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User's Manual

Multiplex Human Cytokine ELISA Kit

REF **DEIA10237**

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

This multiplex ELISA kit for pro-inflammatory cytokines is designed for semi-quantitative and simultaneous determination of pro-inflammatory cytokines including interleukin-1α (IL-1α), interleukin 1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), granulocyte macrophage colony stimulating factor (GM-CSF), monocyte chemotactic and activating factor (MCAF), and tumor necrosis factor-α (TNF-α) in cell culture supernatant and other biological samples. The kit is intended FOR LABORATORY RESEARCH USE ONLY and is not for use in diagnostic or therapeutic procedures.

General Description

Inflammation is body's protective response to foreign subjects, pathogens, tissue damage, autoimmune and other harmful stimuli. It is initiated by production of a cascade of chemicals and cytokines in affected area. These pro-inflammatory mediators result in vasodilation, increased vascular permeability, influx of blood, plasma leakage, neutrophil and macrophage infiltration and activation. Inflammatory reaction plays an important role in limiting foreign substance and engulfing pathogens and tissue debris.

Acute inflammation is the initial phase to eliminate invaded foreign substances, pathogens and other harmful stimuli. If the stimuli persist, chronic inflammation will evolve. Progressive tissue destruction and shifting cell types occurs simultaneously during chronic inflammation. The cytokine profiles that regulate the procedure are different depending on the causes, location and progress of the inflammation.

IL-1α is constitutively produced as precursor by epidermal cells at large amount in healthy individual. It is likely that the cytokine is secreted through microvesicle formation since the precursor doesn't have a signal peptide. Both the unprocessed form and processed form of IL1α possess biological activity. IL-1α is important for controlling the invasion of pathogen through skin and wound healing. IL-1α has been found to stimulate its own production, fibroblast proliferation and collagen production, increase neutrophil count in blood, stimulate IL-2 production, B lymphocyte proliferation and maturation, increase the concentration of copper and lower the iron and zinc level, stimulate hepatocytes to produce acute phase protein, and act in synergy with TNF-α to stimulate the production of GM-CSF, G-CSF and IL-6. IL-1α was also found to contribute to the generation of type IV hypersensitivity reactions.

IL-1β has been found to shares the IL-1R receptor with other cytokines in IL-1 family. It is produced by activated macrophage as a proprotein, which is cleaved by caspase I to become active. Like IL-1α, IL-1β is an important pro-inflammatory mediator. IL-1β can stimulate the production of IL-6 and TNF-β. Persistent IL-1β signaling was found to contribute to the chronic inflammatory reaction in brain by sustained activation of NFκB in human glial cells, which leads to prolonged induction of selective pro-inflammatory genes.

IL-6, also called B-cell stimulatory factor-2 (BSF-2) and interferon beta-2, plays an essential role in the final differentiation of B-cells into immunoglobulin-secreting cells. IL-6 can be produced by macrophage through Toll-like receptors in response to pathogenic molecular stimuli. Toll-like receptors are pattern recognition receptors recognize pathogen associated molecule patterns. IL-6 causes increased body temperature in acute inflammatory phase by initiating synthesis of Prostaglandin E2 in hypothalamus. The cytokine is also involved in inducing myeloma and plasmacytoma growth, nerve cell differentiation, and acute phase reactant production.

Like IL-6, IL-8 can be secreted by cells with Toll-like receptors in response to the stimulation of pathogens. IL-8's primary function is to recruit neutrophils and other target cells through chemotaxis. Neutrophils, then, phagocytose the antigen which triggers the antigen pattern Tolllike receptors.

GM-CSF is an extracellular homodimer polyprotein, functioning as a hematopoietic growth factor and immune modulator. It can be produced and act upon a variety of cell types, including T-lymphocytes, B-lymphocytes, monocytes/macrophages, endothelial cells, fibroblasts, stromal cells, mesothelial cells, kerotinocytes, osteoblasts, uterine epithelial cells, synoviocytes, mast cells and various solid tumors. GM-CSF stimulates stem cells to produce granulocytes and monocytes to cope with infection. Recombinant GM-CSF is used to boost white blood cell count of cancer patients after chemotherapy. rGM-CSF may also be useful as an immune tonic for anemia and AIDS patients.

