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# Flow-cytometry-based in vitro assay for assessing T-cell-mediated cytotoxicity against a target cell line (24-well plate, pmel-1 or OT-I T cells, MC38 cell line) V.3

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**1** Works for me dx.doi.org/10.17504/protocols.io.8d2hs8e

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## ABSTRACT

In vitro co-cultures of cytotoxic T cells with their target cells are important assays to assess the functionality of the T cells in a scalable way. These assays rely on co-culturing CD8 T-cells, often times genetically modified to express a specific TCR or CAR, with another type of cell line that can be recognized by T cells. Co-cultures are typically run for 6-24 hours and then the amount of cells that were killed in the co-culture can be assessed through different techniques – e.g. radioactive Cr or non-radioactive LDH release assays. Here, we outline another alternative to these release assays which relies on flow cytometry to estimate the number of target cells left in the culture after a certain period of time.

## THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Viable and efficient electroporation-based genetic manipulation of unstimulated human T cells Pinar Aksoy, Bulent Arman Aksoy, Eric Czech, Jeff Hammerbacher bioRxiv 466243; doi: <https://doi.org/10.1101/466243>

## MATERIALS

NAME ▾	CATALOG # ▾	VENDOR ▾
<a href="#">Trypsin 0.05% 1X Solution</a>	16777-202	VWR Scientific
<a href="#">CytoOne 24-well TC plate</a>	CC7682-7524	USA Scientific
<a href="#">APC anti-mouse CD3 Antibody</a>	100235	BioLegend
<a href="#">PerCP anti-mouse CD8a Antibody</a>	100731	BioLegend
<a href="#">pmel-1 mouse (B6.Cg-Thy1a/Cy Tg(TcrαTcrβ)8Rest/J)</a>	005023	Jackson Laboratory
<a href="#">OT-I mouse (C57BL/6-Tg(TcrαTcrβ)1100Mjb/J)</a>	003831	Jackson Laboratory

## STEPS MATERIALS




NAME ▾	CATALOG # ▾	VENDOR ▾
<a href="#">Trypsin 0.05% 1X Solution</a>	16777-202	VWR Scientific
<a href="#">PerCP anti-mouse CD8a Antibody</a>	100731	BioLegend
<a href="#">APC anti-mouse CD3 Antibody</a>	100235	BioLegend

## BEFORE STARTING





- Make sure you have enough activated (for at least 3 days), healthy (>50% viability), and cytotoxic (CD8) T cells in culture before starting
- Make sure you have access to a flow-cytometer after the co-culture is done
- When in doubt, use 24-well plates for the co-culture
- Make sure the cell line expresses the target protein (for CAR) or presents the relevant peptide (for TCRs) up-front

- Make sure the cell line can grow and sustain viability in T cell media throughout the co-culture
- Make sure the final T cell concentration doesn't go higher than 2 million per mL since this can cause stress on the T cells and the cell line
- This protocol assumes the assay is carried out at 8:1 T-cell:Cell-line ratio. Please scale the numbers up if you would like to assay at a different scale/ratio
- When in doubt, use OT-I CD8 T cells against MC38s that are pulsed with the SIINFEKL peptide as a positive control
- When in doubt, use OT-I CD8 T cells against MC38s that are NOT pulsed with the SIINFEKL peptide as a negative control
- This protocol assumes the T cells and the cancer cells are of mouse origin. If you are using a different organism or the channels are not appropriate for your flow-cytometer, please customize your antibodies accordingly

#### Day 0 - Seeding the target cells

- 1 Collect at least 3 million MC38s by trypsinizing them from an on-going culture
- 2 Spin them down at  **200 x g** for  **00:05:00** at  **4 °C** and re-suspend them in fresh media at a 250,000 cells per mL concentration
- 3 Seed each 24-well-plate well with 500 uL of the cell suspension (i.e. 125,000 MC38 cells per plate)
- 4 Incubate overnight and allow cells to adhere to the plate

