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Application Note

# Lentiviral vs. Non-Viral Delivery for CAR-T Manufacturing

## De-risking Cell Therapy Manufacturing with the TcBuster™ Non-Viral Transposon System

Chemistry, manufacturing, and controls (CMC) are a core consideration when developing and manufacturing a genetically modified cell therapy product. Specialized reagents are required, including cell culture media, activation and selection reagents, cytokines, cell expansion platforms, and gene transfer reagents.

### Challenges of Using Lentiviral Vectors for Gene Transfer

Lentiviral vectors are an established gene transfer method for cell therapies. However, there are significant drawbacks to using viral vectors, such as batch-to-batch variability, long manufacturing lead times, and high costs. Manufacturing considerations, safety, and multiplexed editing are driving developers to explore next-generation, non-viral gene transfer methods for manufacturing cell therapies.

### Non-Viral Gene Editing with TcBuster

TcBuster is a non-viral gene editing system for stable gene transfer in any cell type and is based on DNA transposition. The TcBuster reagents, comprised of the mRNA transposase and DNA transposon, are electroporated into the cell. Once inside the cell, the translated TcBuster transposase binds to specific sites known as inverted terminal repeats (ITRs) which flank the genes of interest (GOIs) on the DNA transposon. TcBuster then excises and inserts the DNA cargo into the host cell genome at AT sites, where it is stably expressed (**FIGURE 1**).

This application note describes a comparison between TcBuster and lentivirus in a CAR-T manufacturing workflow, detailing gene transfer efficiency, cell viability and fold expansion, memory phenotype, and functionality. Additionally, the work discusses the genetic characterization and T cell receptor diversity after genetic modification.

### Key Takeaways

- The TcBuster system offers an efficient non-viral solution for integrating GOIs, such as CARs.
- TcBuster offers a superior insertional profile and T cell phenotype compared to lentiviral vectors.
- TcBuster is comparable to lentiviral vectors in other key performance areas, including CAR expression, fold expansion, and functionality, while providing a faster time to clinic and lower cost of goods sold (COGS).

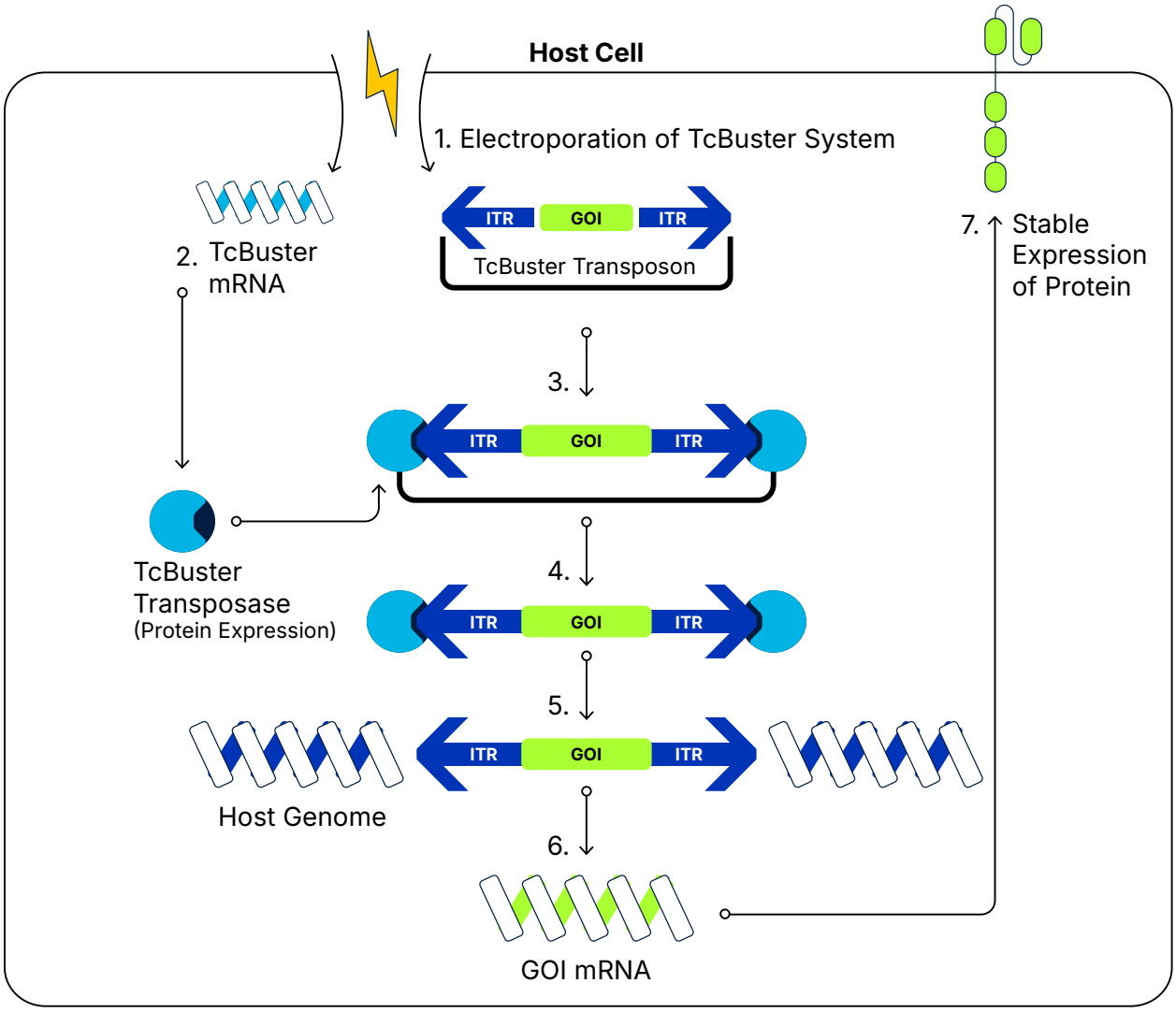



**FIGURE 1.** OVERVIEW OF THE TCBUSTER TRANSPOSITION MECHANISM

1. TcBuster system components introduced into cells via electroporation.
2. TcBuster-M mRNA translated into transposase enzyme.
3. TcBuster-M transposase binds to ITRs on DNA transposon.
4. TcBuster-M transposase excises GOIs from transposon.
5. TcBuster-M inserts GOI into the host genome.
6. The GOI mRNA transcribed from the host genome.
7. The translated protein is now stably expressed in cells.

# Materials and Methods

| Material | Supplier | Catalog Number |
|---|---|---|
| GMP Human T Cell Media | Bio-Techne | CCM038-GMP |
| Recombinant Human IL-7 GMP Protein | Bio-Techne | BT-007-GMP |
| Recombinant Human IL-15 GMP Protein | Bio-Techne | BT-015-GMP |
| Human AB Serum | BioIVT | HUMANABSRMP-HI-1 |
| G-Rex® 6 Well Plate | ScaleReady | 80660 |
| T Cell TransAct™ | Miltenyi | 130-111-160 |
| TcBuster-M Compatible DNA Plasmid | Bio-Techne | TCBP001 for 4.1kb or Custom |
| TcBuster-M 001.1 mRNA | Bio-Techne | TCB-001.1 |
| Hyclone™ Electroporation Buffer | Cytiva or MaxCyte | EPB-1 |
| OC-100x2™ Processing Assembly | MaxCyte | SOC-1x2 |
| MaxCyte® Electroporation System | MaxCyte | GTx |
| Simple Plex™ Cell Activation Panel 2 | Bio-Techne | ST01C-CS-007366 |
| Simple Plex Controls | Bio-Techne | 894977, 899077, 898137, 897018 |
| Ella™ Automated Immunoassay System | Bio-Techne | 600-100 |
| Incucyte® SX5 | Sartorius | 4816 |

**TABLE 1.** MATERIALS AND REAGENTS USED IN THIS STUDY

## Experimental Workflow

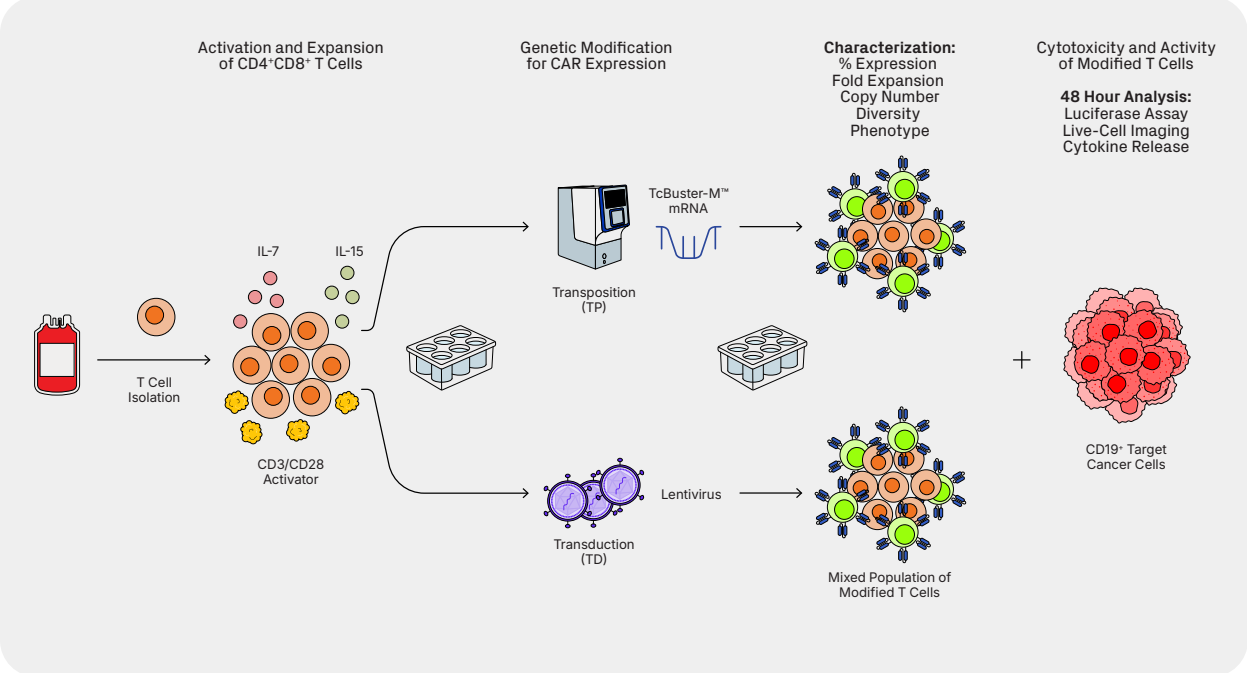



**FIGURE 2A.** A TYPICAL WORKFLOW FOR GENETIC ENGINEERING OF IMMUNE CELLS

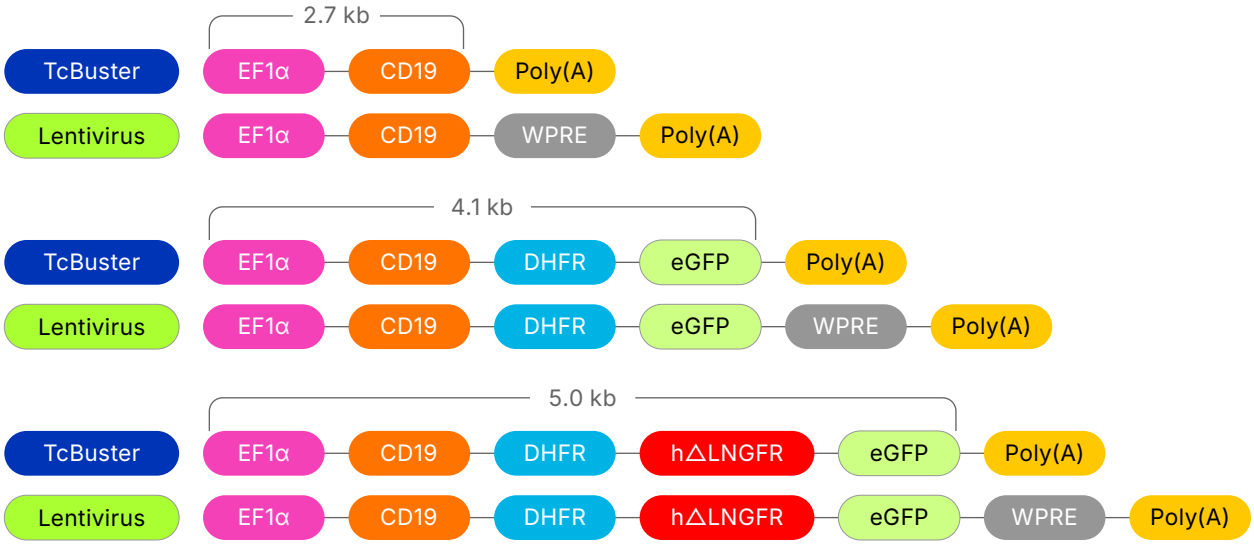



**FIGURE 2B.** A DEPICTION OF THE THREE PLASMIDS USED IN EXPERIMENTATION

The GOI denoted in the following figures is reported as the size of the relevant genes integrated into the genome, which is equal between the TcBuster transposon and the lentivirus.

