arigoPLEX® Human Proinflammatory Cytokine Multiplex ELISA Kit (IFN gamma, IL1 beta, IL6, TNF alpha)

arigoPLEX® Human Proinflammatory Cytokine Multiplex ELISA Kit (IFN gamma, IL1 beta, IL6, TNF alpha) is an Enzyme Immunoassay kit for the quantification of Human Proinflammatory Cytokine (IFN gamma, IL1 beta, IL6, TNF alpha) in serum, plasma and cell culture supernatants.

Catalog number: ARG82862

Package: 96 wells

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INTRODUCTION

An inflammatory cytokine or proinflammatory cytokine is a type of signaling molecule that is secreted from immune cells like helper T cells (Th) and macrophages, and certain other cell types that promote inflammation. They include interleukin-1 (IL-1), IL-6, IL-12, and IL-18, tumor necrosis factor alpha (TNF-α), interferon gamma (IFNγ), and granulocyte-macrophage colony stimulating factor (GM-CSF) 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.

Excessive chronic production of inflammatory cytokines contribute to inflammatory diseases, that have been linked to different diseases, such as atherosclerosis and cancer. Dysregulation has 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. [Provide by Wikipedia: proinflammatory cytokines]

IFNγ, or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral, some bacterial and protozoan infections. IFNγ is an important activator of macrophages and inducer of major histocompatibility complex class II molecule expression. Aberrant IFNγ expression is associated with a number of autoinflammatory and autoimmune diseases. The importance of IFNγ in the immune system stems in part from its ability to inhibit viral replication directly, and most importantly from its immunostimulatory and immunomodulatory effects. IFNγ is produced

predominantly by natural killer cells (NK) and natural killer T cells (NKT) as part of the innate immune response, and by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops as part of the adaptive immune response. IFNγ is also produced by non-cytotoxic innate lymphoid cells (ILC), a family of immune cells first discovered in the early 2010s. [Provide by Wikipedia: Interferon gamma]

Interleukin 1 beta (IL-1β) also known as leukocytic pyrogen, leukocytic endogenous mediator, mononuclear cell factor, lymphocyte activating factor and other names, is a cytokine protein that in humans is encoded by the IL1B gene. There are two genes for interleukin-1 (IL-1): IL-1 alpha and IL-1 beta (this gene). IL-1β precursor is cleaved by cytosolic caspase 1 (interleukin 1 beta convertase) to form mature IL-1β. [Provide by Wikipedia: IL-1bata]

IL-6 is secreted by macrophages in response to specific microbial molecules, referred to as pathogen-associated molecular patterns (PAMPs). These PAMPs bind to an important group of detection molecules of the innate immune system, called pattern recognition receptors (PRRs), including Toll-like receptors (TLRs). These are present on the cell surface and intracellular compartments and induce intracellular signaling cascades that give rise to inflammatory cytokine production. IL-6 is an important mediator of fever and of the acute phase response. [Provide by Wikipedia: IL-6]

Tumor necrosis factor (TNF, cachexin, or cachectin; often called tumor necrosis factor alpha or TNF-α) is a cytokine – a small protein used by the immune system for cell signaling. If macrophages (certain white blood cells) detect an infection, they release TNF to alert other immune system cells as part of an

inflammatory response. TNF is a member of the TNF superfamily, which consists of various transmembrane proteins with a homologous TNF domain. [Provide by Wikipedia: TNF-alpha]

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. The antibodies specific to IFN gamma, IL1 beta, IL-6 and TNF alpha has been pre-coated onto a microtiter plate. Standards Mixture and samples are pipetted into the wells and any target cytokines present is bound by the immobilized specific antibodies. After incubation, added Mixed Antibody-Conjugate to each well and incubate. After washing away any unbound substances, the HRP Solution is added to the wells and incubated. The wells are thoroughly washed to remove all unbound HRP Solution. A TMB substrate is added to the wells and color develops in proportion to the amount of target cytokines in the initial step. The color development is stopped by the addition of STOP solution and the intensity of the color is measured at a wavelength of O.D. 450 nm. The concentration of human proinflammatory cytokines in the samples is then determined by comparing the O.D. 450 nm of samples to the standard curve.e.

MATERIALS PROVIDED & STORAGE INFORMATION

Use the kit before expiration date. Opened kits retain activity for 8 weeks if stored as described above.

