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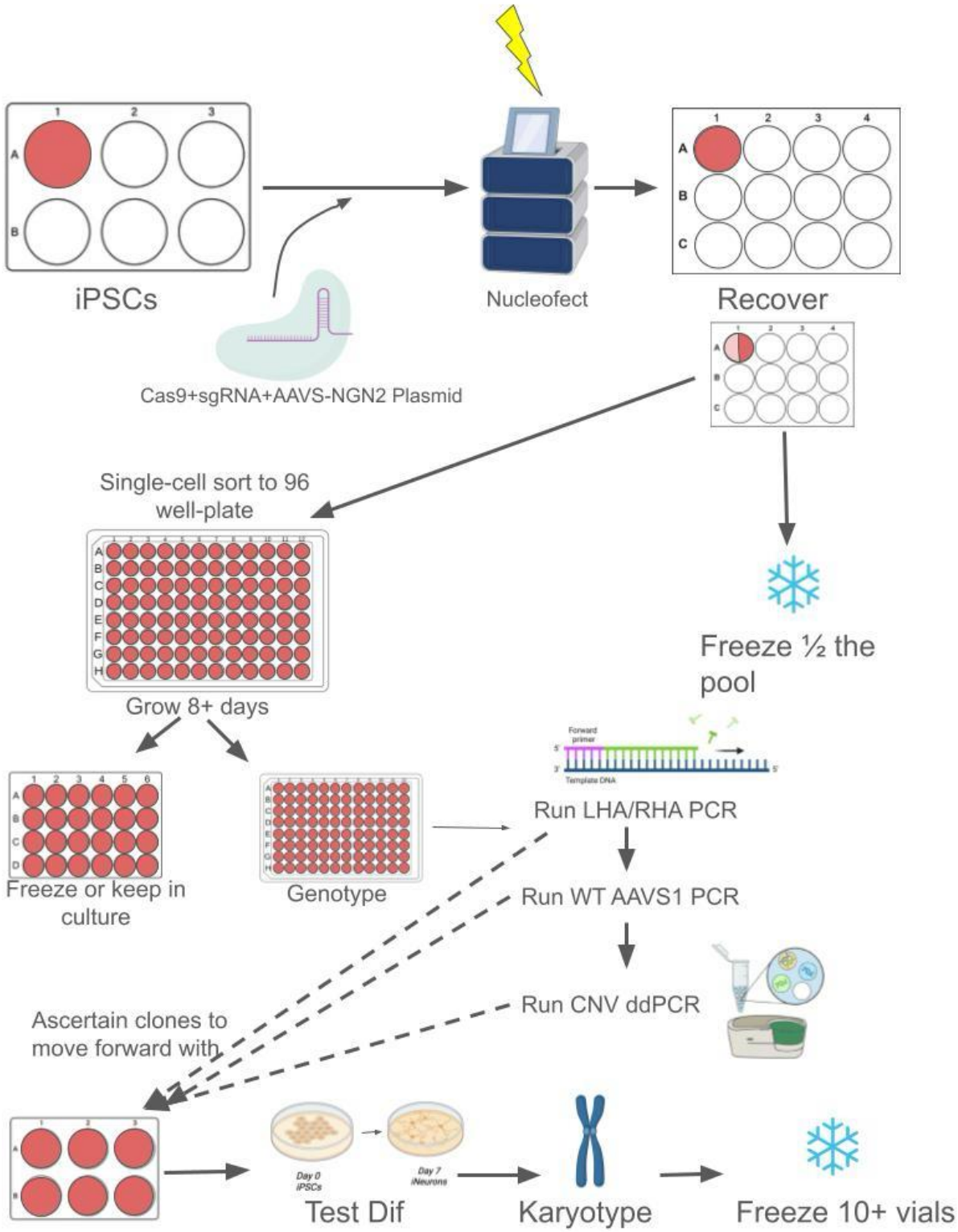
Integrating NGN2 into iPSC lines with Ribonucleoprotein (RNP): Nucleofection Single-cell Sorting, Genotyping, and Line Maintenance Protocol

