

## Speedy™ Human CD69 One-Step ELISA Kit Datasheet

Please read it entirely before use

**Catalogue Number:** SE50109

**Size:** 96T

**Sensitivity:** 5.9 pg/mL

**Range:** 23.4-1500 pg/mL

**Usage:** For the quantitative detection of human CD69 concentrations in serum, plasma and cell lysate.

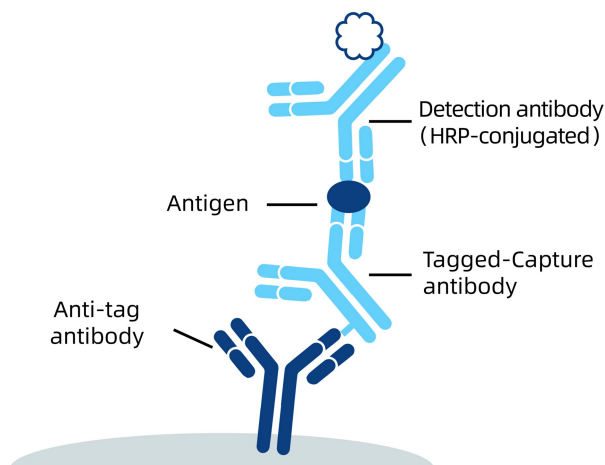
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## 1. Background

CD69 is a type II C-type lectin receptor that serves as an early activation marker for lymphocytes. It is rapidly expressed on the surface of T cells, B cells, and natural killer (NK) cells following activation. CD69 plays a crucial role in lymphocyte proliferation and functions as a signal-transmitting receptor, influencing cell cycle progression and cytokine production. It also regulates the differentiation of T helper (Th) subsets, including Th1, Th2, Th17, and regulatory T cells (Tregs), by modulating intracellular signaling pathways and cytokine secretion.

## 2. Principle



An anti-tag antibody is pre-coated onto the bottom of wells. After adding antigen or samples, Tagged-Capture antibody and HRP-conjugated detection antibody, a sandwich complex is formed in the solution. TMB acts as a HRP substrate, and the solution color will change from colorless to blue. A stop solution containing sulfuric acid turns the solution yellow. The color intensity is proportional to the quantity of bound protein, which is measurable at 450 nm with the correction wavelength set at 630 nm.

## 3. Required Materials

- 3.1 A microplate reader capable of measuring absorbance at 450 nm with the correction wavelength set at 630 nm.
- 3.2 Calibrated, adjustable precision pipettes and disposable plastic tips. A manifold multi-channel pipette is recommended for large assays.
- 3.3 Plate washer: automated or manual.
- 3.4 Absorbent paper towels.
- 3.5 Glass or plastic tubes to prepare standard and sample dilutions.
- 3.6 Beakers and graduated cylinders.
- 3.7 Log-log or semi-log graph paper or computer and software for ELISA data analysis. A four-parameter logistic (4-PL) curve-fit is recommended. Proteintech data analysis website, <https://www.ptgcn.com/products/elisa-kits/>.
- 3.8 Microplate thermostatic shaker.

## 4. Kit Components and Storage

|  |           |   |
|--|-----------|---|
| <b>Microplate</b> - 96 well microplate precoated an anti-tag antibody (8 well × 12 strips) | 1 plate   | <b>Unopened Kit:</b><br>Store at 2-8°C for 6 months or -20°C for 12 months.<br><br><b>Opened Kit:</b><br>All reagents stored at 2-8°C for 7 days.<br><br><b>Please use a new standard for each assay.</b> |
| <b>Protein standard</b> - 3000 pg/bottle; lyophilized                                      | 2 bottles |   |
| <b>Capture antibody (100×)</b> - 60 µL/vial*   | 1 vial    |   |
| <b>Detection antibody, HRP-conjugated (100×)</b> - 60 µL/vial*                             | 1 vial    |   |
| <b>Sample Diluent PT 3-ef</b> - 30 mL/bottle. For human serum and plasma.                  | 1 bottle  |   |
| <b>Sample Diluent PT 4B1</b> - 30 mL/bottle. For cell lysate.                              | 1 bottle  |   |
| <b>Detection Diluent</b> - 15 mL/bottle  | 1 bottle  |   |
| <b>Wash Buffer Concentrate (20×)</b> - 30 mL/bottle  | 1 bottle  |   |
| <b>Extraction Reagent</b> - 15 mL/bottle   | 1 bottle  |   |
| <b>Tetramethylbenzidine Substrate (TMB)</b> - 12 mL/bottle                                 | 1 bottle  |   |
| <b>Stop Solution</b> - 12 mL/bottle  | 1 bottle  |   |
| <b>Plate Cover Seals</b>   | 4 pieces  |   |