#### Day 1 - Co-culture

- 5 Collect 2 million cytotoxic T cells per sample (i.e. per well) from an on-going culture
- 6 Spin them down at  **350 x g** for  **00:05:00** at  **4 °C** and re-suspend them in fresh media at 1 million per mL concentration
- 7 Supplement T cells with 200 IU/mL rIL2
- 8 Aspirate the culture media from each of the 24-well-plate wells that contain a sample. Try to aspirate as much as possible but make sure you don't disturb the adherent cells during this process
- 9 Add  **2 ml** of the T cell suspension onto each of the sample wells. Assuming that the cancer cell line doubled overnight, this would result in a 8:1 (2 million:250K) T-cell:MC38 ratio.

- 10 For positive controls (samples that are expected to get killed), if the cancer cell line doesn't express or present the target protein/epitope, make sure to supplement the co-culture with the target peptide.



- For pmel-1 CD8 cells, the co-culture should be supplemented with the hgp100 at 0.96 ug/mL (i.e. **[M]1 Micromolar (μM)**) at the beginning of the culture
- For OT-I CD8 cells, the co-culture should be supplemented with the SIINFEKL at 9.63 ug/mL (i.e. **[M]10 Micromolar (μM)**) at the beginning of the culture

- 11 Incubate the co-culture for at least 16 hours (overnight) before assaying.

#### Day 2 - Flow-cytometry-based cytotoxicity assesment

- 12 Prepare and label **2 ml** eppendorf tubes for each of your samples
- 13 Using a P1000, thoroughly pipette up and down each well and transfer everything in the well over to the corresponding **2 ml** tube
- 14 Without letting the well to dry out too much (> 2 minutes), add **200 μl** of trypsin and incubate at **Room temperature** for **00:02:00**.



**Trypsin 0.05% 1X Solution**  
by VWR Scientific  
Catalog #: **16777-202**

- 15 Stop the trypsinization by adding **800 μl** of culture media into each well
- 16 Spin down the tubes at **350 x g** for **00:05:00** and aspirate **1 ml** from the top without distrubing the cell pellet. Then transfer the **1 ml** of trypsinized cells over to the corresponding tube. Spin down the tubes at **350 x g** for **00:05:00** and aspirate the supernatant.

- 17 Re-suspend the cell pellet in  **1 ml** flow buffer and add  **5 µl** from each of the antibodies.



**PerCP anti-mouse CD8a Antibody**

by BioLegend

Catalog #: [100731](#)











**APC anti-mouse CD3 Antibody**

by BioLegend

Catalog #: [100235](#)



Make sure to pick two markers that will distinguish one cell from the other. We have found CD3 and CD8 staining, together, can help easily distinguish T cells from the cancer cells. Given that it is not easy to gate cells out only using the FSC/SSC channels, having these extra stains increase the specificity.

- 18 Stain the cells for  **00:20:00** at  **Room temperature** or  **00:30:00** at  **4 °C**
- 19 Spin the cells down at  **350 x g** for  **00:05:00** , remove the supernatant, and re-suspend them in  **400 µl** PBS.
- 20 Run  **200 µl** of the sample fully on flow (i.e. do not limit the number of events).



Since the cancer cells co-cultured with lots of T cells can easily clog the flow cytometer, having half of the sample as a back-up always helps.

**Analysis**

- 21 To count the number of MC38s left in the dish,
- Using your MC38 samples that were cultured on their own first, define the viable population as your initial gate (G0)
  - Within your G0, open the CD3/CD8 stain channels and gate the double-negative population (MC38s)

- 22 Normalize the positive samples against their corresponding negative controls to estimate the fraction of MC38 that are left in the plate. Subtracting this from 100% would give you an estimate of how much cytotoxicity happened for that particular sample.



At 8:1 OTI:MC38 ratio, 16 hours co-culture should yield >70% cytotoxicity when the number of MC38s left in the SIINFEKL-pulsed well is normalized against the unpulsed well.



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