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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) to integrate the NGN2 casette into the AAVS1 locus and how to isolate the lines with the desired insertion. 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 steps to integrate NGN2 into 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 1 well of a 12-well plate for each nucleofection reaction with 0.5 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 ice-cold)
    b. Incubate the plate at 37ºC 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	1:10	0.3 mL	2 mL
10 mM ROCK Inhibitor (RI)	1:1000	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 4ºC for up to 3 months
6. Reconstitute 1.5 nmol sgRNA in a BSC
    a. AAVS-NGN2 sgRNA Sequence: GUCCCUAGUGGCCCCACUGU
        i. Add 15 µL TE to make 100 µM sgRNA
        ii. Store -20ºC for long term storage
7. Prepare RNP
    a. For each nucleofection, mix a ratio of 1:3 (spCas9:sgRNA) in a PCR tube
    b. For each reaction:
        i. 0.5 µL spCas9 (40 µM)
        ii. 0.6 µL AAVS-NGN2 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
8. 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
    NOTE: We use Accutase to obtain single cells
    ii. Incubate plates for 3-7 min at 37ºC
    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 350k cells to a 1.5 mL Eppendorf tube (per reaction)

9. 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+AAVS-sgRNA) to Eppendorf tube
    d. Add 500 ng of the AAVS-NGN2 plasmid
        i. pUCM-AAVS1-TO-hNGN2 - Addgene #105840
        NOTE: The volume of the plasmid should not exceed 5 µL; if more is needed to reach 500 ng, a more concentrated stock of plasmid is required
        NOTE: The plasmid is toxic to cells and there will be much more cell death than in a normal nucleofection
    e. Pipette to mix and transfer each sample to one well of a 16 strip nucleofector strip
    f. Pop bubbles in sample by tapping strip on surface or using a P20 pipette tip
10. 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

11. Place nucleofector strip in the hood (do not spray with EtOH) and incubate at RT for 5-10 mins
12. Prepare plate
    a. Obtain the 12 well-plate coated with Matrigel earlier
    b. Aspirate Matrigel
    c. Replace with 1 mL media
13. 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
14. Transfer cells to plate
    a. For each nucleofection, transfer the entire 100ul reaction to 1 well of the 12 well-plate
    b. Gently rock the plate and incubate overnight at 37ºC
15. Maintain cells
    a. Day 1 post nucleofection
        i. Feed with mTeSR Plus with ROCK inhibitor and Clone R

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

    b. Day 2 post nucleofection
        i. Feed with mTeSR Plus with Clone R

Reagent	Dilution Factor	For 1 rxn	20 mL recipe
mTesR Plus	1	2 mL	18 mL
10 mM ROCK Inhibitor (RI)	1:1000	2 µL	20 µL

        ii. When there are colonies of ~10 cells, remove from Clone R
    c. Continue feeding daily with mTeSR Plus until cells are 70-80% confluent (approximately 5-7 days)
    d. When cells are at 70-80% confluency, 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)

Harvesting Cells for Freezing

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 37ºC
    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

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 37ºC 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. Sort for mCherry fluorescence (excitation 561 nm, emission 610 nm)
    NOTE: There will likely be 2 populations of red cells, one higher on the side scatter axis than the other. Sort on the lower side scatter population as this will give you a single integration of the NGN2 cassette

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	10x	1.2 mL
Anti-Anti	100x	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.

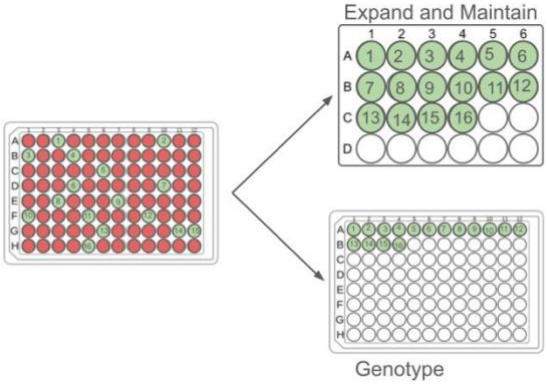
Reagent	Dilution Factor	25 mL recipe
mTesR Plus	1	25 mL
ROCK Inhibitor	1:1000	25 µL

   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.

