Cell engineering

Streamlined nonviral genome editing in activated primary human T cells

Optimizing the Neon NxT Electroporation System with 8-Channel Pipette for enhanced efficiency and scalability to the CTS Xenon Electroporation System

Introduction

Electroporation is a widely used technique in molecular biology and genetic engineering for delivering exogenous genetic material into cells. It involves the application of short, high-voltage electrical pulses to create temporary pores in the cell membrane, allowing for the efficient delivery of DNA, RNA, or other molecules of interest. This method has proven to be highly effective for introducing genetic modifications such as knockouts (KOs) and knock-ins (KIs) in a variety of cell types.

The Invitrogen™ Neon™ Transfection System is a trusted instrument for electroporation. The Invitrogen™ Neon™ NxT Electroporation System is an enhanced version of this system, building upon its success. The Neon NxT system combines the established design of its exceptional pipette tip, where electroporation occurs within the pipette tip chamber, with the security of Thermo Scientific™ ClipTip™ tip attachment technology. The system allows precise control of electrical parameters and offers the Invitrogen™ TransfectionLab™ Cloud Application for experimental design.

The Invitrogen™ Neon™ NxT 8-Channel Pipette was introduced to increase the throughput capabilities of the Neon NxT Electroporation System, enabling the processing of up to eight samples in a single run with different electroporation parameters, such as voltage, pulse width, and pulse number. This level of customization helps ensure optimal conditions for a wide range of experimental needs. By enabling eight electroporation conditions to be performed in each run, with full customization of up to 96 individualized programs for a 96-well-plate, this system offers a substantial increase in throughput and efficiency. This capability is particularly valuable when working with large sample sizes for optimization or screening applications.

Optimization results obtained using the Neon NxT 8-Channel Pipette are highly valuable for clinical translation of chimeric antigen receptor (CAR) T cell therapies. To better serve the cell therapy industry, Thermo Fisher Scientific created the Gibco™ CTS™ Xenon™ Electroporation System to enable closed, scalable transfection of cells for cell therapy applications. For streamlined optimization, the system is compatible with processes and protocols developed with the Neon NxT Electroporation System.

Here, we demonstrate the ease of optimizing electroporation parameters using the Neon NxT 8-Channel Pipette for activated primary T cells. By systematically varying the electroporation program and buffer type, we identify the optimal conditions for successful genome editing. We also show the reproducibility of optimized results when scaling from the Neon NxT system to the CTS Xenon system. This scalability of the electroporation parameters allows researchers to seamlessly transition from small-scale experiments to large-scale production. The results highlight the utility of the Neon NxT 8-Channel Pipette for rapid optimization of electroporation that is translatable to clinical process development of CAR T cell therapies with the CTS Xenon system.

Materials and methods

The overall workflow used in this study is summarized in Figure 1. Details of the equipment, media, and reagents used are provided in Table 1.

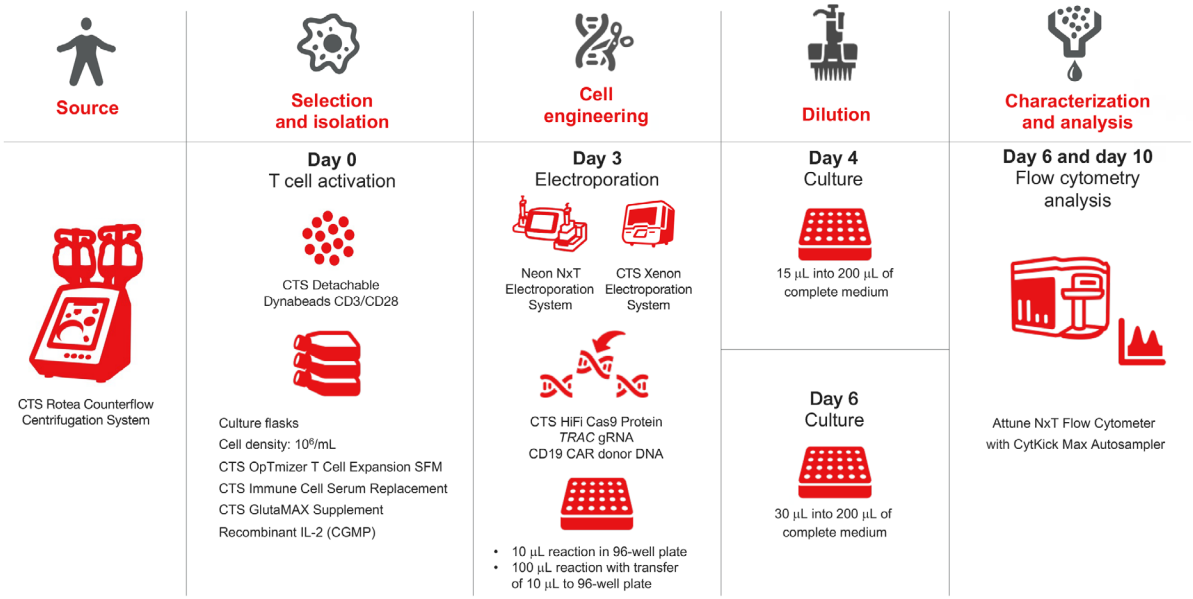

Figure 1. Genome editing workflow with the Neon NxT and CTS Xenon Electroporation Systems.

