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Cell therapy

Gene modification for cell therapy applications

Achieving efficiency with CTS TrueCut Cas9 Protein and the CTS Xenon System

Introduction

The advanced therapeutics industry has been undergoing intense change, with the clinical success of CAR T cell therapy spurring increased interest and investments. A critical component of any therapeutic developer's risk mitigation strategy is to ensure that the manufacturing workflow incorporates equipment and reagents suitable for clinical manufacturing. Thermo Fisher Scientific is focused on providing manufacturing solutions that are appropriate for long-term commercialization of therapeutic products, through the addition of new innovative technologies to the Gibco™ Cell Therapy Systems™ (CTS™) product suite.

Here we showcase how a large-volume electroporation system, the Gibco™ CTS™ Xenon™ Electroporation System, can be used with the Gibco™ CTS™ TrueCut™ Cas9 Protein to provide a streamlined path to clinical cell therapy manufacturing.

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Materials and methods

Overview

Peripheral blood mononuclear cells (PBMCs) were previously isolated from an apheresis product using the Gibco™ CTS™ Rotea™ Counterflow Centrifugation System and frozen for future use (Figure 1). On day 0, PBMCs were thawed and activated using Gibco™ CTS™ Dynabeads™ CD3/CD28 beads. The T cells were then expanded in Gibco™ CTS™ OpTmizer™ T Cell Expansion Serum-Free Medium (SFM) supplemented with Gibco™ CTS™ Immune Cell Serum Replacement (SR) and other components as instructed per the product insert. On day 3, activated T cells were debeaded and electroporated using either the Invitrogen™ Neon™ Transfection System or the CTS Xenon Electroporation System in conjunction with CTS TrueCut Cas9 Protein and Invitrogen™ TrueGuide™ Synthetic gRNA (sgRNA). The T cells were divided into two populations per electroporation condition for downstream analysis, then put back into culture for expansion in complete CTS OpTmizer medium. On days 6 and 10, cells were analyzed for expansion, viability, and phenotype as well as knock-in and knockout efficiency, using the Invitrogen™ Attune™ NxT Flow Cytometer. Cells were frozen (cryopreserved) on day 9, then later thawed, to simulate clinical practice before testing functionality in a CAR T killing assay on day 12. Imaging was conducted on the Invitrogen™ EVOS™ M5000 Imaging System.

Electroporation experimental setup

To demonstrate electroporation efficiency across different scales, we tested 100 µL of cell suspension on the Neon system and 1 mL of cell suspension on the CTS Xenon system using the Gibco™ CTS™ Xenon™ SingleShot Electroporation Chamber across three different donors (donors A, B, and C). In addition, donor C was used for scale-up to an 18 mL electroporation volume on the CTS Xenon system using the Gibco™ Xenon™ Electroporation Cartridge, MultiShot™ format, 5–25 mL. Cell density was 5 x 10^7 cells/mL in Gibco™ CTS™ Xenon™ Genome Editing Buffer across all donors and scale conditions. A "no electroporation" control was also tested. To demonstrate a genetic modification strategy that is clinically relevant, we used the CTS Xenon electroporation system to deliver CTS TrueCut Cas9 Protein and *TRAC*-encoded TrueGuide sgRNA along with a donor dsDNA chimeric antigen receptor (CAR) construct to the cells. Respective payload quantities and electroporation conditions are listed in Figure 2.

