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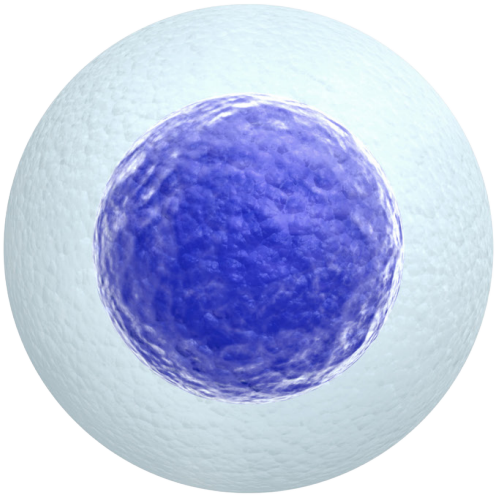
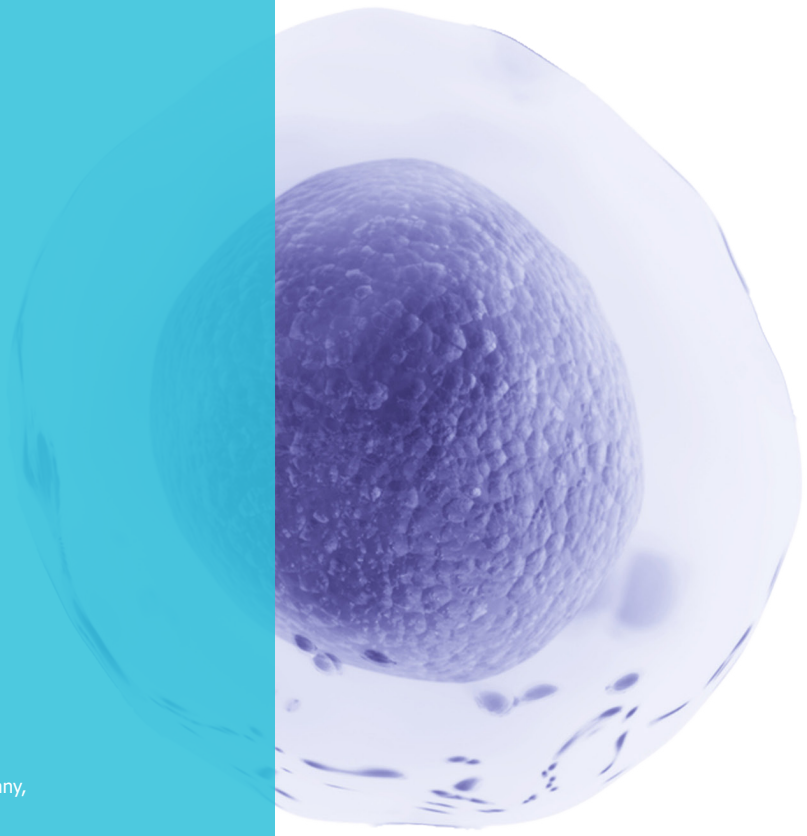
Genome Editing of Human CD34+ Hematopoietic Stem and Progenitor Cells with Lipid Nanoparticles: Discovery to Preclinical





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Document ID: HSCLNPKIT-AN-MAR24

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Introduction

Genome editing of CD34+ hematopoietic stem cells (HSCs) is increasingly being acknowledged for its therapeutic potential in addressing inherited hematological disorders. As HSCs are difficult to source and maintain in culture, this exacerbates the shortcomings of conventional transfection methods such as electroporation, especially in the context of maintaining sufficient cell viability and yield for effective therapeutics. Lipid nanoparticle (LNP) technology enables gentle and highly efficient delivery of genetic material to HSCs and is highly scalable to support acceleration to the clinic. Here, we demonstrate the biological performance of the Cytiva™ CD34+ HSC LNP kits, evaluated *in vitro* using metrics such as gene editing efficiency, cell surface phenotype, clonogenic activity and cell viability and yield.

Background

Hematopoietic stem cells (HSCs) are essential for proper hematological maintenance. Through multipotent differentiation and self-renewal, HSCs replenish the blood system during an individual's lifespan [1]. Naturally, HSCs are an attractive target for gene therapy for permanent correction of hematological disorders. These cells are especially suitable for *ex vivo* gene editing and for use as therapies, with standard methods available for cell harvesting and culture [2].

Gene editing in HSCs can be achieved through a number of enzymes, including transcription activator–like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats (CRISPR) Cas9 for genetic disease corrections such as hemoglobinopathies, Fanconi anemia and hereditary immunodeficiencies [3]. However, CRISPR-Cas9 is increasingly preferred due to the ease of target selection and optimization [4, 5]. In CRISPR-Cas9 mediated gene editing, the Cas9 protein is directed to the target DNA by a synthetic, single guide RNA (sgRNA), where it induces double strand breaks that are mostly resolved through non-homologous end joining (NHEJ). The RNA-guided nature of CRISPR-Cas9 system enables flexibility of target selection and multiplexed gene editing, creating tremendous potential in HSC therapies.

For genome engineering, delivery of Cas9 in messenger RNA (mRNA) format is increasingly being adopted due to rapid production, scalability and the ability for repeat administration for multiplexed knockouts [6]. Gene editing has traditionally involved the use of viral vectors or electroporation for the delivery of genetic materials to target cells. However, these conventional methods possess potential drawbacks in safety, such as the immunogenicity and cytotoxicity associated with viral vectors [7] or the cell death resulting from electrical pulses required for electroporation [8].