Table 1. Materials and methods used in this study.

Unit operation	Details
Cell source and isolation	Peripheral blood mononuclear cell (PBMC) isolation was performed from fresh leukapheresis product from healthy human donors using the Gibco™ CTS™ Rotea™ Counterflow Centrifugation System and cryopreserved for later use.
Activation	Cryopreserved PBMCs were thawed then activated with Gibco™ CTS™ Detachable Dynabeads™ CD3/CD28 at a 1:3 (cells:beads) ratio; cells were de-beaded 72 hours post-thaw using the Invitrogen™ DynaMag™-50 Magnet prior to electroporation.
Electroporation	Cell density: 50 x 10^6 cells/mL. Buffer: Invitrogen™ Neon™ NxT Resuspension Genome Editing Buffer (GE buffer) was used for all experiments unless specified otherwise in the relevant section. Payload: 120 µg/mL Gibco™ CTS™ HiFi Cas9 Protein, 32 µg/mL Invitrogen™ TrueGuide™ gRNA targeting the *TRAC* gene, and 240 µg/mL anti-CD19 CAR donor DNA.
Cell expansion	Media composition: 92% Gibco™ CTS™ OpTmizer™ T Cell Expansion SFM (89.4% basal medium, 2.6% supplement), 5% Gibco™ CTS™ Immune Cell Serum Replacement, 3% Gibco™ GlutaMAX™ Supplement (6 mM), 100 UI/mL IL-2. Pre-electroporation culture: PBMCs activated with Dynabeads CD3/CD28 were cultured in T-175 flasks at 10^6 cells/mL for 3 days. Post-electroporation culture: Post-electroporation cells were cultured in 96-well plates at ~2.5 x 10^6 cells/mL. Cell density dilution with fresh complete medium was performed on days 4 and 6 (24 hours and 72 hours post-electroporation).
Characterization and analysis	Flow cytometry: Cells were analyzed for viability, recovery, and KO and KI efficiency using the Invitrogen™ Attune™ NxT Flow Cytometer on days 6 and 10. The flow cytometry gating strategy is shown in the appendix (Figure S1). Percent viability was defined as the viable cells out of the "Lymphocytes" gate. Percent recovery was defined as the viable cells out of the "All Cells" gate. Percent KO efficiency was normalized using the following formula: 100 – (100 x (percent TCR^+ in electroporation sample/percent TCR^+ in no-electroporation sample)). Percent KI efficiency was defined as the total percentage of TCR^- CAR^+ cells out of the total live cells gate.

Results and discussion

Neon NxT 1-Channel and 8-Channel Pipettes: same performance on one device

Both the Neon NxT 1-Channel and 8-Channel Pipettes are operated with the same device. This study presents a side-by-side comparison of their performance with 10 µL and 100 µL tips. The program 2,300 V/3 ms/4 pulses was used throughout the study. Genome editing performance on day 6 is illustrated in Figure 2. For both reaction volumes, the Neon NxT 1-Channel and 8-Channel Pipettes demonstrated similar KO and KI efficiency with minimal impact on cell viability.

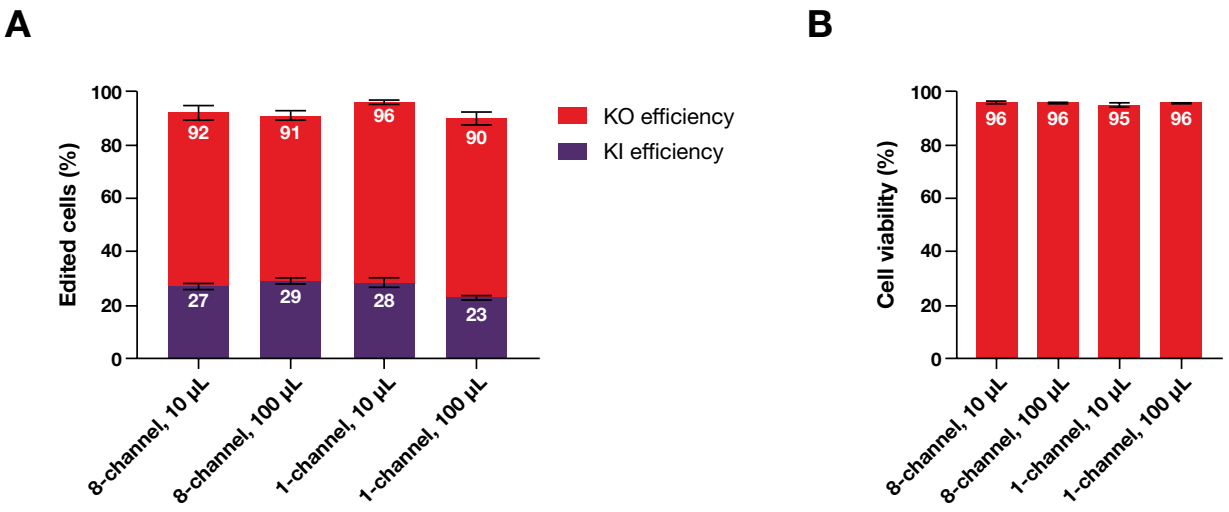

Figure 2. Genome editing performance of Neon 1-Channel and 8-Channel Pipettes. (A) KO and KI efficiency. **(B)** Cell viability.

