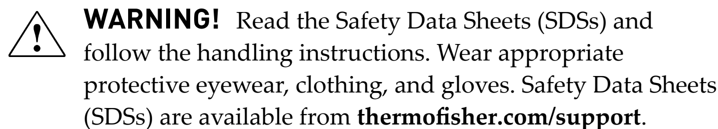
Gene editing of feeder-dependent mouse pluripotent stem cells (mPSCs) using CRISPR/Cas9

Pub. No. MAN0016689 **Rev.** A.0



WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Introduction

Genome editing of mouse embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) using the CRISPR/Cas9 system enables the efficient creation of mouse models that carry one or more alleles- or mutations-of-interest. Such models serve as valuable tools for interrogating nucleic acid and protein function, and consequently, for elucidating biological processes and disease mechanisms. To successfully perform CRISPR/Cas9-mediated genome editing of mouse pluripotent stem cells (mPSCs), many factors need to be considered, such as choice of growth media, genome editing tools, and delivery methods. Here we provide a protocol for the culture and cryopreservation of feeder-dependent mPSCs prior to editing, followed by the optimization and execution of gene editing as well as the isolation and characterization of mPSC clones.

Required materials

Item	Source
DMEM, high glucose, pyruvate	11995065
Attachment Factor Protein (1X)	S006100
MEM Non-Essential Amino Acids Solution (100X)	11140050
KnockOut™ Serum Replacement–Multi-Species	A3181501
KnockOut™ DMEM	10829018
LIF Recombinant Mouse Protein	PMC9484
GlutaMAX™ Supplement	35050079
DPBS, no calcium, no magnesium	14190144
Trypsin-EDTA, (0.05%), phenol red	25300054
PSC Cryopreservation Kit	A2644601
2-Mercaptoethanol	21985023
Neon™ Transfection System	MPK5000
Neon™ Transfection System 10 µL Kit	MPK1025
GeneArt™ CRISPR Nuclease Vector (OFP or CD4)	A21174 or A21175
GeneArt™ Genome Cleavage Detection Kit	A24372
Flow cytometer (e.g. Attune™ NxT Acoustic Focusing Cytometer)	A29001
C57BL/6 Mouse Embryonic Fibroblasts	MTI-GlobalStem GSC-6202G
37°C water bath	MLS[1]
Appropriate tissue culture plates and supplies	MLS
Cell sorter with 96-well plate sort capability (Optional)	MLS

[1] MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Prepare media and materials

Prepare MEF Medium (100 mL of complete medium)

Aseptically mix the following components:

Reagent	Volume
DMEM, high glucose, pyruvate	80 mL
KnockOut™ Serum Replacement–Multi-Species	20 mL

Complete MEF Medium can be stored at 2–8°C for up to 1 week.

Prepare mouse pluripotent stem cell (mPSC) Culture Medium (100 ml of complete medium)

Aseptically mix the following components:

Reagent	Volume
KnockOut™ DMEM	78 mL
KnockOut™ Serum Replacement–Multi-Species	20 mL
MEM Non-Essential Amino Acids Solution (100X), 10 mM	1 mL
GlutaMAX™ Supplement	1 mL
2-Mercaptoethanol, 1000X	100 µL
LIF Recombinant Mouse Protein, 10 µg/mL	100 µL

Complete mPSC Culture Medium can be stored at 2–8°C for up to 1 week.

Prepare MEF dishes

Coat culture vessels with Attachment Factor (optional)

Attachment Factor (AF) is a sterile solution (1X) containing 0.1% gelatin.

1. Cover the whole surface of each new culture vessel with Attachment Factor (AF) solution and incubate the vessels for 30 minutes at 37°C or for 2 hours at room temperature.
2. Use sterile technique in a laminar flow culture hood, and completely remove the AF solution from the culture vessel by aspiration just prior to use. It is not necessary to wash the culture surface before adding cells or medium. Coated vessels may be used immediately or stored at room temperature for up to 24 hours.

