R&D systems
by bio-techne

Reagents

Feeder-Free Expansion of Peripheral Blood NK Cells From PBMCs

Using ExCellerate™ Human NK Cell Media

This protocol describes how to expand peripheral blood NK cells from PBMCs without feeder cells in standard culture vessels. This is accomplished using ExCellerate Human NK Cell Expansion Media and soluble R&D Systems cytokines. Expanded NK cells can reach >80% purity in 14 days or less when using this method.

Disclaimer: This protocol is a work in progress reflective of the most up-to-date experimental conditions used. This document is meant to be a starting point for continued optimization.

Materials Needed

Material	Catalog Number
ExCellerate Human NK Cell Expansion Media	CCM037
Human IL-2	BT-002-GMP
	BT-002-AFL
	BT-002-GMP-050/LQ
	BT-002-AFL-050/LQ
Human IL-12	10018-IL
Human IL-18	BT-018-GMP
	BT-018-AFL
	9124-IL
Human IL-21	BT-021-GMP
	BT-021-GMP-025/LQ
	BT-021-AFL
	BT-021-AFL-025/LQ
	BT-021
Human NKp46 Antibody	MAB1850
	AF1850
	MAB1850R
Optional: Human IL-15	BT-015-GMP
	BT-015-AFL
	BT-015-GMP-025/LQ
	BT-015-AFL-025/LQ
Optional: Human AB Serum	HABS001-GMP
Phosphate Buffered Saline (PBS)	Various Vendors
96 Well and 24 Well Tissue Culture Plates	Various Vendors
T25 and T75 Flasks	Various Vendors

General Guidelines

- Maintain sterile technique. Use nuclease-free reagents and sterile pipette filter tips for best results. This protocol should be performed in a BSC.
- All R&D Systems reagents should be stored according to the manufacturer's recommendations.
- Avoid multiple freeze-thaws of reconstituted cytokines.
- This protocol has been optimized for expansion of NK cells from whole PBMCs. Follow the PBMC isolation procedure that is best for your workflow.
- Donor variability may affect expansion rates of NK cells. Monitor cell health during the culture period to avoid overgrowth.

Timeline

NK Cell Activation	Scale Up	Split Flasks	Harvest
Day 0	**Days 3, 7, and 9**	**Days 10 and 11**	**Day 14**
• Thaw PBMCs OR isolate PBMCs from whole blood • Determine NK cell % in PBMC population • Incubate PBMCs with cytokine-supplemented media	• Prepare media with fresh cytokines • Reseed and restimulate NK cells • Monitor cell growth	• Prepare media with fresh cytokines • Split flasks to increase yield • Monitor cell growth	• Collect cells for desired application • Analysis

Preparation of Complete Media

Mix and sterile filter complete media:

1. 1X ExCellerate NK cell media.
2. 150 IU/mL IL-2.
3. 2 ng/mL IL-12.
4. 10 ng/mL IL-15 (optional), IL-18, and IL-21.

Protocol

Day 0: NK Cell Activation

Prepare Plates

1. Prepare a solution of 10 µg/mL Human NKp46 antibody in 1X PBS (50 µL per well of a 96 well tissue culture plate; see plate coating chart for suggested volumes for each culture vessel).
2. Add NKp46 antibody solution to each well and incubate at 37 °C for two hours.
    a. Note: Plates may also be prepared by incubating at 4 °C overnight.
3. Wash plates with sterile 1X PBS immediately prior to seeding cells.
    a. Note: Do not allow plate coating to dry out.

Prepare Media

4. Mix and sterile filter complete media.
Refer to Preparation of Complete Media Above →

Isolate/Thaw PBMCs and Stimulate with Cytokines

5. Either:
    a. Isolate PBMCs following desired protocol.
    b. Or Thaw frozen isolated PBMCs and wash with complete media.
6. Aspirate supernatant.
7. Resuspend cell pellet in complete media.
8. Count resuspended cells.
9. Perform flow cytometry to determine the percentage of NK cells present.
10. Seed PBMCs to yield a total of 50,000 NK cells per well (0.156×10^6 NK cells/cm^2) into prepared wells of a 96 Well Plate.
    a. See Seeding and Plate Coating Reference table for recommended seeding densities.
11. Add NK cell media with supplemented cytokines to each well to a final volume of 250 µL.
12. Incubate cells for 3 days at 37 °C, 5% CO_2.
    a. Note: Seeding density and timing of expansion may require further optimization in-house.

Day 3: Scale Up

Prepare Plates

1. Prepare a solution of 10 µg/mL Human NKp46 antibody in 1X PBS (250 µL per well of a 24 well tissue culture plate; see plate coating chart for suggested volumes for each culture vessel).
2. Add NKp46 antibody solution to each well and incubate at 37 °C for two hours.
    a. Note: Plates may also be prepared a day in advance and stored at 4 °C overnight.
3. Wash plates with sterile 1X PBS immediately prior to seeding cells.
    a. Note: Do not allow plate coating to dry out.

Prepare Media

4. Mix and sterile filter complete media.
Refer to Preparation of Complete Media on page 2 →

Transfer Cells

5. Mix each well of cells and transfer volume to a prepared well of a 24 well plate.
6. Wash each well with complete media and transfer residual cells to the 24 well plate.
7. Add complete media to each well for a total volume of 2 mL per well.
8. Incubate cells for 4 days at 37 °C, 5% CO_2.
    a. Note: This step may require further optimization in-house.

Day 7: Scale Up

Prepare Flasks

1. Prepare a solution of 10 µg/mL Human NKp46 antibody in 1X PBS (2 mL per T25 flask; see plate coating chart for suggested volumes for each culture vessel).
2. Add NKp46 antibody solution to each T25 flask and incubate at 37 °C for two hours.
    a. Note: Flasks may also be prepared a day in advance and stored at 4 °C overnight.
3. Wash flasks with sterile 1X PBS immediately prior to seeding cells.
    a. Note: Do not allow flask coating to dry out.

Prepare Media

4. Mix and sterile filter complete media.
Refer to Preparation of Complete Media on page 2 →

Transfer Cells

5. Mix each well of cells and transfer volume to a prepared T25 flask.
6. Wash each well with complete media and transfer residual cells to the flask.
7. Add complete media to each flask for a total volume of 8 mL per flask.
8. Incubate cells for 2 days at 37 °C, 5% CO_2.
    a. Note: This step may require further optimization in-house.

Day 9: Scale Up

Prepare Flasks

1. Prepare a solution of 10 µg/mL Human NKp46 antibody in 1X PBS (3 mL per T75 flask; see plate coating chart for suggested volumes for each culture vessel.)
2. Add NKp46 antibody solution to each T75 flask and incubate at 37 °C for two hours.
    a. Note: Flasks may also be prepared a day in advance and stored at 4 °C overnight.
3. Wash flasks with sterile 1X PBS immediately prior to seeding cells.
    a. Note: Do not allow flask coating to dry out.

Prepare Media

4. Mix and sterile filter complete media.
Refer to Preparation of Complete Media on page 2 →

Transfer Cells

5. Mix each well of cells and transfer volume to a prepared T75 flask.
6. Wash each T25 flask with complete media and transfer residual cells to the T75 flask.
7. Add complete media to each T75 flask for a total volume of 16 mL per flask.
8. Incubate cells overnight at 37 °C, 5% CO_2.
    a. Note: This step may require further optimization in-house.

Day 10: Split Flasks

Prepare Media

1. Mix and sterile filter complete media.
Refer to Preparation of Complete Media on page 2 →

Split Flasks:

2. Mix the cells in each T75 flask and transfer half of the volume into a new T75 flask.
3. Add complete media to each T75 flask to bring the total volume to 16 mL.
4. Incubate cells overnight at 37 °C, 5% CO_2.
    a. Note: This step may require further optimization in-house.

Day 11: Split Flasks

Prepare Media

1. Mix and sterile filter complete media.
Refer to Preparation of Complete Media on page 2 →

Split Flasks:

2. Mix each T75 flask and transfer the cells to four T75 flasks.
3. Add complete media to each T75 flask to bring the total volume to 16 mL.
4. Incubate cells for 3 days at 37 °C, 5% CO_2.
    a. Note: This step may require further optimization in-house.

Day 14: Harvest

Collect Cells and Analyze

1. Mix the cells and sample each flask for final cell counts and for desired flow cytometry applications.
2. Cryopreserve remaining cells or use directly in functional assays.

Seeding and Plate Coating Reference

Plate Format	cm^2	NKp46 Antibody Solution	NK Cells Plated Day 0	Media
96 Well Plate	0.32 cm^2	50 µL	50,000	250 µL
24 Well Plate	2 cm^2	250 – 300 µL	300,000	1 – 2 mL
6 Well Plate	10 cm^2	1.5 mL	1.5 × 10^6	3 mL
T25 Flask	25 cm^2	2 mL	4 × 10^6	8 mL
T75 Flask	75 cm^2	3-4 mL	12 × 10^6	16 mL

Troubleshooting Guide

Problem	Possible Cause	Solution
Low Fold Expansion and/or Low Viability	Starting material	• Rest PBMCs for one hour in cytokine-free media prior to plating. Recount total viable cells after resting (many cells die post-thaw). • Ensure starting material viability is above 90%.
	Donor variability	• Some donors respond poorly to feeder-free expansion protocols. If a different donor is not an option, the other listed solutions may increase expansion and viability.
	Culture conditions	• More frequent media exchanges may be required based on the rate of growth. • Human AB serum may be added at 2-5% to increase expansion and viability. If using serum, decrease seeding density and/or increase frequency of culture scale-ups. • Monitor growth and viability to determine optimal timing for scale-ups.
	Seeding density too low/too high	• Adjust seeding density. • Lower seeding densities on Day 0 and Day 3 may improve viability and expansion.

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