Multiplex Human Cytokine ELISA Kit

(Th1/Th2/Th17 Cytokines)

For Simultaneous Quantitative Determination of T helper Cell Differentiation Cytokines, Including Interferon-γ, Interleukin-2, Interleukin-4, Interleukin-10, Interleukin-13, Interleukin-17, Interleukin-22, and Tumor Necrosis Factor-α, in Cell Culture Supernatant and Other Biological Samples

Catalogue Number: EM10003

96 tests

FOR LABORATORY RESEARCH USE ONLY
NOT FOR DIAGNOSTIC USE

ANOGEN

2355 Derry Road East, Unit 23
Mississauga, Ontario
CANADA L5S 1V6
Tel: (905) 677-9221 or (877) 755-8324
Fax: (905) 677-0023

Email: info@anogen.ca ♦ Web Site: www.anogen.ca

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INTENDED USE

This multiplex ELISA kit for Th1/Th2/Th17 cytokines is designed for semi-quantitative and simultaneous determination of cytokines relevant to T helper cell differentiation. The kit simultaneously determines interferon-γ(IFN-γ), interleukin-2(IL-2), interleukin-4(IL-4), interleukin-10(IL-10), interleukin-13(IL-13), interleukin-17A(IL-17A), interleukin-22(IL-22), and tumor necrosis factor-α (TNF-α) in cell culture supernatant and other biological samples. In combination with other Anogen quantitative cytokine ELISA kits, the Th1/Th2/Th17 cytokine multiplex ELISA kit is expected to be useful for the investigation of the relationship of cytokine expression, T helper cell differentiation in various disease models.

The kit is intended FOR LABORATORY RESEARCH USE ONLY and should not be used in any diagnostic or therapeutic procedures.

INTRODUCTION

T helper cells (Th cells) are a diversified group of CD4+ T cells that play an essential role in immune-response through cytokine secretion and cell to cell contact (surface molecule expression). The importance of Th cells is manifested by the acquired immunodeficiency in HIV patients whose CD4+ T cells are selectively infected and destroyed by HIV virus. The function of Th cells is multifaceted, including stimulation of CD8+ cytotoxic cells, promoting B cell proliferation, regulating antibody subclass switching, and activation of macrophages and other cells in innate immune system.

T helper cells are originated from Naïve CD4+ T cells, which are generated in bone marrow and differentiate into effector Th cells in peripheral lymphoid organs and blood upon contact with cognate antigen that matches with the complementarity-determining region on T cell receptor. Th cells were classified into T helper 1 cells (Th1) and T helper 2 cells (Th2) in the mid-1980s based on their unique cytokine secretion patterns and their roles in cellular and humoral immunity. Th1 cells secrete IFN-γ as their signature cytokine, and are also the primary producer of IL-2. Th2 cells fail to make IFN-γ, but produce IL-4, IL-5, and IL-13 as their signature cytokines. Th1 cells are needed in host defense against intracellular bacteria and virus, while Th2 cells are essential for humoral responses against extracellular parasites. Cytokines play important roles in the development and maintenance of T helper cell lineage. In the presence of IL-12, Naïve CD4+ cells tend to differentiate into Th1, whereas IL-4 stimulates Th2 development. Interestingly, cytokines produced by one Th subset tends to inhibit the development of other Th subset(s). For example, IL-10 secreted by Th2 can counteract the effect of IL-12 through suppressing Th1 and stimulating Th2 differentiation.

In 2005, Harrington et al described a new subset of IL-17 secreting CD4+ T cells as T helper 17 (Th17) cells, which is involved with autoimmunity and clearance of extracellular bacteria and fungi. It was observed that Th17 cells can be induced from Naïve CD4+ T cells by IL-23 in the absence of IFN-r and IL-4. More recently, T follicular helper cells (Tfh), regulatory T cells (Treg), T helper 9 cells (Th9) and T helper 22 cells (Th12) were proposed.