   Note: Coating the vessel with AF improves attachment and spreading of MEFs but this step is dispensable.

Thaw and plate mitotically inactivated MEFs

Mitotically inactivated MEF feeder layers are ideally used a day after seeding.

1. Remove the vial of inactivated MEFs from liquid nitrogen storage using metal forceps.
2. Roll the vial between your gloved hands until the outside is free of frost.

   This should take ~10–15 seconds.
3. Immerse the vial in a 37°C water bath without submerging the cap, and swirl the vial gently.
4. When only an ice crystal remains, remove the vial from the water bath.
5. Spray the outside of the vial with 70% ethanol and place it in hood.
6. Pipet the thawed cells gently into a sterile 50-mL conical tube.
7. Slowly add 10 mL of pre-warmed MEF Medium drop-wise to cells in the 50-mL conical tube.

   While adding the medium, gently move the tube back and forth to mix the inactivated MEFs. This reduces osmotic shock to the cells. Aseptically transfer the contents to a 15-ml conical tube.
8. Centrifuge the cells at 200 × g for 5 minutes.
9. Aspirate the supernatant, then resuspend the cell pellet in MEF Medium to a density of approximately 5×10^6 cells/mL.
10. Aspirate the AF solution from the pre-coated culture vessels.
11. Add the appropriate volume of MEF Medium into each pre-coated culture vessel and add the appropriate amount of inactivated MEF suspension. See Table 1.

    Depending on the mouse line of origin, the required feeder density can vary. For C57 strain cells, the recommended inactived MEF plating density is 1.5×10^4 cells/cm^2.

    Table 1 MEF seeding densities and culture volumes

Culture vessel	Surface area (cm^2)	Number of MEFs	Optimal volume
6-well plate	10 cm^2/well	1.5×10^5/well	2 mL/well
12-well plate	4 cm^2/well	6.0×10^4/well	1 mL/well
24-well plate	2 cm^2/well	3.0×10^4/well	0.5 mL/well
96-well plate	0.32 cm^2/well	5.0×10^3/well	0.2 mL/well
35-mm dish	10 cm^2	1.6×10^5	2 mL
60-mm dish	20 cm^2	3.3×10^5	5 mL
100-mm dish	60 cm^2	1.0×10^6	10 mL

12. Move the culture vessels in several quick figure eight motions to disperse the cells across the surface of the vessels.
13. Place inactivated MEF culture vessels into a 37°C, 5% CO_2 incubator.

14. Maintain inactivated MEF culture by changing medium every 2 days. See Figure 1 for the typical appearance of inactivated MEF cultures.

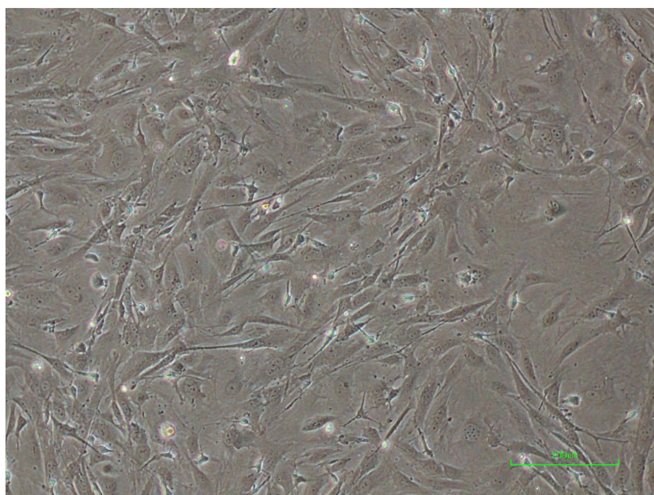


Figure 1 Typical appearance of inactivated MEF culture

Mitotically inactivated MEFs plated on culture vessels coated with Attachment Factor, shown here 24 hours after plating. Scalebar is 200 micrometers.

