

ACT1™

Assay for CytoToxicity

Non-radioactive cytotoxicity assay for flow cytometry

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Part A: Introduction

The immune system is capable of recognizing and destroying target cells, such as tumor or intracellular pathogen infected cells, through a process known as cell mediated cytotoxicity (CMC) or antibody-dependent cell-mediated cytotoxicity (ADCC) (1). Evaluation of this CMC/ADCC activity is one of the most important immunoassays to monitor the status of the immune system.

The most commonly used method to measure CMC/ADCC is a radioactive chromium-51 (⁵¹Cr) release assays (2). There are several disadvantages with this assay in that it is expensive, difficult to load certain cell types, strict environmental regulations makes waste disposable expensive and spontaneous release of ⁵¹Cr results in high background. With the use of flow cytometry, it is now possible to eliminate the need for radioactive material and increased the ability to quantify cytolytic activity on a single cell bases. Various groups have demonstrated that measuring CMC/ADCC activity by flow cytometry has a strong (95%) correlation with the traditional ⁵¹Cr release assay (3,4,5,6).

Part B: Assay Principal

A cell tracking dye CFSE analog (7,8,9) is utilized to label the target cell population and thus separating them from the effector cell population. After the assay has run its experimental protocol, 7AAD (live/dead) (10,11) is added to measure cell death. 7AAD only enters membrane compromised cells and binds to DNA.

Flow cytometry is utilized to gate on the target cells and measure 7AAD negative vs. 7AAD positive cells. % cytotoxicity is calculated by the following equation (see experimental example below):

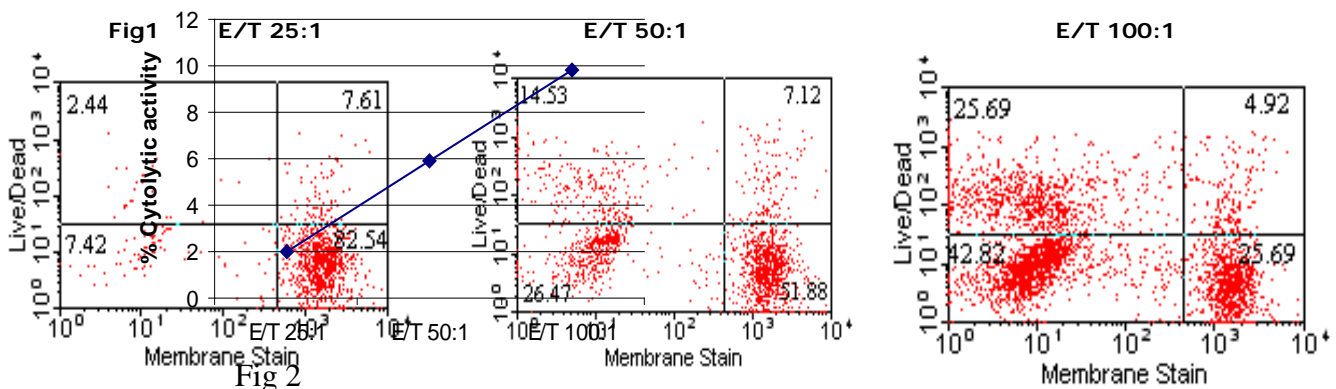
7AAD positive (upper right quadrant)=R1/ 7AAD Positive (upper right quadrant)= R1 + 7AAD negative (lower right quadrant)=R2 x100 (R1/(R1+R2)*100)

Membrane Stain (CFSE analog): excitation: 488nm Laser Emission: FL1.

Live/Dead Stain (7AAD): excitation: 488nm Laser Emission: FL3.

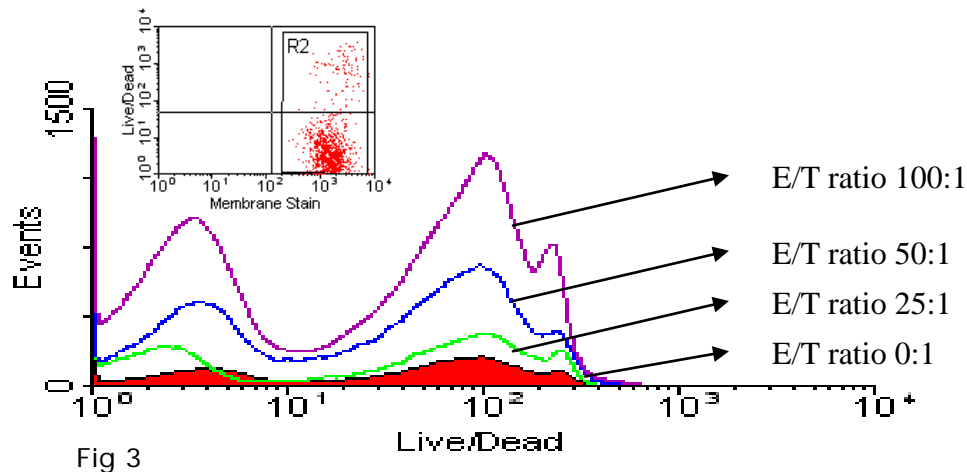
Part C: Experimental example

Optimization of the cytolytic assay needs to be determined by individual researchers. For example to test natural killer ability of swine $\gamma\delta$ lymphocytes (12,13), K562 cells were stained with CFSE analog and adjusted to a final concentration of 1×10^4 cells/100 ul in RPMI containing 10 % FBS. $\gamma\delta$ lymphocytes were added at: effector (E)/ target (T) ratios of 25:1, 50:1, and 100:1 and adjusted to a total volume of 400 ul RPMI, then incubated for 4 hours at 37° C in a sterile capped facs tube. Following incubation 7AAD (live/dead) stain was added directly to each tube, incubated for 15 min on ice and analyzed via flow cytometry as shown in **Figure 1**.