IL-2 is one of the key cytokines that drive adaptive immunity. It is secreted by Naïve CD4+ cells immediately after T cell receptor is activated through binding with antigens presented on MHC

by antigen-presenting cells. IL-2 stimulates Th1 differentiation, clone expansion and acts as autocrine to stimulate its own production. It also promotes the survival of antigen specific memory T cell, and stimulates the maturation of regulatory T-cells. The cytokine also influences the activity of B cells, CD8+ T cells, gamma delta T cells, NK cells and LAK cells.

IL-10 is expressed by a variety of cell types and plays an important role in inflammatory and immune responses. It down-regulates the expression of Th1 cytokines. IL-10 also suppresses the production of pro-inflammatory cytokines by monocytes and neutrophils, down-regulates the expression of activating and co-stimulatory molecules on monocytes and dendritic cells, and stimulates the growth of B cells and mast cells.

Produced by Th1, $CD8^+$ T cells and natural killer (NK) cells, IFN-γ is known to stimulate Naïve CD4+ T cells to differentiate into Th1 cells. It is both an inhibitor of viral replication and a regulator of numerous immunological functions. IFN-γ has been reported to induce its own expression. IFN-γ has documented antiviral, antiprotozoal and immunomodulatory activities. The immunomodulatory effects of IFN-γ are extensive and diverse. In monocyte/ macrophages, IFN-γ increases expression of class 1 MHC antigens; increases the production of IL-1, platelet-activating factor, H_2O_2, and pterin, protects monocytes against LAK cell-mediated lysis; and down-regulates IL-8 mRNA expression. IFN-γ selectively enhances IgG_{2a} secretion by LPS-stimulated B cell activation. IFN-γ has been shown to up-regulate ICAM-1, but not E-selectin or VCAM-1 expression on endothelial cells. IFN-γ has also been reported to implicate in the development of a cholinergic phenotype in embryonic septal neurons.

IL-4 was initially characterized as a B cell stimulatory factor (BSF-1) for its crucial role in Th2 cell differentiation, B cell expansion and differentiation into plasma cells. It was subsequently revealed that IL-4 is a pleiotropic cytokine with multiple immune response modulating functions on diverse cell types. IL-4 induces antibody class switching to IgE, up-regulates MHC class II production and plays a role in airway allergy. IL-4 stimulates protective immunity against parasites. It is also reported that IL-4 is implicated with metastasis. IL- 4 is produced by naïve $CD4^+$ T cells, Th2 cells, fetal thymocytes, $CD8^+$ T cells, mast cells and basophils. IL- 4 exhibits approximately 25% amino acid sequence homology to IL-13 that shares a number of biological functions with IL-4. Human IL-4 gene, composed of four exons and three introns, have been localized to a chromosome that contains tandem-arranged genes for IL-3, IL-5, IL-9, IL-13 and GM-CSF.

IL-13 is secreted mainly by activated T helper 2 cells. IL-13 binds to IL-4Rα in addition to IL-13Rα1 and IL-13Rα2. The function of IL-13 partially overlaps with IL-4. Both IL-4 and IL-13 link to signal transducer and activator of transcription 6 (STAT6) through the receptor activation. IL-13 can induce B-cell proliferation and IgE classs switching. However, IL-13 is less competent in this function comparing with IL-4. Unlike IL-4, IL-13 does not play an important role in hematopoietic cell shift, such as naïve T helper differentiation to T helper 2. IL-13 is also associated with many features of the allergic airway diseases, including mucus hypersecretion, goblet metaplasia, chemokine induction and recruitment of effector cells. All of them contribute to airway obstruction. Polymorphisms in IL-13 gene have been found to link to increased eosinophil count, serum total IgE and high risk of asthma. It is reported that IL-I3 signaling is significant in inducing physiological changes to non-immuno cells in parasitized organs.