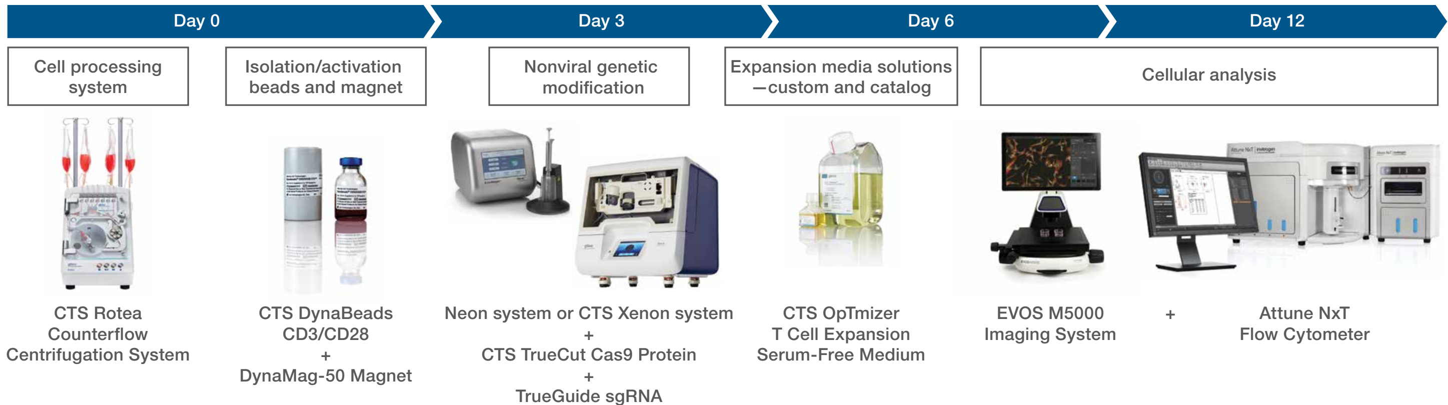

Figure 1. Autologous T cell therapy workflow.

A

	Experimental details	
Cells	PBMCs isolated from fresh leukapheresis products from 3 healthy human donors	
	Cell concentration during electroporation	5 x 10^7 cells/mL
Payload	CTS TrueCut Cas9 Protein	120 µg/mL
	Custom *TRAC* sgRNA	30 µg/mL
	Linear CAR donor dsDNA	240 µg/mL
Electroporation protocol	Neon system electroporation protocol #24	1,600 V; 10 ms; 3 pulses
	CTS Xenon system electroporation protocol	2,300 V; 3 ms; 4 pulses

B

Samples tested

- No-electroporation control
- Neon system, 100 µL, donors A–C
- CTS Xenon system with CTS Xenon SingleShot chamber, 1 mL, donors A–C
- CTS Xenon system with Xenon cartridge, MultiShot format (5–25 mL), 18 mL, donor C

Figure 2. Experimental and sample details. (A) Experimental conditions and electroporation parameters. **(B)** Sample information.

Flow cytometry gating strategy

The Attune NxT Flow Cytometer was used to assess gene editing efficiency and phenotype. Figure 3 shows the gating strategies that were applied: A represents the parental gate, B represents the unedited population, and C represents the edited cell population. The V5 antibody was used to detect a part of the CAR antigen on the T cells to quantify how many cells expressed the CAR on their membrane.

CAR T killing assay

CAR T cells from donors A and B were tested for functionality in a cytotoxicity assay (Figure 4). Briefly, cells were frozen (cryopreserved) on day 9, then later thawed and grown for 3 days post-thaw to simulate clinical practice. Effector CAR T cells or control cells (unmodified cytotoxic T lymphocytes (CTLs)) were thawed and seeded into 96-well plates containing Green Fluorescent Protein (GFP)-labeled Nalm6 target cells at effector to target (E:T) ratios ranging from 10:1 to 0:1. The E:T cell mixtures were incubated for 6 hours and analyzed for percent cytotoxicity using the EVOS M5000 Imaging System and the Attune NxT Flow Cytometer.

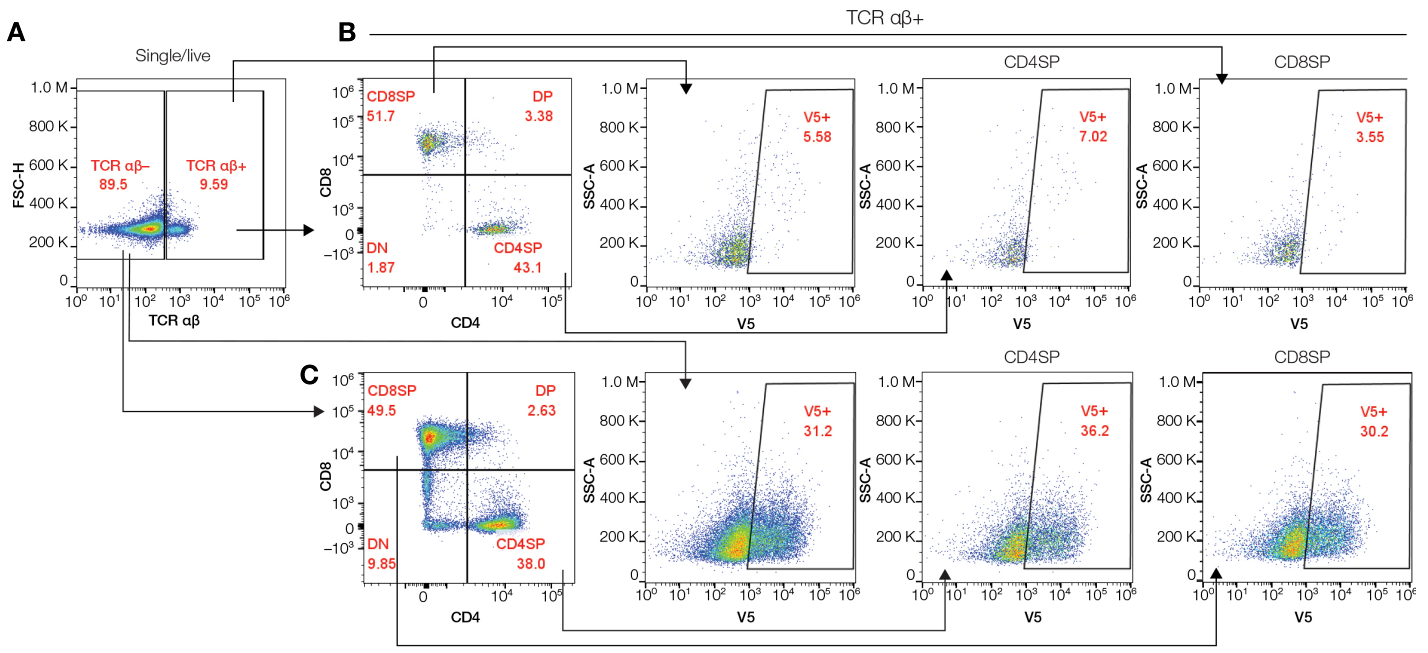

Figure 3. Gating strategy for the Attune NxT Flow Cytometer.

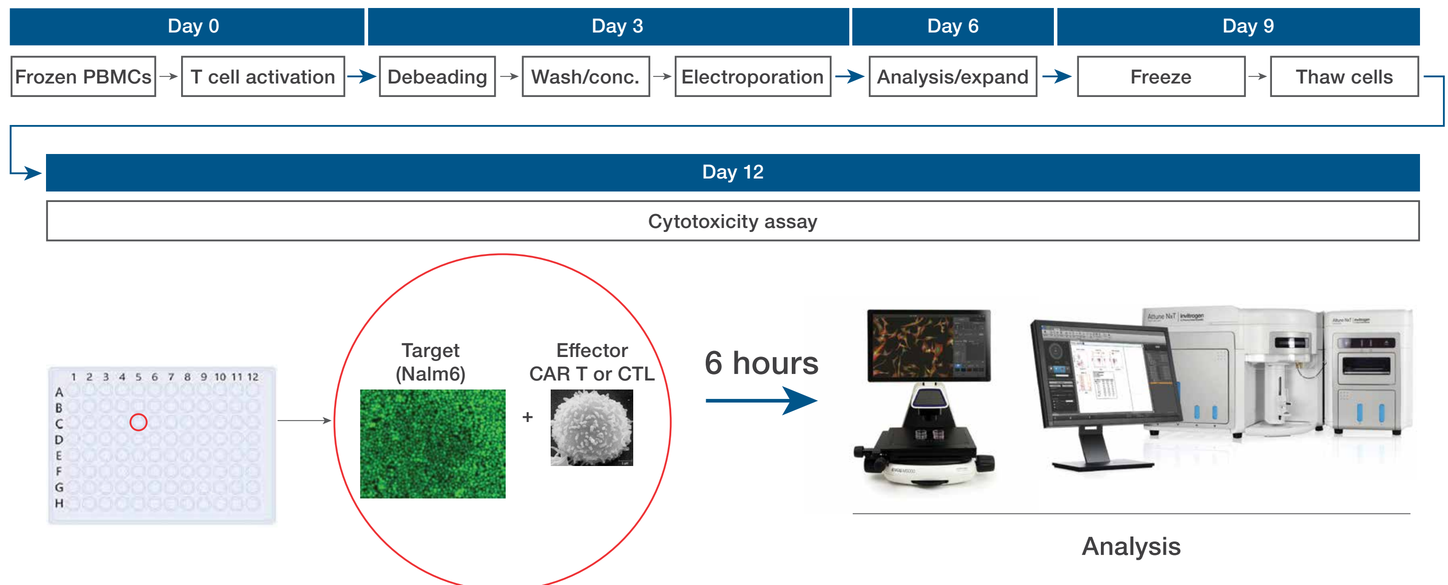

Figure 4. Cytotoxicity assay workflow.

Results

Viability and cell expansion

Electroporation success is a balance between transfection efficiency and viability, and post-electroporation viability greater than 70% is generally considered to be acceptable. Viability was assessed at 72 hours post-electroporation and was consistent across various electroporation volume scales, including the no-electroporation controls. This consistency demonstrated that electroporation itself, as well as the electroporation volume, did not significantly alter cell viability (Figure 5). In addition, viability following electroporation by the CTS Xenon system was well above 70% for all donors. As seen in the experimental setup outlined in Figure 2, we show a no-electroporation control (0 μL), electroporation of 100 μL using the Neon system, and electroporation of 1 mL using a CTS Xenon™ SingleShot Electroporation Chamber in the CTS Xenon system, for all donors.

Cell numbers were recorded immediately prior to electroporation (day 3), and cell growth was assessed at 3 and 7 days post-electroporation (days 6 and 10, respectively), to ensure modified cells were capable of expansion. The electroporation-only control cells and the modified cells that received payload were able to expand 25- to 35-fold over the course of 7 days (Figure 6). As expected, modified cells exhibited slightly lower expansion due to internalization of the payload and the physical effects of electroporation. Cell viability on day 10 was roughly 90% for all donors and electroporation conditions.

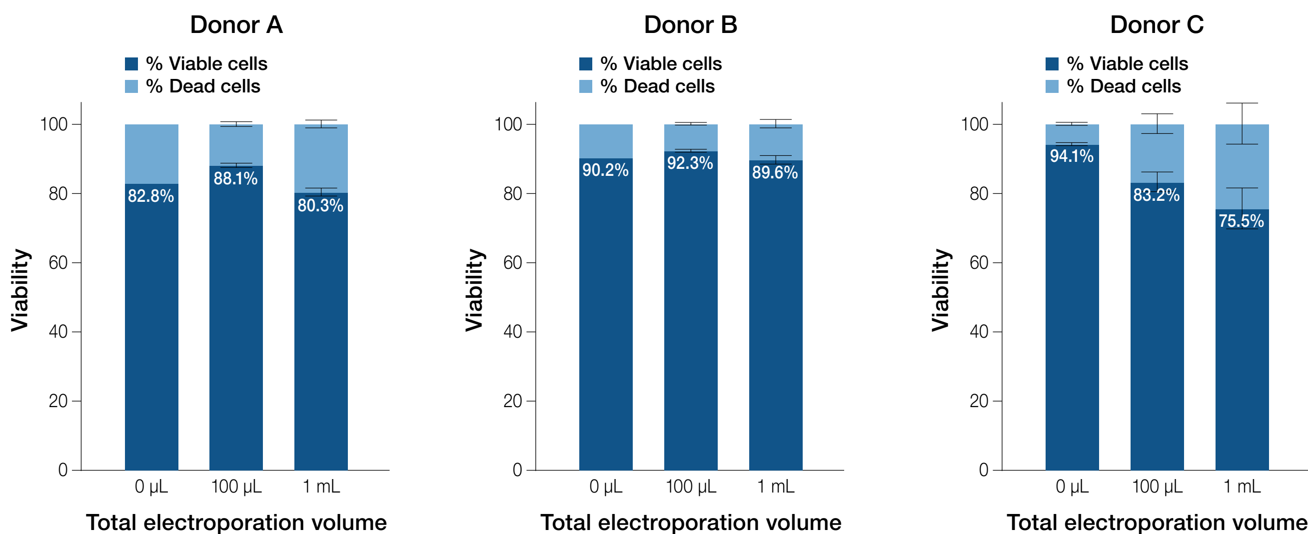

Figure 5. Cell viability measured on day 6, 3 days post-electroporation with the CTS Xenon system.

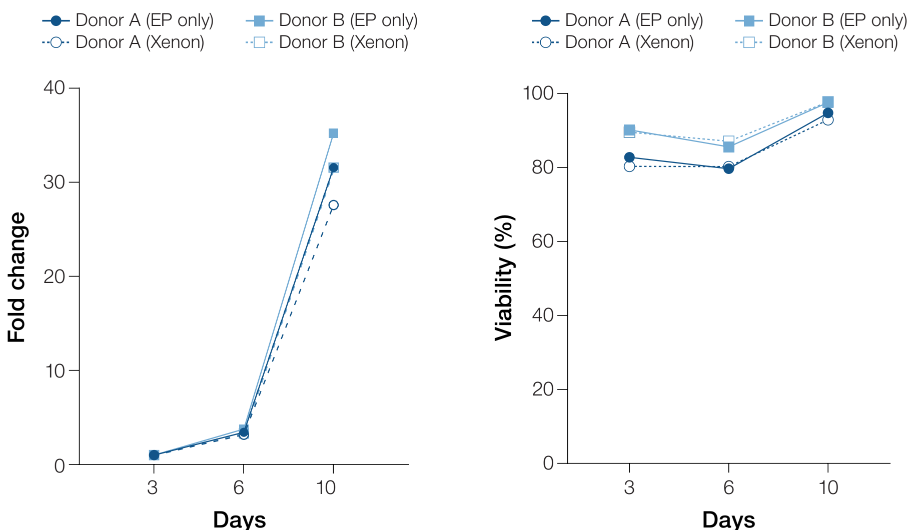

Figure 6. Cell expansion and viability were tested on the day of electroporation (day 3) as well as 3 and 7 days post-electroporation (days 6 and 10, respectively).

