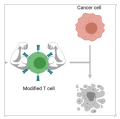

Nov 06, 2019

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.4

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1 *Works for me* dx.doi.org/10.17504/protocols.io.83bhyin

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ABSTRACT

In vitro co-cultures of cytotoxic T cells with their target cells are important assays to asses 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 assesed 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, Bülent Arman Aksoy, Eric Czech, Jeff Hammerbacher bioRxiv 466243; doi: https://doi.org/10.1101/466243

MATERIALS

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

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Trypsin 0.05% 1X Solution	16777-202	VWR Scientific
PerCP anti-mouse CD8a Antibody	100731	BioLegend
APC anti-mouse CD3 Antibody	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. **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. **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.

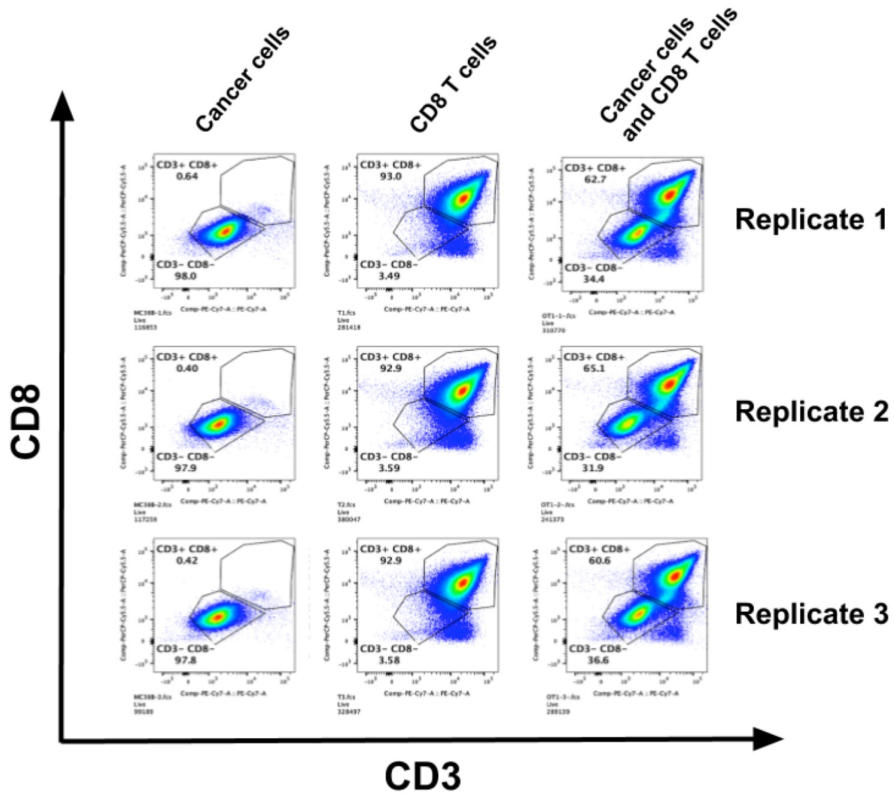
20 Run **200 µl** of the sample fully on flow (i.e. do not limit the number of events).

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. Substracting 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.