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We use this protocol and it's working

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CRISPR/Cas9 generation of knock-out iPSCs

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ABSTRACT

This protocol describes RNP-based CRISPR/Cas9 gene-editing to generate knock-out iPSCs. iPSCs are transfected with sgRNA, recombinant Cas9, and GFP using Lipofectamine Stem. After FACS sorting to enrich for successfully transfected cells, individual clones are picked and expanded for further analysis.

ATTACHMENTS

553-1149.pdf

MATERIALS

Materials

- 10 cm cell culture dishes
- 6-well, 24-well, and 96-well plates
- Cell culture microscope for picking colonies

Reagents

- Synthetic sgRNA (for example Gene Knockout Kit v2 from Synthego = multi-guide mix for knockout).
- Alt-R® S.p. HiFi Cas9 Nuclease V3 **IDT Catalog #1081060**
- Lipofectamine™ Stem Transfection Reagent **Thermo Fisher Catalog #STEM00003**

- Opti-MEM (Reduced Serum Medium) **Thermo Fisher Scientific Catalog #31985062**
- mTeSR™1 1 L Kit **STEMCELL Technologies Inc. Catalog #85857**
- Growth Factor Reduced (GFR) Matrigel® **Corning Catalog #354230**
- DMEM/F-12 **Thermo Fisher Catalog #11320033**
- Accutase® solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A6964**
- Y-27632 ROCK Inhibitor **Selleckchem Catalog #S1049**
- PBS buffer **Thermo Fisher Scientific Catalog #10010023**
- Penicillin-Streptomycin (10,000 U/mL) **Thermo Fisher Scientific Catalog #15140122**
- Falcon 40 µm Cell Strainer **Corning Catalog #352340**
- KnockOut™ Serum Replacement **Thermo Fisher Catalog #10828028**

Transfection with sgRNA and recombinant Cas9

1 Accutase-dissociate iPSCs on the morning of transfection.

1.1 Plate 800,000 iPSCs onto one well of a Matrigel-coated 6-well plate in mTeSR medium

supplemented with [M] 10 micromolar (µM) ROCK inhibitor (RI).

2 1 - 2 hours prior to transfection, change media to fresh, pre-warmed mTeSR + RI (2 mL /well).

Note

Replacing the media is important because remaining Accutase can interfere with Lipofectamine Stem transfection.

3 **Prepare sgRNA:**

Resuspend [M] 1.5 nanomolar (nM) sgRNA in 15 µL of RNAse-free TE buffer to make a [M] 100 micromolar (µM) stock solution.

3.1 Dilute required amount of sgRNA to [M] 1 micromolar (µM) final concentration in TE buffer.

4 Flick tube of HiFi Cas9 enzyme (IDT) to mix and briefly spin.

4.1 Dilute required amount of HiFi Cas9 ([M] 62 micromolar (µM) stock solution) to [M] 1 micromolar (µM) in Room temperature OptiMEM.

Note

e.g. 0.5 µL of [M] 62 micromolar (µM) HiFi Cas9 in 30.5 µL OptiMEM. Leftover diluted Cas9 can be stored at -20 °C.

5 For transfecting a single well of a 6-well plate, set up **Tube 1** with

	A	B
	OptiMEM	76 µL
	1 µM HiFi Cas9	12 µL
	1 µM sgRNA	12 µL

5.1 Mix by pipetting up and down gently, then incubate for 00:05:00 at Room temperature to allow RNP complexes to form. 5m

6 Add 0.4 µg GFP plasmid to RNP complex in **Tube 1** and mix by pipetting.

7 Set up Lipofectamine Stem mix in **Tube 2** by combining 100 µL OptiMEM and 4 µL Lipofectamine Stem.

8 Add **Tube 2** solution to **Tube 1** and flick tube to mix. Incubate mixture for 00:10:00 at Room temperature, then add mixture dropwise to iPSCs. 10m

9 On the following day, individualize iPSCs with Accutase and plate onto 6 Matrigelcoated wells of a 6-well plate in mTeSR + RI.

9.1 3d

Feed cells daily with mTeSR without RI until cells are about 70% confluent (after ~ 72:00:00).

FACS-sorting

10 Coat 1-2x 10 cm dishes per condition with Matrigel and add 10 mL mTeSR (+RI +**Penicillin/Streptomycin**) on the day of FACS sorting. Keep 10 cm dishes in cell culture incubator at 37 °C .

Note

Because of the increased risk for microbiological contamination during FACS sorting, we recommend supplementing mTeSR medium with Penicillin/Streptomycin (P/S).

11 Collect transfected iPSCs with an Accutase split and resuspend into mTeSR (+RI +P/S).

Note

The media volume depends on size of the cell pellet; we have used 1.5 mL for resuspending iPSCs from 4 wells of a 6-well plate.

12 Filter iPSCs through a 40 µm cell strainer into a 50 mL conical tube to individualize cells and avoid clumping.

12.1 Move single-cell suspension to a 15 mL tube.

12.2 Prepare an additional 15 mL conical tube with 1.5 mL mTeSR (+P/S, +RI) for collecting cells after FACS sorting

.

12.3 Keep both tubes On ice until FACS sorting.

13 Perform FACS-sorting for GFP-expressing iPSCs using standard FACS parameters.

> **Note**
>
> Transfection efficiency with Lipofectamine Stem should be about 30%.

13.1 Plate ~10,000 GFP-expressing cells onto one 10 cm dish.

13.2 Move 10 cm dish back-and-forth and left to-right to distribute cells evenly.

14 Exchange media on the next day to mTeSR + P/S, but without RI. Keep feeding cells daily with mTeSR + P/S.

Picking Colonies

15 Pick clones when individual colonies reach a size of ~ 1 mm.

> **Note**
>
> This should be ~ 9 days after FACS sorting for KOLF2.1 iPSCs.

16 For the first attempt of CRISPR/Cas9 editing, we recommend picking 24 colonies per condition.

Note

If none of the picked clones has the desired genotype, the editing protocol may need to be optimized and/or more clones should be picked during the next attempt.

16.1 

In two 96-well plates, coat 12 wells for each condition with Matrigel; then add 200 µL mTeSR per well (+ P/S, + RI).

16.2 

Put 96-well plates in cell culture incubator and allow some time to equilibrate.

16.3 

Aspirate old mTeSR media off 10 cm dishes and replace with 15 mL fresh media per dish.

Note

Add 15 mL instead of 10 mL mTeSR to each 10 cm dish for picking iPSC clones, because aspirating colonies will remove media.

Note

We find that leaving iPSCs at Room temperature for a prolonged time after picking clones seems to decrease chances of survival. Therefore, we recommend equilibrating 96-well plates before starting to pick colonies and using two 96-well plates per condition.

17 To pick individual clones, visualize colonies under a tissue culture microscope.

Note

Ideally, the microscope is placed in an open hood. We find that it's also feasible to place the microscope in a regular cell culture hood and lift the glass front all the way up.

18 Use a P200 pipette to scratch each colony from the dish and aspirate the colony in 100 µL media.

18.1

Pipette up- and down four times in one well of an additional empty (third) 96-well plate to break up cell clusters.

18.2

Then add all 100 µL to one well of one of the pre-equilibrated 96-well plates.

19 Pick 12 colonies into the first 96-well plate. Then return the first 96-well plate to the cell culture incubator and use the second 96-well plate for picking clones 13-24.

Note

While picking clones, try to avoid colonies that look as if two different colonies have grown together (e.g., looking like the number 8). We recommend picking a random mix of smaller, medium-sized, and larger colonies.

Note

Optional: The remaining clones may be kept in culture for 1-2 more days and can then be used to make pooled cell lysates. These lysates can be analyzed per Western Blot to get a general sense of the knock-out efficiency.

Expanding individual clones

1w 3d 0h 29m

20 Feed iPSCs in 96-well plates daily with mTeSR + P/S. The cells are ready to be split when iPSCs in a well are almost confluent and/or individual colonies grow to a size >half of the well.

Note

For KOLF2.1 iPSCs, this should be ~ 168:00:00 after picking individual colonies. Some clones may have to be split earlier than other clones.

21 Splitting cells from 96-well plates to 24-well plates using Accutase:

21.1

Prepare 24-well plate(s) by coating with Matrigel GFR, then adding 500 µL mTeSR + RI + P/S per well. Place 24-well plate(s) in cell culture incubator to pre-equilibrate.

21.2 14m

- Aspirate mTeSR from all wells of the 96-well plate and wash with PBS.
- Then add 25 µL Accutase per well. Return to incubator and let sit for 00:06:00 - 00:08:00 .
- Add 200 µL mTeSR (+RI + P/S) to each well and break up bigger cell clusters by pipetting up and down 3-4 times in each well.
- Transfer cells from one well of the 96-well plate to one well of a 24-well plate.

Note

Optional: To obtain cellular DNA for screening successful CRISPR edits, pipette ~ ¼ of the volume into an Eppendorf tube.

22 Splitting cells from 24-well plates to 6-well plates using Accutase:

22.1 iPSCs should be ready to split within 2-4 days. Prepare 6-well plates by coating with Matrigel

GFR, then adding 2 mL mTeSR + RI per well.

22.2 14m

- Aspirate mTeSR from wells in 24-well plate and wash with PBS.
- Add 100 µL Accutase per well and incubate at 37 °C for 00:06:00 - 00:08:00 .
- While cells are at 37 °C , prepare one 1.5 mL Eppendorf tube with 200 µL mTeSR + RI for each clone.

22.3

- Aspirate cells in Accutase by pipetting up and down a few times in each well.
- Then add cells to prepared Eppendorf tube.

22.4 3m

- Spin cells down using a tabletop centrifuge (00:03:00 at 200 rcf and Room temperature .
- Resuspend each pellet in 1 mL mTeSR + RI (taken from the prepared 6-well plate) and plate individual clones onto separate wells of 6-well plates.

23 Feed cells daily with mTeSR. Cells should be ready to be split and cryopreserved within 2-3 days.

23.1

For freezing down a higher number of clones, cells can again be Accutase-split into Eppendorf tubes, then resuspended in cryopreservation media:

	A	B
	DMSO	10%
	Knockout Serum Replacement	20%
	mTeSR	70%

24 Individual clones can then be thawed and expanded to confirm knock-out via Western Blot.

Note

All clones to be used in future studies should be confirmed to have a normal karyotype by cytogenetic analysis of G-banded metaphase cells.