four freeze–thaw cycles without significantly affecting the performance of the assay. Once reconstituted, the multi-analyte calibrator may be frozen and thawed three times. Controls are not freeze–thaw stable. 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. The plates cannot be stored after removing from the pouch, hence we do not recommend testing partial plates when running this panel.

Calibration

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

Tested Samples

Normal Samples

Normal rat serum, EDTA plasma, heparin plasma, citrate plasma, and urine samples from a commercial source were diluted 4-fold and tested. Results for each sample set are displayed in Table 10. 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 rat samples tested in the Proinflammatory Panel 1 (rat) Kit

Sample Type	Statistic	IFN-γ	IL-1β	IL-4	IL-5	IL-6	KC/GRO	IL-10	IL-13	TNF-α
Serum (N=18)	Median (pg/mL)	6.72	ND	ND	75.2	51.5	387	118	25.6	14.1
	Range (pg/mL)	2.20–10.8	ND	ND	–	33.4–55.6	44.9–778	67.8–291	11.7–28.5	3.60–22.4
	% Detected	50	0	0	6	28	100	83	33	89
EDTA Plasma (N=16)	Median (pg/mL)	15.3	ND	12.7	124	148	41.9	170	19.4	19.6
	Range (pg/mL)	–	ND	–	–	55.9–285	5.69–761	–	–	8.51–179
	% Detected	6	0	6	6	50	94	6	6	81
Heparin Plasma (N=16)	Median (pg/mL)	13.4	ND	13.3	67.7	84.3	156	165	18.6	14.0
	Range (pg/mL)	3.77–21.0	ND	10.8–18.9	–	26.6–170	12.2–325	114–298	11.8–43.9	5.39–31.7
	% Detected	88	0	100	6	69	100	100	94	100
Citrate Plasma (N=10)	Median (pg/mL)	10.8	395	15.2	58.3	72.9	44.0	290	24.5	30.6
	Range (pg/mL)	3.67–20.0	–	3.22–20.8	–	24.8–422	16.2–327	100–340	11.0–43.6	6.84–3,070
	% Detected	50	10	80	10	70	100	100	90	90
Urine (N=11)	Median (pg/mL)	2.47	ND	12.1	58.3	165	22.8	188	4.80	7.18
	Range (pg/mL)	1.80–11.2	ND	–	–	–	4.30–58.6	–	4.33–13.8	1.65–196
	% Detected	27	0	9	9	9	73	9	27	64

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

Stimulated Samples

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

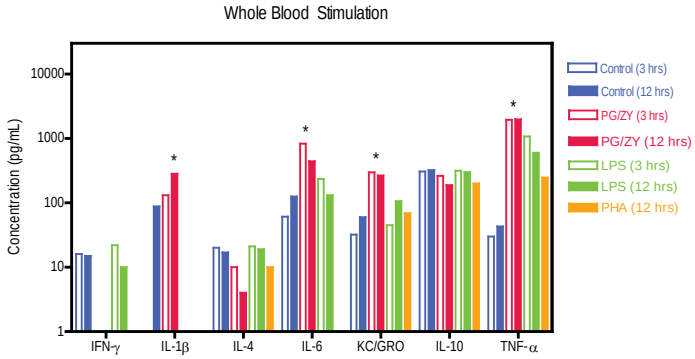

Figure 6. *Normal rat whole blood stimulated with LPS, PHA, PG and ZY.*

A rat alveolar macrophage cell line (NR8383) was stimulated for 4 hours with 5 µg/mL of each LPS and pokeweed mitogen (PWM). The lysates were then collected and tested. The concentrations were normalized for 50 µg of lysate per well. IL-1β, IL-6, KC/GRO and TNF-α show significant stimulation with both LPS and PWM.

