HSCs Engineering

Non-viral genome editing in Hematopoietic Stem Cells (HSCs) for cell-therapy applications - from discovery to manufacturing

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Key takeaways

- ✓ **Optimizing electroporation conditions with the right parameters is crucial to ensure efficient and successful transfection of cells, maximizing the desired outcomes of gene editing or other applications.**
- ✓ **The Invitrogen™ Neon™ NxT Electroporation System scalability to the large-scale Gibco CTS™ Xenon™ electroporation system offers researchers a versatile platform for small-scale optimization, enabling a smooth transition from initial experiments to large-scale applications**
- ✓ **The cell processing and delivery instruments together with gene editing reagents and optimized gene delivery protocols developed through this work offers a robust solution for efficient and precise engineering of hematopoietic stem cells (HSCs) in various research and therapeutic applications.**

Introduction

Transfection of cells to alter their genotype or phenotype is crucial in a variety of life science applications. A diverse array of transfection methods are available, and selecting the best approach often depends on compatibility with the specific application. Electroporation is a physical transfection strategy that uses an electrical pulse to create temporary pores in cell membranes through which nucleic acids or proteins can pass into cells. It is a highly efficient and powerful tool that has been shown to have superior performance with gene editing-based payloads, such as CRISPR-Cas9 system and TALEN. Thermo Fisher offers research scale Invitrogen™ Neon™ NxT Electroporation System with either 10uL or 100uL kit and the GMP-compliant CTS™ Xenon™ Electroporation System with either the 1mL SingleShot or the closed single use 5-25 mL MultiShot consumable. Both instruments share the same core technology. In this study, we have demonstrated that these platforms are highly flexible and show compatibility with a wide range of mammalian cell types with different payloads. Moreover, we have demonstrated the scalability of electroporation conditions between the two instruments. Gene editing conditions on the Neon NxT System can be scaled up to the Xenon System, which is a platform designed for GMP-compliant cell therapy manufacturing.

HSCs were isolated from mobilized leukopak through positive selection. The electroporation parameters, payload concentration, culture conditions, culture period, payload type, and knock-out locus were systematically fine-tuned in the small-scale setting to identify the optimal conditions for genome editing. A minimum of three healthy donors were employed to ascertain the knock-out/knock-in efficiency at the B2M locus. Subsequently, we leveraged the insights gained from Neon NxT optimization to scale up the process using large-scale Xenon system. Remarkably, the identified conditions translated from Neon NxT to Xenon, exhibiting up to 90% knock-out efficiency and 10-20% knock-in efficiency in Xenon, with negligible impact on cell viability. These findings highlight the successful scalability and robustness of the optimized workflow across the two electroporation systems; enabling a smooth transition from bench to manufacturing scale for clinical application developments.

Invitrogen™ Neon™ NxT Electroporation System

Figure 1: Invitrogen™ Neon™ NxT Electroporation System

- **Proven transfection efficiency and cell viability**
  Pipette technology featured in >11,200 publications
- **Flexibility when you need it**
  Electroporation parameters, cell types, and applications
- **Save valuable research time**
  Simple 3-step workflow, intuitive user interface
- **Preserve sample**
  Minimize sample transfer loss and contamination risk
- **Scalability to large-volume GMP manufacturing**
  Gibco™ CTS™ Xenon™ Electroporation System*

Innovation highlights

- **Invitrogen™ TransfectionLab™ Cloud App**
  Experiment design with cloud connectivity
- **User-friendly**
  Intuitive interface

CTS™ Xenon™ Electroporation System

Highlights

System based on core Neon technology

Open platform that allows tailoring of electroporation parameters

- voltage, pulse width, pulse number (Neon & Xenon)
- pulse interval (Xenon only)

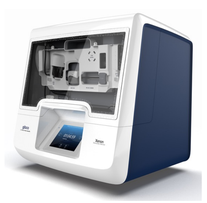
Modular design

Consistent performance across various scales
(100 uL – 1 mL – 5-25 mL)

Connectivity and tracking

- Software upgrade to enable compliance with 21 CFR part 11
- Cloud, OPC-UA* & DeltaV connectivity

