Human M1/M2/MDSC Cytokine Multiplex ELISA Kit (GM-CSF, IFN gamma, IL4, IL6, IL10, IL12, MCP1, TNF alpha)

Multiplex Enzyme Immunoassay for the semi-quantification of GM-CSF, IFN gamma, IL4, IL6, IL10, IL12, MCP1, TNF alpha in serum, plasma cell culture supernatant and other biological samples.

Catalog number: ARG80930

Package: 96 wells

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INTRODUCTION

GM-CSF is a cytokine that stimulates the growth and differentiation of hematopoietic precursor cells from various lineages, including granulocytes, macrophages, eosinophils and erythrocytes. [UniProt]

IFN gamma is produced by lymphocytes activated by specific antigens or mitogens. IFN-gamma, in addition to having antiviral activity, has important immunoregulatory functions. It is a potent activator of macrophages, it has antiproliferative effects on transformed cells and it can potentiate the antiviral and antitumor effects of the type I interferons. [UniProt]

IL4 participates in at least several B-cell activation processes as well as of other cell types. It is a costimulator of DNA-synthesis. It induces the expression of class II MHC molecules on resting B-cells. It enhances both secretion and cell surface expression of IgE and IgG1. It also regulates the expression of the low affinity Fc receptor for IgE (CD23) on both lymphocytes and monocytes. [UniProt]

IL6 is a cytokine with a wide variety of biological functions. It is a potent inducer of the acute phase response. Plays an essential role in the final differentiation of B-cells into Ig-secreting cells Involved in lymphocyte and monocyte differentiation. Acts on B-cells, T-cells, hepatocytes, hematopoietic progenitor cells and cells of the CNS. Required for the generation of T(H)17 cells. Also acts as a myokine. It is discharged into the bloodstream after muscle contraction and acts to increase the breakdown of fats and to improve insulin resistance. It induces myeloma and plasmacytoma growth and induces nerve cells differentiation. [UniProt]

IL10 inhibits the synthesis of a number of cytokines, including IFN-gamma, IL-2, IL-3, TNF and GM-CSF produced by activated macrophages and by helper T-cells. [UniProt]

MCP-1 is a chemotactic factor that attracts monocytes and basophils but not neutrophils or eosinophils. Augments monocyte anti-tumor activity. Has been implicated in the pathogenesis of diseases characterized by monocytic infiltrates, like psoriasis, rheumatoid arthritis or atherosclerosis. May be involved in the recruitment of monocytes into the arterial wall during the disease process of atherosclerosis. [UniProt]

TNF alpha is a cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. It is potent pyrogen causing fever by direct action or by stimulation of interleukin-1 secretion and is implicated in the induction of cachexia, Under certain conditions it can stimulate cell proliferation and induce cell differentiation. Impairs regulatory T-cells (Treg) function in individuals with rheumatoid arthritis via FOXP3 dephosphorylation. Upregulates the expression of protein phosphatase 1 (PP1), which dephosphorylates the key 'Ser-418' residue of FOXP3, thereby inactivating FOXP3 and rendering Treg cells functionally defective (PubMed:23396208). The TNF intracellular domain (ICD) form induces IL12 production in dendritic cells. [UniProt]

PRINCIPLE OF THE ASSAY

This is a multiplex enzyme immunoassay for the quantification of M1/M2/MDSC macrophagy Cytokines GM-CSF, IFN gamma, IL4, IL6, IL10, IL12, MCP1, TNF alpha. This assay employs the quantitative sandwich enzyme immunoassay technique. Monoclonal antibody specific for GM-CSF, IFN gamma, IL4, IL6, IL10, IL12, MCP1 and TNF alpha has been pre-coated onto a wells of microtiter plate. Standards or samples are pipetted into the wells and any cytokine present is bound by the immobilized antibody. Then a biotin-conjugated antibody mixture is added to each well and incubate. After washing away any unbound substances, an HRP-conjugated avidin is added to each well and incubate. A substrate solution (TMB) is added to the wells and color develops in proportion to the amount of cytokine bound in the initial step. The color development is stopped by the addition of acid and the intensity of the color is measured at a wavelength of 450nm ±2nm. The concentration of Cytokine in the sample is then determined by comparing the O.D. of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody-coated microplate	8 well X 12 strips coated with 8 different antibodies. The arrangement of antibody coating is shown below	4°C. Unused strips should be sealed tightly in the air-tight pouch.
Standard mixtures	2 vials (Lyophilized). Each vial contains a buffered protein base and 8 pro-inflammatory cytokines at different concentrations: GM-CSF:1500 pg IFN gamma: 1900 pg IL4: 2150 pg IL6: 450 pg IL10: 1100 pg IL12: 860 pg MCP1: 2300 pg TNF alpha: 1680 pg	4°C
Biotin antibody conjugate mixture	6 ml (Ready-to-use)	4°C
HRP conjugate mixture	11 ml (Ready-to-use)	4°C
Standard Diluent I (for serum/plasma samples)	25 ml (Ready-to-use)	4°C
Standard Diluent II (for cell culture supernatant samples)	25 ml (Ready-to-use)	4°C
20X Wash buffer	60 ml	4°C
Substrate A	10 ml	4°C (Protect from light)
Substrate B	10 ml	4°C (Protect from light)