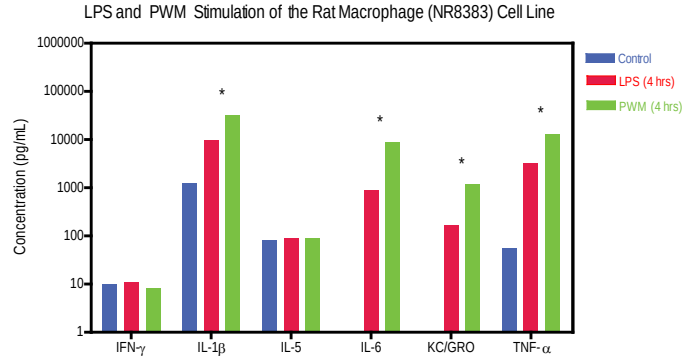

Figure 7. *NR8383 rat macrophage cell line stimulated with LPS or PWM.*

Assay Components

Calibrators

The assay calibrator blend uses the following recombinant rat proteins:

Table 11. Recombinant rat proteins used in the Calibrators

Calibrator	Expression System
IFN-γ	*E. coli*
IL-1β	*E. coli*
IL-4	*E. coli*
IL-5	*E. coli*
IL-6	Sf21 insect cells
KC/GRO	*E. coli*
IL-10	*E. coli*
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	Goat Polyclonal	A
IL-1β	Mouse Monoclonal	Goat Polyclonal	A
IL-4	Mouse Monoclonal	Goat Polyclonal	A
IL-5	Rat Monoclonal	Rat Monoclonal	A
IL-6	Mouse Monoclonal	Goat Polyclonal	A
KC/GRO	Rabbit Polyclonal	Goat Polyclonal	A
IL-10	Mouse Monoclonal	Goat Polyclonal	A
IL-13	Mouse Monoclonal	Goat Polyclonal	B
TNF-α	Hamster Monoclonal	Goat Polyclonal	A

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Appendix A

Calibration curves below illustrate the relative sensitivity of each assay under **Alternate Protocols**: Reference Protocol (2-hour sample incubation/3 wash steps, blue curve), Alternate Protocol 1 (overnight sample incubation, red curve), and Alternate Protocol 2 (tissue culture: reduced wash, green curve).

