# TECHNICAL MANUAL

# Cytokine Multiplex PharmaGenie ELISA Kit

- **SKU CODE:** AEGE00007
- **SIZE:** 96T
- **DETECTION PRINCIPLE:** Sandwich
- **RUO:** Research-Use-Only

Revised: 08/2025

# Cytokine Multiplex PharmaGenie ELISA Kit

*Please read entire manual carefully before starting experiment.*

## Table of Contents

# 1. Key Features

**Assay Range:**

IL2: 15.625 - 500 pg/mL; IL6: 31.25 - 1000 pg/mL; IL10: 15.625 - 500 pg/mL; IFN gamma: 15.625 - 500pg/mL

**Sensitivity:**

IL2: 15.625 pg/mL; IL6: 31.25 pg/mL; IL10: 15.625 pg/mL; IFN gamma: 15.625 pg/mL

**Detection Method:**

Sandwich

**Sample Type:**

Serum, plasma and other biological samples

# 2. Storage & Expiry

Assay Genie ELISA Kits are shipped on ice packs. Please store this ELISA Kit as indicated in section 4. Validity for 12 months . Date of expiration is on the ELISA Box label.

# 3. Product Description

The Cytokine Multiplex PharmaGenie ELISA Kit is a semi-quantitative multiplex enzyme immunoassay designed for the simultaneous detection and measurement of key cytokines associated with Cytokine Release Syndrome (CRS) in CAR-T cell therapy. This kit enables concurrent analysis of human IL-2, IL-6, IL-10, and IFN-γ in serum, plasma, and cell culture supernatants.

Each well is pre-coated with a specific capture antibody designed to bind a single cytokine (IL-2, IL-6, IL-10, and IFN-γ). Standards for IL-2, IL-6, IL-10, and IFN-γ are provided, along with a single mixed solution of cytokine-specific detection antibodies.

When standards and samples are added to the wells, any cytokines present bind to the immobilized capture antibodies. After incubation and washing, the mixed cytokine-specific detection antibodies are added, forming a sandwich complex. Following a second incubation and wash, an HRP-conjugated reagent is introduced, enabling colour development upon substrate addition.

Subsequent incubation with the TMB substrate results in a colorimetric reaction proportional to the concentration of each cytokine. The reaction is stopped with a stop solution, and absorbance is measured at 450 nm. Cytokine concentrations are determined by comparing the optical density of each sample to the provided standard curve.

As a semi-quantitative assay, the kit generates a standard curve for each cytokine, enabling accurate estimation of cytokine concentrations in the same assay well. The multiplex format streamlines workflow, conserves sample volume, and provides rapid, reliable cytokine screening/profiling.

# 4. Kit Contents

| No | Component Name | Size | Storage |
|---|---|---|---|
| 1 | Coated microtiter plate | 8 wells x 12 strips | Store 2 ~ 8°C |
| 2 | Standard Mixture | 3 vials (lyophilized) | |
| 3 | 100X Antibody Conjugate Mixture | 120 µ L | Store at - 20°C |
| 4 | 1000X HRP-Streptavidin Solution | 15 µ L | Store 2 ~ 8°C |
| 5 | Standard / Sample Diluent Buffer | 20 mL | |
| 6 | 10X Antibody Diluent Buffer | 10 mL | |
| 7 | 10X Wash Buffe | 2 X 50 mL | |
| 8 | TMB substrate | 12 mL (ready to use) | Store 2 ~ 8°C (Protect from light) |
| 9 | STOP solution | 12 mL (ready to use) | Store 2 ~ 8°C |
| 10 | Plate sealer | 3 pieces | Store 2 ~ 8°C |

## Additional materials required:

1. 37°C incubator.
2. Plate Reader with 450nm filter.
3. Precision pipettes and disposable pipette tips.
4. Distilled water.
5. Disposable tubes for sample dilution.
6. Absorbent paper.

# 5. Precautions

1. Store all reagents according to the instructions on the product label. Before use, allow all reagents to equilibrate to room temperature.

2. Before opening the secondary packaging, bring the pre-coated strip plates to room temperature. Return any unused strips immediately to the original packaging and reseal tightly. Store unused plates at 4°C for up to one month. All other unused reagents should be properly sealed or covered.

3. The volumes of the standard, biotinylated antibody, and enzyme conjugate are small. Perform a quick centrifugation prior to use to ensure that any liquid adhering to the tube walls or caps collects at the bottom.

4. Always use disposable pipette tips during the assay to prevent cross-contamination.

5. Inspect all kit components before use. To ensure accurate results, mix thoroughly when preparing dilutions, loading samples, or adding stop solution.

6. During the washing steps, after removing Wash Buffer, tap the plate dry on clean absorbent paper until no residual droplets or watermarks are visible. Do not insert tissue directly into the wells.