In contrast, lipid nanoparticles (LNPs) are a promising technology that enables gentle and efficient cellular transfection of RNA. During formulation, LNPs encapsulate and protect the RNA cargo prior to cytoplasmic delivery. The cellular uptake of the RNA-LNP complex is mediated by the endogenous ApoE-LDLR pathway [9], enabling a gentle delivery method with minimal toxicity. The overwhelming success of COVID-19 mRNA vaccines has demonstrated the wide potential of LNPs to enable highly efficient and safe delivery of RNA. Furthermore, recent studies have focused on the advantages of LNPs over traditional methods like electroporation for *ex vivo* HSC and CAR T-cell therapies. Vavassori et al. (2023) and Kitte et al. (2023) have highlighted the benefits of LNPs in terms of cell viability and sustained mRNA expression, among other advantages [10, 11]. Specifically for HSC gene modifications, Vavassori et al. demonstrated that LNP-based editing dampened the induction of the p53 pathway, supporting higher clonogenic activity and similar or higher reconstitution by long-term repopulating HSCs, as opposed to electroporation [10].

We aim to enable LNP-based HSC gene engineering through accessible, easy to use reagents and instruments for production of high quality RNA-LNPs. Here, we showcase a novel method for the genetic engineering of HSCs using the Cytiva™ CD34+ HSC LNP kits, which were designed to deliver various RNA payloads and demonstrate seamless

integration of LNP treatment into HSC cell culture workflows. RNA-LNPs produced with the CD34+ HSC LNP kits on the NanoAssemblr Spark or Ignite showed highly efficient target knockout (> 80–90%), while maintaining high cell viability (> 95%) and cell proliferation (> 20-fold increase). Furthermore, we show the scalability of LNP production and cell culture optimization.

Materials & Methods

Description	Recommended Supplier
Cytiva™ CD34+ HSC LNP kit, 100 µL	Cytiva, 1003000 or 100400 (with cartridges)
Cytiva™ CD34+ HSC LNP kit, 2 mL	Cytiva, 1005000
Cas9 mRNA	Trilink, CleanCap® Cas9 mRNA, L-7606
sgRNA targets	CD45 target sequence: GAGUUUAAGCCACAAAUACA CD33 target sequence: AUCCCUGGCACUCUAGAACC
Quant-iT™ RiboGreen® RNA Assay Kit, incl. 20X TE Buffer, RNase-free	Thermo Fisher Scientific, R11490
Mobilized Human Peripheral Blood CD34+ Cells, Frozen	STEMCELL Technologies Inc., 70060.1
Human Cord Blood CD34+ Cells, Frozen	STEMCELL Technologies Inc., 70008.1
StemSpan™ SFEM II	STEMCELL Technologies Inc., 09655
StemSpan™ CD34+ Expansion Supplement (10X)	STEMCELL Technologies Inc., 02691
UM729	STEMCELL Technologies Inc., 72332
RPMI 1640 Medium	Thermofisher Scientific, 11875093
Fetal Bovine Serum (FBS)	Thermofisher Scientific, A3840302
Antibodies	FVS575V: BD Biosciences, 565694 • αCD34: BioLegend, 343608 • αCD38: BioLegend, 303530 • αCD90: BioLegend, 328110 • αCD45: BD Biosciences, 563879 • αCD133: BioLegend, 372810 • αCD33: BioLegend 366620
MethoCult™ H4435 Enriched	STEMCELL Technologies Inc., 04445
HyCryo-STEM cryopreservation media	Cytiva, SR30002.02

Equipment

Description	Recommended Supplier
NanoAssemblr™ Spark™	Precision NanoSystems ULC (Now part of Cytiva), NIS0003
NanoAssemblr™ Ignite™	Precision NanoSystems ULC (Now part of Cytiva), NIN0001
Fluorescence plate reader	BioTek™ Synergy™ H1, or similar
Flow cytometer	CytoFLEX V3-B3-R0, Beckman Coulter, C09747
Automated CFU Counter	STEMvision™ Automated Colony-Forming Unit (CFU) Assay Reader instrument, STEMCELL Technologies Inc

Methods

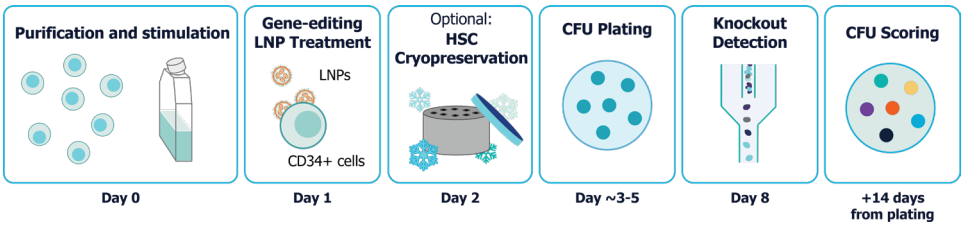

Figure 1. Schematic diagram of the LNP treatment and HSC cell culture workflow*. RNA-LNPs can be produced during or prior to cell culture workflow, allowing for greater flexibility. The RNA-LNPs can be stored short-term at 4 °C for 1 week or long-term at –80 °C for at least 1 month.*

A. CD34+ HSC culture

1. Cryopreserved CD34+ HSCs isolated from mobilized peripheral blood (~1 million cells) were thawed and washed once with 2% FBS (v/v) supplemented RPMI 1640 media by centrifugation at 300 x g for 10 minutes at room temperature (Day 0).
2. Cells were diluted to 0.3 million cells/mL in serum-free medium (StemSpan SFEM II) containing 1x CD34+ expansion supplement and 1 µM UM729 (complete media).

   Note: *The CD34+ HSC LNP kit shows optimal performance in serum-free media. Low serum (≤1%) at the time of LNP addition may be acceptable, but should be experimentally validated. Further, serum-free media are recommended for optimal retention of stemness and long-term repopulating HSC phenotype (CD34+ CD38- CD90+ CD133+).*
3. Cells were incubated at 37 °C/5% CO_2 for 1 day prior to LNP treatment.

B. LNP Preparation

1. CD45 or CD33 targeted RNA-LNPs were prepared with the 100 µL or 2 mL CD34+ HSC LNP Kit based on their respective Instructions for Use (IFU).