Thaw and plate mPSCs

1. Aspirate the MEF Medium from a dish containing inactivated MEFs and add pre-warmed mPSC Culture Medium to the dish.

2. Label the dish containing MEF cells with the passage number from the vial, the date, and user initials.

3. Remove the vial of mPSCs from liquid nitrogen storage using metal forceps.

   Note: If the vial is going to be exposed to ambient temperatures for more than 15 seconds between removal and thawing, transfer the vial into a container containing a small amount of liquid nitrogen or dry ice.

4. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.

5. When only an ice crystal remains, remove the vial from the water bath.

6. Spray the outside of the vial with 70% ethanol and place it in hood.

7. Pipet cells gently into a sterile 50-mL conical tube using a 5-mL sterile pipette.

8. Slowly add 10 mL of mPSC Culture Medium drop-wise to cells in the 50-mL conical tube.

   While adding the medium, gently move the tube back and forth to mix the mPSCs. This reduces osmotic shock to the cells.

9. Rinse the vial with 1 mL of mPSC Culture Medium and add to the 50-mL conical tube with cells.

10. Transfer cell suspension to a 15-mL conical tube and centrifuge the cells at 200 × g for 5 minutes.

11. Aspirate the supernatant and gently resuspend the cell pellet in an appropriate volume of pre-warmed mPSC Culture Medium.

12. Remove 20 µL of the cell suspension and determine the viable cell count manually using the Trypan Blue exclusion method.

    Frozen vials of mPSCs may also contain MEFs if the mPSCs were cultured on MEF feeder layers before cryopreservation. We recommend determining the viable cell count manually, because automatic cell counters cannot distinguish between mPSCs and MEFs. The mPSCs will be smaller and rounder than the feeder cells, and more uniform in shape.

13. Add a sufficient volume of mPSC Culture Medium according to Table 2 by gently pipetting the cells up and down in the tube a few times.

Table 2 Volume of mPSC Culture Medium required

Culture vessel	Surface area (cm^2)	Number of mPSCs	mPSC Culture Medium
6-well plate	10 cm^2/well	4.0×10^5/well	2 mL/well
12-well plate	4 cm^2/well	1.6×10^5/well	1 mL/well
24-well plate	2 cm^2/well	8.0×10^4/well	0.5 mL/well
96-well plate	0.32 cm^2/well	1×10^3/well	0.2 mL/well
35-mm dish	10 cm^2	4.0×10^5	2 mL
60-mm dish	20 cm^2	8.0×10^5	4 mL
199-mm dish	60 cm^2	2.4×10^6	12 mL

14. Aspirate the spent medium from the MEF dish and slowly add the thawed cells onto the dish at a plating density of approximately 4×10^4 cells/cm^2.

15. Gently place dish into a 37°C, 5% CO_2 incubator.

    Tilt the plate in the X and Y directions to disperse cells across the surface of the dish.

16. Incubate the cells overnight.

17. The next day, aspirate the spent medium to remove floating dead cells, and add fresh mPSC Culture Medium to the dish according to the volumes in Table 2.

    Return plate gently into the incubator.

18. Examine cells under the microscope and replace spent medium daily.

    Cells should be ready for passage in 2–3 days. See Figure 2.

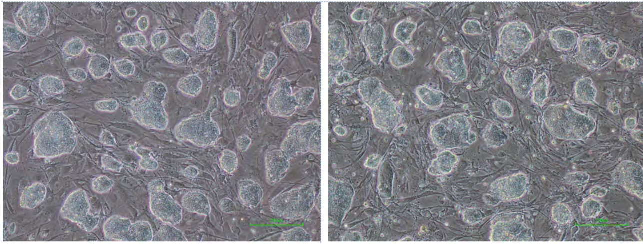


Figure 2 Examples of confluent mouse ESC cultures grown on inactivated MEFs

Colonies are large and growing close to each other, indicating that the cultures are ready to be passaged. Left 129S2/SvPas line, Right C57BL/6N line. Scalebar is 200 micrometers.

