RNP Transfection in WTCs

Required reagent list:

- Complete mTeSR1 culture media, referred to in this protocol as simply "mTeSR1": 400 mL basal media with provided 100 mL 5X supplement (catalog # 05850, Stem Cell Technologies) with added 5 mL (1% v/v) Penicillin/Streptomycin (catalog # 15140-122, Gibco) *Refer to page 16 of the Stem Cell Technologies technical manual about preparation, storage and shelf life of this media*.
- Matrigel (catalog # 354230, Corning)
- DMEM/F12 media, phenol red-free (catalog # 11039-021, Gibco Life Technologies)
- ROCK inhibitor (Ri) $[10mM]_{stock}$ reconstituted in DMSO per manufacturer's instructions (Stemolecule™ Y27632, catalog # 04-0012-10, Stemgent)
- DPBS, without Ca^{+} or Mg^{++} (catalog # 14190-144, Gibco Life Technologies)
- StemPro® Accutase® (catalog # A11105-01, Gibco Life Technologies)
- Tissue Culture Treated 6-well Plates (catalog #657-160, CellStar)
- crRNA (custom synthesis from Dharmacon, or other vendor, unique to each target site)
- tracrRNA (constant sequence of RNA used with any Dharmacon crRNA, catalog #U-002000)
- PCR tubes (preferred brand)
- 1.5 mL Eppendorf DNA LoBind Microcentrifuge Tubes, or similar (catalog #13-698-791, Fisher Scientific)
- Duplex Buffer: 100 mM Potassium Acetate; 30 mM HEPES, pH 7.5
- TE pH 7.5 (catalog #11-01-02-02, IDT)
- Cas9 Protein (Berkeley QB3 MacroLab, http://qb3.berkeley.edu/macrolab/)
- Donor plasmid maxi-prep, eluted in Endotoxin-Free Water
- Neon 100 µL Kit (catalog #MPK10096, Fisher Scientific)

Required instruments:

- Thermocycler or heat block capable of reaching 95˚C
- Neon Electroporation Device
- Micro-centrifuge
- Equipment necessary for sterile cell culture of hiPSC, see "Culture and Freezing Methods for WTC" protocol

Steps before starting:

1. Check that the morphology of your cells is consistent with known, good hiPSC morphology (Fig. 1). Ideally, cells should be at ~75% confluency, and should be fully recovered from previous passage. Some dead cells in the media is normal, but this should not be more than 1-5%.
2. If necessary, prepare fresh mTeSR1 media:
    a. Thaw 5X supplement at room temperature (RT) for ~4-6 h, or at 4˚C overnight. Do not thaw 5X supplement at 37˚C.
    b. Combine 5X supplement with 400 mL mTeSR1 and 5 mL Pen/Strep.
    c. Sterile filter media with a 0.22 µM media filter before first use.
3. Bring mTeSR1 media to RT on the bench. Do not warm mTeSR1 in a 37˚C water bath.
4. Pre-warm Accutase in a 37˚C water bath.
5. Label vessel(s) (culture dish or CryoVials, etc.) with cell line name, clone, passage number, date, and attach barcode (as applicable). Bring any Matrigel-coated vessels to room temperature.
    a. If necessary, prepare Matrigel-coated vessels (refer to "Culture and Freezing Methods for WTC" protocol)
6. Prepare mTeSR1 +ROCK inhibitor (Ri) media. mTeSR1 + Ri should always be used with cells for 24 h after they are treated with Accutase to promote cell survival.
    a. Add Ri at 1000X dilution to mTeSR1 media.
    b. Mix well by pipetting.
    e.g., for 100 mL mTeSR1, add 100 µl Ri.
    Note: Lyophilized Ri stock is reconstituted in DMSO at 10 mM, per manufacturer's instruction. We recommend making 200 µL aliquots in 1.5 mL Eppendorf tubes and storing at -20˚C for up to 6 months.