   Note: *The 100 µL CD34+ HSC LNP kit is designed to work on the NanoAssemblr Spark and the 2 mL Kit is designed to work on NanoAssemblr Ignite.*
2. Briefly, RNA solutions containing sgRNA and Cas9 mRNA were prepared in a 1:1 weight ratio. LNPs were prepared on either the NanoAssemblr Spark (for 100 µL kit) or NanoAssemblr Ignite (for 2 mL kit). The IFUs include specific workbooks for two-component RNA encapsulation protocols.
3. After formulation, the encapsulated RNA was quantified using the RiboGreen Assay for accurate dose determination.

C. LNP Treatment

1. The recommended timepoint for LNP treatment is around 24 hours post-thaw and stimulation. To ensure success, we recommend flow cytometric assessment of LDLR prior to treatment and proceed if > 50% LDLR expression is observed in the live cell population.
2. Cells were counted 24 hours post-thaw then diluted to ≤ 0.2 million cells/mL in complete media supplemented with 1 µg/mL ApoE.
3. Diluted HSCs were seeded as follows:
   a. 100 µL per well in 96-well u-bottom plate
   b. 250–500 µL per well in 48-well flat-bottom plate
   c. 2 mL per well in 6-well flat-bottom plate
4. LNPs were added at the optimal dose for each target: 3.2 µg/million cells or 6.4 µg/million cells. An optimal treatment dose is likely to fall between 2–6 µg RNA per million cells, but will depend on the design, size and quality of the RNA. A dose titration is recommended to determine the optimal dose for each payload.
5. Treatment plates were incubated at 37 °C/5% CO_2 for 72–96 hours, then maintained by 1:1 dilution with complete media to sustain cell growth. Plates were further incubated up to 7 days after the initial LNP treatment, then analyzed by flow cytometry (unless LNP-treated HSCs were cryopreserved, see below).

D. HSC cryopreservation post LNP treatment

Cryopreservation of LNP-treated HSCs may be desired to preserve stemness and/or for extended cell storage. The following method may be implemented to a standard culture workflow. LNP-treated HSCs may be split such that the majority undergoes cryopreservation while a portion of the cells are cultured in parallel to assess gene editing efficiency.

1. 24 hours post-LNP treatment, HSCs were cryopreserved following the Cytiva HyCryo-STEM cryopreservation media instructions.

   Note: *HyCryo-STEM cryopreservation media is not equivalent to the LNP cryopreservation buffer provided in the kit.*

   a. LNP-treated cells were counted, then centrifuged at 300 x g for 10 minutes at room temperature.

   b. Supernatant was removed and retained for 1:1 dilution of HyCryo-STEM cryopreservation media in spent complete media.

   c. Cells were resuspended in HSC cryobuffer to 1 million cells/mL.

   d. Cells were chilled at 4 °C for 10 minutes, then frozen to –80 °C in a temperature-controlled manner prior to liquid nitrogen storage.

2. Cells were thawed following Step A (above) and cultured for 4 days prior to analysis by flow cytometry.

E. Colony-forming unit (CFU) assay

1. LNP-treated cells were used to perform CFU assays from days 3–5 (see **Figure 1**) with enriched MethoCult media following the manufacturer instructions.
2. Cells were seeded and incubated at 37 °C/5% CO_2 for 14 days.
3. Colony formation was evaluated using STEMVision as per the manufacturer instructions.

F. Target knockout and phenotype analysis by flow cytometry

1. Target knockout and phenotype were assessed 7 days post-treatment (Day 8).
2. Cells were first stained for live/dead discrimination, then subsequently stained for target and biomarker surface expression.
3. Data was acquired using a CytoFLEX flow cytometer then analyzed using FlowJo™ V10.7.

Note: *The biological implications of the LNP treatment and the culture conditions on the selected editing target must be considered when designing analytical methods. For instance, cell culture media components can play a critical role in the expression pattern and/or the availability of binding sites for a given biomarker. As such, detection methods should be adequately validated using the experimental conditions.*

Results & Discussion

CD34+ HSCs were cultured and treated with LNPs following the schematic shown in **Figure 1** of the Material and Methods. Two biological knockouts, CD45 and CD33, were evaluated as indicated within the figures and figure legends below.

CD45 was selected as a model antigen due to its ubiquitous expression in all leukocytes, including HSCs [12]. Recently, CD45 was shown as a promising antigen for a universal CAR T cell therapy, leveraging precisely the feature that CD45 is found on all immune cells, including malignant cells of various blood cancers [13]. By CRISPR base-editing HSCs, this innovative approach protects healthy HSC-derived cells from CD45-targeted CAR T cell clearance, while still allowing the CAR T cells to target and eliminate cancer cells. The development of a universal CAR T cell therapy could streamline the treatment of various hematological cancers without the need to individually design and test therapies for each condition.

Similarly, CD33 was chosen for its therapeutic relevance in a hematological disease model: acute myeloid leukemia (AML). Challenges in targeted immunotherapy for AML arise from overlapping surface antigen expression between the diseased and healthy cells and the lack of cancer specific antigens [14]. Knockout of CD33 in HSCs confers resistance against CD33-targeted therapies for AML (both antibody and CAR T therapies) to bypass issues stemming from common antigen expression [15].

