Multiplex Human Cytokine ELISA Kit

(M1/M2/MDSC Cytokines)

For Simultaneous Quantitative Determination of M1/M2/MDSC
Cytokines Including Granulocyte Macrophage Colony Stimulating
Factor, Interferon-γ, Interleukin-4, Interleukin-6, Interleukin-10,
Interleukin-12, Monocyte Chemotactic and Activating Factor,
and Tumor Necrosis Factor-α, in Cell Culture Supernatant
and Other Biological Samples

Catalogue Number: EM10002

96 tests

FOR LABORATORY RESEARCH USE ONLY
NOT FOR DIAGNOSTIC USE

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INTENDED USE

This multiplex ELISA kit for M1/M2/MDSC cytokines is designed for semi-quantitative and simultaneous determination of cytokines relevant to the proliferation of Myeloid Derived Suppressor Cells (MDSCs) and their differentiation toward M1 or M2 phenotype. The kit simultaneously determines granulocyte macrophage colony stimulating factor (GM-CSF), interferon-γ (IFN-γ) interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12), monocyte chemotactic and activating factor (MCAF, also known as MCP-1), 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 M1/M2/MDSC cytokine multiplex ELISA kit is expected to be useful for the investigation of the relationship of cytokine expression and MDSC induced inmmunosuppression 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

Myeloid derived suppressor cells (MDSCs) are myelogenous cells that are capable of negatively regulating T cell immunity. Increased numbers of MDSCs are found in pathological conditions such as malignancy, transplantation, chronic infection and inflammation. MDSCs cannot be classified by a standard leukocyte linage marker since MDSCs are comprised of various myeloid originated cells at immature status including myeloid progenitor cells, immature monocytes, dendritic cells, and granulocytes. MDSCs in human are broadly defined as lin(-/low)CD33(+) HLA-DR(-)CD11b(+) cells with altered enzyme and cytokine profile and immunosuppressive function. While CD33 (+) and CD11b (+) denote myeloid origin in human, Gr-1(+) and CD11b (+) define myeloid origin in MDSCs in mouse.

In chronic inflammation caused by cancer, the interaction between tumor cells and MDSCs causes MDSCs to expand and increase its potential in T cell inhibition (Sevko et al. 2013). MDSCs have been recognized as one of the major mechanisms for tumor evasion from host immunity and are recently evaluated as target for cancer treatment (Sinha et al. 2005).

Cytokines are believed to play a critical role in MDSC development and differentiation. GM-CSF and IL-6 have been shown to stimulate MDSC expansion in vivo and in vitro ((Lechner et al. 2010, Morales et al. 2010). T-helper 2 cytokines, IL-4 and IL-13, are the major polarization signals for MDSC to differentiate toward the more T-cell inhibitory M2 phenotype of MDSCs (Bronte et al. 2003, Sinha et al. 2005). Additionally, interleukin 4 receptor alpha (IL-4Rα), the common receptor for IL-4 and IL-13, has been found to be up-regulated in MDSCs (Mandruzzato et al. 2009). One of the main characteristics of M2 MDSCs is the up-regulation of IL-10 and down-regulation of IL-12 (Bunt et al. 2007). IL-10 inhibits cell immunity by decreasing the secretion of T helper 1 type cytokines and the expression of MHC class II antigens and co-stimulatory molecules. It has also been postulated that M2 MDSC-mediated T- cell inhibition is the consequence of increased production of arginases and reactive oxygen species by MDSCs (Zea et al. 2005, Rodriguez et al. 2006) and the enzymes secreted by MDSCs block the synthesis of zeta chain in T-cell receptor complex and sequester cystine and limit the availability of cysteine to T-cells. Signal transduction through calcium binding protein S100A8/A9 and

signal transduction and activator of transcription (STAT) is likely implicated in MDSC activities (Zhao et al. 2012).

M2 MDSCs inhibit effector T cells but promote Regulatory T cells. The increased expression of cytokines and chemokines such as VEGF, MCP-1 and MIF in the tumor microenvironment is believed to promote the infiltration of MDSCs and stimulate tumor angiogenesis and metastasis (Bellamy et al. 2001, Huang et al. 2007, and Simpson et al. 2013).