Passage mPSCs

When to split cells

In general, split cells when one of the following occurs:

- Culture has reached ~70-80% confluency.
- Increased differentiation occurs.

Split ratio

The split ratio can vary, though it is generally between 1:3 and 1:10. Occasionally, cells will grow at a different rate and the split ratio will need to be adjusted. A general rule is to observe the last split ratio and adjust the ratio according to the appearance of the mPSC colonies.

If the cells look healthy and colonies have enough space, split using the same ratio. If they are overly dense and crowding, increase the ratio. If the cells are sparse, decrease the ratio. Cells will need to be split every 2–4 days based upon appearance.

Enzymatically passage with Trypsin-EDTA Solution

1. Aspirate the spent medium from the dish and rinse the dish twice with DPBS, no calcium, no magnesium. Refer to Table 3 for recommended volumes.

2. Aspirate DPBS and add enzyme solution to the dish containing mPSCs.

   Adjust the volume of enzyme solution for various dish sizes. See Table 3.

Table 3 Reagent volumes (mL per well or per dish)

Culture vessel	Surface area	DPBS	Enzyme solution	mPSC Culture Medium
6-well plate	10 cm^2/well	2 mL/well	1 mL/well	2 mL/well
12-well plate	4 cm^2/well	1 mL/well	0.5 mL/well	1 mL/well
24-well plate	2 cm^2/well	0.5 mL/well	0.3 mL/well	0.5 mL/well
96-well plate	0.32 cm^2 /well	0.2 mL/well	50 µL/well	150 µL/well
35-mm dish	10 cm^2	2 mL	1 mL	2 mL
60-mm dish	10 cm^2	4 mL	2 mL	4 mL
100-mm dish	60 cm^2	12 mL	5 mL	12 mL

3. Incubate the dish for 3–5 minutes in a 37°C, 5% CO_2 incubator until individual single cells start to round up.

4. Gently pipet inactivated MEF and mPSC sheet up and down sufficiently to disperse the colonies from the feeder layers into a single-cell suspension using a 1-mL pipette.

   Make sure to pipet gently to minimize the formation of bubbles. Add an appropriate amount of mPSC Culture Medium to the dish to stop the dissociation reaction.

5. Transfer the mPSC suspension into a 15-mL conical tube and centrifuge the tube at 200 × g for 5 minutes to pellet the cells.

6. Carefully aspirate the supernatant from the mPSC pellet.

7. Resuspend the pellet with an appropriate amount of mPSC Culture Medium. See Table 3. This is dependent on the split ratio and the number of dishes used.

8. Mix the cell suspension well with a 10-mL pipette.

   Be careful not to cause bubbles in the medium.

9. Add appropriate volume of cell suspension to each dish (e.g. 2–3 x 10^6 cells per 100-mm dish).

10. Return the dish to the incubator.

11. Move the dish in several quick left-right top-bottom motions to disperse cells across the surface of the dish.

12. Incubate cells overnight in a 37°C, 5% CO_2 incubator. Replace spent medium daily.

    Note: While cells are attaching, be careful when opening and closing the incubator doors to avoid disturbing the even distribution of cells.

Freeze mPSCs

We recommend cryopreserving and banking mPSCs before editing in order to serve as a backup. mPSCs can also be expanded and banked after editing in order to create a pool of cells that can be used for downstream applications or testing.

1. Follow step 1 through 4 in "Enzymatically passage with Trypsin-EDTA Solution" on page 4 to harvest cells for cryopreservation.
2. Transfer the mPSC suspension into a 15-mL conical tube and count the cells.
3. Centrifuge the tube at 200 × g for 5 minutes to pellet the cells.
4. Calculate the volume of cryopreservation solution required to give cell density of 1×10^6 viable cells/mL.
5. Resuspend the pellet in pre-warmed PSC Cryopreservation medium. Immediately dispense suspension into cryovials (1 mL/vial).
6. Achieve cryopreservation in an automated or manual controlled rate freezing apparatus following standard procedures (1°C decrease per minute).
7. Transfer frozen cells to liquid nitrogen; we recommend vapor phase storage at –200°C to –150°C.