IFN-γ is produced predominantly by natural killer and natural killer T cells as part of the innate immune response, and by CD4 and CD8 cytotoxic T lymphocyte (CTL), effector T cells once antigen-specific immunity develops. IFN-γ primarily stimulates its own expression and upregulates other genes to stimulate and modulate immunity through the Jak-Stat signaling pathway. It promoters T helper 1 differentiation and cell associated immunity, and also suppresses T helper 2 differentiation and humoral immunity.

IFN-γ is released from viral infected cells and acts upon neighboring cells to produce large amount protein kinase R, which phosphorylates transcription initiation factor eIF in response to viral infection. As a results, enzymes critical to mRNA replication is reduced and viral mRNA replication inhibited. In conjunction with CD40, IFN-γ binds to and activates macrophages, which are then able to kill intracellular pathogens. Bound IFN-γ causes the macrophage to produce elevated amounts of both MHC class I and II molecules, thus increasing the macrophage's presentation of foreign peptides. It also stimulates the production of antigenprocessing associated transportors and enzymes. IFN-γ is critical for controlling viral and other intracellular pathogen infection and tumor development. Aberrant IFN-γ expression is also associated with a number of autoimmune diseases.

MCAF is also called monocyte chemotactic protein-1 (MCP-1) and chemokine (C-C motif) ligand 2 (CCL2). It is primarily secreted by monocytes, macrophages and dendritic cells. It can be induced by platelet derived growth factor (PDGF). This cytokine displays chemotactic activity for monocytes and basophils but not for neutrophils or eosinophils. MCAF causes the degranulation of basophils and mast cells and augments the activity of monocyte and macrophage. MCAF plays an important role in inflammation, and also implicated with angiogenesis, auto-immune diseases, renal diseases, chronic infection and granuloma formation.

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic inflammatory cytokine. It has both growth promotion and inhibition effect to some cells. Secretion of the cytokine at low level is beneficial to body's normal function as it maintains homeostasis by regulating the body's circadian rhythm and promotes the remodeling or replacement of injured and senescent tissue by stimulating fibroblast growth. TNF-α plays a role in the immune response to bacterial, and certain fungal, viral, and parasitic invasions as well as in the necrosis of specific tumors. In the local inflammatory immune response, TNF-α initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection. TNF-α also stimulates blood clotting which serves to contain the infection. TNF-α hyper-expression in response to the components of some bacteria such as LPS can cause septic shock.

This ELISA assay is a 3.5 hour solid phase immunoassay readily applicable to measure the levels of eight cytokines in cell culture supernatant, and other biological fluids. It showed no cross reactivity with various other proteins. This Multiplex ELISA is expected to be effectively used for investigations of inflammatory cytokine expression in various experimental, pathological and physiological conditions.

Principles of Testing

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 precoated with monoclonal antibodies specific to IL-1α, IL-1β, L-6, IL-8, INF-γ, GM-CSF, MCAF, and TNF-α respectively. Standards or samples are then added to the strips, and the biotinconjugated 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.

Reagents And Materials Provided

1. ANTIBODY COATED MICROTITER PLATE 96 wells, The plate contains twelve 8-well ELISA strips. Each of the eight wells has been coated with a different monoclonal antibodies specific to one of the 8 cytokines as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	IL-1 α	IL-1 α	IL-1 α	IL-1 α	IL-1 α	IL-1 α	IL-1 α	IL-1 α	IL-1 α	IL-1 α	IL-1 α	IL-1 α
B	IL-1β	IL-1β	IL-1β	IL-1β	IL-1β	IL-1β	IL-1β	IL-1β	IL-1β	IL-1β	IL-1β	IL-1β
C	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6
D	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8
E	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF
F	INF-γ	INF-γ	INF-γ	INF-γ	INF-γ	INF-γ	INF-γ	INF-γ	INF-γ	INF-γ	INF-γ	INF-γ
G	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF
H	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNFα	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α

2. BIOTIN CONJUGATE MIXTURE, Mixture of Biotin conjugated anti-human cytokine antibodies, 6 mL

3. HRP CONJUGATE MIXTURE, Mixture of Horseradish Peroxidase Conjugates, 11 mL

4. STANDARD MIXTURE, 2 vials, Two vials of lyophilized standard mixture. Each vial contains a buffered protein base and eight pro-inflammatory cytokines at different concentrations: IL-1α 860pg, IL-1β 1400pg, IL-6 680pg, IL-8 1700pg, GM-CSF 950pg, IFN-γ 550pg, MCAF 2000pg, TNFα 1050pg.

