



 $m{\ell}$ Obtaining of NK cell clones using IL-2 and gene-modified K562 cells expressing membrane-

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

Maria Streltsova¹, Sofya Erokhina¹, Leonid Kanevskiy¹, Dean Lee², William Telford³, Elena Kovalenko¹

¹Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow, 117997, Russian Federation, ²Center for Childhood Cancer and Blood Disorders, The Research Institute, Nationwide Children's Hospital, Columbus, OH, USA, ³National Cancer Institute, National Institute of Health, Bethesda, 20892, USA

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Sofya Erokhina



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 IFNy. 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 $10-20\times10^6$ NK cells suitable to study phenotype, proliferative and functional activity of a certain NK cell clone.

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EXTERNAL LINK

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PROTOCOL STATUS

Working

We use this protocol in our group and it is working

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^{bright}, CD56^{dim}CD57⁻, CD56^{dim}CD57⁺, also in certain experiments these subsets were additionally sorted into HLA-DR⁺ and HLA-DR⁻. 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.

STEPS MATERIALS

NK Cell Isolation Kit, human 130-092-657 Miltenyi Biotec NK Cell Isolation Kit, human 130-092-657 Miltenyi Biotec NK Cell Isolation Kit, human 130-092-657 Miltenyi Biotec Fetal calf serum SH3007103 HyClone DMSO D1435 Sigma Aldrich RPMI-1640 medium supplemented with 10% FBS recombinant human IL-2 000000011011456001 Sigma Aldrich Thermo Fisher Scientific	NAME ~	CATALOG #	VENDOR ~
NK Cell Isolation Kit, human 130-092-657 Miltenyi Biotec Fetal calf serum SH3007103 HyClone DMSO D1435 Sigma Aldrich RPMI-1640 medium supplemented with 10% FBS recombinant human IL-2 000000011011456001 Sigma Aldrich AIM V™ Medium, liquid 12055091	NK Cell Isolation Kit, human	130-092-657	Miltenyi Biotec
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RPMI-1640 medium supplemented with 10% FBS recombinant human IL-2 O00000011011456001 Sigma Aldrich Thermo Fisher	Fetal calf serum	SH3007103	HyClone
recombinant human IL-2 000000011011456001 Sigma Aldrich AIM V TM Medium, liquid 12055091 Thermo Fisher	DMSO	D1435	Sigma Aldrich
AIM V™ Medium, liquid 12055091 Thermo Fisher	RPMI-1640 medium supplemented with 10% FBS		
AIM V™ Medium. liquid 12055091	recombinant human IL-2	00000011011456001	Sigma Aldrich
	AIM V TM Medium, liquid	12055091	

BEFORE STARTING

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 Ca²⁺/Mg²⁺-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- μ m Millipore filter and store up to 2 months at 4°C.



NK Cell Isolation Kit, human

by 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 μ l of separation buffer per 10⁷ of total cells and transfer the suspension into a new FalconTM 15 ml polypropylene conical centrifuge tube.



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

4 Add 10 μl of NK Cell Biotin-Antibody Cocktail (The NK Cell Isolation Kit, human, by Miltenyi Biotech) per 10⁷ of total cells and mix gently.

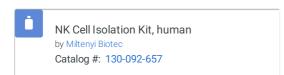


NK Cell Isolation Kit, human

by 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 Biotech) per 10⁷ of total cells.



- 7 Mix gently and incubate for 10 min at 4°C.
- 8 Adjust the volume of the separation buffer up to $500 \, \mu l$ per 10^8 of total cells.
- 9 Install MACSTM 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 MACSTM 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

Column for magnetic separation

Miltenyi Biotech 130-042-901 👄

DEQUIPMENT

 $\label{eq:midimacstar} \begin{aligned} & \text{MidiMACS}^{\text{\tiny{TM}}} \; \text{Separator} \\ & \text{Magnet for magnetic separation} \end{aligned}$

Miltenyi Biotech 130-042-302 👄

- Prepare a new FalconTM 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.

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- 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 5×10⁶ 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 tube

Corning 352063 👄

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.

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.
- Resuspend the pellet in 300-500 μ l of separation buffer per 5×10^6 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.

PEQUIPMENT

FACSVantage DiVa cell sorter

Beckton Dickinson -

 $20\,$ Cultivate the plates with cells in a CO2-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 CO₂-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 CO₂-incubator for one more week.

Preparation of feeder cells

22 Cultivate the K562mbIL-21 feeder cells using RPMI-1640 medium in 25cm² cell culture flask at the concentration of 2-6×10⁵ cells/ml (to maintain cell growth in log-phase).

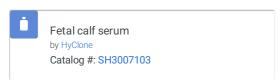


RPMI-1640 medium supplemented with 10% FBS

23 Irradiate feeder cells with γ radiation (100 Gy). Cells can be used immediately or frozen in 90% FCS (HyClone) containing 10% DMSO (Sigma) in a refrigerator (-150°C - -135°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 5×10^5 K562-mbIL21 cells in cell culture flask for 3-5 days. The number of irradiated cells should not increase.





Preparation of plates for cell sorting

24 Add 100 unit/ml of recombinant IL-2 (Sigma) and 10⁴ of irradiated feeder cells per ml into warm AIM-V medium (Gibco).





- 25~ Put 200 μl of the medium into central 60 wells of a 96-well plate.
- 26~ Put 200 μl 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.

During cell sorting, the medium in the plates should be warm. For this purpose, the prepared plates can be put in a CO₂-incubator for a few hours.

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