arigoPLEX® Mouse Th1/Th2/Th17/Treg Cytokine Multiplex ELISA Kit (IFN gamma, IL4, IL17, IL10)

arigoPLEX® Mouse Th1/Th2/Th17/Treg Cytokine Multiplex ELISA Kit (IFN gamma, IL4, IL17, IL10) is an Enzyme Immunoassay kit for the quantification of IFN gamma, IL4, IL17, IL10 in serum, plasma and cell culture supernatant.

Catalog number: ARG82914

Package: 96 wells

Lot. 354872

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

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INTRODUCTION

T cells are grouped into a series of subsets based on their function. CD4 and CD8 T cells are selected in the thymus, but undergo further differentiation in the periphery to specialized cells which have different functions. T cell subsets were initially defined by function, but also have associated gene or protein expression patterns.

T helper cells (TH cells) assist other lymphocytes, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages. These cells are also known as $CD4^+$ T cells as they express the CD4 glycoprotein on their surfaces. Helper T cells become activated when they are presented with peptide antigens by MHC class II molecules, which are expressed on the surface of antigen-presenting cells (APCs). Once activated, they divide rapidly and secrete cytokines that regulate or assist the immune response. These cells can differentiate into one of several subtypes, which have different roles. Cytokines direct T cells into particular subtypes.

Cell type	Cytokine Produced	Role in immune defense
Th1	IFN gamma, IL2	Produce an inflammatory response, key for defense against intracellular bacteria, viruses and cancer.
Th2	IL4, IL5, IL13	Immunologically important against extracellular pathogens, such as worm infections
Th17	IL17A, IL17F, IL22	Defense against gut pathogens and at mucosal barriers
Treg	TGF-beta, IL10	Tregs are able to inhibit T cell proliferation and cytokine production and play a critical role in preventing autoimmunity.

Cytotoxic T cells (TC cells, CTLs, T-killer cells, killer T cells) destroy virus-infected cells and tumor cells, and are also implicated in transplant rejection. These cells are defined by the expression of the CD8 protein on their cell surface. Cytotoxic T cells recognize their targets by binding to short peptides (8-11 amino acids in length) associated with MHC class I molecules, present on the surface of all nucleated cells. Cytotoxic T cells also produce the key cytokines IL2 and IFNγ. These cytokines influence the effector functions of other cells, in particular macrophages and NK cells.

Regulatory T cells are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell–mediated immunity toward the end of an immune reaction and to suppress autoreactive T cells that escaped the process of negative selection in the thymus.

Two major classes of $CD4^+$ Treg cells have been described—$FOXP3^+$ Treg cells and $FOXP3^-$ Treg cells.

Regulatory T cells can develop either during normal development in the thymus, and are then known as thymic Treg cells, or can be induced peripherally and are called peripherally derived Treg cells. These two subsets were previously called "naturally occurring" and "adaptive" (or "induced"), respectively. Both subsets require the expression of the transcription factor FOXP3 which can be used to identify the cells. Mutations of the FOXP3 gene can prevent regulatory T cell development, causing the fatal autoimmune disease IPEX.

Several other types of T cells have suppressive activity, but do not express FOXP3 constitutively. These include Tr1 and Th3 cells, which are thought to originate during an immune response and act by producing suppressive molecules. Tr1 cells are associated with IL10, and Th3 cells are associated with TGF-beta. [Provide by Wikipedia: T cell]

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. The antibodies specific to mouse IFN gamma, IL4, IL17 and IL10 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 and washing step, 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 cytokines produced by subsets of help T cell and Treg cell 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	2 X 35 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	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	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4
C	IL17	IL17	IL17	IL17	IL17	IL17	IL17	IL17	IL17	IL17	IL17	IL17
D	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10
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	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4
G	IL17	IL17	IL17	IL17	IL17	IL17	IL17	IL17	IL17	IL17	IL17	IL17
H	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10

2. Standards Mixture each vial contains a buffered protein base and four cytokines at different amount: IFN gamma: 1000 pg; IL4: 500 pg; IL17: 500 pg; IL10: 500 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 100X Antibody Conjugate Mixture 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 (22-26°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.
- 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). Cell culture supernatant samples maybe used directly. 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 Mixture	1:2	1:4	1:8	1:16	1:32 (Low Conc. Standard Mixture)	1:64
IFN γ	1000	500	250	125	62.5	31.25	15.6
IL4	500	250	125	62.5	31.25	15.6	7.8
IL17	500	250	125	62.5	31.25	15.6	7.8
IL10	500	250	125	62.5	31.25	15.6	7.8

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (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

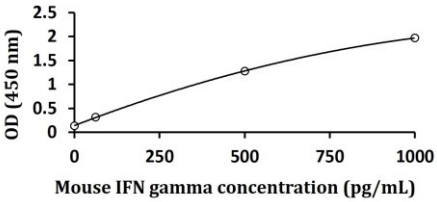
2. Cover the plate and incubate for **2 hours** at **room temperature**.
3. Aspirate each well and wash, repeating the process 5 times for a **total 6 washes.** Wash by filling each well with **1X Wash Buffer (300 µL)** using a squirt bottle, manifold dispenser, or autowasher. Keep the wash buffer in 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 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, but wash for a total 7 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. 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.
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.

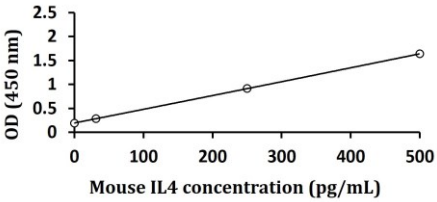
EXAMPLE OF TYPICAL STANDARD VALUES

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

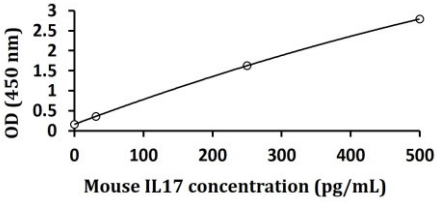
Example of Mouse IFN gamma standard curve



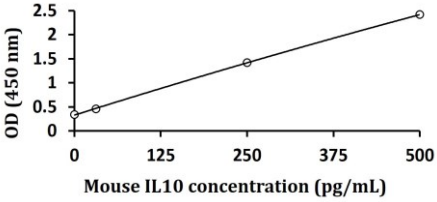
Example of Mouse IL4 standard curve



Example of Mouse IL17 standard curve



Example of Mouse IL10 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.