TECHNICAL BULLETIN

Genome Editing of Human CD34+ Hematopoietic Stem and Progenitor Cells Using the ArciTect™ CRISPR-Cas9 System and StemSpan™ Media

Introduction

CRISPR-Cas9, an RNA-guided genome editing technology, is revolutionizing cell biology due to the ease and efficiency by which it enables genetic manipulation of mammalian cells. The ability to genetically manipulate hematopoietic stem and progenitor cells (HSPCs) in particular has significantly advanced our understanding of the mechanisms that regulate hematopoiesis and is contributing to the development of novel cellular therapies. This Technical Bulletin provides instructions for CRISPR-Cas9 genome editing and maintenance of human CD34+ cells, including optimized pre- and post-editing culture conditions and methods to evaluate genome editing efficiency. This document also includes a case study detailing optimization of pre- and post-editing culture conditions to support a high efficiency genome editing workflow in CD34+ HSPCs.

While CRISPR-Cas9 technology has been successfully applied in numerous cell lines, its application in primary human CD34+ cells has been hampered by challenges in efficient expression and delivery of CRISPR-Cas9 components. Early attempts to apply CRISPR-Cas9 for genome editing in CD34+ HSPCs used plasmids for Cas9 and guide RNA (gRNA) expression, which resulted in low targeting efficiency and high toxicity.[1] These expression systems also pose concerns about safety in clinical translation due to the risk of unwanted genetic mutations and immunogenicity.[2]

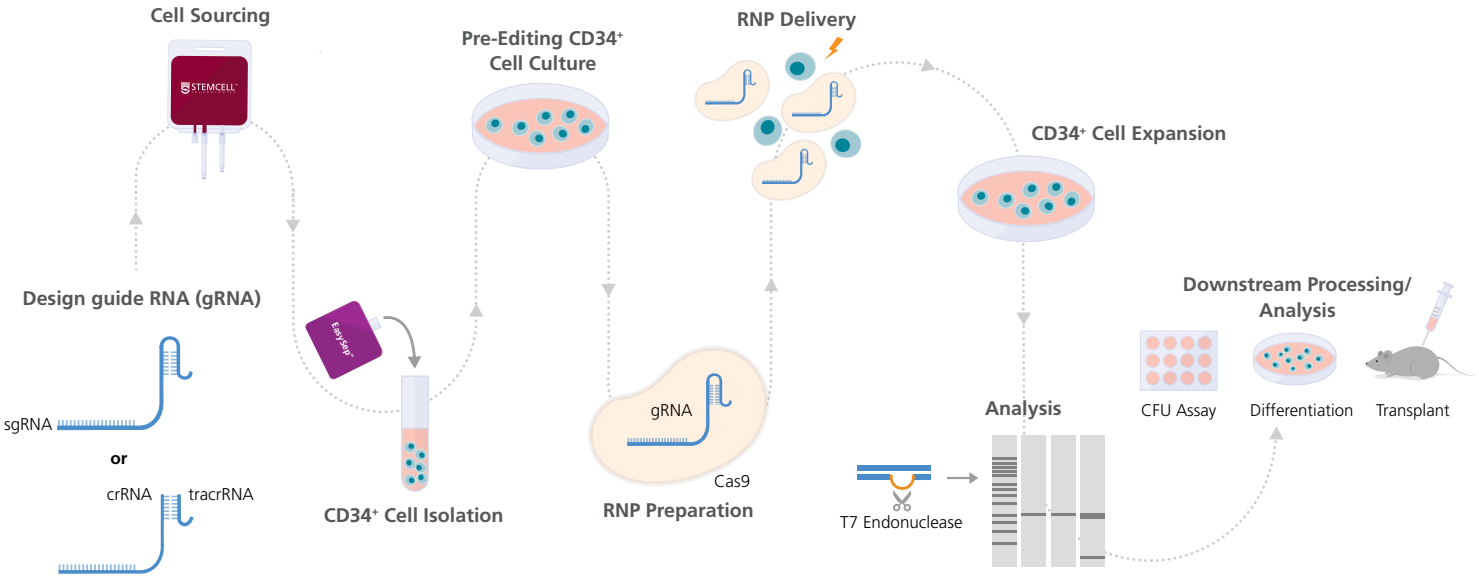

Figure 1. Experimental Workflow for CD34+ Human Hematopoietic Stem and Progenitor Cell (HSPC) Genome Editing

