arigoPLEX® Human CTL/NK activation Multiplex ELISA Kit (IFN gamma, TNF alpha, Perforin, Granzyme B)

arigoPLEX® Human CTL/NK activation Multiplex ELISA Kit (IFN-gamma, TNF-alpha, Perforin, Granzyme B) is an Enzyme Immunoassay kit for the quantification of Human Cytotoxic T cell Cytokine (IFN-gamma, TNF-alpha, Perforin, Granzyme B) in serum, plasma and cell culture supernatants.

Catalog number: ARG83005

Package: 96 wells

Lot. 373876

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

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INTRODUCTION

IFN-gamma: This gene encodes a soluble cytokine that is a member of the type II interferon class. The encoded protein is secreted by cells of both the innate and adaptive immune systems. The active protein is a homodimer that binds to the interferon gamma receptor which triggers a cellular response to viral and microbial infections. Mutations in this gene are associated with an increased susceptibility to viral, bacterial and parasitic infections and to several autoimmune diseases.

TNF-alpha: This gene encodes a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. This cytokine is mainly secreted by macrophages. It can bind to, and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. This cytokine has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, psoriasis, rheumatoid arthritis ankylosing spondylitis, tuberculosis, autosomal dominant polycystic kidney disease, and cancer. Mutations in this gene affect susceptibility to cerebral malaria, septic shock, and Alzheimer disease. Knockout studies in mice also suggested the neuroprotective function of this cytokine.

Perforin: This gene encodes a protein with structural similarities to complement component C9 that is important in immunity. This protein forms membrane pores that allow the release of granzymes and subsequent cytolysis of target cells. Whether pore formation occurs in the plasma membrane of target cells or in an endosomal membrane inside target cells is subject to

debate. Mutations in this gene are associated with a variety of human disease including diabetes, multiple sclerosis, lymphomas, autoimmune lymphoproliferative syndrome (ALPS), aplastic anemia, and familial hemophagocytic lymphohistiocytosis type 2 (FHL2), a rare and lethal autosomal recessive disorder of early childhood.

Granzyme B: This gene encodes a member of the granzyme subfamily of proteins, part of the peptidase S1 family of serine proteases. The encoded preproprotein is secreted by natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) and proteolytically processed to generate the active protease, which induces target cell apoptosis. This protein also processes cytokines and degrades extracellular matrix proteins, and these roles are implicated in chronic inflammation and wound healing. Expression of this gene may be elevated in human patients with cardiac fibrosis.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. The antibodies specific to IFN-gamma, TNF-alpha, Perforin and Granzyme B 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 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]	2 vial (lyophilized)	4°C
100X Antibody Conjugate Mixture	120 µL	≤ -20°C
1000X HRP-Streptavidin concentrate	15 µL	4°C
Standard / Sample Diluent Buffer	50 mL	4°C
10X Wash Buffer	50 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	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	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	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α
C	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin
D	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB
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	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α
G	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin	Perforin
H	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB	GranzymeB

2. Standards Mixture each vial contains a buffered protein base and four cytokines at different amount: IFN-gamma 1000 pg; TNF-alpha: 1500 pg; Perforin: 6000 pg; Granzyme B: 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.
- Upon received, store 100X 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 **0.2 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 Standard / Sample Diluent Buffer to yield 1X detection antibody solution. (e.g. 10 µl of 100X Antibody Conjugate Mixture concentrate + 990 µl of 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 concentrate solution into Standard / Sample Diluent Buffer to yield 1X HRP-Streptavidin Solution buffer. (e.g. 1 µl of 1000X HRP-Streptavidin concentrate solution + 999 µl of Diluent Buffer)
- Sample: Before assay, serum and plasma are recommended to dilute with equal volume of Diluent Buffer. (eg. Premix 250 µL sample with 250 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. 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
IFN-gamma	1000	500	250	125	62.5	31.3	15.6
TNF-alpha	1500	750	375	187.5	93.8	46.9	23.4
Perforin	6000	3000	1500	750	375	187.5	93.8
Granzyme B	1000	500	250	125	62.5	31.25	15.63

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.

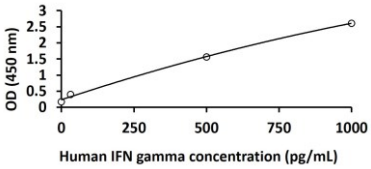
   Note: To obtain the approximate concentrations of 4 cytokines on 22 test samples, the low concentration standard mixture (**S1, 1:32 from high concentration mixture**), the high concentration Standards Mixture (**S2, stock**) and test samples (T1 to T22) can be added as the scheme as below:

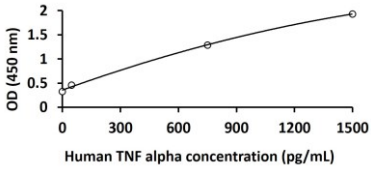
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
B	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
C	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
D	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
E	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
F	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
G	S2	T2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

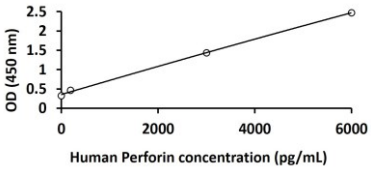
2. Cover the plate and incubate for **2 hours** at **room temperature**.
3. Aspirate each well and wash, repeating the process 4 times for a **total 5 time washes**. Wash by filling each well with **1× Wash Buffer (300 µL)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of 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 **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 **10-20 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 30 minutes** after adding the stop solution.

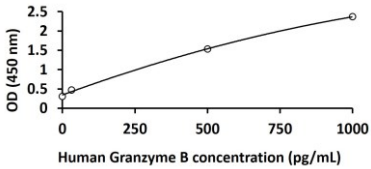
EXAMPLE OF TYPICAL STANDARD VALUES

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. 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. To obtain more accurate results, more dilution points can be used when 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.