Optimize mPSC transfection for CRISPR/Cas9 delivery

GeneArt™ CRISPR Nuclease Vector encodes both the Cas9 protein and the CRISPR gRNA sequence of choice, thereby providing the reagents necessary for genome editing. Plasmid delivery in mPSCs can be achieved most efficiently through electroporation. Before proceeding with genome editing, we recommend optimizing plasmid delivery into your mPSC-of-choice using a reporter under the control of a constitutive promoter.

1. Prepare a 24-well MEF plate the day before electroporation as described in "Thaw and plate mitotically inactivated MEFs" on page 2.
2. On the day of electroporation, change the medium to 1 mL mPSC Culture Medium, then set aside for after the electroporation has been carried out.
3. Also on the day of electroporation, prepare AF-coated wells as described in "Coat culture vessels with Attachment Factor (optional)" on page 2.

   Make sure to have twice the number wells compared to the original mPSC cultures.
4. Dissociate mPSC cultures following steps 1–4 in "Enzymatically passage with Trypsin-EDTA Solution" on page 4.
5. Add mPSC Culture Medium to the collected cells in order to double the volume.
6. Divide the cell suspension, placing the cells into twice the number of AF-coated wells compared to before.
7. The cell suspension will contain both dissociated mPSCs and MEFs. Eliminate the MEFs by allowing them to attach to the AF-coated wells for 15 minutes at 37°C.
8. Collect the supernatant containing only mPSCs into a 15-mL conical tube.
9. Count the cells using a hemocytometer or an automated cell counter.
10. Centrifuge at 200 × g for 5 minutes to pellet the cells.
11. Set up the Neon™ Transfection System with the electroporation conditions optimized for mPSCs. See Table 4.

    Note: It is possible that none of these conditions permit efficient transfection or survival. In that case, we recommend testing all the preset conditions on the Neon™ Transfection System and selecting the best one for your cell line.
12. Resuspend the cells in electroporation buffer R at a concentration of 1×10^7 cells/mL.
13. Distribute 10 µL of cell suspension in microfuge tubes and add up to 2 µL of DNA.

    For electroporation optimization we recommend trying different amounts of plasmid DNA ranging from 200 ng to 2 µg. Adjust DNA volume to 2 µL with buffer R if needed.
14. Electroporate the prepared aliquots using the Neon™-10 µL tip and the two conditions listed in Table 4.
15. Eject the contents of each tip into the 24-well MEF plate and place the plate in a 37°C incubator.

    Distribute the cells evenly by making several quick left-right top-bottom motions.
16. Maintain the mPSCs as described above and follow reporter expression and cell survival.

    Reporter expression can typically be quantified 48–72 hrs after electroporation using a flow cytometer.

Table 4 Recommended Neon™ Transfection System electroporation settings for mPSCs

Pulse voltage	Pulse width	Pulse number	Cell density	Neon™ tip type
1400 V	10 ms	3	1×10^7 cells/mL	10 µL
1200 V	20 ms	2	1×10^7 cells/mL	10 µL

Perform genome editing in mPSCs

1. Follow steps 1–15 from "Optimize mPSC transfection for CRISPR/Cas9 delivery" on page 5, now with the GeneArt™ CRISPR Nuclease Vector containing the CRISPR-of-interest and using the ideal electroporation condition and DNA concentration.

2. Set up two wells per GeneArt™ CRISPR Nuclease Vector condition. One will be for testing genome editing efficiency; the other will be used for expansion and banking.

3. Cells for testing genome editing efficiency can be collected 72 hrs after electroporation using enzymatic dissociation as in steps 1–4 of "Enzymatically passage with Trypsin-EDTA Solution" on page 4.

