Version 06-05/25

User's Manual

CRS Cytokine Multiplex ELISA Kit

REF **DEIA-PX0003**

Σ **96T**

RUO

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

The kit is an Enzyme Immunoassay kit for the semi-quantification of Human CAR-T / CRS (Cytokine Release Syndrome) Cytokine (IL2, IL6, IL10, IFN gamma) in serum, plasma and cell culture supernatants.

Principles of Testing

The antibodies specific to human IL-2, IL-6, IL-10, and IFN-gamma has been pre-coated onto a 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 step, 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 O.D. 450 nm. The concentration of CART / CRS cytokines in the samples is then determined by comparing the O.D. 450 nm of samples to the standard curve.

Reagents And Materials Provided

1. Antibody Coated Microplate, 8 × 12 strips, 4°C
2. Standards Mixture, 3 vials (lyophilized), 4°C
3. 100× Antibody Conjugate Mixture, 120 µL, ≤ -20°C
4. 1000× HRP-Streptavidin Solution, 15 µL, 4°C
5. Standard / Sample Diluent Buffer, 20 mL, 4°C
6. 10× Antibody Diluent Buffer, 10 mL, 4°C
7. 10× Wash Buffer, 2 × 50 mL, 4°C
8. TMB substrate, 12 mL (ready to use), 4°C (protect from light)
9. STOP solution, 12 mL (ready to use), 4°C
10. Plate sealer, 3 pieces, 4°C.

Notes: Use the kit before expiration date. Opened kits retain activity for 8 weeks if stored as described above

Materials Required But Not Supplied

(1) Microplate reader capable of reading at 450 nm

(2) Deionized or distilled water

(3) Pipettes and pipette tips

(4) Multichannel micropipette reservoir

Reagent Preparation

1. 1× Wash Buffer: Dilute 10× Wash Buffer into distilled water to yield 1X Wash Buffer. (E.g. add 50 mL of 10× Wash Buffer into 450 mL of distilled water to a final volume of 500 mL) The 1X Wash Buffer is stable for up to 4 weeks at 2-8°C. Mix well before use.

2. 1× Antibody Diluent Buffer: Dilute 10× Antibody Diluent Buffer into distilled water to yield 1× Antibody Diluent Buffer.(E.g., add 10 mL of 10× Wash Buffer into 90 mL of distilled water to a final volume of 100 mL) The 1× Antibody Diluent Buffer is stable for up to 4 weeks at 2-8°C. Mix well before use.

3. 1× Antibody Conjugate Mixture: It is recommended to prepare this reagent immediately prior to use and use it within 20 min after preparation. Dilute 100× Antibody Conjugate Mixture concentrate into 1× Antibody Diluent Buffer to yield 1× detection antibody solution. (e.g. 12μL of 100× Antibody Conjugate Mixture concentrate + 1188μL of Diluent Buffer).

4. 1× HRP-Streptavidin Solution: It is recommended to prepare this reagent immediately prior to use and use it within 20 min after preparation. Dilute 1000× HRP-Streptavidin concentrate solution into 1× Antibody Diluent Buffer to yield 1× HRP Streptavidin Solution buffer. (e.g. 1μL of 1000× HRPStreptavidin concentrate solution + 999μL of Diluent Buffer).

5. Sample: Diluent serum and plasma samples with equal volume of Standard/ Sample Diluent Buffer before assay (1:1, dilution factor=2). If the initial assay found samples contain proteins higher than the highest standard, the samples can be diluted with Standard / Sample Diluent Buffer and then re-assay the samples. For the calculation of the concentrations this dilution factor has to be taken into account. Cell culture supernatantscould be assayed directly. (It is recommended to do pretest to determine the suitable dilution factor).

6. Standards Mixture:

A. Add 1 mL of Standard / Sample Diluent Buffer to reconstitute the lyophilized Standard Mixture to obtain the high concentration stock of 4 cytokines at different concentrations (see table below). Brief vortex and allow the stock standard to sit for at least 15 minutes with gentle agitation to make sure the standard is dissolved completely before making serial dilutions.

B. For quantitative assay, use the above high concentration Standards Mixture and a 32-fold diluted low concentration Standards Mixture to test together with up to 22 test samples. If more accurate results are required, a two-fold serial dilution with Standard / Sample Diluent Buffer can generate a more accurate standard curve. However, the number of test samples will be reduced

The concentrations of the 4 cytokines in different dilutions of the Standards Mixture are listed as below:

Cytokines (pg / mL)	High Conc. Standard Mixture	1:2	1:4	1:8	1:16	1:32 (Low Conc. Standard Mixture)	1:64
IL-2	500	250	125	62.5	31.25	15.6	7.8
IL-6	1000	500	250	125	62.5	31.25	15.6
IL-10	500	250	125	62.5	31.25	15.6	7.8
IFN-γ	500	250	125	62.5	31.25	15.6	7.8

Assay Procedure

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

1. Add 100 μ L of the Standards Mixture or diluted samples to the Antibody Coated microplate. **Note:** To obtain the approximate concentrations of 4 cytokines on 22 test samples, the low concentration standard mixture (S1, 1:32 from high concentration mixture), the high concentration Standards Mixture (S2) and test samples

(T1 to T22) can be added as the scheme below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
B	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
C	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
D	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
E	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
F	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
G	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
H	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22

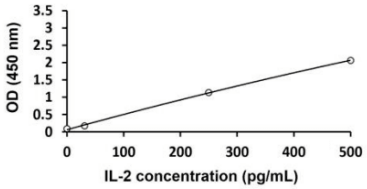
2. Cover the plate and incubate for 2 hours at room temperature.
3. Aspirate each well and wash, repeating the process 4 times for a total 6 washes. Wash by filling each well with 1× Wash Buffer (300 µL) using asquirt bottle, manifold dispenser, or autowasher. Keep the wash buffer in the wells for 30-60 sec before remove. 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.
4. Add 100 µ L of 1× Antibody Conjugate Mixture to each wells.
5. Cover the plate and Incubate for 1 hour at room temperature.
6. Aspirate each well and wash as step 3.
7. Add 100 µL of 1× HRP-Streptavidin Solution to each well. Cover the plate and incubate for 1 hour at room temperature.
8. Aspirate each well and wash as step 3, but wash for a total 7 washes.
9. Add 100 µL of TMB Substrate to each well. Cover and incubate for 10-20 minutes at room temperature in the dark.
10. Immediately Add 100 µL of Stop Solution to each well, gently tap the plate to mix well.The color of the solution should change from blue to yellow.
11. Read the OD with a microplate reader at 450 nm immediately. It is recommended reading the absorbance within 30 minutes after adding the stop solution.

Calculation

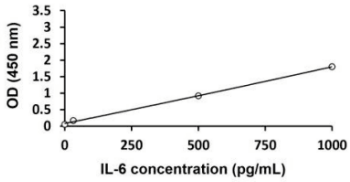
1. Calculate the average absorbance values for each set of standards and samples.
2. For semi-quantitative assay, 4 rough curves for 4 cytokines can be generated from OD readings of high concentration standard and low concentration standard mixture. The approximate cytokine concentration can be obtained 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. 4 Parameter Logistics is the preferred method for the result calculation. Other data reduction functions may give slightly different results.
4. If the samples have been diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure.

Typical Standard Curve

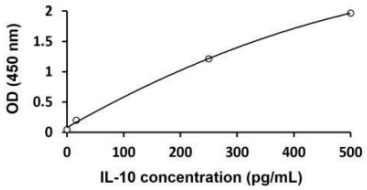
The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



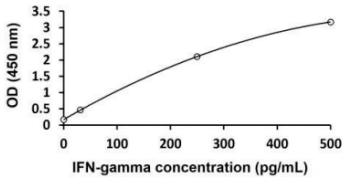
Example of Human IL-2 standard curve



Example of Human IL-6 standard curve



Example of Human IL-10 standard curve



Example of Human IFN-gamma standard curve

Precautions

1. Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
2. Upon received, store 100× Antibody Conjugate at ≤ -20°C. Store other component 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. All reagents must be mixed without foaming and briefly spin down the all vials before use.
6. If crystals are observed in the 10X Wash Buffer or Diluent Buffer warm to 37°C until the crystals are completely dissolved.
7. Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay
8. Ensure complete reconstitution and dilution of reagents prior to use.
9. 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.
10. All reagents must be mixed without foaming before use.
11. Change pipette tips between the addition of different reagent or samples.
12. 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.

Note

1. Do not use haemolytic, icteric or lipaemic specimens.
2. Samples containing sodium azide should not be used in the assay.
3. Avoid disturbing the white buffy layer when collection serum / plasma Sample.
4. To obtain the data of each cytokine, > 0.2 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.