Enhanced genome editing with GE buffer

The objective of this experiment was to determine the optimal buffer for KO and KI of a CD19 CAR in activated primary T cells. The Neon NxT 8-Channel Pipette enabled rapid testing of the three buffer options, which include GE, R, and T buffers, across multiple electroporation programs. We expected that GE buffer would perform best as it was designed to increase genome editing efficiency by driving homology-directed repair (HDR) after transfection. This work confirmed that performing T cell editing in GE buffer resulted in the highest editing efficiency compared to R and T buffers (Figure 3) with the average KO of the TCR reaching 97% and average KI of the CD19 CAR reaching as high as 60%.

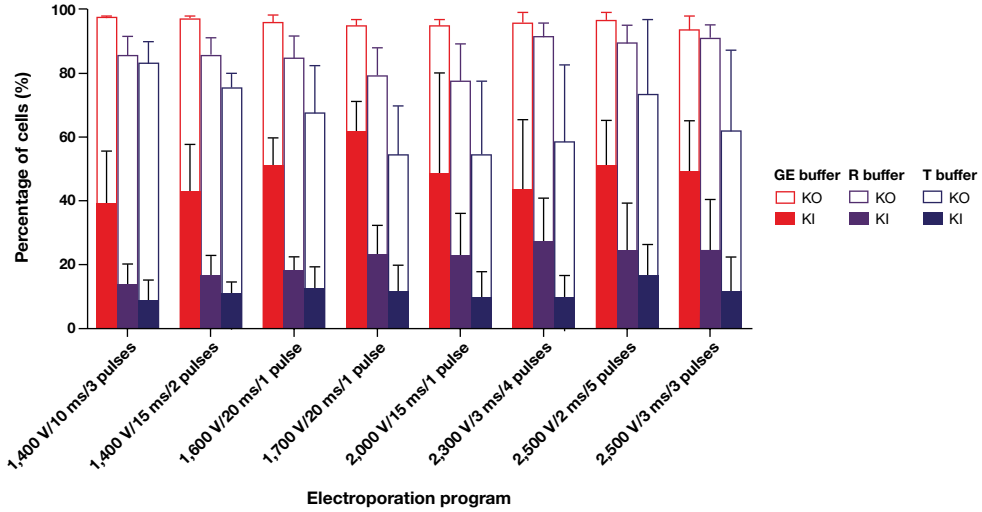

Figure 3. Editing performance in GE, R, or T buffer on day 10.

Reproducibility of electroporation program performance across multiple donors

Donor-dependent differences in editing efficiency are a known source of variability in cell and gene therapy workflows. Using a range of electroporation programs, the T cell editing efficiency was determined for PBMCs originating from 3 different donors. Across the programs tested, clear trends in editing efficiency were observed (Figure 4). Program 2,000 V/15 ms/1 pulse showed the highest KI efficiency across all 3 donors and optimal tradeoff in terms of cell viability. Importantly, there was significant variability in editing efficiency across donors, with donor 3 exhibiting much lower knockout efficiency compared to the other two. These results illustrate the importance of the PBMC material source for achieving high editing efficiency.

