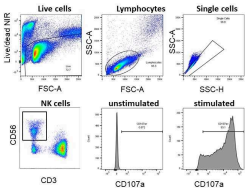
Jul 11, 2023 Version 2

Degranulation and cytokine production (functional assay) V.2



DOI
dx.doi.org/10.17504/protocols.io.8epv51me6l1b/v2

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.8epv51me6l1b/v2

Protocol Citation: Philippa R Kennedy 2023. Degranulation and cytokine production (functional assay). **protocols.io** **https://dx.doi.org/10.17504/protocols.io.8epv51me6l1b/v2**Version created by **Philippa R Kennedy**

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

Created: December 15, 2022

Last Modified: July 11, 2023

Protocol Integer ID: 74080

Keywords: natural killer cell, NK cell, CD107a, flow cytometry, cytokine, functional, cytotoxic granlue release, primary functions of nk cell, nk cell, direct measure of cytotoxicity, accumulation of the cytotoxic granule membrane protein, cytotoxic granule membrane protein, surface of the nk cell, cytotoxicity, cytokine production, tumor cell, cytotoxcity, cell, functional assay, cytometry, based assay, responses at the single cell level, endosomes with the lysosome, recycling endosome, single cell level, killing assay

Abstract

Two primary functions of NK cells - their cytotoxcity and ability to produce cytokines, are measured in a flow-based assay that assesses these responses at the single cell level when NK cells are challenged with tumor cells. Cytotoxicity is not directly measured, instead it is inferred from cytotoxic granlue release. For this reason, this assay should be accompanied by a direct measure of cytotoxicity, such as a *Time -lapse killing assay*. Cytotoxic granlue release is measured by the accumulation of the cytotoxic granule membrane protein, CD107a (LAMP1), at the surface of the NK cell, when fusion of recycling endosomes with the lysosome is blocked by monensin. Cytokines accumulate in NK cells when Golgi transport is blocked by brefeldin A. At the end of an effector:target co-culture, these proteins and other lineage surface markers are detected by flow cytometry.

This version takes less time than the previous protocol without affecting the quality of the result.

Guidelines

The day of the assay (after effectors are plated) is long because of the 5 h incubation. Be sure to start early in the day.

During the setup of the assay, to avoid shocking the cells, pre-warm media and pipette gently, up until the staining steps. After the 5 h incubation, the aim is to stop all reactions, so pre-cool centrifuges and staining buffers to 4°C. Once cells are fixed, this cooling is no longer necessary.

The manufacturer recommend live/dead staining in PBS, but a side-by-side comparison of staining in PBS or flow buffer revealed no difference in performance of the NIR dye in our hands.

The manufacturer recommends washing in perm buffer after staining, but a side-by-side comparison of washing in perm buffer or flow buffer revealed no difference in performance of the staining in our hands.

When working with tissue samples, blocking prior to staining is required; filtering through a 70µm filter prior to running through a flow cytometer is required; and counting beads can be beneficial for accurate assessment of cell number.

Materials

R10 media recipe:

- RPMI cat. 2240-089, Gibco
- 10% heat inactivated fetal calf serum cat. 26140079, Gibco
- 100 U/ml Penicillin & 100 µg/ml Streptomycin cat. 15140122, Gibco

B0 media recipe:

- 60% DMEM cat. 10-017-CV Corning
- 30% Hams F12 cat. 10-080-CV Corning
- 10% heat inactivated AB serum cat. HP1022 Valley Biomedical Inc
- 100 U/ml Penicillin & 100 µg/ml Streptomycin cat. 15140122, Gibco
- 20 µM β-mercaptoethanol cat. M7522, Sigma Aldrich
- 50 µM Ethanolamine cat. E0135, Sigma Aldrich
- 20 µg/ml Ascorbic Acid cat. A4544, Sigma Aldrich
- 5 ng/ml Sodium Selenite (Na_2SeO_3) cat. S5261, Sigma Aldrich

Flow buffer:

- 1% heat inactivated AB serum, cat. HP1022 Valley Biomedical Inc
- 0.5mM EDTA
- PBS cat. 21-040-CV, Corning

Inhibitors

- GolgiStop Cat. No. 554724, BD Biosciences
- GolgiPlug Cat. No. 555029, BD Biosciences

Stains:

- anti-CD107a, clone H4A3, cat. 328606, BioLegend, RRID:AB_1186036
- anti-CD56-PE-Cy7, clone HCD56 cat. 318318, Biolegend, RRID:AB_604107
- anti-CD3-PECF594, clone UCHT1, cat. 562280, BD Horizon, RRID:AB_11153674
- anti-IFNγ-Bv650, clone 4S.B3, cat. 502538, BioLegend, RRID:AB_2563608
- anti-TNFa-Bv421, clone Mab11, cat. 502932, Biolegend, RRID:AB_10960738
- Live/Dead Fixable Near-IR Staining Kit, cat. L-34976, Thermo Fisher

Fixation:

- 2% Paraformaldehyde/PBS

Permeabilization:

- Permeabilization Buffer(10x), cat. 00-8333-56 eBioscience

For tissue samples:

- Human TruStain FcX™ Fc Receptor Blocking Solution (cat. 422302, BioLegend)
- CountBright™ Absolute Counting Beads, for flow cytometry (cat. C36950, ThermoFisher Scientific)

- EZflow cell strainer 70µm, (cat. 1170C02, Thomas Scientific)

Troubleshooting

Before start

If working with frozen cells, these must be thawed and prepared the day before the assay. Book flow cytometers in advance to avoid delays in acquiring the data. Ideally, samples should be run the day after the assay was performed, but can be stored up to three days e.g. if assay was performed on a Friday.

