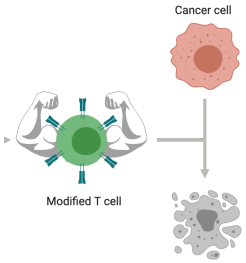
Oct 17, 2019 Version 3

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



DOI
dx.doi.org/10.17504/protocols.io.8d2hs8e

Bulent Arman Aksoy[1], Pinar Aksoy[1], Elinor Gottschalk[1], Jeff Hammerbacher[1]

[1]Medical University of South Carolina

Hammer Lab
Tech. support phone: **+18437924527** email: **arman@hammerlab.org**

Bulent Arman Aksoy
Medical University of South Carolina

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.8d2hs8e

Protocol Citation: Bulent Arman Aksoy, Pinar Aksoy, Elinor Gottschalk, Jeff Hammerbacher 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). **protocols.io https://dx.doi.org/10.17504/protocols.io.8d2hs8e**

Manuscript citation:
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

Protocol status: Working
We use this protocol and it's working

Created: October 17, 2019

Last Modified: October 17, 2019

Protocol Integer ID: 28826

Keywords: T cells, cytotoxicity, co-culture, MC38, TCR, CAR, flow cytometry, CD3, CD8, mediated cytotoxicity, cytometry, amount of cell, number of target cell, target cell, alternative to these release assay, mc38 cell line, release assay, important assay, target cell line, cell, cell line, type of cell line, assay

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.

Materials

MATERIALS

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

STEP MATERIALS

- Trypsin 0.05% 1X Solution **VWR International (Avantor)** **Catalog #**16777-202
- PerCP anti-mouse CD8a Antibody **BioLegend** **Catalog #**100731
- APC anti-mouse CD3 Antibody **BioLegend** **Catalog #**100235

Protocol materials

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

Troubleshooting

Before start

- 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.

Note

- 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 **VWR International (Avantor) 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 **BioLegend Catalog #**100731

APC anti-mouse CD3 Antibody **BioLegend Catalog #**100235

Note

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).

Note

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.

Expected result

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.