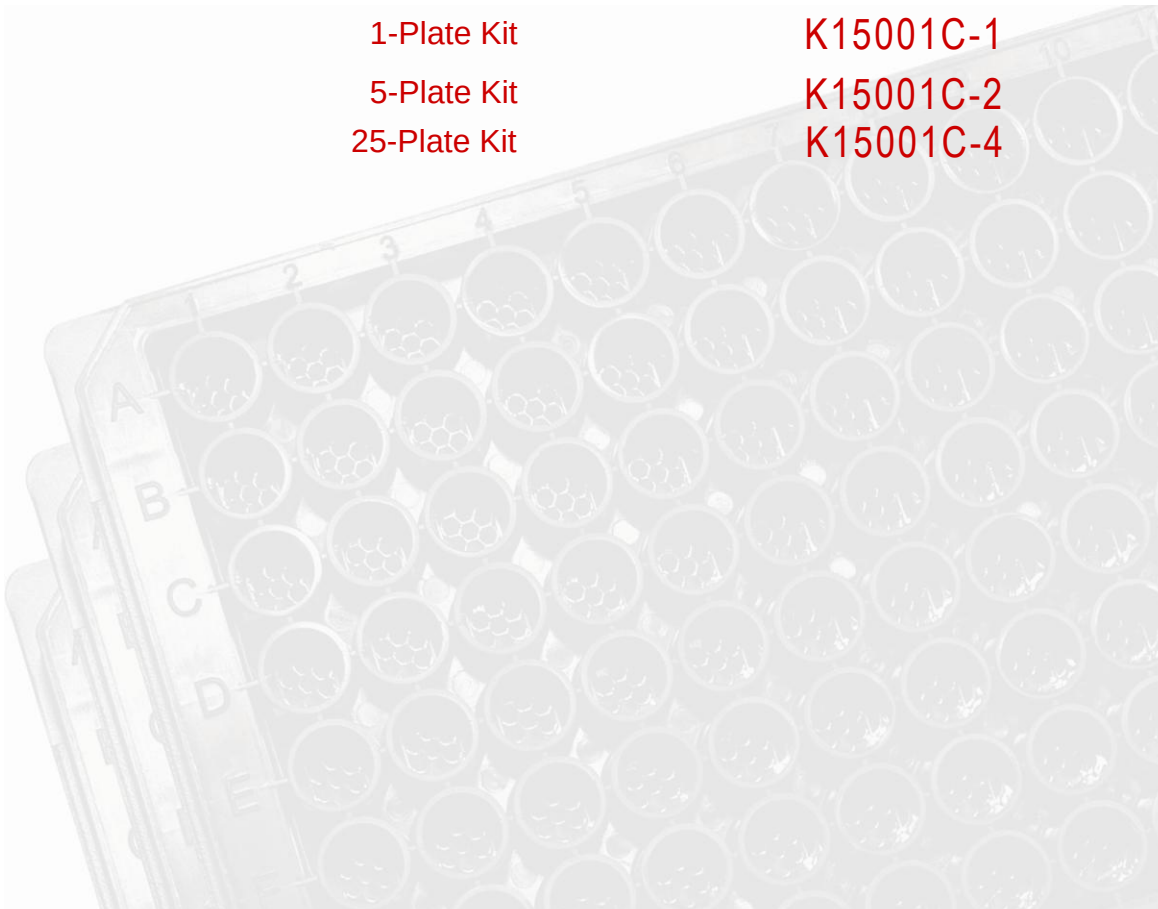
MESO SCALE DISCOVERY

MULTI-SPOT Assay System

Human Chemokine 9-Plex Assay
Ultra-Sensitive Kit

1-Plate Kit	K15001C-1
5-Plate Kit	K15001C-2
25-Plate Kit	K15001C-4



MSD MULTI-SPOT Assays

Ultra-Sensitive Kit

Human Chemokine 9-Plex Assay

Eotaxin, MIP-1β, Eotaxin-3, TARC, IP-10, IL-8, MCP-1, MDC, MCP-4

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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Ordering Information

ordering information

MSD Customer Service

Phone: 1-301-947-2085
Fax: 1-301-990-2776
Email: CustomerService@mesoscale.com

MSD Scientific Support

Phone: 1-301-947-2025
Fax: 1-240-632-2219 attn: Scientific Support
Email: ScientificSupport@mesoscale.com

