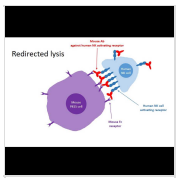


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# Redirected lysis (P815 functional assay)

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ABSTRACT

In order to test the function of specific activating receptors on the surface of human natural killer (NK) cells, 1) mouse antibodies that cross-link those human receptors and 2) mouse cell line P815 that has Fc receptors to gather those antibodies at the immune synapse are co-incubated with human NK cells. The effective cross-linking of specific human NK cell receptors at the synapse causes 'redirected' lysis of the P815 cells. Cytokine production and cytotoxic granule release ('degranulation' as measured by CD107a appearance at the surface of the NK cell) are two readouts of human NK cell activation that can be measured by flow cytometry. This assay can be combined with cell trace labeling to examine how different populations of NK cells respond to specific receptor triggering.

The P815 assay is adapted from: https://www.ncbi.nlm.nih.gov/pubmed/20033652
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GUIDELINES

**Antibody purity**

NK cells magnetically isolated from peripheral blood mononuclear cells (PBMC) can have very small proportions of myeloid cells contaminating the isolation. These are very sensitive to contaminants and can greatly enhance NK cell functional responses if present (http://www.ncbi.nlm.nih.gov/pubmed/24337374). For this reason it is best to use highly purified ('LEAF' or 'NA/LE') antibodies for triggering in these studies.

**Control antibodies**

In addition to the antibodies against specific human receptors to be investigated, this assay should include non-specific control antibodies (an isotype control antibody) and surface-bound non-activating antibodes (e.g. anti-CD56) to see the background levels of functional response when NK cells form contacts with P815, but activating receptors are not triggered. Receptor-independent activation (e.g. PMA + ionomycin) can serve as a useful positive control for degranulation and cytokine production.

**Titrating antibodies**

Antibody concentrations should be titrated to find a dose that induces robust functional responses when NK cells are co-incubated with P815, but not when then are incubated with the antibody alone. The antibodies listed were all titrated to 1 microgram/ml for the current assay.

**Variations**

*1. Pre-incubating the NK cells with antibodies, rather than pre-incubating the P815 cells with antibodies*

The binding of the specific anti-human receptor antibodies is generally stronger to the human receptor than it is to the mouse Fc receptor, so the original protocol had the pre-incubation step with NK cells. However, our assays involve a single target cell line, but multiple NK cell conditions that take a significant amount of time to prepare. Given the duration of the assay, for time saving reasons, we changed the incubation step to be with the P815 so we could start the incubation while the NK cells were being prepared. Preliminary optimization assays showed similar results were obtained by this method as to when the antibodies were first pre-incubated with the NK cells.

*2. Washing off antibodies after a pre-incubation step*

Some published methods wash antibodies away after the initial pre-incubation step on NK cells. A comparison of these two variations (washing or not washing) prior to addition of P815 showed a titrable response from the NK cells in both cases. The washing step greatly increased the amount of antibody that was required to get maximal responses without obviously providing any other benefit. For this reason the wash step was removed from our assay.

MATERIALS TEXT

MATERIALS

Penicillin-Streptomycin **Gibco - Thermo Fisher Catalog #15140122**

Recombinant Human IL-15 Protein **R&D Systems Catalog #247-ILB**

RPMI 1640 Medium, HEPES **Thermo Fisher Catalog #22400089**

Fetal Bovine Serum, qualified, United States **Thermo Fisher Catalog #26140079**

CellTrace™ Violet Cell Proliferation Kit, for flow cytometry **Thermo Fisher Catalog #C34557**



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LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation **Thermo Fisher Catalog #L34976**

anti-human TNF-alpha AlexaFluor647 (clone MAb11) **BioLegend Catalog #502916**

P815 **ATCC**

anti-human CD107a FITC (clone H4A3) **BioLegend Catalog #93937**

anti-human IFN-gamma BrilliantViolet 650 (clone 4S.B3) **BioLegend Catalog #93705**

Golgi Stop (Protein Transport Inhibitor Monensin) **BD Biosciences Catalog #554724**

Golgi Plug (Protein Transport Inhibitor Brefeldin) **BD Biosciences Catalog #555029**