4. Transfer dissociated cells to a 1.5 mL microfuge tube and centrifuge at 200 × g for 5 minutes to pellet the cells.

5. Process cells according to the GeneArt™ Cleavage Detection Kit manual to assess CRISPR editing efficiency (Figure 3) or store pellets at –80°C for analysis at a later time.

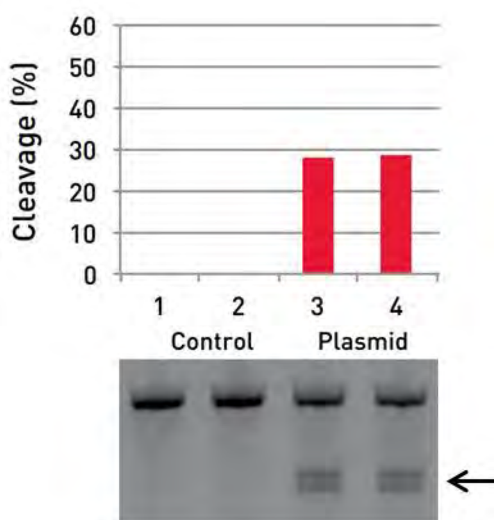


Figure 3 CRISPR/Cas9 mediated genomic cleavage efficiency using the GeneArt™ Genomic Cleavage Detection Kit

Mouse PSCs transfected with the GeneArt™ CRISPR Nuclease Vector (plasmid) were assayed for CRISPR/Cas9 mediated genomic cleavage efficiency using the GeneArt™ Genomic Cleavage Detection Kit. A typical result and quantification are shown.

6. Cells for expansion should be maintained and transferred to a larger well or plate size, then banked as described in "Passage mPSCs" on page 4 and "Freeze mPSCs" on page 5.

Isolate and expand edited mPSC clones

When genome editing is confirmed, single cell clones can be isolated using either single cell sorting or limited cell dilution cloning (LDC). Typically done in 96-well plates, the number of plates depends on the editing efficiency. The lower the efficiency, the more plates will need to be generated. After single cell plating, formed colonies will then be expanded and screened by Sanger sequencing or targeted next generation sequencing.

Grow clones from single cells

1. Thaw edited pool of mPSCs as described in "Thaw and plate mPSCs" on page 3.

2. When edited mPSCs have recovered, prepare an appropriate number of MEF-coated 96-well plates as described in "Prepare MEF dishes" section.

3. The day of single cell seeding, if cell sorting is used, change the medium in the MEF-coated 96-well plates to 200 µL mPSC Culture Medium. For LDC, change the medium to 150 µL mPSC Culture Medium.

4. Prepare a single cell suspension of the mPSCs by following steps 1–7 in "Passage mPSCs" on page 4.

5. For sorting, use the single cell suspension and sort 1 cell into each well of the 96-well plate. For LDC, dilute the cells to 20 cells per mL of medium and add 50 µL to each well.

6. Allow the single cells to grow into colonies for about 5–7 days, changing the medium every 2 days with 200 µL fresh mPSC Culture Medium.

7. At Day 5–7, screen the plates for wells with only one colony, which most likely emerged from a single cell and is thus more likely to be clonal.

   Mark clonal cultures on the lid of the plate.

Consolidate clonal cultures to facilitate screening

1. Prepare fresh 96-well plates with MEFs, keeping in mind the number of clonal cultures identified in step 7 of "Grow clones from single cells" on page 6.

   Allow the MEFs to attach overnight.

2. Replace medium in each well with 100 µL mPSC Culture Medium.

3. Follow steps 1–3 of "Enzymatically passage with Trypsin-EDTA Solution" on page 4, targeting the clonal cultures identified in step 7 and processing only one 96-well plate at a time.

4. Gently triturate each dissociated colony in the Trypsin solution, then add 50 µL of PSC medium.

   Mix and transfer to the new 96-well MEF plate already containing mPSC Culture Medium.

5. When all clonal cultures are consolidated and transferred into fresh plates, place the plates in a 37°C, 5% CO_2 incubator.