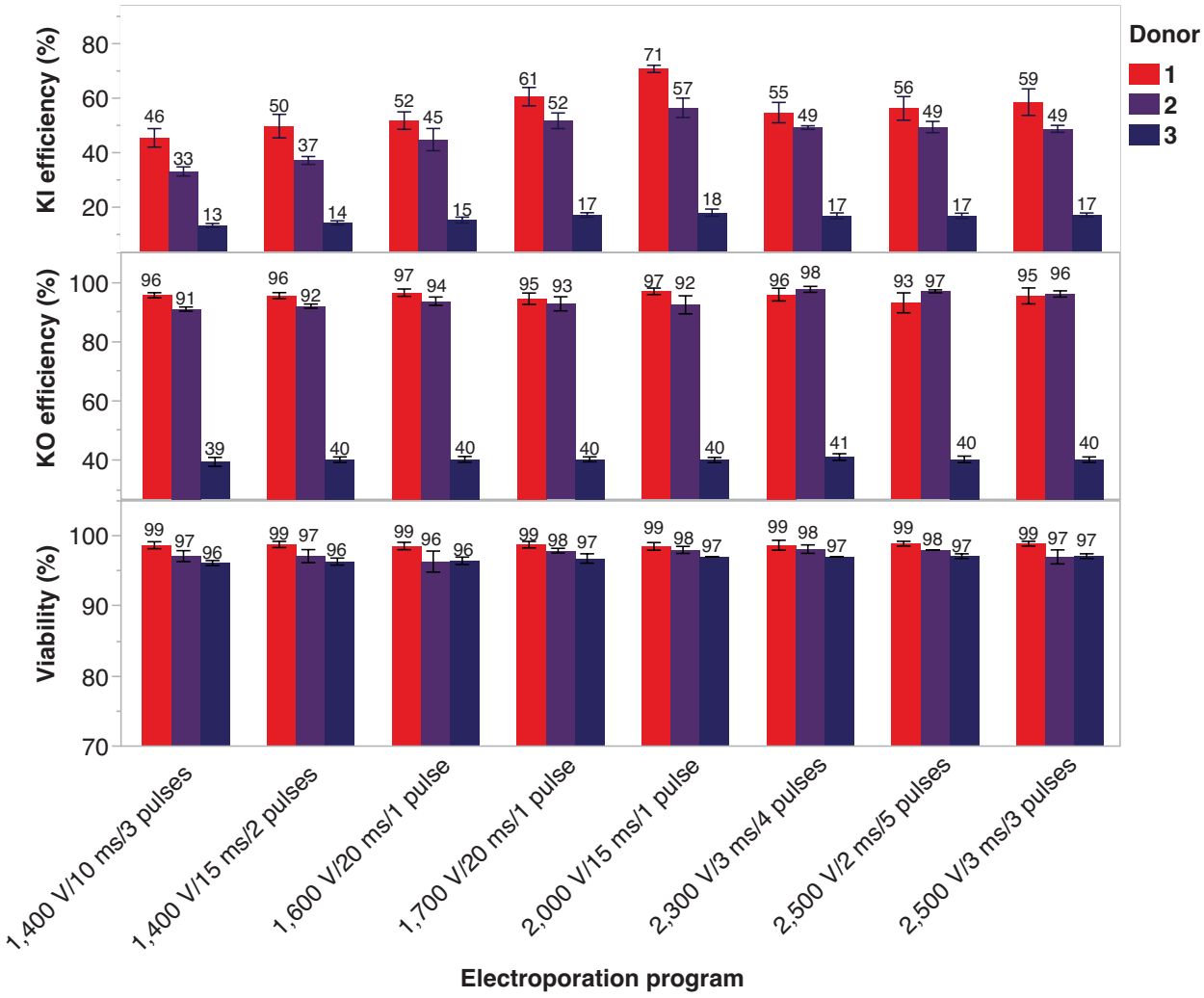

Figure 4. Editing efficiency across 3 different donors for 8 different programs on day 10.

Scalability assessment from Neon NxT to CTS Xenon systems

In a previous study, we demonstrated alignment of gene editing performance in primary activated T cells across a range of electroporation conditions using the Neon NxT and CTS Xenon Electroporation Systems (Appendix Figure S2). To broaden this analysis, we screened 94 programs using the Neon NxT 8-Channel Pipette, as listed in Table 2.

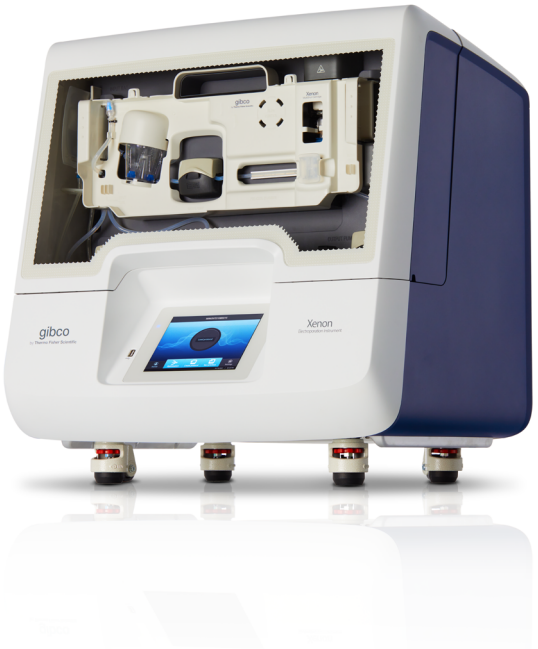

Table 2. List of programs screened with the Neon NxT 8-Channel Pipette.

