Suggested Protocols Enable-NK™ Duo and Enable-NK™ Duo 10X

- This document provides detailed cell culture methodology information; please refer to the "Instructions for Use" document for simple guidelines for optimal use of the product. Enable-NK Duo Onkologic Inc. Part #: 1210-xxxxx, 1260-xxxxx, 1215-xxxxx, 1265-xxxxx

- These protocols also apply to Enable-NK™ Duo 10X, once custom Enable-NK™ Grow and custom Enable-NK™ Activate have been made per Instructions For Use (IFU). Enable-NK Duo 10X Onkologic Inc. Part #: 1710-xxxxx, 1760-xxxxx, 1715-xxxxx, 1765-xxxxx

Protocol 1: NK cell expansion using IL-2 (without feeder cells)

Initial setup of NK cell cultures in Enable-NK™ Grow

1) Prepare Complete Enable-NK™ Grow medium. Supplement Enable-NK™ Grow medium with the following additives.
    a) For culturing KHYG-1 cells and other cell lines:
        i) 10% fetal bovine serum (FBS)
        ii) 100 U/mL IL-2
        iii) 1% Penicillin/Streptomycin
    b) For culturing primary NK cells:
        i) 10% AB human serum (HS)
        ii) 100 U/mL IL-2
        iii) 1% Penicillin/Streptomycin
2) Centrifuge the desired number of cells for initial culture setup, and resuspend in Complete Enable-NK™ Grow medium, at the following densities.
    a) For KHYG-1 cells and other cell lines:
        i) 0.75×10^6 cells/mL
    b) For primary NK cells:
        i) 1×10^6 cells/mL
    c) Note that these seeding densities will also be the "maintenance densities" used during continued expansion of the cultures (see Steps 6-8 below).
3) Seed plates or flasks, depending on the total size of the culture. Incubate at 37°C (5% CO_2).

Enable-NK™ Grow: Gradual reduction of serum content – THESE STEPS ARE OPTIONAL

- If serum reduction is a goal, follow steps 4-5; otherwise, skip to step 6.

4) After 1-3 days at full (initial) serum content, the serum may be reduced in a stepwise manner.
    a) Prepare serum-free Enable-NK™ Grow with the following additives:
        i) 100 U/mL IL-2

ii) 1% Penicillin/Streptomycin

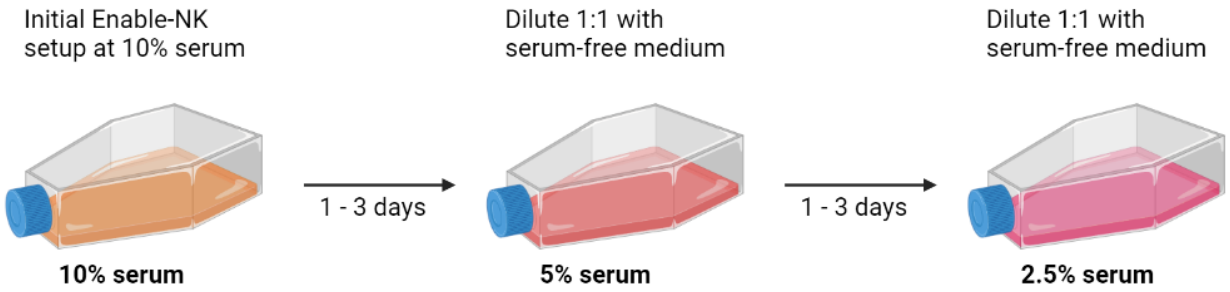
b) Add the IL-2-supplemented serum-free Enable-NK™ Grow medium such that the initial volume of the culture is doubled.

i) This dilutes the serum content, reducing it by half.

ii) Serum content will now be 5%.

5) Serum may be further reduced by half, every 1-3 days, until serum reaches the desired (reduced) level. It is <u>not</u> recommended to reduce serum content below 2% v/v.

a) See schematic below.



Enable-NK™ Grow: Expansion of NK cells

6) As the NK cell culture expands, perform cell counts every 2-3 days.

7) Each day that a count is performed (every 2-3 days), add Complete Enable-NK™ Grow medium (or serum-reduced medium if desired) such that the density is brought back down to the initial seeding density.

a) For KHYG-1 cells and other cell lines:

i) 0.75×10^6 cells/mL

b) For primary NK cells:

i) 1×10^6 cells/mL

8) Continue to maintain the cultures at these “maintenance densities” until one or more of the following criteria are met, at which point the cultures can be transitioned into Enable-NK™ Activate.

