

Transposition of Primary Human T Cells With the TcBuster™ Non-Viral Transposon System

Using the Lonza 4D-Nucleofector® Electroporation System

Introduction

This protocol describes how to genetically modify purified CD4⁺ and CD8⁺ primary human T cells using the TcBuster transposon system. This can be accomplished using the Lonza 4D-Nucleofector X unit with 100 µL Nucleocuvettes™. TcBuster-M is a commercially available hyperactive transposase that efficiently integrates multi-cistronic CAR constructs into many different cell types while consistently providing robust integration and high editing efficiency.

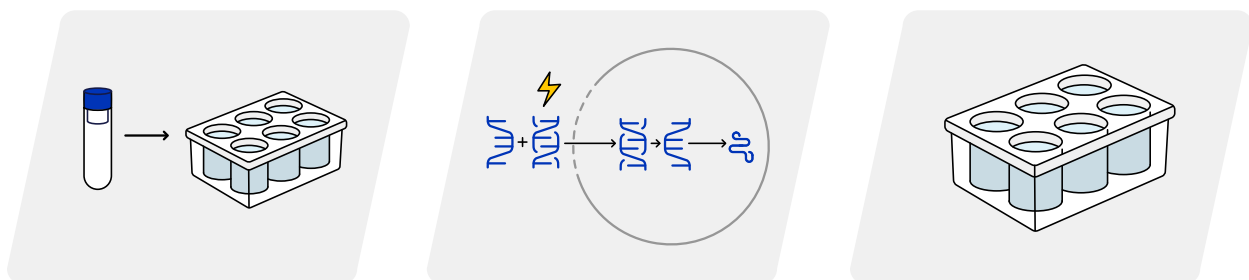
Note: When working with transposons, the end user should always titrate the transposon to determine optimal electroporation conditions for the construct. Continued optimization in the end user's experimental environment is recommended. If you have any technical questions about the TcBuster DNA transposon system or would like to design a custom transposon, 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 T Cell Transposition Procedure



Step 1

Thaw and activate

Step 2

Electroporate with TcBuster-M mRNA transposase and TcBuster specific DNA Transposon

Step 3

Expand and grow transposed cells

TABLE // 01

Materials Required

Material	Catalog Number
GMP T Cell Media	CCM038-GMP
Human IL-7*	BT-007-GMP / BT-007-AFL
Human IL-15*	BT-015-GMP / BT-015-AFL
TcBuster-M Compatible DNA Transposon (Plasmid)	TCBP001-100 TCBP002-100 Custom, if applicable
TcBuster-M mRNA	TCB-001.1-100
G-Rex® 6 Well Plate M-Series	Wilson Wolf
P3 Primary Cell 4D-Nucleofector X Kit L	Lonza
4D-Nucleofector Core Unit and X Unit	
CD3/CD28 T Cell Activator	<ul style="list-style-type: none"> • Miltenyi (T Cell TransAct™) • StemCell Technologies (ImmunoCult™ Human CD3/CD28 T Cell Activator) • ThermoFisher (Dynabead™ Human T-Activator CD3/CD28)
Human AB Serum	Multiple vendors
DNase I	
RNAse Inhibitor	
15 and 50 mL Centrifuge Tubes	
1.5 mL Eppendorf Tubes	
Nuclease-Free Water	
Cell Counter	

*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.
- 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 plasmid or TcBuster-M mRNA, do so in sterile nuclease-free water. The total volume of nucleic acids added should not exceed 10% of the total reaction volume.
- Work quickly and carefully; avoid leaving T cells in the Lonza P3 buffer for extended periods of time as this leads to reduced transposition efficiency and viability.
- If using the recommended Bio-Techne media and G-Rex bioreactor, cells will be confluent after 9 days of culture. If using a different media or reaction vessel, follow manufacturer's protocol to determine optimal culture time.

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 + Electroporation Only	No TcBuster-M mRNA added to reaction, only plasmid + electroporation	Used as control to anticipate background signal caused by episomal expression of the plasmid. 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

Activation	Transposition	Expansion
Day 0 <ul style="list-style-type: none"> • Prepare media • Isolate/thaw CD4⁺ and CD8⁺ T cells • Stimulate with CD3/CD28 activator • Incubate (2 days) 	Day 2 <ul style="list-style-type: none"> • Mix and count cells • Prepare reagents and reaction tubes • Transpose cells • Incubate (7 days) 	Day 6 (Optional) <ul style="list-style-type: none"> • Check transposition efficiency and cell growth progress Day 9 <ul style="list-style-type: none"> • Harvest cells for desired application • Analysis

Protocol

Activation

Day 0 of Total Culture Time

Prepare Media

- Mix and sterile filter complete media. Provided below is a recipe using Bio-Techne's GMP Human T Cell Media:
 - GMP Human T Cell Media
 - 5% Human AB Serum
 - 10 ng/mL IL-7
 - 10 ng/mL IL-15

Isolate/Thaw T Cells

- Either:
 - Isolate CD4⁺ and CD8⁺ primary human T cells following desired protocol or
 - Thaw CD4⁺ and CD8⁺ purified primary human T cells and wash with complete media.
- Aspirate supernatant and resuspend cell pellet in complete media.

