Workflow Optimization of Large-Scale Genome Editing of Hematopoietic Stem/Progenitor Cells (HSPCs)

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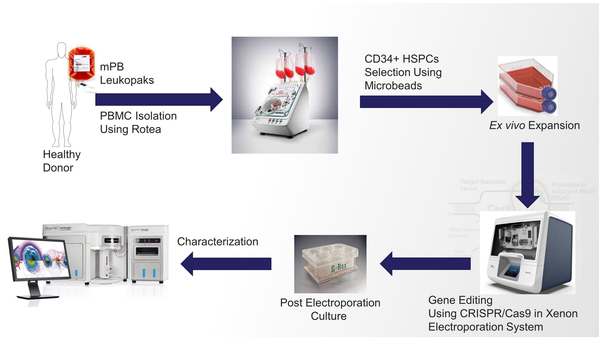
Introduction

Hematopoietic stem cell gene therapy (HSC GT) has generated significant interest in recent years to treat genetic blood cell disorders. The use of allogeneic hematopoietic stem cells (HSCs) has become clinical standard in this field yet it bears considerable risks for the patient for potential immunologic complications. Autologous HSC GT is a promising approach that potentially avoid these limitations and thus could be safer for the patients [1,2].

In this work, we describe a workflow optimization for the genome engineering of hematopoietic stem/progenitor cells. We have used the Gibco™ CTS™ Rotea™ Counterflow Centrifugation System for the CD34+ cell pre-isolation leukocyte enrichment from mobilized leukopak [3]. CD34 tagged microbeads and column-based method was used for the positive selection of HSPCs. We have optimized a culture condition for the CD34+ cells using the culture media developed by Sei et al. which supports superior expansion of human HSCs [4]. A flow panel has been designed for the phenotyping of HSCs/progenitor cells to identify the CD34+, CD38-, CD45- and CD90+ population. Initially, CD34+ cells were transfected with GFP plasmids using Invitrogen™ Neon™ Transfection system. In order to optimize the electroporation (EP) condition for the HSPCs, we have electroporated CD34+ cells with GFP Plasmids using a set of optimization electroporation parameters with varying voltages, pulse widths and pulse numbers. CRISPR/Cas9 technique have been used for the genetic engineering of the HSPCs. We have optimized a set of sgRNAs to obtain the best knock-out efficiency targeting B2M gene in CD34+ cells. Optimal EP condition was further evaluated using the best performing sgRNA. We have designed and synthesized a double stranded donor DNA for the GFP Knock-In at the B2M gene knocked-out site of the CD34+ cells. We have obtained some promising data from our workflow optimization which could potentially lead to a large-scale genetic modification of CD34+ cells using Gibco™ CTS™ Xenon™ Electroporation System.

There are already numerous evidences that autologous HSC GT could be a promising strategy for the future [2, 5]. Despite all the recent progress, the clinical application of this HSC gene therapy remains extremely challenging. CRISPR/cas9 based autologous HSC GT will probably require considerable optimization and will require further safety evaluations. Despite all the challenges ahead, we strongly deem that, autologous HSC GT will facilitate future advancements in treating genetic blood disorders [2,5].

Figure 1. HSCs Gene Therapy Workflow



Materials and methods

CRISPR sgRNAs were designed using Invitrogen™ TrueDesign™ Genome Editor tool (https://apps.thermofisher.com/apps/genome-editing-portal/). Mobilized leukopak was purchased from ALLCELLS (mLP, RegF, FR). To obtain CD34+ cells from mLP, initially RBC elutriation method was used in CTS Rotea Counterflow System to isolate PMBCs. Human CD34+ cells were isolated from PBMCs by QuadroMACS™ LS columns (Miltenyi Biotec) using CD34 microbeads. CD34+ cells were electroporated using Neon Transfection System and Gibco CTS Xenon Electroporation System. Cells were characterized after electroporation using Attune NxT Flow Cytometer.

Results

CD34+ Cell Isolation from Mobilized Leukopak

Gibco CTS Rotea Counterflow Centrifugation System has been used to isolate lymphocytes from mobilized leukopak. Two different Rotea protocols were used to separate RBCs and monocytes fractions from the lymphocyte fraction. Almost all the CD34+ cells were recovered in the lymphocyte fraction of the RBC elutriation protocol. Miltenyi CD34 microbeads and LS columns were used to isolate CD34+ cells from the lymphocyte, monocyte and RBC fractions of the mobilized leukopak.

Figure: 2: Schematic of Rotea Centrifugation System

Figure 3. % CD34+ Cell Recovery from different Rotea Protocols

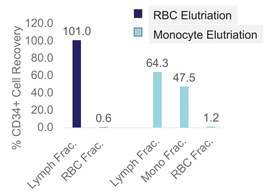
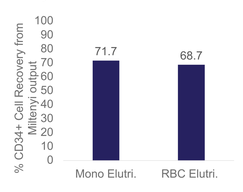


Figure 4. %CD34+ Cell Recovery from Miltenyi Columns



Phenotypic Analysis of CD34+ Cells

Cells were analyzed for their phenotype using Attune™ Nxt Flow cytometer. CD34+ cells were stained with CD34, CD38, CD45RA and CD90 antibodies.

Figure 5: lineage hierarchies of human hematopoietic stem cells

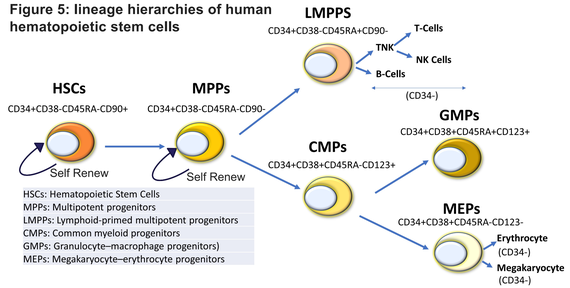
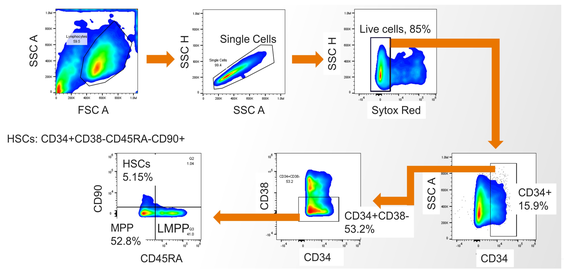


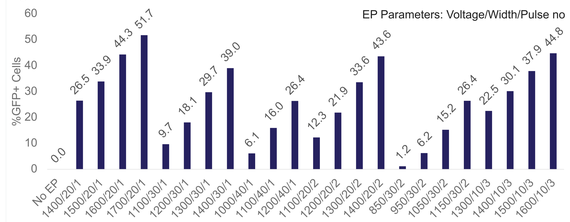
Figure 6. Flow panel for the phenotypic analysis of HSPCs.



Results

EP Parameter Optimization with GFP Plasmid

Figure 7. % GFP+ cells in CD34+ cells



Payload Optimization

The CRISPR/Cas9 payload was optimized for CD34+ cells several sgRNAs were designed and tested using Neon EP system prog. 24 (1600V/10ms/3-pulses).

Figure 8. KO efficiency in CD34+ cells using different sgRNAs.

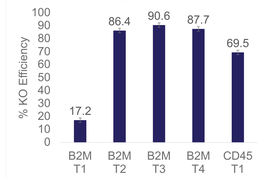


Table 1. sgRNA sequences for the KO experiments.

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

EP Parameter Optimization with B2M T3 KO

Neon 24 well plate optimization was performed by transfecting GFP Plasmids into CD34+ cells.

Figure 9. B2M KO efficiency in the total live cells and CD34+ cells.

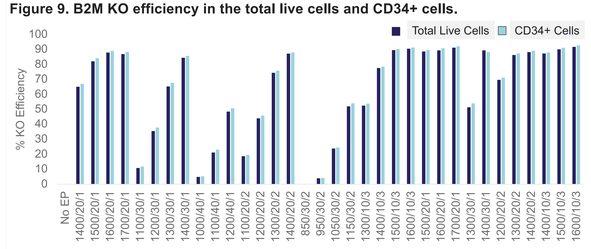
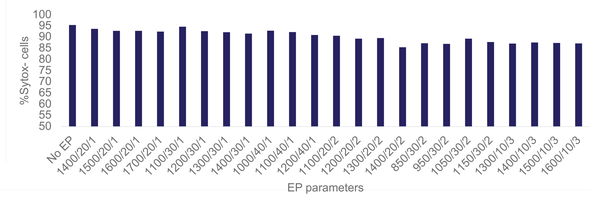


Figure 10. Viability of cells in 24 well Plate EP Optimization with B2M KO.



Results

Neon vs Xenon performance for B2M KO in CD34+ cells

Neon-100uL tip and Xenon-1mL SingleShot were compared for B2M KO efficiency in HSCs

Figure 11. B2M KO efficiency in Neon vs Xenon EP system

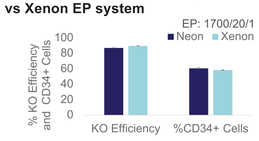
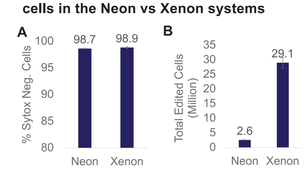


Figure 12. A. Viability B. Total edited cells in the Neon vs Xenon systems



Conclusions

Electroporation process scales seamlessly from research and discovery on the Neon Transfection System to large-volume on the Xenon Electroporation System. In this Cas9/gRNA genome editing experiment, knock-out efficiency on the Xenon system was comparable to the Neon system for B2M KO in CD34+ Cells. 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.

References

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Acknowledgements

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