Flow-Based NK Cell Killing Assay Against Tumour Target Cells

Ashkar Lab
McMaster Immunology Research Centre
Department of Pathology and Molecular Medicine
McMaster University, Hamilton, ON, Canada

Supplies

- Tumour target cells
- 15 mL falcon tubes
- 50 mL falcon tubes
- Ice container
- CFSE (Sigma Cat#21888)
- Sterile PBS
- DMSO
- 10% RPMI Media
- 1.5mL tubes
- Hemacytometer and trypan blue
- Centrifuge

Labeling of Target Cells with CFSE

Note regarding number of tumour cells to stain with CFSE: After each spin approximately ~ 10% of cells are lost. Ensure you have grown up enough cells in culture and choose enough cells to label i.e. there are 6 spins in this protocol, so if you need $5x10^6$ CFSE stained tumour cells total, you will need to start this protocol with **at least** $10x10^6$ tumour cells.

1) Calculate the number of CFSE labeled and unlabeled target cells you will require using sample plate layout and calculations found on page 5.
2) Wash target cells with 20 mL PBS, re-suspend in low volume of media, count
3) Re-suspend target cells in media at desired concentration for plating. Transfer the cells required for the unstained controls into another tube and put aside on ice.
4) Wash remaining target cells with 20 mL of PBS 2x

All following steps should be conducted in light-sensitive conditions

5) Prepare CFSE during the washes:
   CFSE Stock Concentration 50mM; Final Concentration 2.5uM
   a) Take 10uL of stock and add to 990uL DMSO (0.5mM) => vortex
   b) Take 300uL of mixture from a) and add to 2700uL PBS (0.05mM=50uM) => vortex
   c) Take 1mL of mixture from b) and add to 9mL PBS (5uM). => vortex
      a. Note: if you have more than 10mL of cells to label, at this step you could instead take 2mL of b) and add to 18mL PBS. Keep on ice.
6) After 2nd wash, resuspend cells in a VERY low volume of PBS, the goal is a concentration of ~$10x10^6$ cells/mL.
7) Count cells and re-suspend to $10x10^6$ cells/mL in PBS
8) Add an equal volume of CFSE mixture c) to cells (i.e. for 2mL of cells add 2mL of mixture 3c). Cap tube, then immediately vortex (mid-speed) to mix.
9) Incubate 15 minutes at 37 degrees Celsius.

10) Dilute up to 50mL with 10% FBS in PBS to quench any unbound CFSE and incubate at room temperature for 10 minutes protected from light. Spin 1500rpm, 5 minutes, low brake.
11) Dump supernatant and resuspend cells in 20 mL PBS. Mix well by pipetting. Spin 1500rpm, 5 minutes, low brake.
12) Dump supernatant and resuspend in media and count
13) Re-suspend cells to desired final concentration in media. Cells are now ready for use

NK Cell Killing Assay with Tumour Target Cells

Supplies

- 96 well round bottom plate
- FACS buffer (0.2% BSA in PBS, 0.2um filtered)
- Centrifuge with plate spinners
- FACS tubes
- Fixable Viability Dye. We use Fixable Viabity Dye eFluor 780 from eBioscience (cat#: 65-0865-18)
- Incubator

Plate Layout Preparation

1) Choose two or more ratios to use for killing assay:
effector (NK cells):target (CFSE labeled MDA-231) ratio
i.e. 1:1, 5:1, 10:1 are typical choices. Choose based on what is known about the killing ability of the cells. All killing wells should be in triplicate if possible.

2) Calculate the number of labeled tumour target cells required:
Ideally, $2x10^5$ target cells/well should be used.
i.e. a 5:1 killing well would contain $1x10^6$ NK cells:$2x10^5$ CFSE labeled tumour target cells.

3) Calculate the number of NK (effector) cells required:
Ideally, there will be $2x10^5$ NK cells and $2x10^5$ CFSE labeled tumour target cells in each 1:1 ratio killing well. The total number of NK cells you will require depends on the ratios you chose and the total number of killing wells you will have.