- When the target cell number / fold expansion has been achieved, or cell numbers plateau
- When cells have fully adapted to desired (lower) serum concentration
- At minimum, when cells have spent at least 3 days in Enable-NK™ Grow medium

Enable-NK™ Activate: For further expansion and activation of cytotoxicity

9) Prepare <u>Complete Enable-NK™ Activate</u> medium. Supplement Enable-NK™ Activate medium with the following additives.

a) For culturing KHYG-1 cells and other cell lines:

i) 10% fetal bovine serum (FBS) or, if using reduced serum, the desired final serum content

ii) 100 U/mL IL-2

iii) 1% Penicillin/Streptomycin

b) For culturing primary NK cells:

i) 10% AB human serum (HS) or, if using reduced serum, the desired final serum content

ii) 100 U/mL IL-2
iii) 1% Penicillin/Streptomycin

10) For transitioning to Enable-NK™ Activate, there are 2 distinct options:
    a) Option 1 – Full switch to Enable-NK™ Activate by centrifuging cells
        i) Collect all cells in centrifuge tubes, and centrifuge (recommended 300 g for 5 minutes).
        ii) Do not let cells remain pelleted for more than 30 seconds after spin is completed.
        iii) Resuspend pellets in Enable-NK™ Activate at desired density; distribute to new flasks.
    b) Option 2 – Gradual switch to Enable-NK™ Activate by stepwise addition to Enable-NK™ Grow
        i) This method avoids centrifugation of the NK cells, which we recommend.
        ii) Add Enable-NK™ Activate directly to the existing Enable-NK™ Grow culture.
        iii) After counting cells, add enough Enable-NK™ Activate to achieve desired density.
        iv) Repeat over the course of several additions, over several days.
        v) Activation effect of Enable-NK™ Activate does not require 100% Enable-NK™ Activate.
11) Culture at 37°C (5% CO_2) for anywhere between 2-5 days.
12) Monitor cell numbers every 2-3 days, and add more Enable-NK™ Activate medium (at the desired serum content, plus 100 U/mL IL-2 and 1% Penicillin/Streptomycin) as necessary as the cells proliferate.
    a) It is recommended to culture the NK cells between 2–5 days in Enable-NK™ Activate. Following these suggestions ensure the product works optimally; however, feel free to experiment with the procedures as desired.
13) The NK cells can now be used in experiments.

Protocol 2: NK cell expansion using K562 feeder cells and IL-2

Protocol 1 can be adapted to combine the use of **Enable-NK™ Duo** with the use of feeder cells for even more robust proliferation and activation. To employ a feeder cell-based protocol, we provide the following recommendations:

Recommended feeder cell line:

- K562-mb15-41BBL
    - These are K562 cells engineered to express membrane-bound IL-15 as well as 4-1BBL.
    - Cellosaurus link: https://www.cellosaurus.org/CVCL_C7IM
    - Reference: Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. Blood 2005;106:376–83.

Recommended range of NK-to-feeder ratio:

- Between 1:1 and 3:1

NOTE: Feeder cells should be irradiated prior to use in NK cell cultures.

Protocol 3: NK cell expansion with neither IL-2 nor feeder cells (following cytokine stimulation)

Note: All centrifugation steps should be performed at RCF of 320 g for 10 minutes.

Cytokine-based Pre-Expansion Stimulation[1]

1) Make Stimulation Medium. This is normal complete medium[2] supplemented with:
    a) 10 ng/mL IL-12
    b) 50 ng/mL IL-15
    c) 50 ng/mL IL-18
2) After isolating primary human NK cells (e.g. using immunomagnetic separation), centrifuge and resuspend NK cells in Stimulation Medium at a density of 2.5×10^6 cells/mL.
3) Distribute cells into a 24-well plate, 2 mL per well (i.e. 5×10^6 NK cells per well).
4) Incubate for 18 hours at 37°C with 5% CO_2.

Enable-NK™ Grow: For cell expansion

5) Make Complete Enable-NK™ Grow medium. Supplement Enable-NK™ Grow medium using:
    a) 10% human AB serum
    b) 10 ng/mL IL-15
    c) 1% Penicillin/Streptomycin
6) Centrifuge cells, and wash 3 times with Enable-NK™ Grow medium (supplemented with 10% serum, but without any added cytokines). Then resuspend NK cells in Complete Enable-NK™ Grow medium at a density of 1×10^6 cells/mL.
    a) (Cells from different wells can be pooled together if desired.)
7) Distribute cells into flasks, normal culture plates, or G-Rex plates as desired[3].
8) Incubate at 37°C with 5% CO_2.
9) Every 2 - 3 days, determine the cell density in each flask; also measure the volumes. Add Complete Enable-NK™ Grow to each flask as necessary to bring their densities back down to 1×10^6 cells/mL.

