May 20, 2022

Creating iPSC lines with Ribonucleoprotein (RNP): Nucleofection, Single-cell Sorting, Genotyping, and Line Maintenance Protocol

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This protocol describes how to perform gene editing on human induced pluripotent stem cells (iPSCs) via ribonucleoprotein (RNP) and how to the isolate lines with the desired excision. It describes nucleofection, single cell sorting via FACS, genotyping, and the maintenance of the cell lines throughout the process. This protocol is optimized for spCas9.

Creating iPSC lines with Ribonucleoprotein (RNP) - Nucleofection, Single-cell Sorting, Genotyping, and Line Maintenance Protocol_ClellandLab.pdf

DOI

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iPSC lines , Ribonucleoprotein (RNP), gene editing , nucleofection, single-cell sorting, FACS, genotyping, spCas9, Line Maintenance, Stem Cells, excision

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May 18, 2022 Kamaljot Gill University of California, San Francisco

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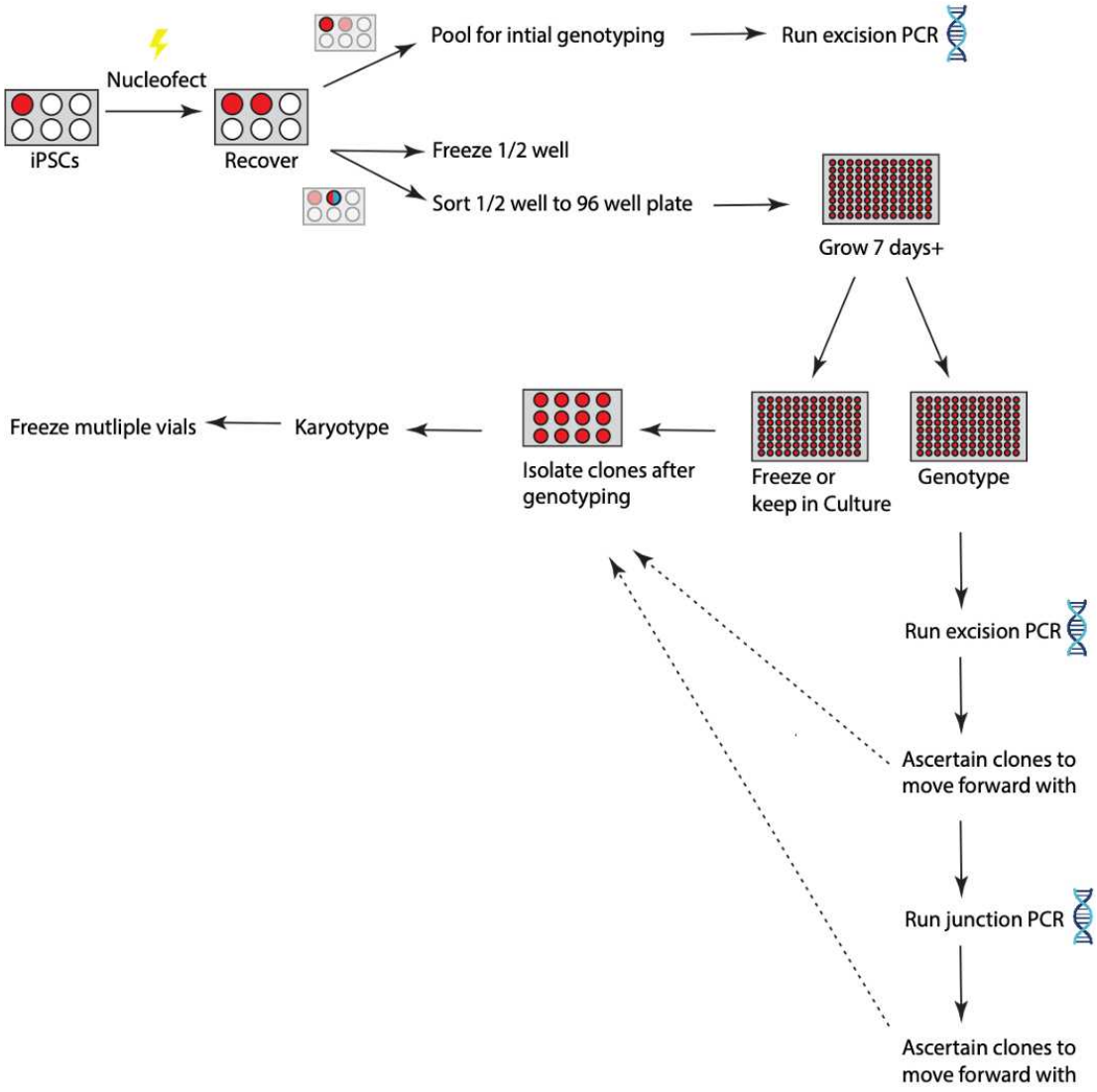