Program ID	Voltage	Pulse width	Pulse number
1	1,600	1	2
2	1,650	16	1
3	2,500	11	1
4	2,500	1	5
5	2,500	1	4
6	2,100	16	1
7	2,500	1	5
8	1,400	17	2
9	2,300	1	4
10	1,850	4	5
11	2,300	5	1
12	1,400	4	5
13	2,300	1	5
14	1,600	1	1
15	1,400	12	3
16	1,600	5	5
17	1,600	1	4
18	1,600	14	2
19	1,400	1	1
20	2,500	4	2
21	1,600	20	1
22	1,400	17	2
23	1,650	16	1
24	1,600	4	1
25	1,400	17	2
26	2,300	3	4
27	1,400	1	2
28	2,250	7	2
29	2,100	16	1
30	1,400	18	2
31	1,950	4	4
32	2,500	1	4
33	2,500	4	1
34	1,600	5	5
35	1,850	20	1
36	2,500	11	1
37	1,950	4	4
38	2,000	1	5
39	2,300	1	5
40	1,600	1	3
41	1,400	18	2
42	1,950	9	2
43	2,100	16	1
44	1,400	20	1
45	2,300	5	2
46	1,600	14	2
47	2,500	11	1
48	2,300	1	5
49	1,400	20	1
50	1,400	20	1
51	1,400	1	1
52	1,400	9	4
53	2,300	5	1
54	1,600	5	5
55	2,500	5	2
56	2,500	11	1
57	2,500	1	4
58	1,600	1	2
59	1,850	20	1
60	2,250	14	1
61	1,400	1	1
62	1,400	9	4
63	2,500	1	5
64	1,600	7	4
65	1,400	7	5
66	2,500	4	2
67	1,600	1	1
68	1,400	1	1
69	1,850	20	1
70	2,300	13	1
71	1,850	20	1
72	1,400	4	1
73	1,400	7	5
74	2,300	4	2
75	2,000	1	2
76	2,500	1	5
77	2,500	1	4
78	1,950	1	2
79	1,600	20	1
80	1,400	9	4
81	2,300	4	3
82	1,400	4	1
83	1,400	20	1
84	2,300	1	4
85	2,500	4	2
86	1,400	1	1
87	1,400	7	5
88	1,600	7	4
89	1,400	10	3
90	1,400	15	2
91	1,700	20	1
92	2,000	15	1
93	2,500	2	5
94	2,500	3	3

Note: some repeated programs were utilized to confirm reproducability

Editing performance—measured by KO efficiency, KI efficiency, and percent cell recovery—is summarized in Figure 5A. Program 10 (1,850 V/4 ms/5 pulses) emerged as the top-performing condition, providing a favorable balance between editing efficiency and viability. Program 26 (2,300 V/3 ms/4 pulses) is a commonly used protocol for editing primary activated T cells on the CTS Xenon system. Therefore, the two programs were chosen for a comparative evaluation on the CTS Xenon system (Figures 5B–D) in terms of recovery and editing efficiency. Program 10, the top-performing program on the Neon NxT system, outperformed program 26, indicating the value of the Neon NxT system for optimization and screening when identifying and transferring optimal editing conditions to large-scale workflows.

