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User's Manual

Multiplex Human Cytokine ELISA Kit(M1/M2/MDSC Cytokines)

REF **DEIA-MX001**

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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 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-γinterleukin-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.

General Description

Myeloid derived suppressor cells (MDSCs) are myelogenous cells that are capable of negatively regulating T cell immunity. MDSCs are important components of tumor micro-environment. Increased numbers of MDSCs are found in pathological conditions such as malignancy, 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, immature dendritic cells, and immature 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 of 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-4R), 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 costimulatory 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 to 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 some tumors.

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.

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 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.

Reagents And Materials Provided

1. ANTIBODY COATED MICROTITER PLATE, 96 wells
2. BIOTIN CONJUGATE MIXTURE, 6ml
3. HRP CONJUGATE MIXTURE, 11ml
4. STANDARD MIXTURE, 2 vials
5. CALIBRATOR DILUENT I, 25ml
6. CALIBRATOR DILUENT II, 25ml

7. WASH BUFFER (20X), 60ml
8. SUBSTRATE A, 10ml
9. SUBSTRATE B, 10ml
10. STOP SOLUTION, 14ml

Materials Required But Not Supplied

1. Single or multi-channel precision pipettes with disposable tips: 10-100 µL and 50-200 L 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

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 -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.

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 (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. We provide a table for correct amounts of Substrate Solution to prepare.
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 25 folders (1:25) 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.

Assay Procedure

1. Prepare Wash Buffer (1X) and dilute the Standard Mixture before starting assay procedure (see Preparation of Reagents).
2. Add 100 µL of Standard or Sample to the appropriate well of the antibody pre-coated Microtiter Plate and incubate 1 hour at room temperature.
3. Without discarding the standards and samples, add 50 µL 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 uL/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 100 µL 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 100 µL Substrate Solution to each well. Cover and incubate for 15 minutes at room temperature.
9. Add 100 µL 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

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.

Specificity

The kit shows no cross reactivity with other proteins.