Enable-NK™ Activate: For further expansion and activation of cytotoxicity

10) Three to five days prior to desired enhanced cytotoxic activity, make Complete Enable-NK™ Activate. This is Enable-NK™ Activate supplemented with:
    a) 10% human AB serum
    b) 10 ng/mL IL-15
    c) 1% Penicillin/Streptomycin
11) Determine cell density and volume, as done previously.
12) Add Complete Enable-NK™ Activate to each flask as necessary to bring their densities back down to 1×10^6 cells/mL. This addition of Activate Medium to Grow Medium is okay; indeed, this method is preferable to using centrifugation of cells in order to completely change the medium from Grow to Activate.

13) Addition of Complete Enable-NK™ Activate can be performed 2 - 3 times over the 3 - 5 days of cytotoxicity activation.
14) Following this 3 - 5 day activation period, NK cells can be used.

Protocol 3 Notes:

1. Stimulation protocol adapted from: Romee R, et al. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. Science Translational Medicine. 2016;8(357). doi: 10.1126/scitranslmed.aaf2341.

2. Recipe for "normal complete medium" to be used in the pre-expansion stimulation step:
    a. RPMI-1640
    b. 10% human AB serum
    c. 1% L-glutamine
    d. 1% Penicillin/Streptomycin
    e. 1% HEPES
    f. 1% Non-essential amino acids

3. Different experimenters will have different preferences for what type of culture vessels to use during expansion. The following is a suggested procedure using T-flasks:
    a. Distribute cells into T-25 flasks (positioned upright with cap facing upward), 2 mL per flask (i.e. 2×10^6 NK cells per flask). As expansion proceeds, when the volume of any T-25 flask reaches 10 mL, move that flask's full volume of culture to a T-75 flask (also positioned upright with cap facing upward). Then, when the volume reaches 40 mL, move that flask's culture to a T-175 flask (also positioned upright with cap facing upward). Alternatively, the culture could be split into multiple T-75 flasks.

Protocol 4: Transduction of NK cells using retrovirus

Note: The following transduction protocol is adapted from the original protocol, which can be found in the following publication: Miah SM, Campbell KS. Expression of cDNAs in human Natural Killer cell lines by retroviral transduction. Methods Mol Biol. 2010;612:199-208. doi: 10.1007/978-1-60761-362-6_13. PMID: 20033642; PMCID: PMC2798139.

Day 1

1) **Preparation of virus vial, Step 1:** Add 10 µl PLUS Reagent to the 1 mL of supernatant in the vial, mix well using a 1000 µl micropipette, and incubate for 15 mins at RT.
2) Label two 15 mL tubes:
    a) Untransduced
    b) Transduced
3) Spin down 0.5 million cells in each of the 2 tubes (300 *g* for 5 min). Discard the supernatants.

4) **Preparation of virus vial, Step 2:** Add 4 µl Lipofectamine, mix well using a 1000 µl micropipette, and incubate for 15 mins at RT.
5) Resuspend the cell pellets in 5 mL each of serum-free Opti-MEM to wash them.
6) Spin down the two tubes at 300 *g* for 5 min. Discard the supernatants.
7) Resuspend the "transduced" pellet in the virus/Lipofectamine/PLUS mixture.
8) Resuspend the "untransduced" pellet in 1 mL serum-free Opti-MEM.
9) Move these 2 samples to two 24-well plates – one well in the center of each plate.
    a) **IMPORTANT: Choose a well in the center of the plate** – same well for each.
10) Centrifuge the plates at 700 *g* for 60 min.
11) Incubate the plates in the incubator for at least 3 hours.
12) Centrifuge the plates at 700 *g* for 60 min.
13) Incubate the plates in the incubator overnight.

Day 2

14) In the morning, centrifuge the plates once again at 700 *g* for 30 min.
15) Incubate the plates in the incubator for 3 hours.
16) Make 5 mL complete medium with 2x serum and 2x IL-2:
    a) 4 mL RPMI
    b) 1 mL serum
    c) Pen/Strep as desired
    d) Add IL-2 at 200 U/mL
17) Add 1 mL of the (2x serum and IL-2) complete RPMI to each of the 2 wells, and incubate overnight. Discard the remaining ~3 mL of the 2x medium.

Day 3

18) The following day, transfer the well contents to 2 wells of a 6-well plate. Add 3 mL of IL-2-containing complete RPMI (with the normal concentrations) to each well.
19) After 6 days, check the transduction efficiency using flow cytometry.

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