MSD Advantage

MSD advantage

MESO SCALE DISCOVERY'S MULTI-ARRAY® Technology is a multiplex immunoassay system that enables the measurement of biomarkers utilizing the next generation of electrochemiluminescent detection. In an MSD® assay, specific Capture Antibodies for the analytes are coated in arrays in each well of a 96-well carbon electrode plate surface. The detection system uses patented SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of the MULTI-ARRAY and MULTI-SPOT® plates. The electrical stimulation is decoupled from the output signal, which is light, to generate assays with minimal background. MSD labels can be conveniently conjugated to biological molecules, are stable and are non-radioactive. Additionally, only labels near the electrode surface are detected, enabling non-washed assays.

One of the advantages of MSD assays is the minimal sample volume required as compared to a traditional ELISA, which is also limited by its inability to measure more than a single analyte. With an MSD assay, ten different biomarkers can be analyzed simultaneously using as little as 10-25 µL of sample. These assays have high sensitivity, up to five logs of linear dynamic range, and excellent performance in complex biological matrices. Combined, these advantages enable the measurement of native levels of biomarkers in normal and diseased samples without multiple dilutions. Further, the simple and rapid protocols of MSD assays provide a powerful tool to generate reproducible and reliable results. The MSD product line offers a diverse menu of assay kits for profiling biomarkers, cell signaling pathways, and other applications, as well as a variety of plates and reagents for assay development.

Introduction

introduction

Eotaxin is a 73 amino acid CC chemokine that was first discovered in broncheoalveolar lavage fluid of allergen sensitized guinea pigs. It signals through a single receptor, CCR3, which is highly expressed on eosinophils. Thus, eotaxin plays an important role in the recruitment of eosinophils to sites of inflammation. Eotaxin 2 and **Eotaxin 3** also belong to the CC chemokine group and signal through the same receptor as eotaxin. There exists about 60% homology between human, mouse and guinea pig eotaxins.

IL-8 (Interleukin-8), also known as CXCL8, is a chemokine responsible for the attraction of neutrophils to vascular endothelium and extravasation into inflammed tissues. It is produced primarily by activated macrophages in response to toll-like receptor agonists and certain bacterial pathogens.

IP-10 (Interferon-inducible protein-10) is a CXC cytokine that was originally identified as an IFN-γ inducible gene in monocytes, fibroblasts and endothelial cells. It functions by binding to its receptor, CXCR3. It is chemotactic for monocytes, T cells, dendritic cells and NK cells, and has antitumor as well as antiangiogenic activities in vivo.

MCP-1 (Monocyte chemotactic protein-1) **and MCP-4** are members of CC subgroup of chemokine family. MCPs are chemoattractants for monocytes, T cells, NK cells and hepatic stellate cells. These proteins are expressed in a variety of pathophysiological conditions characterized by mononuclear or eosinophilic infiltrates. MCP-1 functions by binding to its receptor, CCR2. MCP-4 shares CCR2 receptor and also binds to CCR3 receptor.

MDC (macrophage derived chemokine) is a CC chemokine that was isolated by random sequencing of cDNA clones from human monocyte derived macrophages. It acts on NK cells, T cells, dendritic cells, and is a potent chemoattractant for CCR4 expressing TH2 cells. It is highly expressed in the thymus, macrophages and monocyte derived dendritic cells.

MIP-1α (Macrophage Inflammatory Protein-1α) and **MIP-1β** are highly homologous, but distinct CC chemokines that independently regulate specific aspects of the host inflammatory response to various external stimuli. Both peptides are produced by macrophages and are associated with type I immune response. They induce the synthesis as well as release of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α.