\* Centrifugation immediately before use

## 5. Safety Notes

- 5.1 Avoid any skin and eye contact with Stop Solution and TMB. In case of contact, wash thoroughly with water.
- 5.2 Do not use the kit after the expiration date.
- 5.3 Do not mix or substitute reagents or materials from other kit lots or other sources.
- 5.4 Be sure to wear protective equipment such as gloves, masks and goggles during the experiment.
- 5.5 When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer to improve assay precision

## 6. Sample Collection and Storage

6.1 Serum: Allow blood samples to clot for 30 minutes, followed by centrifugation for 15 minutes at 1000xg. Clear serum can be assayed immediately or aliquoted and stored at -20°C. Avoid repeated freeze-thaw cycles.

6.2 Plasma: Use EDTA, heparin, or citrate as an anticoagulant for plasma collection. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. The plasma can be assayed immediately or aliquoted and stored at -20°C. Avoid repeated freeze-thaw cycles.

6.3 Cell Lysate:

1) Collect cells and wash by centrifuging at 500 x g for 5 minutes before resuspension in pre-cooled PBS buffer. Perform this step three times.

2) Count cells and then discard the supernatant.

3) Add protease inhibitor cocktail to the Extraction Reagent to a final concentration immediately prior to performing cell lysis.

4) Add 1 mL of Extraction reagent (containing protease inhibitor cocktail) Per  $1 \times 10^7$  cells, Incubate cell suspension on ice for 30 minutes, use ultrasound to treat the samples.

5) Centrifuge cell lysate at 10,000 x g for 5 minutes at 4°C.

6) Measure the concentration of total protein in cell lysate using BCA assay. Where possible, keep samples on ice to avoid protein degradation.

## 7. Reagent Preparation

**7.1 Wash Buffer (1X):** If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 30 mL of Wash Buffer Concentrate(20X) to 570 mL deionized or distilled water to prepare 1X Wash Buffer.

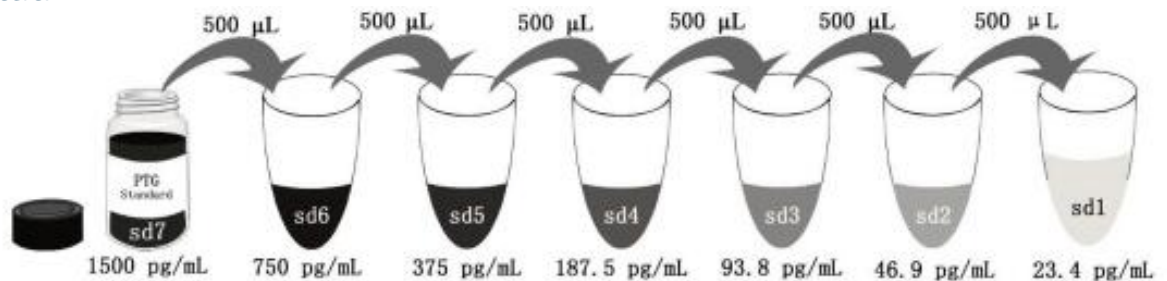
**7.2 Antibody Cocktail (1X):** Dilute 100X capture antibody and 100X HRP-conjugated detection antibody using Detection Diluent prior to assay. Suggested 1:100 dilution: 50  $\mu$ L 100X capture antibody + 50  $\mu$ L 100X Detection Antibody, HRP-conjugated + 4,900  $\mu$ L Detection Diluent. Mix gently but thoroughly.

