Transposition of Peripheral Blood NK Cells With the TcBuster-M™ Non-Viral Transposon System

Using the Neon™ Electroporation System

Introduction

This protocol describes how to genetically modify purified, peripheral blood NK cells with TcBuster-M mRNA transposase and DNA transposon plasmid. This is accomplished using the Neon electroporation system with the 100 µL kit. Robust NK cell expansion after genome editing is achieved by culturing NK cells in ExCellerate™ Human NK Cell Expansion Media in G-Rex® Bioreactors. TcBuster-M is a commercially available hyperactive transposase that integrates multi-cistronic CAR constructs into multiple cell types with high editing efficiency. This protocol works with the Neon and the Neon NxT Electroporation System. *Please ensure the electroporation kit matches the electroporation system.*

When working with transposons, the end user should always titrate the DNA transposon to determine optimal electroporation conditions for the construct. Continued optimization in the end user's experimental environment is recommended. If you have any technical questions about the TcBuster DNA transposon system, please contact our technical support team at techsupport@bio-techne.com for assistance.

Abbreviations:

CAR: Chimeric Antigen Receptor

GMP: Good Manufacturing Practice

DNAse I: Deoxyribonuclease I

RNAse: Ribonuclease

BSC: Biosafety Cabinet

FIGURE // 01

TcBuster-M NK Cell Transposition Procedure

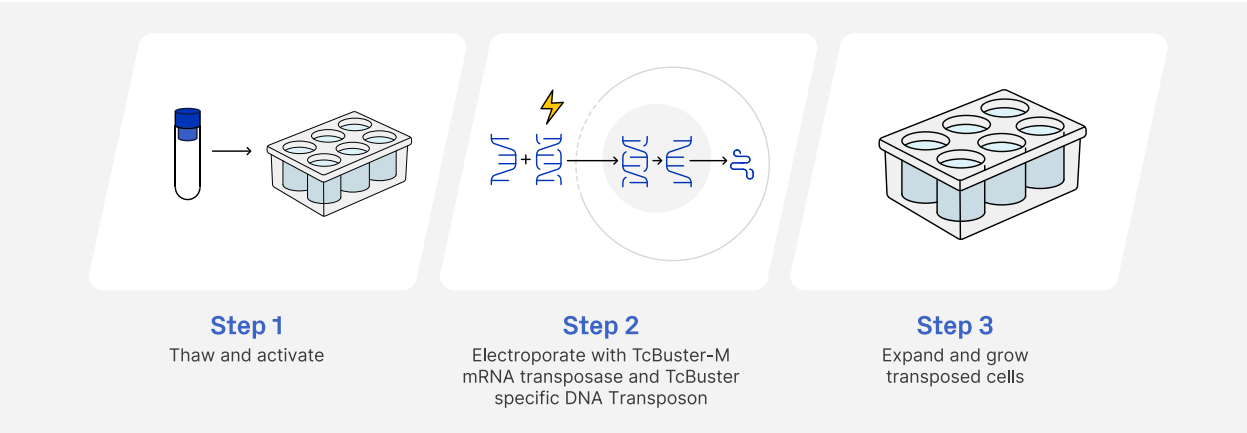


TABLE // 01

Materials Required

Material	Catalog Number
ExCellerate Human NK Cell Media	CCM037
Human IL-2 *	BT-002-GMP / BT-002-AFL
Human IL-15 *	BT-015-GMP / BT-015-AFL
TcBuster-M mRNA	TCB-001.1-100
TcBuster-M Compatible DNA Transposon (Plasmid)	TCBP001-100 TCBP002-100 Custom, if applicable
G-Rex 6 Well Plate	ScaleReady
Neon Electroporation System or Neon NxT Electroporation System	ThermoFisher
100 µL Neon Transfection Kit or 100 µL Neon NxT Kit (Kit must correspond to system)	
Feeder Cell Line	In-house
DNase I	Multiple vendors
RNase Inhibitor	
15 and 50 mL Centrifuge Tubes	
1.5 mL Eppendorf Tubes	
Nuclease-Free Water	
Cell Counter	
Cold Metal Beads	

*Note that IL-7 and IL-15 are available Animal-Free and GMP in lyophilized and liquid formulations.

General Guidelines

- Maintain sterile technique, wearing gloves, using nuclease-free reagents and sterile pipette filter tips for best results. This protocol should be performed in a BSC.
- Avoid pre-mixing the TcBuster-M mRNA and plasmid within the reaction tubes by placing each reagent on opposite sides of the tube.
- All reagents should be stored according to the manufacturer's recommendations.
- Avoid multiple freeze-thaws of the TcBuster-M mRNA and store as single-use aliquots.
- If diluting the plasmid transposon or TcBuster-M mRNA, do so in sterile nuclease-free water.
- Work quickly and carefully; avoid leaving resuspended NK cells in the Neon R buffer for longer than 15 minutes prior to electroporation.
- This protocol has been optimized for transposition of CD3-depleted, CD56-selected PBMCs. Follow the purification and activation protocols that are best for your workflow.
- It is recommended to refresh the media and to check cell growth every 3-4 days to avoid overgrowth and cell death. If more culture time is required, repeat the maintenance steps.

TABLE // 02

Suggested Controls

Control	Description	Purpose
No Electroporation	Standard grow out	Wild type cells for comparison to experimental samples. Used as control for toxicity from TcBuster-M and/or plasmid, cell death from electroporation, and viability/growth after genome modification.
Electroporation Only	No TcBuster-M or plasmid	Used as control to anticipate cell death caused by electroporation alone.
Plasmid Only	No TcBuster-M mRNA added to reaction, only plasmid + electroporation	Used as a control to anticipate background signal caused by plasmid replication within the cytosol. This signal is typically transient and is expected to disappear after one week of culture.
Positive Control	Transposon plasmid that has validated high editing efficiency	Ensures that all reactions, protocol, and equipment are properly functioning.

TABLE // 03

Timeline

NK Cell Activation	Electroporation	Maintenance	Harvest
Day -4	**Day 0**	**Day 2 and Day 6**	**Day 9**
• Thaw PBMCs and isolate NK cells OR thaw purified NK cells • Incubate NK cells with irradiated feeder cells	• Prepare media • Mix and count NK cells • Prepare reagents and reaction tubes • Transpose cells	• Monitor growth and transposition • Prepare fresh media and cytokines • Restimulate NK cells (day 2 only)	• Collect cells for desired application • Analysis • Restimulate NK cells (optional)

Protocol

NK Cell Activation

Day -4

Prepare Media

1. Mix and sterile filter complete media:
   a. 1X ExCellerate NK Cell Expansion Media
   b. 50 IU/mL IL-2

Isolate/Thaw T Cells

2. Either:
   a. Isolate $CD56^+$ and $CD3^-$ primary human NK cells from PBMCs following desired protocol, or
   b. Thaw $CD56^+$ and $CD3^-$ primary human NK cells and wash with complete media.

3. Aspirate supernatant and resuspend cell pellet in complete media.

4. Count resuspended cells.

5. Seed isolated NK cells at a density of 5 x 10^5 cells/cm^2 into wells of a G-Rex 6 Well Plate.
    a. See G-Rex Plating Reference for NK cells at the end of this protocol for recommended seeding densities.
        i. This step may require further optimization in-house.

Stimulate With Irradiated Feeder Cells

6. Stimulate NK cells with irradiated feeders following desired protocol.
    a. Seeding at a 2:1 ratio of irradiated K562 feeder cells to isolated NK cells has been optimized for this protocol. Please optimize NK to feeder cell ratio and time of activation for your needs.

7. Add NK cell media with IL-2 (50 IU/mL final concentration) to each well to a final volume of 4 mL/cm^2.
    a. Refer to G-Rex capacity in the G-Rex Plating Reference.

8. Incubate cells for 4 days at 37 °C, 5% CO_2.
    a. This step may require further optimization in-house.

Electroporation

Day 0

Prepare Media

1. Mix and sterile filter complete media:
    a. 1X ExCellerate NK cell media
    b. 50 IU/mL IL-2
    c. 10 ng/mL IL-15

Mix and Count Cells

2. Mix each well of expanded NK cells and transfer entire volume to a 50 mL conical tube.

3. Count cells and set aside while preparing other reagents.
    a. Each Neon electroporation requires 5.5 x 10^6 cells per reaction.
    b. Use this value to determine how many reactions can be performed.

Prepare Reagents and Reaction Tubes

4. Prepare recovery media with DNase I mixture:
    a. Each sample will require 1 mL of complete media with DNase I, including controls. Add DNase I to the media to a final concentration of 10 µg/mL and mix to combine.
    b. Add 1 mL of DNase I mixture to each well of G-Rex 6 well and place G-Rex 6 well in a 37 °C, 5% CO_2 incubator to equilibrate.
        i. If using a G-Rex 24 well, each sample will require 0.5 mL of complete media with DNase I.
        ii. If using alternative media, it is recommended that recovery media with DNase I should be free of any serums or supplements as these reagents have been shown to affect transposition efficiency.

5. Aliquot complete media needed after cell recovery in appropriately sized container.
    a. Each well of a G-Rex 6 well will require 10 mL of media.
        i. If electroporated cells will be placed in a G-Rex 24 well plate, 7.5 mL of media per well is required.
    b. Pre-warm complete media in a water bath until ready for use. Only pre-warm the volume of media to be used the same day.

6. Prepare Neon R resuspension buffer with RNase Inhibitor:
    a. Each electroporation reaction will require 110 µL of Neon R buffer. Use this value to determine how much buffer is needed.
    b. Aliquot volume of Neon R buffer into 1.5 mL Eppendorf tube for electroporation reactions.
    c. Add RNase Inhibitor to a final concentration of 400 U/mL and mix to combine.

7. Prepare electroporation reactions in Eppendorf tubes according to the guidelines given in Table 4:
    a. Place the plasmid and the TcBuster-M mRNA on opposite sides of the reaction tube to avoid pre-

mixing these reagents.
   b. Store on cold beads until use.

8. Prepare the Neon electroporation system within a BSC and maintain sterility throughout the procedure.
9. Insert the electroporation tube within the pipette station.
10. Add 3 mL of room temperature E2 buffer (E100 buffer if using the Neon NxT system).

TABLE // 04

Component	Concentration (Per 110 µL Reaction)
TcBuster-M mRNA	1.1 µg
Small Plasmid (3 kb-5 kb)	2.2-3.3 µg
Medium Plasmid (5.5 kb-6.5 kb)	4.4 µg
Custom Plasmid	Titrate plasmid to determine

Transpose Cells

11. Centrifuge cells at 150 x g for 10 minutes at room temperature with accelerator at 9 and brake set to 5.
12. After centrifugation, carefully aspirate supernatant.
13. Resuspend cells by quantum sufficit (Q.S.) technique in the appropriate volume of prepared Neon R buffer with RNase Inhibitor. Each reaction will require 110 µL of resuspended cells. The additional 10 µL of volume when using the 100 µL Neon kit provides a volume cushion to ensure bubbles are not introduced into the Neon electroporation pipette tip.
    a. Q.S.: Start with half of the final volume of prepared Neon R buffer with RNase Inhibitor needed and resuspend cell pellet. Determine the new volume after incorporating cell pellet and add remaining volume of total Neon R buffer mix needed.
        i. Example: 5 reactions are planned. Final volume desired is 550 µL.
            1. Resuspend cell pellet with half of final desired volume of Neon R buffer with RNase Inhibitor: 275 µL.
            2. Measure volume after incorporation of cell pellet: 325 µL.
            3. 550 µL – 325 µL = 225 µL.
            4. Add 225 µL of Neon R buffer with RNase Inhibitor to 325 µL of cells to reach 550 µL final volume.
14. Preparing one reaction at a time, mix 110 µL of cells in Neon R buffer with the prepared plasmid and TcBuster-M mRNA in a reaction tube. Mix 5-7 times without introducing bubbles.
15. Insert a Neon 100 µL tip into the Neon pipette. Ensure that the clamp of the Neon pipette fully grasps the mount stem of the 100 µL tip.
16. Depress the Neon pipette to the first stop, immerse the tip into the prepared reaction mixture, then slowly release the Neon pipette to aspirate the reaction mixture into the tip.
    a. It is very important to ensure that no bubbles are present within the Neon 100 µL tip when electroporating as this will cause arcing.
        i. It has been observed that the Neon tip may aspirate slowly. Keep the pipette tip submerged in the reaction mixture until certain the tip has been filled.

17. Insert the Neon pipette into the Neon tube on the pipette station until a click is heard.
    a. Ensure that air has not entered the 100 µL tip after placing into the E2 buffer.

18. Electroporate this reaction mixture using the Neon system at the following settings: 1600V, 8ms, 3 pulses.
    a. A program using 1850V, 10ms, 2 pulses has also been shown to successfully transpose NK cells with a medium sized plasmid. These programs are a starting point; parameters may need to be optimized.

19. Immediately after electroporation, gently dispense cells into the prepared G-Rex 6 well containing 1 mL of pre-warmed DNase I media mixture.

20. Gently rock plate to distribute cells in the media with DNase I.

21. Repeat steps 14-20 for each reaction.

22. Once all reactions have been completed, place G-Rex 6 well plate in the incubator at 37 °C for 15 minutes.

23. Following incubation with DNase I, add 10 mL of pre-warmed media with IL-2 (50 IU/mL) and IL-15 (10 ng/mL) to each well of G-Rex, for a total volume of 11 mL.
    a. If using a 24 well G-Rex, add 7.5 mL of complete media with cytokines, for a total volume of 8 mL.

24. Return G-Rex to incubator at 37 °C, 5% CO_2.

Maintenance

Day 2

1. Mix and sterile filter complete media:
    a. 1X ExCellerate Human NK Cell Expansion Media
    b. 50 IU/mL IL-2
        i. Note that IL-15 is not required for NK cell maintenance moving forward.

2. Mix the cells and sample each well for cell counts.
    a. It is optional to perform flow cytometry applications to assess transposition efficiency.

3. Re-seed NK cells, if needed, to 0.25 x 10^6 cells/cm^2.
    a. To avoid cell death caused by overgrowth, reseeding densities less than 0.5 x 10^6 cells/cm^2 are recommended. Cells can be split into additional wells for restimulation or discarded.

4. Restimulate NK cells with irradiated feeder cells following established protocol.
    a. A ratio of 1:1 irradiated K562 feeder cells to NK cells has been optimized for this protocol.

5. Add media to each well to a final volume of 4 mL/cm^2.

Day 6

1. Mix and sterile filter complete media:
    a. 1X ExCellerate Human NK Cell Expansion Media
    b. 50 IU/mL IL-2

2. Before mixing the wells, reduce the volume of each well to contain ~2 mL media per cm^2.

3. Mix the cells and sample each well for cell counts.
    a. If desired, may perform flow cytometry applications to assess transposition efficiency at this time.

4. Re-seed NK cells, if needed, to 2 x 10^6 cells/cm^2.
    a. To avoid cell death caused by overgrowth, maintain cell density below 20 x 10^6 cells/cm^2. Cells can be split into additional wells or discarded.

5. Refresh media in each well by topping up to a final media volume of 4 mL/cm^2.

Harvest

Day 9

Collect Cells and Analyze

1. Mix the cells and sample each well for final cell counts and for desired flow cytometry applications.
2. Cryopreserve remaining cells or use directly in functional assays.
3. If more culture time is required, it is recommended to reseed cell density to 0.25-0.5 x 10^6 cells/cm^2 and to refresh at least half the media in each well. Continue culture by restimulation with irradiated feeder cells following established protocol.

TABLE // 05

Troubleshooting Guide

Problem	Possible Cause	Solution
Low Transposition Efficiency	Increase plasmid concentration.	Titrate plasmid up to 2 µg higher than suggested concentration.
	Electroporation program is too low of intensity.	Try an electroporation program with higher voltage or longer pulse duration.
	Not enough cells in transposition reaction.	Try up to 10 x 10^6 cells per electroporation reaction.
	NK cell RNases degraded TcBuster-M mRNA.	Add RNase inhibitor directly to NK cell pellet prior to resuspension in Neon R Buffer.
High Copy Number	Lower plasmid concentration.	Titrate plasmid up to 2 µg lower than suggested concentration.
No Transposition	Not all reagents were added to reaction tube.	Ensure TcBuster-M mRNA and transposon plasmid are both added to reaction.
	Arcing occurred during electroporation.	Ensure there are no pockets of air within tip.
	Serum present in media.	If serum was added to NK cell media, activate NK cells in the absence of serum. Serum can be added to culture after electroporation.
Low Viability and/ or Low Fold Expansion	Too few electroporated NK cells plated in well of G-Rex.	Refer to table above for minimum cells plated. If NK cell recovery is poor after electroporation, add cells into a G-Rex 24 well plate with 0.5 mL of media containing DNase I.
		Try 10 x 10^6 cells in 100 µL per electroporation reaction.
	Cell growth exceeded maximum capacity of G-Rex.	Count cells at day 6 after electroporation and split if necessary.
		Decrease day 2 reseed density.
	Arcing occurred during electroporation.	Ensure there are no pockets of air within tip.
	Electroporation program may be too harsh.	Try electroporation program with lower voltage or shorter pulse durations.

TABLE // 06

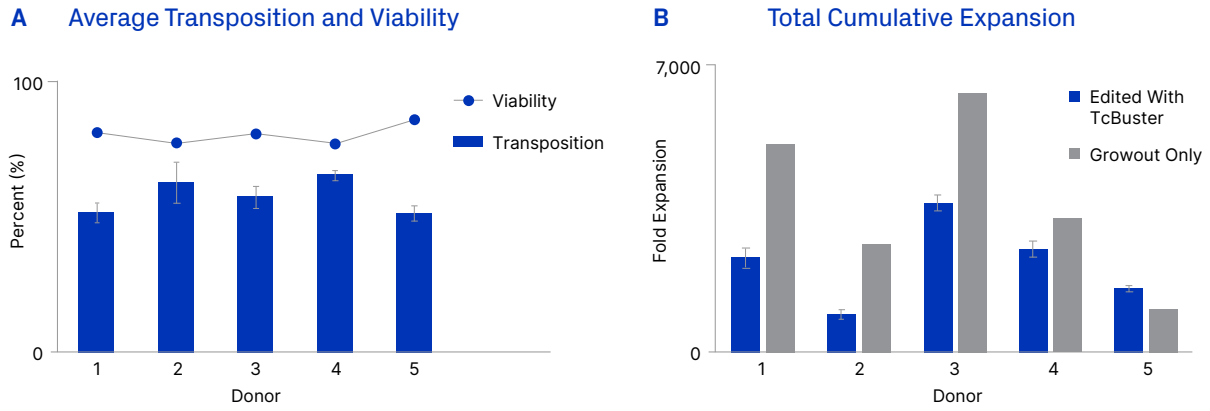
G-Rex Plating Reference for NK Cells

G-Rex Format	cm^2	Cells Plated Day -4	Cells Plated Post-EP*	Capacity	Confluency
G-Rex 24 Well Plate	2 cm^2	1-3 x 10^6	2-5 x 10^6	8 mL	40 x 10^6
G-Rex 6 Well Plate	10 cm^2	5-15 x 10^6	5-25 x 10^6	40 mL	150-200 x 10^6
G-Rex 6M Well Plate	10 cm^2	5-15 x 10^6	5-25 x 10^6	100 mL	150-200 x 10^6
G-Rex 100M	100 cm^2	50-150 x 10^6	100 x 10^6	1,000 mL	1.5-2 x 10^9
G-Rex 500M	500 cm^2	250-750 x 10^6	500 x 10^6	5,000 mL	7.5-10 x 10^9

*EP designates electroporation

FIGURE // 02

Representative Data



TcBuster effectively edits NK cells with minimal impacts to viability and cell growth. Purified peripheral blood NK cells from five donors were grown in ExCellerate Human NK Cell Expansion Media (Catalog # CCM037) and gene edited with TcBuster-M Transposase mRNA (Catalog # TCB-001.1-100) and a 5.8 kB TcBuster transposon plasmid on the Neon electroporation platform. On day 9 post-electroporation, NK cells were assessed for transposition, cell viability, and cell counts. A) TcBuster-mediated gene editing achieved > 50% transposition efficiency (bar) across 5 NK cell donors and maintained cell viability (line). B) Cumulative fold expansion from day -4 to day 9 post-electroporation demonstrates TcBuster gene editing minimally impacts cell growth. NK cell purity (CD56+ CD3-) was > 95%. Each donor has three electroporation replicates and one unedited (growout) control. Error bars represent ± SD.