TARC / CCL17 (Thymus- and activation-regulated chemokine) is a lymphocyte-directed CC chemokine, that attracts CC chemokine receptor 4–positive (CCR4+) or CCR8+ cells. It was first cloned from PBMCs stimulated with phytohemagglutinin. TARC is constitutively and selectively expressed in the thymus. It has four conserved cysteines with a molecular weight of approximately 8kDa.

Principle of the Assay

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. The assays are available in both singleplex and multiplex formats. In a singleplex assay, an antibody for a specific protein target is coated on one electrode (or "spot") per well. In a multiplex assay, an array of capture antibodies against different targets is patterned on distinct spots in the same well. The Human Chemokine 9-Plex Assay detects Eotaxin, MIP-1β, Eotaxin-3, TARC, IP-10, IL-8, MCP-1, MDC and MCP-4 in a sandwich immunoassay format (Figure 1). MSD provides a plate that has been pre-coated with capture antibody on spatially distinct spots – antibodies for Eotaxin, MIP-1β, Eotaxin-3, TARC, IP-10, IL-8, MCP-1, MDC and MCP-4. The user adds the sample and a solution containing the labeled detection antibodies— anti-Eotaxin, anti-MIP-1β, anti-Eotaxin-3, anti-TARC, anti-IP-10, anti-IL-8, anti-MCP-1, anti-MDC and anti-MCP-4 labeled with an electrochemiluminescent compound, MSD SULFO-TAG label—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 labeled detection antibodies by bound analytes completes the sandwich. The user adds an MSD read buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD SECTOR® instrument for analysis. Inside the SECTOR instrument, a voltage applied to the plate electrodes causes the labels bound to the electrode surface to emit light. The instrument measures intensity of emitted light to provide a quantitative measure of Eotaxin, MIP-1β, Eotaxin-3, TARC, IP-10, IL-8, MCP-1, MDC and MCP-4 present in the sample.

1 = Eotaxin
2 = MIP-1β
3 = Eotaxin-3
4 = TARC
5 = IP-10
6 = *BSA Blocked*
7 = IL-8
8 = MCP-1
9 = MDC
10 = MCP-4

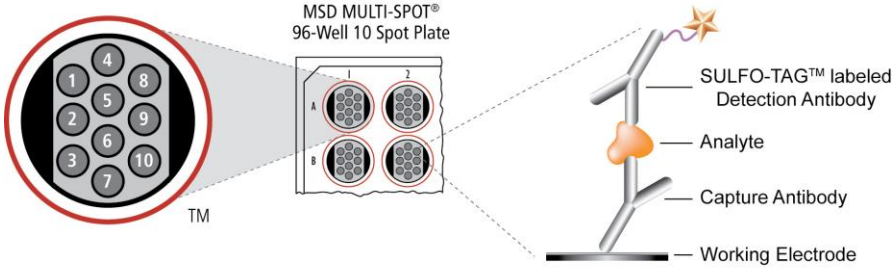


Figure 1. *Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing records.*

IV Reagents Supplied

reagents supplied

Product Description	Storage	Quantity per Kit K15001C-1	K15001C-2	K15001C-4
MULTI-SPOT 96-well 10 Spot Human Chemokine 9-Plex Plate N05001B-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG Detection Antibody Blend[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Human Chemokine 9-Plex Calibrator Blend (Ultra-Sensitive) (20X)	≤-70°C	1 vial (15 µL)	5 vials (15 µL ea)	25 vials (15 µL ea)
Diluent 2 R51BB-4 (8 mL) R51BB-3 (40 mL)	≤-10°C	1 bottle (8 mL)	1 bottle (40 mL)	5 bottles (40 mL ea)
Diluent 3 R51BA-4 (5 mL) R51BA-5 (25 mL)	≤-10°C	1 bottle (5 mL)	1 bottle (25 mL)	5 bottles (25 mL ea)
Read Buffer T (4X) R92TC-3 (50 mL) R92TC-2 (200 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	2 bottles (200 mL ea)

