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

## Using the Neon™ 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 Neon electroporation system with the 100 µL kit. 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.

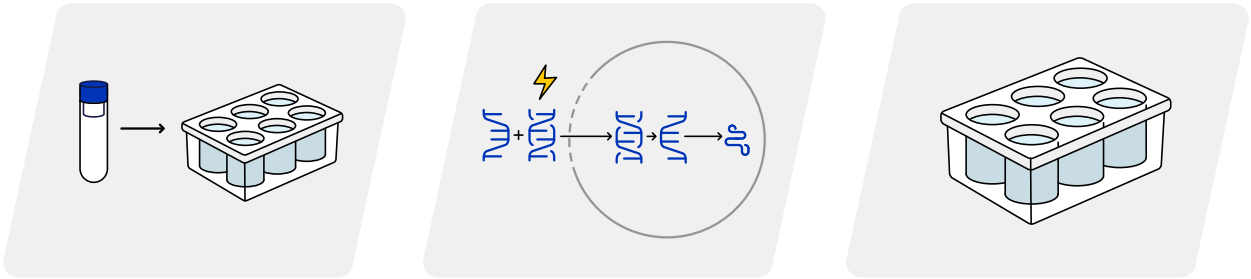
Note: When working with transposons, the end user should always titrate the transposon to determine optimal electroporation conditions for the construct. Continued optimization in the end user's experimental environment is recommended. If you have any technical questions about the TcBuster DNA transposon 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<br>TCBP002-100<br>Custom, if applicable |
| **TcBuster-M mRNA** | TCB-001.1-100 |
| **G-Rex® 6 Well Plate M-Series** | Wilson Wolf |
| **CD3/CD28 T Cell Activator** | • Miltenyi (T Cell TransAct™)<br>• StemCell Technologies (ImmunoCult™ Human CD3/CD28 T Cell Activator)<br>• ThermoFisher (Dynabead™ Human T-Activator CD3/CD28) |
| **100 µL Neon Transfection Kit** | ThermoFisher |
| **Neon Electroporation System** | |
| **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 Neon R 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**<br>• Prepare media<br>• Isolate/thaw $CD4^+$ and $CD8^+$ T cells<br>• Stimulate with CD3/CD28 activator<br>• Incubate (2 days) | **Day 2**<br>• Mix and count cells<br>• Prepare reagents and reaction tubes<br>• Transpose cells<br>• Incubate (7 days) | **Day 6 (Optional)**<br>• Check transposition efficiency and cell growth progress<br>**Day 9**<br>• Harvest cells for desired application<br>• Analysis |

## Protocol

### Activation

**Day 0 of Total Culture Time**

#### Prepare Media

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

#### Isolate/Thaw T Cells

2. Either:
   a. Isolate $CD4^+$ and $CD8^+$ primary human T cells following desired protocol or
   b. Thaw $CD4^+$ and $CD8^+$ purified primary human T cells and wash with complete media.

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

4. Count resuspended cells.

5. Dilute to 2-3 x $10^6$ cells per mL and plate 1 mL per $cm^2$ in G-rex 6M.
    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 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 µg/mL of DNAse I to the media and vortex.
    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.

6. Prepare an RNAse Inhibitor + R buffer mixture:
    a. Each reaction will require 110 µL of RNAse Inhibitor + R buffer mixture. Use this value to determine how much mixture is needed total.
    b. Mix 400 U/mL of RNAse Inhibitor with R buffer.
    c. Allow to come to room temperature.

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. Prepare the Neon electroporation system in a way that maintains sterility throughout the procedure (preferably within a BSC).
    a. Insert the electroporation tube within the pipette station until a click is heard. Add 3 mL of room temperature E2 (Neon) or E100 (Neon NxT) buffer.

**TABLE // 04**

TcBuster-M mRNA Transposase and DNA Transposon Concentration Guide

| Component | Neon (Per 110 µL Reaction) |
|---|---|
| **TcBuster-M mRNA** | 1.1 µg |
| **Small to Medium Plasmid (4 – 6.5 kb)** | 1.1 µg |
| **Large Plasmid (6.5 - 9 kb)** | 3.3 µg |
| **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 RNAse inhibitor + R buffer mixture at a concentration of 100 x $10^6$ 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: 550 µL
        ii. Resuspend cell pellet with half that volume of buffer mixture: 275 µL
        iii. New volume after resuspension: 325 µL
        iv. Add R buffer mixture to reach 550 µL final volume: 225 µL

11. Move prepared G-Rex 6M with DNAse I mixture added to BSC.

12. Only preparing one reaction at a time, mix 110 µL of cells in R buffer mixture with the prepared plasmid and TcBuster-M mRNA in the 1.5 mL reaction tube. Mix 3-5 times without introducing bubbles.

13. 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.

14. Depress the Neon pipette to the first stop, immerse the tip into the prepared reaction mixture, slowly release the Neon pipette to aspirate the reaction mixture into the tip.
    a. Note: It is very important to ensure that no bubbles are present within the Neon 100 µL tip when electroporating as this will cause arcing. We thus recommend including an extra 10 µL in each reaction to minimize bubbles in the tip.
    b. Note: It has been observed that the Neon tip may aspirate slowly. It is recommended to keep the pipette tip submerged in the reaction mixture until certain the tip has been filled.

15. Insert the Neon pipette into the Neon tube on the pipette station until a click is heard.
    a. Note: Ensure that air has not entered the 100 µL tip after placing into the E2 (Neon) or E100 (Neon NxT) buffer.

16. Electroporate this reaction mixture using the Neon system with the programs found in Table 05.
    a. Note: These programs are a starting point; parameters may need to be optimized depending on plasmid construct.

TABLE // 05

| Component | Programs |
|---|---|
| **Small/Medium Plasmid (4 – 6.5 kb)** | 1600 V, 12 ms, 3 pulses |
| **Large Plasmid(6.5 – 9 kb)** | 1600 V, 8 ms, 3 pulses |
| **Custom Plasmid** | Optimize programs |

17. Immediately after electroporation, gently dispense cells into the prepared G-Rex 6M containing 1 mL of pre-warmed DNAse I media mixture.
    a. Note: Do not mix cells once plated.
    b. Note: Do not place cells against the vessel walls. Aim to dispense directly into media.
18. Repeat this process for each reaction.
19. Once all reactions have been completed, gently rock the G-Rex to evenly distribute cells.
20. Place G-Rex 6M in the incubator at 37 °C for 15 minutes.
21. Following incubation, fill G-Rex 6M to final volume of 10 mL/cm$^2$ with pre-warmed complete media.
22. Place the G-Rex into incubator at 37 °C, 5% $CO_2$.

## 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

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

TABLE // 06

## 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 // 07

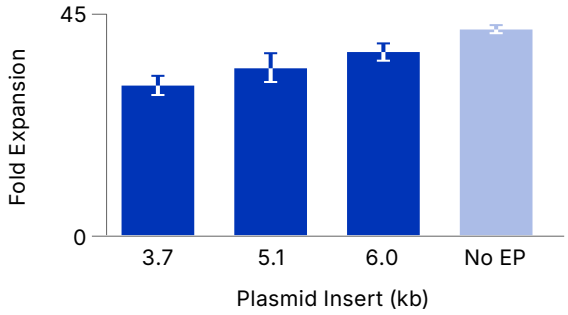
## Troubleshooting

| Problem | Possible Cause | Solution |
|---|---|---|
| **Low Transposition Efficiency** | Increase plasmid concentration | Titrate plasmid up to 2 µg higher than suggested concentration above |
| | Increase program intensity | Increase voltage by 50 and/or duration by 2 |
| | 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 | Decrease voltage by 50 and/or duration by 2 |
| **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 |

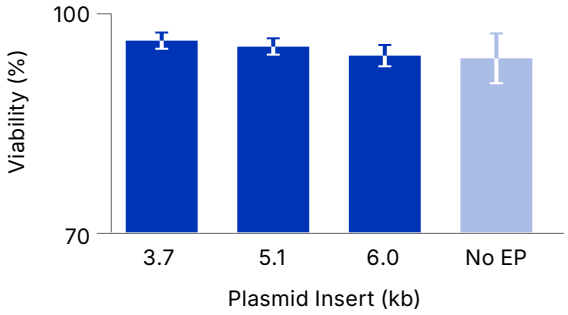
## Representative Data

**FIGURE // 02**

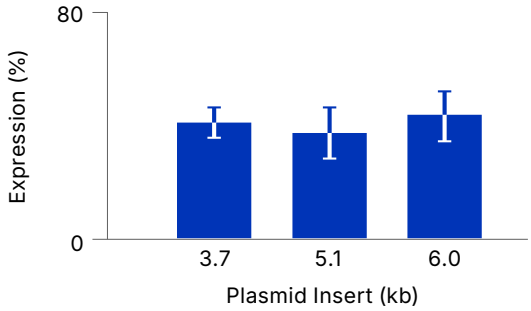
A. Fold Expansion



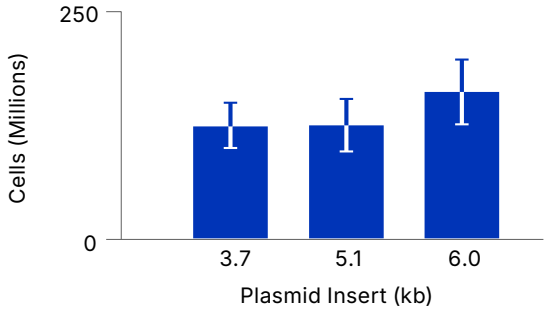

B. Viability




C. Expression of Select Genes of Interest




D. Total Number of CAR⁺ T Cells




**T cells modified with different sized cargos on the Neon, 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 > 30-fold expansion, (A) and remain highly viable within the G-Rex after genome modification and electroporation (B). TcBuster achieved > 40% 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 ± SD.