Following analysis, data can be imported into dot plots to calculate percentages of cells separated into various quadrants. % Cytotoxicity can be determined by an increase of target cells (lower right quadrant) moving into upper right quadrant and calculated by $(R1/(R1+R2))*100$ (Fig 1). Target cells only (E/T ratio 0:1 or uninfected target cells(for intracellular pathogen infected targets) should be used to measure spontaneous cell death. Spontaneous death can be calculated and subtracted as background control. By graphing results it can be determined if the assay parameters are setup properly Fig 2. As demonstrated in the graph, a linear increase indicates the assay is optimized and an E/T ratio will be optimal for the experiments Fig 2.

Cytolytic activity by $\gamma\delta$ lymphocytes were analyzed by gating target cells (upper and lower right quadrants) R2 (see below) and overlaid in a histogram fig 3.



Part D: Kit Components

Part#: 4002 Membrane stain, 4 vials of lyophilized powder.

Part#: 4003 Live/Dead stain, 3 vial containing of lyophilized powder.

Part#: 3003 1 bottle 10X PBS with Ca^{2+} and Mg^{2+} .

Part E: Materials needed to be supplied by individual user

1. DMSO
2. PBS without Ca^{2+} and Mg^{2+}
3. FACS tubes
4. Flow Cytometer
5. Methanol
6. Pipettors and tissue culture materials.

Part F: Reconstitution and storage of reagents.

1. Membrane Stain Part #: 4002

1. Keep lyophilized vials at $-20^{\circ}C$. Reconstitute CFSE analog vial with 300 μ l DMSO (100X), aliquot into 50-100 μ l per vial and store at $-20^{\circ}C$.

2. Live Dead Stain Part#: 4003

1. Keep lyophilized vials at $-20^{\circ}C$.
2. Reconstitute Live/Dead stain with 100 μ l absolute methanol, aliquot and store at $-20^{\circ}C$.

3. Prior to use dilute 1:10 dilution with 1 X PBS with Ca^{2+} and Mg^{2+} (this equals a **20X Live/Dead stain**) and add 20 μl of this 20X live/dead stain to your 400 μl samples. The 20x 7AAD solution can be stored for several months at 4°C when stored tightly closed and protected from light. **Note:** *We recommend that each individual investigator titrate out the 7AAD to optimize it for their protocol.*

3. 10X PBS with Ca^{2+} and Mg^{2+} Part#:3003

1. Make a 1X solution prior to use. For example to 1 mL of 10X PBS add 9mL of double distilled water. Store at 4°C.

Part G: Special Characteristics

The approximate excitation and emission peaks of the membrane stain are 492 nm and 517 nm. Labeled membrane stain can be visualized by fluorescence microscopy using a standard fluorescein filter set FL1. The optimum excitation and emission peaks of 7AAD live/dead stain are 503 nm and 675 nm FL3, however 7AAD live/dead stain excites with a 488nm laser.

Part H: Directions.

Target Cell Labeling

1. Take target cells and wash them 2 times with 1 X PBS with or without Ca^{2+} or Mg^{2+} .
2. Adjust to a final concentration of 1-2 X 10^6 cells in a total volume of 1 mL of 1X PBS without Ca^{2+} and Mg^{2+} .
3. Following reconstitution of membrane stain CFSE analog (as described above in Part F) take 1-15 μl of membrane stain (100X) and add it to the 1 mL of target cells. You may need to first further dilute out the membrane stain 1:10 – 1:100, in PBS, prior to staining your cells. **Note:** *Each investigator should titrate out the CFSE to optimize staining for their cell lines. Both CFSE (titrated) membrane stained target cells and non-stained effector cells should be run together in the same tube so to determine optimum staining and separation conditions. Do not stain target cells, which are capable of proliferating, more than 24 hours prior to the assay. Proliferation will decrease fluorescent intensity. If staining intensity is very bright please see technical note 1 below.*
4. Vortex and incubate for 15 minutes at room temp (keep protected from light).
5. Centrifuge cells and re-suspended in 3 ml of media containing FBS and incubate at 37°C for 30 minutes.
6. Centrifuge and wash the labeled target cells 2 times in the media of your choice.
7. Labeled target cell can are now ready for your assay. Adjust the cells concentration according to your experimental protocol.

