

Electroporation of primary human CD34⁺ hematopoietic stem and progenitor cells

Delivery of ribonucleoprotein complexes using the Alt-R CRISPR-Cas9 System and Amaxa[®] Nucleofector[®] System

Contributed by Ayal Hendel, PhD; Jenny Shapiro, PhD; Adi Tovin, PhD; Ortal Iancu; Keren Mizrahi, PhD

The Mina and Everard Goodman Faculty of Life Sciences and Advanced Materials and Nanotechnology Institute, Bar-Ilan University, Ramat-Gan 52900 Israel

The method presented here is provided by customers who have used the Alt-R CRISPR-Cas9 System. This can serve as a starting point for using the Alt-R CRISPR-Cas9 System in similar biological systems but may require optimization for your gene or application. IDT does not guarantee these methods, and application specialists at IDT can only provide general guidance with limited troubleshooting and support.

Overview

This protocol describes the delivery of a CRISPR-Cas9 ribonucleoprotein (RNP) containing Alt-R Cas9 nuclease complexed with an Alt-R CRISPR-Cas9 guide RNA (gRNA, such as crRNA:tracrRNA duplex or sgRNA), into CD34⁺ hematopoietic stem and progenitor cells (HSPCs) using electroporation with the Amaxa Nucleofector System (Lonza).

Delivery of Cas9 and guide RNA as an RNP complex to CD34⁺ HSPCs was shown to yield high editing levels in the specific target site, along with reduced cell toxicity and off-target cleavage activity [1–3].

Important considerations

1. Both fresh and cryopreserved CD34⁺ cells, derived from umbilical cord blood or mobilized peripheral blood, can be used.
2. Alt-R Cas9 Electroporation Enhancer, a non-targeting carrier DNA*, can be used to improve electroporation efficiency in CD34⁺ cells (Figure 1).
3. We recommend including predesigned gRNAs targeting the human *EMX1* gene as positive controls in your experiment (Figure 1).
4. We suggest limiting the duration of **steps D** and **E** in this protocol (see Figure 2). In experiments that include many samples, we recommend that one lab member prepares RNP complexes (**step D**) while another lab member simultaneously prepares cells for electroporation (**step E**):

* This oligo is designed to avoid homology to the human, mouse, or rat genome (100 nt sequence: TTAGCTCTGTTTACGTCCCAGCGGGCAT GAGAGTAACAAGAGGGTGTGGTAATATTACGGTACCGAGCACTATCGATACAATATGTGTGCATACGGACACG).

- *Step D (prepare RNP complexes):* Leaving RNP complexes at room temperature (15–25°C) for longer than 20 min may allow degradation of complex components. It is also advisable to standardize complex preparation time among samples and experiments.
- *Step E (wash and resuspend cells before electroporation):* Leaving cells in Nucleofector Solution for extended periods of time may lead to reduced transfection efficiency and viability [4].

