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## Obtaining of NK cell clones using IL-2 and gene-modified K562 cells expressing membrane-bound IL-21

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**Protocol status:** Working

**We use this protocol and it's working**

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## Abstract

Here we present the simple and effective method for obtaining clones of NK cells, sorted according to a given set of surface markers. The lifespan of clones grown by this method can reach 14 weeks and cloning efficiency is up to 50%, depending on the original NK cell subset. Obtained clones are functionally active; they are able to lyse target cells and produce IFN $\gamma$ . The principle of method includes single cell sorting and further clonal expansion in the presence of IL-2 and gene-modified K562 feeder cells. Our method allows to expand the progeny of a single NK cell up to  $10\text{--}20 \times 10^6$  NK cells suitable to study phenotype, proliferative and functional activity of a certain NK cell clone.

The work was supported by Russian Science Foundation, grant #16-15-00309.

## Guidelines

The purpose of this protocol is to obtain clonal populations of NK cells for their further study and expansion. This method is based on sorting single NK cells using the "Single Cell" mode on a cell sorter. In this protocol, we work with isolated human NK cells, but the clones can be obtained from PBMC too, though it will require more time and additional antibodies, and the results can be less pure.

According to our protocol, clones were obtained from subpopulations at different differentiation stages:

CD56<sup>bright</sup>, CD56<sup>dim</sup>CD57<sup>-</sup>, CD56<sup>dim</sup>CD57<sup>+</sup>, also in certain experiments these subsets were additionally sorted into HLA-DR<sup>+</sup> and HLA-DR<sup>-</sup>. Obtained clones expressed the NKG2D receptor and high levels of CD56. Normally, NK cells remained homogeneous during clonal growth in expression of such markers as NKG2A, KIR2DL2/DL3, NKp46, NKp30, NKG2C. Other markers, such as CD57, CD16, can be heterogeneously distributed inside a single clone.


## Materials


### STEP MATERIALS


 NK Cell Isolation Kit, human **Miltenyi Biotec Catalog #130-092-657**

 NK Cell Isolation Kit, human **Miltenyi Biotec Catalog # 130-092-657**

 Fetal calf serum **HyClone Catalog #SH3007103**

 DMSO **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D1435**

 RPMI-1640 medium supplemented with 10% FBS

 recombinant human IL-2 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #000000011011456001**

 AIM V<sup>™</sup> Medium, liquid **Thermo Fisher Scientific Catalog #12055091**

 NK Cell Isolation Kit, human **Miltenyi Biotec Catalog #130-092-657**

## Protocol materials

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## Before start

First of all it is necessary to obtain fresh human PBMC (e.g., with the help of standard procedure of ficoll-paque gradient centrifugation of peripheral blood samples). Blood should be collected into heparin- or EDTA-containing tubes, and  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate buffer (PBS) should be used during the procedure to avoid cell clumping. Next, after PBMC isolation, the magnetic separation should be performed to enrich NK cells for cell sorting. The current protocol is described for Miltenyi Biotec NK cell isolation kit (cat. #130-092-657).

## NK cell isolation

- 1 Resuspend PBMC with ice-cold separation buffer in 15 or 50 ml Falcon™ tube. Determine cell number.

### Note

All further steps should be done in sterile conditions and as fast as possible. Keeping cells cold and using pre-cooled solutions is needed to prevent capping of antibodies and non-specific labelling. The NK Cell Isolation Kit, human (Miltenyi Biotec) is used for negative magnetic NK cell separation.

### Note

Separation buffer:  
Dilute 0.5% BSA (Serva) and 2 mM EDTA (Serva) in PBS. Adjust pH to 7.2. Sterilize by filtering through 22-µm Millipore filter and store up to 2 months at 4°C.

 NK Cell Isolation Kit, human **Miltenyi Biotec Catalog #130-092-657**

- 2 Centrifuge at 200×g for 10 min at 4°C. Remove the supernatant by decanting or with an automatic pipette.
- 3 Resuspend the pellet in 40 µl of separation buffer per 10<sup>7</sup> of total cells and transfer the suspension into a new Falcon™ 15 ml polypropylene conical centrifuge tube.

### Note


This step allows to avoid the intrusion of non-labelled cells from the previous 15/50 ml Falcon™ tube walls into the separation column.

- 4 Add 10 µl of NK Cell Biotin-Antibody Cocktail (The NK Cell Isolation Kit, human, by Miltenyi Biotec) per 10<sup>7</sup> of total cells and mix gently.

 NK Cell Isolation Kit, human **Miltenyi Biotec Catalog #130-092-657**



- 5 Incubate for 5 min at 4°C.
- 6 Add 30 µl of separation buffer and 20 µl of NK Cell MicroBead Cocktail (The NK Cell Isolation Kit, human, by Miltenyi Biotec) per  $10^7$  of total cells.

 NK Cell Isolation Kit, human **Miltenyi Biotec Catalog # 130-092-657**
- 7 Mix gently and incubate for 10 min at 4°C.
- 8 Adjust the volume of the separation buffer up to 500 µl per  $10^8$  of total cells.
- 9 Install MACS™ LD column into the magnetic field of a MidiMACS Separator and rinse it with 3 ml of separation buffer.

#### Note

It is convenient to begin rinsing the column, when you have put the cells into the ice for 10 min (step 7), as rinsing takes approximately the same time.

Other MACS™ columns (MS, LS) can be used for this procedure, but with the LD column, we obtained NK cells with the highest purity.