4) Calculate the number of unlabeled tumour target cells required for *control wells*:
See sample plate layout below. The ideal number of cells per control well is $2x10^5$.

5) Determine the plating concentrations and volumes for the NK cells and target cells.
i.e. Target cells: $2x10^5$ cells/100uL and plate 100 uL/well.
NK cells: (# NK cells in highest effector:target ratio well)/100uL
i.e. 10:1 highest effector:target (E:T) ratio = $2x10^6$ NK cells/well => NK plating concentration
= $2x10^6$ NK cells/100 uL
Plate 100uL NK cells in highest E:T well and corresponding NK cell volumes in lower E:T ratio wells. Add appropriate amount of media to lower E:T ratio wells to bring the final volume to 200 uL/well.

Setting up Killing Assay

1) Label your target cells as previously outlined.
Note: *you can also label your effector cells, however tumor cells are often autofluorescent and that can make it harder to differentiate between CFSE labeled NK cells and autofluorescent tumor cells during analysis*

2) Wash NK cells in media, count, and re-suspend to the concentration desired for plating.

3) In a round bottom plate, plate the desired number of NK cells (effector cells) in each killing well. (please find an example plate layout below)
4) Note which wells should receive CFSE-labeled or unlabeled target cells. Plate desired number of labeled or unlabeled target cells in each killing or control well. Mix the cells well upon adding the target cells to the effector cells.
5) Incubate for 5 hours in a 37C, 5% CO_2 incubator.
6) After the 5 hours, spin the plate at 1500 for 5 min.
7) Remove supernatant (can freeze supernatant for cytokine analysis). Add PBS (200µL per well) to each well to wash the cells, spin at 1500rpm for 5min
8) During the spin prepare your Fixable Viability Dye mix. Dilute the dye at a 1 in 1000 in PBS (1 µL per 1mL). Prepare enough to stain using 100 µL per well. Be sure not to add your viability dye to certain controls (unstain, viability dye FMO, and CFSE compensation control wells) – just add 100 uL PBS to these wells.
9) After the cells are spun down, remove supernatant.
10) Add 100 µL of your viability dye stain to each well. Incubate in fridge or on ice for 30min protected from light.
11) After the incubation, spin down the plate at 1500 for 5 min.
12) Remove supernatant, then wash with 100 µL of PBS to each well and spin again
13) Fix the cells using 1% PFA for a minimum of 1 hour. Do not exceed 4 hours in PFA. After fixation, spin the plate at 1500 for 5 min, remove PFA and resuspend in FACS buffer.
14) Samples are now ready for data acquisition on a cytometer.
15) Gating strategy: Gate on CFSE+ cells to exclude NK cells from analysis. From the CFSE+ cells, gate the dead cells.

Sample plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	1:1	1:1	1:1									
B	5:1	5:1	5:1									
C	10:1	10:1	10:1									
D												
E												
F	Basal Death	Basal Death	Basal Death	Basal Death								
G	Viability Dye (V.D.) FMO (labeled target cells + NK cells) No VD stain (just PBS)	CFSE FMO (unlabeled target cells + NK cells) Stained with V. D.	Unstained (unlabeled target cells + NK cells) No VD stain (just PBS)									
H	Labeled + Unlabeled target cells. No VD stain (just PBS)	10 NK cells: 1 target cell ratio, Unlabeled target cells only Stained with VD										

Effector: Target Ratio
These wells will have CFSE Labelled Target cells and will be stained with a Viability Dye

Basal Cell Death 3- 4 wells (Labelled target Cells only, no NK cells). Stained with Viability Dye.

Control Wells

CFSE Compensation

Viability Dye Compensation

How to calculate specific lysis:

Average the % dead cells across the basal cell death wells. This average will be the % basal cell death.

$$\% \text{ specific lysis} = \frac{100 \times (\% \text{ lysis in sample} - \% \text{ basal death})}{100 - \% \text{ basal death}}$$