Transfection efficiency

Activated T cells were genetically modified with either the Neon (100 µL) or CTS Xenon (1 mL) transfection systems. The cells were modified with ribonucleoprotein complexes comprising CTS TrueCut Cas9 Protein and TrueGuide sgRNA encoding the *TRAC* site. Figure 7 showcases consistent gene editing efficiency; superior efficiency was observed with the CTS Xenon system (22–44% knock-in efficiency) compared to the Neon system (15–23% knock-in efficiency), which suggests that the CTS Xenon system can be used to easily scale and optimize the transfection process in a closed system.

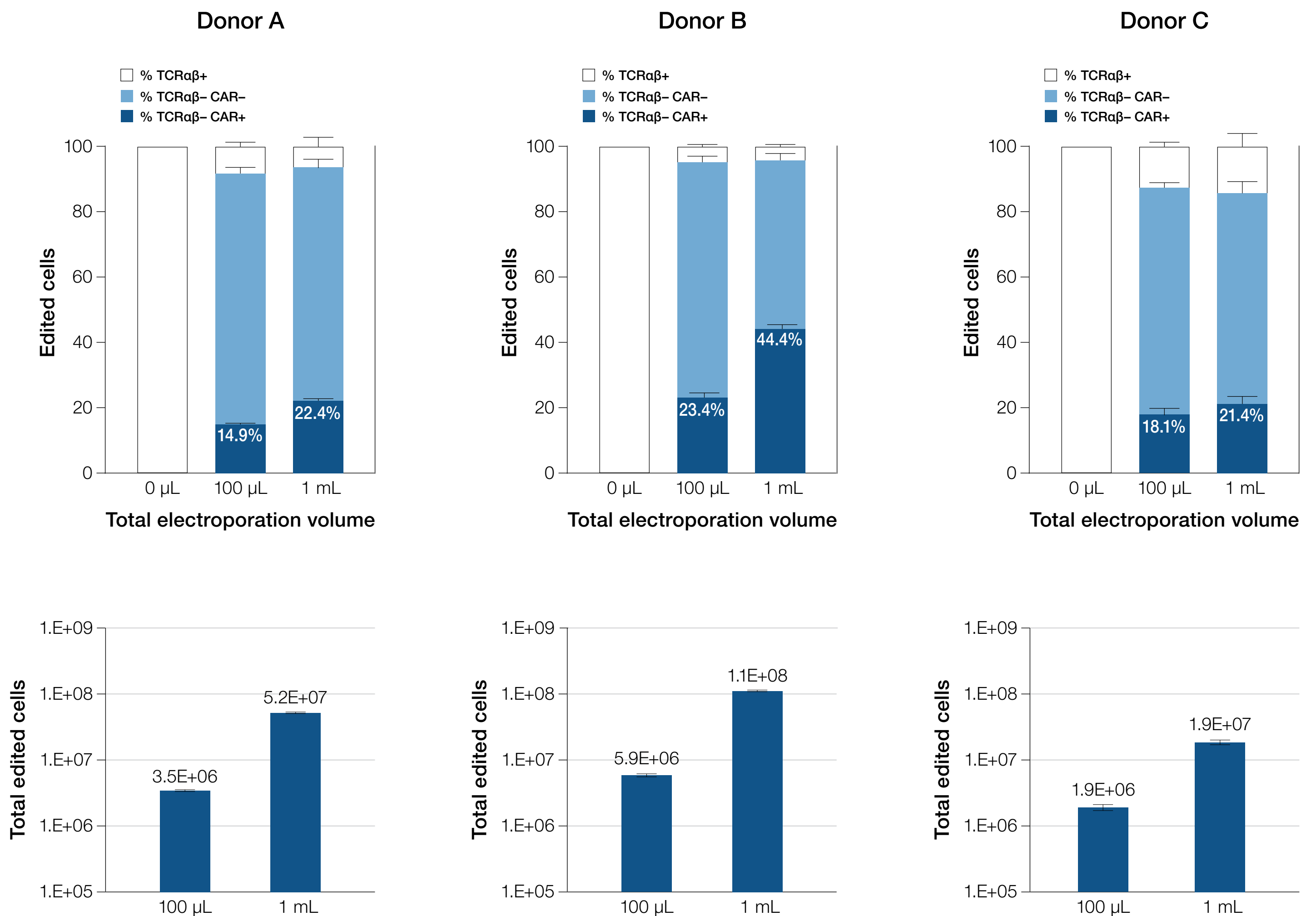

Figure 7. Gene editing efficiency. The light blue bars represent total cells for the no-electroporation control, and the dark blue bars represent total edited cells for the two electroporation volumes (100 µL and 1 mL). Data were collected 3 days post-electroporation (day 6).

Phenotype

T cell phenotype was assessed on the Attune NxT Flow Cytometer. Compared to no-electroporation controls, there is minimal or no phenotypic change across the electroporation volumes tested.

There was a shift in phenotype when comparing cells before and after activation, electroporation, and expansion, but no significant impact from electroporation alone (Figure 8).

Cytotoxicity

Cytotoxicity was assessed using a CAR T cell killing assay. After thaw, CAR T cells exhibited sustained expression of the CAR construct and demonstrated the ability to efficiently kill GFP-labeled target cells (Figure 9).

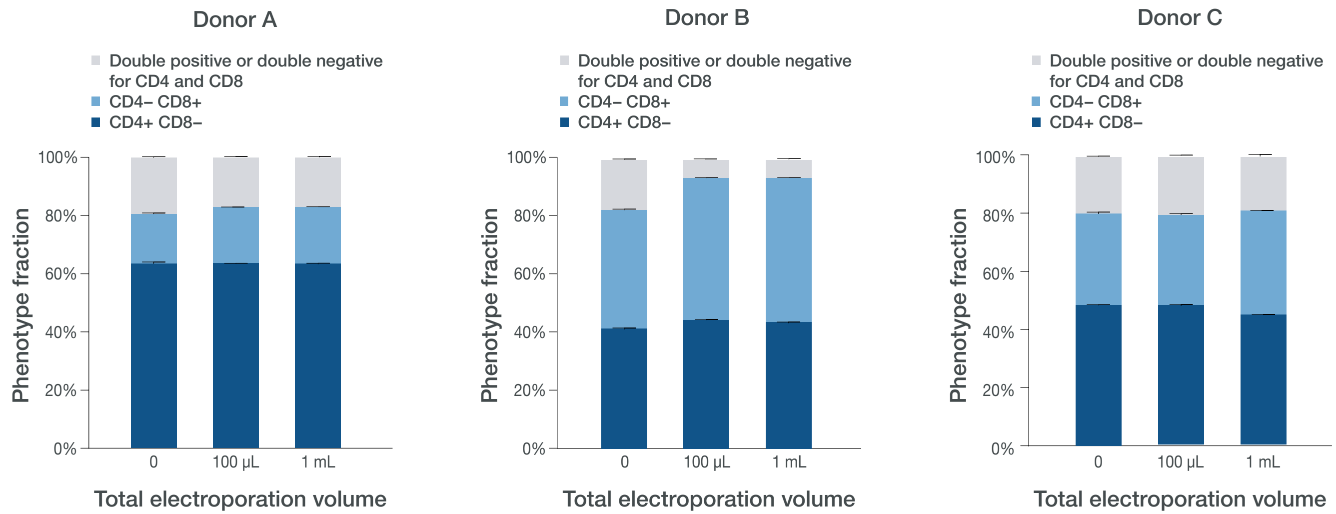

Figure 8. Phenotypic data post-electroporation. The dark blue bars represent CD4⁺ cells, the light blue bars represent CD8⁺ cells, and the grey bar represents either double positive or double negative CD4/CD8 cells.