Mouse IgG1 κ Isotype Ctrl Antibody (clone MOPC-21) LEAF™ **BioLegend Catalog #400124**

anti-human CD314 (NKG2D) antibody (clone 1D11) LEAF **BioLegend Catalog #320810**

anti-human CD337 (NKp30) antibody (clone P30-15) LEAF™ **BioLegend Catalog #325204**

anti-human CD2 antibody (clone RPA-2.10) LEAF™ **BioLegend Catalog #300212**

anti-human CD226 (DNAM1) antibody (clone DX11) NA/LE **BD Biosciences Catalog #559786**

anti-human CD16 antibody (clone 3G8) purified **BD Biosciences Catalog #550383**

anti-human CD56 PE-Cy7 (clone HCD56) **BioLegend Catalog #92189**

anti-human CD3 PE-CF594 (clone UCHT1) **BD Biosciences Catalog #562280**

1 *Optional:* Label specific cell subsets prior to this assay with Cell Trace Violet (CTV) according to the manufacturer's instructions to examine the behavior of a particular cell subset.
CellTrace™ Violet Cell Proliferation Kit, for flow cytometry **Thermo Fisher Catalog #C34571**

1.1 *Option 1:* Label effector cells (peripheral blood mononuclear cells 'PBMC' or NK cells) with CTV before combining them with unlabeled subsets (e.g. monocytes) to see how the effector cells perform in combination. Care must be taken when determining effector: target cell ratios.

1.2 *Option 2:* Label effector cells with CTV to combine this assay with a proliferation assay. Perform



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this functional assay at the end of the proliferation assay.

1.3 *Option 3:* Label P815 cells with CTV to combine this assay with a direct measure of target cell death. At the end of the assay, when analyzing samples on a flow cytometer, count the relative number of live P815 for each treatment condition by running the flow cytometer at a constant flow rate and analyzing the number of live CTV+ cells obtained within a specified time limit e.g. 60s.

2 P815 cells are counted and resuspended at $2.5x10^6$ cells/mL in **R10 (RPMI + 10% fetal bovine serum + 1% penicillin streptomycin).** 100 µl ($2.5x10^5$ cells) per well are added to a 96 well U-bottom plate. Anti-human receptor mAb (e.g. anti-CD16, anti-NKp30, anti-NKG2D, anti-DNAM1, anti-CD2, mIgG1) at 1 µg/ml are added to individual wells containing P815 and to the same number of wells containing R10 alone. The plate is incubated for 30 minutes at room temperature.

3 During the incubation, effector cell populations are counted and resuspended in R10. At the end of the incubation, $5x10^5$ effector cells and anti-CD107a-FITC antibody (5 µl/well) are added to each well, into a final volume of 200 µl. The plate is incubated at 37°C 5% $CO_2$ for 1 h.

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% $CO_2$.

5 Cells are washed twice in PBS, as defined below. This definition also applies to subsequent washes.

5.1 The plate is spun in a centrifuge at 300*g* for 5 min. The supernatant is removed and replaced with 200 µL PBS/well (first wash).

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

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

6 Cells are resuspended in 200 µL PBS with a dead cell marker (1/1000 dilution; Live/Dead Fixable Aqua Staining Kit, Cat. No: L-34966, Thermo Fisher) and incubated for 30 min at 4°C in the dark.

7 Cells are washed twice in **flow buffer (1% AB serum, 0.5mM EDTA in PBS).**

8 After the final spin, wells are resuspended in 50 µL of flow buffer containing anti-CD56-PE-Cy7 (2 µl / well) and anti-CD3-PECF594 (1 µL / well). The plate is incubated at 4°C for 15 min in the dark. After the incubation, wells



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are topped up to 200 µL with flow buffer and washed once with flow buffer.

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

10 Cells are resuspended in 100 µL 0.1% Triton X/PBS and incubated at room temperature in the dark for 5 min to permeabilize them. Afterwards, wells are topped up to 200 µL with flow buffer and washed once with flow buffer.

11 Permeabilized cells are resuspend in 100 µL of flow buffer containing anti-IFNγ-BV650 (3 µL / well) and anti-TNFα-AF647 (5 µL / well). The plate is incubated for 30 min at 4°C in the dark.

12 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.

13 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).

14 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 and subdivided according to cell trace labeling.



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