**7.3 Sample Dilution:** Different samples should be diluted with corresponding Sample Diluent, samples may require further dilution if the readout values are higher than the highest standard OD reading. Variations in sample collection, processing and storage may affect the results of the measurement .

Recommended Dilution for different sample types: 1:2 or 1:4 is recommended for human serum and plasma; 1:2 to 1:64 is recommended for cell lysate.

### 7.4 Standard Serial Dilution:

For human serum and plasma, add 2 mL Sample Diluent PT 3-ef in protein standard. For cell lysate, add 2 mL Sample Diluent PT 4B1 in protein standard.



|  |              |             |             |             |             |             |             |
|--|--------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Add # $\mu$ L of Standard diluted in the previous step | —            | 500 $\mu$ L | 500 $\mu$ L | 500 $\mu$ L | 500 $\mu$ L | 500 $\mu$ L | 500 $\mu$ L |
| # $\mu$ L of Sample Diluent PT 3-ef or PT 4B1          | 2000 $\mu$ L | 500 $\mu$ L | 500 $\mu$ L | 500 $\mu$ L | 500 $\mu$ L | 500 $\mu$ L | 500 $\mu$ L |
|  | "sd7"        | "sd6"       | "sd5"       | "sd4"       | "sd3"       | "sd2"       | "sd1"       |

## 8. Assay Procedure Summary

Bring all reagents to room temperature before use (Detection antibody, HRP-conjugated antibody can be used immediately). To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

8.1 Preset the layout of the microplate, including control group, standard group and sample group, take out the required number of microplate strips and return excess strips to the foil pouch containing the drying reagent pack and reseal; store at 4°C immediately. Microplate strips should be used in one week.

8.2 Add 50 µL standard or sample to appropriate wells. To avoid high background always add samples or standards to the well before the addition of antibody cocktail.

8.3 Add 50 µL 1× Antibody Cocktail solution (refer to Reagent Preparation 7.2) to each well. Seal plate with cover seal and incubate at 37°C on a microplate thermostatic shaker set at 400 rpm for 1 hour (incubate at 37°C for 2 hours is recommended if thermostatic shaker is not available) .

### 8.4 Wash

1) Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting. Remove any residual solution by tapping the plate a few times on fresh paper towels.

2) Wash 4 times with 1× Wash Buffer, using at least 350-400 µL per well. Following the last wash, firmly tap plates on fresh towels 10 times to remove residual Wash Buffer. Avoid getting any towel fibers in the wells or wells drying out completely.

8.5 Add 100 µL TMB substrate solution to each well, protected from light. Incubate at 37°C on a microplate thermostatic shaker set at 400 rpm for 15 to 20 minutes. (Substrate Solution should remain colorless until added to the plate.)

8.6 Add 100 µL Stop Solution to each well in the same order as addition of the TMB substrate. Note: Avoid skin and eye contact with the Stop solution.

8.7 Read results immediately on a microplate reader at a wavelength of 450 nm. If possible, perform a double wavelength readout (450 nm and 630 nm).

8.8 Data analysis: Calculate the average of the duplicate readings (OD value) for each standard and sample, and subtract the average of the zero standard absorbance. Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, four-parameter logistic curve-fit (4-PL) analysis is recommended. If the samples have been diluted, the fitting result must be multiplied by the dilution factor used.