STOP solution	14 ml (Ready-to-use)	4°C

ANTIBODY COATING PATTERN IN MICROTITER PLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF
B	IFN gamma	IFN gamma	IFN gamma	IFN gamma	IFN gamma	IFN gamma	IFN gamma	IFN gamma	IFN gamma	IFN gamma	IFN gamma	IFN gamma
C	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4
D	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6
E	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10
F	IL12	IL12	IL12	IL12	IL12	IL12	IL12	IL12	IL12	IL12	IL12	IL12
G	MCP-1	MCP-1	MCP-1	MCP-1	MCP-1	MCP-1	MCP-1	MCP-1	MCP-1	MCP-1	MCP-1	MCP-1
H	TNF alpha	TNF alpha	TNF alpha	TNF alpha	TNF alpha	TNF alpha	TNF alpha	TNF alpha	TNF alpha	TNF alpha	TNF alpha	TNF alpha

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Pipettes and pipette tips
- Deionized or distilled water
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times.
- All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens. Do not use water baths to thaw

samples or reagents.

- If crystals are observed in the 20X Wash buffer, warm to 37°C until the crystals are completely dissolved.
- Ensure complete reconstitution and dilution of reagents prior to use.
- Samples could be disposed of in a manner as below to inactivate human viruses. (optional)

  Solid Wastes: Autoclave 60 min. at 121°C.

  Liquid Wastes: Add sodium hypochlorite to a final concentration of 1.0%. The waste should be allowed to stand for a minimum of 30 minutes to inactivate the virus before disposal.
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

To obtain the data of each cytokine, at least 0.8 ml of the sample is needed to complete one run of the assay. It is recommended to have a larger volume available in case that the experiments have to be repeated. The unused samples should be stored frozen at ≤-20°C or ≤-70°C to avoid sample degradation. For long term storage, store at ≤-70°C is recommended.

Cell Culture Supernatants - Remove particulates by centrifugation and aliquot & store samples at ≤ -20 °C or ≤-80°C. Avoid repeated freeze-thaw cycles.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30

minutes before centrifugation for 10 minutes at 1000 x g at 4°C. Collect serum and assay immediately or aliquot and store samples at ≤ -20 °C or ≤-80°C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g at 4°C within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C or ≤-80°C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

- 1X Wash buffer: Dilute 20X Wash buffer into distilled water to yield 1X Wash buffer. 1X Wash buffer is stable for 1 month at 2-8 °C. Mix well before use.
- Substrate solution: Substrate A and Substrate B (containing TMB) should be mixed together in equal volumes up to 15 minutes before use. Refer to the table below for correct amounts of substrate solution to prepare:

Strips Used	Substrate A (ml)	Substrate B (ml)	Total volume (ml)
2 (16 wells)	1.5	1.5	3.0
4 (32 wells)	3.0	3.0	6.0
6 (48 wells)	4.0	4.0	8.0
8 (64 wells)	5.0	5.0	10.0
10 (80 wells)	6.0	6.0	12.0
12 (96 wells)	7.0	7.0	14.0

- Standards: Please select appropriate Diluent buffer for each sample type. Two vials of Standards are provided in this kit to allow both serum/plasma and cell culture supernatant testing. Standard diluent I contains animal serum and PBS for serum/plasma testing. Standard diluent II contains animal serum and RPMI 1640 for cell culture supernatant testing. The standards provided in the kit are for customers

to use at own discretion. If samples generate values higher than the highest standard, dilute the samples with appropriate standard diluent and repeat assay.

High concentration standard stock:

Reconstitute standards with either 2ml Standard diluent I (for serum/plasma testing) or Standard diluent II (for cell culture supernatant testing) to obtain high concentration standard stock. Allow solution to sit for 15 minutes with gentle agitation prior to making dilutions. This stock solution can be aliquoted and stored frozen at -70 °C for up to 30 days.