7. The TMB substrate is photosensitive, protect it from prolonged light exposure. Avoid contact with metal surfaces, as this may interfere with the reaction.

8. This kit is intended for single use and should be used within its stated shelf life.

# 6. Sample Preparation

The procedures outlined in this document are provided as general recommendations for sample preparation in ELISA assays. Due to the variability of biological samples and specific assay requirements, users are advised to optimize protocols based on their own experimental conditions.

***Note**: For information regarding validation data in specific samples, please contact our Technical Support Team at techsupport@assaygenie.com.*

## General Considerations

To prevent denaturation or degradation of target proteins, it is recommended to process samples promptly and store them under appropriate conditions.

- **Storage Conditions**:
    - **Short-term**: 2-8 °C for up to 5 days.
    - **Medium-term**: -20 °C for up to 6 months.
    - **Long-term**: -80 °C or cryopreservation in liquid nitrogen.
- **Thawing Protocol**: Frozen samples should be thawed rapidly in a 15-25 °C water bath to minimize ice crystal-induced damage. Thawed samples can be analyzed immediately or stored temporarily at 2-8 °C.
- **Freeze-Thaw Cycles**: Repeated freeze-thaw cycles should be strictly avoided due to their detrimental effect on protein stability.

### A. Blood-Derived Samples

- **Serum:** Allow whole blood to coagulate at room temperature (2 h) or 2-8 °C overnight. Centrifuge at 1000 × g for 20 min and collect the supernatant. Store or use immediately.
- **Plasma:** Collect in anticoagulant tubes (EDTA, citrate, or heparin), mix gently, and centrifuge within 30 min at 1000 × g, 2-8 °C for 15 min. Store or assay as needed.

- **Anticoagulant Guidance:** The document provides detailed recommendations on the selection and properties of EDTA, citrate, and heparin for various analytical requirements.

**B. Tissue Homogenates**

Tissue samples should be homogenized prior to use. Avoid buffers containing NP-40, Triton X-100, or DTT, as these strongly inhibit the assay. We recommend using 50 mM Tris + 0.9% NaCl + 0.1% SDS, pH 7.3.

The recommended protocol is as follows:

- **Sample Collection and Washing**
  - Place the target tissue on ice.
  - Rinse the tissue with pre-cooled PBS buffer (0.01 M, pH 7.4) to remove residual blood.
  - Weigh the tissue for further processing.
- **Homogenization**
  - Grind the tissue on ice using an appropriate lysate.
  - The lysate volume should correspond to the tissue weight; typically, 9 mL PBS is used per 1 g of tissue. It is recommended to add protease inhibitors to the PBS (e.g., 1 mM PMSF). ***Note:*** *PBS buffer or mild RIPA lysis buffer can be used for homogenization. When using RIPA, adjust pH to 7.3.*
- **Cell Disruption**
  - Further disrupt the tissue using ultrasonic homogenization or freeze–thaw cycles.
    - Ultrasonic homogenization: Keep samples on an ice bath during sonication to avoid overheating.
    - Freeze–thaw cycles*:* Repeat twice for effective lysis.
- **Centrifugation and Storage**
  - Centrifuge the homogenate at 5000 × g for 5 minutes.

- Collect the supernatant for immediate analysis, or aliquot and store at –20°C or –80°C for future assays.

- **Protein Concentration Measurement**
  - Determine total protein concentration using a BCA assay.
  - For ELISA assays, the total protein concentration should generally be 1–3 mg/mL.
  - Tissues with high endogenous peroxidase levels (e.g., liver, kidney, pancreas) may react with TMB substrate, causing false positives. If this occurs, treat samples with 1% $H_2O_2$ for 15 minutes before repeating the assay.

***Note:*** *Liver, kidney, and pancreas samples often contain high levels of endogenous peroxidase, which may react with the chromogenic substrate at elevated sample concentrations, potentially resulting in false positive signals.*

*If analysis of these tissues is required, a gradient dilution assay is recommended. A proportional decrease in signal with increasing dilution typically indicates minimal interference and supports the accuracy of the results.*

*To further minimise potential interference, samples can be pre-treated with 1% hydrogen peroxide ($H_2O_2$) for 15 minutes prior to testing. To prepare the treatment solution, add 1 µl of pure $H_2O_2$ to 100 µl of sample (1% v/v).*

**C. Cell Culture Supernatant**

Centrifuge the sample at 2500 rpm for 5 minutes at 2–8°C. Carefully collect the clarified cell culture supernatant for immediate analysis, or aliquot and store it at –80°C for future assays.