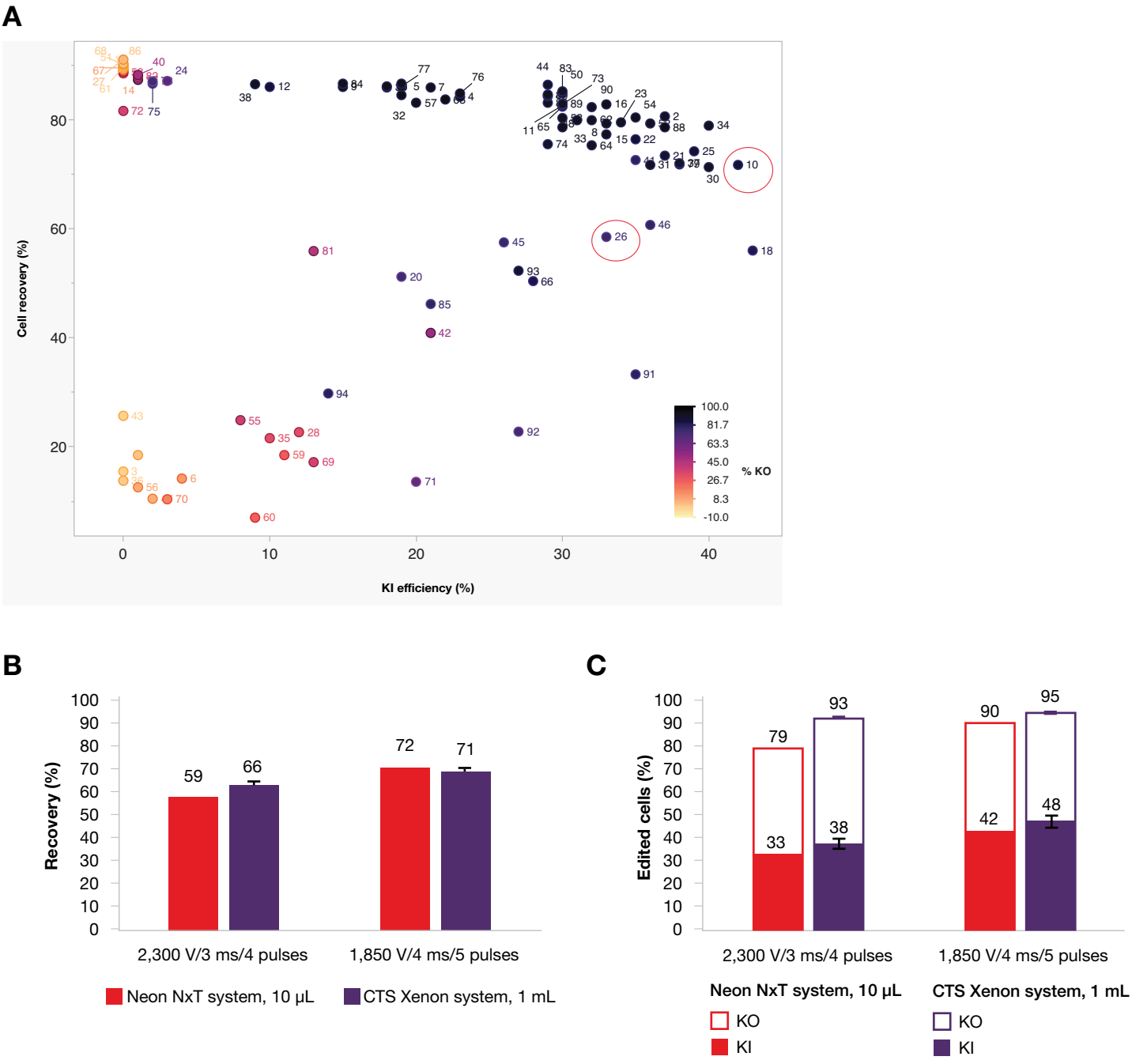

Figure 5. Assessment of scalability from the Neon NxT to CTS Xenon systems on day 6. (A) Performance screening of electroporation programs with the Neon NxT 8-Channel Pipette in terms of KO and KI efficiency and percent cell recovery. **(B)** Cell recovery (see Figure S1), **(C)** KO and KI efficiency comparisons between the Neon NxT and CTS Xenon systems. The scalability of electroporation parameters from the Neon NxT to CTS Xenon systems helps enable reliable translation from small-scale experiments to larger-scale manufacturing processes.

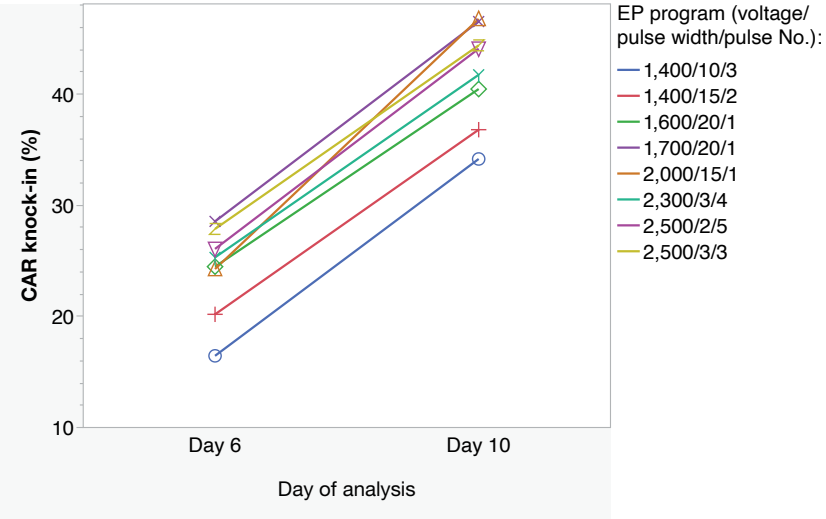

Figure 6. KI efficiency in day 6 vs. day 10 analysis.

Day 6 vs. day 10 flow cytometry analysis

To enable accurate assessment, it is important to follow the cells beyond the initial edited population. We show that from day 6 to day 10 post-isolation (days 3 and 7 post-electroporation, respectively), there is an average 2-fold increase in the percentage of CAR+ T cells (Figure 6). Importantly, this increase in KI over time was consistent across all electroporation programs tested, demonstrating the benefit of characterizing editing efficiency at later time points.

No toxicity from payload and GE buffer

The Neon NxT 8-Channel Pipette was designed for efficient processing of large samples with different conditions. In some experiments, cells are resuspended in a resuspension buffer (e.g., GE buffer) containing a payload (Cas9 protein, gRNA, DNA) for an extended period of time to process a large sample size. We aimed to determine if there is any toxicity from the GE buffer and payload with extended incubation times.

To investigate this, we incubated cells with the GE buffer and payload at the room temperature for up to an hour before electroporation. We then assessed genome editing performance (Figure 7). The findings indicate that up to an hour of incubation does not negatively impact cell recovery or editing performance. Interestingly, KI efficiency slightly increases with longer incubation.