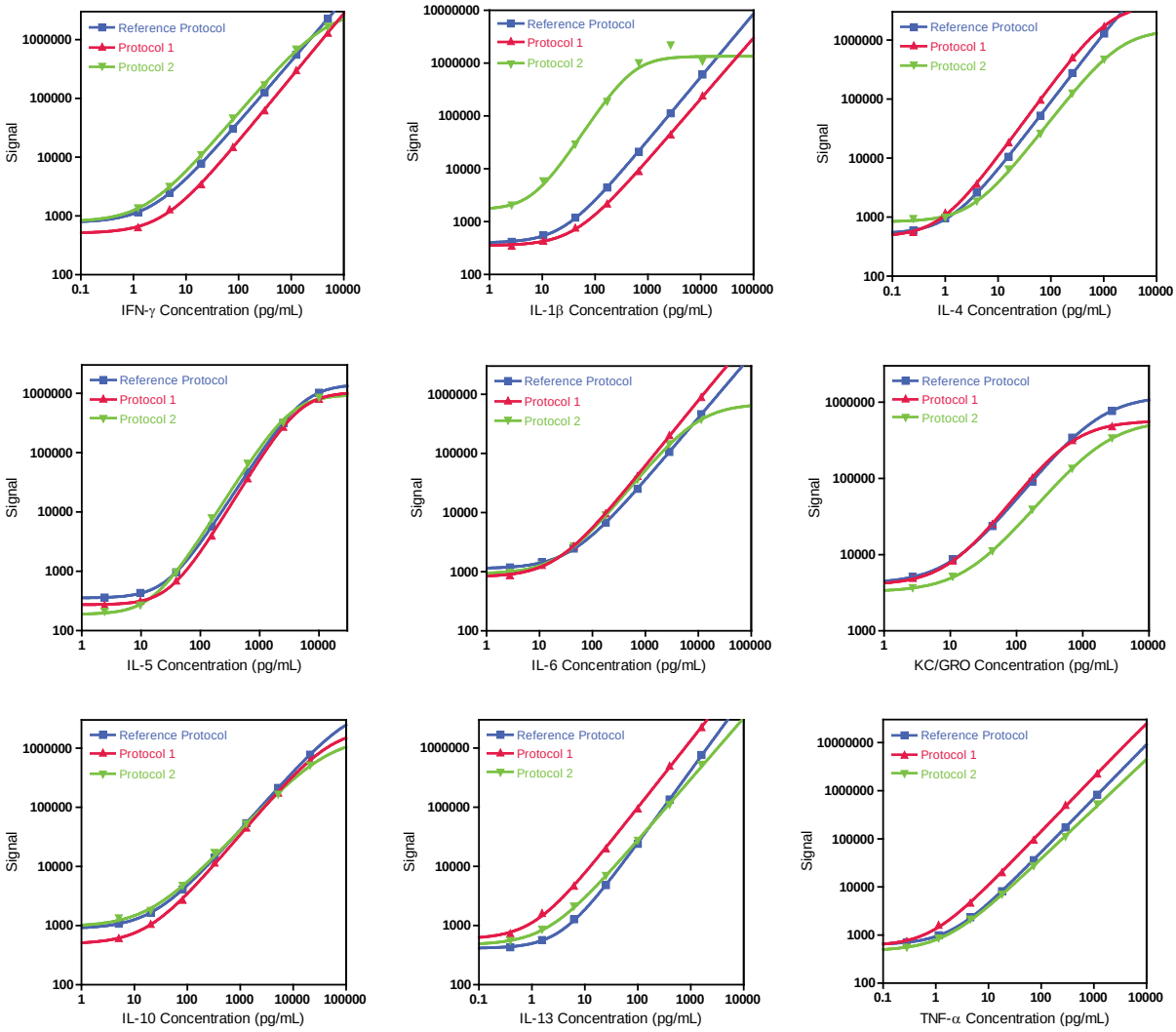

Table 13. Relative sensitivity when using alternate protocols

	LLOD Comparison (pg/mL)		
	Reference Protocol	Protocol 1	Protocol 2
IFN-γ	0.65	0.66	0.18
Il-1β	6.92	15.4	0.83
IL-4	0.69	0.20	0.50
IL-5	14.1	16.3	12.8
IL-6	13.8	2.33	2.48
KC/GRO	1.04	4.12	2.25
IL-10	16.4	8.81	2.09
IL-13	1.97	0.99	0.81
TNF-α	0.72	0.12	0.22

