Electroporation of human B cell lines with CRISPR reagents

Delivery of ribonucleoprotein complexes using the Alt-R CRISPR-Cas9 System and the Neon® Transfection System into Ramos, BJAB, and DG75 cells

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Adjust cell culture density one day before electroporation

1. Follow these general cell culture guidelines:
   - Do not use freshly thawed cells for electroporation
   - Use cells with the lowest passage number possible
   - Use cells dividing in log phase
2. Change the cell culture media or split cells to obtain good confluency for electroporation.

   Note: For Ramos, BJAB, and DG75 B cell lines, good culture density is between 1 x 10^5 and 7.5 x 10^5 cells/mL one day before electroporation (accordingly, the cells will have doubled by the day of electroporation).

Anneal the crRNA:tracrRNA duplex and prepare the electroporation enhancer

1. Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA and tracrRNA) in IDTE Buffer to a final concentration of 200 µM.

   Note: Use the IDT Resuspension Calculator at SciTools Web Tools for assistance. Store unused, resuspended RNAs at –20°C.

2. Mix the two RNA oligos in equimolar concentrations in a nuclease-free, sterile microcentrifuge tube to a final duplex concentration of 44 µM. The following table shows examples for 2.5 and 10 µL final volume (5 and 20 electroporations, respectively):

Component	Amount (µL)	Amount (µL)
200 µM Alt-R CRISPR-Cas9 crRNA	0.55	2.2
200 µM Alt-R CRISPR-Cas9 tracrRNA	0.55	2.2
Nuclease-Free IDTE Buffer	1.4	5.6
Total volume	2.5	10
# of electroporations	5	20

3. Heat at 95°C for 5 minutes.
4. Remove from heat, briefly centrifuge, then cool to room temperature (20–25°C) on the bench top.
5. Prepare the Alt-R CRISPR-Cas9 Electroporation Enhancer.

   Note: Use the IDT Resuspension Calculator at SciTools Web Tools for assistance.

   a. At first use, resuspend the Alt-R CRISPR-Cas9 Electroporation Enhancer to 100 µM in IDTE and store at –20°C.

   b. On the day of experiment, dilute the 100 µM stock to a 10.8 µM working solution. You will need 2 µL of working solution for each electroporation.

Assemble the RNP complex

1. For each electroporation, dilute the Alt-R Cas9 (61 µM stock) to 37 µM as follows.

Component	Amount (µL)	Amount (µL)
61 µM Alt-R S.p. Cas9 enzyme (nuclease or nickase)	0.3	1.5
Resuspension Buffer R (from the Neon System Kit)	0.2	1
Total volume	0.5	2.5
# of electroporations (5 recommended)	1	5

2. For each electroporation, combine the crRNA:tracrRNA duplex and Cas9 enzyme by gently swirling the pipette tip while pipetting:

   Note: Create a mixture for 5 electroporation samples by adding 2.5 µL of diluted Cas9 enzyme to the same volume of crRNA:tracrRNA duplex.

3. Incubate the mixture at room temperature for 15–20 minutes.

   Note: During this time, prepare the Neon device as well as the recovery medium and plate(s).

Prepare the Neon Transfection System

1. Turn on the Neon device.
2. Enter an electroporation setting.

   Note: For optimal setting for Ramos, DG75, and BJAB cell lines, see Enhanced electroporation conditions for B cell lines.

3. Set up the Neon pipette station by filling a Neon vial with 3 mL of Buffer E (included in the Neon System Kit) and insert it into the station.

   Note: Prepare 1 Neon vial per cell line, or per 10 electroporations.

Prepare the recovery medium and plates

1. Prepare recovery medium without antibiotics [e.g., RPMI media, 20% fetal calf serum, and if needed, GlutaMAX™ supplement, 1X (Thermo Fisher), 10 mM HEPES].
2. Using 24-well plates, fill the number of wells (one per electroporation) needed with 0.5–1 mL of recovery medium.
3. Fill the number of wells needed for "no electroporation but with RNP" controls with 0.5–1 mL of recovery medium.

   Note: It is recommended to include at least one control sample per experiment that does not undergo electroporation to set up knockout screening conditions.

4. Place the plate(s) in a cell culture incubator (e.g., 37°C, 5% CO_2).

Collect and transfect the cells

1. Pipette cells up and down to dissociate cell clumps.
2. Count the cells in suspension culture.
3. Determine the total number of cells necessary for your experiment.

   Note: For B cell lines, use 5 x 10^5 to 10 x 10^5 cells per electroporation.

4. Centrifuge the required number of cells for all electroporations at 300 x *g* at room temperature for 5 minutes.

   Note: Centrifuge cells for no more than 20 electroporations at one time.

5. Remove as much supernatant as possible without disturbing the pellet.

   Optional: Wash cells in 5 mL of 1X PBS (centrifuge at 300 x*g* at room temperature for 5 minutes).

6. Resuspend cells by adding 9 µL of Resuspension Buffer R per electroporation.
7. For each electroporation and control sample, mix the following in a microcentrifuge tube:

Component	Amount (µL)
crRNA:tracrRNA:Cas9 RNP complex	1
Cell suspension	9
10.8 µM Alt-R CRISPR-Cas9 Electroporation Enhancer	2
Total volume	12
# of electroporations	1

8. With a Neon pipette, transfer 10 µL of cell:RNP mixture (from step 7 above) into the pipette tip, avoiding air bubbles.
9. Insert the Neon pipette with the tip into the pipette station. Verify the presence of electrolytic buffer in the Neon vial.
10. Press Start.
11. After electroporation, immediately transfer the cells to a recovery well.

    Note: Cells can be split to 96-well plates for single-cell cloning by limiting dilution.

12. Allow cells to recover in a cell culture incubator (e.g., 37°C, 5% CO_2).

Analyze survival and knockout efficiency in B cell lines

1. After 24 hr, stain a subset of cells in a 24-well plate using a viability dye (e.g., propidium iodide).
2. After 4–5 days, stain cells for the targeted marker.

Note: For single-cell cloning, the time may vary; use plating efficiency for survival.

Enhanced electroporation conditions for B cell lines

In Ramos cells, the most consistent combined survival and knockout efficiency was achieved by electroporation with 1 pulse of 20 ms at 1450 V (Figure 1).

For the less extensively tested DG75 and BJAB cell lines, the best working settings were 1 pulse of 20 ms at 1550 V (DG75) and 1 pulse of 40 ms at 1350 V (BJAB) (data not shown).

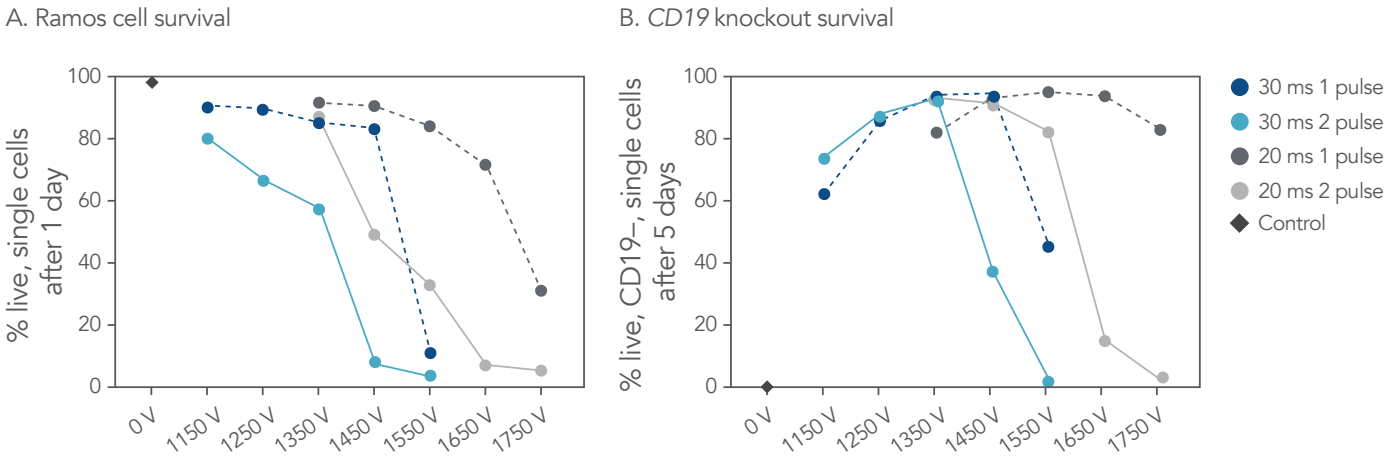

Figure 1. Enhanced conditions were based on cell survival and on-target knockout rates. One million Ramos cells per electroporation were transfected with the RNP containing a crRNA targeting human *CD19* (selected from a screen of more than 30 crRNAs for intra- and extra-cellular markers in different B cell lines) according to the above protocol. (**A**) The day after electroporation, part of the cells were stained with 2.5 µg/mL propidium iodide (BioLegend, catalog #421301) and analyzed by flow cytometry. (**B**) Five days after electroporation, part of the cells were stained with 2.5 µg/mL Alexa Fluor® 647 anti-human CD19 (BioLegend, catalog #302220, clone HIB19) – plus propidium iodide – and analyzed by flow cytometry. Control = cell:RNP mixture without electroporation. Data courtesy of Dr Marco Cavallari, University of Freiburg, Germany.

Revision history

Version	Release date	Description of changes
2	July 2022	Corrected document for internal MAPSS compliance
1	January 2020	Initital release.

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