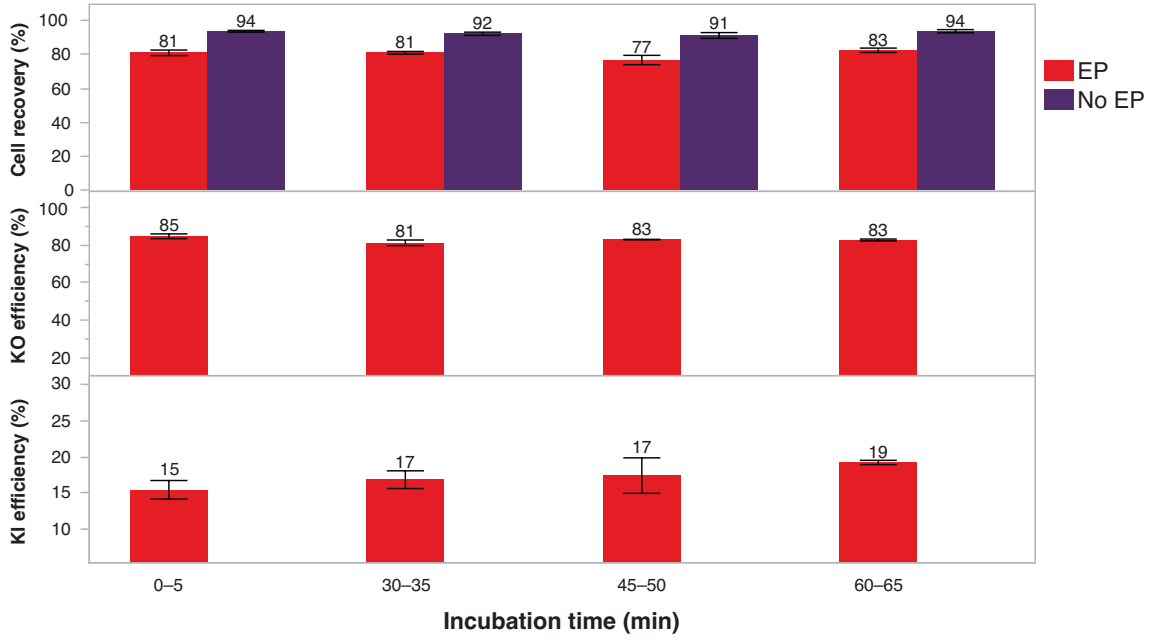

Figure 7. Editing efficiency following different incubation times in GE buffer with payload.

Conclusion

The Neon NxT Electroporation System demonstrates remarkable versatility for fast optimization of electroporation parameters. The scalability of the system from small-scale to larger-scale applications with the CTS Xenon Electroporation System helps ensure consistent and efficient workflows. The ability to achieve high editing efficiency, particularly with the GE buffer, and the reproducibility of results across multiple donors underscores the system's robustness and reliability. This positions the Neon NxT system and CTS Xenon system as a powerful and efficient platform for both scaled-out research and scaled-up clinical process development in cell therapy workflows.

Ordering information

Product	Cat. No.
Neon NxT Electroporation System Starter Kit with 1-Channel and 8-Channel Pipettes	NEON18SK
CTS Detachable Dynabeads CD3/CD28	A56996
DynaMag-50 Magnet	12302D
PeproGMP Human IL-2 Recombinant Protein, PeproTech	GMP200-02
CTS Rotea Counterflow Centrifugation System	A47679
CTS Xenon Electroporation System	A50301
CTS Xenon SingleShot Electroporation Chamber	A50305
CTS Xenon Genome Editing Buffer	A4998001
CTS HiFi Cas9 Protein	A54224
TrueGuide Synthetic gRNA	thermofisher.com/trueguide
CTS OpTmizer T Cell Expansion SFM	A1048501
CTS Immune Cell Serum Replacement (SR)	A2596102
GlutaMAX Supplement	35050061
Attune CytPix Flow Cytometer	A51841
CytKick Max Autosampler	A42973
eBioscience Flow Cytometry Staining Buffer	00-4222-57
TCR alpha/beta Monoclonal Antibody (IP26), FITC, eBioscience	11-9986-42
V5 Tag Monoclonal Antibody (TCM5), PE, eBioscience	12-6796-42
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation	L34975
Brilliant Stain Buffer	00-4409-42
UltraComp eBeads Plus Compensation Beads	01-3333-42