A typical workflow for genetic engineering in primary human T cells is depicted in **(FIGURE 2A)**. In this study, T cells from 3 healthy donors were isolated by CD4/CD8 magnetic selection and activated for 2 days, in the presence of CD3 and CD28 activation reagents and 10 ng/mL each of IL-7 and IL-15 in human T cell media. Using the MaxCyte® electroporation system, TcBuster was used to insert a range of different plasmid sizes (2.7 kb, 4.1 kb, and 5 kb), all containing an anti-CD19 CAR, into activated T cells **(FIGURE 2B)**. Lentivirus payloads of equivalent size were used for transduction. Flow cytometry was used to evaluate the efficiency of cargo insertion and the resulting T cell phenotypes. Both transposed and transduced cells were activated and expanded in a G-Rex® 6 well plate post-genetic modification. Cell counts were used to track fold expansion and viability, and gDNA of each population was collected to determine the copy number via digital droplet PCR (ddPCR). T cell receptor repertoire diversity analysis was performed by iRepertoire. To evaluate cell activity, transposed and transduced anti-CD19 CAR-T cells from 3 donors were separately co-cultured with $CD19^+$ or $CD19^-$ target cells, after which supernatants were collected for cytokine quantification using Ella. Furthermore, each modification method was evaluated by luciferase assay and live cell imaging, which was completed on the Incucyte® SX5.

# Results

## Lentiviral Transduction vs. TcBuster Transposition Efficiency and Cell Health

**FIGURE 3**

### Comparing TcBuster and lentivirus impact on cell health and modification efficiency of primary human T cells

T cells from 3 donors were expanded for 9 days in GMP Human T Cell Media, supplemented with 5% hAB serum and 10 ng/mL each of IL-7 and IL-15, in a 6 well G-Rex. The GOI is reported as the promoter and genes integrated in the genome and is depicted in Figure 2B. Figure 3 shows the efficiency of genetic modification for both TcBuster and lentivirus in T cells **(3A)**. After nine days of culture, fold expansion **(3B)** and total number of CAR+ cells **(3C)** are illustrated, as is cell viability after genome modification **(3D)**. Data points represent the average of 3 donors ± SD.

Primary human T cells from 3 donors were genetically modified by either TcBuster or lentivirus. Three GOIs, referred to as small (2.7 kb), medium (4.1 kb), and large (5 kb) **(FIGURE 2B)**, were evaluated for both methods. Editing efficiency when delivering the small and medium sized GOIs was comparable between the two methods. TcBuster maintained the same high integration efficiency when delivering the large GOI, while lentivirus transduction rates were reduced at the 5 kb insert size **(FIGURE 3A)**.

TcBuster and lentivirus achieved >27-fold expansion and produced a similar total number of CAR⁺ cells after 9 days of culture in a 6 well G-Rex for all three GOIs **(FIGURE 3B, C)**. Viability remained high for all conditions after genetic modification by TcBuster or lentivirus **(FIGURE 3D)**. Overall, TcBuster was comparable to or outperformed lentivirus at efficiently integrating cargos in T cells, while sustaining a similar fold expansion and viability.

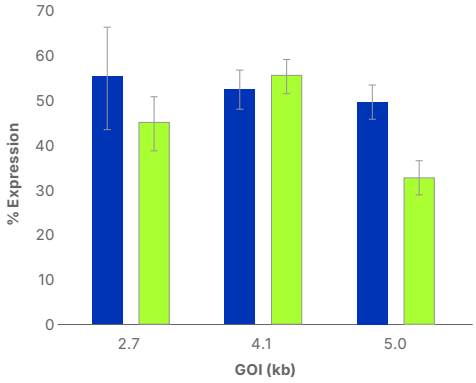



**FIGURE 3A.** EXPRESSION OF SELECT GENES OF INTEREST

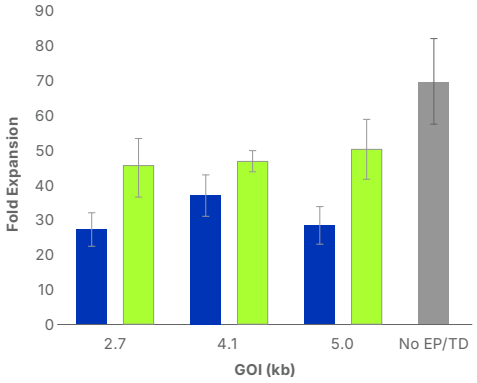



**FIGURE 3B.** FOLD EXPANSION

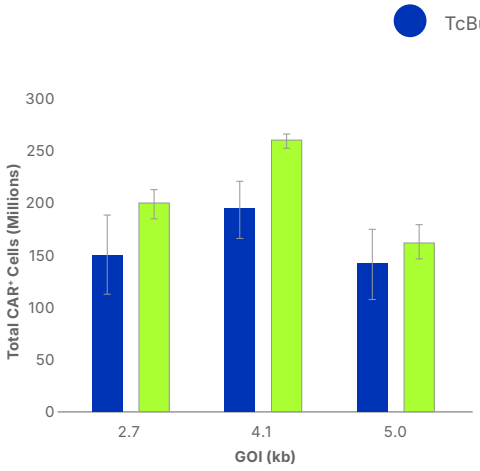



**FIGURE 3C.** TOTAL NUMBER OF CAR⁺ T CELLS

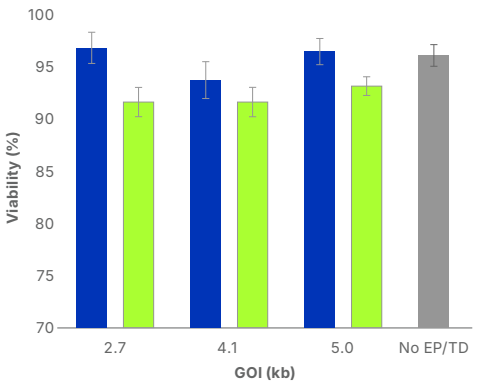



**FIGURE 3D.** VIABILITY

## Genetic Analysis

**FIGURE 4**

### Genetic characterization and T cell receptor repertoire analysis of modified cells

**(4A)** T cells from 3 donors were collected after 9 days of culture for digital PCR analysis of the population's average vector copy number (VCN). **(4B)** Integration site analysis (ISA) was performed on 3 different T cell donors that were modified with a different range of plasmid sizes. DNA libraries were Illumina sequenced by GeneWerk (now ProtaGene). **(4C)** A representative donor was selected to display immune repertoire changes before and after genetic modification. Each rectangle represents a unique CDR3 sequence where the size of that rectangle represents the abundance of that CDR3 sequence. **(4D)** The diversity index (Di) is a proprietary qualitative value used to describe and compare the relative diversity of T cell receptors within a population. The higher the Di value, the more relative diversity amongst the TCR chains within that submitted T cell population. Data points represent the average of 3 donors ± SD.

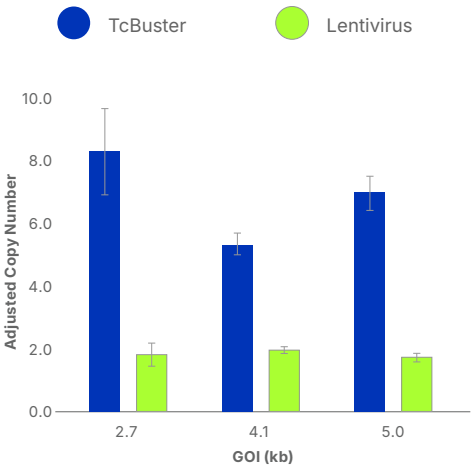



**FIGURE 4A.** AVERAGE ADJUSTED COPY NUMBER OF INSERTED GENES IN T CELL POPULATION

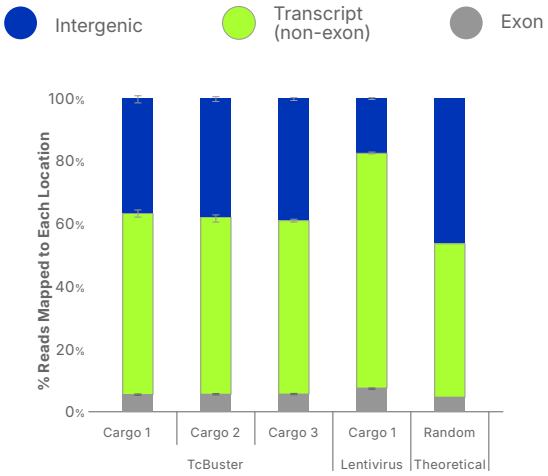



**FIGURE 4B.** INTEGRATION SITE ANALYSIS OF INSERTED GENES AFTER GENETIC MODIFICATION

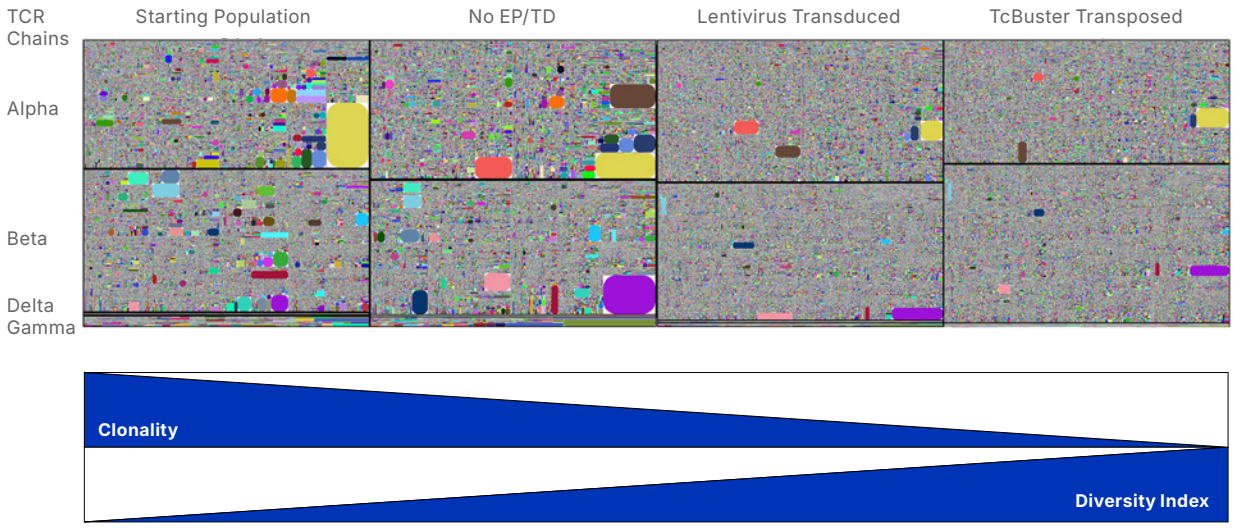



**FIGURE 4C.** VISUALIZATION OF T CELL REPERTOIRE CHANGES BEFORE AND AFTER GENETIC MODIFICATION

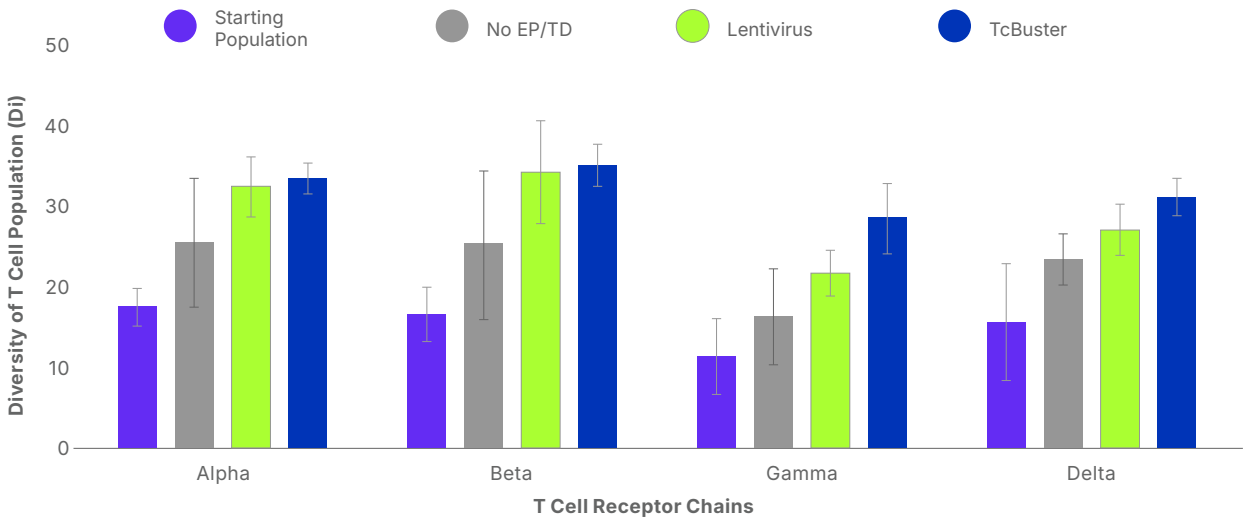



**FIGURE 4D.** RELATIVE DIVERSITY OF T CELL POPULATIONS BEFORE AND AFTER GENETIC MODIFICATION

For all GOIs, VCN averaged 8 or lower for TcBuster-transposed T cell populations. In comparison, lentivirus-transduced T cell populations averaged 2 or lower **(FIGURE 4A)**. While more copies were inserted using transposition, a superior insertional profile during genetic modification was observed. Using ISA, T cell populations were assessed for cargo integration by genomic location. Using multiple donors and cargos, TcBuster-transposed T cells showed an insertion profile nearly consistent with that of a theoretically random profile, favoring intergenic regions. Conversely, lentiviral vectors have been shown to preferentially insert within transcriptional units[1]. These findings are consistent with the ISA performed here, where lentivirus-transduced T cells exhibit an increased preference for transcript regions **(FIGURE 4B)**. Concerns of insertional mutagenesis generally depend on two key factors: the VCN in the population and the location of the integrant. As TcBuster-mediated gene transfer displays a nearly unbiased insertional profile, this system can allow for a wider range of VCN than lentivirus while minimizing the risk of insertional mutagenesis.

T cell repertoire diversity was measured before and after genetic modification **(FIGURE 4C, TOP)**. Starting populations displayed a larger degree of clonality due to T cell reactivity (i.e. infection) from the donor. After genetic modification, both TcBuster and lentivirus T cell populations showed decreased clonality. Clonality and diversity are intrinsically related and have an inverse relationship such that when clonality increases in each T cell population, the inherent diversity in TCR chains (α, β, γ, δ) decreases **(FIGURE 4C, BOTTOM)**. TCR chain Di score was calculated for both TcBuster and lentivirus, with starting population and no EP/TD controls **(FIGURE 4D)**. Genetically modified populations had increased Di scores, and thus low clonality, compared to starting populations. However, no EP/TD controls had lower Di scores. Therefore, it is possible that the process of genetic modification (electroporation and transposition or transduction) leads to the death of major clonal populations, consisting mainly of effector or effector memory T cells. Without these processes, these mature effector populations can persist longer in culture and are observed in the No EP/TD control.

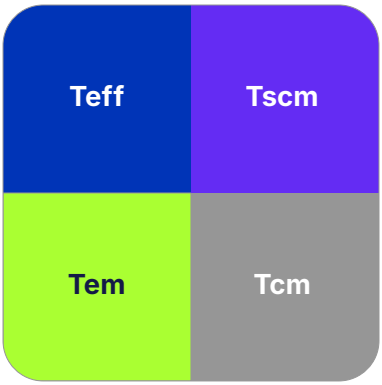
Overall, T cells edited with TcBuster show an increased VCN in these electroporation conditions. However, the superior and unbiased insertional profile plausibly allows for this increase in VCN without increased risk of insertional mutagenesis, which is reflected in the comparable Di scores for both methods of gene transfer.

## T Cell Phenotype Analysis

**FIGURE 5**

T cell phenotype following 9 days of culture

**(5A)** A representative flow cytometry gating strategy for determining phenotype based on CD45RA and CCR7 expression. The phenotypes have been previously described[3] as stem cell memory (Tscm, CD45RA$^+$/CCR7$^+$), central memory (Tcm, CD45RA$^-$/CCR7$^+$), effector memory (Tem, CD45RO$^-$/CCR7$^-$), and effector (Teff, CD45RA$^+$/CCR7$^-$). T cells from 3 donors were expanded for 9 days and phenotype was determined as described. **(5B)** CD4$^+$ and **(5C)** CD8$^+$ populations were broken out by T cell phenotype. Data points represent the average of 3 donors ± SD.



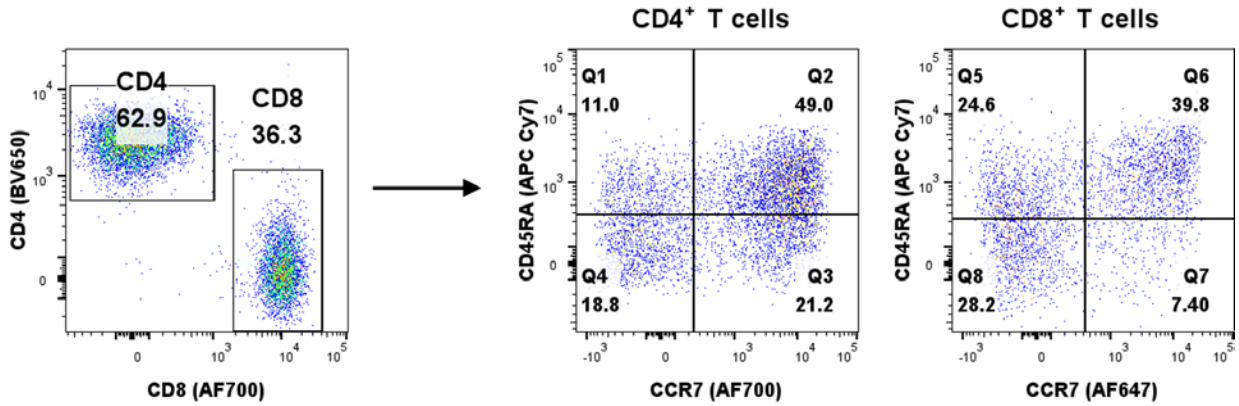




**FIGURE 5A.** FLOW CYTOMETRY GATING STRATEGY FOR DETERMINING T CELL PHENOTYPE

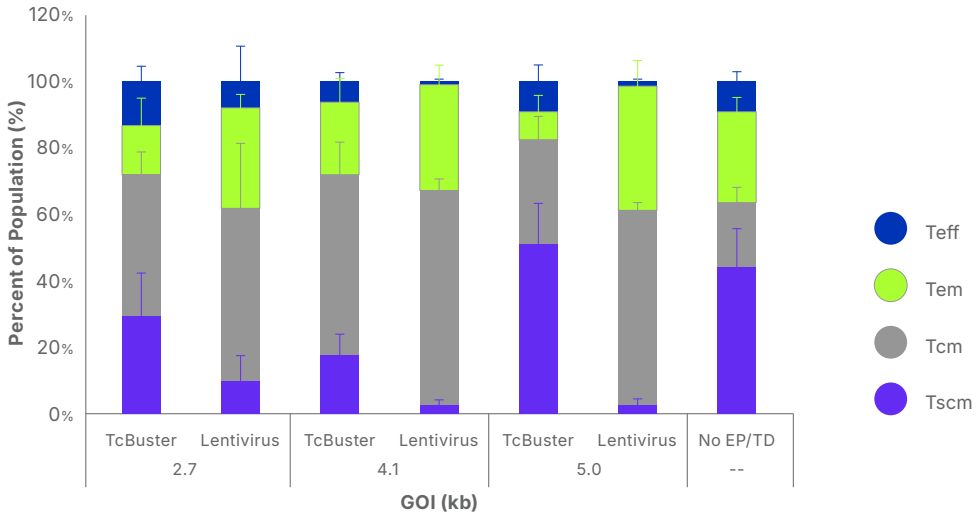



**FIGURE 5B.** CD4$^+$ T CELL PHENOTYPE

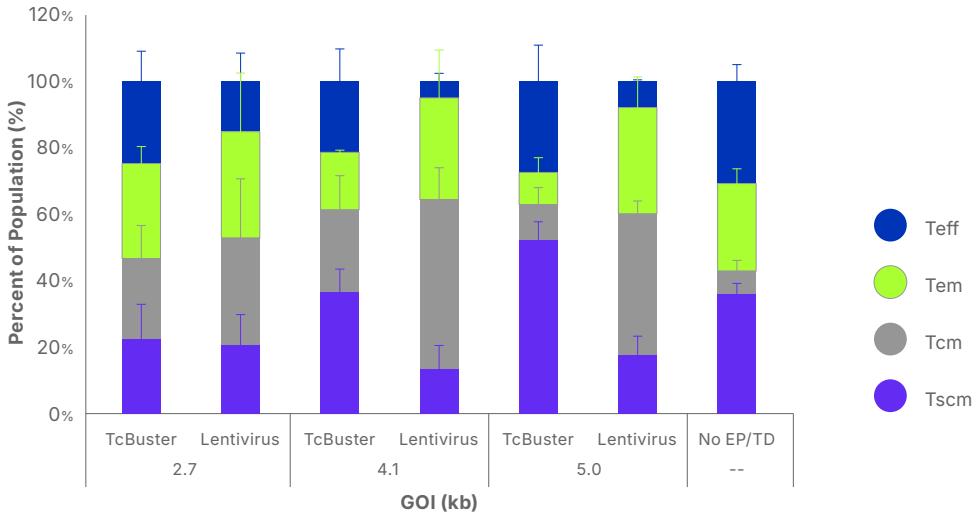



**FIGURE 5C.** CD8⁺ T CELL PHENOTYPE

A variety of factors influence the functionality of T cell-based therapies and are therefore critical for determining their effectiveness in the clinic. One of these factors is T cell phenotype, as T cell populations consisting of a more naïve phenotype have displayed an enhanced antitumor immunity and persistence *in vivo*[3]. This study employed a typical T cell phenotype panel; the gating strategy for which is described in Figure 5A, to evaluate the presence of effector and memory T cells.

For all conditions, TcBuster-modified T cell populations yielded a higher percentage of naïve Tscm cells on average compared to lentivirus. This effect is especially pronounced when delivering the medium (4.1 kb) or large (5 kb) cargo sizes. These increases correspond to respective fold change in Tscm cells of 7.0 and 20.4 for the CD4⁺ subset **(FIGURE 5B)**, and 1.8 and 2.0 in the CD8⁺ subset **(FIGURE 5C)**.

While TcBuster-transposed cells were composed of a larger percentage of Tscm cells, lentivirus had a higher percentage of Tcm cells. When compared to no EP/TD controls at these cargo sizes, similar levels of Tscm cells were seen in the TcBuster edited populations, while lentivirus transduced cells were consistently lower. This observation suggests lentivirus transduction negatively impacts T cell phenotype, promoting Tcm and Tem cells over Tscm cells. Overall, T cell editing with TcBuster maintains a greater percentage of naïve phenotypes compared to lentivirus.

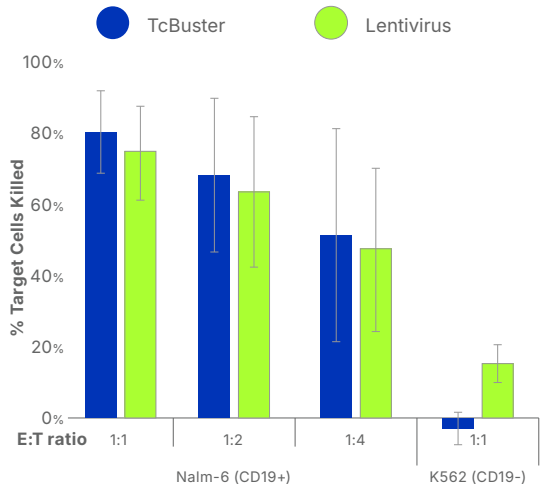



**FIGURE 6A.** ANTI-CD19 CAR MODIFIED T CELLS CO-CULTURED WITH EITHER CD19⁺ OR CD19⁻ TARGET CELLS FOR 24 HOURS

## Functional Assays

**FIGURE 6**

### Comparing the functionality of TcBuster- and lentivirus-modified T cells

T cells from 3 donors modified with the 4.1 kb plasmid were cryopreserved in CS10 following 7 days of culture after modification. These genetically modified T cells were thawed and immediately added to either CD19⁺ or CD19⁻ target cells at the specified E:T ratios. **(6A)** Luciferase activity of remaining target cells was measured after 24 hours of co-culture. **(6B)** Target cell viability was monitored over a 48-hour period using an IncuCyte® SX5 at a set E:T ratio (1:1). Differences in target cell counts after 48 hours between TcBuster- and lentivirus-modified T cells in each donor were <1.3 fold. **(6C)** Supernatants were collected after co-culture and analyzed by ELISA using the Ella platform for secretion of IFNγ, TNFα, Granzyme B, and Perforin. Data points represent the average of 3 donors each containing technical triplicates ± SD.

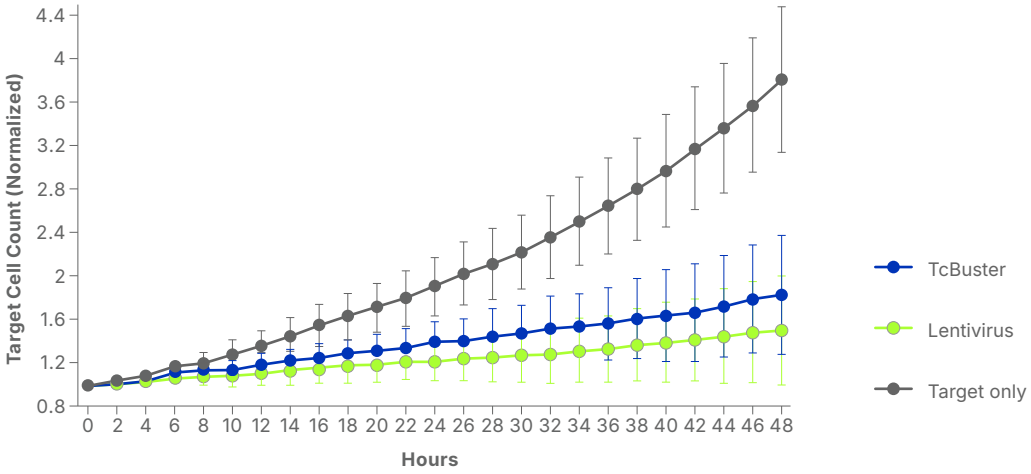



**FIGURE 6B.** ANTI-CD19 CAR MODIFIED T CELLS CO-CULTURED WITH CD19⁺ TARGET CELLS FOR 48 HOURS AT 1:1 E:T RATIO

Potency is a critical quality attribute for all CAR-T cell therapies and directly relates to the functional bioactivity of the drug product[4]. Manufacturing decisions for CAR-T cells (e.g. gene transfer method) have the potential to impact the potency, and therefore its clinical performance.

Cytotoxicity and cytokine release assays were performed to understand if differences in gene transfer method affected potency. Cells modified with the 4.1 kb cargo were chosen for functional analyses. Anti-CD19 CAR-modified T cells were co-cultured with CD19⁺ Nalm-6 or CD19⁻ K562s. All three effector-to-target cell ratios (E:T) revealed TcBuster- and lentivirus-edited cells were similarly efficacious at CD19⁺ cell killing **(FIGURE 6A)**. Both TcBuster and lentivirus time course killing at the 1:1 E:T ratio demonstrated efficient killing compared to controls and <1.3-fold difference in target cell counts by 48 hours **(FIGURE 6B)**. Using the Ella platform, anti-CD19 CAR-T cell secretion of inflammatory cytokines (IFNγ and TNFα), as well as markers of degranulation (Granzyme B and Perforin) were measured **(FIGURE 6C)**. For all three E:T ratios, similar levels of enzyme secretion were detected for both TcBuster and lentivirus. Thus, TcBuster- and lentivirus-modified T cells show comparable levels of functionality, as measured by target cell killing and cytokine release.

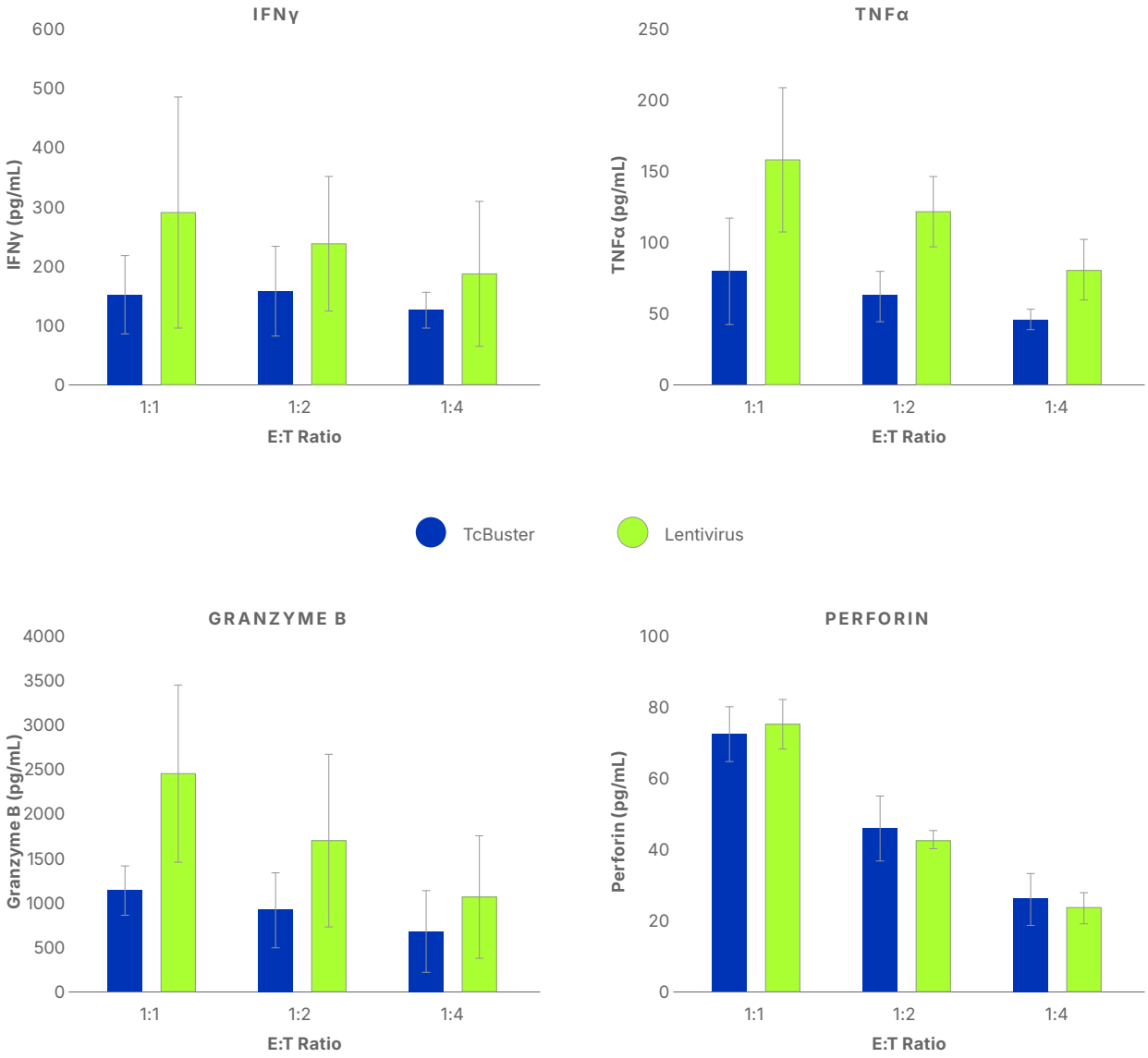



**FIGURE 6C. ANTI-CD19 CAR-T CELL SECRETION OF INFLAMMATORY CYTOKINES AND DEGRANULATION MARKERS**

This study utilized the Ella automated immunoassay platform to assess cytokine secretion in TcBuster-engineered CAR-T cells, as a measure of functionality.

**Discover How Ella Can Characterize CAR-T Cell Signaling and Activation**

Scan the QR Code or Visit:
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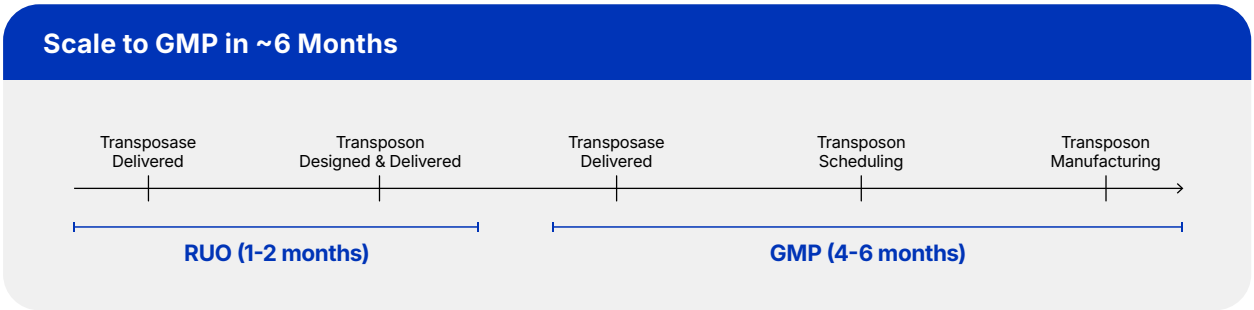



**FIGURE 7.** TIMELINE FOR THE TCBUSTER SYSTEM

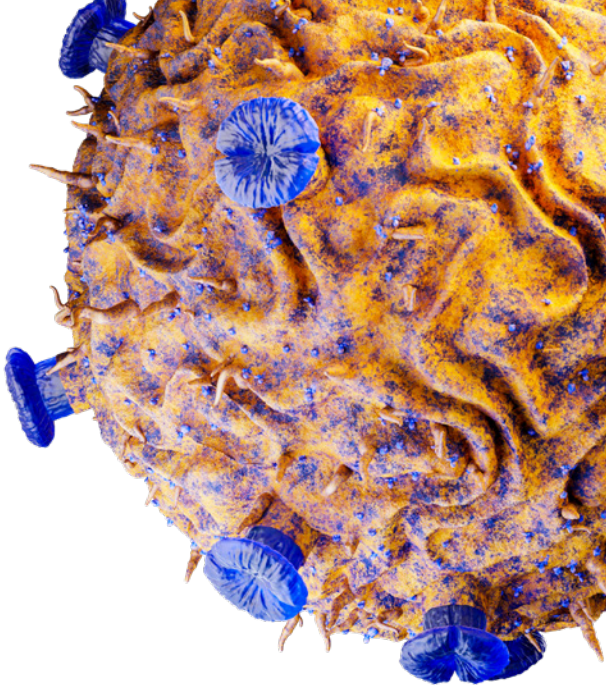
Prospective custom manufacturing and delivery timelines for research grade and GMP transposase and transposon materials.

| Transposon Batch Size | Patients Treated at 30 µg/mL EP | Patients Treated at 60 µg/mL EP | Patients Treated at 100 µg/mL EP |
|---|---|---|---|
| 100 mg | 666 | 333 | 200 |
| 250 mg | 1666 | 833 | 500 |
| 500 mg | 3333 | 1666 | 1000 |
| 1 g | 6666 | 333 | 2000 |
| | Recommended minimum dose for small cargos | | Recommended max dose for large cargos |

**TABLE 2.** DOSING SCHEMES FOR THE TCBUSTER SYSTEM

GMP transposon batch size with three different dosing regimens. Batch assumes 2E8 cells/mL and 1E9 total cells processed (5 × 1 mL electroporation reactions). TcBuster-M transposase vialed appropriately for 1E9 total cells electroporated.

Materials manufacturing lead times are a crucial logistical consideration when developing a cell therapy product. For initial evaluation of the system, Bio-Techne provides research grade transposase mRNA, as well as transposon controls that is available in as little as 1-2 weeks. Through a streamlined process, custom transposons, with your genes of interest, may be ordered within 1-2 months. Clinical grade TcBuster reagents are backed by a strong and scalable nucleic acid supply chain, and proper planning can ensure GMP-grade TcBuster reagents will be ready for the clinic in less than 6 months **(FIGURE 7)**. Bio-Techne stocks GMP transposase mRNA, and GMP transposon can be ordered while undergoing research or IND-enabling studies from a variety of nucleic acid manufacturers, for maximum flexibility to meet your specific needs. Prospective batches from these manufacturers can be produced in quantities as little as 100 mg to as large as 1 g **(TABLE 2)**. Depending on cargo size, a single batch can potentially treat thousands of patients.



## Conclusion

Gene transfer is a critical step in the CMC process of CAR-T drug products and therefore has significant logistical and technical ramifications for potential therapy. Lentiviral vectors have been the historical choice for gene transfer during CAR-T manufacturing. However, accessible and timely manufacturing of consistent, high-quality lentivirus is not possible for many labs and companies. Access to newer technologies, such as non-viral-based platforms like the TcBuster DNA transposon system, provide cost-effective solutions that also yield technical improvements. This application note discussed how the TcBuster system is built with nucleic acid-based reagents for high consistency batches that can potentially treat thousands of patients in a short amount of time. In a small-scale format, this study compared TcBuster and lentivirus head-to-head. TcBuster transposition yielded T cells with a superior insertional profile, more naïve-like phenotype, and the ability to insert large DNA cargo without a loss in efficiency. At the same time, TcBuster was comparable to lentivirus in measurements of cell health and functional performance.

**Learn More About TcBuster**

Scan the QR Code or Visit:
**bio-techne.com/tcbuster**

## References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