The results and discussion of this Application Note are arranged in the following sections:

A. Cell Culture Optimization
B. CRISPR-Cas9 Gene Knockouts
C. Cell Proliferation and Viability Following LNP Treatment
D. HSC Cryopreservation and Freeze-Thawing Post LNP Treatment
E. LNP Scale Up and Robust LNP Performance

A. Cell Culture Optimization

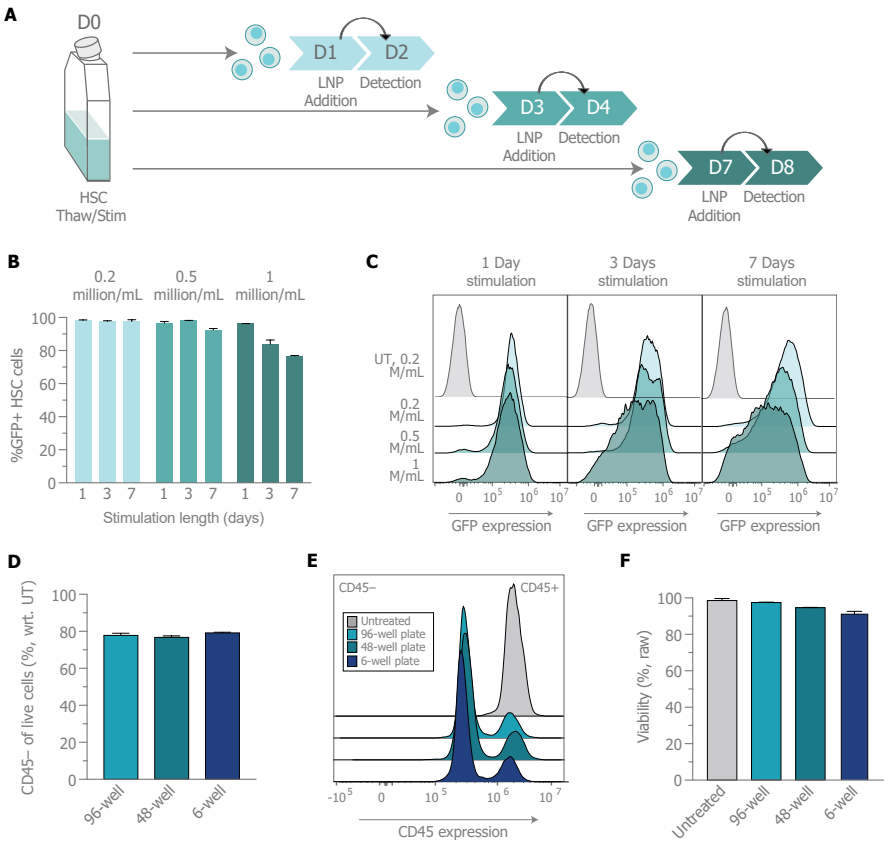

Figure 2. Cell culture condition optimization for RNA-LNP delivery. A) *Schematic timeline of the experimental setup illustrating the process of HSC thawing and stimulation, LNP addition for RNA delivery, and subsequent detection of transfection efficiency.* ***B)*** *Percentage of GFP+ HSCs following RNA delivery across varying cell densities (0.2, 0.5, and 1 million cells/mL) and stimulation lengths (1, 3, and 7 days).* ***C)*** *Corresponding flow cytometry histograms of GFP expression for B.* ***D)*** *CD45 knockout of HSCs cultured and treated in 96-well, 48-well and 6-well formats.* ***E)*** *Corresponding flow cytometry histograms of CD45 expression for D.* ***F)*** *Cell viability in different well plate formats post RNA-LNP delivery. For all, NanoAssmblr Spark was used to formulate LNPs. HSCs were dosed at 3.2 µg RNA/million for GFP, and 6.4 µg RNA/million for CD45 knockout.*

Initially, we investigated the optimal cell culture conditions for LNP-mediated RNA delivery into HSCs. We evaluated multiple parameters, including cell density, stimulation length, and well-plate format. We used both GFP mRNA expression and CD45 gene knockouts as readout of transfection efficiency.

Figure 2A outlines the experiment timeline for optimal cell density (0.2, 0.5, and 1 million/mL) and stimulation lengths (1, 3, and 7 days) prior to RNA-LNP treatment. This timeline aims to identify the culture density and stimulation length that results in the highest and most homogenous GFP expression, as indicators of successful RNA delivery. By varying the HSC densities (0.2, 0.5, and 1 million cells/mL), we observed that increased cell density corresponded with decreased GFP transfection efficiency post-LNP mediated RNA delivery, particularly at prolonged stimulation lengths (**Figure 2B**). We theorize that crowding and insufficient nutrient supply may limit cellular ability to translate synthetic RNAs in high quantities at higher cell densities. GFP expression profiles obtained via flow cytometry (**Figure 2C**) highlight an increase in heterogenicity and broadening of GFP expression at both prolonged stimulation and higher cell densities. Consequently, we recommend reduced cell densities (0.2 million/mL or lower) and short stimulation time prior to LNP addition (24 hours).

Cell culture scale is another critical parameter that can influence LNP-mediated RNA delivery and cell viability. The compatibility of LNP treatment workflow with various cell culture scales was assessed using different well-plate formats (96, 48, 6-wells). CD45 expression was measured to evaluate the effectiveness of LNP delivery for CRISPR-Cas9 mRNA-mediated gene knockouts. RNA-LNPs were prepared using the CD34+ HSC LNP kit, in which Cas9 mRNA and sgRNA (mixed in 1:1 wt. ratio) were encapsulated. **Figures 2D** and **2E** show successful and consistent CD45 KO performance across all cell culture scales tested. Furthermore, cell viability was above 90% for all LNP treatment conditions, comparable to that of untreated cells (**Figure 2F**).

The results of these experiments show culture condition optimizations for LNP-mediated RNA delivery, particularly highlighting the importance of cell density and HSC stimulation time on RNA transfection. The results suggest that lower cell densities and shorter stimulation periods prior to LNP addition are preferred for strong protein expression. Additionally, we see consistent performance of CD45 knockout across different cell culture scales, coupled with high cell viability post-LNP treatment, emphasizing the benefits of LNP technology.