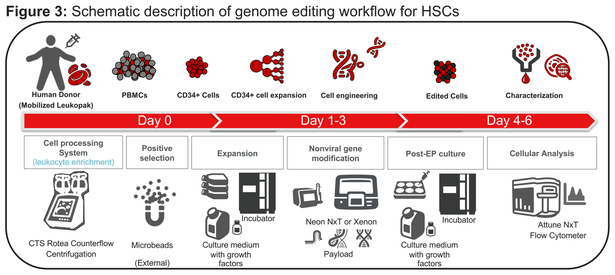
Figure 2: CTS Xenon Electroporation System



Materials and methods

Human Mobilized Leukopaks (mLPs) were used as the source of HSCs. Both regimens, RegF or RegH were used in this study. To obtain CD34+ cells from mLPs, initially CTS Rotea Counterflow System (ThermoFisher) was used to isolate PMBCs. CD34+ cells were isolated from PBMCs by positive selection. Pre-electroporation and post-electroporation cell were cultured in CellGenix SCGM medium supplemented with cytokines IL-6, SCF, FLT3L, IL-3 and TPO. Non-viral CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 (CRISPR-associated protein 9) system was used for genome editing. CD34+ cells were electroporated using Neon NxT Electroporation System and CTS Xenon Electroporation System (ThermoFisher). Invitrogen™ TrueCut™ Cas9 enzyme (ThermoFisher) along with Invitrogen™ TrueGuide™ sgRNA targeting B2M locus and dsDNA coding for GFP with flanking sequences to integrate within B2M locus were used for gene editing. Cells were characterized after electroporation using Attune™ NxT Flow Cytometer (ThermoFisher). The locus specific sgRNAs were designed using Invitrogen™ TrueDesign™ Genome Editor tool: (https://apps.thermofisher.com/apps/genome-editing-portal/). For electroporation, cells were resuspended in Neon NxT Genome Editing Buffer (GE buffer), which enhances homology-directed repair (HDR) pathway in repairing DNA double-strand breaks (DSBs) during gene editing to improve KO/KI efficiency. For flow cytometry analysis, cells were stained with CD34, CD38, CD45RA, CD90 and B2M antibodies.

Figure 3: Schematic description of genome editing workflow for HSCs



Results

24-well plate electroporation optimization with GFP plasmid

Cells were electroporated using the Neon NxT system with GFP plasmid mapped in **Figure 6A** using the conditions described in **Table 1**. Electroporation (EP) efficiency with different parameters is described in **Figure 6B**.

Figure 6: A. Plasmid map. **B.** EP parameter optimization results

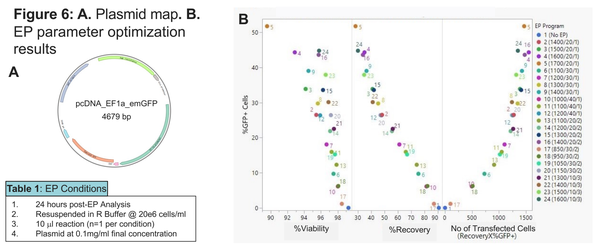


Table 1: EP Conditions

1.	24 hours post-EP Analysis
2.	Resuspended in R Buffer @ 20e6 cells/ml
3.	10 µl reaction (n=1 per condition)
4.	Plasmid at 0.1mg/ml final concentration

Payload optimization

The CRISPR/Cas9 payload was optimized for CD34+ cells and several sgRNAs (**Table 2A**) were designed and tested using the conditions described in **Table 2B**.

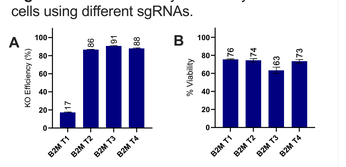
Table 2A. sgRNA sequences

sgRNA	Sequence	PAM
B2M T1	CGCGAGCACAGCTAAGGCCA	CGG
B2M T2	GGCCGAGATGTCTCGCTCCG	TGG
B2M T3	GGCCACGGAGCGAGACATCT	CGG
B2M T4	GAGTAGCGCGAGCACAGCTA	AGG

Table 2B: EP Conditions

1.	72 hours post-EP Analysis
2.	72 hours Pre-EP Culture
3.	Resuspended in GE Buffer @ 20e6 cells/ml
4.	100 µl reaction (n=3 per condition)
5.	sgRNA 30µg/ml and 120mg/ml Cas9
6.	Program 1700/20/1

Figure 7: A. KO efficiency **B.** Viability in CD34+ cells using different sgRNAs.