Schematic describing the flow of steps in creating iPSC cell lines with RNP

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Reagents:

- P3 Primary Cell 96-well Nucleofector™ Kit (960 RCT) **Lonza Catalog #V4SP-3096**
- ReLeSR™ 100 mL **Stemcell Technologies Catalog #5872**
- PBS, pH 7.4 **Thermo Fisher Catalog #10010023**
- ACCUTASE™ 100 mL **Stemcell Technologies Catalog #7920**
- mTeSR™ Plus **Stemcell Technologies Catalog #100-0276**
- CloneR™ 10 mL **Stemcell Technologies Catalog #5888**
- KnockOut™ DMEM **Thermo Fisher Scientific Catalog #10829018**
- Growth Factor Reduced (GFR) Matrigel® phenol red-free **Corning Catalog #356231**
- CryoStor® CS10 **Stemcell Technologies Catalog #07930**
- QuickExtract DNA Extraction Solution **Lucigen Catalog #QE09050**
- Antibiotic-Antimycotic (100X) **Thermo Fisher Catalog #15240062**
- Y-27632 **Selleckchem Catalog #S1049**
- Olympus Plastics 28-154 35µm Strainer Caps **Genesee Scientific Catalog #28-155**
- Sigma Isopropanol **Contributed by users Catalog #190764-4L**
- Mineral Oil **Sigma Aldrich Catalog #M8410-100ML**

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A	B	C
Reagent	**Company**	**Catalog #**
P3 Primary Cell 96-well Nucleofector™ Kit (96 RCT)	Lonza	V4SP-3096
ReLeSR™	Stemcell	5872
PBS, pH 7.4	ThermoFisher	10010023
Accutase™	Stemcell	7920
mTeSR™ Plus	Stemcell	100-0276
CloneR™	Stemcell	5888
KnockOut™ DMEM	ThermoFisher	10829018
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free, 10 mL	Corning	356231
CryoStor® CS10	Stemcell	7930
QuickExtract™ DNA Extraction Solution	Lucigen	QE09050
Antibiotic-Antimycotic (100X)	ThermoFisher	15240062
ROCK1 Inhibitor (Y-27632 2HCl)	Selleckchem	S1049
Olympus Plastics 28-154, 35µm Strainer Caps	Genesee	28-155
2-Propanol	Sigma	190764-4L
Mineral Oil	Sigma	M8410-100ML
Cas9-NLS Purified Protein	MacroLabs	

Equipment:

4D-Nucleofector Core Unit
Control system for performing nucleofection
Lonza **AAF-1002B**

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4D-Nucleofector X Unit
Supports Nucleofection® of various cell numbers in 100 µL cuvettes or 20 µL 16-well strips

Lonza **AAF-1002X**

Mr. Frosty Freezing Container
freezing container

ThermoFisher Scientific **5100-0001**

Costar® vacuum aspirator

MilliporeSigma **CLS4931**

A	B	C
Equipment	**Company**	**Catalog #**
BD FACS Aria Fusion	Beckton Dickinson	-

Nucleofecting

1 Cell Culture

1.1 Grow iPSCs in 1 well of a 6 well-plate until ~70-80% confluency.

At this confluency in a 6 well-plate, there should be at least 1.5 million cells.

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2 Coating plates

2.1

Coat 1 x 6 well-plate with Matrigel.

- Matrigel is diluted in KO DMEM to make a working concentration of [M]**80 µl/ml** (keep the Matrigel ice-cold)
- Matrigel coating volumes

1. 6 well plate: 1 mL
2. 12 well plate: 0.5 mL
3. 24 well plate: 0.25 mL
4. 48 well plate 0.125 mL
5. 96 well plate: 0.100 mL

2.2

30m

Incubate the plate at **37 °C** for **00:30:00** .

3 Turn on the Lonza 4D-Nucleofector.

4 Prepare Media

4.1

For 20 mL:

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A	B
mTeSR Plus	18 mL
Clone R (10X)	2 mL
10mM ROCK Inhibitor (RI)	20 µL

5 Prepare P3 Buffer.

5.1 

For 12 reactions (**250 µL**) in a 16 well nucleofector strip.