The ArciTect™ sgRNA (single guide RNA) or ArciTect™ crRNA (CRISPR RNA) sequences can be designed using the CRISPR Design Tool once a target locus for editing is identified. Human CD34+ primary cells can be purchased or isolated from a number of sources, such as cord blood, bone marrow, and mobilized peripheral blood, using column-free cell separation technology, including EasySep™. Next, CD34+ cells are cultured for 2 days prior to ribonucleoprotein (RNP) delivery in StemSpan™ SFEM II medium (Catalog #09605) supplemented with StemSpan™ CD34+ Expansion Supplement (Catalog #02691) in the presence or absence of the small molecule UM729 (Catalog #72332; prepared to a final concentration of 1 µM). The ArciTect™ CRISPR-Cas9 RNP is then prepared and delivered into CD34+ cells using electroporation, and cells are plated in StemSpan™ SFEM II supplemented with StemSpan™ CD34+ Expansion Supplement post-electroporation. Editing efficiency can be analyzed using ArciTect™ T7 Endonuclease I Kit (Catalog #76021) or flow cytometry, if the experimental design permits. Colony-forming unit (CFU) assays may also be used to assess editing efficiency by plucking and isolating DNA from individual colonies, followed by PCR analysis. Progenitor cell function may be measured post-modification by in vitro CFU assays. Additionally, edited CD34+ cells can be further cultured in conditions stimulating lineage-specific differentiation by combining StemSpan™ media with expansion supplements designed for the expansion and differentiation of progenitors to erythroid, myeloid, megakaryocyte, natural killer (NK), or T cell lineages.

More recently, electroporation of HSPCs with ribonucleoprotein (RNP) complexes made from recombinant Cas9 protein and synthetic chemically modified gRNAs has achieved high efficacy across a number of targets.[3-5] The RNP-based ArciTect™ CRISPR-Cas9 expression system includes custom synthetic chemically modified gRNA (sgRNA or crRNA) and purified Cas9 protein to fully support genome editing of human HSPCs. In addition to effective genome editing tools, manipulation of HSPCs requires standardized culture conditions for optimal editing efficiency and progenitor cell expansion. StemSpan™ media supports standardized culture conditions for high-efficiency genome editing, while allowing researchers the flexibility to choose from serum-free, xeno-free, and animal component-free formulations to establish specific conditions for their experiments.

The following protocol is for the preparation and subsequent delivery of CRISPR-Cas9 RNP complexes into human CD34$^+$ cells via electroporation using either the Neon® Transfection System or Lonza® 4D Nucleofector™ X Unit (Figure 1). It is important to first isolate CD34$^+$ cells, as their frequency in cord blood (CB) or other hematopoietic tissues is low, and cell quality and viability can be variable between different primary samples. Despite the small sample volume and relatively low frequency of CD34$^+$ cells (typically 0.1 - 1% of nucleated cells), CB is a good source of HSPCs for techniques such as gene editing. CD34$^+$ cells can be isolated from whole CB using EasySep™ Human Cord Blood CD34 Positive Selection Kit II (Catalog #17896) in a simple, two-step procedure (Document #DX20181). This protocol was validated using both fresh and cryopreserved CB-derived CD34$^+$ cells and may need to be further optimized for CD34$^+$ cells isolated from other sources. Following the protocol, we present a comprehensive study of pre- and post-editing culture conditions that led to identification of optimal conditions for efficient CD34$^+$ genome editing and maintenance of the HSPC phenotype.

Delivery of CRISPR-Cas9 Ribonucleoprotein Complexes into Human CD34$^+$ HSPCs Using Electroporation

Materials Required

Product	Catalog #
ArciTect™ sgRNA[†] or ArciTect™ crRNA[†] ArciTect™ tracrRNA Kit • ArciTect™ tracrRNA • ArciTect™ Annealing Buffer (5X)	200-0013 or 76010/76011/76012 76016/76017/76018
ArciTect™ Cas9 Nuclease*	76002/76004
EasySep™ Human Cord Blood CD34 Positive Selection Kit II	17896
StemSpan™ SFEM II	09605
StemSpan™ CD34$^+$ Expansion Supplement (10X)	02691
Falcon® 24-Well Flat-Bottom Plate, Tissue Culture-Treated	38021
Neon® Transfection System 10 µL Kit • Resuspension Buffer T • Electrolytic Buffer E • 10 µL Neon® pipette tips or P3 Primary Cell 4D-Nucleofector™ X Kit S • 16-well Nucleocuvette™ Strips • P3 Primary Cell Nucleofector™ Solution • Supplement 1	Thermo Fisher MPK1025 or Lonza V4XP-3032
Costar® 24-Well Flat-Bottom Plate, Tissue Culture-Treated	38017
DNase- and RNase-free microcentrifuge tubes	38089
Falcon® Conical Tubes, 15 mL	38009
Heating block or thermocycler	---

[†]ArciTect™ sgRNA is only available in Australia, Austria, Belgium, Canada, China, Denmark, Finland, France, Germany, Iceland, Ireland, Luxembourg, the Netherlands, New Zealand, Norway, Poland, Portugal, Singapore, Spain, Sweden, Switzerland, the United Kingdom, and the United States.