IL-17A is the proto-type of a newly discovered pro-inflammatory cytokine family which consists of IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F. All the IL-17s have similar structure with four highly conserved cysteine residues and same 3 dimensional structures, which distinguish this family from other cytokines. IL-17 is produced by a group of CD4+ T help cells termed Th17 cells. In addition to previous discovered Th1 and Th2 cells, the Th17 cells consist in the third group of T helper cells. IL-23, a growth and stabilization factor was the first cytokine that was found to involve with the development of Th 17 cells. Later on, other cytokines including TGF-beta, IL-6, and IL-21, were also found to contribute to the procedure. The transcription factors implicated in the procedure were identified as STAT3, RORgt, and RORa. IL-17, like IFN-γ, is a potent mediator of delayed-type reaction. IL-17 induces the secretion of many other pro-inflammatory cytokines and chemokines such as IL-6, IL-8, GM-CSF, G-CSF, TGF-beta, TNF-alpha, GRO-alpha, MCP-1, and stimulates the expression of NF-kappaB, mitogen activated protein kinase, and prostaglandings from many cell types. By stimulating cytokine production and recruiting monocytes neutrophils to site of inflammation, the cytokine exerts its effect in response to tissue damage and invasion.

IL-22 is produced by dendritic cells, T-cells and natural killer cells during bacterial infection, auto-immunity and tissue inflammation. IL-22 acts upon innate immunity cells through its receptors expressed exclusively on these cells. In CD4+ T helper cells, IL-22 expression has been found to be associated with Th17 and Th1. Recently, an IL-22 expressing T helper cell subset (Th22) was characterized which is distinct from other T cells by co-expression of the chemokine receptor CCR6 and the skin-homing receptors CCR4 and CCR10. IL-22 expression is elevated in psoriatic skin inflammation, atopic dermatitis, and inflammatory bowel disease. In cutaneous T-cell lymphoma, IL-22 dominates the tumor microenvironment and STAT3 phosphorylation was observed. IL-22 was also found to promote murine hepatocyte survival and ameliorate intestinal inflammation in mouse ulcerative colitis model. A natural antagonist of IL-22, IL-22 binding protein, was found to down-regulate IL-22 function.

Tumor Necrosis Factor alpha (TNF-α), also known as cachectin was initially named for its remarkable ability to cause hemorrhagic necrosis of tumors in mice. The primary source of TNF-α is thought to be the monocyte/macrophage but various cell types are known to express this cytokine. TNF-α is produced upon stimulation with cytokines such as IL-1, 1L-2, GM-CSF, TNF-α itself and with bacterial lipopolysaccharide. Th1 cells secrete IFN-γ, IL-2, IL-10, and TNF-alpha/beta. Various pathological conditions are associated with the production of high levels of TNF-α. These include septic shock syndrome, cachexia, autoimmune diseases, hepatitis, leukemia, myocardial ischaemia, organ transplantation rejection, multiple sclerosis, rheumatoid arthritis, and meningococcal septicemia. TNF-α is a pleiotropic cytokine that can induce disease through TNF-α toxicity (tissue injury, catabolic illness, and mediating septic shock) and improve host defense mechanisms (stimulating inflammation and increasing immune cell function). Since TNF-α has such a complex and important function in immune-response, studying TNF-α can help understanding its role in T cell differentiation.

This ELISA assay is a 3.5 hour solid phase immunoassay readily applicable to measure the levels of eight cytokines relevant to the differentiation of T helper cells in cell culture supernatant, and other biological fluids. It showed no cross reactivity with other proteins.

