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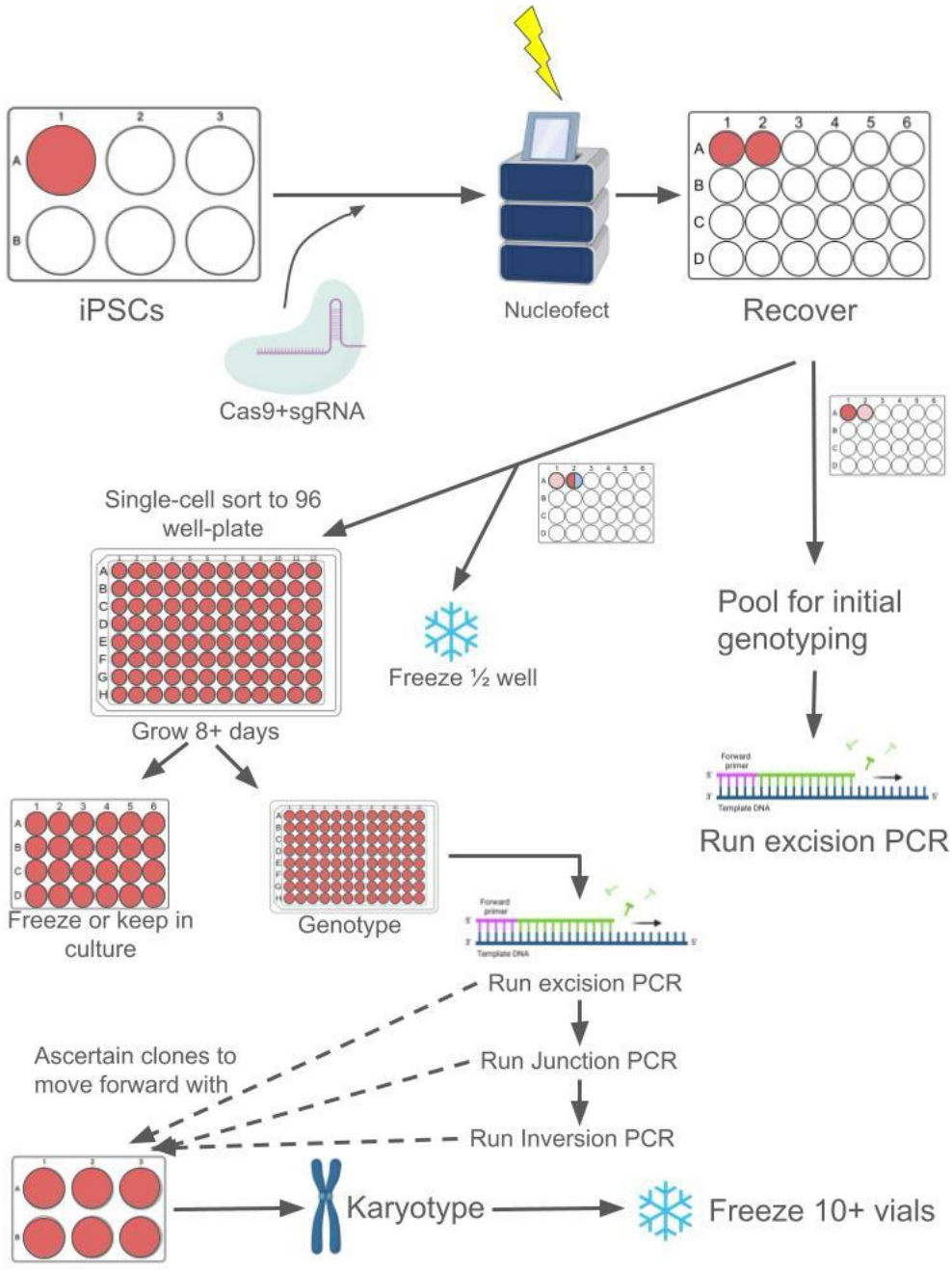
# Creating iPSC lines with Ribonucleoprotein (RNP): Nucleofection Single-cell Sorting, Genotyping, and Line Maintenance Protocol

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This protocol describes how to perform gene editing on human induced pluripotent stem cells (iPSCs) or human embryonic stem cells (ESCs) via ribonucleoprotein (RNP) and how to isolate the 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.