The materials required are indicated on a per-well basis. These values will need to be scaled up for the actual number of wells in an experiment. Multiple gRNA targeting sequences are often tested when targeting a new gene, as different gRNAs can exhibit a range of targeting/editing efficiencies at both on- and off-target sites.

A. Isolation of CD34+ Cells from Human Cord Blood

Isolate human CD34+ HSPCs from fresh (< 48 hours old) umbilical cord blood (CB) using EasySep™ Human Cord Blood CD34 Positive Selection Kit II (Catalog #17896). Refer to the Product Information Sheet for details.

Optional: *Use frozen primary human CB CD34+ cells (e.g. Catalog #70008).*

1. Count cells and adjust to 1 - 3 x 10^5 cells/mL in StemSpan™ SFEM II supplemented with StemSpan™ CD34+ Expansion Supplement. Add 1 mL cell suspension per well of a 24-well plate.

   Note: *UM729 (Catalog #72332) may be added at a concentration of 1 µM to increase CD34+ cell expansion.*

2. Incubate at 37°C and 5% CO_2 for 48 hours.

B. Preparation of ArciTect™ sgRNA and ArciTect™ crRNA and ArciTect™ tracrRNA Stock Solutions

1. Briefly centrifuge the vials before opening.

2. Add nuclease-free water to each vial to give a final concentration of 200 µM (crRNA and tracrRNA) or 100µM (sgRNA), as indicated in Table 1 and Table 2, respectively.

Table 1. Preparation of 200 µM* ArciTect™ crRNA or ArciTect™ tracrRNA

ArciTect™ crRNA or ArciTect™ tracrRNA	Volume of Nuclease-Free Water (µL)
2 nmol	10
5 nmol	25
10 nmol	50
20 nmol	100

**200 µM is equal to 200 pmol/µL*

Table 2. Resuspension Volume for 100 µM* ArciTect™ sgRNA

ArciTect™ sgRNA	Volume of Nuclease-Free Water (µL)
4 nmol	40

**100 µM is equal to 100 pmol/µL*

3. Mix thoroughly. If not used immediately, aliquot and store at -20°C for up to 6 months or at -80°C for longer than 6 months. After thawing the aliquots, use immediately. Do not re-freeze.

C. Annealing of crRNA:tracrRNA Duplexes

RNP complexes can be prepared using either the two-part crRNA:tracrRNA gRNA, which requires pre-annealing (see below), or sgRNA. If working with sgRNA, skip this section and continue to section D.

1. Prepare an 80 µM crRNA:tracrRNA solution by combining components in a DNase- and RNase-free microcentrifuge tube as indicated in Table 3. Volumes are sufficient for 4 (using the 4D-Nucleofector™ system) to 17 (using the Neon® system) electroporation reactions; adjust as required. Mix thoroughly.

Table 3. Preparation of 80 µM (80 pmol/µL) crRNA:tracrRNA Duplex

Reagent	Volume (µL)
200 µM ArciTect™ crRNA	4
200 µM ArciTect™ tracrRNA	4
ArciTect™ Annealing Buffer (5X)	2
Total	**10**

2. In a thermocycler or heating block, incubate crRNA:tracrRNA mixture at 95°C for 5 minutes followed by 60°C for 1 minute. Cool to room temperature (15 - 25°C). If not used immediately, store at -80°C for up to 1 month.

D. Preparation of CD34+ Cell Suspension for Electroporation

1. Add 1 mL StemSpan™ SFEM II supplemented with StemSpan™ CD34+ Expansion Supplement to one well of a 24-well culture plate per electroporation condition. Incubate at 37°C and 5% CO_2 to pre-warm.

2. Add CD34+ cell suspension (from section A) to a 15 mL conical tube. Centrifuge at 300 x *g* for 5 minutes. Count cells. Prepare 75,000 - 100,000 cells per electroporation reaction.

   Note: *The number of cells per electroporation reaction can be reduced to 30,000 cells, if necessary.*

3. Wash cells once with sterile PBS prior to electroporation. Centrifuge at 300 x *g* for 5 minutes.

E. Preparation of ArciTect™ CRISPR-Cas9 RNP Complex Mix for Electroporation

1. To prepare the RNP Complex Mix, combine the components in the order listed in Table 4 in a microcentrifuge tube. Adjust component volumes according to the desired number of transfections.
2. Mix thoroughly.