5. CALIBRATOR DILUENT I, 25 mL, PBS buffer containing bovine serum albumin and preservative. For serum/plasma testing.

6. CALIBRATOR DILUENT II, 25 mL, Cell culture medium RPMI 1640 with newborn calf serum and

preservative. For cell culture supernatant testing.

7. WASH BUFFER (20×), 60 mL, 20-fold concentrated solution of buffered surfactant.

8. SUBSTRATE A, 10 mL, Buffered solution with urea hydrogen peroxide

9. SUBSTRATE B, 10 mL, Buffered solution with TMB.

10. STOP SOLUTION, 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: 10-100uL and 50-200uL 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.

Storage

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

Specimen Collection And 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 -70°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 -70°C for future testing.

Reagent Preparation

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 (1×): Add 60 mL of Wash Buffer (20×) and dilute to a final volume of 1200 mL with distilled or deionized water. Mix thoroughly. If a smaller volume of Wash Buffer (1×) is desired, add 1 volume of Wash Buffer (20×) to 19 volumes of distilled or deionized water. Wash Buffer (1×) 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, containing bovine serum albumin and PBS, is for serum/plasma testing. Calibrator Diluent II, containing 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 10 minutes with gentle agitation prior to making further dilutions. The diluted standards should be used immediately. The high concentration standard stock can be aliquoted and stored frozen at -70°C for future use.

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	1:64
A. IL-1α	430	215	6.7
B. IL-1β	700	350	10.9
C. IL-6	340	170	5.3
D. IL-8	850	425	13.3
E. GM-CSF	475	237.5	7.4
F. IFN-γ	275	137.5	4.3
G. MCAF	1000	500	15.6
H. TNF-α	525	262.5	8.2

Assay Procedure

1. Prepare Wash Buffer (1X) and dilute the Standard Mixture before starting assay procedure (see Preparation of Reagents).
2. Add 100uL 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 50uL 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 (1×) 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 (1×). Always adjust your washer to aspirate as much liquid as possible and set fill volume at 350µL/well/wash (range: 350-400µL). 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 HRP 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 within 10 minutes.

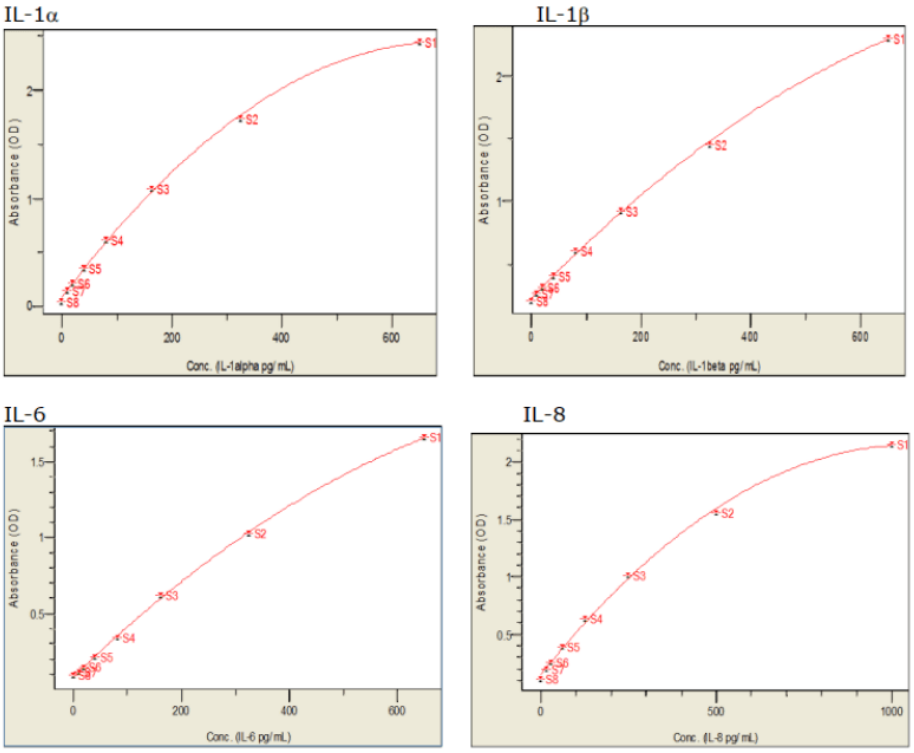
Calculation

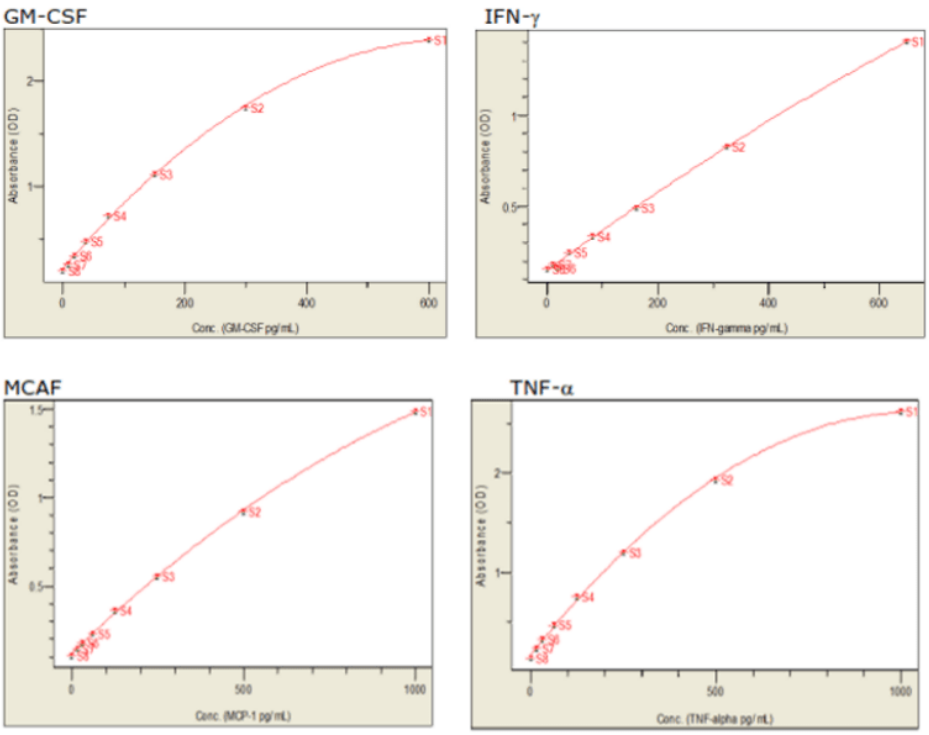
Form row A to row H, the OD readings of each well on the strip reflect the concentrations of each of the eight cytokines: IL-1α, IL-1β, IL-6, IL-8, INF-γ, GM-CSF, MCAF, and TNF-α in order. In semi-quantitative assay, 8 rough curves for the 8 cytokines can be generated from the OD readings of the high 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 CD.

Typical Standard Curve

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





Performance Characteristics

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.

Cytokine (pg/ml)	1	1:2	1:64	Cal I
A. IL-1α	1.681	1.095	0.087	0.041
B. IL-1β	1.384	0.951	0.107	0.065
C. IL-6	1.711	1.109	0.095	0.059
D. IL-8	1.299	0.910	0.151	0.113
E. GM-CSF	1.177	0.785	0.148	0.126
F. IFN-γ	1.499	0.833	0.194	0.175
G. MCAF	1.459	0.933	0.107	0.087
H. TNF-α	2.018	1.406	0.188	0.119

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 (20×) is stored at a lower temperature (2-5°C), crystals may form, which must be dissolved by warming to 37°C prior to use.

Limitations

1. THE KIT IS FOR LABORATORY RESEARCH USE ONLY, IS NOT FOR USE IN DIAGNOSTIC PROCEDURES.
2. 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.
3. 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 performation.
4. 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.