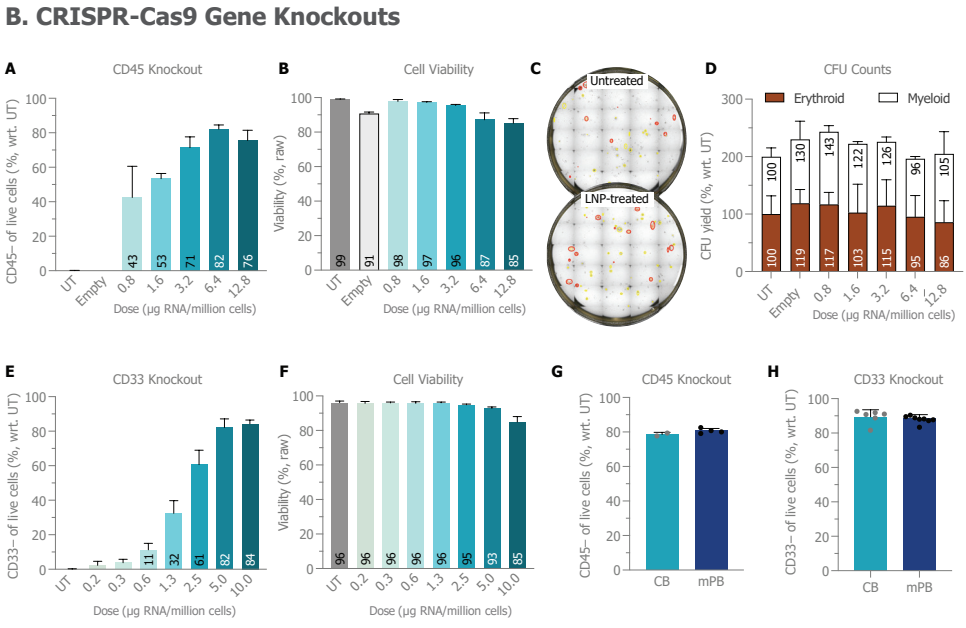

Figure 3. CD45 and CD33 knockout in HSCs. ***A)*** *CD45 dose response of CRISPR-Cas9 mRNA and sgRNA LNPs produced on the NanoAssemblr Spark instrument.* ***B)*** *Corresponding cell viability.* ***C)*** *Representative images of untreated and CRISPR RNA-LNP treated CFU plates.* ***D)*** *Normalized erythroid and myeloid colony yields of untreated (UT), empty LNP treated, and CRISPR RNA-LNP treated samples.* ***E)*** *CD33 dose response of CRISPR-Cas9 mRNA and sgRNA LNPs produced on the NanoAssemblr Spark instrument and* ***F)*** *corresponding cell viability.* ***G)*** *CD45 knockout of CRISPR RNA-LNP treated HSCs sourced from human mobilized peripheral blood (mPB) and human cord blood (CB), and* ***H)*** *CD33 knockout of CRISPR RNA-LNP treated HSCs sourced from mPB and CB.* ***G*** *and* ***H****, LNPs were made on the Spark, and a dose of 3.2 µg RNA/million was used for CD33 and 6.4 µg RNA/million for CD45.*

For gene editing, dose-response analysis is critical for balancing gene knockout efficacy with cellular viability and off-target editing. Precise modulation of gene editing components, Cas9 mRNA and sgRNA, is essential to achieve optimal editing outcomes. This study explores the dose-dependent effects of CRISPR RNA-LNP delivery using the CD34+ HSC LNP kit, evaluating knockout efficiency, cell viability and clonogenic activity. A dose response should be evaluated for new payloads and knockout targets to determine the appropriate therapeutic window.

For the experiments, RNA-LNPs were produced on the NanoAssemblr Spark (CD34+ HSC LNP kit, 100 µL) encapsulating Cas9 mRNA and sgRNA targeted to either CD45 or CD33. **Figure 3A** shows CD45 knockout efficiency of the untreated (UT), empty LNPs (no RNA control), and CD45 targeted CRISPR RNA-LNPs at the indicated doses. We observe a dose-dependent increase in knockout efficiency, with the highest dose yielding the most substantial effect. Cell viability post-editing was measured with flow cytometry (**Figure 3B**), demonstrating that viability remains high across all LNP doses. Further, we performed colony-forming unit (CFU) assay for these doses using a standard method recognized in the field, with example images shown in **Figure 3C**. Quantification of the CFU results (**Figure 3D**) reveals the differentiation potential into erythroid and myeloid lineages, normalized to the untreated controls. We observed no significant impact of LNP treatment on the differentiation potential of the HSCs at all tested doses. Similarly, we show dose-dependent knockout efficiency for CD33 targeted editing (**Figure 3E**), as well as the maintenance of high cell viability across all tested doses (**Figure 3F**).

Additionally, we evaluated LNP performance across major donor sources of HSCs: mobilized peripheral blood (mPB) and cord blood (CB). Each source has its utility, such that mPB is preferred for adult transplants due to higher HSC yields and faster engraftment, while CB is advantageous for pediatric transplants and for lower incidence of graft-versus-host disease (GvHD). We tested both CD45 and CD33 knockout with the CD34+ HSC LNP kit for both mPB and CB (**Figures 3G, H)**, showing identical LNP performance in both HSC sources.

The dose-response results confirm that CRISPR RNA-LNP mediated gene editing is effective across a range of doses for gene knockouts in CD34+ HSCs, using our models for CD45 and CD33 knockout. Cells maintained high cell viability and differentiation capacity at all tested doses. LNP-mediated gene editing is applicable across diverse HSC sources, both mPB and CB.