V Required Materials and Equipment - not supplied

required materials and equipment — not supplied

- Deionized water for diluting concentrated buffers
- 50 mL tubes for reagent preparation
- 15 mL tubes for reagent preparation
- Microcentrifuge tubes for preparing serial dilutions
- Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing
- Appropriate liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL into a 96-well microtiter plate
- Plate washing equipment: automated plate washer or multichannel pipette
- Adhesive plate seals
- Microtiter plate shaker

VI Safety

safety

Safe laboratory practices and personal protective equipment such as gloves, safety glasses, and lab coats should be used at all times during the handling of all kit components. All hazardous samples should be handled and disposed of properly, in accordance with local, state, and federal guidelines.

[1] Some SULFO-TAG labeled detection antibodies may be light-sensitive, so they should be stored in the dark.

VII Reagent Preparation

Bring all reagents to room temperature and thaw the Calibrator stock on ice.

> **Important:** Upon first thaw, separate Diluent 2 and Diluent 3 into aliquots appropriate to the size of your assay needs. These diluents can go through up to three freeze-thaw cycles without significantly affecting the performance of the assay.

Prepare Calibrator and Control Solutions

Calibrator for the Human Chemokine 9-Plex Assay is supplied at 20-fold higher concentration than the recommended highest Calibrator. Prepare the highest Calibrator point by diluting the stock Calibrator blend 20-fold in Diluent 2. MSD recommends the preparation of an 8-point standard curve consisting of at least 2 replicates of each point. Each well requires 25 µL of Calibrator. For the assay, MSD recommends 4-fold serial dilution steps and Diluent 2 alone for the 8th point:

	Human Chemokine 9-Plex Calibrator Blend (pg/mL)									
Standard	Eotaxin	MIP-1β	Eotaxin-3	TARC	IP-10	IL-8	MCP-1	MDC	MCP-4	Dilution Factor
20X Stock	800000	800000	200000	800000	200000	50000	200000	8000000	200000	
STD-01	40000	40000	10000	40000	10000	2500	10000	400000	10000	20
STD-02	10000	10000	2500	10000	2500	625	2500	100000	2500	4
STD-03	2500	2500	625	2500	625	156	625	25000	625	4
STD-04	625	625	156	625	156	39	156	6250	156	4
STD-05	156	156	39	156	39	9.8	39	1560	39	4
STD-06	39	39	9.8	39	9.8	2.4	9.8	390	9.8	4
STD-07	9.8	9.8	2.4	9.8	2.4	0.61	2.4	98	2.4	4
STD-08	0	0	0	0	0	0	0	0	0	N/A

To prepare this 8-point standard curve for up to 4 replicates:

1) Prepare the highest Calibrator point (STD-01) by transferring 10 µL of the Chemokine 9-Plex Calibrator blend to 190 µL Diluent 2.
2) Prepare the next Calibrator (STD-02) by transferring 50 µL of STD-01 to 150 µL of Diluent 2. Repeat 4-fold serial dilutions 5 additional times to generate 7 Calibrators.
3) The recommended 8th Standard is Diluent 2 (i.e. zero Calibrator).

Notes:

a. Alternatively, Calibrators can be prepared in the sample matrix or diluent of choice to verify acceptable performance in these matrices. In general, the presence of some protein (for example, 1% BSA) in the sample matrix is helpful for preventing loss of analyte by adsorption onto the sides of tubes, pipette tips, and other surfaces. If your sample matrix is serum-free tissue culture media, then the addition of 10% FBS or 1% BSA is recommended.

b. The standard curve can be modified as necessary to meet specific assay requirements.

Dilution of Samples

Serum and Plasma

All solid material should be removed by centrifugation. Plasma prepared in heparin tubes commonly displays additional clotting following the thawing of the sample. Remove any additional clotted material by centrifugation. Avoid multiple freeze/thaw cycles for serum and plasma samples. Normal serum or plasma samples may not require a dilution prior to being used in the MSD Human Chemokine 9-Plex Assay. Serum or plasma with high levels of these analytes may require a dilution.