#### Equipment

MACS LD column	NAME
Column for magnetic separation	TYPE
Miltenyi Biotec	BRAND
130-042-901	SKU
<a href="https://www.miltenyibiotec.com/AD-en/products/macs-cell-separation/columns/ld-columns.html">https://www.miltenyibiotec.com/AD-en/products/macs-cell-separation/columns/ld-columns.html</a>	LINK



## Equipment

**MidiMACS™ Separator**

NAME

Magnet for magnetic separation

TYPE

Miltenyi Biotec

BRAND

130-042-302

SKU

<https://www.miltenyibiotec.com/AD-en/products/macs-cell-separation/separators/midimacstm-separator-and-starting-kits.html#130-042-302>

LINK

- 10 Prepare a new Falcon™ 15 ml polypropylene conical centrifuge tube and put it under the column to collect flow-through containing unlabeled NK cells. Apply cell suspension onto the column.
- 11 Wash the column two times with 3 ml of separation buffer. The buffer should be added only when the column reservoir is empty.
- 12 Take the 15 ml tube from under the column, mix well and determine cell number.

## Fluorescence-activated single cell sorting

- 13 Centrifuge the tube with NK cells at 200×g for 10 min at 25°C. Remove the supernatant by decanting or with automatic pipette.
- 14 Resuspend the pellet in 100 µl of separation buffer per 5×10<sup>6</sup> of total cells and transfer suspension into a new Falcon® 5mL round bottom polypropylene tube, with snap cap.



### Equipment

Falcon® 5mL round bottom polypropylene tube, with snap cap, sterile	NAME
tube	TYPE
Corning	BRAND
352063	SKU
<a href="https://www.corning.com/worldwide/en/products/life-sciences/keymatch/352063.html">https://www.corning.com/worldwide/en/products/life-sciences/keymatch/352063.html</a>	LINK

### Note

If needed, add larger volume of separation buffer and take some cells to prepare controls, e.g., unstained control or single-stain controls, in the additional 5 ml polypropylene centrifuge tubes.

- 15 Add anti-CD56 and anti-CD3 monoclonal antibodies (and any other antibodies needed) according to the manufacturer's instructions into the experimental tube and control tubes.

### Note

It is possible to expand the panel of antibodies for staining and subsequent sorting of different NK cell subpopulations.

- 16 Mix gently and incubate for 30 min on ice.
- 17 Add 1 ml of staining solution, centrifuge at 300×g for 4 min at 4°C, and decant the supernatant. Repeat one more time.
- 18 Resuspend the pellet in 300–500 µl of separation buffer per 5×10<sup>6</sup> of total cells.



- 19 Sort the cells into the 96 well plates, prepared in advance (see below "Preparation of plates for cell sorting" and "Preparation of feeder cells"), using "single cell" mode on your cell sorter.

#### Equipment

FACSVantage DiVa

NAME

cell sorter

TYPE

Beckton Dickinson

BRAND

-

SKU

- 20 Cultivate the plates with cells in a CO<sub>2</sub>-incubator for 2-3 weeks.

#### Note

It is important not to move the plates during this period. Stirring of the medium can adversely affect the growth of the clones.

- 21 After 2 weeks of cultivation in a CO<sub>2</sub>-incubator, a visual assessment of the clone growth is carried out using optical microscope.

#### Note

Cells should be attached to the bottom on the plate and located in the center forming a circular clump. If the accumulation of cells is barely noticeable, the plates should be returned to the CO<sub>2</sub>-incubator for one more week.

## Preparation of feeder cells

- 22 Cultivate the K562mbIL-21 feeder cells using RPMI-1640 medium in 25cm<sup>2</sup> cell culture flask at the concentration of  $2-6 \times 10^5$  cells/ml (to maintain cell growth in log-phase).

⊗ RPMI-1640 medium supplemented with 10% FBS



- 23 Irradiate feeder cells with  $\gamma$  radiation (100 Gy). Cells can be used immediately or frozen in 90% FCS (HyClone) containing 10% DMSO (Sigma) in a refrigerator ( $-150^{\circ}\text{C}$  –  $-135^{\circ}\text{C}$ ) or in liquid nitrogen.

#### Note


Using non-irradiated feeder cells will result in the death of NK cells due to much faster proliferation of K562 cells and subsequent lack of nutrients in the medium. Therefore, if you are not sure about the quality of feeder cell irradiation, you can cultivate  $5 \times 10^5$  K562-mbIL21 cells in cell culture flask for 3-5 days. The number of irradiated cells should not increase.

 Fetal calf serum **HyClone Catalog #SH3007103**

 DMSO **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D1435**

## Preparation of plates for cell sorting

- 24 Add 100 unit/ml of recombinant IL-2 (Sigma) and  $10^4$  of irradiated feeder cells per ml into warm AIM-V medium (Gibco).

 recombinant human IL-2 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #000000011011456001**

 AIM V™ Medium, liquid **Thermo Fisher Scientific Catalog #12055091**

- 25 Put 200  $\mu\text{l}$  of the medium into central 60 wells of a 96-well plate.

- 26 Put 200  $\mu\text{l}$  of the RPMI-1640 medium into 36 marginal wells.

#### Note

Marginal wells are prone to rapid evaporation. This leads to early death of NK cells clones. Therefore, wells with medium only should always surround the wells with cultivated cells. This approach minimizes evaporation from the central wells.

- 27 During cell sorting, the medium in the plates should be warm. For this purpose, the prepared plates can be put in a  $\text{CO}_2$ -incubator for a few hours.