All the data represents % of parent population

24-well plate electroporation optimization with B2M T3 knock-out (KO)

Cells were electroporated with B2M T3 sgRNA using the conditions described in **Table 3**. The relationship between KO efficiency and cell recovery or total viable edited cells (TVEC) with different parameters is described in **Figure 8**.

Figure 8: EP parameter optimization results

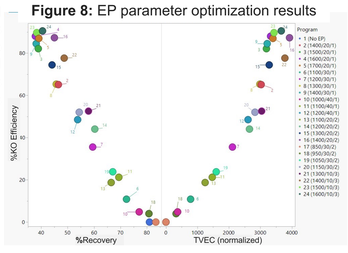


Table 3: EP Conditions

1.	72 hours post-EP Analysis
2.	Resuspended in GE Buffer @ 20e6 cells/ml
3.	10 µl reaction (n=3 per condition)
4.	sgRNA 30µg/ml and 120µg/ml Cas9
5.	72 hours Pre-EP Culture

Results

Electroporation cell concentration optimization

Cells were electroporated in different concentrations using the conditions described in **Table 4**. Two different donors of HSCs were used in this experiment.

Figure 9: The results obtained is shown as cell concentration vs **A.** KO in total live cells, %viability, %recovery, and normalized TVEC **B.** KO in CD34+ cells, %CD34+ cells, KO in CD34+CD90+ cells and % CD34+CD90+ cells

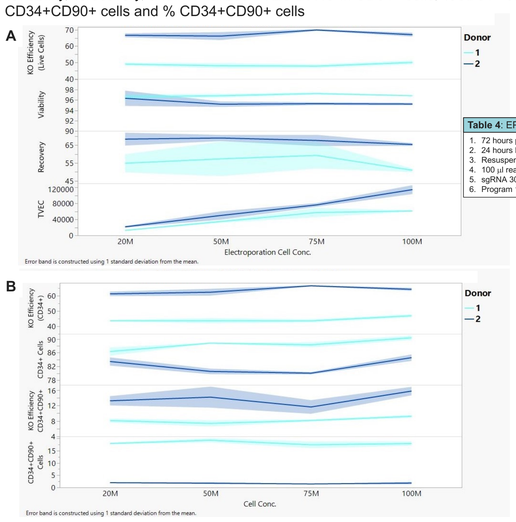


Table 4: EP Conditions

1.	72 hours post-EP Analysis
2.	24 hours Pre-EP Culture
3.	Resuspended in GE Buffer
4.	100 µl reaction (n=3 per condition)
5.	sgRNA 30µl/ml and 120µg/ml Cas9
6.	Program 1700/20/1

Pre-electroporation culture time optimization

Cells were cultured for different periods before electroporation using the conditions in **Table 5**. The effect of different pre-EP culture time on EP efficiency and stemness is analyzed.

Figure 9: Effect of *ex-vivo* expansion of HSCs on electroporation efficiency

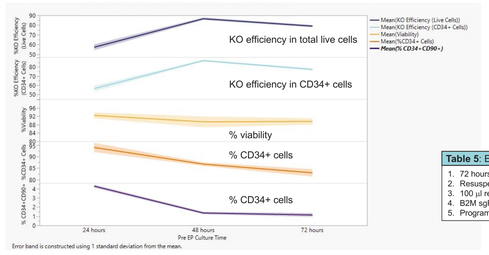


Table 5: EP Conditions

1.	72 hours post-EP Analysis
2.	Resuspended in GE Buffer @ 20e6 cells/ml
3.	100 µl reaction (n=3 per condition)
4.	B2M sgRNA 30µg/ml and 120µg/ml Cas9
5.	Program 1700/20/1

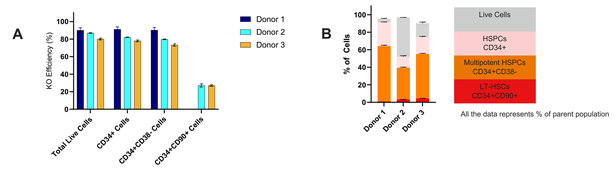
Donor to donor variation in KO efficiency

Three different donors of HSCs electroporated using the conditions in **Table 6**. have been described in Figure 10 A and Figure 10B

Table 6: EP Conditions

1.	72 hours post-EP Analysis
2.	48 hours Pre-EP Culture
3.	Resuspended in GE Buffer @ 20e6 cells/ml
4.	100 µl reaction (n=3 per condition)
5.	B2M sgRNA 30µg/ml and 120µg/ml Cas9
6.	Program 1700/20/1

Figure 10: Donor to donor variation in terms of **A.** KO efficiency in different subset of HSC progenitors. **B.** Percentage of different subset of HSC progenitors in total cells and viability.