4. Count resuspended cells.
5. Dilute to $2-3 \times 10^6$ cells per mL and plate 1 mL per cm^2 in G-Rex.
 - a. Note: Additional details on G-Rex formats, volumes, and cell plating can be found at the end of this protocol in the "G-Rex Plating Reference" table.

Stimulate With CD3/CD28 Activator

6. Stimulate T cells with CD3/CD28 activation reagent according to manufacturer's protocol.
7. Thoroughly mix suspended cells.
8. Incubate cells for 2 days at 37°C , 5% CO_2 .

Transposition

Day 2 of Total Culture Time

Mix and Count Cells

1. Gently mix the activated cell complexes to break them apart, approximately 10-15 times, and transfer entire volume to a 50 mL conical tube.
2. If using an activation reagent that requires removal, do so following manufacturer's instructions.
3. Count resuspended cells and set aside while preparing other reagents.

Prepare Reagents and Reaction Tubes

4. Prepare and pre-warm 100 mL of complete media per electroporation reaction, including controls.
5. Prepare a DNase I mixture:
 - a. Warm 1.5 mL of basal media per reaction including controls.
 - i. Note: This media should be free of any serums or supplements as these reagents have been shown to affect transposition efficiency.
 - b. Add $10\ \mu\text{g/mL}$ of DNase I to the media and mix to combine.
 - c. Add 1 mL of DNase I mixture to each well of G-Rex 6M, one well per reaction including controls.
 - d. Transfer plate into a 37°C , 5% CO_2 incubator to acclimate the media.
 - e. Keep remaining DNase I mixture in conical for use in step 16. Keep conical warm in an incubator.
6. Prepare an RNase Inhibitor + P3 buffer mixture:
 - a. Prepare P3 buffer according to manufacturer instructions.
 - b. Each reaction will require $100\ \mu\text{L}$ of RNase Inhibitor + P3 mixture. Use this value to determine how much mixture is needed total.
 - c. Mix $400\ \text{U/mL}$ of RNase Inhibitor with P3 buffer.
 - d. Allow to come to room temperature.
 - i. Note: If arcing occurs, we have observed a benefit in keeping the RNase Inhibitor + P3 buffer mixture cool prior to electroporation.
7. Prepare reactions in 1.5 mL tubes according to the guidelines in the TcBuster-M mRNA Transposase and DNA Transposon Concentration Guide for your specific plasmid size.
 - a. Try not to pre-mix mRNA and DNA in 1.5 mL tube at this step as DNA could contain RNases. Place DNA and RNA on separate sides on the bottom of the tube.
 - b. Note: These concentrations are a starting point; parameters may need to be optimized for your specific desires.

8. Set out desired number of Nucleocuvettes and sterile transfer pipettes from P3 Primary Cell 4D-Nucleofector X Kit L, one each per electroporation reaction.

TABLE // 04

TcBuster-M mRNA Transposase and DNA Transposon Concentration Guide

Component	Lonza (Per 110 μ L Reaction)
TcBuster-M mRNA	1 μ g
Plasmid (4 - 9 kb)	3 μ g
Custom Plasmid	Titrate plasmid to determine

Transpose Cells

9. Centrifuge cells at 150 x g for 10 minutes with accelerator at 9 and brake at 7.
 - a. Note: Slower spin speeds and lower brake have been optimized to maintain cell shape and maximize electroporation efficiency.
10. Aspirate supernatant and resuspend cells in the appropriate volume of prepared RNase Inhibitor + P3 buffer mixture at a concentration of 100×10^6 cells/mL. Be sure to account for cell pellet volume during resuspension. Follow the example given below for proper resuspension technique.
 - a. Example: I have 5 reactions planned.
 - i. Final volume desired: 500 μ L
 - ii. Resuspend cell pellet with half that volume of P3 buffer mixture: 250 μ L
 - iii. New volume measured after cell resuspension: 305 μ L
 - iv. Add P3 buffer mixture to reach 500 μ L final volume: 195 μ L
11. Before continuing, read steps 12 through 17 entirely to ensure movements are completed quickly and efficiently to maximize transposition efficiency and cell viability.
12. Move prepared G-Rex and conical with DNase I mixture from the incubator to the BSC.
13. Only preparing one reaction at a time, mix 100 μ L of cells in P3 buffer with the prepared plasmid and TcBuster-M mRNA in the 1.5 mL tube. Mix 3-5 times without introducing bubbles.
14. Transfer the entire reaction mixture to an electroporation cuvette.
 - a. Note: It is very important to ensure that the reaction mixture is sitting entirely on the bottom of the cuvette. Tapping or flicking the cuvette gently may help to adjust volume to evenly sit on the cuvette bottom.
15. Electroporate this reaction mixture using the 4D Nucleofector system at the following setting:

DN-107.

 - a. Note: This program is a starting point; parameters may need to be optimized depending on plasmid construct.
16. While the cells are in the Nucleofector, draw up a small amount of DNase I mixture from the conical with the sterile transfer pipette included in the kit.
17. Keeping media in the transfer pipette, draw up electroporated cells from cuvette so that there is a layer of media sitting on top of the cells.

18. Gently dispense the entire volume into the DNase I mixture in the prepared G-Rex 6M without getting any cells on the vessel walls.
19. Repeat this process for each reaction.
20. Once all reactions have been completed, gently rock G-Rex to evenly distribute cells.
21. Place G-Rex in the incubator at 37 °C for 15 minutes.
22. Following incubation, fill G-Rex 6M to final volume of 10 mL/cm² with pre-warmed complete media.
23. Place G-Rex into incubator at 37 °C, 5% CO₂.

Expansion

Optional: Day 6 of Total Culture Time

Check transposition efficiency and cell growth

1. At this point, cells and media do not need handling and can be left untouched until day 9 of total culture time. However, if checking transposition efficiency is desired, it is recommended to do this after 6 days of total culture time and not to handle electroporated cells prior to this.
 - a. Note: If using a vessel smaller than a G-Rex 6M, such as the 24 well (Catalog # 80192M) or the 6 well (Catalog # 80240M) G-Rex, at least half of a media exchange must be performed on day 6.
2. Mix cells thoroughly and sample each well for cell counts and for desired flow cytometry applications.
 - a. Anti-CD19 CAR expression can be detected using Recombinant Human CD19 Fc Chimera Atto 647 protein (Catalog # [ATM9269](#)).

Day 9 of Total Culture Time

Harvest Cells

3. Volume reduce the G-Rex 6M to ~2-4 mL/cm² (about 1/3 of the volume capacity).
4. Mix the cells and sample each well for final cell counts and for desired flow cytometry applications.
5. Cryopreserve remaining cells or use directly in functional assays.

TABLE // 05

G-Rex Plating Reference

G-Rex Format	cm ²	Cell # for Activation	Activation Volume	Cells Plated Post-EP*	Volume Post-EP*	Confluency
G-Rex 24 Well Plate	2 cm ²	5 x 10 ⁶	2 mL	2 x 10 ⁶	8 mL	60-80 x 10 ⁶
G-Rex 6 Well Plate	10 cm ²	25 x 10 ⁶	10 mL	10 x 10 ⁶	40 mL	350-400 x 10 ⁶
G-Rex 6M Well Plate	10 cm ²	25 x 10 ⁶	10 mL	10 x 10 ⁶	100 mL	350-400 x 10 ⁶
G-Rex 100M	100 cm ²	250 x 10 ⁶	100 mL	100 x 10 ⁶	1,000 mL	3.5-4 x 10 ⁹
G-Rex 500M	500 cm ²	1250 x 10 ⁶	500 mL	500 x 10 ⁶	5,000 mL	15-20 x 10 ⁹