IFN-γ is a potent activator for MDSCs to develop into the more tumoricidal and virucidal M1 phenotype. IFN-γ and other M1 polarizing signals up-regulate IL-12 and TNF-α in M1 MDSC. M1-polarized MDSCs express elevated signature markers such as inducible Nitric Oxide Synthase (iNOS), nitric oxide (NO), TNF-α, IFN-γ (Yang et al. 2013). Nitric oxide (NO) and TNF-α play important roles in clearing bacterial, and certain fungal, viral, and parasitic invasions as well as in the necrosis of specific tumors and nitric oxide (NO)

This ELISA assay is a 3.5 hour solid phase immunoassay readily applicable to measure the levels of eight cytokines relevant to the generation and differentiation of MDSCs 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 GM-CSF, IFN-γ, IL-4, IL-6, IL-10, IL-12, MCAF (also known as MCP-1), 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 EM02-1) _____ *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	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF
B	IFN-γ	IFN-γ	IFN-γ	IFN-γ	IFN-γ	IFN-γ	IFN-γ	IFN-γ	IFN-γ	IFN-γ	IFN-γ	IFN-γ
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-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6
E	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10
F	IL-12	IL-12	IL-12	IL-12	IL-12	IL-12	IL-12	IL-12	IL-12	IL-12	IL-12	IL-12
G	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF	MCAF
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 EM02-2) _____ *6 mL*
    Mixture of Biotin conjugated anti-human cytokine antibodies.

3. **HRP CONJUGATE MIXTURE** (Part EM02-3)_____ *11 mL*
    Mixture of Horseradish Peroxidase Conjugates

4. **STANDARD MIXTURE** (Part EM02-4) _____ *2 vials*
    Two vials of lyophilized standard mixture. Each vial contains a buffered protein base
    and eight pro-inflammatory cytokines at different concentrations: GM-CSF 1000pg, IFN-
    γ 3200pg, IL-4 2000pg, IL-6 800pg, IL-10 3200pg, IL-12 2000pg, MCAF 3200pg, TNF-α
    2000pg.

5. **CALIBRATOR DILUENT I** (Part EM02-5)_____ *25 mL*
    Newborn calf serum with PBS buffer and preservative. *For serum/plasma testing.*

6. **CALIBRATOR DILUENT II** (Part EM02-6)_____ *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 EM02-7)_____ *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°C to - 80°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°C to - 80°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 (-20°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. GM-CSF	500	250	125	62.5	31.25	15.6	7.8
B. IFN-γ	1600	800	400	200	100	50	25
C. IL-4	1000	500	250	125	62.5	31.25	15.6
D. IL-6	400	200	100	50	25	12.5	6.25
E. IL-10	1600	800	400	200	100	50	25
F. IL-12	1000	500	250	125	62.5	31.25	15.6
G. MCAF	1600	800	400	200	100	50	25
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 <u>1 hour at room temperature.</u>

    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: GM-CSF, IFN-γ, IL-4, IL-6, IL-10, IL-12, 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 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. GM-CSF	1.849	1.186	0.748	0.469	0.299	0.212	0.171	0.122
B. IFN-γ	1.790	1.070	0.606	0.387	0.255	0.176	0.141	0.107
C. IL-4	1.977	1.270	0.752	0.443	0.277	0.179	0.139	0.083
D. IL-6	2.018	1.309	0.821	0.465	0.271	0.155	0.114	0.072
E. IL-10	1.814	1.169	0.728	0.438	0.274	0.176	0.126	0.068
F. IL-12	2.231	1.512	0.972	0.577	0.371	0.246	0.185	0.111
G. MCAF	1.932	1.328	0.806	0.451	0.265	0.168	0.122	0.078
H. TNF-α	2.249	1.511	0.993	0.591	0.395	0.264	0.199	0.135

STANDARD CURVES

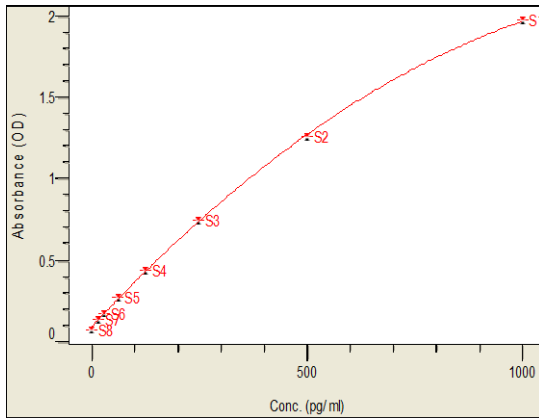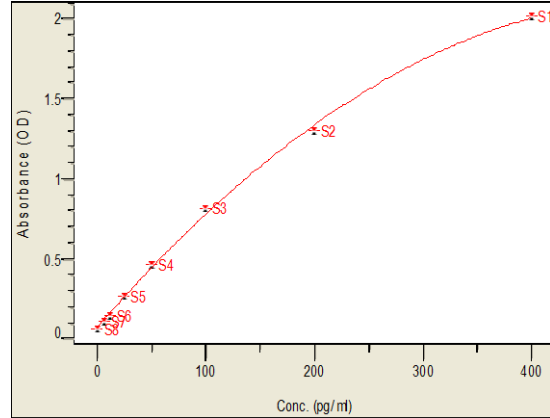The cytokine standard curves were generated from the above data are for illustration only.