Tissue Culture

Tissue culture supernatant samples may not require dilution prior to being used in the MSD Human Chemokine 9-Plex Assay. 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. Samples from experimental conditions with extremely high levels of cytokines may require a dilution.

Other Matrices

Information on preparing samples in other matrices, including sputum, CSF, and tissue homogenates can be obtained by contacting MSD Scientific Support at 1-301-947-2025 or ScientificSupport@mesoscale.com.

Prepare Detection Antibody Solution

The Detection Antibody Blend is provided at 50X stock solution. The final concentration of the working Detection Antibody Solution should be at 1X. For each plate used, dilute a 60 µL aliquot of the stock Detection Antibody Blend into 2.94 mL of Diluent 3.

Prepare Read Buffer

The Read Buffer should be diluted 2-fold in deionized water to make a final concentration of 2X Read Buffer T. Add 10 mL of 4X Read Buffer T to 10 mL of deionized water for each plate.

Prepare MSD Plate

This plate has been pre-coated with antibody for the analyte shown in Figure 1. The plate can be used as delivered; no additional preparation (e.g., pre-wetting) is required. The plate has also been exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies.

VIII Assay Protocol

assay protocol

1. **Addition of Diluent 2:** Dispense 25 µL of Diluent 2 into each well. Seal the plate with an adhesive plate seal and incubate for 30 min with vigorous shaking (300–1000 rpm) at room temperature.
2. **Addition of the Sample or Calibrator:** Dispense 25 µL of Sample or Calibrator into separate wells of the MSD plate. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
3. **Wash and Addition of the Detection Antibody Solution:** Wash the plate 3 times with PBS-T. Dispense 25 µL of the 1X Detection Antibody Solution into each well of the MSD plate. Seal the plate and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
4. **Wash and Read:** Wash the plate 3 times with PBS-T. Add 150 µL of 2X Read Buffer T to each well of the MSD plate. Analyze the plate on the SECTOR Imager. Plates may be read immediately after the addition of Read Buffer.

Notes

Shaking a 96-well MSD plate typically accelerates capture at the working electrode.

Bubbles in the fluid will interfere with reliable reading of plate. Use reverse pipetting techniques to insure bubbles are not created when dispensing the Read Buffer.

IX Analysis of Results

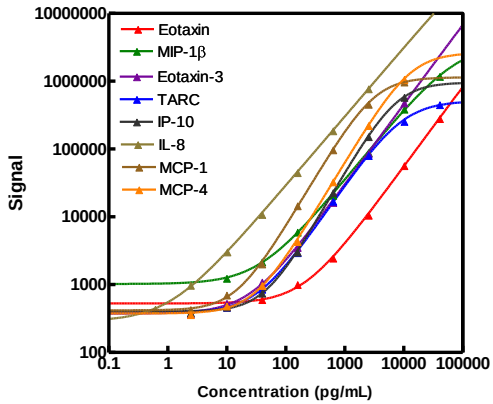
analysis of results

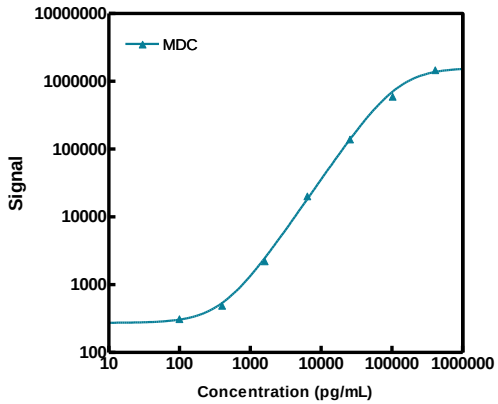
The Calibrators should be run in duplicate to generate a standard curve. The standard curve is modeled using least squares fitting algorithms so that signals from samples with known levels of the analyte of interest can be used to calculate the concentration of analyte in the sample. The assays have a wide dynamic range (3–4 logs) which allows accurate quantitation in many samples without the need for dilution. The MSD DISCOVERY WORKBENCH® analysis software utilizes 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 Standard Curve

typical standard curve

The following standard curves are an example of the dynamic range of the assay. The actual signals may vary and a standard curve should be run for each set of samples and on each plate for the best quantitation of unknown samples.