Preparing effector and target cells

1 Effector cells (PBMCs or enriched NK cells) are resuspended in **R10**.
Induced pluripotent stem cell (iPSC)-derived NK cells should be resuspended in **B0**.

1.1 **R10 media recipe:**

- RPMI cat. 2240-089, Gibco
- 10% heat inactivated fetal calf serum cat. 26140079, Gibco
- 100 U/ml Penicillin & 100 µg/ml Streptomycin cat. 15140122, Gibco

B0 media recipe:

- 60% DMEM cat. 10-017-CV Corning
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- 10% heat inactivated AB serum cat. HP1022 Valley Biomedical Inc
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- 20 µM β-mercaptoethanol cat. M7522, Sigma Aldrich
- 50 µM Ethanolamine cat. E0135, Sigma Aldrich
- 20 µg/ml Ascorbic Acid cat. A4544, Sigma Aldrich
- 5 ng/ml Sodium Selenite (Na2SeO3) cat. S5261, Sigma Aldrich

2 Target cells are added to each well at an effector:target ratio of 2:1. At the same time, FITC conjugated anti-CD107a (clone H4A3, BioLegend; batch specific, but normally **3 µL/well**) is added to each well. The cells are then incubated at 37°C 5% CO_2.

01:00:00

1h

3 5×10^5 cells/well are added to a U-bottomed 96 well plate.

1d

These numbers can be decreased to 1×10^5 effector cells/well if the number of effector cells is limited, so long as target numbers are decreased accordingly.

If thawing effector cells, these should be plated one day in advance in 200 µL **R10** (PBMC) or 200 µL **B0 with 50 U/mL IL-2** (iPSC-NK cells) and cultured at 37°C 5% CO_2 overnight Overnight . This allows recovery of surface proteins and functionality. In this case, plates are spun at 300*g* for 5 min the following day in order to remove excess media.

Adding inhibitors

4 One hour after the addition of anti-CD107a, cells are given monensin (GolgiStop Cat. No. 554724, BD Biosciences) and brefeldin A (GolgiPlug Cat. No. 555029, BD Biosciences). GolgiStop (1/150) and GolgiPlug (1/100) are diluted in R10 and 20 µL is added to each well. Cells are incubated for a further 4 h at 37°C 5% CO_2. 04:00:00 — 4h

Live/dead and surface staining of cells — 30m

5 Cells are washed twice, as defined below, in **flow buffer (1% AB serum, 0.5mM EDTA in PBS)**. This definition also applies to subsequent washes.

5.1 The plate is spun in a centrifuge at 300*g* for 5 min *(this is approximately 1200rpm in a large benchtop centrifuge)*. The supernatant is removed and replaced with 200 µL flow buffer/well (first wash). — 5m

5.2 The plate is spun again in the centrifuge at 300*g* for 5 min. The supernatant is removed and replaced with 200 µL flow buffer/well (second wash). — 5m

5.3 The plate is spun for a final time at 300*g* for 5 min and the supernatant is removed. — 5m

6 *Optional: If working with processed tissues, prepare 5µL Fc Receptor Blocking Solution (cat. 422302, BioLegend) in 100µL flow buffer. Resuspend each well in 20µL of this blocking solution and incubate for 5 min* 00:05:00 *at room temperature to block non-specific staining. Proceed to surface staining without washing.* — 5m

7 Cells are resuspended in 50 µL flow buffer containing:
1. **anti-CD56-PE-Cy7** (clone HCD56, Biolegend; batch specific, but normally **5 µL/test**)
2. **anti-CD3-PECF594** (clone UCHT1, BD Horizon, batch specific, but normally **1 µL/test**)
3. **Live/Dead Fixable Near-IR** Staining Kit, cat. L-34976, Thermo Fisher (**1/1000 dilution**)

Incubate for 15 min at 4 °C 00:15:00 . After the incubation, wells are topped up to 200 µL with flow buffer and washed once with flow buffer. 00:10:00 — 25m

8 - Cells are resuspended in 100 µL 2% paraformaldehyde/PBS and incubated at room temperature in the dark for 10 min to fix them 00:10:00 . Afterwards, wells are topped up to 200 µL with flow buffer and washed once with flow buffer. 00:10:00 — 20m

Note

The assay can be paused here overnight. If doing so, wash once more in flow buffer, topping up to 100 µL/well, then store at 4 °C. Seal the edge of the plate with parafilm to avoid evaporation.

Intracellular staining of cells

30m

9 ▪ Cells are washed in 150 µL **perm buffer (10% Permeabilization Buffer, cat. 00-8333-56 eBioscience/distilled H_2O)**.

10 Permeabilized cells are resuspended in 50 µL perm buffer containing:
1. BV650 conjugated IFNγ (4S.B3, BioLegend; batch specific, but normally **3 µL/test**)
2. BV421 conjugated TNFa (Mab11, Biolegend; batch specific, but normally **5 µL/test**).
They are incubated for 30 min at 4 °C in the dark. 00:30:00

30m

11 After staining, wells are topped up to 200 µL with flow buffer and washed once with flow buffer. Cells are resuspended in 200 µL of flow buffer and transferred to bullet tubes. 00:10:00

10m

12 The tubes are covered and stored in the dark at 4 °C until they are ready to be run on a flow cytometer (LSR II, BD Biosciences).

To be able to compare NK cell numbers or tumor cell numbers between samples, run the flow cytometer at a consistent speed (e.g. high) and acquire data for a set time (e.g. 60s)

13 Data is analyzed using FlowJo software (Tree Star Inc., RRID:SCR_008520)

Degranulation (CD107a+) and cytokine production (IFNγ+ or TNFα+) are assessed for the live (dead cell marker-) NK cell (CD56+ CD3-) population.