## Procedure summary

Add 50  $\mu$ L standard or sample to appropriate wells



Add 50  $\mu$ L antibody cocktail solution (1x) to each well



Incubate at 37  $^{\circ}$ C on a microplate thermostatic shaker set at 400 rpm for 1 hour



Aspirate and wash each well four times with 400 $\mu$ L 1 x Wash Buffer



Add 100  $\mu$ L TMB substrate solution to each well



Incubate at 37  $^{\circ}$ C on a microplate thermostatic shaker set at 400 rpm for 15minutes

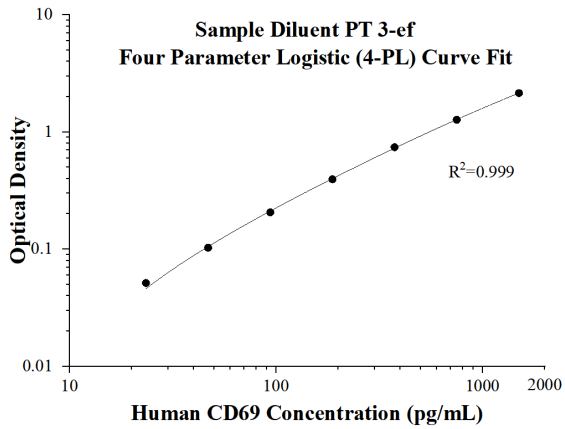


Add 100  $\mu$ L Stop Solution to each well and Read OD immediately

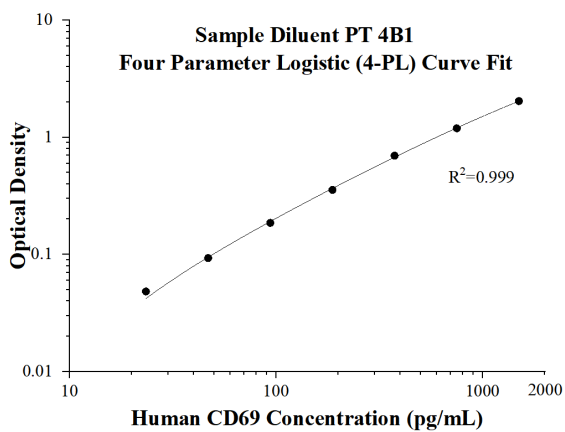
## 9. Validation Data

### 9.1 Standard curve

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.



| (pg/mL) | O.D              | Average | Corrected |
|---------|------------------|---------|-----------|
| 0       | 0.0167<br>0.0194 | 0.01805 | -         |
| 23.4    | 0.0672<br>0.0719 | 0.06955 | 0.0515    |
| 46.9    | 0.1171<br>0.1249 | 0.121   | 0.10295   |
| 93.8    | 0.2231<br>0.2252 | 0.22415 | 0.2061    |
| 187.5   | 0.418<br>0.4087  | 0.41335 | 0.3953    |
| 375     | 0.748<br>0.7722  | 0.7601  | 0.74205   |
| 750     | 1.272<br>1.3065  | 1.28925 | 1.2712    |
| 1500    | 2.1755<br>2.1633 | 2.1694  | 2.15135   |



| (pg/mL) | O.D              | Average | Corrected |
|---------|------------------|---------|-----------|
| 0       | 0.0244<br>0.0232 | 0.0238  | -         |
| 23.4    | 0.0724<br>0.0716 | 0.072   | 0.0482    |
| 46.9    | 0.1198<br>0.1136 | 0.1167  | 0.0929    |
| 93.8    | 0.2099<br>0.2092 | 0.20955 | 0.18575   |
| 187.5   | 0.3849<br>0.374  | 0.37945 | 0.35565   |
| 375     | 0.7311<br>0.7105 | 0.7208  | 0.697     |
| 750     | 1.2147<br>1.2211 | 1.2179  | 1.1941    |
| 1500    | 2.0658<br>2.0622 | 2.064   | 2.0402    |

## 9.2 Precision

**Intra-assay Precision** (Precision within an assay) Three samples of known concentration were tested 8 times on one plate to assess intra-assay precision.