Figure 1: Schematic describing the flow of steps in creating iPSC cell lines with RNP



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## Nucleofecting

1. Cell Culture
    a. Grow iPSCs in 1 well of a 6 well-plate until ~70-80% confluency
        i. In a 6 well-plate at 70-80% confluency, there should be at least 1.5 million cells
2. Coating plates
    a. Coat 2 wells of a 24 well plate for each nucleofection with 0.25 mL of Matrigel per well
        i. Matrigel is diluted in KO DMEM (1X) to make a working solution of 80 µg/mL (keep the Matrigel stock ice-cold)
    b. Incubate the plate at 37°C for at least 30 minutes
3. Turn on the Lonza 4D-Nucleofector
4. Prepare nucleofection media
    a.

| Reagent | Dilution Factor | For 1 rxn | 20 mL recipe |
|---|---|---|---|
| mTesR Plus | 1 | 2.7 mL | 18 mL |
| Clone R | 10x | 0.3 mL | 2 mL |
| 10 mM ROCK Inhibitor (RI) | 1000x | 3 µL | 20 µL |

5. Prepare P3 buffer → KEEP ON ICE
    a. For 12 reactions (250 ul)
        i. 45.45 µL Nucleofector™ Supplement
        ii. 204.54 µL Nucleofector™ Solution
    b. Once made, you may store at 4°C for up to 3 months
6. Reconstitute 1.5 nmol sgRNA in a BSC
    a. Add 15 µL TE to make 100 µM sgRNA
    b. Store -20C for long term storage
7. (Optional) Reconstitute 2 nmol repair oligo in a BSC
    a. Add 20 µL TE to make 100 µM solution
    b. Store -20C for long term storage
8. Prepare RNP
    a. For each nucleofection, mix a ratio of 1:3 (spCas9:sgRNA)
        i. If using 2 guides, prepare the RNPs separately
    b. For each reaction:
        i. 0.5 µL spCas9 (40 µM)
        ii. 0.6 µL sgRNA (100 µM)

1. Pipette to mix and swirl tip in PCR tube to thoroughly combine sgRNA and spCas9

c. Incubate at room temperature for at least 15 mins and up to 1 hour

9. Prepare cells (1 well/reaction)
   a. Wash iPSCs once with 1X DPBS
   b. Detach cells with Accutase
      i. Add 0.5 mL Accutase to each well in a confluent 6 well-plate
      NOTE: We use Accutase to obtain single cells
      ii. Incubate plates for 3-7 min at 37°C
      iii. Quench the Accutase with 2.5 mL 1X DPBS
      iv. Spin down cells in a 15 mL conical tube for 3 mins at 300xg
      v. Remove supernatant and resuspend cells in 1 mL warm nucleofection media
      vi. Count cells and transfer 150k cells to a 1.5 mL Eppendorf tube (per reaction)
10. Prepare Reaction
   a. Centrifuge cells at 300 xg for 3 min. Aspirate supernatant
   b. Resuspend pellets in 20 ul of ice cold P3 buffer
   NOTE: Cells shouldn't stay in P3 buffer for more than 15 min prior to nucleofection
   c. Add 1 µL of RNP (Cas9+sgRNA) to Eppendorf tube
      i. If using 2 different RNPs, only add 0.5 µL of each
   d. (Optional) Add 0.64 µL of repair oligo
   e. Pipette to mix and transfer each sample to one well of a 16 strip nucleofector strip
   f. Bob bubbles in sample by tapping strip on surface or using a P20 pipette tip
11. Nucleofecting
   a. Set up reaction on 4D-Nucleofector Core X Unit
      i. Select 16 well-strip
      ii. Select wells being used in your strip
         1. Ensure correct orientation of nucleofector strip – the side with the uneven shaped pegs and larger hole is the top of the strip
      iii. Select P3 buffer setting
      iv. Select Pulse Code
         1. iPSCs = DS138
         2. Embryonic SCs = CB150
   b. Place nucleofector strip in nucleofector in the correct orientation
      i. The strip should fit cleanly with the green peg holding it in place

c. Hit start
d. Green plus sign should appear if reaction is successful

12. Place nucleofector strip in the hood (do not spray with EtOH) and incubate at RT for 5-10 mins
13. Prepare plate
    a. Obtain the 24 well-plate coated with Matrigel earlier
    b. Aspirate Matrigel
    c. Replace with 0.5 mL media
    d. (Optional) If using a repair oligo: Add 0.7 µL Alt-R HDR enhancer V2/1 mL media
14. Recover cells
    a. After incubation, add 80 µL of media to the well of the nucleofector strip
    b. Slowly pipette up and down to ensure cells are properly dispersed
15. Transfer cells to plate
    a. From each nucleofection reaction of 100 µL, transfer 50 µL of the cell suspensions to each of 2 wells of the 24 well-plate
    b. Gently rock the plate and incubate overnight at 37°C
16. Maintain cells
    a. Grow cells until they are 70-80% confluent (approximately 3-4 days)
    b. When cells are at 70-80% confluent, you may
        i. Harvest one well for sorting
        ii. Harvest one well for freezing and genotyping (lift the well and pellet into 2 separate conical tubes)
    c. For iPSCs:
        i. Edited cells can be taken off ROCK inhibitor and Clone R 1 day after nucleofection

        NOTE: Cells can be taken off of ROCK inhibitor and Clone R when there are colonies of ~ 10 cells, this may take longer if using a plasmid or making a toxic edit
    d. For embryonic Stem Cells:
        i. Edited cells should be kept on ROCK inhibitor and Clone R until FACS sorting

| Reagent | Dilution Factor | For 1 rxn | 20 mL recipe |
|---|---|---|---|
| mTesR Plus | 1 | 1.8 mL | 18 mL |
| Clone R | 10x | 0.2 mL | 2 mL |
| 10 mM ROCK Inhibitor (RI) | 1000x | 2 µL | 20 µL |

## Harvesting Cells for Freezing and Genotyping

1. At 70-80% confluency, collect the pooled population of cells
    a. Remove media from well
    b. Wash once with PBS
    c. Remove PBS and add 250 µL of Accutase
    d. Incubate plate for 5-10 min at 37°C
    e. Quench the Accutase with 1.75 mL of PBS
    f. Transfer 1 mL/conical tube of the cells to 2 x 15 mL conical tube
    g. Centrifuge at 300 xg for 3 min
    h. Aspirate the supernatant
2. Freezing the pool
    a. Prepare freezing media

| Reagent | Dilution Factor | For 1 mL |
|---|---|---|
| mTesR Plus | 1 | 0.9 mL |
| DMSO | 1:10 | 0.1 mL |
| 10 mM Rock Inhibitor (RI) | 1:1000 | 1 µL |

    b. Resuspend one of the pellets in 1 mL of freezing media
    c. Transfer cells into a cryovial and place cryovials in a Mr. Frosty
    d. Place Mr. Frosty at -80°C for 24 hrs
        i. After 24-48 hrs, move the vial to liquid nitrogen
3. Genotyping pool (go to the Genotyping section for more information)
    a. Store remaining pellet in conical tube at -20C until ready to extract DNA
    b. Perform an excision PCR with an unedited line as a negative control
    NOTE: This is an important check that can save you time later on. Genotyping the pool informs you that you have excision or insertion in the pool of cells. If it you do not have excision or insertion in the pool of cells, then there is no need to genotype individual clones

## Single Cell Sorting by FACS

1. Prepare a 96 well-plate
    a. Coat a 96 well-plate with 50 µL/well of Matrigel
    b. Incubate the Matrigel-coated plate at 37°C for at least 30 min up to overnight
    c. Prepare sorting media

| Reagent | Dilution Factor | 20 mL recipe |
|---|---|---|
| mTesR Plus | 1 | 18 mL |
| Clone R | 1:10 | 2 mL |
| Anti-Anti | 1:100 | 200 µL |
| 10 mM Rock Inhibitor (RI) | 1:1000 | 20 µL |

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

d. Aspirate the Matrigel from the 96 well-plate (do not wash) and add 100 µL/well of media to each well
e. Set plate back in incubator

2. Preparing Cells
    a. Wash the well with 1X DPBS
    b. Add 0.25 mL Accutase
    c. Incubate the plate for 5-10 min at 37°C
    d. Quench Accutase with 1.75 mL of PBS
    e. Transfer cells into a 15 mL conical tube
    f. Centrifuge cells at 300 xg for 3 min
    g. Remove supernatant
    h. Resuspend cells in 1 mL of warm sorting media
    i. Transfer cells to a FACS tube with a cell strainer cap (5 mL round-bottom polystyrene test tubes)
        i. Take entire 1 mL cell suspension and pipette cells through the strainer cap into the tube with even, consistent pressure
3. Transport cells to FACS machine
    a. Seal the 96 well-plate with media with parafilm
    b. Clean a large container with ethanol
    c. Place 96 well-plate and FACS tube with cells in the container
4. FACS Sorting
    a. Perform single cell sorting with a BD Aria Fusion (Beckton Dickinson), equipped with 355, 405, 488, 561, and 640 nm lasers
        i. The WC alignment of each laser should be verified with Cytometer Setup and Tracking Beads (Beckton Dickinson) before sample acquisition
    b. Set forward a scatter threshold of 15,000 to eliminate debris from list mode data, and fix the number of events to be collected.

c. In certain experiments mCherry fluorescence (excitation 561 nm, emission 610 nm) can be used to define sorting parameters. If inserting NGN2 into a safe harbor loci for example, then you will sort on medium mCherry fluorescence as very high mCherry signal likely signifies cells with multiple integrations that you do not want to collect.
d. Drop delay determination and 96 well plate set-up setup using Accudrop beads (Becton Dickinson).
e. 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.

NOTE: From this point on, each well contains an individual clone. Be sure to avoid cross-contamination

5. For iPSCs
    a. Quarantining Cells: Day 0
        i. Move cells to the quarantine incubator (or a separate incubator from other cell lines) during duration of 7-day Anti-Anti treatment and confirming that the cells are mycoplasma negative
            1. Perform a mycoplasma test between Day 3 and Day 7
    b. Days 1-3
        i. Do not change media
        ii. Ensure there is 1 cell/well and there is no contamination

NOTE: Cells will be hard to see for the first few days

NOTE: Expect a ~40-50% cell survival for single cells

    c. Day 4
        i. Prepare fresh media without ROCK inhibitor

| Reagent | Dilution Factor | 12 mL recipe |
|---|---|---|
| mTesR Plus | 1 | 10.8 mL |
| Clone R | 1:10 | 1.2 mL |
| Anti-Anti | 1:100 | 120 µL |

        ii. Aspirate spent media with a multichannel aspirator
        iii. Pipette 100 µL/well of new media into each well of the 96 well-plate

NOTE: Avoid cross-contamination. These are individual clones

        iv. Check there is 1 colony/well. Ensure colonies are growing
    d. Day 6
        i. Prepare fresh media without Clone R and ROCK inhibitor

| Reagent | Dilution Factor | 12 mL recipe |
|---|---|---|
| mTesR Plus | 1 | 12 mL |
| Anti-Anti | 1:100 | 120 µL |

ii. Aspirate spent media with a multichannel aspirator
iii. Pipette 100 µL/well of new media into each well of the 96 well-plate
NOTE: Avoid cross-contamination. These are individual clones
iv. Check there is 1 colony/well. Ensure colonies are growing
v. Perform a mycoplasma test at this stage or earlier

e. Day 8+
i. 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.
ii. If the cells are not ready, continue feeding with just mTeSR Plus
1. Aspirate spent media with a multichannel aspirator
2. Pipette 100 µL/well of new media into each well of the 96 well-plate
NOTE: Avoid cross-contamination. These are individual clones
3. Check there is 1 colony/well. Ensure colonies are growing
iii. Keep cells in 96 well-plate for 1-7 more days or until the clones can be passaged to a smaller plate format.

6. For embryonic stem cells
a. Quarantining Cells: Day 0
i. Move cells to the quarantine incubator (or a separate incubator from other cell lines) during duration of 7-day Anti-Anti treatment and confirming that the cells are mycoplasma negative
1. Perform a mycoplasma test between Day 3 and Day 7
b. Days 1-5
i. Do not change media
ii. Ensure there is 1 cell/well and there is no contamination
NOTE: Cells will be hard to see for the first few days
NOTE: Expect a 40-50% cell survival for single cells
c. Day 6
i. Prepare fresh media without ROCK inhibitor

| Reagent | Dilution Factor | 12 mL recipe |
|---|---|---|
| mTesR Plus | 1 | 10.8 mL |
| Clone R | 1:10 | 1.2 mL |

| | | |
|---|---|---|
| Anti-Anti | 1:100 | 120 µL |

ii. Aspirate spent media with a multichannel aspirator
iii. Pipette 100 µL/well of new media into each well of the 96 well-plate
NOTE: Avoid cross-contamination. These are individual clones
iv. Check there is 1 colony/well. Ensure colonies are growing
v. Perform mycoplasma test at this stage or earlier

d. Day 8+
i. 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.
ii. If the cells are not ready, continue feeding with just mTeSR Plus
1. Aspirate spent media with a multichannel aspirator
2. Pipette 100 µL/well of new media into each well of the 96 well-plate
NOTE: Avoid cross-contamination. These are individual clones
3. Check there is 1 colony/well. Ensure colonies are growing
iii. 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 Clones

1. Identify the surviving clones
a. Using a microscope and marker, count and label the wells of the 96 well-plate where there are surviving clones.
b. If there are less than 24 clones, then you will passage the cells in one 96 well-plate and one 24 well-plate
c. If there are between 24 and 48 clones, then you will passage the cells in one 96 well-plate and one 48 well-plate
NOTE: The 96 well-plate is for genotyping and the other plate is for maintaining the clone in culture
2. Prepare the plates
a. Matrigel coat a 96 well-plate and a 24 or 48 well-plate
b. Incubate at 37°C for at least 30 min up to overnight
c. Prepare media
i.

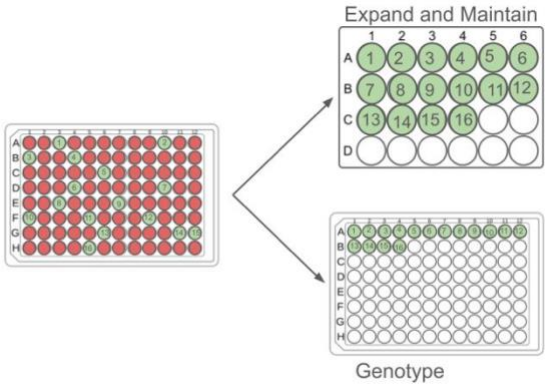
| Reagent | Dilution Factor | 25 mL recipe |
|---|---|---|
| mTesR Plus | 1 | 25 mL |

| | | |
|---|---|---|
| ROCK Inhibitor | 1:1000 | 25 µL |

d. Aspirate the Matrigel from plates.
   i. 96 well-plate: 100 µL/well
   ii. 48 well-plate: 250 µL/well
   iii. 24 well-plate: 500 µL/well