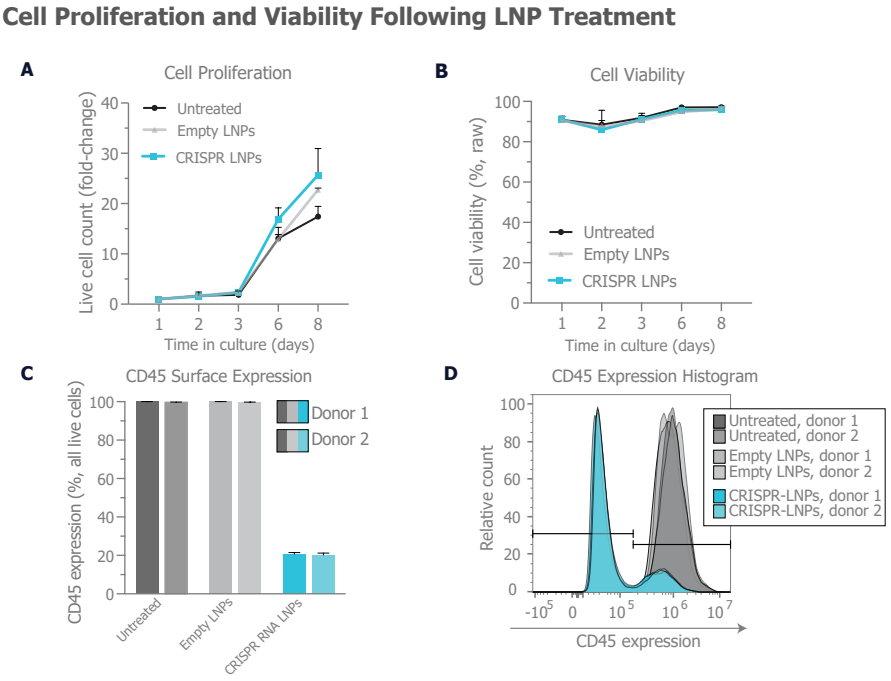

Figure 4. LNP-mediated CD45 targeted CRISPR-Cas9 gene editing of HSCs. A) *Cell proliferation and* ***B)*** *viability monitored for over 1 week after CRISPR-RNA LNP treatment at 6.4 µg RNA/million cells.* ***C)*** *LNP-mediated gene editing compared between 2 individual donors using flow cytometry via CD45 surface expression analysis and* ***D)*** *corresponding histogram.*

Cell viability and yield are critical metrics for the success of engraftment following genetic modification. Typically in clinical practice, the number of HSCs required for transplant is in the order of hundreds of millions or billions. Maintaining high cell viability and cell yield after genetic modification is critical to achieve enough drug product for a therapeutic dose. Electroporation, commonly used for HSC modification, has been reported to adversely affect cell viability and cell yields due to the mechanical and electrical stress that is imposed on the cells [16]. This reduction in viable cell count can significantly impact the *ex vivo* manufacturing process, leading to challenges in achieving sufficient cell numbers for effective therapies. Furthermore, some cell loss is inevitable with cryopreservation of cells, which is frequently used for storing the precious HSCs; this further emphasizes the need for gentle cargo delivery.

The impact of LNP treatment on cell proliferation and viability was evaluated with live/dead staining using acridine orange and propidium iodide (AO/PI) on an automated cell counter. Untreated, empty-LNP or CD45 targeted CRISPR RNA-LNP treated HSCs were monitored over 8 days of *in vitro* culture. Notably, both cell proliferation and cell viability were unaffected by the LNP treatment. Shown in **Figure 4A**, CD34+ HSC LNP kit treated HSCs show equivalent or higher cell yield compared to untreated controls. Likewise, using AO/PI staining, the raw viability of LNP-treated cells was maintained consistently above 85% and was indistinguishable to the untreated cells throughout all monitored days of cell culture (**Figure 4B**). Comparison of the CD45 surface expression in two different cell donors showed consistent, robust performance of LNPs for Cas9 mRNA and sgRNA delivery (**Figures 4C, D**).

This experiment demonstrates that the delivery of CRISPR-Cas9 components via LNPs does not compromise the proliferative capacity and viability of HSCs, allowing users to maintain high cell numbers necessary for successful engraftment. Post-gene editing, unperturbed cell proliferation in RNA-LNP treated cells yielded similar cell counts to untreated cell controls. Additionally, the consistent high efficiency of CD45 editing across different cell donors highlights the reliability of LNPs for *ex vivo* HSC manipulation.

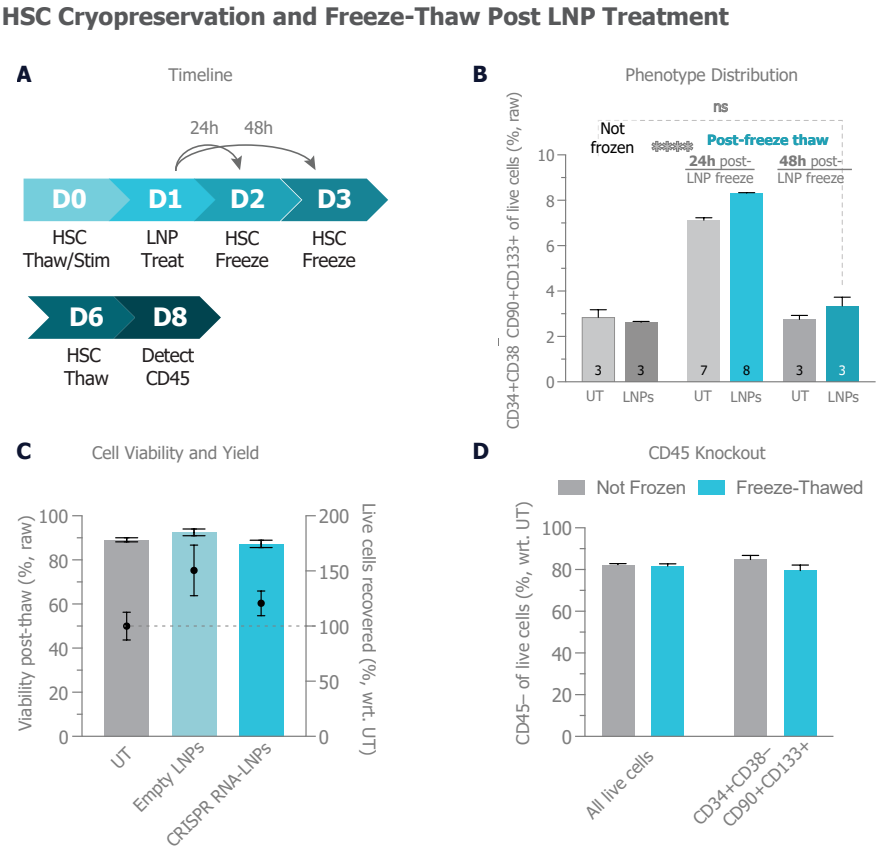

Figure 5. Cryopreservation of HSCs post LNP-mediated CRISPR Cas9 gene editing. A) *Schematic diagram of the freeze-thaw (F/T) cycles of HSCs.* ***B)*** *Post-thaw, or not-frozen long-term repopulating HSC phenotype levels, at either 24h or 48h post-LNP treatment freeze time points.* ***C)*** *Cell viability (bars) and live cell recovery (dots) post 24h-LNP treatment F/T cycle.* ***D)*** *CD45 knockout efficiency observed in freeze-thawed and not frozen LNP-treated HSCs in total live cells and long-term repopulating HSC phenotype (CD34+ CD38- CD90+ CD133+).*

Cryopreservation of cells is essential for long term storage of cells and for transportation between geographically separated facilities [17, 18]. For HSCs, cryopreservation is especially important to maintain the stemness since prolonged *ex vivo* culture will result in the proliferation and differentiation of HSCs, which subsequently decreases the availability of long-term repopulating or primitive, stem-like phenotypes. Freezing cells can suspend the differentiation process and extend the window of stemness. However, for conventional methods of RNA delivery such as electroporation, the mechanical stress and subsequent significant reduction in viable cell numbers make it difficult to freeze HSCs after manipulation [8]. Consequently, users are forced to extend *ex vivo* culture for cell recovery, making a trade-off between stemness and proliferation to achieve adequate cell yields for cryopreservation.

In order to de-risk HSC freezing during the process of LNP-mediated CRISPR-Cas9 editing, LNP treated HSCs were subjected to one cycle of freeze-thaw. Following the experimental timeline in **Figure 5A**, LNP-treated HSCs were frozen at two different intervals (24 and 48 hours post LNP treatment) to determine the optimal timepoint for cryopreservation. Cells were frozen below –80 °C using Cytiva HyClone™ HyCryo-STEM cryopreservation media for long-term HSC storage. After one cycle of freeze-thaw, cells were cultured for 4 days then analyzed by flow cytometry. Phenotype analysis (**Figure 5B**) exemplifies the advantage of freezing cells at an earlier timepoint, as cells frozen after 24 hours showed over 2-fold higher retention of the long-term repopulating phenotype (CD34+CD38–CD90+CD133+) compared to not-frozen cells (maintained in culture for 7 days post-treatment) and cells that were frozen after 48 hour treatment. Furthermore, CRISPR RNA-LNP treatment did not alter the phenotype distribution relative to the untreated counterpart (UT vs. LNPs). Post-freeze thaw, the untreated, empty-LNP or CRISPR RNA-LNP treated HSCs showed high cell viability (bars, **Figure 5C**), as well as viable cell recovery (dots, **Figure 5C**). Finally, both freeze-thawed and not-frozen LNP-HSCs showed high CD45 knockout efficiency, indicating sufficient retention and stability of the RNA payload and its byproducts after the cryopreservation process (**Figure 5D**).

These results demonstrate the compatibility of LNP-mediated CRISPR-Cas9 editing with HSC cryopreservation. The optimal time point of cryopreservation, 24 hours post-LNP treatment, significantly enhances the retention of stemness-associated phenotypes, a key attribute for long-term *in vivo* engraftment. High cell viability and recovery rates post-HSC thaw further highlight the gentle nature of LNPs for RNA delivery. Combined with high CD45 knockout rates post-cryopreservation, this result successfully de-risks practical incorporation of RNA-LNPs into clinically relevant workflows.

E. LNP Scale-up and Robust LNP Performance

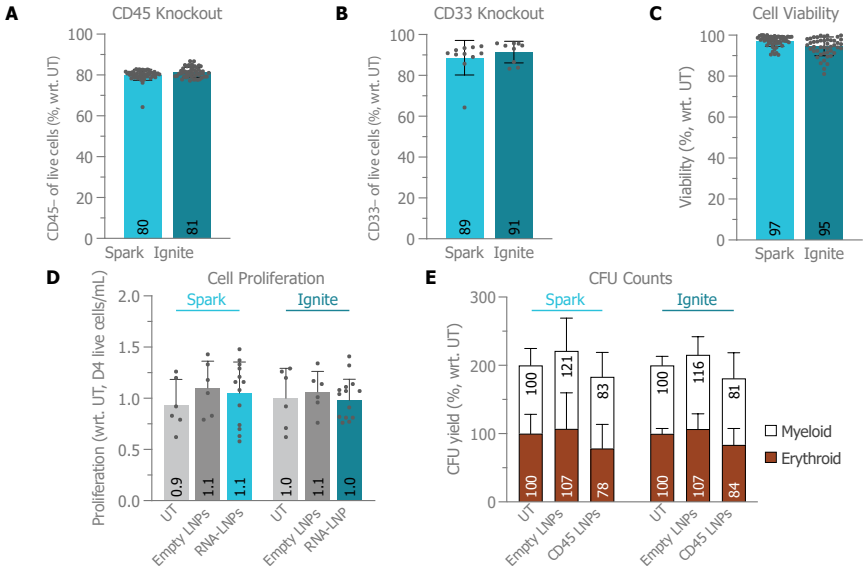

Figure 6. Demonstration of LNP production scale-up. *LNPs produced at Spark and Ignite scales assessed for* ***A)*** *CD45 knockout and* ***B)*** *CD33 knockout of the total live cell population, as well as* ***C)*** *corresponding cell viability. A–C includes >20 unique experiments with >4 unique donors.* ***D)*** *Untreated, empty-LNP and CRISPR RNA-LNP treated HSCs were assessed for proliferation using an automated cell counter.* ***E)*** *Untreated, empty-LNP and CRISPR-Cas9 RNA loaded LNP treated HSCs were assessed for clonogenicity using an automated CFU counter, 5 independent experiments with Spark and 2 independent experiments with Ignite.*

Lipid nanoparticles can be easily scaled-up to production batch sizes suitable for clinical trials. Often with conventional RNA delivery methods, bottlenecks in production arise from challenges in scale-up. Such examples can be seen with viral vectors where challenges in scale-up arise in several major points of the production line, such as establishing stable cell lines, downstream processing and purification [19]. Similarly, electroporation systems often lack large-scale, GMP-compliant solutions, especially ones that combine high reproducibility, cell viability and efficiency. LNP production on the NanoAssemblr platform allows seamless transition from discovery scales on the Spark to preclinical scales on the Ignite, and clinical and commercial scales with the NanoAssemblr Commercial Formulation System.