**Inter-assay Precision** (Precision between assays) Three samples of known concentration were tested in 16 separate assays to assess inter-assay precision.

| Intra-assay Precision |   |              |      |     | Inter-assay Precision |    |              |      |     |
|-----------------------|---|--------------|------|-----|-----------------------|----|--------------|------|-----|
| Sample                | n | Mean (pg/mL) | SD   | CV% | Sample                | n  | Mean (pg/mL) | SD   | CV% |
| 1                     | 8 | 544.9        | 22.3 | 4.1 | 1                     | 16 | 546.9        | 17.2 | 3.1 |
| 2                     | 8 | 137.0        | 5.1  | 3.7 | 2                     | 16 | 138.0        | 7.1  | 5.1 |
| 3                     | 8 | 64.5         | 2.8  | 4.3 | 3                     | 16 | 64.9         | 2.5  | 3.9 |

## 9.3 Recovery

The recovery of human CD69 spiked to three different levels throughout the range of the assay in various matrices was evaluated.

| Sample Type  |      | Average% of Expected | Range (%) |
|--------------|------|----------------------|-----------|
| Human plasma | 1:4  | 105                  | 98-114    |
|              | 1:8  | 103                  | 97-111    |
| Cell lysate  | 1:16 | 110                  | 102-118   |

## 9.4 Sample values

**Human plasma** - Male plasma samples were evaluated for the presence of human CD69 in this assay.

| Sample Type       | Mean (ng/mL) | Range (ng/mL) |
|-------------------|--------------|---------------|
| Male plasma (n=7) | 2.3          | 0.1-7.0       |

**Cell Lysate** - Human Burkitt lymphoma cells (Daudi) ( $1 \times 10^6$  cells/mL) were cultured unstimulated or stimulated with 50 ng/mL PMA for 1 day. Cells were lysed and measured the total protein, assayed for human CD69.

|              | Human CD69 (ng/mL) | Total protein (mg/mL) |
|--------------|--------------------|-----------------------|
| Unstimulated | 0.7                | 2.7                   |
| Stimulated   | 58.6               | 3.1                   |

|                   | Human CD69 (ng/mL) | Total protein (mg/mL) |
|-------------------|--------------------|-----------------------|
| K-562 cell lysate | 2.4                | 1.2                   |
| U-937 cell lysate | 24.3               | 2.6                   |

## 9.5 Sensitivity

The minimum detectable dose of human CD69 is 5.9 pg/mL. This was determined by adding two standard deviations to the concentration corresponding to the mean O.D. of 20 zero standard replicates.

## 9.6 Linearity

To assess the linearity of the assay, samples were diluted with the appropriate Sample Diluent to produce samples with values within the dynamic range of the assay.

(The cell lysate was initially diluted 1:32.)

|      |                      | Human plasma<br>(Sample Diluent PT 3-ef) | Cell lysate<br>(Sample Diluent PT 4B1) |
|------|----------------------|--|--|
| 1:2  | Average% of Expected | 100                                      | 100                                    |
|      | Range (%)            | -  | -                                      |
| 1:4  | Average% of Expected | 106                                      | 89                                     |
|      | Range (%)            | 104-109                                  | 79-100                                 |
| 1:8  | Average% of Expected | 107                                      | 99                                     |
|      | Range (%)            | 94-119                                   | 94-105                                 |
| 1:16 | Average% of Expected | 78                                       | 92                                     |
|      | Range (%)            | 71-85                                    | 78-106                                 |

## 9.7 Specificity

This assay recognizes natural and recombinant human CD69.

The following factors prepared at 50 ng/mL were assayed and exhibited no cross-reactivity or interference.

Recombinant mouse:

CD69

## 10. References

1. Cibrián, Danay, and Francisco Sánchez-Madrid. European journal of immunology vol. 47,6 (2017): 946-953.
2. Mita, Yuki Yoshi et al. International immunology vol. 30,12 (2018): 559-567.
3. Martín, Pilar et al. Molecular and cellular biology vol. 30,20 (2010): 4877-4889.
4. Shinoda, Kenta et al. Proceedings of the National Academy of Sciences of the United States of America vol. 109,19 (2012): 7409-7414.