Human Inflammatory Cytokine Multiplex ELISA Kit (IL1 alpha, IL1 beta, IL6, IL8, GM-CSF, IFN-gamma, MCAF and TNF-alpha)

Multiplex Enzyme Immunoassay for the semi-quantification of IL1 alpha, IL1 beta, IL6, IL8, GM-CSF, IFN-gamma, MCAF and TNF-alpha in serum, plasma cell culture supernatant and other biological samples.

Catalog number: ARG80929

Package: 96 wells

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INTRODUCTION

A proinflammatory cytokine or more simply an inflammatory cytokine is a type of signaling molecule (a cytokine) that is excreted from immune cells like helper T cells (Th) and macrophages, and certain other cell types that promote inflammation. They include interleukin-1 (IL1), IL12, and IL18, tumor necrosis factor (TNF), interferon gamma (IFN-gamma), and granulocyte-macrophage colony stimulating factor and play an important role in mediating the innate immune response. Inflammatory cytokines are predominantly produced by and involved in the upregulation of inflammatory reactions.

A proinflammatory cytokine or an inflammatory cytokine is a type of cytokine (signaling molecule) that is excreted from immune cells and certain other cell types that promotes inflammation. Inflammatory cytokines are predominantly produced by helper T cells (Th) and macrophages and involved in the upregulation of inflammatory reactions. Therapies to treat inflammatory diseases include monoclonal antibodies that either neutralize inflammatory cytokines or their receptors.

Proinflammatory cytokines include interleukin-1 (IL1), IL12, and IL18, tumor necrosis factor (TNF), interferon gamma (IFN-gamma), and granulocyte-macrophage colony stimulating factor.

Inflammatory cytokines play a role in initiating the inflammatory response and to regulate the host defense against pathogens mediating the innate immune response. Some inflammatory cytokines have additional roles such as acting as growth factors. Pro-inflammatory cytokines such as IL1β, IL6, and TNF-α also trigger pathological pain. While IL1β is released by monocytes and macrophages, it is also present in nociceptive DRG neurons. IL6 plays a role in

neuronal reaction to an injury. TNF-α is a well-known proinflammatory cytokine present in neurons and the glia. TNF-α is often involved in different signaling pathways to regulate apoptosis in the cells. Excessive chronic production of inflammatory cytokines contribute to inflammatory diseases. That have been linked to different diseases, such as atherosclerosis and cancer. Dysregulation of proinflammatory cytokines have also been linked to depression and other neurological diseases. A balance between proinflammatory and anti-inflammatory cytokines is necessary to maintain health. Aging and exercise also play a role in the amount of inflammation from the release of proinflammatory cytokines. [From wikipedia]

PRINCIPLE OF THE ASSAY

This is a multiplex enzyme immunoassay for the semi-quantification of Pro-inflammatory Cytokines Interleukin-1 alpha (IL1 alpha), Interleukin-1 beta (IL1 beta), Interleukin-6 (IL6), Interleukin-8 (IL8), Interferon Gamma (IFN-gamma), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Monocyte Chemotactic and Activating Factor (MCAF), and Tumor Necrosis Factor alpha (TNF-alpha). This assay employs the semi-quantitative sandwich enzyme immunoassay technique. Monoclonal antibody specific for IL1 alpha, IL1 beta, IL6, IL8, GM-CSF, IFN-gamma, MCAF and TNF-alpha has been pre-coated onto 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: IL1 alpha: 860 pg IL1 beta: 1400 pg IL6: 680 pg IL8: 1700 pg GM-CSF: 950 pg IFN-gamma: 550 pg MCAF: 2000 pg TNF-alpha: 1050 pg	4°C
Biotin 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 testing)	25 ml (Ready-to-use)	4°C

Standard Diluent II (for cell culture supernatant testing)	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	IL-1a	IL-1a	IL-1a	IL-1a	IL-1a	IL-1a	IL-1a	IL-1a	IL-1a	IL-1a	IL-1a	IL-1a
B	IL-1b	IL-1b	IL-1b	IL-1b	IL-1b	IL-1b	IL-1b	IL-1b	IL-1b	IL-1b	IL-1b	IL-1b
C	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6
D	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8
E	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF
F	IFN-g	IFN-g	IFN-g	IFN-g	IFN-g	IFN-g	IFN-g	IFN-g	IFN-g	IFN-g	IFN-g	IFN-g
G	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF
H	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a

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 ≤-70°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 ≤-70°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 ≤-70°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 of 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 10 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: IL1a	430	215	107.5	53.75	26.88	13.44	6.72
B:IL1b	700	350	175	87.5	43.75	21.88	10.94
C:IL6	340	170	85	42.5	21.25	10.63	5.31
D:IL8	850	425	212.5	106.25	53.13	26.56	13.28
E:GM-CSF	475	237.5	118.75	59.38	29.69	14.84	7.42
F:IFN-g	275	137.5	68.75	34.38	17.19	8.59	4.30
G:MCAF	1000	500	250	125	62.5	31.25	15.63
H:TNF-a	525	262.5	131.25	65.63	32.81	16.41	8.20

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 10 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.

Detection Range

IL1 alpha: 6.72- 430 pg/ml

IL1 beta: 10.94- 700 pg/ml

IL6: 5.31- 340 pg/ml

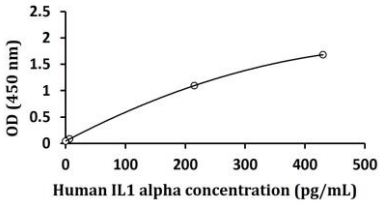
IL8: 13.28- 850 pg/ml

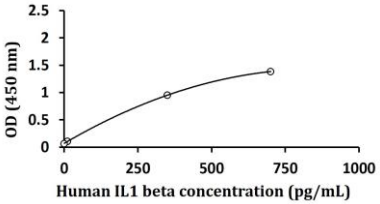
GM-CSF: 7.42- 475 pg/ml

IFN-gamma: 4.3- 275 pg/ml

MCAF: 15.63- 1000 pg/ml

TNF-alpha: 8.2- 525 pg/ml


OD (450 nm)
2.5
2
1.5
1
0.5
0
0
100
200
300
400
500
Human IL1 alpha concentration (pg/mL)


OD (450 nm)
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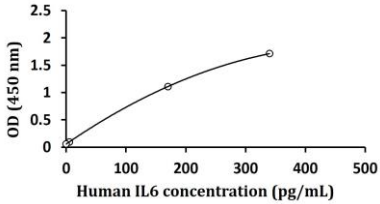
2
1.5
1
0.5
0
0
250
500
750
1000
Human IL1 beta concentration (pg/mL)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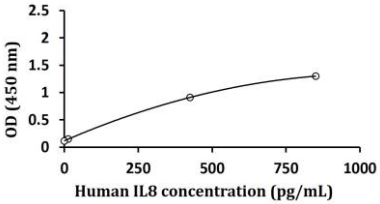
1.5
1
0.5
0
0
100
200
300
400
500
Human IL6 concentration (pg/mL)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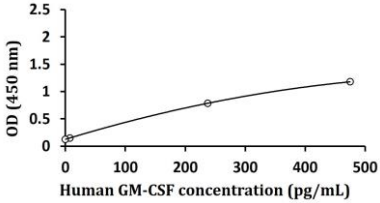
2
1.5
1
0.5
0
0
250
500
750
1000
Human IL8 concentration (pg/mL)


OD (450 nm)
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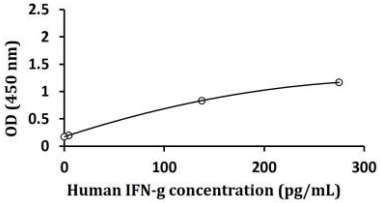
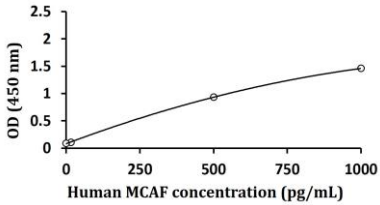
2
1.5
1
0.5
0
0
100
200
300
400
500
Human GM-CSF concentration (pg/mL)


OD (450 nm)
2.5
2
1.5
1
0.5
0
0
100
200
300
Human IFN-g concentration (pg/mL)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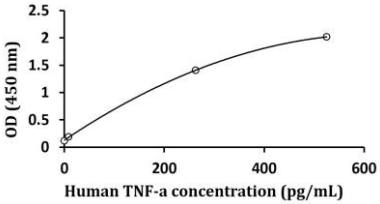
2
1.5
1
0.5
0
0
250
500
750
1000
Human MCAF concentration (pg/mL)


OD (450 nm)
2.5
2
1.5
1
0.5
0
0
200
400
600
Human TNF-a concentration (pg/mL)