PRINCIPLE OF THE ASSAY

This enzyme linked immunosorbent assay (ELISA) applies a technique called a quantitative sandwich immunoassay. The microwells on the 8-well strips enclosed in the kit have been pre-coated with monoclonal antibodies specific to IFN-γ, IL-2, IL-4, IL-10, IL-13, IL-17, IL-22, and TNF-α respectively. Standards or samples are then added to the strips, and the biotin-conjugated detection antibody mixture will be added late on. The above cytokines, if present, will bind and become immobilized by the antibody pre-coated on the wells and then be "sandwiched" by biotin conjugate. The microtiter plate wells are thoroughly washed to remove unbound components of the sample. In order to quantitatively determine the amount of cytokine present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Avidin is a tetramer containing four identical subunits that each has a high affinity-binding site for biotin. The wells are thoroughly washed to remove all unbound HRP-conjugated Avidin. A TMB (3, 3' 5, 5' tetramethyl-benzidine) substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain coating antibody and the specific cytokine, biotin-conjugated antibody and enzyme-conjugated Avidin will develop a blue colour. The intensity of colour development is proportional to the concentration of the specific cytokine presented in the each wells. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour will change to yellow. The intensity is measured spectrophotometrically at a wavelength of 450nm ± 2 nm.

Samples were tested together with standards diluted with a similar matrix, or one of the Calibrator Diluent provided with the kit. This allows the operator to produce Optical Density (O.D) versus cytokine concentration (pg/mL). The concentration of cytokines in the samples is then determined by comparing the O.D. of the samples to the standards.

LIMITATIONS OF THE PROCEDURE

- As manufacturers we take great care to ensure that our products are suitable for use with all validated sample types, as designated in the product insert. However, it is possible that in some cases, high levels of interfering factors may cause unusual results.
- The kit should not be used beyond the expiration date on the kit label.
- Any variation in standard diluent, operator pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in kit performance.
- Soluble receptors or other binding proteins present in biological samples do not necessarily interfere with the measurement of ligands in samples. However, until the factors have been tested, the possibility of interference cannot be excluded.

REAGENTS PROVIDED

All reagents provided are stored at 2-8°C. Refer to the expiration date on the label.

	96 wells

1. **ANTIBODY COATED MICROTITER PLATE** (Part EM03-1) ***96 wells***
The plate contains twelve 8-well ELISA strips. Each of the eight wells has been coated with a different monoclonal antibody specific to one of the 8 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	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2
C	IL-4	IL-4	IL-4	IL-4	IL-4	IL-4	IL-4	IL-4	IL-4	IL-4	IL-4	IL-4
D	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10
E	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13
F	IL-17A	IL-17A	IL-17A	IL-17A	IL-17A	IL-17A	IL-17A	IL-17A	IL-17A	IL-17A	IL-17A	IL-17A
G	IL-22	IL-22	IL-22	IL-22	IL-22	IL-22	IL-22	IL-22	IL-22	IL-22	IL-22	IL-22
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. **BIOTIN CONJUGATE MIXTURE** (Part EM03-2) ***6 mL***
Mixture of Biotin conjugated anti-human cytokine antibodies.

3. **HRP CONJUGATE MIXTURE** (Part EM03-3) ***11 mL***
Mixture of Horseradish Peroxidase Conjugates

4. **STANDARD MIXTURE** (Part EM03-4) ***2 vials***
Two vials of lyophilized standard mixture. Each vial contains a buffered protein base and eight pro-inflammatory cytokines at different concentrations: IFN-γ 2000pg, IL-2 3000pg, IL-4 2000pg, IL-10 1600pg, IL-13 1600pg, IL-17A 2000pg, IL-22 1200pg, TNF-α 2000pg.

5. **CALIBRATOR DILUENT I** (Part EM03-5) ***25 mL***
Newborn calf serum with PBS buffer and preservative. *For serum/plasma testing.*

6. **CALIBRATOR DILUENT II** (Part EM03-6) ***25 mL***
Cell culture medium RPMI 1640 with newborn calf serum and preservative. *For cell culture supernatant testing.*

7. **WASH BUFFER (20X)** (Part 30005) ***60 mL***
20-fold concentrated solution of buffered surfactant.

8. **SUBSTRATE A** (Part EM03-7) ***10 mL***
Buffered solution with H_2O_2

9. **SUBSTRATE B** (Part 30007) ***10 mL***
Buffered solution with TMB.

10. **STOP SOLUTION** (Part 30008) ***14 mL***
2N Sulphuric Acid (H_2SO_4). Caution: Caustic Material!

