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User's Manual

Multiplex Human Cytokine Th1/Th2/Th17 ELISA Kit

REF **DEIA-MX002**

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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

Human Multiplex ELISA Kit (IFN gamma, IL2, IL4, IL10, IL13, IL17A, IL22, TNF alpha) is an Enzyme Immunoassay kit for the semi-quantification of IFN gamma, IL2, IL4, IL10, IL13, IL17A, IL22 and TNF alpha in serum, plasma and cell culture supernatants.

General Description

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-(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 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.

Principles of Testing

This is a multiplex enzyme immunoassay for the semi-quantification of Th1/Th2/Th17/Treg Cytokines: IFN gamma, IL2, IL4, IL10, IL13, IL17A, IL22 and TNF alpha.

This assay employs the semi-quantitative sandwich enzyme immunoassay technique. The Antibodies specific to IFN gamma, IL2, IL4, IL10, IL13, IL17A, IL22 and TNF alpha has been pre-coated onto wells of microtiter plate. Standards Mixture and samples are pipetted into the wells and any target cytokines present is bound by the immobilized specific antibodies. After incubation and washing, added Mixed Antibody-Conjugate to each well and incubate. After washing away any unbound substances, the HRP Conjugate is added to the wells and incubated. The wells are thoroughly washed to remove all unbound HRP Conjugate. A TMB substrate is added to the wells and color develops in proportion to the amount of target cytokines in the initial step. The color development is stopped by the addition of STOP solution and the intensity of the color is measured at a wavelength of 450 nm. The concentration of Th1 / Th2 / Th17 / /Treg cytokines in the samples is then determined by comparing the O.D of samples to the standard curve.

Reagents And Materials Provided

1. Antibody Coated microplate, 8 × 12 strips, 4°C

   The Antibody Coated microplate contains twelve 8-well ELISA strips. Each of the eight wells has been coated with a different antibody specific to one of the 8 cytokines as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ
B	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2
C	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10
D	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	IL4
E	IL13	IL13	IL13	IL13	IL13	IL13	IL13	IL13	IL13	IL13	IL13	IL13
F	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A	IL17A
G	IL22	IL22	IL22	IL22	IL22	IL22	IL22	IL22	IL22	IL22	IL22	IL22
H	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα	TNFα

2. Standards Mixture, 2 vial (lyophilized), 4°C. Each vial contains a buffered protein base and 8 cytokines at different amount: IFN gamma: 1600 pg, IL2: 2000 pg, IL10: 1000 pg, IL4: 1500 pg, IL13: 1000 pg, IL17A: 1280 pg, IL22: 1000 pg, TNF alpha: 1600 pg.
3. Biotin Antibody conjugate mixture, 6 mL (ready to use), 4°C
4. HRP conjugate mixture, 11 mL (ready to use), 4°C
5. Standard Diluent I (for serum / Plasma samples), 25 mL (ready to use), 4°C
6. Standard Diluent II (for cell culture supernatant samples), 25 mL (ready to use), 4°C
7. 20× Wash Buffer, 60 mL, 4°C
8. Substrate A, 10 mL, 4°C
9. Substrate B, 10 mL, 4°C (protect from light)
10. STOP solution, 14 mL (ready to use), 4°C

Materials Required But Not Supplied

1. Microplate reader capable of measuring absorbance at 450nm
2. Pipettes and pipette tips
3. Deionized or distilled water
4. Automated microplate washer (optional)

Storage

Store the unopened kit at 2-8 °C. Use the kit before expiration date

Specimen Collection And Preparation

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

To obtain the data of each cytokine, at least 0.8 ml of the sample is needed to complete one run of the assay. It is recommended to have a larger volume available in case that the experiments have to be repeated. The unused samples should be stored frozen at ≤-20°C or ≤-70°C to avoid sample degradation. For long term storage, store at ≤-70°C is recommended.

1. Cell Culture Supernatants: Remove particulates by centrifugation and aliquot & store samples at ≤ -20 °C or ≤-70°C. Avoid repeated freeze-thaw cycles.

2. Serum: Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation

for 10 minutes at 1000 x g at 4°C. Collect serum and assay immediately or aliquot and store samples at ≤ -20 °C or ≤-70°C. Avoid repeated freeze-thaw cycles.

3. Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g at 4°C within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C or ≤-70°C. Avoid repeated freeze-thaw cycles.

Note:

1. Do not use haemolytic, icteric or lipaemic specimens.
2. Avoid disturbing the white buffy layer when collection serum / plasma sample.
3. Samples containing sodium azide should not be used in the assay.

Reagent Preparation

1. 1× Wash Buffer: Dilute 20× Wash Buffer into distilled water to yield 1× Wash Buffer. (E.g., add 30 mL of 20× Wash Buffer into 570 mL of distilled water to a final volume of 600 mL) The 1× Wash Buffer is stable for up to 4 weeks at 2-8°C. Mix well before use.

2. TMB substrate: Substrate A and Substrate B (containing TMB) 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)	Total volume (ml)
2 (16 wells)	1.5	1.5	3.0
4 (32 wells)	3.0	3.0	6.0
6 (48 wells)	4.0	4.0	8.0
8 (64 wells)	5.0	5.0	10.0
10 (80 wells)	6.0	6.0	12.0
12 (96 wells)	7.0	7.0	14.0

3. Standards Mixture:

Please select appropriate Diluent buffer for each sample type.

Two vials of Standards are provided in this kit to allow both serum/plasma and cell culture supernatant testing. Standard diluent I contains animal serum and PBS for serum/plasma testing. Standard diluent II contains animal serum and RPMI 1640 for cell culture supernatant testing. The standards provided in the kit are for customers to use at own discretion. If samples generate values higher than the highest standard, dilute the samples with appropriate standard diluent and repeat assay.

High concentration standard stock:

Reconstitute standards with either 2ml Standard diluent I (for serum/plasma testing) or Standard diluent II (for cell culture supernatant testing) to obtain high concentration standard stock. Allow solution to sit for 15 minutes with gentle agitation prior to making dilutions. This stock solution can be aliquoted and stored frozen at -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 standards mixture to test together with up to 10 test samples. If more accurate results are required, a two-fold serial dilution with appropriate dilution buffer can generate a more accurate standard

curve. However, the number of test samples will be reduced.

To dilute standards, produce a serial 2-fold dilution series from 1:2 to 1:64 dilutions. The concentration of 8 cytokines in different dilutions of the mixed standard are listed as below:

Cytokine (pg / mL)	High Conc. Standard Stock	1:2	1:4	1:8	1:16	1:32 (Low Conc. Standard)	1:64
A: IFN γ	800	400	200	100	50	25	12.5
B: IL2	1000	500	250	125	62.5	31.25	15.625
C: IL10	500	250	125	62.5	31.25	15.625	7.8125
D: IL4	750	375	187.5	93.75	46.875	23.438	11.719
E: IL13	500	250	125	62.5	31.25	15.625	7.8125
F: IL17A	640	320	160	80	40	20	10
G: IL22	500	250	125	62.5	31.25	15.625	7.8125
H: TNF α	800	400	200	100	50	25	12.5

Assay Procedure

All materials should be equilibrated to room temperature (20-25°C) before use.

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add 100 µl of standards or samples into wells. Cover and incubate for 1h at RT. (Example) To obtain the approximate concentrations of 8 cytokines on 10 test samples (T1-T10), the low concentration standard mixture (1:32 from high concentration mix, S1) and high concentration standard mixture (stock, S2) and test samples can be added as scheme below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
B	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
C	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
D	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
E	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
F	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
G	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
H	S1	S2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10

3. Without discarding the content, add 50 µl of Biotin conjugate mixture into each well. Mix well, cover and incubate for 1 hour at room temperature.
4. Aspirate each well and wash, repeating the process 4 times for a total 5 washes. Wash by filling each well with 1× Wash Buffer (350µl) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.

   Note:

   Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame.

For automated Washing: It is recommended that the washer be set for a soaking time of 10 seconds or shaking time of 5 seconds between washes.

5. Add 100 µl of HRP conjugate mixture into each well. Cover and incubate for 1h at room temperature.
6. Prepare Substrate Solution no more than 15 minutes before end of HRP conjugate mixture incubation.
7. Aspirate each well and wash as step 4.
8. Add 100 µl of Substrate solution mixture to each well. Incubate for 15 minutes at room temperature in dark.
9. Add 100 µl of Stop Solution to each well. The color of the solution should change from blue to yellow.
10. Read the OD with a microplate reader at 450 nm immediately. It is recommended reading the absorbance within 30 min after adding stop solution.

Calculation

1. Calculate the average absorbance values for each set of standards and samples.
2. For semi-quantitative assay, 8 rough curves for 8 cytokines can be generated from OD readings of high concentration standard and low concentration standard mixture. The approximate cytokine concentration can be obtained from the rough curves. As the standard curves might not be perfectly straight, the concentration obtained from a rough curve derived from 2 points would not be very accurate.
3. To obtain more accurate results, more dilution points can be used when generating standard curves.

Typical Standard Curve

EXAMPLE OF TYPICAL STANDARD VALUES

The following data shows the OD readings of a run of this multiplex ELISA with multiple dilutions using standard diluent I. It is for demonstration purpose only and cannot be used to replace standard curve for testing. Each investigators have to assay standards along with test samples.

	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

Detection Range

IFN gamma 12.5 - 800pg/ml; IL13 7.8 - 500pg/ml

IL2 15.6 - 1000pg/ml; IL17A 10 - 640pg/ml

IL10 7.8 - 500pg/ml; IL22 7.8 - 500pg/ml

IL4 11.7 - 750pg/ml; TNF alpha 12.5 - 800pg/ml

Precautions

1. Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
2. Store the kit at 2-8°C at all times.
3. Prior to beginning the assay procedure, bring all reagents and required number of strips to room temperature (20-25°C).
4. Unused wells must be stored at 2-8 °C in the sealed foil pouch and used in the frame provided.
5. Avoid air bubbles in the wells as this could result in lower binding efficiency and higher CV% of duplicate reading.
6. Briefly spin down the all vials before use.
7. If crystals are observed in the 20× Wash Buffer, warm to 37°C until the crystals are completely dissolved.
8. Substrate B contains 20% acetone, keep this reagent away from sources of heat or flame.
9. Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
10. Ensure complete reconstitution and dilution of reagents prior to use.
11. Take care not to contaminate the mixed TMB Substrate. Do not expose the mixed TMB solution to glass, foil or metal. If the solution is blue before use, DO NOT USE IT.
12. Change pipette tips between the addition of different reagent or samples.
13. Use a plate cover for each incubation step.
14. Taping the well strips together with lab tape can be done as an extra precaution to avoid plate strips from coming loose during the procedure.