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

Cytotoxicity Assay Protocol

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ABSTRACT

This is the protocol for cytotoxicity assay in cancer cell lines using flow cytometry.

MATERIALS

1. Tumor cell line
2. Tumor-reactive T cells
3. Tumor cell medium: RPMI 1640 (Gibco 11875) + 10% Fetal Bovine Serum (FBS) complete mixture
4. T cell medium: 50ml complete MEM media (Gibco 11095080) + 500 IU/ml IL2 + add 50 µL of
1000X beta-mercaptoethanol (55mM)
5. 1X Phosphate-buffered saline (PBS)
6. FACS Buffer: 2% FBS in PBS
7. APC-conjugated rabbit anti-active caspase 3 Ab
8. BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit
9. Fixation/permeabilization solution
10.BD Perm/Wash™ Buffer

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1 Begin T cell culturing a week prior. Grow and culture tumor cell lines. Ensure the cells are not over-confluent.

2 Split the tumor cells the day before the assay. Perform the cytotoxic assay as described in next steps.

3 Count tumor cells. Collect 3-5M cells for each cell line.

4 Centrifuge the cells and wash the pellet once with PBS. Ensure that all media is washed off as this can inhibit the staining.

5 Aspirate the supernatant and leave as little PBS as possible

6 Re-suspend cells in culture medium at the appropriate ratio for the experiment (1X10^6 / ml for 50,000 cells/50ul/well or about ~80% confluent) using 96 well round well plate with 50,000 cell tumor and in triplication.

7 Add 50 ul of T cell suspension diluted to achieve the desired E:T ratio. Below is an example of how to set up E:T ratios:

	A	B	C
	ET Ratio(s)	T cells/50ul /well	Tumor cells/50ul/well
	0:1	0 (only with T cell medium)	5 x104 cells
	1:1	5 x104 cells	5 x104 cells
	3:1	1.5x105 cells	5 x104 cells

8 Centrifuge the mixed cells in the tubes at 300 rpm for 5 minutes to gently pellet the cells. Incubate for 3 hours at 37^{0}C.

9 Add 150ul PBS into the plate and then centrifuge at 2000rpm for 5min.

10 Remove the supernatant and add then 100ul/well Trypsin. The plate is incubated at 37C incubator for 3-5mins.

11 Add 100ul FACS buffer to stop the trypsinization and centrifuge at 2000rpm for 5 min.

12 Remove the supernatant carefully.

13 Fix and permeabilize cells using Cytofix/Cytoperm™ Kit (BD BioSciences) for 20 minutes at room temperature (100ul/well – **mix well**).

14 Wash with Perm/Wash™ Buffer (150ul/well) at 2000 rpm for 5 min.

15 Prepare caspase antibody dilution such that each well gets stained with 2.5ul of APC-conjugated rabbit anti-active caspase 3 Ab in 50 ul Perm/Wash™ Buffer. Mix gently and incubate for 30 min in the dark on ice or in the 4°C refrigerator.

16 Wash cells 1Xwith Perm/Wash™ Buffer (150ul).

17 Resuspend the cell pallets into 100ul FACS buffer (2% FBS in 1X PBS).

18 Analyze the samples using flow cytometry.