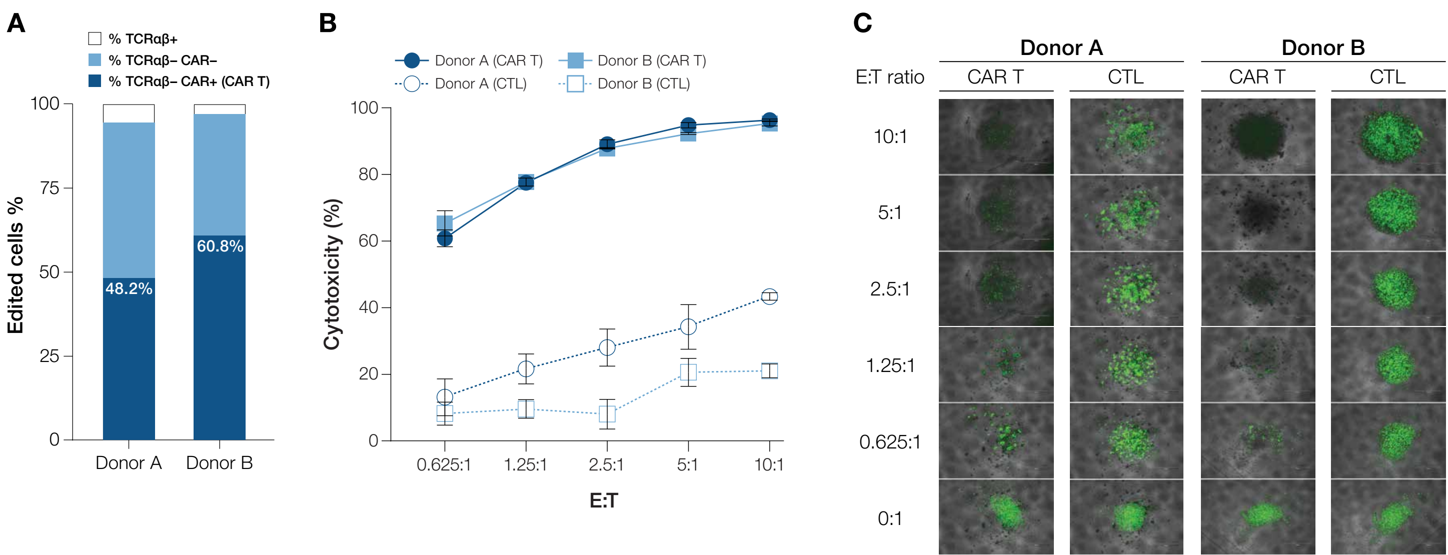

Figure 9. CAR T cells were tested for cytotoxic functionality on day 12 post-electroporation. Effector cells were assessed for editing efficiency or performance **(A)** and incubated with GFP-labeled target cells at various E:T ratios **(B, C)**. Cells effectively killed target cells, exhibiting the desired functionality.

Scale up using the Xenon Electroporation Cartridge, MultiShot format, 5–25 mL

To showcase the scale-up capabilities of the CTS Xenon system, an 18 mL electroporation experiment with cells from donor C was carried out on the Xenon Electroporation Cartridge, MultiShot format, 5–25 mL, using the parameters referenced in Figure 2. Viability, electroporation efficiency, and phenotype fractions were similar across all three scales (Figure 10). In general, the time required for electroporation and cell loss can be challenges when moving to larger scales, and minimizing the amount of time cells are exposed to electroporation buffer is recommended. However, the entire 18 mL electroporation required a time frame spanning fewer than 25 minutes from the time cells were exposed to electroporation buffer to completion of the electroporation protocol, and recovery was well within the expected range.

