MSD® MULTI-SPOT Assay System

Special Order Rat Biomarker Group 1

V-PLEX® assays: IFN-γ, IL-1β, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, TNF-α

Other MSD catalog rat assays: IL-1α, EPO, GM-CSF, MCP-1, VEGF-A

Special Order Kits	5-Plate Kit	25-Plate Kit
Catalog #	K153A4I-2	K153A4I-4

This protocol may be used for combinations of up to 10 of the assays above.

MSD Cytokine Assays

Special Order Rat Biomarker Group 1 Kit

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

Other MSD catalog rat assays: IL-1α, EPO, GM-CSF, MCP-1, VEGF-A

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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Introduction

Special order kits allow customers to combine validated V-PLEX assays in a new configuration or mix V-PLEX assays with selected assays from MSD's standard menu. This allows customers to create unique multiplex assays. The assays listed below have been tested by MSD for analytical compatibility. Data on analytical performance, calibration curves, reproducibility, and specificity are provided in the certificate of analysis (C of A) included with each kit. Special order applications may be unique; therefore, users should test abundance levels in their specific samples and matrices to determine the optimum combination of assays.

Assays that can be combined into a Special Order Rat Biomarker Kit (Group 1) are listed below.

MSD Rat V-PLEX Assays: IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, KC/GRO, TNF-α

Other Analytically Compatible MSD Rat Assays: IL-1α, EPO, GM-CSF, MCP-1, MIP-3α, VEGF-A

The Special Order Rat Biomarker Group 1 kit contains plates pre-coated with specific capture antibodies. Other reagents (calibrators and detection antibodies) are provided based on the assays selected. Reagents for special order V-PLEX assays are the same as those provided in the pre-configured V-PLEX kits. Calibrators for the standard assays are supplied at high concentrations to provide ample material for blending with the multi-analyte V-PLEX calibrator(s). All detection antibodies are provided separately for maximum flexibility in building custom multiplex assays.

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 are all sandwich immunoassays. MSD provides a plate pre-coated with capture antibodies on independent and well-defined spots, as shown in the layout below. The assays are provided on MSD 4-spot, 7-spot, or 10-spot SECTOR® plates. 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 imager where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light to provide a quantitative measure of analytes in the sample.

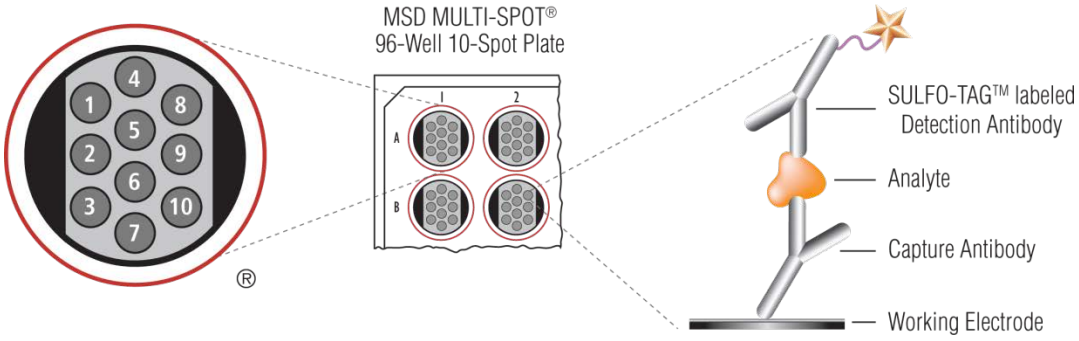

Figure 1: Multiplex plate spot diagram showing possible placement of analyte capture antibodies. A spot map identifying the location of each assay in the special order can be found on the plate packaging. This information will be needed for data analysis. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

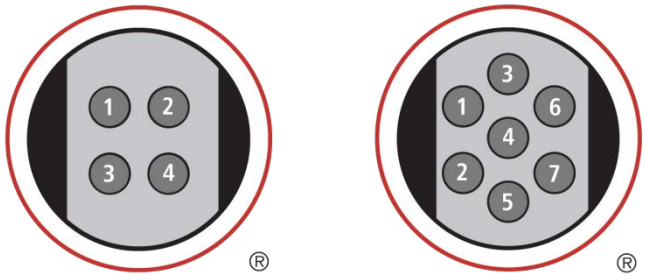

Figure 2: Special order assays can also be provided on 4- or 7-spot plates.

Reagents Supplied

Calibrators

Calibrators for V-PLEX assays are supplied using the same multi-analyte calibrator blends provided with preconfigured V-PLEX kits. Calibrators for other assays are provided separately.

Detection Antibodies

Individual SULFO-TAG detection antibodies are provided for each assay ordered.

Plates

A spot map identifying the location of each assay ordered can be found on the plate packaging. This information will be needed for data analysis.

Reagent	Storage	Catalog #	Description
MULTI-SPOT 96-Well Custom Plate	2–8°C	—	4-, 7-, or 10-spot, 96-well plate, foil sealed, with desiccant.
V-PLEX Proinflammatory Panel 1 (rat) Calibrator Blend	2–8°C	C0044-2	Nine recombinant rat proteins in diluent, buffered and lyophilized, 1 vial per plate. Individual analyte concentration is provided in the lot-specific certificate of analysis.
Additional individual assay calibrators (50 µg/mL)	≤-70°C	—	Calibrator for individual, catalog assays, one 30 µL vial per assay; sufficient for 5 plates.
Blocker H	RT	R93BI-2	Reagent required to block coated plates prior to adding calibrators, controls or samples.
Diluent 42	≤-10°C	R50AK-2	Diluent for samples and calibrator; contains serum, blockers, and preservatives.
Diluent 40	≤-10°C	R50AJ-2	Diluent for detection antibody; contains protein, blockers, and preservatives.
Read Buffer T (4X)	RT	R92TC-3	Buffer to catalyze the electro-chemiluminescence reaction.
Individual detection antibody for each assay ordered (50X)	2–8°C	—	Individual anti-rat detection antibody, SULFO-TAG–conjugated; 1 vial for each assay ordered; sufficient for 5 plates.

Required Materials and Equipment (Not Supplied)

- ❑ 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 300–1000 rpm
- ❑ Phosphate-buffered saline plus 0.05% Tween-20 for plate washing or MSD Wash Buffer catalog # R61AA-1
- ❑ Adhesive plate seals
- ❑ Deionized water

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.

Best Practices and Technical Hints

- Do not mix or substitute reagents from different sources or different kit lots. Lot information is provided in the lot-specific certificate of analysis (COA).
- Bring frozen diluents to room temperature in a 24°C water bath. Thaw other reagents on wet ice and use as directed.
- The lyophilized calibrators are reconstituted in assay diluent with an incubation for 15 to 30 minutes at room temperature.
- Prepare calibrators, samples, and controls in polypropylene microcentrifuge tubes; use a fresh pipette tip for each dilution; vortex after each dilution before proceeding.
- Avoid prolonged exposure of detection antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be shielded from light except for direct sunlight.
- Avoid bubbles in wells at all pipetting steps. Bubbles may lead to variable results; bubbles introduced when adding read buffer may interfere with signal detection.
- Use reverse pipetting when necessary to avoid introduction of bubbles, and for empty wells, pipette to the bottom corner.
- Shaking should be vigorous with a rotary motion between 300 and 1000 rpm.
- When using an automated plate washer, rotating the plate 180 degrees between wash steps may improve assay precision.
- Gently tap the plate to remove residual fluid after washing.
- Read buffer should be at room temperature when added to the plate.
- Keep time intervals consistent between adding read buffer and reading the plate to improve inter-plate precision. Unless otherwise directed, read plate as soon as practical after adding read buffer.
- No shaking is necessary after adding read buffer.
- 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.
- If assay results are above the top of the calibration curve, dilute samples, and repeat the assay.
- When running a partial plate, seal the unused sectors (see sector map in instrument and software manuals) 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, separate Diluent 42 and Diluent 40 into aliquots appropriate for the size of your needs before refreezing.

Prepare Calibrator Dilutions

MSD supplies one vial of multi-analyte Proinflammatory Panel 1 (rat) Calibrator Blend that covers all V-PLEX assays and one vial of frozen individual calibrator(s) at 50 µg/mL concentration for each of the other assays on the plate. Each vial of calibrator is enough for 5 plates. The concentration of each calibrator is provided in the certificate of analysis included with the kit.

To prepare calibrator solutions for up to 4 replicates:

1) Reconstitute one vial of Proinflammatory Panel 1 (rat) Calibrator Blend:
   a) Add 250 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.

      Note: If only V-PLEX assays are part of the Special Order kit, continue to Step 3.
2) Prepare a working stock of each individual frozen calibrator.
   a) Thaw all frozen calibrators on ice then vortex.
   b) Dilute each thawed calibrator into the indicated volume of Diluent 42 as described in Table 1 below. The resulting working stock concentrations of each calibrator are displayed. Separate into at least 5 aliquots and freeze any calibrator not immediately needed. Store at ≤-70°C.

Rat Cytokine Assays	Working Stock Concentration (µg/mL)	Bulk calibrator Volume (µL)	Diluent 42 (µL)
IL-1α	0.25	10	1990
EPO	0.5	10	990
GM-CSF	0.5	10	990
MCP-1	0.25	10	1990
MIP-3α	0.25	10	1990
VEGF-A	0.5	10	990

Table 1: Preparation of Working Stocks

3) Prepare 7 calibrator solutions plus a zero calibrator blank:
   a) Prepare Calibrator 1 by combining 200 µL of Proinflammatory Panel 1 (rat) Calibrator Blend from Step 1 and 16 µL of each working stock calibrator(s) from Step 2 in one vial. Then add Diluent 42 to bring this to a final volume of 800 µL.
   b) Prepare Calibrator 2 by transferring 100 µL of Calibrator 1 to 300 µL of Diluent 42. Mix well. Repeat 4-fold serial dilutions 5 times to generate 7 calibrators.
   c) Use Diluent 42 as Calibrator 8.

d) You may aliquot Calibrator 1 and store at ≤-70°C for 1 month or at 2–8°C for 5 days. Non-V-PLEX calibrators may not have been validated for stability. Discard any unused, diluted calibrators.

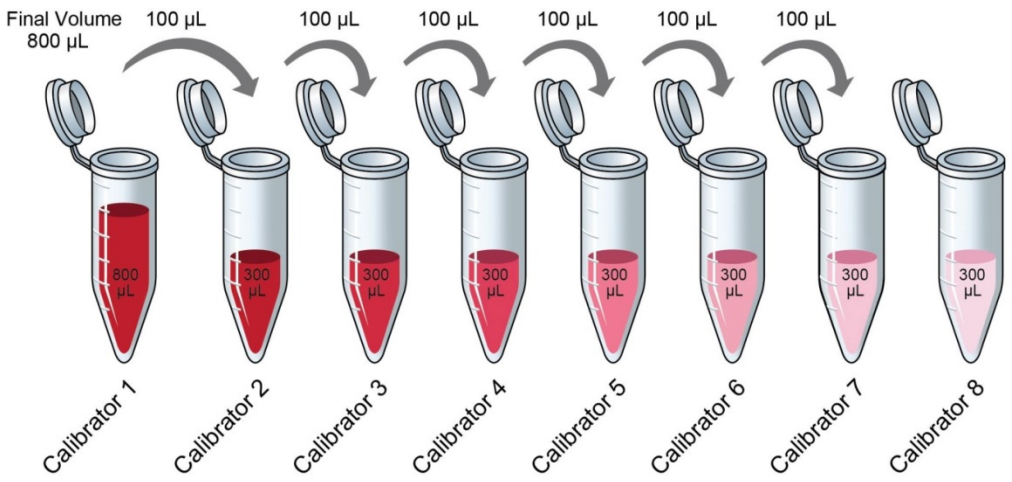

Figure 3: Typical dilution cascade. Adjust calibrator 1 for assays ordered.

Suggested Top Concentrations for Calibration Curve

V-PLEX assays: For the lot-specific concentration of each calibrator in the reconstituted multi-analyte calibrator blend, refer to the C of A supplied with the kit.

Other selected assays: The calibration curve used in the assay will be dependent on the assays and reagents. The calibration curve can be adjusted as needed. Suggested top-of-the-curve concentrations and dilution factors for MSD rat cytokine assays are provided below.

Catalog Assays	Calibrator 1 concentration (pg/mL)	Serial Dilution Factor
IL-1α	5,000	4
EPO	10,000	4
GM-CSF	10,000	4
MCP-1	5,000	4
MIP-3α	5,000	4
VEGF-A	10,000	4

Table 2: Calibrator 1 concentrations for selected assays

Dilute Samples

Dilute samples with Diluent 42. For rat serum, plasma, and urine samples, MSD recommends a 2-4 fold dilution; however, you may adjust dilution factors for the sample set under investigation. For example, to dilute 4-fold, add 30 µL of sample to 90 µL of Diluent 42. You may conserve sample volume by using a higher dilution.

Some assays may require greater dilution (see appendix table for suggested starting dilution for each assay).

The appropriate dilution for other sample matrices may need to be determined. The dilution for supernates may require additional dilution based on stimulation and analyte concentrations in the sample. A 4-fold dilution may be an appropriate starting point.

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.

For 1 plate, combine 60 µL of each supplied 50X detection antibodies, then add Diluent 40 to bring the final volume to 3000 µL.

Prepare Wash Buffer

MSD recommends using phosphate-buffered saline plus 0.05% Tween-20 for plate washing or MSD Wash Buffer catalog # R61AA-1. MSD Wash Buffer is provided as a 20X stock solution. The working solution is 1X.

For 1 plate, combine:

- ❑ 15 mL of wash buffer (20X)
- ❑ 285 mL of deionized water

Prepare Read Buffer

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

For 1 plate, combine:

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

You may keep excess diluted read buffer in a tightly sealed container at room temperature for up to 1 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 (e.g., pre-wetting) is required.