Table 4. Preparation of RNP Complex Mix for Electroporation

Component	sgRNA		crRNA:tracrRNA	
	Neon® Electroporation	4D-Nucleofector™ X Electroporation	Neon® Electroporation	4D-Nucleofector™ X Electroporation
	Volume per reaction (µL)			
Resuspension Buffer T	6.00	--	6.04	--
P3 Primary Cell Nucleofector™ Solution with Supplement 1	--	6.00	--	1.25
ArciTect™ Cas9 Nuclease* (4 µg/µL; 25 µM)	0.90	0.90	0.90	3.75
100 µM sgRNA	0.60	0.60	--	--
80 µM gRNA (prepared in section C)	--	--	0.56	2.50
Total	**7.50**	**7.50**	**7.50**	**7.50**

Note: *May require optimization with different cell sources. A 1:2 (shown) to 1:8 molar ratio of Cas9 to guide gRNA is recommended.*

Note: *These values are for a single electroporation reaction and include pipetting error for Neon® electroporation. Scale up as necessary.*

3. Incubate the RNP Complex Mix at room temperature (15 - 25°C) for 10 - 20 minutes.

F. Electroporation of CD34+ HSPCs with RNP Complex

Perform electroporation using either the Neon® Transfection System (section a) or the Lonza® 4D-Nucleofector™ X Unit (section b).

a) Electroporation Using Neon® Transfection System

1. Aspirate supernatant from the cell pellet (prepared in section D). Resuspend cells in 7.5 µL of Resuspension Buffer T per electroporation condition and pipette up and down to mix.
2. Transfer 7.5 µL of the cell suspension to each 7.5 µL of RNP Complex Mix (prepared in section E) and pipette up and down gently to mix, trying not to form air bubbles.
3. Using a 10 µL Neon® pipette tip, draw up 10 µL of the mixture, check to see if the capillary is free of bubbles, and place into the electroporation chamber containing 3 mL of Electrolytic Buffer E.

Note: *If air bubbles are present in the tip when the cells are electroporated, cell viability and transfection efficiency will be significantly reduced.*

4. Electroporate the mixture using the settings in Table 5.

Note: *Refer to the manufacturer's instructions on electroporation. Electroporation conditions may require optimization for different cell sources.*

Table 5. Recommended Electroporation Conditions for HSPCs Using a Neon® Transfection System

Electroporation Parameter	
Electrical potential	1600 V
Pulse width	10 ms
Number of pulses	3

b) Electroporation Using Lonza® 4D-Nucleofector™ X Unit

1. Aspirate supernatant from the cell pellet prepared in section D. Resuspend cells in 17.5 µL of P3 Primary Cell Nucleofector™ Solution + Supplement 1 per electroporation condition and pipette up and down to mix.
2. Transfer 17.5 µL of the cell suspension to each 7.5 µL RNP Complex Mix (prepared in section E) and pipette up and down gently to mix, trying not to form air bubbles.

Note: *If air bubbles are present in the cuvette when the cells are electroporated, cell viability and transfection efficiency will be significantly reduced.*

3. Transfer 25 µL of the cell suspension + RNP Complex Mix to one well of the 16-well Nucleocuvette™ Strip. Gently tap or use a pipette tip to ensure no air bubbles are present.
4. Set the Lonza® 4D-Nucleofector™ X Unit to program code DZ-100.
5. Place the Nucleocuvette™ Strip in the Shuttle device of the 4D-Nucleofector™ X Unit, select OK to load the strip, and select Start to begin electroporation.

G. Post-Editing Culture

1. Immediately after electroporation, transfer cells to the warm (37°C) plate prepared in section D step 1.
2. Incubate at 37°C and 5% CO_2 for 48 - 96 hours.
3. Harvest cells for assessment of genome editing efficiency.

Note: *Genomic DNA can be amplified by PCR using primers flanking the target region and ArciTect™ High-Fidelity DNA Polymerase Kit (Catalog #76026), followed by sequencing of the PCR products. Alternatively, ArciTect™ T7 Endonuclease I Kit (Catalog #76021) can be used to assess editing efficiency (% INDEL formation) following PCR amplification.*

Case Study: Evaluation of Optimal Culture Methods for High-Efficiency Editing in CD34+ Human Hematopoietic Stem and Progenitor Cells (HSPCs)

To demonstrate genetic knockout with the ArciTect™ CRISPR-Cas9 system, we targeted two genes: *CD45* and β2 Microglobulin (*B2M*) in CD34+ HSPCs. Loss of function due to CRISPR-Cas9-mediated INDEL generation at either locus is readily identifiable by the lack of CD45 or MHC-I expression on the cell surface. Using these experimental systems, we also tested multiple pre- and post-editing media to identify optimal conditions for efficient genome editing and maintenance of the HSPC phenotype.