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



6. Quick extract protocol
    a. Add 25 µL/well of Quick Extract
        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 -80°C 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 37°C
e. Place the frozen plate on paper towels and place it in the 37°C 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 LHA or RHA PCR on all the clones, then only perform the LHA/RHA and WT AAVS1 PCR on clones that have the correct 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 a Left or Right Homology Arm PCR (perform on all clones).
    a. Left Homology Arm:
        i. Forward Primer
            1. CGACTTCCCCTCTTCCGATG
        ii. Reverse Primer
            1. GGGATTCTCCTCCACGTCAC
        iii. Amplicon Size: 1235 bp
        iv. Annealing Temp: 59C
        v. Elongation Time: 1 min 30 sec
    b. Right Homology Arm
        i. Forward Primer
            1. GTGGAATTGCTCCAGGCGAT
        ii. Reverse Primer
            1. CAGTCACCCCACAGTTGGAG
        iii. Amplicon size: 1359 bp
        iv. Annealing Temp: 59C
        v. Elongation Time: 1 min 30 sec
7. Perform WT AAVS1 PCR (perform only on clones that have the correct LHA/RHA band).
    a. Forward Primer
        i. TCTCCTGTGGATTCGGGTCA
    b. Reverse Primer
        i. CTCCAGGAAATGGGGGTGTG
    c. Amplicon size: 432
    d. Annealing Temp: 59C
    e. Elongations Time: 30 sec
8. CNV ddPCR
    a. Perform droplet digital PCR using the “CNV2” setting for the experiment on the Bio-Rad QX200™ Droplet Reader
    b. Target FAM: mCherry
        i. Probe
            1. TAGTCGGGGATGTCGGCGGG
        ii. Forward Primer
            1. GAAGTTCATCACGCGCTCC

iii. Reverse Primer
1. GTTCATGTACGGCTCCAAGG
iv. Annealing Temp: 61.4C
c. Reference HEX: RPP30
i. Bio-Rad: dHsaCP1000485

9. Test Differentiate clones with correct NGN2 integrations
a. See “NGN2 Differentiation” at https://clellandlab.ucsf.edu/protocols

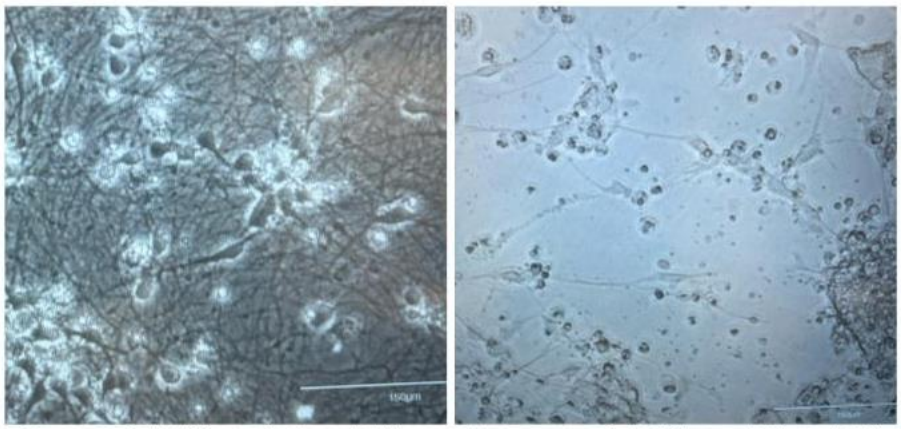


Figure 3: Comparison of a successful vs an unsuccessful NGN2 differentiation

Karyotyping and Freezing

1. Send one clone with correct NGN2 integrations 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 NGN2 integration and normal karyotype.

Materials

sgRNA and Plasmid

sgRNA/Plasmid	**Sequence**
AAVS-NGN2 sgRNA	GUCCCUAGUGGCCCCACUGU
pUCM-AAVS1-TO-hNGN2 plasmid	Addgene #105840

Primers and Probes

Primer/Probe	Sequence
LHA Forward Primer	CGACTTCCCCTCTTCCGATG
LHA Reverse Primer	GGGATTCTCCTCCACGTCAC
RHA Forward Primer	GTGGAATTGCTCCAGGCGAT
RHA Reverse Primer	CAGTCACCCCACAGTTGGAG
WT AAVS1 Forward Primer	TCTCCTGTGGATTCGGGTCA
WT AAVS1 Reverse Primer	CTCCAGGAAATGGGGGTGTG
mCherry Probe	TAGTCGGGGATGTCGGCGGG
mCherry Forward Primer	GAAGTTCATCACGCGCTCC
mCherry Reverse Primer	GTTCATGTACGGCTCCAAGG

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	
ddPCR™ Copy Number Assay: RPP30, Human, Homo sapiens	Bio-Rad	dHsaCP1000485

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
Falcon™ Round-Bottom Polystyrene Test Tubes with Cell Strainer Snap Cap, 5mL	Fisher Scientific	877123
BD FACS Aria Fusion	Becton Dickinson	-