Method: Pre-complex crRNA and tracrRNA *(to be done 2.5 h- 72 h in advance of transfection)*

1. Reconstitute crRNA and tracrRNA to 100 µM in TE. Alternate vortexing 30 sec and pulse-spinning for a minimum of 3 times to ensure all lyophilized RNA is reconstituted.
2. Combine crRNA and tracrRNA in a sterile PCR tube so that each is at a final concentration of 40 µM in a volume appropriate for the experiment. Use Duplex Buffer as the diluent.
   (eg. 8 µL 100 µM crRNA, 8 µL 100 µM tracrRNA, and 4 µL Duplex Buffer).
3. In a thermocycler or heat block, heat the crRNA:tracrRNA mixture(s) from step 2 to 95˚C for 5 min.
4. Remove from thermocycler or heat block and allow tube(s) to cool <u>on the bench top</u> for a *minimum* of 2 h.
5. Store duplexed crRNA:tracrRNA in PCR tubes at -20˚C long-term, or keep on ice if complex is to be used the same day. Always thaw on ice or at 4˚C from -20˚C storage.

Method: Transfection with Neon Electroporation Device

Steps before starting:

1. <u>Pre-treat cells to be transfected with mTESR1 + Ri media for 1-6 hrs prior to transfection to promote cell survival. Cells should appear to have spikey edges around colonies with Ri treatment.</u>
2. Prepare all plate layouts and calculations prior to starting (example of calculations given in Figure 2).
3. See Table 1 for recommendations on transfection controls included with each experiment.
4. Label 1.5 mL Eppendorf tubes for each reaction preparation.
5. Label 1.5 mL Eppendorf tubes for cell aliquots.
6. Prepare 10 µM working concentrations of crRNA:tracrRNA duplex by diluting each in TE.
   a. Keep dilutions and stocks on ice.
   b. Working concentrations of crRNA:tracrRNA duplex can be stored at -20˚C for up to 2 weeks; avoid multiple freeze-thaw cycles (<3 recommended).
7. Prepare 10 µM working concentrations of Cas9 by diluting in TE.
   a. Use a freshly thawed aliquot of Cas9 whenever possible.
   b. Thaw Cas9 from -80˚C storage on ice or at 4˚C until no ice pellet is visible (~2-5 min).
   c. Keep dilutions and stocks on ice.
   d. Working concentrations of Cas9 protein can be stored at -20˚C for up to 2 weeks; avoid multiple freeze-thaw cycles (<2 recommended).
8. Prepare working concentrations of donor plasmid at 1 µg/µL by diluting in TE. Higher concentrations of donor plasmid can be used, but lower concentrations should be avoided (<500 ng/µL). We recommend 1 µg/µL for easy calculations and pipetting.
9. Prepare the destination 6-well plate by aspirating and discarding the excess Matrigel liquid, and adding 4 mL of RT mTeSR1 + Ri media to each well. Keep plate with media in the incubator at 37˚C and 5% CO_2 until ready to plate cells after the transfection procedure (plate should not be stored in incubator for longer than 2 h before use).

Transfection:

1. Set up the Neon device in the hood, per manufacturer's instructions.
2. Passage cells according to "Culture and Freezing Methods for WTC" protocol, proceeding until cells are in suspension and have been counted.
3. <u>Prepare cell aliquots for each transfection reaction (room temperature):</u>
   a. Aliquot cells in mTeSR + Ri into separate 1.5 mL Eppendorf tubes at numbers required for all transfection reactions, including excess
   e.g. if cell suspension is at a concentration of 1 x 10^6 cells/mL and 1.2 x 10^6 cells are required per transfection reaction, then aliquot 1.2 mL cells/tube.
4. <u>Prepare Ribonucleoprotein (RNP) Complex tubes for each transfection reaction (room temperature):</u>
   b. Add the required volume of 10 µM working solution of crRNA:tracrRNA duplex per reaction to the pre-labeled 1.5 mL Eppendorf tubes for each reaction preparation.
   c. Add the required volume of 10 µM working solution of Cas9 protein per reaction to the pre-labeled 1.5 mL Eppendorf tubes already containing crRNA:tracrRNA duplex from step 4a by <u>slowly</u> adding the Cas9 and then gently pipetting up and down three times.

d. Incubate RNP complex at room temperature for a minimum of 10 min and no longer than 1 h prior to addition of cells in step 7, below.

5. Pellet cell aliquots from step 4 as they are ready to be used (we recommend 1-3 reactions at a time), so as to not allow cells to sit pelleted for longer than 5 min. Pellet cells by spinning in a micro-centrifuge or swinging bucket centrifuge at 211 x g for 3 min at RT.
6. Gently aspirate and discard supernatant with a pipette and re-suspend cell pellet in the required volume of Buffer R from the Neon Transfection Kit.