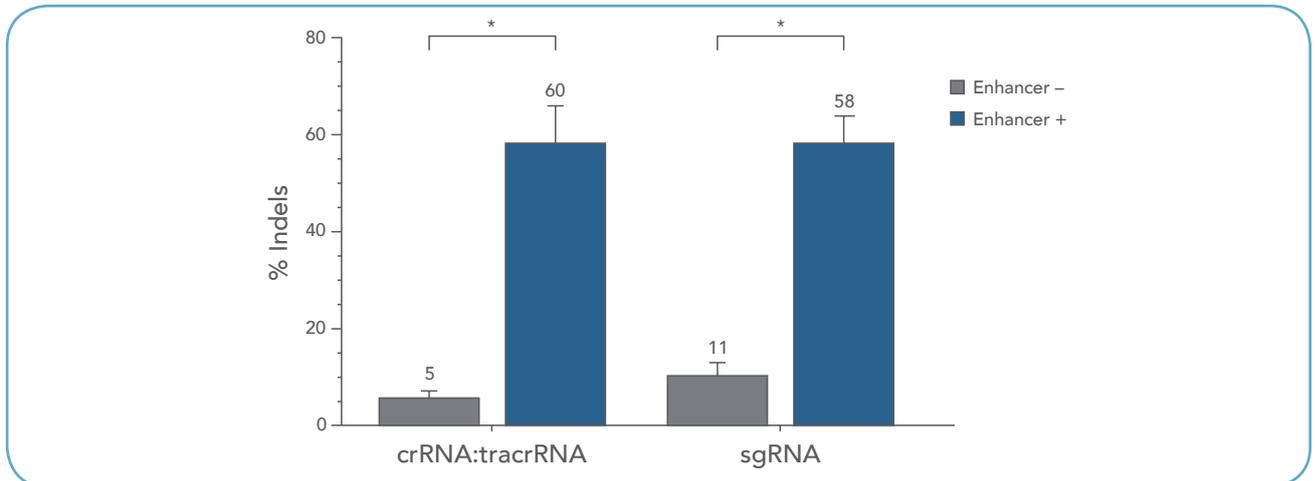


Figure 1. Alt-R Cas9 Electroporation Enhancer increases CRISPR editing efficiency in CD34⁺ cells. An RNP complex that comprised gRNA targeting human *EMX1* (Alt-R CRISPR-Cas9 crRNA:tracrRNA duplex or Alt-R CRISPR-Cas9 sgRNA) and Cas9 nuclease was delivered into CD34⁺ cells (mobilized, 3 independent donors, AllCells, LLC company) by electroporation (4D-Nucleofector system, Lonza). The RNP was delivered with or without Alt-R Cas9 Electroporation Enhancer, as described in this protocol. Genomic DNA was extracted from the cells 48 hr after electroporation, and primers flanking the expected cut site were used for PCR amplification of the *EMX1* gene. The level of NHEJ was assessed by measuring the percentage of insertions and deletions (indels) among the sequenced amplicons of each sample using TIDE analysis software. Error bars represent standard deviation of the mean. * $p < 0.0001$ (Sidak's multiple comparisons test for 2-way ANOVA).

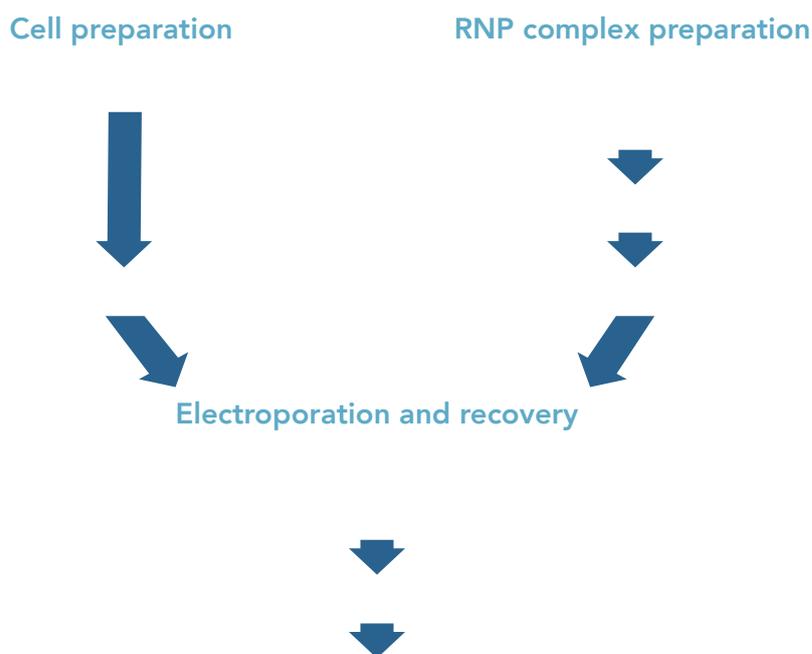


Figure 2. Overview of procedure.

Materials

Kits and reagents	Ordering information
1X Phosphate buffered saline (PBS)	General laboratory supplier
(Optional) StemSpan™ SFEMII	Stemcell Technologies (cat # 9655)
(Optional) Cytokines: Human Hematopoietic Stem Cell Expansion Cytokine Package	PeproTech (cat # HHSC6) or similar
Penicillin-streptomycin	General laboratory supplier
Option 1, 2-part guide RNA (crRNA + tracrRNA):	IDT predesigned and custom crRNA: www.idtdna.com/CRISPR-Cas9
• Alt-R CRISPR-Cas9 crRNA	IDT (cat # 1072532, 1072533, 1072534)
• Alt-R CRISPR-Cas9 tracrRNA or Alt-R CRISPR-Cas9 tracrRNA – ATTO™ 550	IDT (cat # 1075927, 1075928)
Option 2, single guide RNA (sgRNA):	IDT predesigned and custom sgRNA: www.idtdna.com/CRISPR-Cas9
• Alt-R CRISPR-Cas9 sgRNA	
(Optional positive control) Alt-R CRISPR-Cas9 crRNA or sgRNA for human <i>EMX1</i> (Gene ID: 2016, see example in figure 1)	IDT custom gRNA: www.idtdna.com/CRISPR-Cas9
Alt-R S.p. Cas9 Nuclease V3	IDT (cat # 1081058, 1081059)
Alt-R Cas9 Electroporation Enhancer	IDT (cat # 1075915, 1075916)
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	IDT (cat # 11-01-02-02)
P3 Primary Cell 4D-Nucleofector X Kit S (32 RCT), which includes 16-well Nucleocuvette™ Strips	Lonza (cat # V4XP-3032)
(Optional) DNA extraction kit or solutions	General laboratory supplier
Additional equipment	
Low-oxygen tissue culture incubator (5% O ₂)	General laboratory supplier
4D-Nucleofector System	Lonza (www.lonza.com)
Thin, sterile, disposable, plastic pipettes*	General laboratory supplier
(Optional) Multichannel pipette (for 25 µL and 75 µL volumes)	General laboratory supplier
(Optional) Sterile reservoirs	General laboratory supplier
(Optional) Sterile PCR tubes	General laboratory supplier
(Optional) PCR thermal cycler	General laboratory supplier
96-well tissue culture plate, U-bottom shaped†	General laboratory supplier