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Single or multi-channel precision pipettes with disposable tips: 10-100µL and 50-200µL for running the assay.
2. Pipettes: 1 mL, 5 mL 10 mL, and 25 mL for reagent preparation.
3. Multi-channel pipette reservoir or equivalent reagent container.
4. Test tubes and racks.
5. Polypropylene tubes or containers (25 mL).
6. Erlenmeyer flasks: 100 mL, 400 mL, 1 L and 2 L.
7. Microtiter plate reader (450 nm ± 2nm)
8. Automatic microtiter plate washer or squirt bottle.
9. Sodium hypochlorite solution, 5.25% (household liquid bleach).
10. Deionized or distilled water.
11. Plastic plate cover.
12. Disposable gloves.
13. Absorbent paper.

PRECAUTIONS

1. Do not substitute reagents from one kit lot to another. Standard, conjugate and microtiter plates are matched for optimal performance. Use only the reagents supplied by manufacturer.
2. Allow kit reagents and materials to reach room temperature (20-25°C) before use. Do not use water baths to thaw samples or reagents.
3. Do not use kit components beyond their expiration date.
4. Use only deionized or distilled water to dilute reagents.
5. Do not remove microtiter plate from the storage bag until needed. Unused strips should be stored at 2-8°C in their pouch with the desiccant provided.
6. Use fresh disposable pipette tips for each transfer to avoid contamination.
7. Do not mix acid and sodium hypochlorite solutions.
8. Human serum and plasma should be handled as potentially hazardous and capable of transmitting disease. Disposable gloves must be worn during the assay procedure, since no known test method can offer complete assurance that products derived from human blood will not transmit infectious agents. Therefore, all blood derivatives should be considered potentially infectious and good laboratory practices should be followed.
9. All samples should be disposed of in a manner that will inactivate human viruses.
   Solid Wastes: Autoclave 60 min. at 121°C.
   Liquid Wastes: Add sodium hypochlorite to a final concentration of 1.0%. The waste should be allowed to stand for a minimum of 30 minutes to inactivate the virus before disposal.
10. Substrate Solution is easily contaminated. If bluish prior to use, *do not use*.
11. Substrate B contains 20% acetone, keep this reagent away from sources of heat or flame.
12. If Wash Buffer (20X) is stored at a lower temperature (2-5°C), crystals may form, which must be dissolved by warming to 37°C prior to use.

SAMPLE PREPARATION

1. **Minimal Sample Volume**:
   To obtain the data of each cytokine, 0.8ml 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 should be stored frozen at -20^{o}C to - 80^{o}C to avoid sample degradation.

2. **Sample Preparation:**
   a). Cell Culture Supernatant: Centrifuge to remove any visible particulate material.
   b). Serum: Blood should be drawn using standard venipuncture techniques and anti coagulation reagents. Samples should be allowed to clot for one hour at room temperature, centrifuged for 10 minutes (4°C), and serum extracted. Serum should be separated from the blood cells as soon as possible.
   c). Plasma: Blood should be drawn using standard venipuncture techniques and anticoagulant to ensure optimal recovery and minimal platelet contamination.
   d). Sample storage: Samples should be stored at -20^{o}C to - 80^{o}C for future testing.

PREPARATION OF REAGENTS

Remove all kit reagents from refrigerator and allow them to reach room temperature (20-25°C). Prepare the following reagents as indicated below. Mix thoroughly by gently swirling before pipetting. Avoid foaming.

1. **Wash Buffer (1X):** Add 60 mL of Wash Buffer (20X) and dilute to a final volume of 1200 mL with distilled or deionized water. Mix thoroughly. If a smaller volume of Wash Buffer (1X) is desired, add 1 volume of Wash Buffer (20X) to 19 volumes of distilled or deionized water. Wash Buffer (1X) is stable for 1 month at 2-8°C. Mix well before use.