A	B
Nucleofector™ Supplement	45.45 µL
Nucleofector™ Solution	204.54 µL

5.2 Place **On ice**

Once made, you may store P3 buffer at **4 °C** for up to 3 months.

6 Reconstitute **1.5 nanomolar (nM)** sgRNA in a BSC.

6.1 

Add **15 µL** TE buffer to make **100 micromolar (µM)** sgRNA.

6.2 Store **-20 °C** for long term storage.

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7 Prepare RNP

7.1

For each nucleofection, mix a ratio of 1:3 (spCas9:guide)

sgRNA (**100 micromolar (µM)**) = **1.2 µL** , **spCas9** (**40 micromolar (µM)**) = **1 µL**

7.2

Incubate RNPs at **Room temperature** for 10-15 mins.

8 Prepare cells (below are the instructions for 1 well of a 6-well plate)

8.1

Wash the well with PBS.

8.2

3m

Detach cells from plate with Accutase.

- Incubate cells in **0.5 mL** Accutase. Incubate the plate for 10-15 mins at **37 °C** . Quench the Accutase with **2.5 mL** of PBS.
- Transfer media into a 15 mL conical tube.
- Spin down the cells in a 15 mL conical for **00:03:00** at **800 rpm** .
- Remove supernatant.
- Resuspend the cells in 1-2 mL of warm media.

We use Accutase to obtain single cells.

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8.3 Count cells.

8.4 Transfer ~350k cells in an Eppendorf tube (per reaction).

9 Prepare reaction.

9.1 3m

Centrifuge the cells at **800 rpm** for **00:03:00** . Aspirate supernatant.

9.2

Resuspend pellet in **20 µL** of P3 buffer.

9.3

Add **2 µL** of RNP (Cas9+sgRNA) to the Eppendorf tube.

9.4

If using two different RNPs, only add **1 µL** of each because only half of each RNP is needed.

9.5

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Transfer each sample to one well of a 16 strip nucleofector cuvette/strip. Check lid to make sure it is in the correct orientation.

9.6 Tap nucleofector strip on surface to distribute sample and pop any bubbles.
- If needed, use a P20 pipette tip to gently pop any bubbles.

10 Nucleofecting

10.1 Set up the reaction on 4D-Nucleofector Core X - Unit

- Select wells being used in your script
- Select P3 buffer setting
- Select 16-well strip
- Select Pulse Code = DS138

10.2 Place the nucleofector strip in the nucleofector and hit start.

10.3 Green plus sign should appear if reaction is successful.

11 

Place the nucleofector strip in the hood (do not spray it with EtOH) and incubate at **Room temperature** for 5-10 mins.

12 Prepare plate

12.1 Obtain the 6 well-plate that was coated with Matrigel earlier.

12.2 Aspirate Matrigel.

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12.3

Replace with **2 mL** of media.

13 Recover Cells

13.1

Add **80 µL** of media to each well of the nucleofector strip to recover the cells.

13.2

Slowly pipette up and down to ensure cells are properly dispersed.

14

Transfer cells to plate.

14.1

For each nucleofection, transfer **50 µL** /well of the cells to 2 wells of a 6 well-plate.

14.2 3m

Gently rock the plate and incubate **Overnight** at **37 °C** .

15 Maintain cells.

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15.1 Grow the cells until they are 70-80% confluency (approximately 3-4 days).

15.2 When cells are at 70-80% confluency, you may

- Harvest one well for sorting.
- Harvest one well for freezing and genotyping (lift the well and pellet into 2 separate conical tubes).

Harvesting Cells for Freezing and Genotyping:

16 At 70-80% confluency, collect the pooled population of cells.

16.1 

Incubate cells in **0.5 mL** Accutase. Incubate the plate for ~5-10 mins at **37 °C** .

16.2 

Quench the Accutase with **2.5 mL** of PBS.

16.3 

Transport **1.5 mL** /conical tube of the cells to 2 x 15 mL conical tube.

16.4 

3m

Spin down the cells at **800 rpm** for **00:03:00** .

16.5 Aspirate the supernatant.

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17 Freezing the pool.

17.1 

Resuspend 1 of the pellets in **1 mL** of ice-cold CryoStor.

17.2 

Transfer cells into a cryovial.

17.3 Transport vial to a Mr. Frosty with 2-propanol.

17.4 Place Mr. Frosty at **-80 °C** to freeze for **24:00:00** . 1d

- After 24-48 hours, move the vial to liquid nitrogen.

18 Genotyping pool (go to the Genotyping section for more information)

18.1 Store the remaining pellet in the conical tube at **-20 °C** until ready to extract DNA.

18.2 

Perform an excision PCR with an unedited line as a negative control.

18.3

This is an important check that can save you time later on. Genotyping the pool informs you that you have any excision in any cells.

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Single Cell Sorting by FACS

19 Preparing a 96 well-plate

19.1 

Coat a 96 well-plate with **100 µL** /well of Matrigel.

19.2 

30m

Allow the plate to incubate wi for at least **00:30:00** at **37 °C** .

19.3 

Prepare **20 mL** of Media (mTeSR Plus + ROCK Inhibitor (1,000X) + Clone R (10X)+ Anti-Anti (100X)).

A	B
mTeSR Plus	18 mL
CloneR (10X)	2 mL
Anti-Anti (100X)	200 µL
10mM ROCK Inhibitor	20 µL

If using alternative media, it must NOT be more than 2% FBS

19.4 

Aspirate the Matrigel from the 96 well-plate and add **100 µL** /well of media to each well.

19.5 

Set the plate back into the incubator.

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20 Preparing Cells

20.1 

Wash the well with PBS.

20.2 

Add **0.5 mL** Accutase.

20.3 

Incubate the plate for ~10-15 mins at **37 °C** .

20.4 

Quench the Accutase with **2.5 mL** of PBS.

20.5 Transport cells into a 15 mL conical tube.

20.6 

3m

Spin down the cells for **00:03:00** at **800 rpm** .

20.7 Remove supernatant.

20.8 

Resuspend the cells in **1-2 mL** of warm media.

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20.9 Count cells.

20.10 

Dilute cells to a 1.5 million cells/ **500 µL** .

For single - sorting, 1.5 million cells in **500 µL** of media is an appropriate concentration.

20.11 Pass cells through a filter mesh (strainer cap) using a P1000.

- Place a filter mesh on top of a FACS collection tube.
- Replace filter mesh with cap for collection tube.

You can directly press the tip against the mesh and pipette the cell solution into the collection tube.

21 Transport cells to FACS machine.

21.1 Seal the 96 well-plates with media with parafilm.

21.2 Seal the collection tube with parafilm.

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21.3 Clean a large container with ethanol.

21.4 Place the 96 well-plate and collection tube into the container.

22 FAC Sorting

22.1 Perform single cell sorting with aBD FACS Aria Fusion (Beckton Dickinson), equipped with 355, 405, 488, 561 and 640 nm lasers.

> The QC alignment of each laser should be verified with Cytometer Setup and Tracking Beads (Becton Dickinson) before sample acquisition.

22.2 Set forward a scatter threshold of 15,000 to eliminate debris from list mode data, and fix the number of events to be collected.

22.3 In certain experiments mCherry fluorescence (excitation 561 nm, emission 610 nm) can be used to define sorting parameters.

22.4 Drop delay determination and 96 well plate set-up setup using Accudrop beads (Becton Dickinson).

22.5 Use forward scatter area versus height and side scatter area versus height gates to make the single cell determination. The specifications of the sort layout include single cell precision, 96 well collection device and target event of 1.

23 Quarantining Cells (Day 0)

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23.1 

Move cells to the quarantine incubator (or a separate incubator from other cell lines) during the duration of 7-day Anti-Anti treatment and before confirming the cells are mycoplasma negative

- Perform a mycoplasma test between Day 3 and Day 7.

24 Days 1-3

24.1 Do not change media.

24.2 Ensure there is 1 cell/well and there is no contamination.

> Note 1: Cells will be hard to see for the first few days.
> Note 2: Expect a ~40% cell survival for single cells.

25 Day 4

25.1 

Prepare fresh media without ROCK Inhibitor. For **12 mL**

A	B
mTeSR Plus	10.8 mL
Clone R (10X)	1.2 mL
Anti-Anti (100X)	120 µL

25.2 Aspirate spent media using a multichannel aspirator.

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25.3 

Pipette **100 µL** / well of the new media into each well of a 96 well-plate.

Be careful not to cross contaminate. These are individual clones.

25.4 Check there is 1 colony/well. Ensure colonies are growing.

26 Day 6

26.1 Prepare fresh media without ROCK Inhibitor and Clone R. For **12 mL** .

A	B
mTeSR Plus	12 mL
Anti-Anti (100x)	120 µL

26.2 Aspirate spent media using a multichannel aspirator.

26.3 

Pipette **100 µL** / well of the new media into each well of a 96 well-plate.

