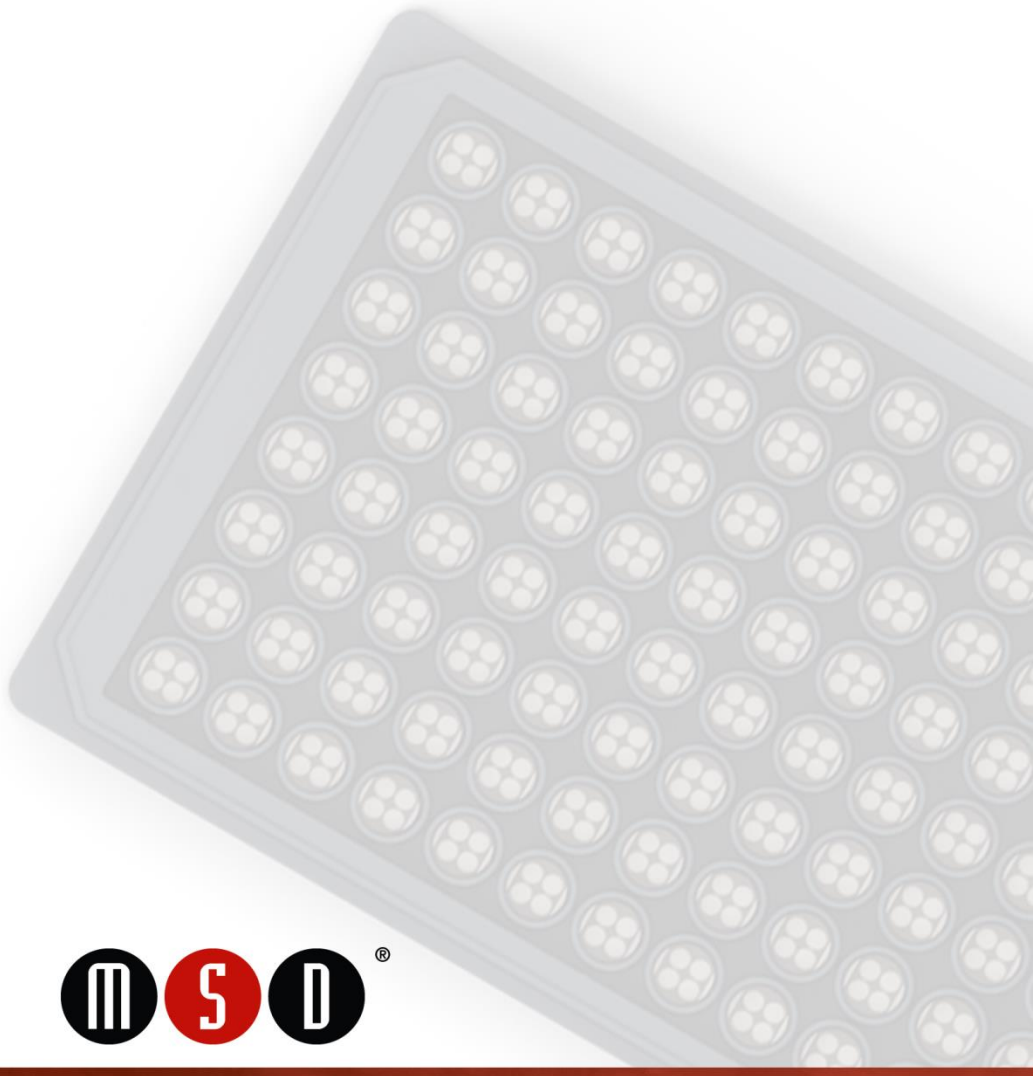
MSD® MULTI-SPOT Assay System

Human MIP-4 Kit

1-Plate Kit	K151RLD-1
5-Plate Kit	K151RLD-2
25-Plate Kit	K151RLD-4



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MSD Cytokine Assays

Human MIP-4 Kit

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

Macrophage inflammatory protein 4 (MIP-4) (CCL18/PARC/AMAC-1/DC-CK-1/SCYA18) is a C-C chemokine strongly expressed in the lung and to a lesser extent in the thymus and lymph nodes.[1,2] To date, the functional receptor and role of MIP-4 has been difficult to ascertain as it appears to exist only in primates, rendering rodent models unusable. MIP-1α is the most closely related chemokine to MIP-4, but CCR1 and CCR5 (receptors for MIP-1α) do not bind MIP-4.[1] *In vitro*, MIP-4 is particularly chemotactic for naive T-lymphocytes, Th2 cells, B cells, and immature dendritic cells.[1,2] Despite MIP-4's absence in rodents, it is functionally active in humans as a T-lymphocyte chemoattractant.[3]