2. **Substrate Solution:** Substrate A and Substrate B 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)	Substrate Solution (mL)
2 strips (16 wells)	1.5	1.5	3.0
4 strips (32 wells)	3.0	3.0	6.0
6 strips (48 wells)	4.0	4.0	8.0
8 strips (64 wells)	5.0	5.0	10.0
10 strips (80 wells)	6.0	6.0	12.0
12 strips (96 wells)	7.0	7.0	14.0

3. **Dilution of Standard Mixture:**

   Standard and Sample Diluent: Selection of appropriate Diluent is important for the standard curve be consistent with the samples being assayed. Two vials of Standard Diluents are provided in the kits. Calibrator Diluent I contains animal serum and PBS is for serum/plasma testing. Calibrator Diluent II contains animal serum and RPMI 1640 is for

cell culture supernatant testing. The two calibrators provided in the kit are for customers to use at own discretion. To obtain more accurate results, an appropriate medium that is used for the particular cell culture experiment is recommended to be used for the dilution of the Standard Mixture. If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent that was used to dilute standard and repeat the assay to obtain result.

High Concentration Standard Stock: Add 2ml of appropriate Diluent to reconstitute the lyophilized standard Stock to obtain the high concentration standard stock of 8 cytokines at different concentrations (see table below). Allow solution to sit for at least 15 minutes with gentle agitation prior to making further dilutions. This high concentration standard stock can be stored frozen (-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 standard mixture to test together with up to 10 test samples. If more accurate results are required, a two fold serial dilution with the appropriate 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 mixed standard are listed as below:

Cytokine (pg/mL)	High Conc. Std Stock	1:2	1:4	1:8	1:16	1:32	1:64
A. IFN-γ	1000	500	250	125	62.5	31.25	15.6
B. IL-2	1500	750	375	187.5	93.75	46.8	23.4
C. IL-4	1000	500	250	125	62.5	31.25	15.6
D. IL-10	800	400	200	100	50	25	12.5
E. IL-13	800	400	200	100	50	25	12.5
F. IL-17A	1000	500	250	125	62.5	31.25	15.6
G. IL-22	600	300	150	75	37.5	18.7	9.3
H. TNF-α	1000	500	250	125	62.5	31.25	15.6

ASSAY PROCEDURE

1. Prepare Wash Buffer (1X) and dilute the Standard Mixture before starting assay procedure (see Preparation of Reagents).

2. Add 100μL of Standard or Sample to the appropriate well of the antibody pre-coated Microtiter Plate and incubate 1 hour at room temperature.

   Note: To obtain the approximate concentrations of 8 cytokines on 10 test samples, the low concentration standard mixture (S1, 1:32 from high concentration mixture), the high concentration standard mixture (S2) and test samples (T1 to T10) can be added as the 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 standards and samples, add 50μL Biotin conjugate mixture to each wells. Mix well. Cover and incubate for 1 hour at room temperature.

4. Wash the Microtiter Plate using one of the specified methods indicated below:

   Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Using a squirt bottle, fill each well completely with Wash Buffer (1X) then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure four more times for a **total of FIVE washes**. After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *Note*: Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame.

   Automated Washing: Aspirate all wells, then wash plates **FIVE times** using Wash Buffer (1X). Always adjust your washer to aspirate as much liquid as possible and set fill volume at 350 μL/well/wash (range: 350-400 μL). After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *It is recommended that the washer be set for a soaking time of 10 seconds or shaking time of 5 seconds between washes.*

5. Dispense 100μl of Avidin Conjugate Mixture to each well Mix well. Cover and incubate for 1 hour at room temperature.

6. Prepare Substrate Solution no more than 15 minutes before end of second incubation (see Preparation of Reagents).

7. Repeat wash procedure as described in Step 4.

8. Add 100μL Substrate Solution to each well. Cover and incubate for 15 minutes at room temperature.

9. Add 100μL Stop Solution to each well. Mix well.

10. Read the Optical Density (O.D.) at 450 nm using a microtiter plate reader set within 30 minutes.

CALCULATION OF RESULTS

Form row A to row H, the OD readings of each well on the strip reflect the concentrations of each of the eight cytokines: IFN-γ, IL-2, IL-4, IL-10, IL-13, IL-17A, IL-22 and TNF-α in order. In semi-quantitative assay, 8 rough curves for the 8 cytokines can be generated from the OD readings of the high concentration standard and low concentration, the approximated cytokine concentration in the samples can be obtained by plotting the OD reading of the samples in each of the 8 wells to the its standard curves. As shown in the standard curve section, the real standard curves are not necessary perfectly straight, therefore, the concentration obtained from a rough curve derived from two points cannot be very accurate.