Appendix B

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

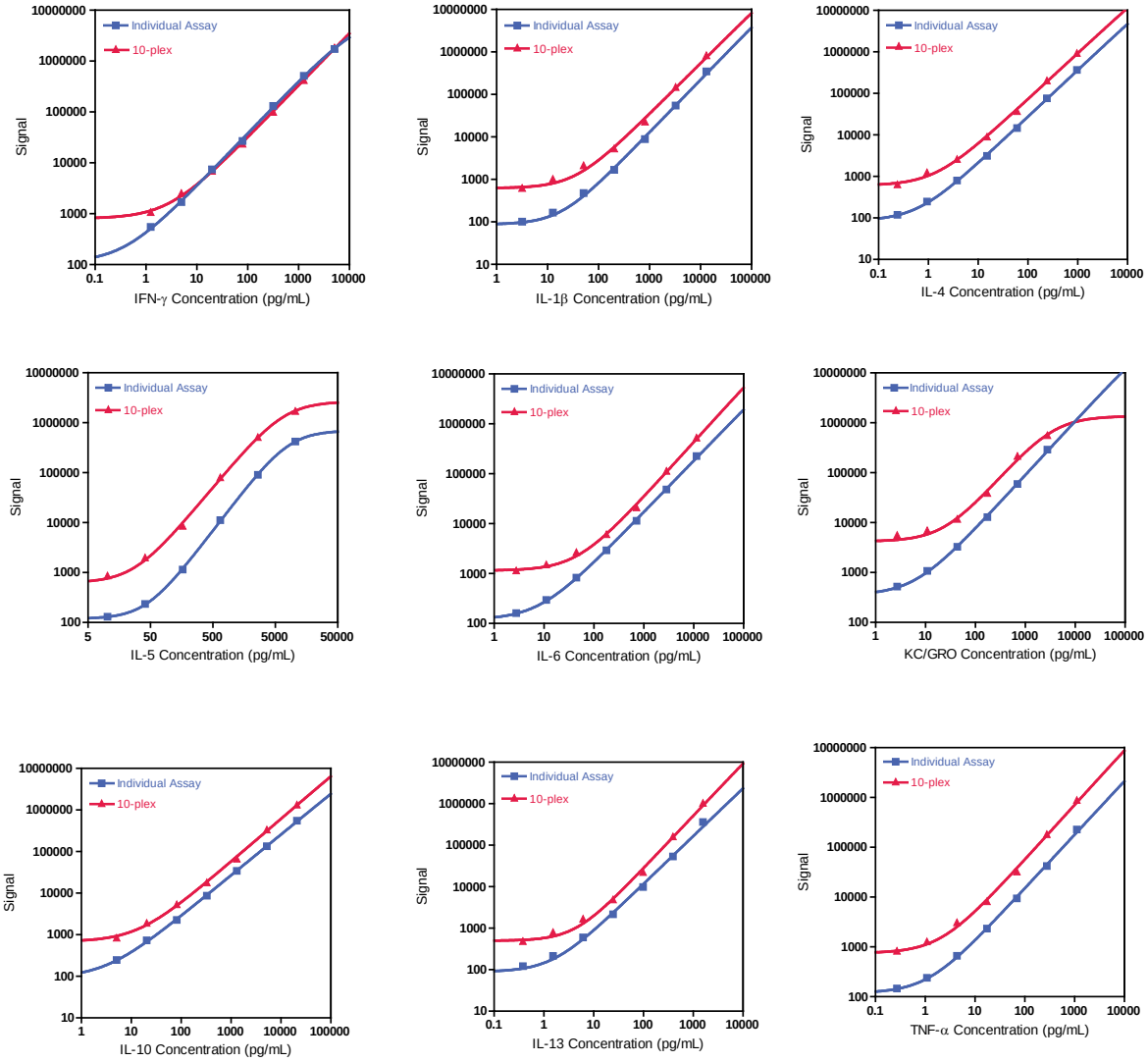

Table 14. Assay performance for individual and 10-plex assays

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

	LLOD (pg/mL)	
Assay	Individual	10-plex
IFN-γ	0.09	0.65
IL-1β	6.58	6.92
IL-4	0.19	0.69
IL-5	18.1	14.1
IL-6	1.83	13.8
KC/GRO	0.82	1.04
IL-10	1.02	16.4
IL-13	0.43	1.97
TNF-α	0.21	0.72

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).