*EP designates electroporation

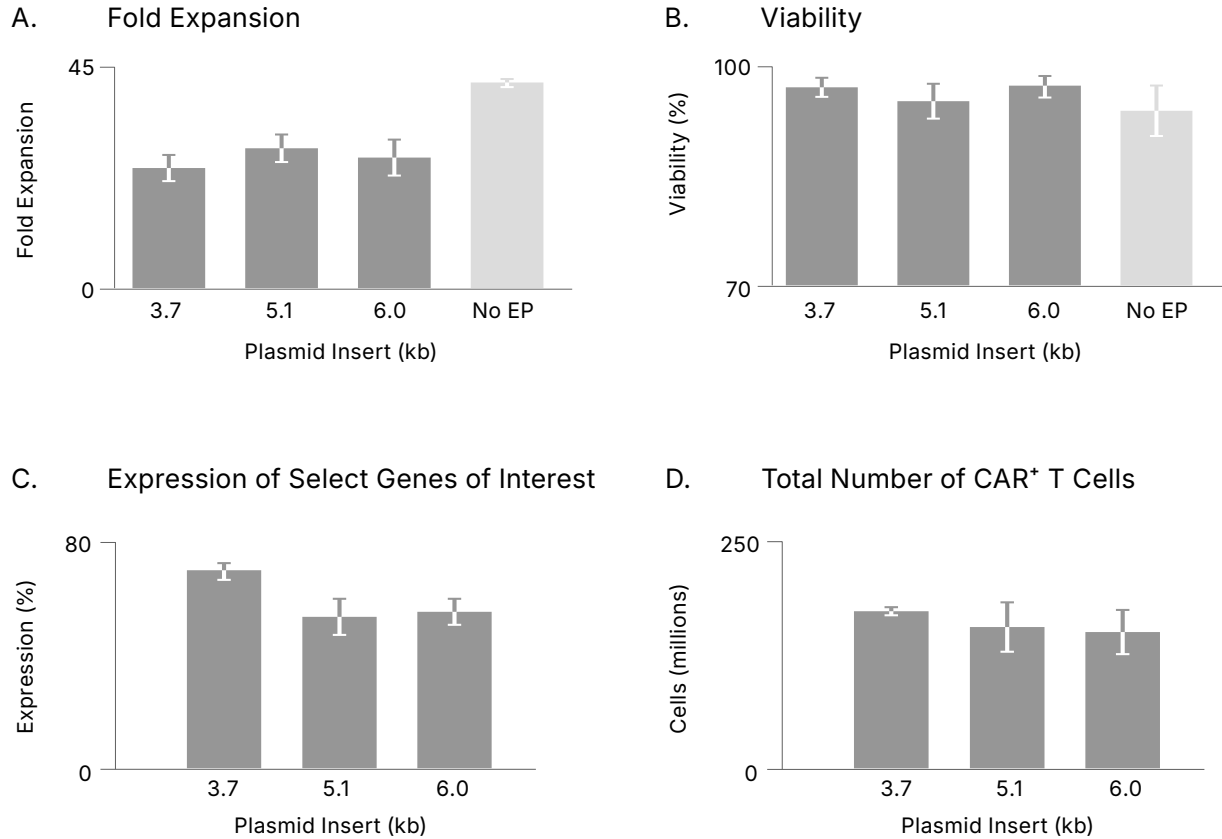
TABLE // 06

Troubleshooting

Problem	Possible Cause	Solution
Low Transposition Efficiency	Increase plasmid concentration	Titrate plasmid up to 2 µg higher than suggested concentration above
	Increase program intensity	Use program EO-115 or EO-142.
	Not enough cells in transposition reaction	Ensure 10 x 10 ⁶ ± 1 x 10 ⁶ cells are used per reaction
High Copy Number	Lower plasmid concentration	Titrate plasmid up to 2 µg lower than suggested concentration above
	Electroporation program is too strong	Use program FI-110 or EO-115
No Transposition	Not all reagents were added to reaction tube	Ensure TcBuster-M mRNA and plasmid are both added to reaction
	Arcing occurred during electroporation	Ensure there are no pockets of air within cuvette
	Degraded TcBuster-M mRNA	Ensure proper handling of TcBuster-M mRNA, and work in a RNase-free environment
Low Viability and/or Low Fold Expansion	Not enough cells plated in G-Rex well	Refer to table above for minimum cells plated
	Cell growth exceeded maximum capacity of G-Rex	Count cells at day 6 of total culture time and split if necessary
	Arcing occurred during electroporation	Ensure there are no pockets of air within cuvette

Representative Data

FIGURE // 02



T cells modified with different sized cargos on the Lonza electroporation system achieve robust cell growth in the G-Rex bioreactor and remain highly viable 7 days after genome modification. Primary human T cells from 3 donors were expanded for 7 days after genetic modification in GMP Human T Cell Media (Catalog # [CCM038-GMP](#)) supplemented with 5% hAB serum and 10 ng/mL each of IL-7 (Catalog # [BT-007-GMP](#)) and IL-15 (Catalog # [BT-015-GMP](#)) in 6 well G-Rex plates. The plasmid insert size is reported as the cargos integrated in the genome (ITR to ITR). TcBuster modified T cells achieve > 24-fold expansion (A) and remain highly viable within the G-Rex after genome modification and electroporation (B). TcBuster achieved > 53% transposition efficiency regardless of plasmid insert size (C) which resulted in > 100 million modified T cells across all tested conditions 7 days after genome modification (D). Data points represent the average of 3 donors with technical duplicates \pm SD.

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