arigoPLEX® Human CART / CRS Cytokine Multiplex ELISA Kit (IL2, IL6, IL10, IFN gamma)

arigoPLEX® Human CAR-T / CRS Cytokine Multiplex ELISA Kit (IL2, IL6, IL10, IFN gamma) 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.

Catalog number: ARG82969

Package: 96 wells

Lot. 354157

For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Cytokine release syndrome (CRS) is a form of systemic inflammatory response syndrome (SIRS) that can be triggered by a variety of factors such as infections and certain drugs. It refers to cytokine storm syndromes (CSS) and occurs when large numbers of white blood cells are activated and release inflammatory cytokines, which in turn activate yet more white blood cells. CRS is also an adverse effect of some monoclonal antibody medications, as well as adoptive T-cell therapies. When occurring as a result of a medication, it is also known as an infusion reaction.

The term cytokine storm is often used interchangeably with CRS but, despite the fact that they have similar clinical phenotype, their characteristics are different. When occurring as a result of a therapy, CRS symptoms may be delayed until days or weeks after treatment. Immediate-onset CRS is a cytokine storm, although severe cases of CRS have also been called cytokine storms.

Symptoms of CRS include fever that tends to fluctuate, fatigue, loss of appetite, muscle and joint pain, nausea, vomiting, diarrhea, rashes, fast breathing, rapid heartbeat, low blood pressure, seizures, headache, confusion, delirium, hallucinations, tremor, and loss of coordination.

Adoptive cell transfer of autologous T-cells modified with chimeric antigen receptors (CAR-T cell therapy) also causes CRS. Serum samples of patients with CAR-T associated CRS have elevated levels of IL-6, IFN-γ, IL-8 (CXCL8), IL-10, GM-CSF, MIP-1α/β, MCP-1 (CCL2), CXCL9, and CXCL10 (IP-10). The most predictive biomarkers 36h after CAR-T infusion of CRS are a fever ≥38.9 °C (102

°F) and elevated levels of MCP-1 in serum. Many of the cytokines elevated in CRS are not produced by CAR-T cells, but by myeloid cells that are pathogenically licensed through T-cell-mediated activating mechanisms. For example, in vitro co-culture experiments have demonstrated IL-6, MCP-1, and MIP-1 are not produced by CAR-T cells, but rather by inflammatory myeloid lineage cells. In vivo models have demonstrated NSG (NOD/SCID/γ-chain deficient mice) with defects of both lymphocyte and myeloid lineage compartments do not develop CRS after CAR-T cell infusion. [Provide by Wikipedia: Cytokine release syndrome]

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. 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.

MATERIALS PROVIDED & STORAGE INFORMATION

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

Component	Quantity	Storage information
Antibody Coated Microplate [1]	8 X 12 strips	4°C
Standards Mixture [2]	3 vials (lyophilized)	4°C
100X Antibody Conjugate Mixture	120 µL	≤ -20°C
1000X HRP- Streptavidin Solution	15 µL	4°C
Standard / Sample Diluent Buffer	30 mL	4°C
10X Antibody Diluent Buffer	8 mL	4°C
10X Wash Buffer	60 mL	4°C
TMB Substrate	12 mL (ready to use)	4°C (protect from light)
STOP Solution	12 mL (ready to use)	4°C
Plate sealer	4 adhesive strips	4°C

Note:

1. 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 4 cytokines as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2
B	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6
C	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10
D	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ
E	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2	IL2
F	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6	IL6
G	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10	IL10
H	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ	IFN γ

2. Standards Mixture each vial contains a buffered protein base and four cytokines at different amount: IL-2, 500 pg / IL-6, 1000 pg / IL-10, 500 pg / IFN-gamma, 500 pg.

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of reading at 450 nm
- Deionized or distilled water
- Pipettes and pipette tips
- Multichannel micropipette reservoir
- Microtiter plate washer

TECHNICAL NOTES AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Upon received, store 100X Antibody Conjugate at ≤ -20°C. Store other component at 2-8°C at all times.
- Prior to beginning the assay procedure, bring all reagents and required number of strips to room temperature (20-25°C).
- Unused wells must be stored at 2-8 °C in the sealed foil pouch and used in the frame provided.
- All reagents must be mixed without foaming and briefly spin down the all vials before use.
- If crystals are observed in the 10X Wash Buffer or Diluent Buffer warm to 37°C until the crystals are completely dissolved.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.

- Ensure complete reconstitution and dilution of reagents prior to use.
- 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.
- All reagents must be mixed without foaming before use.
- Change pipette tips between the addition of different reagent or samples.
- 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.

SAMPLE COLLECTION & STORAGE INFORMATION

The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design.

Cell Culture Supernatants - Remove particulates by centrifugation for 10 min at 1500 x g at 4°C and aliquot & store samples at-20°C up to 1 month or-80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Collect serum and assay immediately or aliquot & store samples at-20°C up to 1 month or-80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g. within 30 minutes of collection. Collect the supernatants and assay immediately or aliquot and store samples at -20°C up to 1 month or -80°C up to 6 months. Avoid repeated freeze-thaw cycles.

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.

REAGENT PREPARATION

- **1X Wash Buffer**: Dilute **10X** Wash Buffer into distilled water to yield 1X Wash Buffer. (E.g., add 50 mL of 10X 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.
- **1X Antibody Diluent Buffer**: Dilute **10X** Antibody Diluent Buffer into distilled water to yield 1X Antibody Diluent Buffer. The 1X Antibody Diluent Buffer is stable for up to 4 weeks at 2-8°C. Mix well before use.
- **1X Antibody Conjugate Mixture**: It is recommended to prepare this reagent immediately prior to use and use it within 20 min after preparation. Dilute **100X** Antibody Conjugate Mixture concentrate into **1X Antibody Diluent Buffer** to yield 1X Antibody Conjugate Solution.
- **1X HRP-Streptavidin concentrate**: It is recommended to prepare this reagent immediately prior to use and use it within 20 min after preparation. Dilute **1000X** HRP-Streptavidin concentrate into **1X Antibody Diluent Buffer** to yield 1X HRP-Streptavidin Solution buffer.

- **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 supernatants could be assayed directly. **(It is recommended to do pre-test to determine the suitable dilution factor).**
- **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 µL** 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 5 times for a **total 6 washes.** Wash by filling each well with **1X Wash Buffer (300 µL)** using a

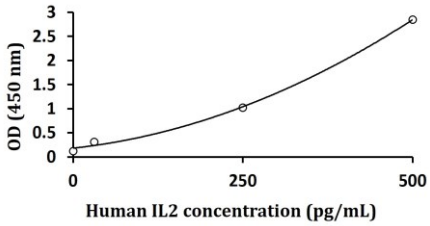
squirt bottle, manifold dispenser, or autowasher. Keep the wash buffer in the wells for 30-60 sec before remove. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.

4. Add **100 µL** of **1X 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 **1X 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 **15-25 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.

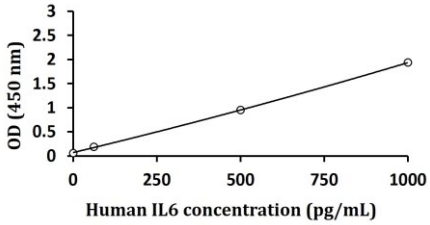
EXAMPLE OF TYPICAL STANDARD VALUES

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

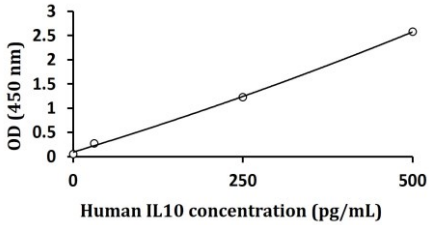
Example of Human IL-2 standard curve



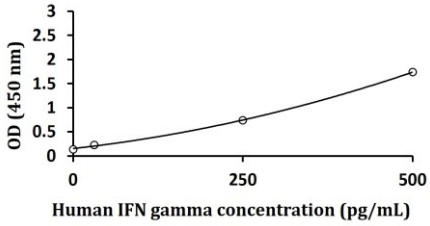
Example of Human IL-6 standard curve



Example of Human IL-10 standard curve



Example of Human IFN-gamma standard curve



CALCULATION OF RESULTS

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. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for details. (https://www.arigobio.com/elisa-analysis)
5. 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.