   Note- when aspirating, it is okay to leave behind a *small* (<10 µL) residual volume of mTESR + Ri with the cell pellet to ensure the pellet is not disturbed.
7. Transfer re-suspended cells in Buffer R into the tube containing the RNP complex to be transfected. Mix the entire volume gently 3 times by pipetting.
8. Add the required volume of plasmid to the cell-RNP mixture. The mixture is now ready for electroporation.
9. Using the Neon pipette and pipette tip provided with the kit, *gently* pipette the cell-RNP-plasmid mixture 2 times before aspirating the 100 µL necessary for the reaction, being careful not to introduce bubbles.
10. Load the tip containing the cell-RNP-plasmid mixture into the Neon chamber, making sure the tip fully engages with the Neon chamber (should click in). Apply the voltage. We recommend setting 8 for WTC RNP transfection: 1300 V, 30 ms, and 1 pulse.

    Note- visually inspect the Neon tip for any bubbles that were introduced during the loading process. If bubbles appear, re-aspirate cell-RNP-plasmid mixture into tip without ANY bubbles and try again. When applying the voltage, watch for tiny bubbles to appear near the Neon electrode at the bottom of the chamber, and listen for a "popping" noise to indicate that the voltage has been properly applied.
11. Quickly unload the tip from the Neon chamber and gently dispense cells into the prepared 6-well plate containing mTeSR + Ri. Gently agitate plate to evenly distribute cells.
12. Repeat Steps 5-11 for each reaction. When all reagents are properly prepared ahead of time, the electroporation process (steps 5-11) for 5-6 samples should take less than 30 min.
13. Incubate cells for 24 h before changing media to mTeSR1 without Ri, then feed daily as usual.
14. Cells can be FACS sorted or used in downstream applications once they reach a healthy confluency and maturity, usually in 3-4 days.

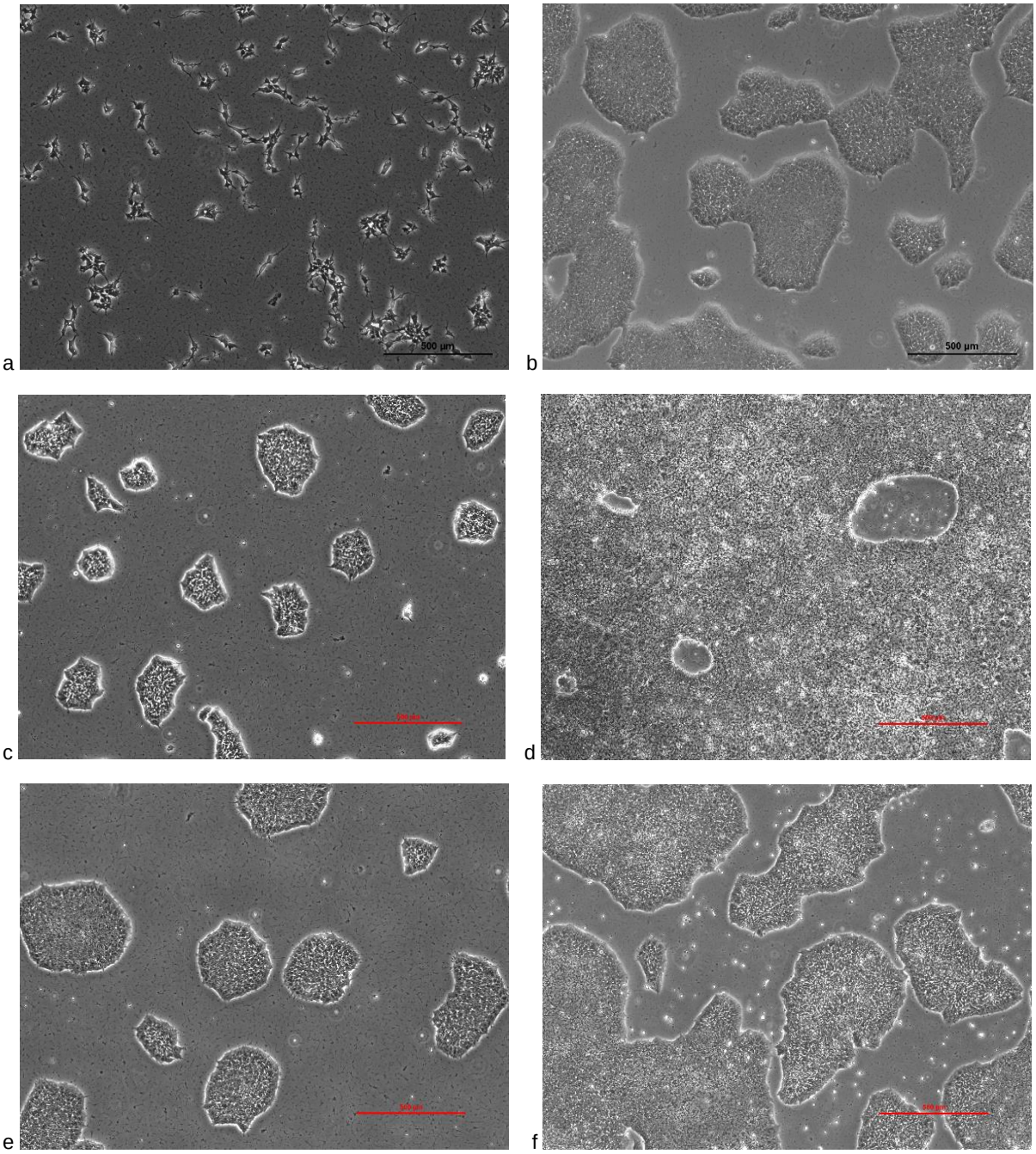

Figure 1. Examples of Confluency: (a) 1 x 10^6 cells plated in 10 cm dish, 24 h after seeding. Cells maintain a "spikey" morphology due to RI treatment. Cells should be allowed to grow 3-4 days before subsequent passaging. **(b)** Same cells from (a) after 3 days of growth. Cells have good mature stem cell morphology and are at an ideal density to be passaged again. **(c)** Immature colonies have a slightly spikey edge and are loosely packed in colony interior. Passaging immature cells should be avoided. **(d)** Overgrown culture that is too confluent to continue to use. Future genomic integrity and/or morphology may be compromised. **(e)** Mature colonies at low density, can be picked as individual colonies or passaged. **(f)** Similar to (b), another example of a culture that has good mature stem cell morphology and is at an ideal density to be passaged again.

	Example Reaction (rxn) Volumes	
	Vol per rxn	**x # rxns (1.3)**
crRNA:tracrRNA Duplex(10 µM)	1.25	**1.63**
Cas9 Protein (10 µM)	1.25	**1.63**
Plasmid (1 µg/uL)	2.00	**2.60**
Constant Reaction Volume (µL)	4.50	**5.86**
	800,000	cells/rxn
	1.3	rxns
	1,040,000	**cells/aliquot**
	124.1	**µL Buffer R/aliquot**

Figure 2. Example calculations for a reaction using 2 µg plasmid and 2 µg Cas9 protein with a 1:1 molar ratio of Cas9:RNA. crRNA:tracrRNA duplex and Cas9 Protein are at a working concentration of 10 µM for ease of pipetting, while still keeping the overall reaction volume less than 10 µL (a requirement per Neon manufacturer's protocol that the reagent be < 10% of total volume). All volumes given in µL. For ease of use of Neon tips, a minimum of 1.3x reaction volume is recommended. In grey-shaded cells: Row 1 is the desired number of cells transfected, Row 3 is Row 1 multiplied by the number of reactions calculated for excess (shown in Row2), and Row 4 is the amount of Buffer R required to re-suspend cells based on a 100 µL reaction at 1.3x excess and takes into account the reaction volume of RNP complex.

Positive Control	pMax-GFP + RNP with target crRNA sequence	pMax-GFP or other constitutively expressed FP delivered as a plasmid. Confirms transfection was successful in the presence of RNP. Sample can be preserved for T7 Assay (or similar) to determine % editing of crRNA sequence.
Negative Control 1	Donor Plasmid + RNP with NTC crRNA sequence	Controls for viability and will account for any toxicity associated with the donor plasmid preparation since the crRNA is non-targeting and therefore should not cause DNA-cutting related toxicity.
Negative Control 2	Buffer only	Cells are re-suspended in Buffer R and transfected with the Neon device but are not treated with RNP or plasmid. Accounts for toxicity associated with electroporation method or buffer.

Table 1. Recommended Controls.