Research implicates MIP-4 involvement in tumor malignancy and various inflammatory and allergic pulmonary, skin, and joint diseases.[4] In addition to its chemotactic function, MIP-4 is able to induce production of adaptive regulatory T cells from $CD4^{+}CD25^{-}$ memory T cells.[2] However, this phenomenon is only present in individuals with non-allergic asthma. Taken in conjunction with elevated MIP-4 levels in allergic diseases (asthma and atopic dermatitis), this suggests MIP-4 desensitization or decreased feedback regulation.[2] In chronic obstructive pulmonary disease, an elevated level of MIP-4 coincides with clinical outcome and can serve as a potential biomarker.[5] MIP-4 is also expressed in abundance in tumor-associated macrophages found in breast tissue and is associated with increased breast cancer metastasis.[6] MIP-4 is thought to cause increased ECM adherence of cancerous cells to fibronectin in the stroma allowing subsequent invasion.[6]

Principle of the Assay

MSD assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. Human MIP-4 is a sandwich immunoassay (Figure 1). MSD provides a plate pre-coated with capture antibodies. 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 provides 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 to provide a quantitative measure of analytes in the sample.

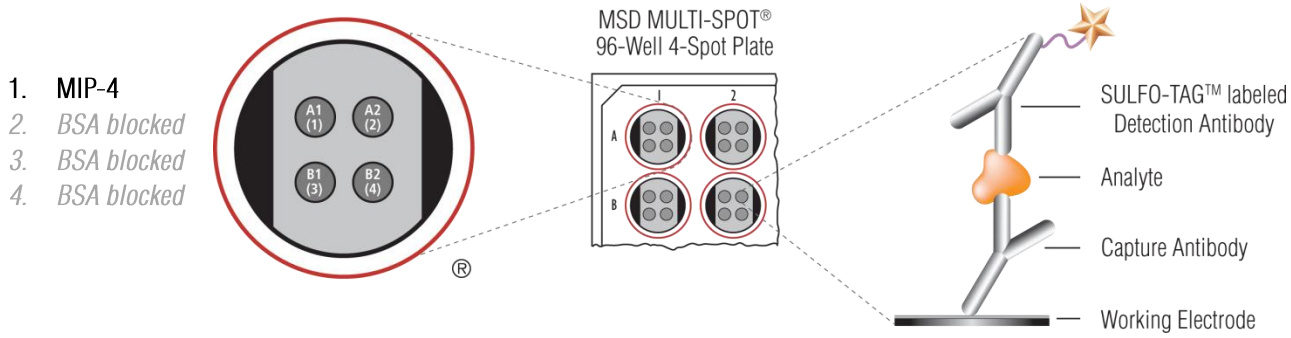


Figure 1. *Spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.*

Reagents Supplied

Product Description	Storage	Quantity per Kit K151RLD-1	K151RLD-2	K151RLD-4
MULTI-SPOT® 96-Well 4-Spot Human MIP-4 Plate N451RLA-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG Anti-hu MIP-4 Antibody[1] (50X) D21RL-2 (75 µL), D21RL-3 (375 µL)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Human MIP-4 Calibrator (0.1 µg/mL) C01RL-2	≤-70°C	1 vial (60 µL)	5 vials (60 µL ea)	25 vials (60 µL ea)
Diluent 43 R50AG-1 (10 mL), R50AG-2 (50 mL)	≤-10°C	1 bottle (10 mL)	1 bottle (50 mL)	5 bottles (50 mL ea)
Diluent 3 R51BA-4 (5 mL), R51BA-5 (25 mL)	≤-10°C	1 bottle (5 mL)	1 bottle (25 mL ea)	5 bottles (25 mL ea)
Read Buffer T (4X) R92TC-3 (50 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	5 bottles (50 mL ea)

Additional Materials and Equipment

- ❑ Appropriately sized tubes for reagent preparation
- ❑ Polypropylene microcentrifuge tubes for preparing serial dilutions
- ❑ Phosphate-buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing or MSD Wash Buffer (Catalog No. R61AA-1)
- ❑ 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
- ❑ Adhesive plate seals
- ❑ Microtiter plate shaker (rotary) capable of shaking at 500-1,000 rpm
- ❑ 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 or at www.mesoscale.com.

[1] SULFO-TAG–conjugated detection antibodies should be stored in the dark.

