Electroporation of primary human CD34$^+$ hematopoietic stem and progenitor cells.

✧ Experimental preparation

1. Guide RNA
   1) Form the crRNA:tracrRNA duplex
      i. Resuspend the Alt-R CRISPR-Cas9 crRNA and Alt-R CRISPR-Cas9 tracrRNA in TE buffer to final concentrations of 200 µM. (For assistance, use the Resuspension Calculator at www.idtdna.com/SciTools.
      ii. 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

      iii. Heat at 95℃ for 5 min.
      iv. Remove from heat, and allow to cool to RT on the bench.
      v. Keep at RT for about 10 min, the place on ice. (Store crRNA:tracrRNA duplex at -20℃ after use).
      Note: Alt-R CRISPR-Cas9 crRNA need predesign and custom in www.idtdna.com/CRISPR-Cas9
   2) Resuspend sgRNA
      i. Order sgRNA from www.idtdna.com/CRISPR-Cas9, the product is a 2 µM powder.
      ii. Add 20 µL TE into sgRNA powder (100 pmol/µL).
      iii. Place on ice (Store crRNA:tracrRNA duplex at -20℃ after use).
2. Cas9 Nuclease
   1) Alt-R™ S.p. Cas9 Nuclease V3 dilution.
   Stock is 500 µg or 100 µg, add 50/10 µL PBS make the concentration into 10 µg/µL
   2) Guide-it™ Recombinant Cas9
   Stock is 3 µg/µL.
3. Cell culture preparation
   Medium: StemSpan™ SFEMII (STEMCELL Technologies) supplemented with 100 ng/ml rhSCF (#255-SC, R&D Systems), 10 ng/ml rhIL-6 (#206-IL, R&D Systems), 1 ng/ml rhIL-3 (#203-IL, R&D Systems) and 1% penicillin–streptomycin (PS, #09367-34, Nacalai).
   CB Cells thawing amount: 2.5x10^5 cells/sample (before 48 hours thawing and culture)

✧ Day 1

1. Thaw CB cells and start pre-culture 48 hours.
2. Adjust sgRNA and Cas9 protein concentration.

✧ Day 3

1. Add each well 500 µL culture medium into 24 well plate and put it into the incubator.
2. Pick up P3 Primary Cell 4D-Nucleofector™ X Kit S | Lonza kit box.
3. Add the entire supplement to Nucleofector Solution P3 as below (each sample):

Component	Amount (µL)
P3 Primary Cell Nucleofector® Solution	16.4
Supplement 1	3.6
Total volume	20

4. Let the supplemented Solution P3 reach room temperature.
5. Prepare RNP complexes as below:

Component	Amount (µL)

100 pmol/μL Alt-R CRISPR-Cas9 sgRNA	2.6
10 μg/μL Alt-R™ S.p. Cas9 Nuclease V3	1.7
Total volume	4.3

Or for Takara Cas9 nuclease

Component	**Amount (μL)**
100 pmol/μL Alt-R CRISPR-Cas9 sgRNA	2.6
3 μg/μL Guide-it™ Recombinant Cas9	5.6
Total volume	8.2

Gently swirling the pipet tip while pipetting.

6. Incubate the mixture in RT 15 min.
7. Collect CB cells into 50 mL falcon tube and centrifuge (1500 rpm, 5 min, 4℃).
8. Aspirate supernatant, use cold PBS wash 2 times.
9. Aspirate PBS and add supplemented Solution P3 20 μL into CB cells. Add supplemented Solution P3 with CB cells into RNP complexes. Use tip gently mix and lightly pipet 3 times (Be careful with the bubbles).
10. Transfer 25 μL to the relevant well of Amaxa plate, gently tap after adding and make sure no air bubbles are present.
11. Open 4D-Nucleofector System, insert plate into the machine. Enter the Protocol and chose DZ-100. Select OK to load the strip, and select Start.
12. After electroporation, remove the plate from the instrument, quickly add 100 μL prewarmed (before experiment incubate in incubator) CB cells culture medium, resuspend cells by gently pipetting up and down, and transfer cells into 24 well plate.

✧ Day 5

Collect cells and check KO efficiency.