6. The next day, replace the medium with 200 µL of fresh mPSC Culture Medium.

   Replace medium every two days thereafter.

7. Three to five days after consolidation, the wells should be confluent and ready for passaging. Follow steps 1–4 of "Enzymatically passage with Trypsin-EDTA Solution" on page 4, processing only one 96-well plate at a time.

8. Set aside 150 µL of each cell suspension for colony screening.

9. Transfer into a 96-well PCR plate and centrifuge at 200 × g for 5 minutes to pellet the cells.

10. Aspirate the supernatant and store at −80°C for further analysis.

11. Plate the remaining 50 µL of each cell suspension in a new 96-well MEF plate with each well already containing 150 µL mPSC Culture Medium.

12. Allow the cells to reach confluency and dissociate the cells by following steps 1–3 in the “Enzymatically passage with Trypsin-EDTA Solution“ on page 4.

13. Gently pipet the cells up and down, dispersing them in the Trypsin solution.

14. Add 150 µL of PSC Cryopreservation medium onto the Trypsin solution and mix.

15. Wrap the plates with Parafilm™ film and store at −80°C until colonies have been screened by sequencing.

Screen clones by sequencing

The clones that have emerged from single cells should be screened for the desired genome modification via sequencing. Sequencing analysis can be done by Sanger sequencing of PCR-amplified regions of the edited genome. Alignment of the sequenced clones can then identify the clones with the desired edit. Decomposition of the sequence traces can also identify the insertions or deletions introduced by the CRISPR. See “Reference“ on page 8.

Sanger sequencing has several limitations, including the inability to assess clonality and the inability to resolve differences between two alleles. Next generation sequencing (NGS) allows simultaneous sequencing of thousands of DNA fragments, providing the sequences and a quantitative assessment of the ratio of the different fragments in a sample. Either targeted NGS for the region of interest or whole genome sequencing can be performed to look at edited DNA. For experimental information on NGS using the Ion Torrent™ platform refer to *Decision Tree for DNA Sequencing on the Ion PGM™ System* (Pub. No. MAN0007998).

Expand edited clones from the 96-well plate

1. Once the desired edited clones are identified, prepare a 24-well inactivated MEF plate as described in “Prepare MEF dishes“ on page 2. On the day of thawing the 96-well plates, change the medium to 500 µL mPSC Culture Medium.

2. To thaw the 96-well plate(s) generated in “Consolidate clonal cultures to facilitate screening“ on page 6, place the plate in a 37°C incubator for 20–30 minutes.

3. When the wells are thawed, transfer the cell suspension from each chosen well into a 15-mL conical tube containing 2 mL of DPBS.

4. Centrifuge at 200 × g for 5 minutes to pellet the cells.

5. Aspirate the supernatant and resuspend the pellet in 250 µL mPSC Culture Medium.

6. Add the cell suspensions to the 24-well MEF plate prepared in step 1.

7. Place the plate in a 37°C incubator and expand cells as needed, see “Passage mPSCs“ on page 4.

   Note: Cells can now be banked or used for characterization.

Characterize mPSCs

After the genome is edited, it is important to check that pluripotency was maintained during the process. Pluripotency can be checked by immunocytochemistry or RT-PCR.

Check pluripotency by immunocytochemistry

1. Replate mPSCs on MEFs in a 24-well plate and process for immunocytochemistry (ICC) analysis 48 hrs after plating.

2. Fix, permeabilize and block the cells using standard protocols or using the Image-iT™ Fixation/Permeabilization Kit (Cat. No. R35602).

3. Stain cells overnight at 4°C with rabbit anti-Sox2 (Cat. No. 48-1400) or rabbit anti-Oct4 (Cat. No. A13998) primary antibodies.

4. Perform several washes and incubate the cells with an Alexa Fluor™ 488- or Alexa Fluor™ 594-conjugated anti-rabbit secondary antibody (Cat. No. A21206 or A21207) and NucBlue™ Fixed ReadyProbes™ Reagent (Cat. No. R37606) at room temperature for 1 hour.