To obtain more accurate results, operator can test more dilution points simultaneously with the test samples. For quantitative measuring single cytokine concentration in multiple samples, quantitative ELISA assay kits for individual cytokine are also available from Anogen, Yes Biotech.

PERFORMANCE DATA

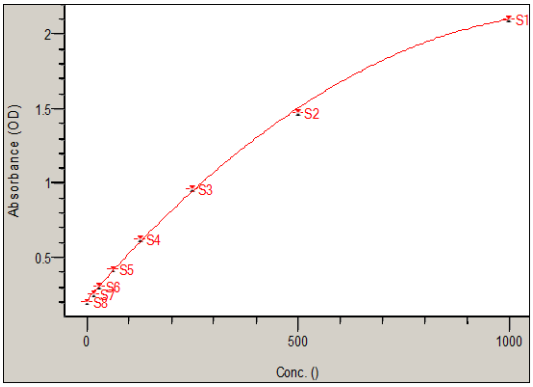
The following table shows the OD readings of a run of this multiplex ELISA with two fold-serial diluted standards using Calibrator Diluent I. It is for demonstration purpose only and cannot be used to replace the standard curve for testing. For each investigation, standards have to be assayed along with test samples and only the curve generated from the same test can be used.

Cytokines	1	1:2	1:4	1:8	1:16	1:32	1:64	Cal I
A. IFN-γ	2.112	1.486	0.971	0.629	0.429	0.310	0.261	0.206
B. IL-2	1.772	1.051	0.674	0.446	0.353	0.295	0.290	0.266
C. IL-4	1.968	1.173	0.762	0.472	0.312	0.226	0.208	0.161
D. IL-10	1.962	1.507	1.036	0.720	0.451	0.316	0.247	0.169
E. IL-13	2.617	1.991	1.339	0.839	0.537	0.340	0.259	0.150
F. IL-17A	2.496	1.598	0.973	0.594	0.394	0.270	0.223	0.147
G. IL-22	2.380	1.534	0.859	0.461	0.257	0.158	0.118	0.071
H. TNF-α	2.120	1.369	0.886	0.595	0.418	0.326	0.293	0.237

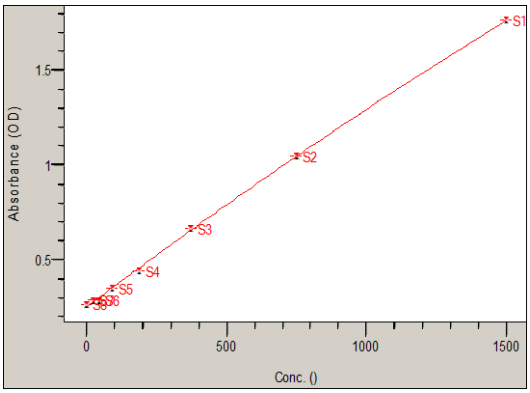
STANDARD CURVES

The cytokine standard curves were generated from the above data are for illustration only.