Dilution of standard mixture:

For semi-quantitative assay, use the above high concentration standard mixture and a 32-fold diluted low concentration standards mixture to test together with up to 10 test samples. If more accurate results are required, a two-fold serial dilution with appropriate dilution buffer can generate a more accurate standard curve. However, the number of test samples will be reduced.

To dilute standards, produce a serial 2-fold dilution series from 1:2 to 1:64 dilutions. The concentration of 8 cytokines in different dilutions of the mixed standard are listed as below:

Cytokine (pg/ml)	Stock	1:2	1:4	1:8	1:16	1:32	1:64
A: GM-CSF	750	375	187.5	93.75	46.88	23.44	11.72
B: IFN gamma	950	475	237.5	118.75	59.38	29.69	14.84
C: IL4	1075	537.5	268.75	134.38	67.19	33.59	16.80
D: IL6	225	112.5	56.25	28.13	14.06	7.03	3.52
E: IL10	550	275	137.5	68.75	34.38	17.19	8.59
F: IL12	430	215	107.5	53.75	26.88	13.44	6.72
G: MCP1	1150	575	287.5	143.75	71.88	35.94	17.97

H: TNF alpha	840	420	210	105	52.5	26.25	13.13

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 20-25°C) before use.

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **100 µl of standards or samples** into wells. Cover and incubate for **1h at RT.**

   (Example) To obtain the approximate concentrations of 8 cytokines on 10 test samples (T1-T10), the low concentration standard mixture (**1:32 from high concentration mix, S1**) and high concentration standard mixture (**stock, S2**) and test samples can be added as scheme below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
B	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
C	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
D	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
E	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
F	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
G	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
H	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10

3. *Without discarding the content*, add **50 µl** of **Biotin conjugate mixture** into each well. Mix well, cover and incubate for **1 hour at room temperature**.
4. Aspirate each well and wash, repeating the process 4 times for a total **5 washes**. Wash by filling each well with **1× Wash Buffer (350µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean

paper towels.

Note:

- Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame.

- For automated Washing: It is recommended that the washer be set for a soaking time of 10 seconds or shaking time of 5 seconds between washes.

5. Add **100 µl** of **HRP conjugate mixture** into each well. Cover and incubate for **1h at room temperature**.
6. Prepare Substrate Solution *no more than 15 minutes* before end of HRP conjugate mixture incubation
7. Aspirate each well and **wash as step 4**.
8. Add **100 µl** of **Substrate solution** mixture to each well. Incubate for **15 minutes at room temperature in dark**.
9. Add **100 µl** of **Stop Solution** to each well. The color of the solution should change from blue to yellow.
10. Read the OD with a microplate reader at **450 nm** immediately. It is recommended reading the absorbance *within 30 min* after adding stop solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards and samples.
2. For semi-quantitative assay, 8 rough curves for 8 cytokines can be generated from OD readings of high concentration standard and low

concentration standard mixture. The approximate cytokine concentration can be obtained from the rough curves. As the standard curves might not be perfectly straight, the concentration obtained from a rough curve derived from 2 points would not be very accurate.

3. To obtain more accurate results, more dilution points can be used when generating standard curves.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data shows the OD readings of a run of this multiplex ELISA with multiple dilutions using standard diluent I. It is for demonstration purpose only and cannot be used to replace standard curve for testing. Each investigators have to assay standards along with test samples.

Cytokines	1	1:2	1:4	1:8	1:16	1:32	1:64	Standard Diluent I
GM-CSF	1.828	1.284	0.826	0.486	0.322	0.218	0.188	0.126
IFN-γ	1.765	1.147	0.698	0.417	0.277	0.212	0.17	0.126
IL-4	0.961	0.493	0.312	0.164	0.125	0.093	0.082	0.066
IL-6	1.99	1.42	0.958	0.59	0.377	0.247	0.18	0.112
IL-10	1.478	1.065	0.689	0.367	0.223	0.152	0.124	0.065
IL-12	1.639	1.133	0.625	0.385	0.264	0.187	0.13	0.087
MCAF	2.115	1.518	0.947	0.533	0.334	0.226	0.188	0.138
TNF-α	1.843	1.231	0.765	0.463	0.332	0.249	0.201	0.163

Detection Range

GM-CSF	750- 23.44 pg/ml	IL10	550- 17.19 pg/ml
IFNgamma	950- 29.69 pg/ml	IL12	430- 13.44 pg/ml
IL4	1075- 33.59 pg/ml	MCP1	1150- 35.94 pg/ml

IL6	225- 7.03 pg/ml	TNFalpha	840- 26.25 pg/ml