A large number of repeat experiments were performed on the Spark and Ignite to demonstrate the robustness and reproducibility of the CD34+ HSC LNP kit. **Figure 6** aims to consolidate LNP performance metrics for both LNP scales. **Figures 6A–C** combine experimental outcome from over 20 unique experiments with at least 4 unique donors for CD45 and CD33 gene knockouts, and their respective cell viabilities. For CD45 gene knockout, the Spark kit achieved 80 ± 2.7% and the Ignite kit achieved 81 ± 2.4% knockout (**Figure 6A**). For CD33 knockout, we observed 89 ± 8.4% for the Spark kit and 91 ± 5.3% for the Ignite kit (**Figure 6B**). For the aforementioned experiments, average cell viability of >95% was maintained, normalized to untreated controls (**Figure 6C**). Cell proliferation and the CFU assay was performed in at least 2 independent experiments for the Spark and Ignite. Cell proliferation was assessed on day 4 for untreated, empty-LNP treated and CD45 targeted RNA-LNP treated HSCs. Shown in **Figure 6D**, for all conditions and both NanoAssemblr instruments, the proliferation of the HSCs remained unchanged. Similarly, CFU assays evaluated the clonogenic activity of the untreated, empty-LNP treated and CD45 targeted RNA-LNP treated HSCs (**Figure 6E**). The empty-LNPs alone showed no impact on the differentiation capacity of the HSCs, yielding consistent erythroid and myeloid colonies with respect to the unedited controls. We observe a slight drop (though not statistically significant) in colony yields for the CRISPR edited samples, for both Spark and Ignite LNPs, which is likely correlated to the CRISPR-Cas9 editing of CD45 and the loss of its function.

The extensive experimental data presented here underscores the robustness and reproducibility of LNPs as a delivery system for CRISPR-Cas9 mediated gene editing of HSCs. The CD34+ HSC LNP kit, tested at different production scales, and over numerous experiments involving multiple donors, shows exceptional performance in terms of knockout efficiency, cell viability, cell proliferation and maintenance of differentiation potential. The high cell viability, paired with the high knockout values demonstrate the gentle yet effective nature of LNP-mediated RNA delivery. These results firmly establish LNPs as a scalable, reproducible, and highly efficient delivery tool for advancing gene therapy applications in HSCs.

Conclusion

This study demonstrates the potential of LNP-mediated RNA delivery as a promising approach for gene editing in HSCs. The advantage of the CD34+ HSC LNP kit is exemplified through consistent and highly efficient knockout of CD45 and CD33, tested over multiple experiments and donors. The compatibility of LNPs with various HSC sources highlights their applicability to a wide range of therapeutics. A key finding is the maintenance of high cell viability and cell proliferation post-LNP treatment, both consistently above 90% with respect to untreated controls, showing an improvement over traditional gene delivery methods. The lack of mechanical stress on cells with LNP treatment is particularly relevant for successful cryopreservation of HSCs post treatment, as this allows optimal retention of stemness through eliminating the need for prolonged cultures to aid cellular recovery. Finally, highly scalable LNP production, as demonstrated by a long-standing history of scale-up from discovery to clinical scales on the NanoAssemblr platform, addresses a critical gap in gene therapy manufacturing where conventional methods face significant challenges. This scalability of LNPs permits efficient RNA library screening at small scales and facilitates swift transition to large-scale clinical applications.

Appendix

Troubleshooting Tips

Problem	Probable Cause	Solution/Action
Low transfection efficiency	Media supplemented with >1% serum (HSA, BSA)	HSC kit is not compatible with moderate to high concentrations of serum proteins
	No ApoE3 supplementation	Culture media must contain 1 µg/mL ApoE for appropriate LNP internalization
	Suboptimal seeding density	Use recommended seeding density of 0.1–0.5 million cells/mL, with further optimization as required Too high – nutrient depletion and inhibition of LNP uptake Too low – limited cell-to-cell interaction
	Poor RNA payload quality	Use purified, high-quality RNA for transfection. Maintain sterility and avoid degradation/contamination of the RNA to be used as payload
	Insufficient RNA-LNP dose	Perform dose response to identify optimal RNA dose for each RNA payload used
	Insufficient treatment duration	Perform expression/knock-out kinetics to determine optimal timepoint for downstream analysis
	Inappropriate method of detection	It is critical to consider the biological implications of the LNP treatment and the culture conditions on the target detection in selecting the appropriate bioanalytical method. Detection method should be validated and optimized prior to utilization
	Lack of experimental controls	If needed, use positive controls for experiments. This could be Trilink GFP mRNA (L-7601) or the CRISPR-Cas9 guides and mRNA used in this Application Note (see Material and Methods) Untreated cells, or empty LNPs (mol. bio. water instead of RNA) can be used as negative controls If already using electroporation or other gene transfer methodology, make sure target effect is successful and detection method is appropriate
Low RNA encapsulation efficiency	Error in LNP preparation	Dilution buffer was used instead of Formulation buffer Improper RNA solution preparation, such as adding too much RNA, resulting in poor encapsulation efficiency. We recommend UV-vis quantification of the RNA concentration prior to formulating
	Error in RiboGreen assay	Triton contaminated the TE wells, yielding false positive free-RNA results

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Document ID: HSCLNPKIT-AN-MAR24