We first optimized electroporation conditions by targeting CD45, as previously described.[4] CD34+ cells were isolated from human CB using EasySep™ Human CD34 Positive Selection Kit II. On day 0, approximately 80 - 95% of the cells were CD34+CD45+ (data not shown). The cells were cultured in StemSpan™ SFEM II supplemented with StemSpan™ CD34+ Expansion Supplement for 2 days prior to CRISPR-Cas9 RNP delivery, as post-isolation culture with cytokine exposure for 48 hours prior to RNP delivery has been shown to increase transfection efficiency.[4] After 2 days, cells were electroporated with RNP complexes containing crRNA:tracrRNA duplexes or sgRNA targeting the *CD45* gene, using either the Neon® Transfection System or the Lonza® 4D-Nucleofector™ X Unit. The electroporation settings are described in the protocol section of this document (Section F). Four days after electroporation, cell viability and expression of CD34 and CD45 were assessed using flow cytometry (Figure 2). RNP-electroporated samples exhibited loss of CD45 expression on the cell surface (Figure 2A, B; > 50% average knockout efficiency across all tested donors), while the percentage of CD34-expressing cells were unaffected by RNP delivery (Figure 2C).

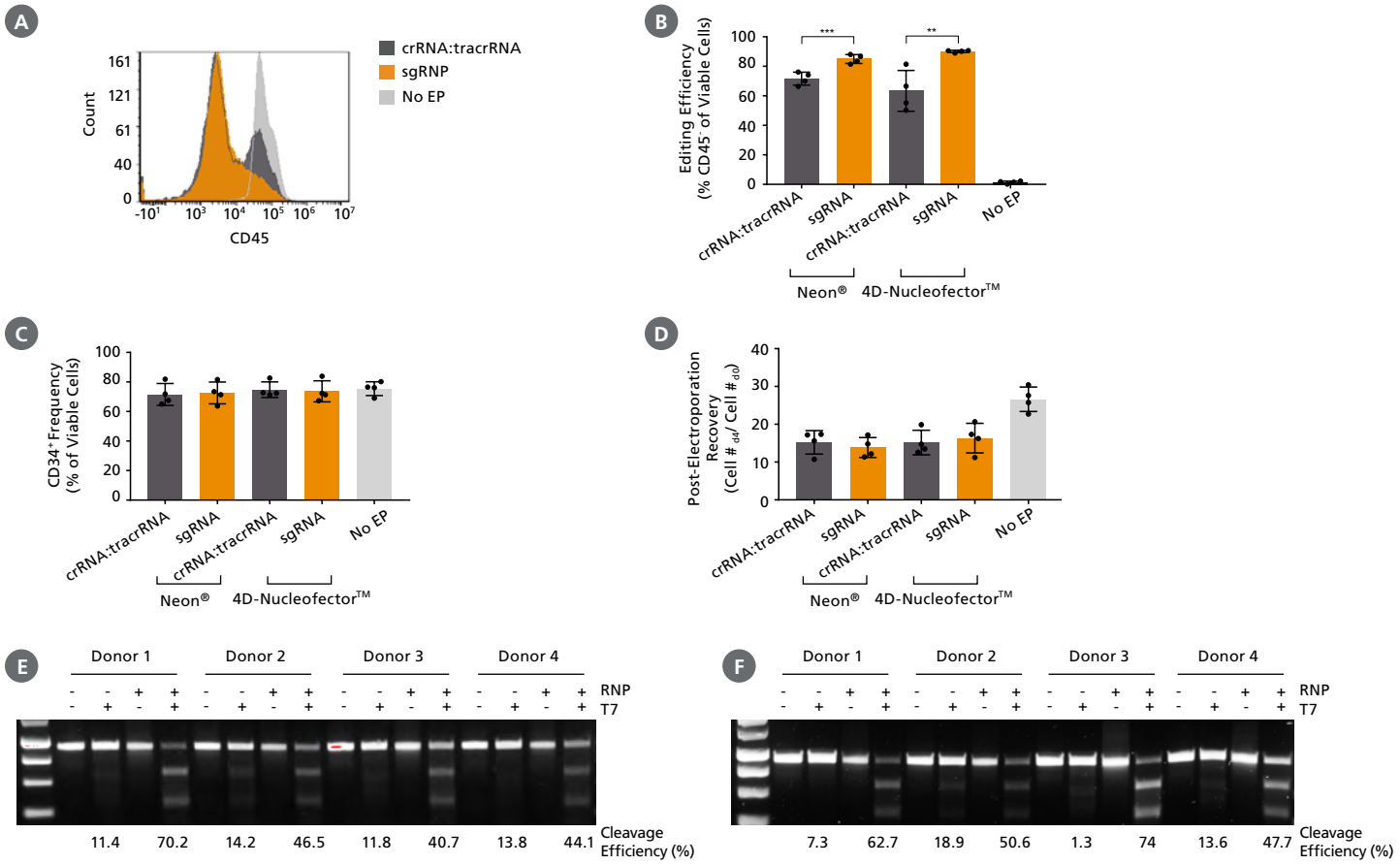

Figure 2. High-Efficiency Genome Editing in CD34+ Cells Using the ArciTect™ CRISPR-Cas9 System