Be careful not to cross contaminate. These are individual clones.

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26.4 Check there is 1 colony/well. Ensure colonies are growing.

26.5 Perform a mycoplasma test during this stage or earlier.

27 Day 8 and later

27.1 If the cells are 70-80% confluent or begin to grow on top of each other, **move onto the next step**. If not, continue with this step.

27.2 

If the cells are not ready, continuing feeding but with just mTeSR Plus

- Aspirate spent media using a multichannel aspirator.
- Pipette **100 µL** / well of mTeSR Plus into each well of a 96 well-plate.

> Be careful not to cross contaminate. These are individual clones.

- Check there is 1 colony/well. Ensure colonies are growing.

27.3 Keep cells in 96 well-plate for 1-7 more days or until the clones can be passaged to a smaller plate format.

Passaging and Maintaining the Clones

28 Identify the surviving clones.

28.1 

Using a microscope and marker, count and label the wells of the 96 well-plate

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where there are surviving clones.

28.2 If there are between 24 and 48 clones, then you will passage the cells in 2 x 48 well-plates

One plate is to keep the clone in culture and the other plate is for genotyping

28.3 If there are less than 24 clones, then you will passage the cells into 2 x 24-well-plates.

One plate is to keep the clone in culture and the other plate is for genotyping

29 Prepare the 24 or 48 well-plates.

29.1 Matrigel coat the 24 or 48 well-plates.

29.2 

30m

Incubate for at least **00:30:00** at **37 °C** .

29.3 

Prepare media. For **25 mL**

A	B
MteSR Plus	25 mL
10mM RI µL	25 µL

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29.4 Aspirate the Matrigel from plates.

29.5 

Add media to plates

- For 48 well-plates: add **250 µL** /well
- For 24 well-plates: add **500 µL** /well

30 Clump passage the clones (row-by-row)

30.1 Aspirate spent media from one row of the 96 well-plates.

30.2 

Pipette **50 µL** /well of ReLeSR.

30.3  45s

Incubate at **Room temperature** for **00:00:45** .

30.4 Aspirate ReLeSR from cells.

30.5  3m

Incubate the 96 well-plate at **37 °C** for **00:03:00** .

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30.6 

Pipette **100 µL** /well of mTeSR Plus w/ ROCK inhibitor to each well in the row.

30.7 

Resuspend cells by pipetting up and down.

30.8 

Move **35 µL** into one well of the new plates and **65 µL** into another well of the other new plate.

Note 1: Be sure that you pipette into the same pattern to ensure the 2 plates are identical.
Note 2: It is possible to add more cells to one plate than the other to harvest the more confluent plate first.

30.9 Once a row is passaged, disperse the cells by rocking in all four directions.

30.10 Continue row-by-row until the entire plate is passaged.

31 Day 1 post passaging

31.1

Most of the time, at least one plate will be 70-80% confluent the next day. This plate can be harvested for genotyping. The other plate may be a little less confluent and should can be maintained until it is 70-80% confluent

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(usually the day after).

31.2

Genotyping (at least performing the excision PCR) can occur within the day and the clones with the preferred edit can be identified.

31.3 If one plate is at least 70-80% confluent, move to the next step. Otherwise, maintain the plates.

32 Harvesting one plate to genotype the clones.

32.1 Aspirate the spent media.

32.2 

Wash the cells with **200 µL** /well of PBS.

32.3 Aspirate the PBS.

32.4 Seal the sides of the plate with parafilm to avoid evaporation

32.5 Store the plate at **-20 °C** and harvest DNA later or extract DNA immediately using Quick Extract.

32.6 

8m 15s

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Quick Extract Protocol.

i. Add **50 µL** /well of Quick Extract.
ii. Scrape the bottom of the well to detach cells.

> Time can be saved by performing the above steps to the entire row before moving to the steps below (one row at a time).

iii. Mix by vortexing for **00:00:15** .
iv. Transfer cells in Quick Extract to labeled PCR tubes.

> If the QE is very viscous at this step, use more QE to obtain a more fluid consistency.

v. Incubate the samples at **65 °C** for **00:06:00** using a Thermocycler.
vi. Mix by vortexing.
vii. Incubate the samples at **98 °C** for **00:02:00** using a Thermocycler.

33

(Optional) Freezing the clones.

33.1

> There is a probability that some clones will be lost via the freezing and thawing process.

33.2

Obtain a Styrofoam box and clean it with ethanol.