Eotaxin		
Conc. (pg/mL)	Average Signal	%CV
0	504	4.1
9.8	522	13.5
39	606	6.4
156	1007	9.9
625	2477	7.0
2500	10660	4.6
10000	57287	9.8
40000	282203	10.5

MIP-1β		
Conc. (pg/mL)	Average Signal	%CV
0	963	14.1
9.8	1245	1.8
39	2174	1.5
156	5968	1.3
625	20939	5.2
2500	88796	4.1
10000	388363	2.2
40000	1181284	3.6

Eotaxin-3		
Conc. (pg/mL)	Average Signal	%CV
0	323	10.6
2.4	373	5.0
9.8	533	11.2
39	1094	1.7
156	3561	1.7
625	16389	4.7
2500	86587	2.2
10000	480213	2.4

TARC		
Conc. (pg/mL)	Average Signal	%CV
0	357	6.1
9.8	465	8.8
39	862	3.2
156	2979	5.7
625	17080	7.2
2500	80821	8.8
10000	257446	6.6
40000	454107	5.8

IP-10		
Conc. (pg/mL)	Average Signal	%CV
0	375	9.6
2.4	389	6.6
9.8	469	4.7
39	750	7.2
156	3033	4.8
625	22934	6.7
2500	152625	2.1
10000	583517	3.6

IL-8		
Conc. (pg/mL)	Average Signal	%CV
0	247	12.7
0.61	433	7.5
2.4	985	3.1
10	3056	3.3
39	10872	3.6
156	45080	7.4
625	187044	3.6
2500	776971	2.9

MCP-1		
Conc. (pg/mL)	Average Signal	%CV
0	316	10.3
2.4	432	4.6
9.8	705	4.7
39	2024	3.7
156	14621	7.9
625	97693	7.3
2500	460947	3.5
10000	979096	7.3

MDC		
Conc. (pg/mL)	Average Counts	%CV
0	298	14.1
98	318	6.8
391	500	8.3
1563	2263	5.0
6250	20583	11.0
25000	142084	7.4
100000	606799	5.9
400000	1486722	5.2

MCP-4		
Conc. (pg/mL)	Average Counts	%CV
0	304	9.9
2.4	363	16.1
9.8	488	1.2
39	979	4.6
156	4346	9.9
625	32988	10.9
2500	223428	3.1
10000	1068598	5.8

XI Sensitivity

sensitivity

The lower limit of detection (LLOD) is the calculated concentration of the signal that is 2.5 standard deviations over the zero Calibrator. The values below represent the average LLOD over multiple kit lots.

	Eotaxin	MIP-1β	Eotaxin-3	TARC	IP-10	IL-8	MCP-1	MDC	MCP-4
LLOD (pg/mL)	23	7.5	6.1	15	12	0.6	8.0	334	8.4

XII Assay Components

assay components

The human Eotaxin, MIP-1β, Eotaxin-3, TARC, IP-10, IL-8, MCP-1, MDC and MCP-4 capture and detection antibodies used in this assay are listed below.

	Source species	
Analyte	**MSD Capture Antibody**	**MSD Detection Antibody**
hEotaxin	Mouse monoclonal	Mouse monoclonal
hMIP-1β	Mouse monoclonal	Goat polyclonal
hEotaxin-3	Mouse monoclonal	Goat polyclonal
hTARC	Mouse monoclonal	Goat polyclonal
hIP-10	Mouse monoclonal	Goat polyclonal
hIL-8	Mouse monoclonal	Goat polyclonal
hMCP-1	Mouse monoclonal	Mouse monoclonal
hMDC	Mouse monoclonal	Rabbit polyclonal
hMCP-4	Mouse monoclonal	Goat polyclonal

Summary Protocol

MSD 96-well MULTI-SPOT Human Chemokine 9-Plex Assay: Ultra-Sensitive Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the
MSD Human Chemokine 9-Plex Assay.