* Ensure the pipette tips reach the bottom of the Nucleocuvette wells without getting stuck [4].

† Scale up plate size, as necessary (see **step B6**).

Methods

A. Culture, evaluate, and count cells

1. Prepare HSPC complete culture medium.

Notes:

- Various media may be used depending on the end purpose of your culture. A cytokine-rich medium based on SFEMII supplemented with SCF (100 ng/mL), TPO (100 ng/mL), Flt3-Ligand (100 ng/mL), IL-6 (100 ng/mL), streptomycin (20 mg/mL), and penicillin (20 unit/mL) is recommended [1–3].
- Store at 4°C for up to 1 week after preparation.

2. Culture freshly isolated or thawed CD34⁺ cells in complete culture medium at a density of 0.25×10^6 cells/mL in a low-oxygen, tissue culture incubator (37°C, 5% CO₂, 5% O₂) for 48 hr before electroporation.
3. On the day of electroporation, count cells:
 - a. Visually inspect the cells using a microscope to ensure healthy appearance.
 - b. Resuspend cells in the culture medium by gently pipetting.
 - c. Transfer cells to a 15 or 50 mL sterile tube.
 - d. Rinse the culture dish surface with an additional few milliliters of prewarmed, complete culture medium and add this volume to the sterile tube.
 - e. Mix cells by gentle pipetting, and count cells.
4. Update your planned experiment according to the available cell number.

Note: The contributors recommend 5×10^4 cells per a well in a 16-well Nucleocuvette Strip [4]. However, 1×10^4 to 1×10^6 cells per well can be used with the same reaction conditions [3].

5. Transfer the exact volume containing the total number of cells needed for your experiment into a new 15 or 50 mL sterile tube.
6. Place the tube containing the cells for your experiment in a low-oxygen incubator.

B. Prepare 4D-Nucleofector System and solutions

4D-Nucleofector System [4]

1. Turn on the 4D-Nucleofector System, and create or upload an experimental parameter file (for details see device manual).
2. Select Nucleofector program **DZ-100**.
3. Add the entire supplement to Nucleofector Solution P3.

Note: Store supplemented Solution P3 at 4°C, and use within 3 months of supplement addition.

- Let the supplemented Solution P3 reach room temperature.

Electroporation enhancer [5]

- Resuspend the Alt-R Cas9 Electroporation Enhancer to 100 μM in IDTE.

Note: For assistance, use the Resuspension Calculator at www.idtdna.com/SciTools.

Recovery plate and media [4]

- Prepare a 96-well, U-bottom culture plate for cells following nucleofection.
 - Fill each well intended for cell culture with 100 μL of complete culture media.
 - Store the plate in a low-oxygen incubator.
- Prewarm (37°C) an additional aliquot of complete culture media with enough for 75 μL/well.

C. Prepare guide RNAs

Option 1: Form the crRNA:tracrRNA duplex

- Resuspend the Alt-R CRISPR-Cas9 crRNA and Alt-R CRISPR-Cas9 tracrRNA in IDTE Buffer to final concentrations of 200 μM.

Note: For assistance, use the Resuspension Calculator at www.idtdna.com/SciTools.

- Mix the two RNA oligos in equimolar concentrations in a sterile PCR tube to a final duplex concentration of 100 μM. The following table shows an example for a final volume of 5 μL:

Component	Amount (μL)
200 μM Alt-R CRISPR-Cas9 crRNA	2.5
200 μM Alt-R CRISPR-Cas9 tracrRNA	2.5
Total volume	5

- Heat at 95°C for 5 min (optional: in a thermal cycler).
- Remove from heat, and allow to cool to room temperature on the bench top.
- Keep at room temperature for about 10 min, then place on ice.