GM-CSF

IFN-γ

IL-4
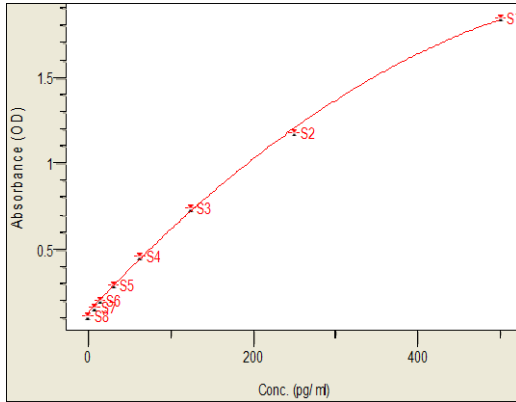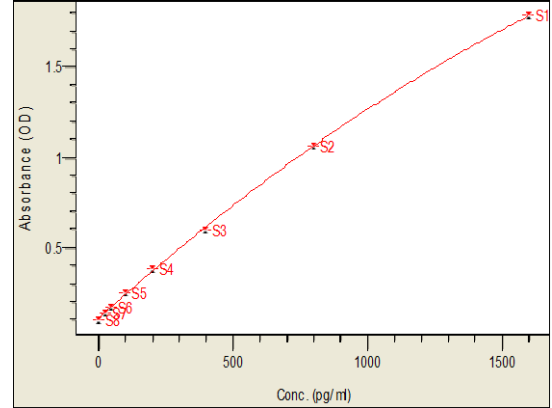
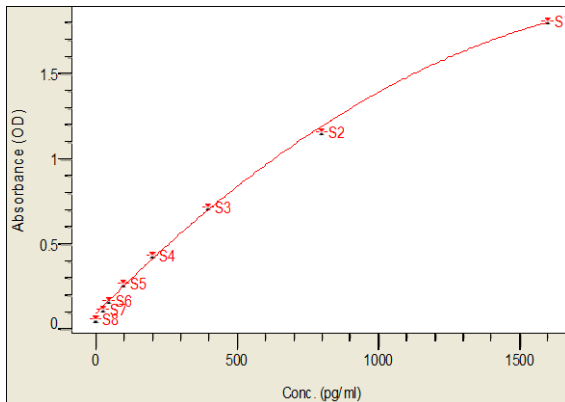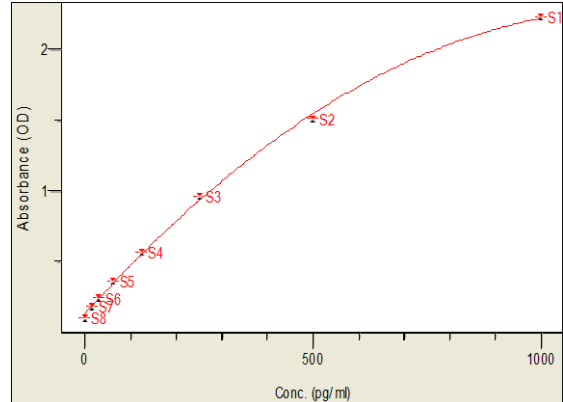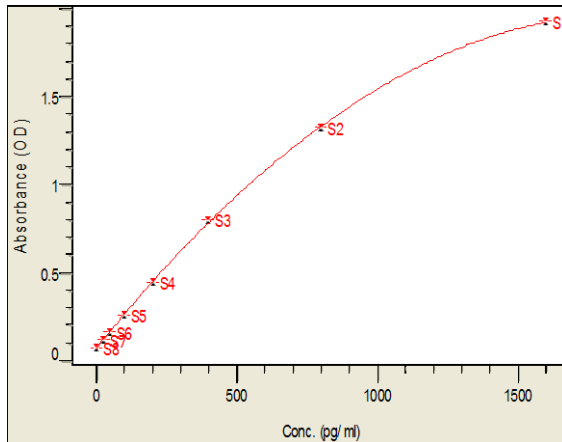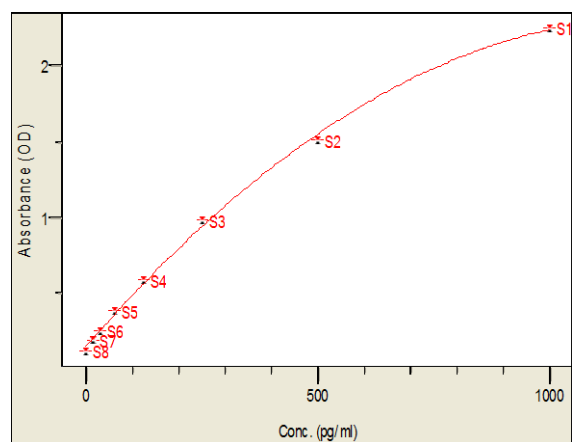**IL-6**

IL-10

IL-12

MCAF

TNF-α

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