arigoPLEX® Rat Inflammatory Cytokine Multiplex ELISA Kit (IL1 beta, IL6, IL10, TNF alpha)

arigoPLEX® Rat Inflammatory Cytokine Multiplex ELISA Kit (IL1 beta, IL6, IL10, TNF alpha) is an Enzyme Immunoassay kit for the semi-quantification of Rat inflammatory Cytokine (IL1 beta, IL6, IL10, TNF alpha) in serum, plasma and cell culture supernatants.

Catalog number: ARG83004

Package: 96 wells

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

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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 (IL1), IL6, IL12, and IL18, tumor necrosis factor alpha (TNF alpha) and interferon gamma (IFN gamma) 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. On the other hand, interleukin-10 (IL10), a cytokine with anti-inflammatory properties, play an important role in infection by regulating the immune responses to pathogens.

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]

Interleukin-1 beta (IL1β) 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 (IL1): IL1 alpha and IL1 beta (this gene). IL1 beta precursor is cleaved by cytosolic caspase 1 (interleukin-1 beta

convertase) to form mature IL1 beta. [Provide by Wikipedia: IL1bata]

IL6 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. IL6 is an important mediator of fever and of the acute phase response. [Provide by Wikipedia: IL6]

Interleukin-10 (IL10), also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. In humans, interleukin 10 is encoded by the IL10 gene. IL10 signals through a receptor complex consisting of two IL10 receptor-1 and two IL10 receptor-2 proteins. Consequently, the functional receptor consists of four IL10 receptor molecules. IL10 binding induces STAT3 signalling via the phosphorylation of the cytoplasmic tails of IL10 receptor 1 + IL10 receptor 2 by JAK1 and Tyk2 respectively. [Provide by Wikipedia: Interleukin 10]

Tumor necrosis factor (TNF, cachexin, or cachectin; often called tumor necrosis factor alpha or TNF alpha) 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 IL1 beta, IL6, IL10 and TNF alpha have 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 Conjugate is added into the wells and incubated. The wells are thoroughly washed to remove all unbound HRP Conjugate. 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 rat proinflammatory 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 10X 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]	2 vial (lyophilized)	4°C
10X Antibody Conjugate Mixture	1.2 mL	≤ -20°C
40X HRP-Streptavidin concentrate	300 µL	4°C
Standard/Sample Diluent Buffer	30 mL	4°C
Antibody Diluent Buffer	50 mL	4°C
Serum Diluent Buffer	10 mL	4°C
10X Wash Buffer	2 X 50 mL	4°C
TMB substrate	12 mL (ready to use)	4°C (protect from light)
STOP solution	6 mL (ready to use)	4°C
Plate sealer	3 strips	RT

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	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β
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	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β	IL1β
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: IL1 beta: 4000 pg; IL6: 8000 pg; IL10: 4000 pg; TNF alpha: 4000 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.
- Upon received, store 10X Antibody Conjugate at ≤ -20°C. Store other component at 2-8°C at all times.
- The reconstituted standard stock should be aliquoted and stored at -80°C.
- 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:

a) Do not use haemolytic, icteric or lipaemic specimens.
b) Samples containing sodium azide should not be used in the assay.
c) Avoid disturbing the white buffy layer when collection serum / plasma sample.

d) To obtain the data of each cytokine, at least **0.1 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 10X Antibody Conjugate Mixture concentrate into Antibody Diluent Buffer to yield 1X detection antibody solution. (e.g. 100 µl of 10X Antibody Conjugate Mixture concentrate + 90 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 40X HRP-Streptavidin concentrate solution into Antibody Diluent Buffer to yield 1X HRP-Streptavidin Solution buffer. (e.g. 10 µl of 40X HRP-Streptavidin concentrate solution + 390 µl of Antibody Diluent Buffer)
- **Sample**: Before assay, serum samples are recommended to premix with Serum Diluent Buffer. (eg. Premix 25 µL serum with 25 µL Serum Diluent Buffer.) Plasma samples are recommended diluent with Standard/Sample Diluent Buffer at least 1:3. (eg. Mix 25 µL serum with 75 µL Diluent Buffer.)

- 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. The reconstituted Standard Mixture high concentration stock could be stored at-80°C for up to 30 days.

  B. For semi-quantitative assay, 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
IL1β	4000	2000	1000	500	250	125	62.5
IL6	8000	4000	2000	1000	500	250	125
IL10	4000	2000	1000	500	250	125	62.5
TNF α	4000	2000	1000	500	250	125	62.5

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 **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, stock**) and test samples (T1 to T22) can be added as the scheme as below:

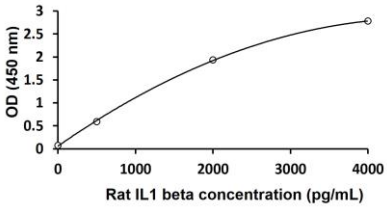
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
B	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
C	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
D	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
E	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
F	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
G	S2	T2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

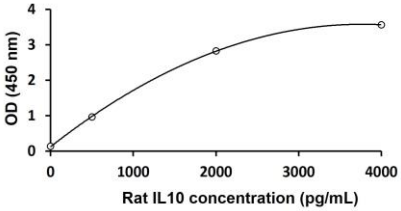
2. Cover the plate and incubate for **2 hours** at **room temperature**.
3. Aspirate each well and wash, repeating the process 2 times for a **total 4 time washes**. Wash by filling each well with **1× Wash Buffer (300 µL)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of 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.
4. Add **100 µL** of **1X Antibody Conjugate Mixture** to each wells.
5. Cover the plate and Incubate for **2 hours** at **room temperature**.
6. Aspirate each well and **wash as step 3** but wash for a **total 6 time washes**.

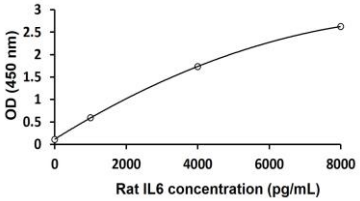
7. Add **100 µL** of **1X HRP-Streptavidin Solution** to each well. Cover the plate and incubate for **20 minutes** at room temperature.
8. Aspirate each well and **wash as step 3** but wash for a **total 8 time washes**.
9. Add **100 µL** of **TMB Substrate** to each well. Cover and incubate for **10-20 minutes** at **room temperature** in the dark.
10. Immediately Add **50 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 30 minutes** after adding the stop solution.

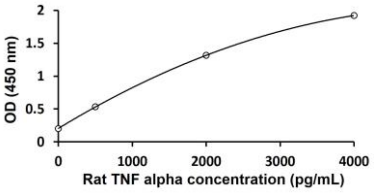
EXAMPLE OF TYPICAL STANDARD VALUES

The following table shows the OD readings of a run of this multiplex ELISA with 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.









CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards and samples.
2. For semi-quantitative assay, 4 rough curves for 4 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. 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. Serum/plasma sample has multiplied the dilution factor (Dilution factor =4). If the samples have been further diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure.