arigoPLEX® Human M1/M2 Cytokines multiplex ELISA Kit (IL4, IL6, IL10, TNF alpha)

arigoPLEX® Human M1/M2 Cytokines multiplex ELISA Kit (IL4, IL6, IL10, TNF alpha) is an Enzyme Immunoassay kit for the quantification of IL4, IL6, IL10, TNF alpha in serum, plasma and cell culture supernatant.

Catalog number: ARG83335

Package: 96 wells

Lot: 354476

For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Macrophages (abbreviated as Mϕ, MΦ or MP) are a type of white blood cell of the immune system that engulfs and digests anything that does not have, on its surface, proteins that are specific to healthy body cells, including cancer cells, microbes, cellular debris, foreign substances, etc. The process is called phagocytosis, which acts to defend the host against infection and injury.

Beyond increasing inflammation and stimulating the immune system, macrophages also play an important anti-inflammatory role and can decrease immune reactions through the release of cytokines. Macrophages that encourage inflammation are called M1 macrophages, whereas those that decrease inflammation and encourage tissue repair are called M2 macrophages. This difference is reflected in their metabolism; M1 macrophages have the unique ability to metabolize arginine to the "killer" molecule nitric oxide, whereas M2 macrophages have the unique ability to metabolize arginine to the "repair" molecule ornithine. However, this dichotomy has been recently questioned as further complexity has been discovered.

M1 "killer" macrophages are activated by LPS and IFN-gamma. M1 macrophages have pro-inflammatory, bactericidal, and phagocytic functions. In contrast, the M2 "repair" designation (also referred to as alternatively activated macrophages) broadly refers to macrophages that function in constructive processes like wound healing and tissue repair, and those that turn off damaging immune system activation by producing anti-inflammatory cytokines like IL10. M2 is the phenotype of resident tissue macrophages, and can be further elevated by IL4. M2 macrophages produce high levels of IL10,

TGF-beta and low levels of IL12. Tumor-associated macrophages are mainly of the M2 phenotype, and seem to actively promote tumor growth.

Macrophages exist in a variety of phenotypes which are determined by the role they play in wound maturation. Phenotypes can be predominantly separated into two major categories; M1 and M2. M1 macrophages are the dominating phenotype observed in the early stages of inflammation and are activated by four key mediators: interferon-γ (IFN-γ), tumor necrosis factor (TNF), and damage associated molecular patterns (DAMPs). These mediator molecules create a pro-inflammatory response that in return produce pro-inflammatory cytokines like Interleukin-6 and TNF. Unlike M1 macrophages, M2 macrophages secrete an anti-inflammatory response via the addition of Interleukin-4 or Interleukin-13. They also play a role in wound healing and are needed for revascularization and reepithelialization. M2 macrophages are divided into four major types based on their roles: M2a, M2b, M2c, and M2d. How M2 phenotypes are determined is still up for discussion but studies have shown that their environment allows them to adjust to whichever phenotype is most appropriate to efficiently heal the wound.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. The antibodies specific to Human IL4, IL6, IL10, 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-Streptavidin Solution is added to the wells and incubated. The wells are thoroughly washed to remove all unbound HRP-Streptavidin 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 M1/M2 Cytokines in the samples is then determined by comparing the O.D. 450 nm of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Upon received, store 100X Antibody Conjugate at ≤-20°C.

Store other components at 2-8°C at all times. Use the kit before expiration date.

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
1000X HRP-Streptavidin Solution	15 µL	4°C
Standard / Sample Diluent Buffer	30 mL (ready to use)	4°C
Antibody Diluent Buffer	35 mL (ready to use)	4°C
10X Wash Buffer	60 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	4 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	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4
B	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6
C	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10
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	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4
F	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6
G	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10
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

2. Standards Mixture each vial contains a buffered protein base and four cytokines at different amount: IL4: 500 pg; IL6: 1500 pg; IL10: 500 pg; TNF alpha: 1500 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

TECHNICAL NOTES AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Upon received, 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 10X 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.
- All reagents must be mixed without foaming before use.
- 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 **10X** Wash Buffer into distilled water to yield 1X Wash Buffer. (E.g., add 50 mL of 10X Wash Buffer into 450 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 1000X HRP-Streptavidin Solution into **Antibody Diluent Buffer** to yield 1X HRP-Streptavidin Solution buffer (e.g. 1 µl of 1000X HRP-Streptavidin Solution + 999 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). Brief vortex and allow the stock standard to sit for at least 15 minutes with gentle agitation to make sure the standard is dissolved completely before making serial 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 Stock	1:2	1:4	1:8	1:16	1:32 (Low Conc. Standard Mixture)	1:64
IL4	500	250	125	62.5	31.3	15.6	7.8
IL6	1500	750	375	187.5	93.9	46.8	23.4
IL10	500	250	125	62.5	31.3	15.6	7.8
TNF alpha	1500	750	375	187.5	93.9	46.8	23.4

ASSAY PROCEDURE

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

1. Add **100 µL** of the **Standards Mixture** and **diluted samples** in the wells of 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

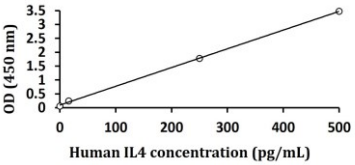
2. Cover the plate and incubate for **2.5 hours** at **room temperature**.

3. Aspirate each well and wash, repeating the process 5 times for a **total 6 washes**. Wash by filling each well with **1X 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 well.
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 **15-25 minutes** at **37°C** in the dark.
   (Note: The incubation time is for reference only, the optimal incubation time should be determined by end user. And the shades of blue color can be seen in the wells with the four highest concentrated standards)
10. Add **100 µL** of **Stop Solution** to each well, gently tap the plate to mix well. The color of the solution should change from blue to yellow. Gently tap the plate to ensure thorough mixing.
11. Read the OD with a microplate reader at **450 nm** immediately. It is recommended reading the absorbance **within 30 minutes** after adding the stop solution.

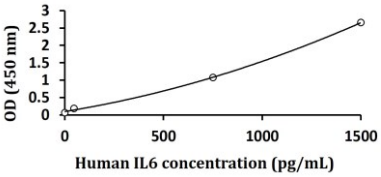
EXAMPLE OF TYPICAL STANDARD VALUES

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.

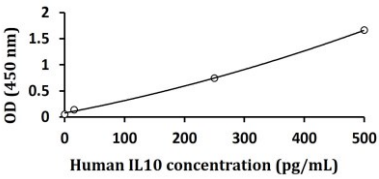
Example of Human IL4 standard curve



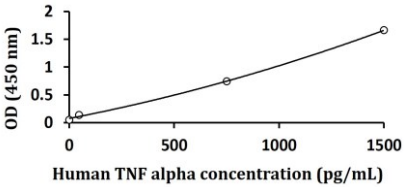
Example of Human IL6 standard curve



Example of Human IL10 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.