(A) Representative histogram of CD45 flow cytometry data from CD34+ cells cultured in StemSpan™ SFEM II plus StemSpan™ CD34+ Expansion Supplement, 4 days after delivery of CRISPR-Cas9 RNP complexes containing either crRNA (dark grey bars) or sgRNA (orange bars) targeting CD45. Non-electroporated (No EP; light grey bars) CD34+ cells served as a control. (B) CD45 editing efficiency and (C) the percentage of CD34+ cells were monitored by flow cytometry using a fluorophore-conjugated CD45 antibody and a fluorophore-conjugated CD34 antibody, respectively, 4 days after electroporation with CRISPR-Cas9 RNP complexes containing Cas9 Nuclease and either crRNA:tracrRNA duplexes or sgRNA using either the Neon® Transfection system or Lonza® 4D-Nucleofector™ X Unit. (D) Post-electroporation cell recovery was measured by dividing the total number of cells collected 4 days post electroporation by the number of electroporated cells. Each data point represents an individual donor; n = 4 - 7 donors, **P < 0.01, ***P < 0.001. Error bars represent standard deviation. (E, F) Genome editing (cleavage) efficiency was assessed 4 days post electroporation with CRISPR-Cas9 RNP complexes containing Cas9 Nuclease and crRNA:tracrRNA duplexes using the Neon® Transfection system (E) or Lonza® 4D-Nucleofector™ system (F) in CD34+ cells using ArciTect™ T7 Endonuclease I Kit. No electroporation: - RNP; RNP electroporated: + RNP.

Post-electroporation cell recovery, as measured by the total number of cells present after 4 days of culture divided by the total number of cells electroporated per condition, was negatively impacted by the delivery of RNP complexes containing crRNA:tracrRNA duplexes or sgRNA compared to non-electroporated (no EP) samples (Figure 2D). This is consistent with previous reports of delayed HSPC proliferation in response to genome editing with programmable nucleases including CRISPR-Cas9.[6] INDEL generation was confirmed by the T7 Endonuclease I Assay (Figure 2E, F).

Next, we targeted a second locus in CD34$^+$ cells to validate the optimized protocol and evaluate the functional impact of genome editing on HSPC lineage commitment using the colony-forming unit (CFU) assay. We chose to target *B2M*, which encodes the accessory chain of major histocompatibility complex (MHC) class I molecules, using a single ArciTect™ crRNA sequence targeting exon 1 of *B2M*, as previously described.[7] This strategy enabled monitoring of editing efficiency by flow cytometry with a fluorophore-conjugated MHC-I antibody, since expression of *B2M* is required for surface expression of MHC-I.[8,9] CD34$^+$ cells were electroporated with RNP complexes using the Neon® Transfection System. Four days after electroporation, cell viability and expression of CD34 and MHC-I were assessed using flow cytometry (Figure 3). RNP-electroporated samples exhibited loss of MHC-I expression on the cell surface (Figure 3A, B; > 50% knockout efficiency across 10 donors). The percentage of CD34-expressing cells and cell viability were unaffected by RNP delivery (Figure 3C and data not shown, respectively). Both RNP-electroporated (RNP) and cells electroporated with Cas9 only (Cas9 Only) conditions exhibited reduced post-electroporation cell recovery relative to non-electroporated (no EP) controls, suggesting that electroporation, rather than loss of target gene expression, is the primary contributor to reduced CD34$^+$ cell recovery/growth in the genome editing workflow (Figure 3D).

Four days after electroporation, a portion of the cells were plated in methylcellulose medium (MethoCult™ H4435 Enriched; Catalog #04435) and a 14-day CFU assay was performed (Figure 3E, F). RNP-electroporated cells showed a similar composition of erythroid (BFU-E), granulocyte/macrophage (CFU-GM), and multilineage (CFU-GEMM) progenitor cells as compared to non-electroporated and Cas9-only conditions (Figure 3E), and plating efficiency was similar between conditions (Figure 3F). Together, this suggests that genome editing of B2M with the ArciTect™ system has minimal impact on the function of myeloid, erythroid, and granulocytic progenitor cells.

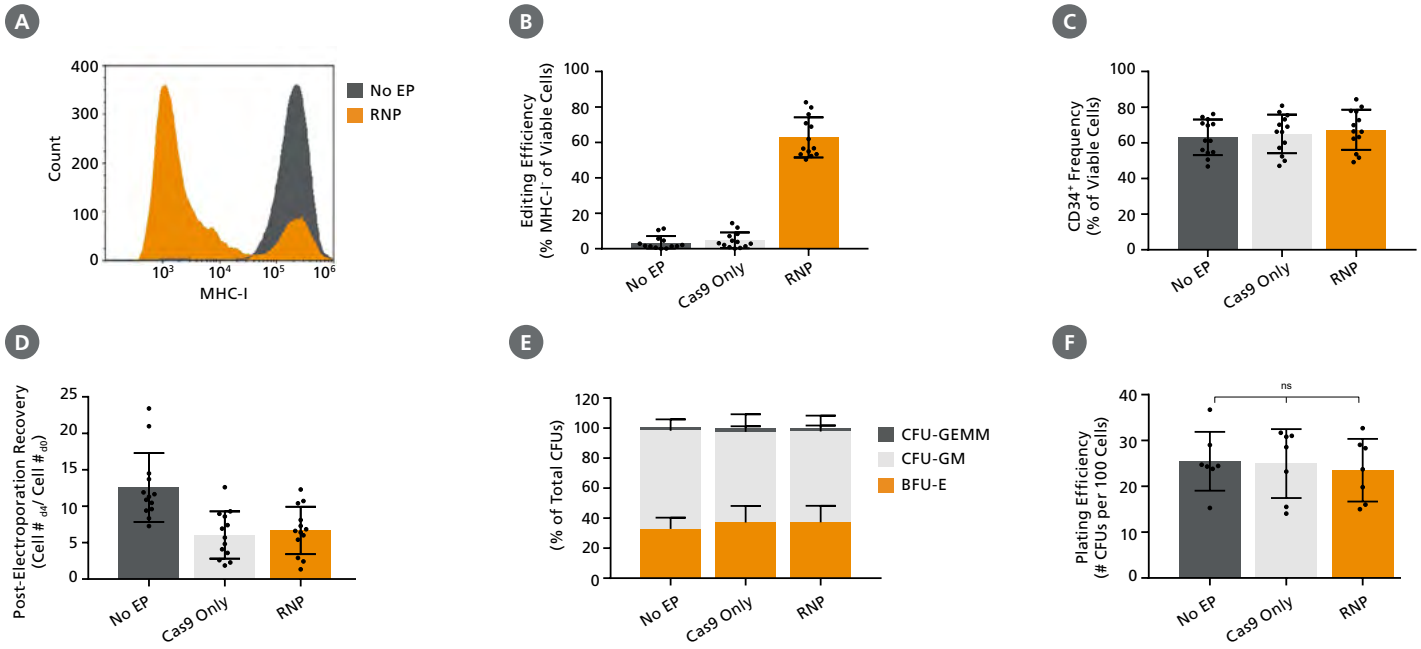

Figure 3. Genome-Edited CD34$^+$ Cells Retain Colony-Forming Potential

(A) Representative histogram of MHC-I flow cytometry data from RNP-electroporated (RNP, orange) and non-electroporated (no EP; dark grey) CD34$^+$ cells cultured in StemSpan™ CD34$^+$ Expansion Supplement, as described in section A of the protocol, 4 days after delivery of the CRISPR-Cas9 RNP complexes containing containing gRNA targeting B2M.(B) *B2M* editing efficiency (% MHC-I$^-$ viable cells) and (C) the percentage of CD34$^+$ cells were monitored by flow cytometry using a fluorophore-conjugated MHC-I antibody and a fluorophore-conjugated CD34 antibody, respectively, in non-electroporated (no EP, dark grey), Cas9 only (cells electroporated with Cas9 only without gRNA, light grey), and RNP-electroporated (RNP) cells. (D) Post-electroporation cell recovery was measured by dividing the total number of cells collected 4 days post electroporation by the number of electroporated cells. (E-F) 4 days post-electroporation, cells were plated in MethoCult™ H4435 Enriched (Catalog #04435) and cultured for an additional 14 days, before counting. Distribution of CFU colony sub-types (E) or the number of colonies per input cell (F) were comparable between edited and non-edited controls. Each data point per condition represents an individual donor; (A-D) n = 10 donors; (E,F) n = 7 donors. Error bars represent standard deviation.

StemSpan™ media promote similar or greater expansion of CD34⁺ cells compared with alternative commercial media products, providing an optimal media system to support CRISPR-Cas9 genome editing in CD34⁺ cells (Figure 4). Using the ArciTect™ CRISPR-Cas9 system, *B2M* editing efficiency (Figure 4A) and cell viability (data not shown) was comparable across medium conditions. However, StemSpan™ media best support maintenance of CD34 expression (Figure 4B) and expansion of more primitive subsets of HSPCs defined by CD34⁺CD90⁺CD45RA⁻ phenotype after genome editing (Figure 4C).

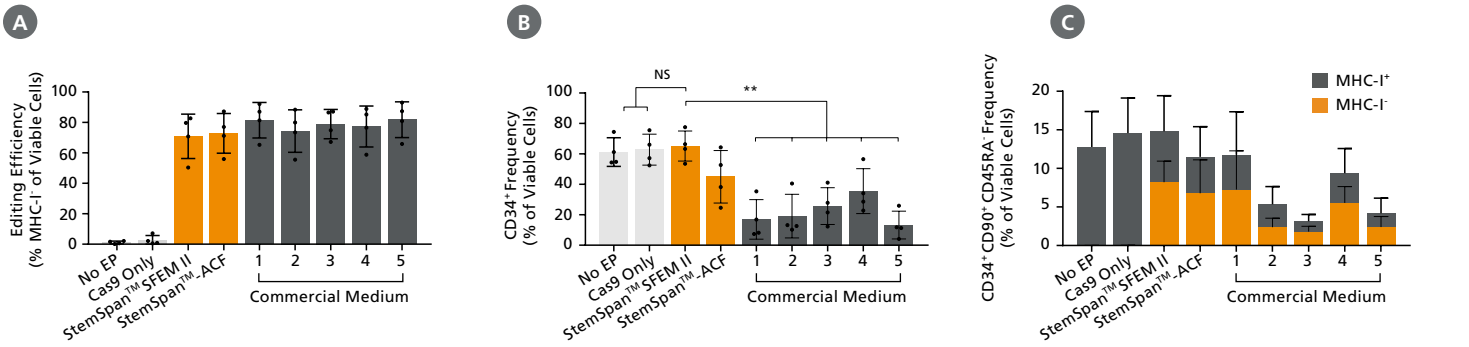

Figure 4. StemSpan™ Media Better Support CD34⁺ and Primitive CD34⁺ CD90⁺ CD45RA⁻ HSPC Expansion for Genome Editing Applications Compared with Alternative Commercial Media

Cells were cultured in the indicated medium supplemented with StemSpan™ CD34⁺ Expansion Supplement plus 175nM UM171* for 2 days, electroporated with CRISPR-Cas9 RNP complexes containing crRNA:tracrRNA targeting *B2M*. Non-electroporated (no EP) and cells electroporated with Cas9 only without gRNA (Cas9 Only) conditions were cultured in StemSpan™ SFEM II supplemented with StemSpan™ CD34⁺ Expansion Supplement plus 175 nM UM171*. (A) *B2M* knockout efficiency (% MHC-I- viable cells) was monitored by flow cytometry using a fluorophore-conjugated MHC-I antibody. (B) The percentage of CD34⁺ cells and (C) CD34⁺CD90⁺CD45RA⁻ cells were quantified by flow cytometry 4 days post-electroporation. Each data point per condition represents an individual donor; n = 4 donors, **P < 0.01. Error bars represent standard deviation.

**The small molecule UM171 was used to generate data in Figure 4. UM171 is no longer licensed for sale by STEMCELL Technologies, however similar results are expected when using UM729 (Catalog #72332) prepared to a final concentration of 1 µM (data not shown). Further titration may be necessary to optimize cell fold expansion in specific conditions. For more information including data comparing UM171 and UM729, see Fares et al.[10]*

Conclusion

Here, we describe an optimized protocol for high efficiency genome editing of CD34⁺ cells using the ArciTect™ CRISPR-Cas9 system and StemSpan™ media for pre- and post-editing culture. We found that sgRNA exhibits higher editing efficiency compared to two-part crRNA:tracrRNA duplexes. While pre- and post-editing culture in all StemSpan™ media and supplements were compatible with the genome editing workflow, we found that StemSpan™ SFEM II supplemented with StemSpan™ CD34⁺ Expansion Supplement best supports maintenance of CD34 expression and expansion of primitive HSPC subsets after genome editing.

The genome editing strategy outlined in this document is typical for most genetic knockout applications. Application-specific protocol modifications, not presented here, might include: use of Cas9-eGFP Nuclease for visualization of positive transfectants; use of the single-strand endonuclease Cas9 Nickase with two flanking gRNAs; or the addition of a DNA donor template for homology-directed repair (HDR)-mediated genetic knock-in.

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