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).
- Assay incubation steps should be performed between 20-26°C to achieve the most consistent signals between runs.
- Bring frozen diluent (if applicable) 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.
- 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.
- Do not touch the pipette tip on the bottom of the wells when pipetting into the MSD plate.
- 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.
- 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 the plate seals prior to reading the plate.
- 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 (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. Thaw the stock calibrator on ice.

> **Important:** Upon first thaw, separate Diluent 43 and Diluent 3 into aliquots appropriate for the size of your needs before refreezing.

Prepare Standards

MSD supplies calibrator for the Human MIP-4 Kit at 20-fold higher concentration than the recommended highest standard. We recommend a 7-point standard curve with 4-fold serial dilution steps and a zero calibrator blank. Thaw the stock calibrator, then add to diluent at room temperature to make the standard curve solutions.

Standard	Human MIP-4 (pg/mL)	Dilution Factor
Stock Calibrator	100,000	
STD-01	5,000	20
STD-02	1,250	4
STD-03	313	4
STD-04	78	4
STD-05	20	4
STD-06	4.9	4
STD-07	1.2	4
STD-08	0	n/a

To prepare 7 standard solutions plus a zero calibrator blank for up to 4 replicates:

1) Prepare the highest standard by adding 50 µL of stock calibrator to 950 µL of Diluent 43. Mix well.
2) Prepare the next standard by transferring 100 µL of the highest standard to 300 µL of Diluent 43. Mix well. Repeat 4-fold serial dilutions 5 additional times to generate 7 standards.
3) Use Diluent 43 as the blank.

Dilute Samples

Serum and Plasma

Plasma prepared in heparin tubes commonly displays additional clotting following thawing of the sample. Remove clots and all solid material by centrifugation. Avoid multiple freeze–thaw cycles for serum and plasma samples. Dilute serum and plasma samples at least 1000-fold* in Diluent 43; however, you may adjust dilution factors for the sample set under investigation.

Tissue Culture

If using serum-free medium, the presence of carrier protein (e.g., 1% BSA) in the solution is helpful to prevent loss of analyte to the labware. Dilute tissue culture supernatant samples at least 1,000-fold* in Diluent 43. Samples with extremely high levels of cytokines may require additional dilution.

* You may use PBS for your initial dilution step (i.e., 1:500) and then use Diluent 43 for the final 1:2 dilution.

Prepare Detection Antibody Solution

MSD provides each detection antibody in a 50X stock solution. The working detection antibody solution is 1X. Avoid exposing 1X detection antibody solution to light to prevent elevated background signals.

For one plate, combine:

- ❑ 60 µL of 50X SULFO-TAG Anti-hu MIP-4 Antibody
- ❑ 2,940 µL of Diluent 3

Prepare Read Buffer

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 prepare diluted read buffer in advance and store it at room temperature in a tightly sealed container.

Prepare Wash Buffer

MSD provides Wash Buffer as a 20X stock solution. 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 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 can be used as delivered; no additional preparation (e.g., pre-wetting) is required.

Protocol

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

STEP 1: **Add Sample or Calibrator**

- ❑ Add 50 µL of Calibrator or diluted sample to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking (500-1,000 rpm) for 2 hours.

STEP 2: **Wash and Add Detection Antibody Solution**

- ❑ Wash the plate three times with at least 150 µL/well of PBS-T or 1X MSD 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 (500-1,000 rpm) for 2 hours.

STEP 3: **Wash and Read**

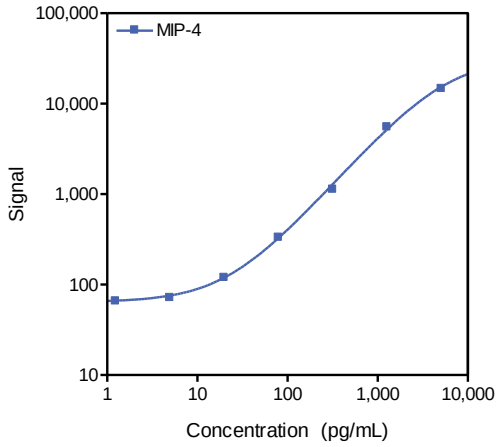
- ❑ Wash the plate three times with at least 150 µL/well of PBS-T or 1X MSD Wash Buffer.
- ❑ Add 150 µL of 2X Read Buffer T to each well. Analyze the plate on an MSD instrument. Incubation in Read Buffer T is not required before reading the plate.

