Human Th1/Th2/Th17/Treg Multiplex ELISA Kit (IFN gamma, IL2, IL4, IL10, IL13, IL17A, IL22, TNF alpha)

Multiplex Enzyme Immunoassay for the semi-quantification of Human IFN gamma, IL2, IL4, IL10, IL13, IL17A, IL22, TNF alpha in serum, plasma and cell culture supernatants.

Catalog number: ARG80933

Package: 96 wells

TABLE OF CONTENTS

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INTRODUCTION

The T helper cells (Th cells), also known as $CD4^+$ cells or CD4-positive cells, are a type of T cell that play an important role in the immune system, particularly in the adaptive immune system. As their name suggests, they "help" the activity of other immune cells by releasing cytokines, small protein mediators that alter the behavior of target cells that express receptors for those cytokines. These cells help to polarize the immune response into the appropriate kind depending on the nature of the immunological insult (virus vs. extracellular bacterium vs. intracellular bacterium vs. helminth vs. fungus vs. protist). They are generally considered essential in B cell antibody class switching, breaking cross-tolerance in dendritic cells, in the activation and growth of cytotoxic T cells, and in maximizing bactericidal activity of phagocytes such as macrophages and neutrophils.

Proliferating helper T cells that develop into effector T cells differentiate into two major subtypes of cells known as Th1 and Th2 cells (also known as Type 1 and Type 2 helper T cells, respectively).

Th1 helper cells lead to an increased cell-mediated response, typically against intracellular bacteria and protozoa. They are triggered by the polarising cytokine IL12 and their effector cytokines are IFN γ and IL2. The main effector cells of Th1 immunity are macrophages as well as CD8 T cells, IgG B cells, and IFN γ CD4 T cells. The key Th1 transcription factors are STAT4 and T-bet. IFN γ secreted by CD4 T cells can activate macrophages to phagocytose and digest intracellular bacteria and protozoa. In addition, IFN γ can activate iNOS (inducible nitric oxide synthase) to produce nitric oxide free radicals to directly

kill intracellular bacteria and protozoa. Th1 overactivation against autoantigens will cause Type IV or delayed-type hypersensitivity reaction. Tuberculin reaction or Type 1 diabetes belong to this category of autoimmunity.

Th2 helper cells lead to a humoral immune response, typically against extracellular parasites such as helminths. They are triggered by the polarising cytokines IL4 and IL2, and their effector cytokines are IL4, IL5, IL9, IL10, IL13 and IL25. The main effector cells are eosinophils, basophils, and mast cells as well as B cells, and IL4/IL5 CD4 T cells. The key Th2 transcription factors are STAT6 and GATA3. IL4 is the positive feedback cytokine for Th2 cells differentiation. Besides, IL4 stimulates B-cells to produce IgE antibodies, which in turn stimulate mast cells to release histamine, serotonin, and leukotriene to cause broncho-constriction, intestinal peristalsis, gastric fluid acidification to expel helminths. IL5 from CD4 T cells will activate eosinophils to attack helminths. IL10 suppresses Th1 cells differentiation and function of dendritic cells. Th2 overactivation against antigen will cause Type I hypersensitivity which is an allergic reaction mediated by IgE. Allergic rhinitis, atopic dermatitis, and asthma belong to this category of overactivation. In addition to expressing different cytokines, Th2 cells also differ from Th1 cells in their cell surface glycans (oligosaccharides), which makes them less susceptible to some inducers of cell death.

Th17 helper cells are a subset of T helper cells developmentally distinct from Th1 and Th2 lineages producing interleukin 17 (IL17). Th cells produce IL17 which is a pro inflammatory substance. This means it is especially good at fighting extracellular pathogens and fungi. [Provide by Wikipedia: T helper cell]

IL2 has essential roles in key functions of the immune system, tolerance and immunity, primarily via its direct effects on T cells. In the thymus, where T cells mature, it prevents autoimmune diseases by promoting the differentiation of certain immature T cells into regulatory T cells, which suppress other T cells that are otherwise primed to attack normal healthy cells in the body. IL2 enhances activation-induced cell death (AICD). IL2 also promotes the differentiation of T cells into effector T cells and into memory T cells when the initial T cell is also stimulated by an antigen, thus helping the body fight off infections. Together with other polarizing cytokines, IL2 stimulates naive CD4+ T cell differentiation into Th1 and Th2 lymphocytes while it impedes differentiation into Th17 and folicular Th lymphocytes. [Provide by Wikipedia: IL2]

IL4 has many biological roles, including the stimulation of activated B-cell and T-cell proliferation, and the differentiation of B cells into plasma cells. It is a key regulator in humoral and adaptive immunity. IL4 induces B-cell class switching to IgE, and up-regulates MHC class II production. IL4 decreases the production of Th1 cells, macrophages, IFN gamma, and dendritic cell IL12. [Provide by Wikipedia: IL4]

IL10 is a cytokine with multiple, pleiotropic, effects in immunoregulation and inflammation. It downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. IL10 can block NF-κB activity, and is involved in the regulation of the JAK-STAT signaling pathway.

Discovered in 1991, IL10 was initially reported to suppress cytokine secretion, antigen presentation and CD4+ T cell activation. Further investigation has shown that IL10 predominantly inhibits lipopolysaccharide (LPS) and bacterial product mediated induction of the pro-inflammatory cytokines TNFα, IL1β, IL12, and IFNγ secretion from Toll-Like Receptor (TLR) triggered myeloid lineage cells. [Provide by Wikipedia: IL10]

IL13 is a cytokine secreted by T helper type 2 (Th2) cells, CD4 cells, natural killer T cell, mast cells, basophils, eosinophils and nuocytes. Interleukin-13 is a central regulator in IgE synthesis, goblet cell hyperplasia, mucus hypersecretion, airway hyperresponsiveness, fibrosis and chitinase up-regulation. It is a mediator of allergic inflammation and different diseases including asthma. [Provide by Wikipedia: IL13]

IL17A regulates the activities of NF-kappaB and mitogen-activated protein kinases. This cytokine can stimulate the expression of IL6 and cyclooxygenase-2 (PTGS2/COX-2), as well as enhance the production of nitric oxide (NO). [Provide by Wikipedia: IL17A]

IL22 is produced by several populations of immune cells at a site of inflammation. Producers are αβ T cells classes Th1, Th22 and Th17 along with γδ T cells, NKT, ILC3, neutrophils and macrophages. IL22 takes effect on non-hematopoietic cells – mainly stromal and epithelial cells. Effects involve stimulation of cell survival, proliferation and synthesis of antimicrobials including S100, Reg3β, Reg3γ and defensins. IL22 thus participates in both wound healing and in protection against microbes. IL22 dysregulation takes

part in pathogenesis of several autoimmune diseases like systemic lupus erythematosus, rheumatoid arthritis and psoriasis. [Provide by Wikipedia: IL22]

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: IFN gamma]

TNF was thought to be produced primarily by macrophages, but it is produced also by a broad variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts. Large amounts of TNF are released in response to lipopolysaccharide, other bacterial products, and interleukin-1 (IL1). In the skin, mast cells appear to be the predominant source of pre-formed TNF, which can be released upon inflammatory stimulus (e.g., LPS). [Provide by Wikipedia: Tumor necrosis factor]

PRINCIPLE OF THE ASSAY

This is a multiplex enzyme immunoassay for the semi-quantification of Th1/Th2/Th17/Treg Cytokines: IFN gamma, IL2, IL4, IL10, IL13, IL17A, IL22 and TNF alpha.

This assay employs the semi-quantitative sandwich enzyme immunoassay technique. The Antibodies specific to IFN gamma, IL2, IL4, IL10, IL13, IL17A, IL22 and TNF alpha has been pre-coated onto wells of 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, added Mixed Antibody-Conjugate to each well and incubate. After washing away any unbound substances, the HRP Conjugate is added to 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 450 nm. The concentration of Th1 / Th2 / Th17 / /Treg cytokines in the samples is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date

Component	Quantity	Storage information
Antibody Coated microplate [1]	8 X 12 strips	4°C
Standards Mixture	2 vial (lyophilized) each vial contains a buffered protein base and 8 cytokines at different amount: IFN gamma: 1600 pg IL2: 2000 pg IL10: 1000 pg; IL4: 1500 pg; IL13: 1000 pg; IL17A: 1280 pg; IL22: 1000 pg; TNF alpha: 1600 pg.	4°C
Biotin Antibody conjugate mixture	6 mL (ready to use)	4°C
HRP conjugate mixture	11 mL (ready to use)	4°C
Standard Diluent I (for serum / Plasma samples)	25 mL (ready to use)	4°C
Standard Diluent II (for cell culture supernatant samples)	25 mL (ready to use)	4°C
20X Wash Buffer	60 mL	4°C
Substrate A	10 mL	4°C
Substrate B	10 mL	4°C (protect from light)
STOP solution	14 mL (ready to use)	4°C

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 8 cytokines as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ
B	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2
C	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10
D	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4
E	IL13	IL13	IL13	IL13	IL13	IL13	IL13	IL13	IL13	IL13	IL13	IL13
F	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A
G	IL22	IL22	IL22	IL22	IL22	IL22	IL22	IL22	IL22	IL22	IL22	IL22
H	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Pipettes and pipette tips
- Deionized or distilled water
- Automated microplate washer (optional)

TECHNICAL NOTES AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit 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.
- Avoid air bubbles in the wells as this could result in lower binding efficiency

and higher CV% of duplicate reading.

- 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.
- Substrate B contains 20% acetone, keep this reagent away from sources of heat or flame.
- 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.
- Use a plate cover for each incubation step.
- 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 sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

To obtain the data of each cytokine, at least 0.8 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. The unused samples should be stored frozen at ≤-20°C or ≤-70°C to avoid sample degradation. For long term storage, store at ≤-70°C is recommended.

Cell Culture Supernatants - Remove particulates by centrifugation and aliquot & store samples at ≤ -20 °C or ≤-70°C. Avoid repeated freeze-thaw cycles.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 10 minutes at 1000 x g at 4°C. Collect serum and assay immediately or aliquot and store samples at ≤ -20 °C or ≤-70°C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g at 4°C within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C or ≤-70°C. Avoid repeated freeze-thaw cycles.

Note:

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

REAGENT PREPARATION

- 1X Wash Buffer: Dilute 20X Wash Buffer into distilled water to yield 1X Wash Buffer. (E.g., add 30 mL of 20X Wash Buffer into 570 mL of distilled water to a final volume of 600 mL) The 1X Wash Buffer is stable for up to 4 weeks at 2-8°C. Mix well before use.

- **TMB substrate**: Substrate A and Substrate B (containing TMB) should be mixed together in equal volumes up to 15 minutes before use. Refer to the table below for correct amounts of substrate solution to prepare:

Strips Used	Substrate A (ml)	Substrate B (ml)	Total volume (ml)
2 (16 wells)	1.5	1.5	3.0
4 (32 wells)	3.0	3.0	6.0
6 (48 wells)	4.0	4.0	8.0
8 (64 wells)	5.0	5.0	10.0
10 (80 wells)	6.0	6.0	12.0
12 (96 wells)	7.0	7.0	14.0

- **Standards Mixture**:

  Please select appropriate Diluent buffer for each sample type.

  Two vials of Standards are provided in this kit to allow both serum/plasma and cell culture supernatant testing. Standard diluent I contains animal serum and PBS for serum/plasma testing. Standard diluent II contains animal serum and RPMI 1640 for cell culture supernatant testing. The standards provided in the kit are for customers to use at own discretion. If samples generate values higher than the highest standard, dilute the samples with appropriate standard diluent and repeat assay.

  High concentration standard stock:

  Reconstitute standards with either 2ml Standard diluent I (for serum/plasma testing) or Standard diluent II (for cell culture supernatant testing) to obtain high concentration standard stock. Allow solution to sit for 15 minutes with gentle agitation prior to making dilutions. This stock solution can be aliquoted and stored frozen at-70 °C for up to 30 days.

Dilution of standard mixture:

For semi-quantitative assay, use the above high concentration standard mixture and a 32-fold diluted low concentration standards mixture to test together with up to 10 test samples. If more accurate results are required, a two-fold serial dilution with appropriate dilution buffer can generate a more accurate standard curve. However, the number of test samples will be reduced.

To dilute standards, produce a serial 2-fold dilution series from 1:2 to 1:64 dilutions. The concentration of 8 cytokines in different dilutions of the mixed standard are listed as below:

Cytokine (pg / mL)	High Conc. Standard Stock	1:2	1:4	1:8	1:16	1:32 (Low Conc. Standard)	1:64
A: IFN γ	800	400	200	100	50	25	12.5
B: IL2	1000	500	250	125	62.5	31.25	15.625
C: IL10	500	250	125	62.5	31.25	15.625	7.8125
D: IL4	750	375	187.5	93.75	46.875	23.438	11.719
E: IL13	500	250	125	62.5	31.25	15.625	7.8125
F: IL17A	640	320	160	80	40	20	10
G: IL22	500	250	125	62.5	31.25	15.625	7.8125
H: TNF α	800	400	200	100	50	25	12.5

ASSAY PROCEDURE

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

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **100 µl of standards or samples** into wells. Cover and incubate for **1h at RT.**

   (Example) To obtain the approximate concentrations of 8 cytokines on 10 test samples (T1-T10), the low concentration standard mixture (**1:32 from high concentration mix, S1**) and high concentration standard mixture (**stock, S2**) and test samples can be added as scheme below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
B	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
C	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
D	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
E	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
F	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
G	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
H	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10

3. *Without discarding the content*, add **50 µl** of **Biotin conjugate mixture** into each well. Mix well, cover and incubate for **1 hour at room temperature**.
4. Aspirate each well and wash, repeating the process 4 times for a total **5 washes**. Wash by filling each well with **1× Wash Buffer (350µl)** 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.

Note:

- Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame.

- For automated Washing: It is recommended that the washer be set for a soaking time of 10 seconds or shaking time of 5 seconds between washes.

5. Add **100 µl** of **HRP conjugate mixture** into each well. Cover and incubate for **1h at room temperature**.
6. Prepare Substrate Solution *no more than 15 minutes* before end of HRP conjugate mixture incubation
7. Aspirate each well and **wash as step 4**.
8. Add **100 µl** of **Substrate solution** mixture to each well. Incubate for **15 minutes at room temperature in dark**.
9. Add **100 µl** of **Stop Solution** to each well. The color of the solution should change from blue to yellow.
10. Read the OD with a microplate reader at **450 nm** immediately. It is recommended reading the absorbance *within 30 min* after adding stop solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards and samples.
2. For semi-quantitative assay, 8 rough curves for 8 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.

EXAMPLE OF TYPICAL STANDARD VALUES

The following data shows the OD readings of a run of this multiplex ELISA with multiple dilutions using standard diluent I. It is for demonstration purpose only and cannot be used to replace standard curve for testing. Each investigators have to assay standards along with test samples.

Cytokines	1	1:2	1:4	1:8	1:16	1:32	1:64	Standard Diluent I
IFN γ	2.043	1.234	0.776	0.454	0.301	0.250	0.201	0.148
IL2	1.489	0.821	0.475	0.305	0.214	0.189	0.168	0.132
IL10	1.734	1.159	0.698	0.428	0.247	0.183	0.129	0.086
IL4	1.621	0.897	0.514	0.280	0.166	0.120	0.081	0.066
IL13	1.327	0.774	0.522	0.313	0.195	0.151	0.124	0.087
IL17A	1.814	0.977	0.530	0.343	0.200	0.143	0.106	0.062
IL22	1.628	1.115	0.702	0.431	0.308	0.216	0.184	0.138
TNF α	1.785	1.090	0.642	0.427	0.294	0.244	0.207	0.193

Detection Range

IFN gamma	12.5 - 800pg/ml	IL13	7.8 - 500pg/ml
IL2	15.6 - 1000pg/ml	IL17A	10 - 640pg/ml
IL10	7.8 - 500pg/ml	IL22	7.8 - 500pg/ml
IL4	11.7 - 750pg/ml	TNF alpha	12.5 - 800pg/ml