**D. Cell Lysates**

- **Suspension Cell Lysate:** Centrifuge the cell suspension at 2500 rpm for 5 minutes at 2–8°C and collect the cell pellet. Wash the pellet with pre-cooled PBS (0.01 M, pH 7.4) and mix gently. Repeat centrifugation and discard the

supernatant. Add 0.5–1 mL of cell lysis buffer containing an appropriate protease inhibitor (e.g., PMSF, final concentration: 1 mM). Lyse the cells on ice for 30–60 minutes or disrupt them using ultrasonic homogenization.

- **Adherent Cell Lysate:** Suspension Cell Lysate Centrifuge the cell suspension at 2500 rpm for 5 minutes at 2–8°C and collect the cell pellet. Wash the pellet with pre-cooled PBS (0.01 M, pH 7.4) and mix gently. Repeat centrifugation and discard the supernatant. Add 0.5–1 mL of cell lysis buffer containing an appropriate protease inhibitor (e.g., PMSF, final concentration: 1 mM). Lyse the cells on ice for 30–60 minutes or disrupt them using ultrasonic homogenization.

Follow next steps for protein extraction and supernatant collection:

- **Protein Release and DNA Disruption**
  - During lysis, pipette gently or intermittently shake the tube to enhance protein extraction.
  - Mucilaginous material formed during lysis is DNA, which can be broken down by ultrasonic disruption (3–5 mm probe, 150–300 W, 3–5 seconds per cycle, with 30-second intervals for 1–2 minutes total).
- **Supernatant collection**
  - After lysis or ultrasonic treatment, centrifuge the lysate at 10,000 rpm for 10 minutes at 2–8°C. Collect the supernatant for immediate use or aliquot and store at –80°C for future assays.

***Notes:*** *Refer to the "Tissue Sample Notes" for additional buffer and inhibitor recommendations.*

**E. Other Sample Types**

For more information about how to process other sample types, (e.g., body fluids, breast milk & more), please contact our Tech Support Team at techsupport@assaygenie.com.

***Important:***

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

# 7. Reagent Preparation

1. **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.
2. **1X Antibody Diluent Buffer:** Dilute 10X Antibody Diluent Buffer into distilled water to yield 1X Antibody Diluent Buffer (e.g., add 10 mL of 10X Wash Buffer into 90 mL of distilled water to a final volume of 100 mL). The 1X Antibody Diluent Buffer is stable for up to 4 weeks at 2-8°C. Mix well before use.
3. **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 detection antibody solution (e.g. 12 L of 100X Antibody Conjugate Mixture concentrate + 1188 L of Diluent Buffer).
4. **1X HRP-Streptavidin Solution**: It is recommended to prepare this reagent immediately prior to use and use it within 20 min after preparation. Dilute 1000X HRP-Streptavidin concentrate solution into 1X Antibody Diluent Buffer to yield 1X HRP-Streptavidin Solution buffer. (e.g. 1 L of 1000X HRP-Streptavidin concentrate solution + 999 L of Diluent Buffer).
5. **Sample:** Dilute 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 pretest to determine the suitable dilution factor).

6. **Preparation of standard:** 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.

   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 Std Conc. | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 (Low) | 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 |

# 8. Assay Procedure

1. **Equilibrate Reagents:** Bring each component in the kit to room temperature for 30 minutes. Take out required strip plates from aluminum foil bags already equilibrated to room temperature, and label the strip plate sequence with a marker. Seal remaining strip plates with a plate sealer, put them back to the aluminum foil bag, then seal the bag, and store at 2 ~ 8°C.
2. **Setup Plate:** Set the standard wells, blank wells and test sample wells, respectively. Recommended to assay samples in duplicate. ***Note:*** *Each individual well detects only one cytokine, the entire 96-well plate enables the simultaneous detection of up to four cytokines. Carefully follow the plate layout to ensure that each well is correctly associated with its corresponding cytokine and to avoid confusion during analysis.*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 |
| B | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 |
| C | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 |
| 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 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 | IL-2 |
| F | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 | IL-6 |
| G | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 | IL-10 |
| 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 |

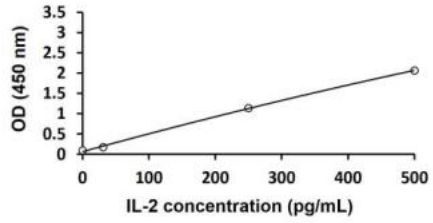
3. **Add Standard, Sample and Blanks:** Add standards at different concentrations (in sequence, blanks and samples to respective wells (100 µL/well). ***Note:*** *To obtain the approximate concentrations of 4 cytokines on 22 test samples, the low and high concentration standard mixture can be added as 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 |