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33.3 Grow the plate until clones are 70-80% confluent.

33.4 Aspirate spent media.

33.5 

Wash cells with **200 µL** /well of PBS. Aspirate

33.6 

Add **100 µL** /well of ReLeSR.

33.7 

45s

Incubate at **Room temperature** for **00:00:45** .

33.8 

3m

Aspirate and incubate plate at **37 °C** for **00:03:00** .

33.9 

Add CryoStor

- For 96 well-plates: **100 µL** /well.
- For 48 well-plates: **200 µL** /well.

33.10 

Add Mineral Oil

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https://dx.doi.org/10.17504/protocols.io.4r3l2oeopv1y/v1

- For 96 well-plates: **100 µL** /well
- For 48 well-plates: **200 µL** /well

33.11 Seal the plate with parafilm.

33.12 Place the plate in the Styrofoam box.

33.13 Carefully transfer the box to a **-80 °C** freezer.

33.14 The cells are stable for up to 1 month at **-80 °C** .

34

(Optional) Thawing clones from frozen 96/48 well-plate.

34.1 30m

Coat 12 well plates with **0.5 mL** /well of Matrigel.

- Incubate the plate at **37 °C** for at least **00:30:00** .

34.2 Label Eppendorf tubes with the clone number of the clones that you will move forward with

34.3

Prepare Media. For **12 mL**

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A	B
mTeSR Plus	12 mL
10mM RI µL	12 µL

34.4 Warm PBS to **37 °C** .

34.5 10m

Place the frozen plate on paper towels and place it in the **37 °C** incubator for **00:10:00** or until edges of the plate are thawed.

34.6

Pipette warm PBS onto wells that you want to thaw.

34.7

Pipette the cells into their respective Eppendorf tube.

34.8 3m

Centrifuge at **800 rpm** for **00:03:00** .

34.9 Aspirate the supernatant

Pellet will be too small to visualize, but it is there!

34.10

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Resuspend the pellet in **1 mL** of media.

34.11 

Pipette the cells into 1 well of a 12 well-plate.

34.12 

Place plate in incubator.

Genotyping

35 

In order to save time and reagents, it is recommended to perform genotyping in various stages and reduce the total number of potential clones throughout the process. Specifically, perform the excision PCR on all the clones, then only perform 5' and 3' cut site PCR on clones that have the correct excision band. Likewise, only maintain clones which pass the various genotyping stages.

36

You may design and manage the primers using a various set of resources, but the following tools are recommended.

36.1 Obtaining gene sequences: National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/)

36.2 Storing and aligning sequences: SnapGene

36.3 Designing primers: Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-

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blast/)

36.4 Ordering primers: Integrated DNA Technologies (www.idtdna.com/)

- Products and Services → Custom DNA Oligos → DNA Oligos (order now)

36.5 Check for off targets: Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

37

Perform an excision PCR (perform on all clones).

37.1 Designing excision primers

- These set of primers should bind outside of both the cut sites.
- Length: After the excision, the expected length of the band should be between 100 bp and 1000 bp.

37.2 Expectations

- Unedited line: A large band or no band because of the size of the excision.
- Homozygous excision: Expected band size.
- Heterozygous excision: Expected band size and the unedited band (unless there is no band).

37.3 Sanger Sequence

- Save some PCR product to sanger sequence at a later time.

38

Perform 5' cut site excision PCR (perform only on clones that have the correct excision band).

38.1 Design 5' cut site primers.

- One of the primers should bind on the 5' outside of the cut site and the other primer should bind within the excision region.

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- Length: If the excision did not occur, the expected length of the band should be between 100 bp and 1000 bp. With the excision, we expect no band

38.2 Expectations

- Unedited line: Expected band size
- Homozygous excision: No band
- Heterozygous excision: Expected band size

39

Perform 3’ cut site excision PCR (perform only on clones that have the correct excision band).

39.1 Design 3’ cut site primers.

- One of the primers should bind 3’ outside of the cut site and the other primer should bind within the excision region.
- Length: If the excision did not occur, the expected length of the band should be between 100 bp and 1000 bp. With the excision, we expect no band.

39.2 Expectations

- Unedited line: Expected band size.
- Homozygous excision: No band.
- Heterozygous excision: Expected band size.

Karyotyping and Freezing

40 Send one or two clones with correct excisions to karyotype.

40.1 Freeze a few vials (3-4) of all clones you are karyotyping.

41 Expand and freeze 10+ vials the clone with the correct excision and normal karyotype.

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