PART I: Assay

1. Add labeled target and effector cells at an E/T ration determined by your experimental protocol. The final volume of each sample should be **400 μl**
2. Cell can be cultured in a sterile FCAS tube or other suitable tissue culture vessel. If using other tissue culture vessel, transfer the sample to a FACS tube prior to analysis.
3. After culturing the samples according to your experimental conditions add 20ul of the **20X 7AAD solution** (from part F above) per 400ul sample, vortex and incubate on ice for 15 minutes. The samples are ready for flow analysis.
4. A tube containing only CFSE membrane stained target cells should be run as a control. This will measure spontaneous cell death and must be subtracted from your data. For antigen specific cytolytic activity such as in vitro infected cells, use autologous non-infected cells plus effector cells as a control.

PART J: Flow Cytometer Setup

1. The following tubes are needed to set up the flow cytometer and compensation:
2. Target cells only
 - i. Membrane stained target cells
 - ii. Live membrane stain target cells + live/dead stain
 - iii. Dead target cells, non membrane stained, + 7AAD (live/dead stain). The cells can be killed using a 56°C water bath. Immerse the tube of cells for 3 to 6 minutes, cool to room temperature and add 7AAD (live/dead stain).
 - iv. Tube containing both membrane stained target and effector cells with 7AAD(live/dead stain) to finalized compensation.
3. Gating strategy: See figure 4a and 4b below. You may gate your target cells using a forward vs side scatter plot (fig 4b) or using a side scatter vs FL-1 plot (fig4a). Run the membrane stained target cells and draw a gate around them (R1), making sure to extend the gate to include dead cells.
4. Next make a FL3 (live/dead) vs FL1 (CFSE) dot plot fig(5a-c) and gate it off R1 (Fig 4)
5. Next run the membrane stained target cells and turn up the FL1 PMT. Make sure the membrane stained target cell population falls within 3rd or 4th log decade scale. This will ensure an adequate separation between effector and target cells (fig 4a). **Note:Staining concentration of target cells may vary depending on cells used. Each investigator should predetermine this step.**
6. Next run the live membrane stained target cells + 7AAD. Adjust the PMT voltages as to obtain the results depicted in Fig 5b.
7. Next run the dead membrane stained target cells + 7AAD and adjust voltages to obtain a picture as depicted in Fig 5c.
8. Place a region R2 (Fig 5b) around the membrane stained target cells and set cytometer to collect 10,000 events within this region Fig 5b.
1. Vortex and analyze your samples. A control sample of membrane stained target cells should be run to determine spontaneous cell death.

Fig 4a

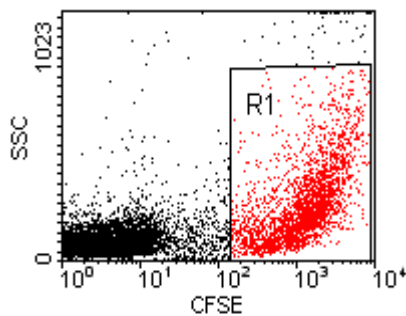
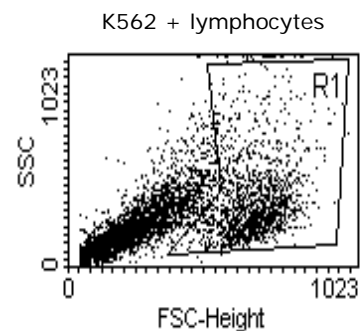
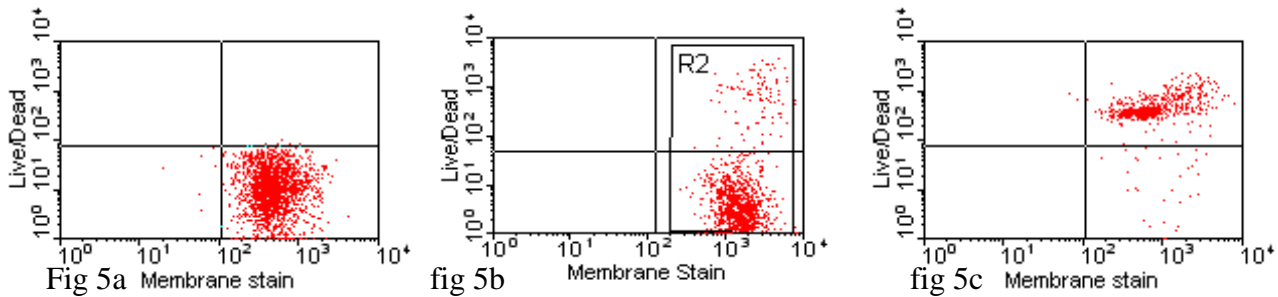


Fig 4b





Analyzing data:

Cytolytic activity can be calculated (upper right quadrant/(upper right quadrant + lower right quadrant)) X 100 (Fig 5a-c).

Technical Notes:

1. To reduce staining intensity, dilute the membrane stain (after reconstitution) 1:50 to 1:100 in PBS. Next adjust the cell staining volume to 1.8mL in PBS. Add 200 μ L of the diluted (1:50 – 1:100) membrane stain. Vortex and incubate for 15 minute in the dark. Wash and proceed as described above.

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