Step 1: Sample and Reagent Preparation

Bring all reagents to room temperature and thaw the Calibrator stock on ice.

If necessary, samples should be diluted in Diluent 2.

Prepare Calibrator solutions and standard curve.

Use the 20X Calibrator stock to prepare an 8-point standard curve by diluting in Diluent 2.

Note: *The standard curve can be modified as necessary to meet specific assay requirements.*

Prepare Detection Antibody Solution by diluting Detection Antibody Blend to 1X in a final volume of 3.0 mL Diluent 3 per plate.

Prepare 20 mL of 2X Read Buffer T by diluting 4X Read Buffer T with deionized water.

SERUM OR PLASMA SAMPLES

Step 2: Add Diluent 2

Dispense 25 µL/well Diluent 2.

Incubate at room temperature with vigorous shaking (300-1000 rpm) for 30 minutes.

Step 3: Add Sample or Calibrator

Dispense 25 µL/well Calibrator or sample.

Incubate at room temperature with vigorous shaking (300-1000 rpm) for 2 hours.

Step 4: Wash and Add Detection Antibody Solution

Wash plate 3 times with PBS-T.

Dispense 25 µL/well 1X Detection Antibody Solution.

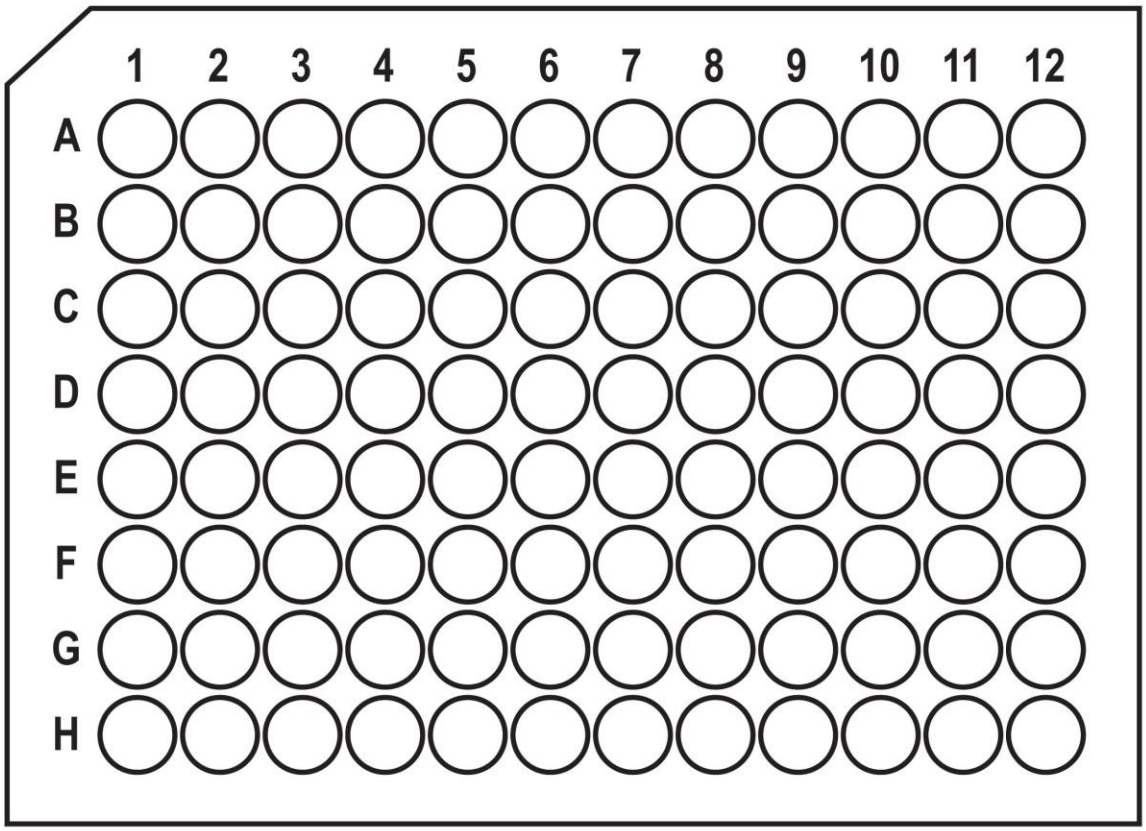
Incubate at room temperature with vigorous shaking (300-1000 rpm) for 2 hours.