Component	Quantity	Storage information
Antibody Coated Microplate [1]	8 X 12 strips	4°C
Standards Mixture [2]	3 vials (lyophilized)	4°C
100X Antibody Conjugate Mixture	120 µL	≤ -20°C
100X HRP-Streptavidin Solution	120 µL	4°C
Standard / Sample Diluent Buffer	30 mL	4°C
Antibody Diluent Buffer	35 mL	4°C
20X Wash Buffer	45 mL	4°C
TMB substrate	12 mL (ready to use)	4°C (protect from light)
STOP solution	12 mL (ready to use)	4°C
Plate sealer	3 adhesive strips	Room temperature

Note:

1. The Antibody Coated Microplate contains twelve 8-well ELISA strips. Each of the eight wells has been coated with a different antibody specific to one of the 4 cytokines as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ
B	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β
C	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6
D	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α
E	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ
F	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β
G	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6
H	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α	TNF α

Standards Mixture each vial contains a buffered protein base and four cytokines at different amount: IFN-gamma, 500 pg / IL-1 beta, 500 pg / IL6, 1000 pg / TNF-alpha, 1000 pg.

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of reading at 450 nm
- Deionized or distilled water
- Pipettes and pipette tips
- Multichannel micropipette reservoir
- Microtiter plate washer (optional)

TECHNICAL NOTES AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store 100X Antibody Conjugate at ≤ -20°C. Store other component at 2-8°C at all times.
- Prior to beginning the assay procedure, bring all reagents and required number of strips to room temperature (20-25°C).
- Unused wells must be stored at 2-8 °C in the sealed foil pouch and used in the frame provided.
- All reagents must be mixed without foaming and briefly spin down the all vials before use.
- If crystals are observed in the 20X Wash Buffer, warm to 37°C until the crystals are completely dissolved.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.

- Ensure complete reconstitution and dilution of reagents prior to use.
- Take care not to contaminate the mixed TMB Substrate. Do not expose the mixed TMB solution to glass, foil or metal. If the solution is blue before use, DO NOT USE IT.
- Change pipette tips between the addition of different reagent or samples.
- Taping the well strips together with lab tape can be done as an extra precaution to avoid plate strips from coming loose during the procedure.

SAMPLE COLLECTION & STORAGE INFORMATION

The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design.

Cell Culture Supernatants - Remove particulates by centrifugation for 10 min at 1500 x g at 4°C and aliquot & store samples at-20°C up to 1 month or-80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Collect serum and assay immediately or aliquot & store samples at-20°C up to 1 month or-80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g. within 30 minutes of collection. Collect the supernatants and assay immediately or aliquot and store samples at -20°C up to 1 month or -80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Note:

1. Do not use haemolytic, icteric or lipaemic specimens.
2. Samples containing sodium azide should not be used in the assay.

3. Avoid disturbing the white buffy layer when collection serum / plasma sample.
4. To obtain the data of each cytokine, **0.2 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.

REAGENT PREPARATION

- **1X Wash Buffer**: Dilute **20X** Wash Buffer into **distilled water** to yield 1X Wash Buffer. (E.g., add 25 mL of 10X Wash Buffer into 475 mL of distilled water to a final volume of 500 mL) The 1X Wash Buffer is stable for up to 4 weeks at 2-8°C. Mix well before use.
- **1X Antibody Conjugate Mixture:** It is recommended to prepare this reagent immediately prior to use and use it within 20 min after preparation. Dilute **100X** Antibody Conjugate Mixture concentrate into **Antibody Diluent Buffer** to yield 1X detection antibody solution. (e.g. 12 µl of 100X Antibody Conjugate Mixture concentrate + 1188 µl of Antibody Diluent Buffer)
- **1X HRP-Streptavidin Solution:** It is recommended to prepare this reagent immediately prior to use and use it within 20 min after preparation. Dilute **100X** HRP-Streptavidin solution into **Antibody Diluent Buffer** to yield 1X HRP-Streptavidin Solution buffer. (e.g. 10 µl of 100X HRP-Streptavidin solution + 990 µl of Antibody Diluent Buffer)
- **Sample:** Diluent serum and plasma samples with equal volume of **Standard / Sample Diluent Buffer** before assay (1:1, dilution factor=2). If the initial assay found samples contain proteins higher than the highest standard, the samples can be diluted with **Standard / Sample Diluent Buffer** and then

re-assay the samples. For the calculation of the concentrations this dilution factor has to be taken into account. Cell culture supernatants could be assayed directly. (It is recommended to do pre-test to determine the suitable dilution factor).

- Standards Mixture:
  A. Add 1 mL of **Standard / Sample Diluent Buffer** to reconstitute the lyophilized Standard Mixture to obtain the high concentration stock of 4 cytokines at different concentrations (see table below). Allow solution to sit for at least **15 minutes** with gentle agitation prior to making further dilutions.
  B. Use the above high concentration Standards Mixture and a 32-fold diluted low concentration Standards Mixture to test together with up to 22 test samples. If more accurate results are required, a two-fold serial dilution with Dilution Buffer can generate a more accurate standard curve. However, the number of test samples will be reduced.