Appendix

Flow cytometry gating strategy

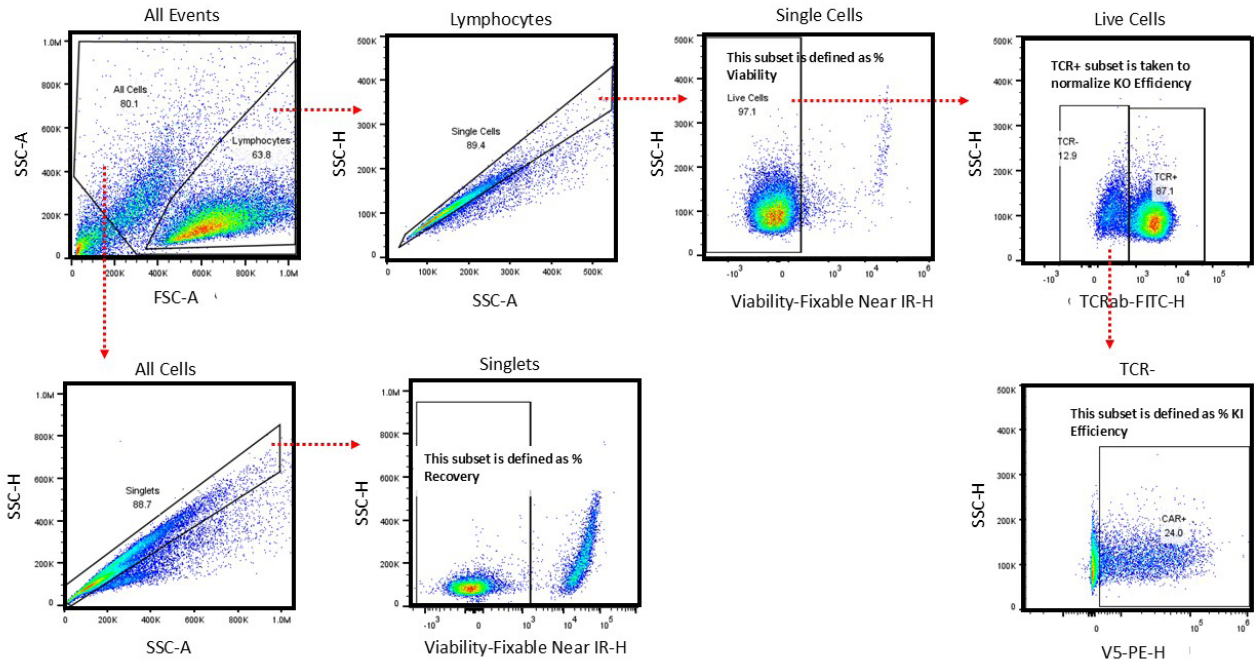

Figure S1. Flow cytometry gating strategy used in this study.

Scalability of the Neon NxT system to the CTS Xenon system

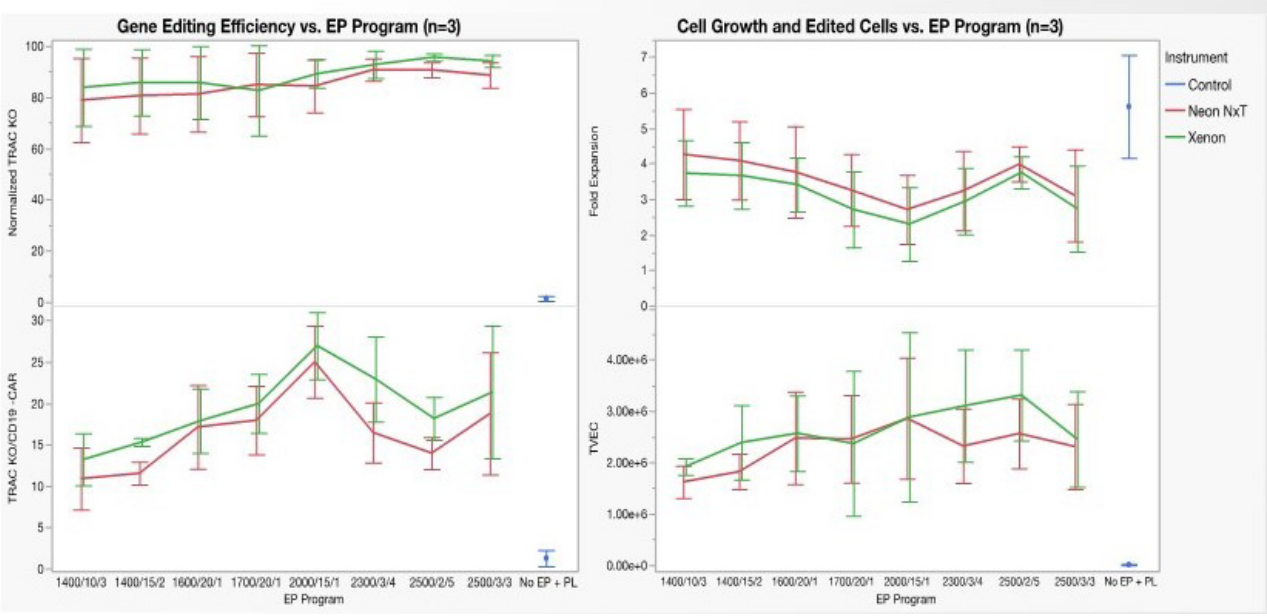

Figure S2. Genome editing performance in the Neon NxT 1-Channel Pipette (100 µL) and Gibco™ CTS™ Xenon™ SingleShot Electroporation Chamber (1 mL). Error bar is constructed using 1 standard deviation from the mean. TVEC: total viable edited cells.