3. Passage Clones
   a. Aspirate spent media from one row of the 96 well-plates.
   b. Pipette 50 µL/well of 0.5 mM EDTA
   c. Incubate at 37°C for 3-5 min
   d. Aspirate EDTA from wells
   e. Pipette 100 µL/well of mTeSR Plus w/ ROCK Inhibitor to each well to be passaged
   f. Resuspend cells by pipetting up and down
   g. Transfer 75 µL to one well of the 24 or 48 well-plate and 25 µL to one well of the 96 well-plate
      NOTE: Be careful to pipette each plate in the same pattern to keep clones organized
   h. Once clones are passaged, disperse cells by rocking plate in all four directions
4. Day 1 post-passaging
   NOTE: 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 be maintained until it is 70-80% confluent (usually the day after).
   NOTE: Genotyping (at least performing the excision PCR) can occur within the day and the clones with the preferred edit can be identified.
   a. If one plate is at least 70-80% confluent, move to the next step. Otherwise, maintain the plates.
5. Harvesting one plate to genotype clones
   a. Aspirate the spent media
   b. Wash the wells with 100 µL/well of PBS
   c. Aspirate the PBS
   d. Seal the sides of the plate with parafilm to avoid evaporation
   e. Store the plate at -20°C and harvest DNA later or extract DNA immediately using Quick Extract.
6. Quick extract protocol
   a. Add 25 µL/well of Quick Extract

Figure 2: Schematic illustrating passaging individual clones to expand and genotype



NOTE: The volume of Quick Extract can be adjusted based on confluency of well

b. Scrape the bottom of the well with a pipette tip to detach the cells
   NOTE: Time can be saved by performing the above steps to the entire row using a multichannel pipette
c. Transfer Quick Extract + cells into labelled PCR tubes
d. Mix by vortexing for ~15s
   NOTE: If Quick Extract is very viscous at this step, add more Quick Extract to obtain a more fluid consistency
   i. Incubate samples at 65°C for 6 min using a Thermocycler
   ii. Mix by vortexing
   iii. Incubate samples at 98°C for 2 min using a Thermocycler

7. (Optional) Freezing the clones
   NOTE: There is a probability that some clones will be lost via the freezing and thawing process, therefore genotyping while the clones remain in culture is preferred.
   a. Obtain a Styrofoam box and clean it with ethanol.
   b. Grow the plate until clones are 70-80% confluent.
   c. Aspirate spent media.
   d. Pipette 50 µL/well of 0.5 mM EDTA
   e. Incubate at 37°C for 3-5 min
   f. Aspirate EDTA from wells
   g. Add CryoStor
      i. For 96 well-plates: 100 µL /well.
      ii. For 48 well-plates: 200 µL /well.
   h. Add Mineral Oil
      i. For 96 well-plates: 100 µL /well
      ii. For 48 well-plates: 200 µL /well
   i. Seal the plate with parafilm.
   j. Place the plate in the Styrofoam box.
   k. Carefully transfer cells to -80C freezer
      i. Cells are stable for ~1 month at -80°C
8. (Optional) Thawing cells from a frozen plate
   a. Coat 12 well-plates with 0.5 mL of Matrigel per well
      i. Incubate plate at 37°C for at least 30 min up to overnight
   b. Label eppendorf tubes with clone numbers of clones you will move forward with
   c. Prepare media

| Reagent | Dilution Factor | 12 mL recipe |
|---|---|---|

| | | |
|---|---|---|
| mTesR Plus | 1 | 12 mL |
| ROCK Inhibitor | 1:1000 | 12 µL |

d. Warm 1X DPBS to 37C
e. Place the frozen plate on paper towels and place it in the 37C incubator for 10 min or until edges of the plate are thawed.
f. Pipette warm 1X DPBS onto wells that you want to thaw.
g. Pipette the cells into their respective Eppendorf tube.
h. Centrifuge at 300 xg for 3 min
i. Aspirate the supernatant
    NOTE: Pellet will be too small to visualize, but it is there!
j. Resuspend cells in 1 mL of media
k. Prepare plate
    i. Obtain the 12 well-plate coated with Matrigel earlier
    ii. Aspirate Matrigel
    iii. Replace with 1 mL media per well
l. Pipette the cells into one well of the 12 well-plate
m. Place plate in incubator

## Genotyping

NOTE: 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, and 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.