The concentrations of the 4 cytokines in different dilutions of the Standards Mixture are listed as below:

Cytokines (pg / mL)	High Conc. Standard Mixture	1:2	1:4	1:8	1:16	1:32 (Low Conc. Standard Mixture)	1:64
IFN-γ	500	250	125	62.5	31.25	15.6	7.8
IL1β	500	250	125	62.5	31.25	15.6	7.8
IL6	1000	500	250	125	62.5	31.25	15.6
TNF-α	1000	500	250	125	62.5	31.25	15.6

The concentrations of the 4 cytokines in different dilutions of the Standards Mixture are listed.

ASSAY PROCEDURE

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

1. Add **100 µL** of the **Standards Mixture** or **diluted samples** to the Antibody Coated microplate.

   Note: To obtain the approximate concentrations of 4 cytokines on 22 test samples, the **low concentration standard mixture** (**S1**, 1:32 from high concentration mixture), the **high concentration Standards Mixture** (**S2**) and test samples (T1 to T22) can be added as the scheme below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
B	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
C	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
D	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
E	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
F	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
G	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
H	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22

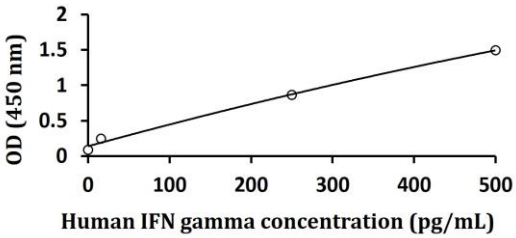
2. Cover the plate and incubate for **2 hours** at **room temperature**.
3. Aspirate each well and wash, repeating the process 4 times for a total **5 washes**. Wash by filling each well with 1× Wash Buffer **(300 µL)** using a squirt bottle, manifold dispenser, or autowasher. Keep the wash buffer in the wells for 30-60 sec before remove. 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.
4. Add **100 µL** of **1X Antibody Conjugate Mixture** to each wells.
5. Cover the plate and Incubate for **1 hour** at **room temperature**.

6. Aspirate each well and **wash as step 3.**
7. Add **100 µL** of **1X HRP-Streptavidin Solution** to each well. Cover the plate and incubate for **1 hour** at **room temperature**.
8. Aspirate each well and **wash as step 3.**
9. Add **100 µL** of **TMB Substrate** to each well. Cover and incubate for **15 minutes** at **room temperature** in the dark.
10. Immediately Add **100 µL** of **Stop Solution** to each well. The color of the solution should change from blue to yellow.
11. Read the OD with a microplate reader at **450 nm** immediately. It is recommended reading the absorbance **within 10 minutes** after adding the stop solution.

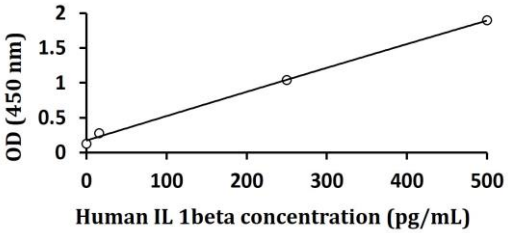
EXAMPLE OF TYPICAL STANDARD VALUES

The following table shows the OD readings of a run of this multiplex ELISA with two fold-serial diluted standards. It is for demonstration purpose only and cannot be used to replace the standard curve for testing. For each investigation, standards have to be assayed along with test samples and only the curve generated from the same test can be used.

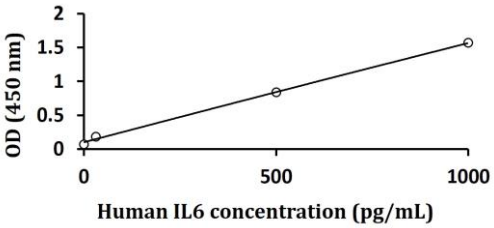
Example of Human IFN gamma standard curve



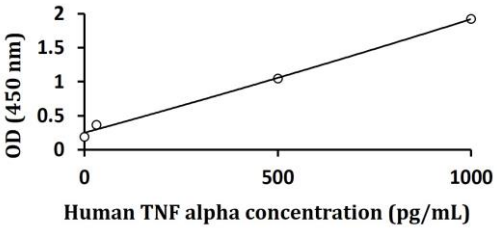
Example of Human IL-1beta standard curve



Example of Human IL6 standard curve



Example of Human TNF alpha standard curve



CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards and samples.
2. The 4 curves for 4 targets can be generated from OD readings of high concentration standard and low concentration standard mixture. The standard curves might not be perfectly straight. To obtain more accurate results, more dilution points can be used when generating standard curves.
3. 4 Parameter Logistics is the preferred method for the result calculation. Other data reduction functions may give slightly different results.
4. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for details. (https://www.arigobio.com/elisa-analysis)
5. If the samples have been diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure.