arigoPLEX®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: ARG83516

Package: 96 wells

Lot. 374273

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

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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]	3 vials	4°C
100X Antibody Conjugate Mixture	120 ul	≤ -20°C
1000X HRP-Streptavidin solution	15 ul	4°C
Standard/Sample Diluent Buffer	30 ml	4°C
Antibody Diluent Buffer	35 ml	4°C
10X Wash Buffer	50 ml	4°C
TMB substrate	12 ml	4°C (protect from light)
STOP solution	12 ml	4°C
Plate sealer	3 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 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	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4
D	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10
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

2. Standards Mixture each vial contains a buffered protein base and eight cytokines at different amount: IFN gamma: 500 pg/ml, IL2: 500 pg/ml, IL4: 500 pg/ml, IL10: 500 pg/ml, IL13: 500 pg/ml, IL17: 1000 pg/ml, IL22: 500 pg/ml, TNF alpha: 1000 pg/ml

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.
- 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 **0.4 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 Standard/Sample Diluent Buffer. (eg. Premix 250 µL

sample with 250 µL Standard/Sample 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 8 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 8 cytokines in different dilutions of the Standards Mixture 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	500	250	125	62.5	31.25	15.63	7.81
B: IL2	500	250	125	62.5	31.25	15.63	7.81
C: IL4	500	250	125	62.5	31.25	15.63	7.81
D: IL10	500	250	125	62.5	31.25	15.63	7.81
E: IL13	500	250	125	62.5	31.25	15.63	7.81
F: IL17A	1000	500	250	125	62.5	31.25	15.63
G: IL22	500	250	125	62.5	31.25	15.63	7.81
H: TNF α	1000	500	250	125	62.5	31.25	15.63

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 **2h 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. 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.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards and samples.
2. The 8 curves for 8 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. Serum/plasma sample has multiplied the dilution factor (Dilution factor =2). 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.

EXAMPLE OF TYPICAL STANDARD VALUES

The following data shows the typical standard curve 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.

Detection Range

IFN gamma: 7.8-500 pg/ml

IL2: 7.8-500 pg/ml

IL4: 7.8-500 pg/ml

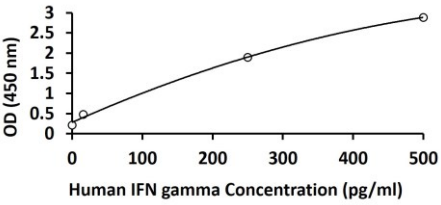
IL10: 7.8-500 pg/ml

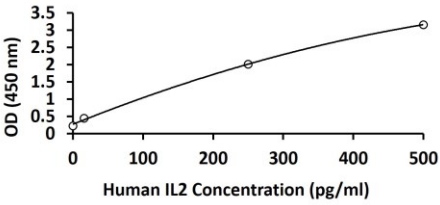
IL13: 7.8-500 pg/ml

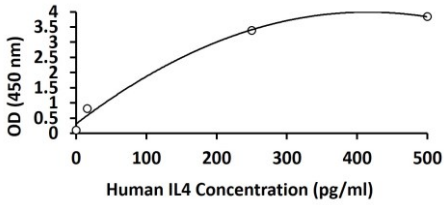
IL17: 15.6-1000 pg/ml

IL22: 7.8-500 pg/ml

TNF alpha: 15.6-1000 pg/ml


OD (450 nm)
0
0.5
1
1.5
2
2.5
3
0
100
200
300
400
500
Human IFN gamma Concentration (pg/ml)


OD (450 nm)
0
0.5
1
1.5
2
2.5
3
3.5
0
100
200
300
400
500
Human IL2 Concentration (pg/ml)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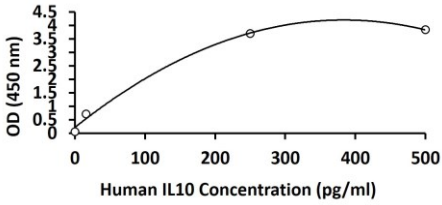
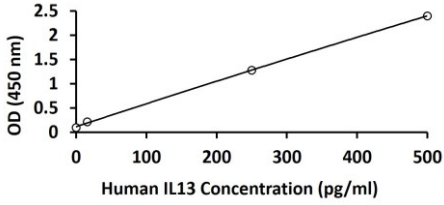
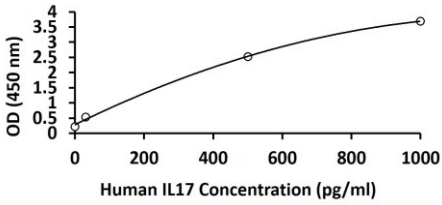
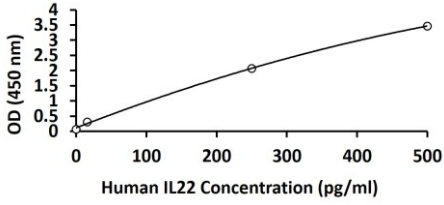
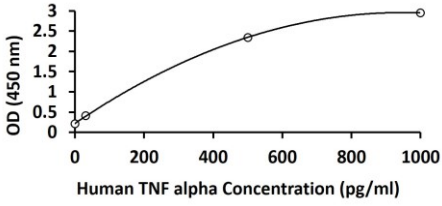
0
0.5
1
1.5
2
2.5
3
3.5
4
0
100
200
300
400
500
Human IL4 Concentration (pg/ml)


OD (450 nm)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
100
200
300
400
500
Human IL10 Concentration (pg/ml)


OD (450 nm)
0
0.5
1
1.5
2
2.5
0
100
200
300
400
500
Human IL13 Concentration (pg/ml)


OD (450 nm)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
200
400
600
800
1000
Human IL17 Concentration (pg/ml)


OD (450 nm)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
100
200
300
400
500
Human IL22 Concentration (pg/ml)


OD (450 nm)
0
0.5
1
1.5
2
2.5
3
0
200
400
600
800
1000
Human TNF alpha Concentration (pg/ml)