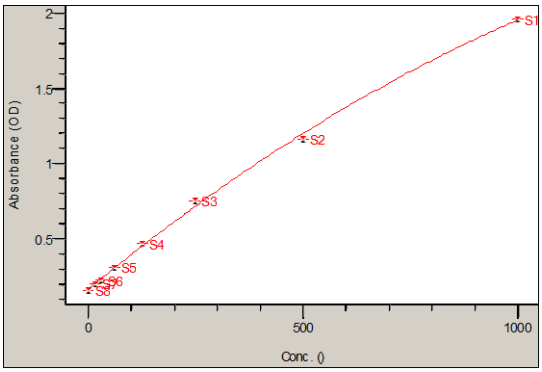
IFN-γ



IL-2



IL-4



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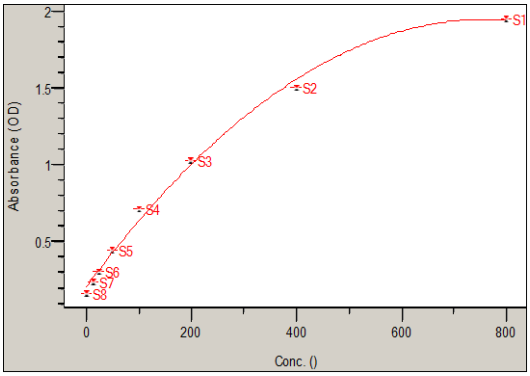

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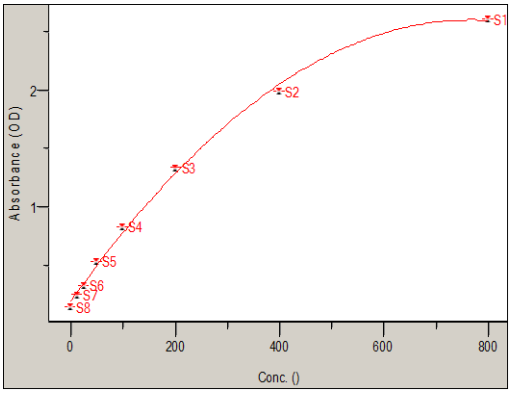
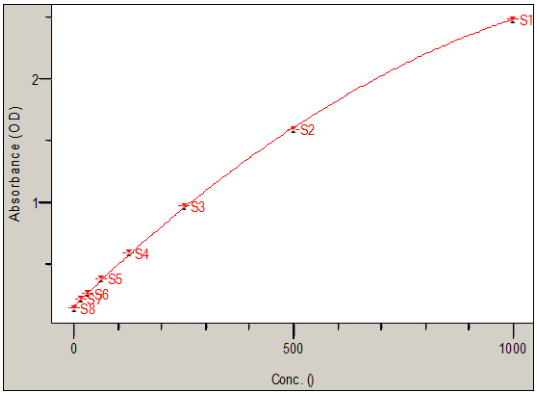
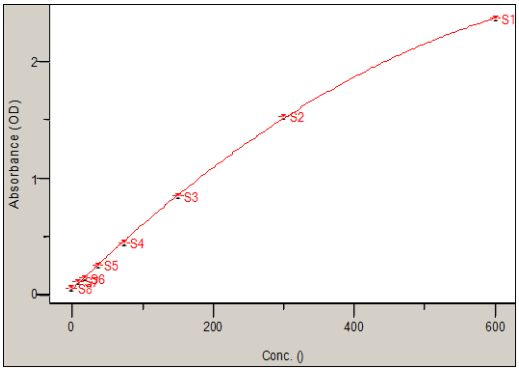


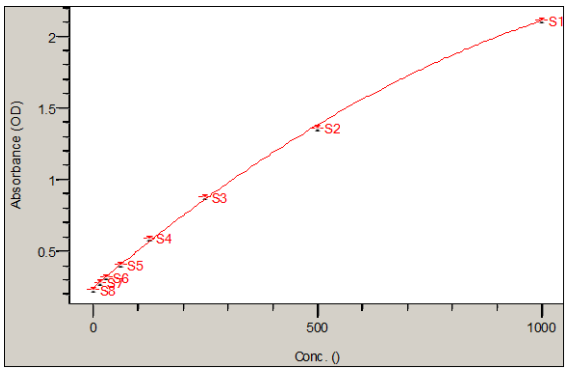
IL-17A



IL-22



TNF-α



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