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In vitro pmel-1 T cell-mediated cytotoxicity assay with CytoTox-ONE Homogenous Membrane Integrity Assay (Promega)

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

[1]Medical University of South Carolina

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

Elinor Gottschalk

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

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Abstract

A plate-based assay to estimate the cytotoxic activity of pmel-1 T cells against target cancer cells. **The CytoTox-ONE Homogeneous Membrane Integrity Assay from Promega** (Madison, WI) is a lactate dehydrogenase (LDH) release-based assay. Dying cells have damaged cell membranes causing cytosolic components, such as LDH, to leak into the culture media. The CytoTox-ONE assay uses LDH in the culture media to convert resazurin to fluorescent resofurin. The resulting fluorescent signal is proportional to the number of non-viable cells in a sample. One advantage to using this assay for T cell-mediated cytotoxicity is that the cancer cells do not need to be removed from the wells to measure cell death, which is especially convenient with adherent cancer cell types that would require trypsinization. However, we have found that the assay produces noisy data and a set of control wells with T cells alone is required to subtract background, overall increasing the number of replicates that are necessary. We had consistent results when culturing the pmel-1 T cells with hgp100 peptide-pulsed MC38 colon cancer cells. But we had inconsistent results using pmel-1 T cells and their natural target, B16-F10 melanoma cells. This setup could be adapted for T cells other than pmel-1 and their target cells. The ratio of T cells to cancer cells and the duration of co-culture would have to be optimized.

Materials

MATERIALS

- CytoTox-ONE(TM) Homogen Membrn Integrity Assay, 200-800 assay **Promega Catalog #**G7890
- CytoOne T75 filter cap TC flask **USA Scientific Catalog #**CC7682-4875
- CytoOne 96-well TC plate **USA Scientific Catalog #**CC7682-7596
- hgp100(25-33) **Genscript Catalog #**RP20344
- B16-F10 cell line (ATCC® CRL-6475™) **ATCC Catalog #**CRL-6475
- MC38 cell line **Catalog #**ENH204-FP

STEP MATERIALS

- CytoTox-ONE(TM) Homogen Membrn Integrity Assay, 200-800 assay **Promega Catalog #**G7890

Protocol materials

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Before start

Start culturing T cells:

1. Day 0: Thaw frozen splenocytes or isolate fresh splenocytes
2. Supplement T cell culture media with 200 IU IL-2.
3. Activate the cells.
4. Day 3 - 6: Maintain the T cells at 1 million cells/ml, splitting when needed. Replenish IL-2 each day at 200 IU/ml.

1 The day before adding T cells to co-culture with the target cancer cells, plate the target cells in a 96 well plate at 25,000 cells per well in DMEM with 10 % FBS.
An example plate layout is below:

- Plate the target cells in columns 2-7. Leave the rest of the wells empty.
- Each column has 6 replicates (we do not recommend reducing the number of replicates because the resulting data is too noisy)
- One plate per condition being tested is required (in this case, the minimum will be two plates, one will have the target cells pulsed with the peptide, one with unpulsed cells as a negative control).

Sample plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12
a												
b		4:1	2:1	1:1	1:2	Target cells alone	Target cells alone	200,000 T cells	100,000 T cells	50,000 T cells	25,000 T cells	culture media
c												
d												
e												
f												
g												
h												

Incubate the target cells overnight to allow them to adhere to the plate.

2 Prepare the target cells for co-culture:

1. If required, pulse the target cells in one plate with 1 uM hgp100 (peptide for 01:00:00 at 37 °C
2. Wash the peptide out 3x with T cell media

Prepare T cells for co-culture (the day post-activation will depend on your experiments).

1. Centrifuge the T cells at 350 x g for 00:05:00 and resuspend in T cell media supplemented with IL-2 (200IU/ml) to obtain a concentration of 2 million cells per ml

Co-culture T cells with target cells:

1. Aspirate culture media from 96 well plate

2. Pipette 100 µL T cell media into columns 3-7 and 9-12 (leaving columns 2 and 8 empty)
3. Pipette 100 µL of T cell suspension into columns 2, 3, 8 and 9
4. Serial dilute the T cells in the plate: there will now be 200 ul total volume in columns 3 and 9. Starting from column 3, pipette up and down 3 times to mix. Then transfer 100 µL to column 4. Pipette up and down to mix in column 4 and transfer 100 µL to column 5. Pipette up and down to mix in column 5 and remove 100 µL and discard. Repeat starting at column 9 to make the T cell dilutions without cancer cells.
5. Incubate plates for 24:00:00 at 37 °C .

3 Perform CytoTox-ONE Homogenous Membrane Integrity Assay:

1. Thaw assay buffer and stop solution from CytoTox-ONE Homogenous Membrane Integrity Assay kit and bring to room temperature.

CytoTox-ONE(TM) Homogen Membrn Integrity Assay, 200-800 assay **Promega Catalog #**G7890

1. Thaw lysis solution and make a 1:5 dilution of lysis solution in PBS - total volume 100 ul per plate; this is good for 1 plate (80 ul PBS + 20 ul lysis solution)
2. Take plate/s out of incubator and place in hood
3. As soon as taking plates out of the incubator add 10 µL diluted lysis solution to each well in **column 7** to lyse the cells for the maximum LDH release control. Make sure to mix the lysis buffer into the well by pipetting up down.
4. Allow plates to sit in hood for 00:30:00 to equilibrate to room temperature
5. Add 11 ml Assay Buffer into one substrate bottle and mix
6. Pour the assay buffer/substrate mixture into a reagent reservoir and using multichannel pipette, pipette 100 µL into each well to assay in the plate, **shake plate gently to mix**
7. Cover from light in hood and incubate for 00:10:00
8. Add 50 µL stop solution to each well in the same order as the assay buffer was added, shake plate gently to mix
9. Directly read plates on a fluorescence plate reader (excitation 560, emission 590) in the order that they were prepared in

4 Calculate percent cytotoxicity:

$$((experimental - CMB) - (Tcellbackground - CMB)/(maxLDHrelease - CMB))X100$$

where experimental is the co-culture well (columns 2-6), CMB (culture medium background) is culture media with no cells (column 12), T cell background is T cells only

at the different concentrations corresponding to the co-culture ratios (columns 8-11), and max LDH release is MC38 cells lysed with a detergent to release all of the LDH in the cells into the culture media (column 7).