Step 5: Wash and Read Plate

Wash plate 3 times with PBS-T.

Dispense 150 µL/well 2X Read Buffer T.

Analyze plate on SECTOR Imager instrument.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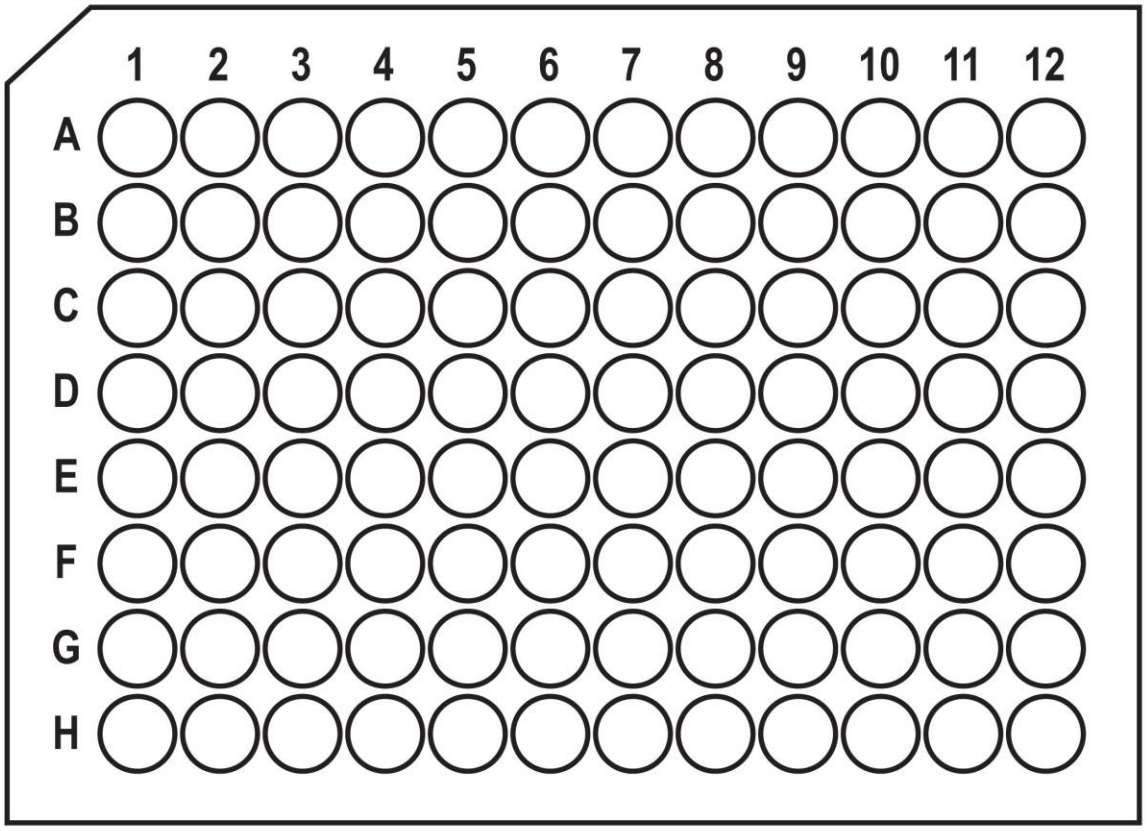
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