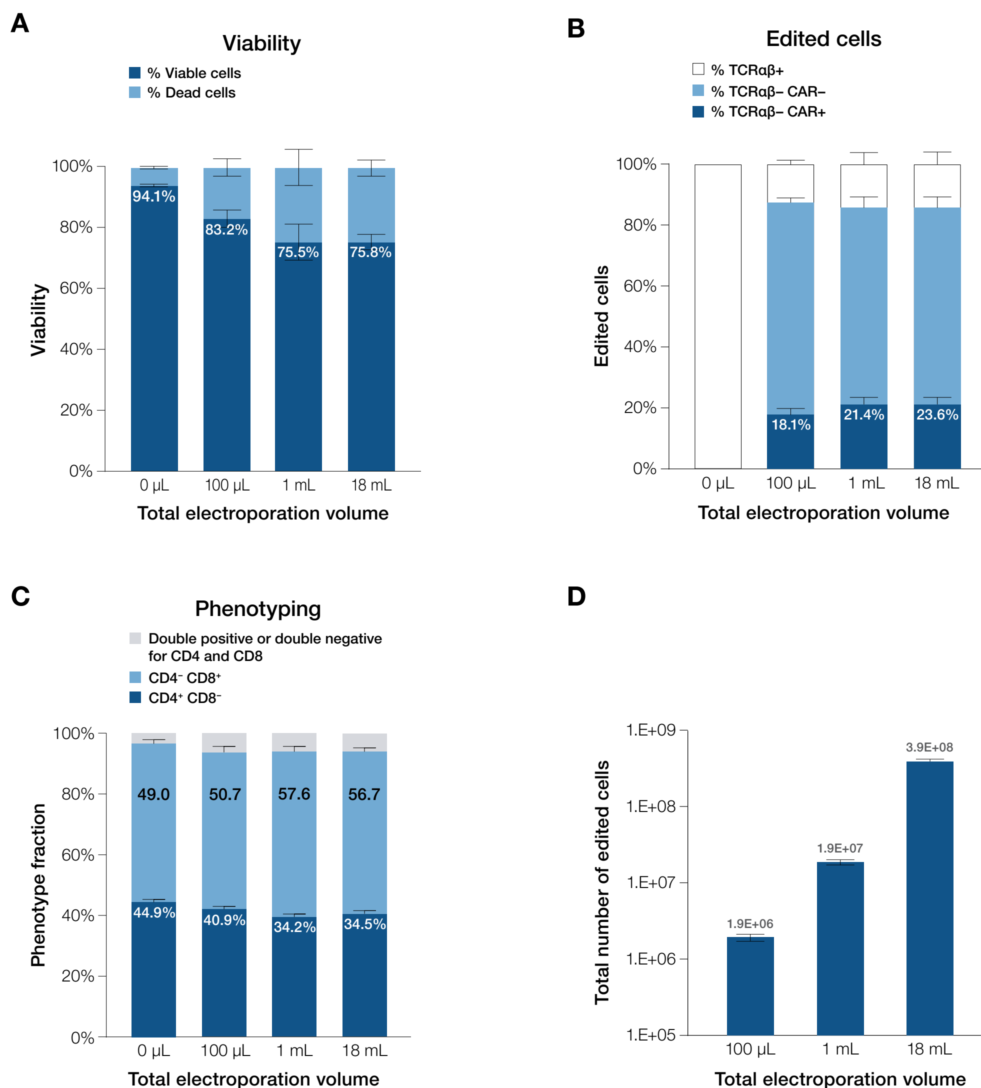

Figure 10. Scale-up of electroporation volumes on the CTS Xenon system. The resulting cells were assessed for viability **(A)**, electroporation efficiency **(B)**, phenotype **(C),** and total number of edited cells **(D)**. Data were collected 3 days post-electroporation (day 6).

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Conclusions

Cell therapy manufacturing guidelines have been evolving as regulatory agencies implement more stringent requirements in an effort to standardize these therapeutics. Ensuring suitability of instruments and reagents for cell therapy manufacturing is a core foundation of our Gibco™ CTS™ product design.

As demonstrated through electroporation efficiency and functionality of the resulting CAR T cells, the CTS Xenon system has been designed to scale up and meet requirements for commercial manufacturing processes. The system can be integrated with reagents such as CTS TrueCut Cas9 Protein as well as a range of products and tools designed for use in cell therapy manufacturing to close and optimize the cell engineering step of your manufacturing process.

Upon customer request, Thermo Fisher can supply regulatory support files for instruments and consumables to assist in regulatory filings. It is the responsibility of the end user to ensure regulatory compliance.

Ordering information

Description	Quantity	Cat. No.
Nonviral genetic modification		
CTS Xenon Electroporation System	1	A52727
CTS Xenon SingleShot Electroporation Chamber, 1 mL	6 pk	A53444
Xenon Electroporation Cartridge, MultiShot format, 5–25 mL	1 pk	A53445
CTS Xenon Genome Editing Buffer, bottle	100 mL	A4998001
CTS Xenon Genome Editing Buffer, bag	100 mL	A4998002
CTS TrueCut Cas9 Protein, 10 mg/mL	2.5 mg	A45220
	5 mg	A45221
Custom *TRAC*-encoded TrueGuide sgRNA	Custom	Please inquire
T cell culture and activation		
CTS OpTmizer T Cell Expansion SFM, bottle	1,000 mL	A1048501
CTS OpTmizer T Cell Expansion SFM, bag	1,000 mL	A1048503
CTS Immune Cell Serum Replacement (ICSR)	50 mL	A2596101
CTS GlutaMAX-I Supplement	100 mL	A1286001
CTS GlutaMAX Supplement, bag	100 mL	A4737001
Human IL-2 (Interleukin-2) Recombinant Protein	1 mg	PHC0023
CTS Dynabeads CD3/CD28	10 mL	40203D
T cell analysis		
Evos M5000 Imaging System	1	AMF5000
Attune NxT Flow Cytometer	1	A29004
Attune NxT Flow Cytometer Autosampler	1	4473928
V5 Tag Monoclonal Antibody (TCM5), PE	100 tests	12-6796-42
CD4 Monoclonal Antibody (RPA-T4), PE-Cyanine7, eBioscience	100 tests	25-0049-42
CD8 Monoclonal Antibody (RFT-8), APC-Cyanine7	100 µg	A15448
SYTOX Red Dead Cell Stain, for 633 or 635 nm excitation	1 mL	S34859
eBioscience Flow Cytometry Staining Buffer	200 mL	00-4222-57

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