Analysis of Results

Run at least one set of calibrators in duplicate to generate the standard curve. The standard curve is modeled using least squares fitting algorithms so that signals from the calibrators can be used to calculate the concentration of analyte in the samples. The assays have a wide dynamic range (3–4 logs) which allows accurate quantification without the need for multiple dilutions or repeated testing. The MSD DISCOVERY WORKBENCH® analysis software uses a 4-parameter logistic model (or sigmoidal dose-response) and includes a $1/Y^2$ weighting function. The weighting function is important because it provides a better fit of data over a wide dynamic range, particularly at the low end of the standard curve.

Typical Data

The following standard curve graph illustrates the dynamic range of the assay. Actual signals will vary. Best quantification of unknown samples will be achieved by generating a standard curve for each plate using a minimum of two replicates of standards.



MIP-4		
Conc. (pg/mL)	Average Signal	%CV
0	57	14.8
1.2	67	8.9
4.9	73	7.4
20	122	6.4
78	338	2.1
313	1,153	6.3
1,250	5,628	3.2
5,000	14,921	10.3

Sensitivity

The lower limit of detection (LLOD) is a calculated concentration based on a signal 2.5 standard deviations above the background (zero calibrator blank).

	MIP-4
Average LLOD (pg/mL)	9.06

Specificity

To assess specificity of the MIP-4 assay, the kit was tested with the following recombinant human proteins: fractalkine, 35,000 pg/mL; I-TAC, 1,500 pg/mL; MCP-2, 250 pg/mL; MIP-3β, 275 pg/mL; and MIP-5, 1,200 pg/mL. Less than 0.1% non-specific binding was observed with each protein.

Assay Components

Calibrator

The assay calibrator uses recombinant human MIP-4, (residues 21–89), expressed in *E.coli*.

Antibodies

	Source Species	
Analyte	MSD Capture Antibody	MDS Detection Antibody
MIP-4	Mouse Monoclonal	Goat Polyclonal

References

1. Schraufstatter IU, et al. The chemokine CCL18 causes maturation of cultured monocytes to macrophages in the M2 spectrum. Immunology. 2011 April;135(4):287-98.
2. Chang Y, et al. The chemokine CCL18 generates adaptive regulatory T cells from memory $CD4^+$ T cells of healthy but not allergic subjects. FASEB J. 2010 Dec;24(12):5063-72.
3. Luzina IG; Atamas, SP. CCR6 is not necessary for functional effects of human CCL18 in a mouse model. Fibrogenesis Tissue Repair. 2012 Jan 18;5(1):2.
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5. Sin DD, et al. Serum PARC/CCL-18 concentrations and health outcomes in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2011 May 1;183(9):1187-92.
6. Chen J, et al. CCL18 from tumor-associated macrophages promotes breast cancer metastasis via PITPNM3. Cancer Cell. 2011 Apr 12;19(4):541-55.

Summary Protocol

Human MIP-4 Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the Human MIP-4 assays.

Sample and Reagent Preparation

- ❑ Bring all reagents to room temperature and thaw the calibrator on ice.
- ❑ Prepare standard solutions using the supplied calibrator:
  - o Dilute the stock calibrator 20-fold in Diluent 43.
  - o Perform a series of 4-fold dilution steps and prepare a zero calibrator blank.
- ❑ Dilute samples 1,000-fold in Diluent 43 before adding to the plate.
- ❑ Prepare detection antibody solution by diluting stock detection antibody 50-fold in Diluent 3.
- ❑ Prepare 2X Read Buffer T by diluting stock 4X Read Buffer T 2-fold with deionized water.
- ❑ If using MSD Wash Buffer, dilute stock 20X MSD Wash Buffer 20-fold with deionized water to prepare a 1X working solution.

STEP 1: Add Sample or Calibrator

- ❑ Add 50 µL/well of Calibrator or diluted sample.
- ❑ Incubate at room temperature with shaking (500–1,000 rpm) for 2 hours.

STEP 2: Wash and Add Detection Antibody Solution

- ❑ Wash the plate three times with at least 150 µL/well of PBS-T or 1X MSD Wash Buffer.
- ❑ Add 25 µL/well of 1X detection antibody solution.
- ❑ Incubate at room temperature with shaking (500–1,000 rpm) for 2 hours.

STEP 3: Wash and Read Plate

- ❑ Wash the plate three times with at least 150 µL/well of PBS-T or 1X MSD Wash Buffer.
- ❑ Add 150 µL/well of 2X Read Buffer T.
- ❑ Analyze the plate on an MSD instrument.

Plate Diagrams