Note: Store crRNA:tracrRNA duplex at –20°C after use.

Option 2: Resuspend sgRNA

1. Resuspend each Alt-R CRISPR-Cas9 sgRNA in IDTE Buffer to a final concentration of 100 μM .

Note: For assistance, use the Resuspension Calculator at www.idtdna.com/SciTools.

2. Place on ice.

Note: Store sgRNA at -20°C after use.

D. Prepare RNP complexes

1. For each well undergoing electroporation, dilute the guide RNA and Cas9 protein in PBS, **gently swirling the pipet tip while pipetting:**

Component	Amount (μL)
PBS	2.1
Alt-R gRNA (from step C) (crRNA:tracrRNA duplex or sgRNA)	1.2 [120 pmol]
Alt-R S.p. Cas9 Nuclease V3*	1.7 [105 pmol]
Total volume	5[†]

* Alt-R S.p. Cas9 Nuclease V3 is provided at a stock concentration of 62 μM .

† This 5 μL reaction volume is for a single nucleofection reaction; scale up as necessary for your experiment.

2. Incubate at room temperature for 10–20 min (optional: in a PCR thermal cycler).

Note: To save time, the RNP can be prepared in parallel with cell preparation (**steps E1 and E5**, during centrifugation).

E. Wash and resuspend cells

Note: The PBS wash is intended to remove all traces of RNases that can quickly degrade the critical CRISPR RNA components. It also facilitates transfer of cells into a microcentrifuge tube in which the cell pellet will be visible, thus allowing maximal removal of unnecessary fluid before reconstitution in P3 electroporation solution.

1. Remove cells for electroporation from the incubator, and centrifuge at 300 x g for 5 min at room temperature.
2. Remove as much supernatant as possible without disturbing the pellet.
3. Resuspend the cells in PBS, and transfer into a sterile microcentrifuge tube.

4. Rinse the original tube with PBS, and add rinse to the microcentrifuge tube.
5. Centrifuge the microcentrifuge tube at 300 x g for 5 min at room temperature.
6. Remove as much supernatant as possible without disturbing the pellet.
7. Resuspend cells by adding 20 μL of supplemented Nucleofector Solution P3 (**step B4**) per 5×10^4 cells.
8. Pipet 20 μL of the cell suspension into individual, sterile PCR tubes.

F. Perform electroporation

1. Add RNP complexes and enhancer to cells, as described in the following table:

Component	Volume (μL)	Final concentration
Cell suspension in P3 (from step E8)	20	—
RNP complex (from step D2)	5	4 μM Cas9 nuclease and 4.6 μM guide RNA* (crRNA:tracrRNA duplex or sgRNA)
Electroporation enhancer (from step B5)	1	3.85 μM
Total volume	26	—

* The RNP concentration is determined by Cas9, so in this reaction, the RNP concentration is 4 μM . You may perform a titration experiment of the RNP concentration in the range of 0.5–4 μM to determine sub-saturation conditions. The titration is unique for each guide type and genomic locus.

Note: The total volume allows for easy pipetting of 25 μL intended for electroporation and prevents the formation of air bubbles in the electroporation strip.

2. Pipet the mixture of cells and RNP complex up and down 2 times (optional: use a multichannel pipette and spin down, if necessary), and transfer 25 μL to the relevant well of a 16-well Nucleocuvette Strip (note that the mock sample will have a lower volume).
3. Gently tap the Nucleocuvette module to make sure no air bubbles are present.
4. Place Nucleocuvette module in the Shuttle device of the 4D-Nucleofector System, select **OK** to load the strip, and select **Start**.

G. Promote cell recovery and culture cells

Note: It is crucial for cell viability to perform the recovery of the cells by adding culture media to the Nucleocuvette as quickly as possible!

1. After electroporation, remove the Nucleocuvette module from the instrument.
2. Quickly add 75 μL of prewarmed culture media (from **step B7**; optional: use a multichannel pipette and sterile reservoirs) per each well of the Nucleocuvette, and resuspend cells by gently pipetting up and down.

3. Use sterile, disposable plastic pipettes to transfer the entire volume from each well of the Nucleocuvette to the correct recovery well in the 96-well, U-bottom tissue culture plate.
4. Incubate cells in a low-oxygen incubator. After 48–96 hr in culture, gDNA can be extracted and genome editing levels can be analyzed.

References

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