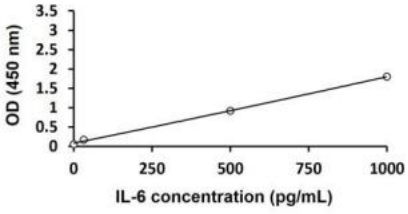
4. **First Incubation:** Seal the reaction wells with a sealing film and incubate at room temperature for 2 hours.
5. **Washing:** Aspirate each well and wash, repeating the process 4 times for a total 6 washes. Wash by filling each well with 1X Wash Buffer (300 µL) using squirt bottle, manifold dispenser, or auto washer. 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.
6. **Antibody Conjugate Mixture:** Add 100 µ L of 1X Antibody Conjugate Mixture to each wells.
7. **Second Incubation:** Seal the reaction wells with a sealing film and incubate at room temperature for 2 hours.
8. **Washing:** Repeat step 5.
9. **1X Streptavidin-HRP**: Add 100 µL of 1X HRP-Streptavidin Solution to each well. Cover the plate and incubate for 1 hour at room temperature
10. **Washing**: Same as Step 5, but 7 times.
11. **Colour development:** Add 100 µL of TMB Substrate to each well. Cover and incubate for 10-20 minutes at room temperature in the dark.
12. **Reaction termination**: Add 100 µL of stop solution to each well and immediately perform the detection.
13. **Signal Reading**: Measure the OD values at 450 nm and 630 nm with a plate reader. The measurement should be completed within 30 minutes after reaction termination.

# 9. Data Analysis

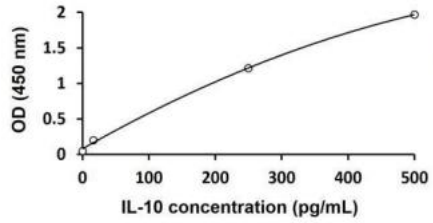
1. Calculate the average absorbance for each standard and sample by averaging the duplicate (or triplicate) OD readings.
2. For semi-quantitative analysis, approximate concentration values for IL-2, IL-6, IL-10, and IFN-γ can be estimated using rough standard curves generated from the high and low concentration standard mixtures. Please note that these curves are based on only two points and may not be linear, which limits the accuracy of the estimated concentrations.
3. For improved accuracy, we recommend including additional standard dilution points to generate full standard curves for each cytokine. Data analysis using a Four Parameter Logistic (4PL) curve-fitting model is recommended for precise quantification. Alternative curve-fitting methods may be used but may yield slightly different results.
4. If samples were diluted during preparation, multiply the concentration obtained from the standard curve by the appropriate dilution factor to determine the actual concentration in the original sample



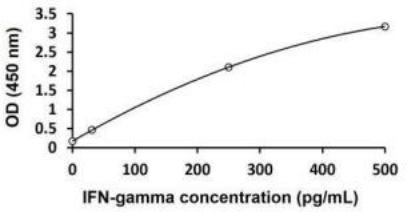

Example of Human IL-2 standard curve




Example of Human IL-6 standard curve




Example of Human IL-10 standard curve




Example of Human IFN-gamma standard curve

# 10. ELISA Troubleshooting

| Problem | Possible Causes | Solutions |
|---|---|---|
| **Standard curve without signal** | Incorrect reagent order; Mixed components from different kits; Missing reagents. | Ensure correct reagent order and use components from the same kit. Verify all reagents are added. |
| **Overflow OD** | Mixed components from different kits; Over-concentrated working solution | Use correct components and prepare solutions at recommended concentrations. |
| **Poor standard curve** | Incorrect curve fitting model. | Try alternative curve fitting models. |
| **Samples without signal** | Sample concentration too low; Incompatible buffer; Incorrect preparation; Sample degradation or excessive freeze-thaw. | Reduce dilution or concentrate sample. Check buffer compatibility and follow proper preparation and storage. |
| **High CV%** | Precipitate formation; Unclean plate; Foaming; Uneven washing; Incomplete reagent mixing; Pipetting inconsistency. | Dilute samples if needed, avoid foaming, ensure uniform washing, mix reagents thoroughly, and use calibrated pipettes. |
| **Low standard signal** | Improperly reconstituted standards; Degraded standards; Incorrect pipetting; Expired kit; Improper storage; Over-dried wells. | Reconstitute standards properly, use fresh kits, follow storage recommendations, and prevent wells from drying. |
| **Slow colour development** | TMB not equilibrated; Incorrect microplate reader wavelength; Over-washing. | Pre-warm TMB (30 min at 37°C), confirm correct wavelength (450 nm), and follow recommended washing times. |
| **High background** | Insufficient washing; Contaminated wash buffer; Excess detection reagents; Delayed reading; TMB exposed to light. | Wash adequately, prepare fresh wash buffer, use correct reagent amounts, read results promptly, and incubate TMB in the dark. |

## Notes:

**Assay Genie 100% money-back guarantee!**

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.