All the data represents % of parent population

GFP knock-in (KI) at the B2M locus of HSCs

A donor DNA was designed targeting B2M locus for knocking-in GFP sequence at B2M locus using the electroporation conditions listed in **Table 7**.

Table 7: EP Conditions

1.	72 hours post-EP Analysis
2.	72 hours Pre-EP Culture
3.	Resuspended in GE Buffer @ 20e6 cells/ml
4.	100 µl reaction (n=3 per condition)
5.	sgRNA 30µg/ml and 120µg/ml Cas9
6.	80µg/ml Donor dsDNA
7.	Program 1700/20/1

Figure 11: A. Design of the B2M locus targeting eGFP flanked by 200 bp homology arm donor DNA construct . **B.** % KI efficiency. **C.** % viability.

Results

Scalability from research use platform, Neon NxT to GMP compatible Xenon electroporation system

Neon NxT 100µL tip and Xenon 1mL SingleShot electroporation chambers were compared for B2M KO efficiency in HSCs using two different programs and conditions listed in **Table 8**. Neon NxT condition is scalable to Xenon with certain EP programs that generates energy within certain range. In high energy programs Xenon electroporation results poor outcome. The formula to calculate total energy from the EP programs is described in **Table 9**.

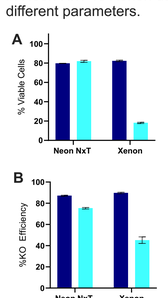
Table 8: EP Conditions

1.	72 hours post-EP Analysis
2.	Resuspended in GE Buffer @ 20e6 cells/ml
3.	100 µl & SS reaction (n=3 per condition)
4.	sgRNA 30ug/ml and 120µg/ml Cas9

Table 9: Total Energy for EP Parameters

Program	Total Energy	Formula
1700/20/1	5.78E+07	(Voltage)^2 X Pulse width X Pulse no
1400/20/2	7.84E+07	

Figure 9: A. % viable cells. **B. %** KO in Neon NxT vs Xenon using two different parameters.



Neon NxT to Xenon scalability for KO/KI application

KO/KI Efficiency in Neon NxT 100µL reaction and and Xenon-1mL SingleShot were compared for B2M Targeted GFP KI in different subsets of HSCs using the conditions listed in **Table 10**.

Figure 10: Neon NxT vs Xenon KO/KI efficiency **A**. In total live cells. **B.** In CD34+ cells. **C**. In CD34+CD38- cells **D**. in CD34+CD90+ cells. **E**. percentage of different subsets of HSCs at the end of day 5. **F**. Total viable edited cells (TVEC) at the end of day 5 in Neon NxT (100µl reaction) vs Xenon (1ml SingleShot).

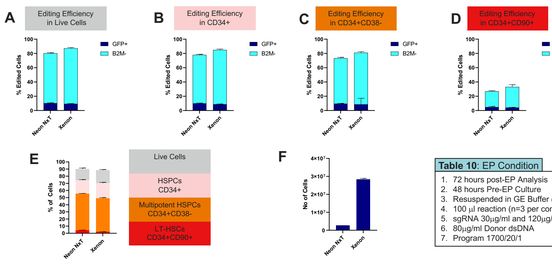


Table 10: EP Condition

1.	72 hours post-EP Analysis
2.	48 hours Pre-EP Culture
3.	Resuspended in GE Buffer @ 20e6 cells/ml
4.	100 µl reaction (n=3 per condition)
5.	sgRNA 30µg/ml and 120µg/ml Cas9
6.	80µg/ml Donor dsDNA
7.	Program 1700/20/1

Conclusions

Electroporation process scales seamlessly from research and discovery on the Neon NxT System to large-volume on the Xenon Electroporation System. The Neon NxT system's scalability to the large-scale CTS Xenon electroporation system offers researchers a versatile platform for small-scale optimization, enabling a smooth transition from initial experiments to large-scale applications, thereby maximizing the efficiency and reproducibility of the electroporation process. While further optimization is needed for Xenon-HSCs gene editing, it is a significant step-forward in overcoming scale-up challenges in HSCs gene therapy manufacturing.

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