Protocol

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.

2. **Add Sample:** Wash the plate 3 times with at least 150 µL/well of wash buffer. Add 50 µL of diluted sample, calibrator, or control per well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.

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.

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. Read the plate on the MSD instrument. No incubation in read buffer is required before reading the plate.

Alternate Protocols

The suggestions below may be useful for simplifying the protocol; however, they have not been tested by MSD Quality Control.

- **Alternate Protocol 1, Extended Sample Incubation:** Incubating samples overnight at 2–8°C may improve sensitivity for some assays. Shaking may improve performance.
- **Alternate Protocol 2, Single Wash (Tissue Culture):** 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.
- **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.

Assay Components

Antibodies: V-PLEX assays

	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-2	Mouse Monoclonal	Goat Polyclonal	C
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

Table 3: Antibodies for V-PLEX assays

Antibodies: Other Assays

	Source Species		
Analyte	MSD Capture Antibody	MSD Detection Antibody	Assay Generation
IL-1α	Mouse Monoclonal	Goat Polyclonal	A
EPO	Mouse Monoclonal	Mouse Monoclonal	A
GM-CSF	Mouse Monoclonal	Goat Polyclonal	A
MCP-1	Goat Polyclonal	Goat Polyclonal	A
MIP-3α	Mouse Monoclonal	Goat Polyclonal	A
VEGF-A	Goat Polyclonal	Goat Polyclonal	A

Table 4: Antibodies for selected assays

Certificate of Analysis for Special Order Cytokine Assays (Rat)

The Certificate of Analysis for Rat Cytokine Special Order Assays will include the following:

- Signal and %CV from calibration curve for each calibrator
- Background signal
- Non-specific binding for all analytes (individual detection antibodies tested with blended calibrator)
- List of components with lot numbers used for QC testing

Appendix

Example: Calibrator Dilution

In this example, the Special Order kit contains IL-2, IL-4, IL-6, IL-10, KC/GRO, GM-CSF, and MCP-1. The kit is provided with the Proinflammatory Panel 1 (rat) lyophilized calibrator blend and individual calibrators for rat GM-CSF and MCP-1.

Step 1:

Reconstitute the lyophilized calibrator with 250 µL of Diluent 42. Mix by inverting at least 3 times. Equilibrate at room temperature for 15-30 minutes and vortex briefly using short pulses.

Thaw GM-CSF and MCP-1 calibrators on wet ice.

Step 2:

Prepare the working stocks. For GM-CSF, the working stock is prepared by adding 10 µL of the bulk calibrator to 990 µL of Diluent 42 (see Table 1). For MCP-1, the working stock is prepared by adding 10 µL of bulk calibrator to 1990 µL of Diluent 42 (see Table 1). Aliquot the working stock into 5 vials and store unused working stock at ≤-70°C.

Step 3:

Combine 200 µL of Proinflammatory Panel 1 (rat) Calibrator Blend, 16 µL each of GM-CSF and MCP-1 working stock in one vial. Add 568 µL of Diluent 42 to bring the blended calibrator to a volume of 800 µL.

The blended calibrator concentration should be at the suggested top of curve. The concentration of GM-CSF in the vial should be 10,000 pg/mL (see Table 2). The concentration of MCP-1 in the vial should be 5,000 pg/mL. The concentration of IL-2, IL-4, IL-6, IL-10, and KC/GRO are provided in the Certificate of Analysis.

Step 4:

To prepare calibrator for up to 4 replicates, use the blended calibrator prepared in step 3 as Calibrator 1, then transfer 100 µL of Calibrator 1 into 300 µL of Diluent 42. Repeat the 4-fold serial dilution 6 times to generate calibrators 2 through 7. Use Diluent 42 as Calibrator 8.

Suggested Sample Dilution

The suggested 4-fold dilution may not be appropriate for all samples and study conditions. These suggestions were determined from normal control animals across multiple studies. If a dilution is not listed below, a suggestion dilution has not been developed. The suggested dilution factors are based on abundance of the analyte in the sample, not on matrix effects.

		Suggested Fold Dilution	
	Assay	Serum/Plasma	Urine
Proinflammatory Panel 1 (rat)	IFN-γ	4	4
	IL-1β	4	4
	IL-2	4	4
	IL-4	4	4
	IL-5	4	4
	IL-6	4	4
	KC/GRO	4	4
	IL-10	4	4
	IL-13	4	4
	TNF-α	4	4

	Suggested Fold Dilution	
Assay	Serum/Plasma	Urine
IL-1α	4	
EPO	4	
GM-CSF	4	
MCP-1	10	
MIP-3α	4	
VEGF-A	4	

Table 5: Suggested sample dilutions