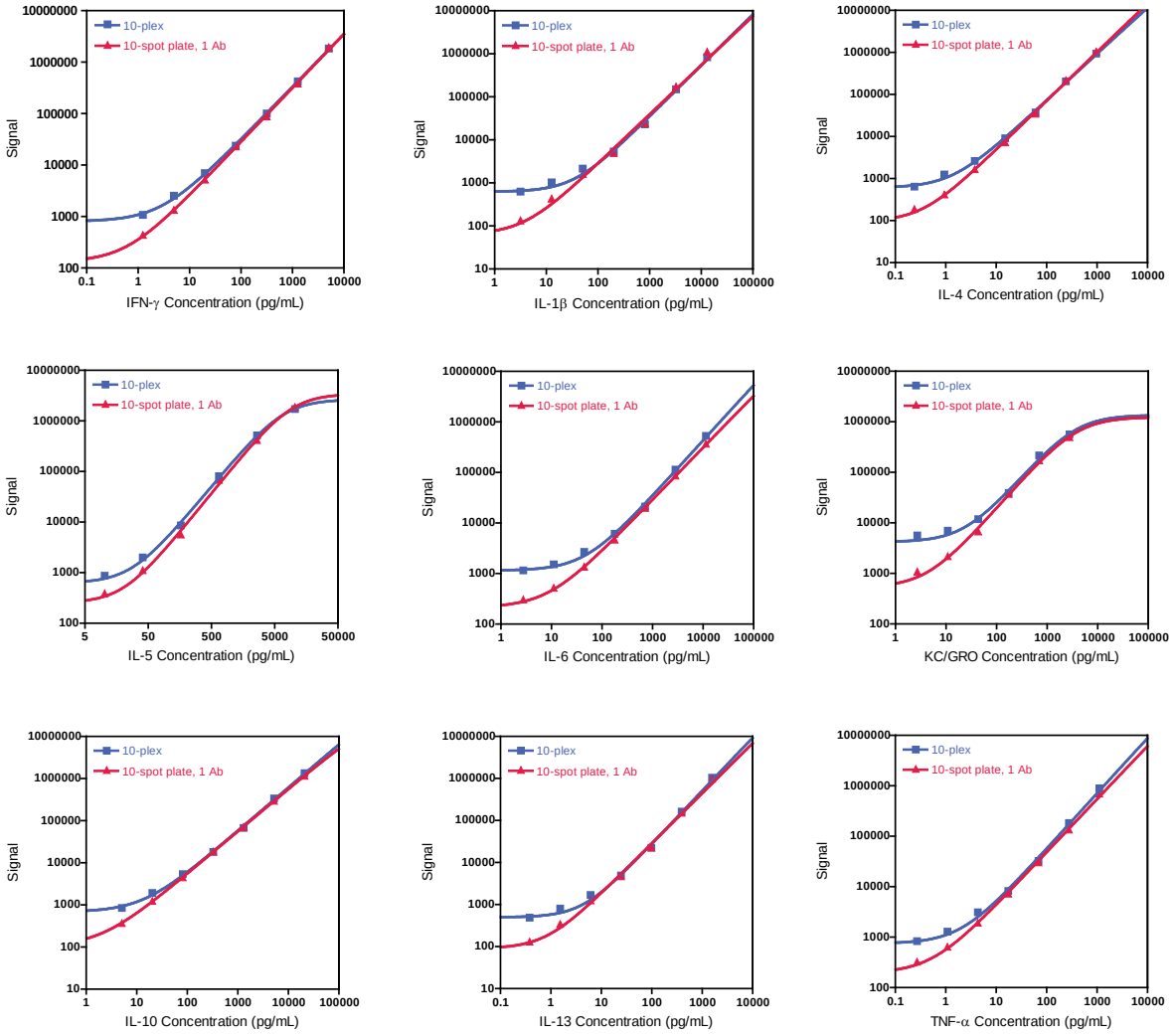

Table 15. LLODs for detection of a single Ab vs. blended Abs

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	0.35	0.65
IL-1β	4.26	6.92
IL-4	0.52	0.69
IL-5	12.8	14.1
IL-6	3.16	13.8
KC/GRO	0.77	1.04
IL-10	1.42	16.4
IL-13	0.96	1.97
TNF-α	0.23	0.72

Summary Protocol

Proinflammatory Panel 2 (rat) Kits

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the proinflammatory panel 2 (rat) assays.

Sample and Reagent Preparation

- ❑ Bring all reagents to room temperature.
- ❑ Prepare calibration solutions in Diluent 42 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 4-fold in Diluent 42 before adding to the plate.
- ❑ Prepare combined detection antibody solution by diluting each 50X detection antibody 50-fold in Diluent 40.
- ❑ Prepare 2X Read Buffer T by diluting 4X Read Buffer T 2-fold with deionized water.

STEP 1: Add Blocker H

- ❑ Add 150 µL/well of Blocker H.
- ❑ Incubate at room temperature with shaking for 1 hour.

STEP 2: 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 3: 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 4: 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.

Catalog Numbers

Table 16. Catalog numbers for V-PLEX and V-PLEX Plus proinflammatory (rat) 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 2 (rat)	K15059D-1	K15059D-2	K15059D-4	K15059G-1	K15059G-2	K15059G-4
Individual Assay Kits						
Rat IFN-γ	K153QOD-1	K153QOD-2	K153QOD-4	K153QOG-1	K153QOG-2	K153QOG-4
Rat IL-1β	K153QPD-1	K153QPD-2	K153QPD-4	K153QPG-1	K153QPG-2	K153QPG-4
Rat IL-4	K153QRD-1	K153QRD-2	K153QRD-4	K153QRG-1	K153QRG-2	K153QRG-4
Rat IL-5	K153QSD-1	K153QSD-2	K153QSD-4	K153QSG-1	K153QSG-2	K153QSG-4
Rat IL-6	K153QXD-1	K153QXD-2	K153QXD-4	K153QXG-1	K153QXG-2	K153QXG-4
Rat KC/GRO	K153QTD-1	K153QTD-2	K153QTD-4	K153QTG-1	K153QTG-2	K153QTG-4
Rat IL-10	K153QUD-1	K153QUD-2	K153QUD-4	K153QUG-1	K153QUG-2	K153QUG-4
Rat IL-13	K153ODD-1	K153ODD-2	K153ODD-4	K153ODG-1	K153ODG-2	K153ODG-4
Rat TNF-α	K153QWD-1	K153QWD-2	K153QWD-4	K153QWG-1	K153QWG-2	K153QWG-4

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

Plate Diagram