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

1. Obtaining gene sequences:
    a. National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/)
2. Storing and aligning sequences:
    a. SnapGene
3. Designing primers:
    a. Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)
4. Ordering primers:
    a. Integrated DNA Technologies (www.idtdna.com/)
        i. Products and Services → Custom DNA Oligos → DNA Oligos (order now)

5. Check for off targets:
    a. Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi)
6. Perform an excision PCR (perform on all clones).
    a. Designing excision primers
        i. This set of primers should bind outside of both the cut sites.
        ii. Length: After the excision, the expected length of the band should be between 100 bp and 1000 bp.
7. Perform 5’ cut site excision PCR (perform only on clones that have the correct excision band).
    a. Design 5’ cut site primers.
        i. One of the primers should bind on the 5’ outside of the cut site and the other primer should bind within the excision region.
        ii. 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
    b. Expectations
        i. Unedited line: Expected band size
    c. Homozygous excision: No band
    d. Heterozygous excision: Expected band size
8. Perform 3’ cut site excision PCR (perform only on clones that have the correct excision band).
    a. Design 3’ cut site primers.
        i. One of the primers should bind 3’ outside of the cut site and the other primer should bind within the excision region.
        ii. 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.
    b. Expectations
        i. Unedited line: Expected band size.
        ii. Homozygous excision: No band.
        iii. Heterozygous excision: Expected band size.
9. Perform inversion PCR (perform on final clones with 3 correct bands)
    a. Used to determine is excised fragment became inverted in DNA sequence
    b. Inversion Primers
        i. Use the 5’ forward primer + 3’ forward primer and 5’ reverse primer + 3’ reverse primer
    c. Expectations
        i. Unedited line: No band
        ii. Proper excision: No band

iii. Edited line with inversion: Expected band size (5’ primer to cutsite + 3’ primer to cutsite)

10. Sanger Sequence
    a. Save some PCR product from all Excision, 5’, and 3’ PCRs to sanger sequence at a later time.

## Karyotyping and Freezing

1. Send one or two clones with correct excisions to karyotype.
2. Freeze a few vials (3-4) of all clones you are karyotyping.
3. Expand and freeze 10+ vials of the clone with the correct excision and normal karyotype.

## Materials

Reagents

| **Reagent** | **Company** | **Catalog #** |
|---|---|---|
| P3 Primary Cell 96-well Nucleofector™ Kit (96 RCT) | Lonza | V4SP-3096 |
| UltraPure™ 0.5 M EDTA, pH 8.0 | ThermoFisher | 15575-038 |
| 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 |
| QuickExtract™ DNA Extraction Solution | Lucigen | QE09050 |
| Antibiotic-Antimycotic (100X) | ThermoFisher | 15240062 |
| ROCK1 Inhibitor (Y-27632 2HCl) | Selleckchem | S1049 |

| | | |
|---|---|---|
| 2-Propanol | Sigma | 190764-4L |
| Mineral Oil | Sigma | M8410-100ML |
| Cas9-NLS Purified Protein | MacroLabs | |

Equipment

| **Equipment** | **Company** | **Catalog #** |
|---|---|---|
| 4-D Nucleofector Core Unit | Lonza | AAF-1002B |
| 4-D Nucleofector X Unit | Lonza | AAF-1002X |
| Mr. Frosty Freezing Container | Thermo Fisher Scientific | 5100-0001 |
| Costar® Vacuum Aspirator | MilliporeSigma | CLS4931 |
| BD FACS Aria Fusion | Becton Dickinson | - |