5. Perform several washes and the store the cells at 4°C in PBS if necessary.

6. Stained cells can be imaged on a fluorescent microscope such as an EVOS™ fluorescent microscope.

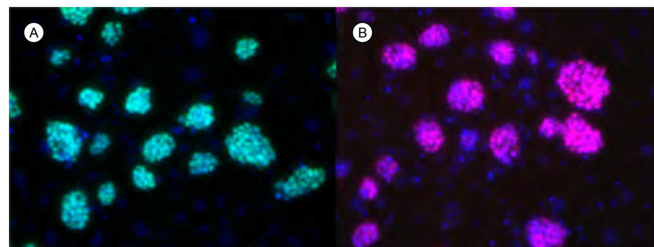


Figure 4 Immunofluorescence analysis of edited mPSC colonies

The mPSC colonies are confirmed to express pluripotency markers Sox2 (green) (A) and Oct4 (red) (B) via immunostaining. Nuclei are counterstained using the NucBlue™ Fixed ReadyProbes™ Reagent (blue).

Check pluripotency by RT-PCR

1. Extract the total RNA from 1×10^6 mPSCs using the PureLink™ RNA Mini Kit (Cat. No. 12183020).

2. Perform a reverse transcription reaction with High-Capacity cDNA Reverse Transcription Kit (Cat. No. 4368813).

   Also include no-reverse transcriptase (no-RT) controls for all samples by leaving out the reverse transcriptase enzyme.

3. Perform the PCR reaction using 1 µL of cDNA from the reverse transcription reaction from step 2 and AmpliTaq Gold™ 360 Master Mix (Cat. No. 4398881), see Table 5. For the RT-PCR primer sequences and the expected product size, see Table 6.

Table 5 PCR conditions following reverse transcription

Step	Temperature	Time	Cycles
Denaturation	95°C	30 sec	25–35 cycles
Annealing	55°C	30 sec	
Elongation	72°C	30 sec	

Table 6 Primers for detection of pluripotency genes in genome-edited mPSCs

Target	Primer sequence	Expected amplicon size
Actb[1]	F:GCTGTATTCCCCTCCATCGTG R:CACGGTTGGCCTTAGGGTTCAG	265 bp
Nanog	F:CCTCAGCCTCCAGCAGATGC R:CCGCTTGCACTTCATCCTTTG	100 bp
Pou5f1 (Oct4)	F:GAAGCAGAAGAGGATCACCTTG R:TTCTTAAGGCTGAGCTGCAAG	129 bp
Sox2	F:GCGGAGTGGAAACTTTTGTCC R:CGGGAAGCGTGTACTTATCCTT	157 bp

[1] A positive control primer set that amplifies a constitutively expressed housekeeping gene (Actb) that encodes a cellular cytoskeleton protein was included.

Appendix

Media substitutions

- In the mPSC Culture Medium, KnockOut™ DMEM (Cat. No. 10829018) can be substituted with DMEM, high glucose, pyruvate (Cat. No. 11995065).
- In the mPSC Culture Medium, KnockOut™ Serum Replacement–Multi-Species (Cat. No. A3181501 or A3181502) can be substituted with KnockOut™ Serum Replacement (Cat. No. 10828028 or 10828010).

Reference

Eva K. Brinkman, Tao Chen, Mario Amendola, and Bas van Steensel; Easy quantitative assessment of genome editing by sequence trace decomposition; Nucl. Acids Res. (16 December 2014) 42 (22): e168 first published online October 9, 2014 doi: 10.1093/nar/gku936

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

The information in this guide is subject to change without notice.

DISCLAIMER: TO THE EXTENT ALLOWED BY LAW, LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Important Licensing Information: These products may be covered by one or more Limited Use Label Licenses. By use of these products, you accept the terms and conditions of all applicable Limited Use Label Licenses.

Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

©2017 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified.