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Protocol status: Working We use this protocol and it's working

Created: Apr 03, 2024

Loss of Function Mutagenesis Protocol

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ABSTRACT

This protocols offers a description of the loss of function mutagenesis procedure for induced pluripotent stem cells.

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MATERIALS

PROTOCOL integer ID: 97721

mTeSR Plus:

Catalog # 100-0276,mTeSRTM Plus cGMP Pluripotent Stem Cell Maintenance Medium | STEMCELL Technologies

- Primocin: (ant-pm-1) https://www.invivogen.com/primocin
- Matrigel
- Geltrex
- DPBS
- ReLeSR
- ROCK-Inhibitor: https://www.tocris.com/products/y-27632-dihydrochloride_1254?
 gad_source=1&gclid=EAIaIQobChMIj83n9vLLhAMVSkt_AB1e5guUEAAYASAAEgLyC_D_BwE&gclsrc=aw.ds
- mFreSR: 05855, https://www.stemcell.com/mfresr-cryopreservation-medium-for-pscs.html
- Cryovials
- Barcoded Cryovials
- Accutase
- Plasmids
- Opti-MEM
- Lipofectamine: STEM00003, https://www.fishersci.com/shop/products/lipofectamine-stem-transfection-reagent-4/STEM00003?
 searchHijack=true&searchTerm=STEM00003&searchType=RAPID&matchedCatNo=STEM00003
- Chroman1: 7163/10, https://www.rndsystems.com/products/chroman-1_7163

- Emricasan: 7310/5, https://www.rndsystems.com/products/emricasan_7310
- Polyamine Supplement: 7739/1, https://www.rndsystems.com/products/polyamine-supplement-x1000-lyophilized_7739
- Trans-ISRIB: 5284/10, https://www.rndsystems.com/products/trans-isrib_5284
- Vacuum Filtration System: S2GPU01RE, https://www.sigmaaldrich.com/US/en/product/mm/s2gpu01re
- 5mL corning round bottom tubes with blue strainer cap
- 5mL corning round bottom sample collection tubes
- DNA Quick Extract: NC9904870, https://www.fishersci.com/shop/products/quick-extract-dna-extrac-50ml/NC9904870#?keyword=NC9904870
- PCR Reagents: 11-495-017, https://www.fishersci.com/shop/products/invitrogen-pcr-sub-x-sub-enhancer-system/11495017?searchHijack=true&searchTerm=11-495-017
- dNTPs: 3622614001, https://www.sigmaaldrich.com/US/en/product/roche/dntpro

F and R Primers

Taq Polymerase

- SAP+ SAP Buffer: 78-390-5000UN, https://www.fishersci.com/shop/products/shrimp-alkaline-phosphatase-sap-4/783905000UN
- Exonuclease: 70-073-X5000U, https://www.fishersci.com/shop/products/exonuclease-i-standard-concentration-10-units-l-2/70073X5000U
- Big Dye: 4336917, https://www.fishersci.com/shop/products/bigdye-terminator-v3-1-cycle-sequencing-kit-6/4337455
- HiDi Formamide: 43-113-20, https://www.fishersci.com/shop/products/hi-di-formamide-5/p-7138586

- RNeasy Plus Mini Kit: 74134, https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/rneasy-plus-kits?
 catno=74134
- Qiashredders: 79654, https://www.qiagen.com/us/products/instruments-and-automation/accessories/qiashredder?catno=79654
- Recombinant Anti-Oct4 antibody: ab181557,
 https://www.abcam.com/products/primary-antibodies/oct4-antibody-epr17929-chip-grade-ab181557.html
- Anti-SSEA4 antibody: ab16287, https://www.abcam.com/products/primary-antibodies/ssea4-antibody-mc813-70-ab16287.html
- Human Nanog Antibody: AF1997-SP, https://www.rndsystems.com/products/human-nanog-antibody_af1997
- Donkey anti-Goat IgG (H+L) Cross Adsorbed Secondary Antibody, Alexa Fluor 568:
 A11057

https://www.fishersci.com/shop/products/donkey-anti-goat-igg-h-l-cross-adsorbed-secondary-antibody-alexa-fluor-568-invitrogen/A11057#?keyword=A-11057

Donkey anti-Chicken IgY (H+L) Highly Cross Adsorbed Secondary Antibody, Alexa Fluor
 647:

A78952

https://www.fishersci.com/shop/products/donkey-anti-chicken-igy-h-l-highly-cross-adsorbed-secondary-antibody-alexa-fluor-647-invitrogen/A78952#?keyword=A78952

Donkey anti-Rabbit IgG (H+L) Cross Adsorbed Secondary Antibody, DyLight 755:
 PISA510043

https://www.fishersci.com/shop/products/donkey-anti-rabbit-igg-h-l-cross-adsorbed-secondary-antibody-dylight-755-invitrogen/PISA510043?

searchHijack=true&searchTerm=SA5-10043&searchType=RAPID&matchedCatNo=SA5-10043

Thermo Scientific DAPI Solution (1mg/mL):

EN62248

https://www.fishersci.com/shop/products/pierce-dapi-nuclear-counterstain-1/EN62248

iPSC Maintenance

- 1 Change media every other day mTeSR Plus with 1ml primocin
 - 1.1 Always warm media to room temperature before use
 - 1.2 Aspirate using glass pipette attached to vacuum and replenish with mTeSR plus

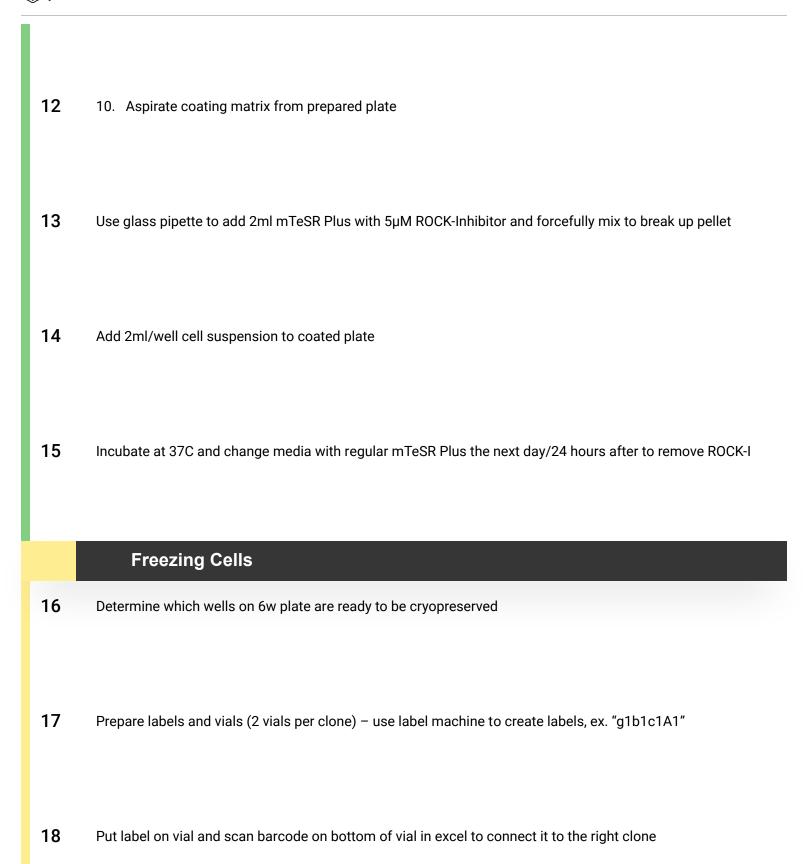
Passaging

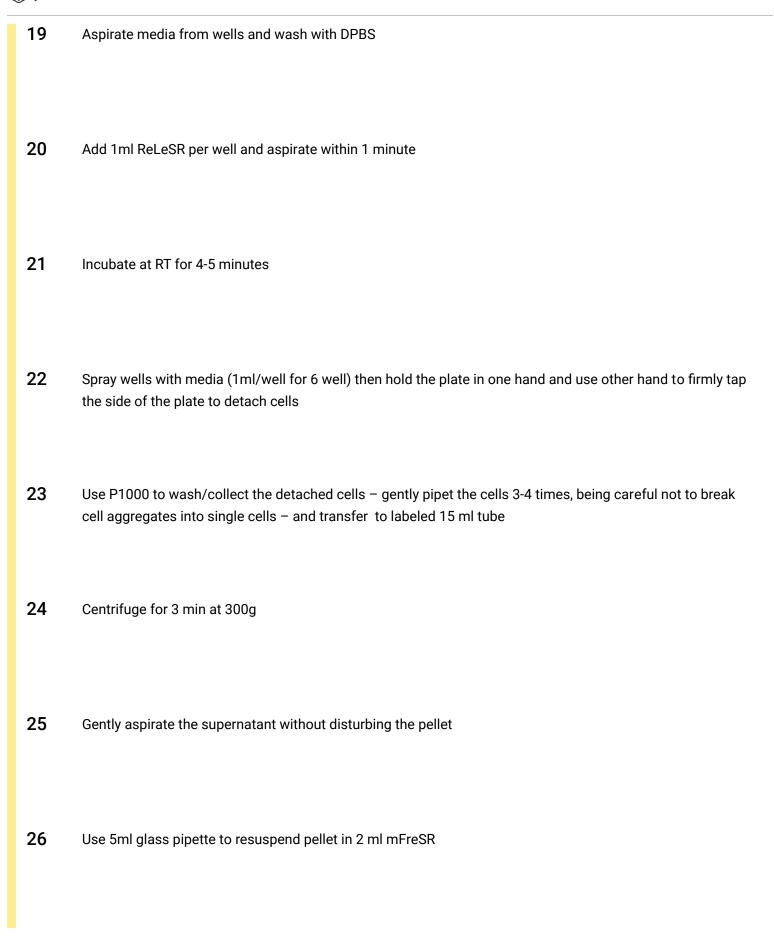
- 2 Passage every 4-6 days (whenever colonies are 70-80% confluent- depends on the density that you plate)
 - 2.1 Coat desired number and size of plates with matrigel/geltrex and incubate overnight or >4hr at 37C
 - 2.2 On day of passage, cool the coated plates at room temperature for >1hr then aspirate matrigel/geltrex then add appropriate amount of media for well size
 - 2.3 Aspirate media from cells and wash with DBPS

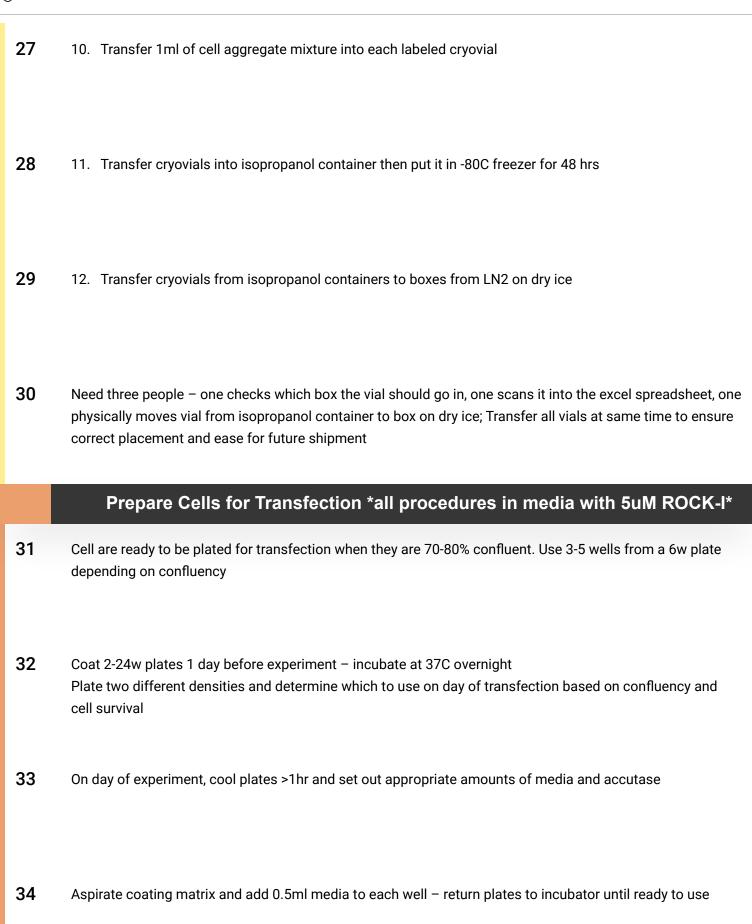
- 2.4 Add appropriate amount of ReLeSR and aspirate within 1 minute 2.5 Incubate at RT for 4-5 minutes 2.6 Spray wells with media (1ml/well for 6w) with p1000 then hold the plate in one hand and use other hand to firmly tap the side of the plate to detach the cells 2.7 Use p1000 to wash/collect the detached cells- gently pipet the cells 2-4 times, being careful not to break the aggregates into single cells and transfer to labeled 15ml tube 2.8 Plate the cell aggregate mixture at the desired density onto the pre-coated wells containing media Usually 1:10 - 1:20 or 1:4-1:5 depending on cell density 2.9 Check that there is an appropriate number of cells under the microscope then place in the incubator at 37C; Move the plate in several quick, short, back-and-forth and side-to-side motions
 - to evenly distribute the cells
- 2.10 Do not disturb the plate for 24 hours

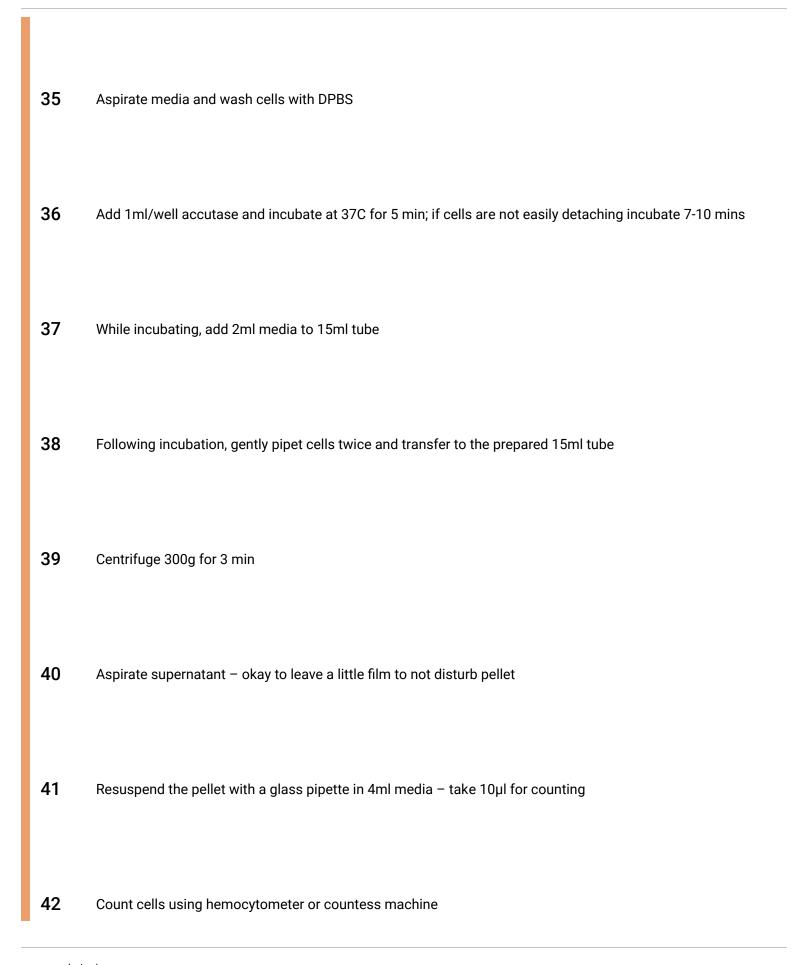
Thawing Cells

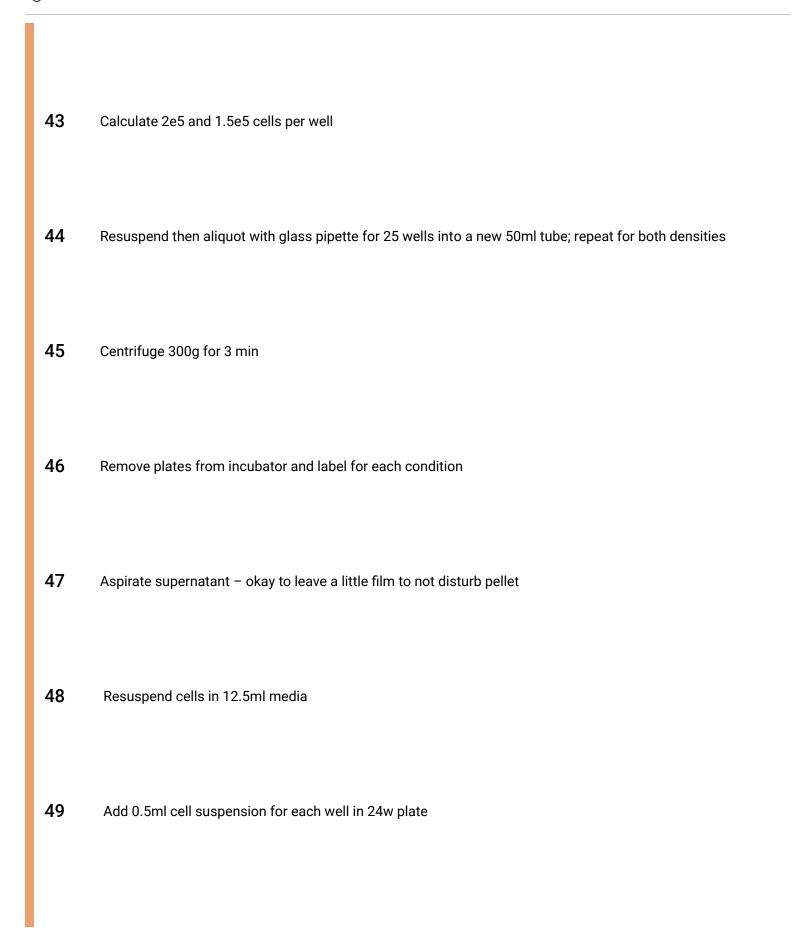
3 Prepare coated 6w plates with 1 well for each cryovial (same as step 1 above) Can prepare 2 wells for each cryovial – depending on how confluent you want cells to be thawed











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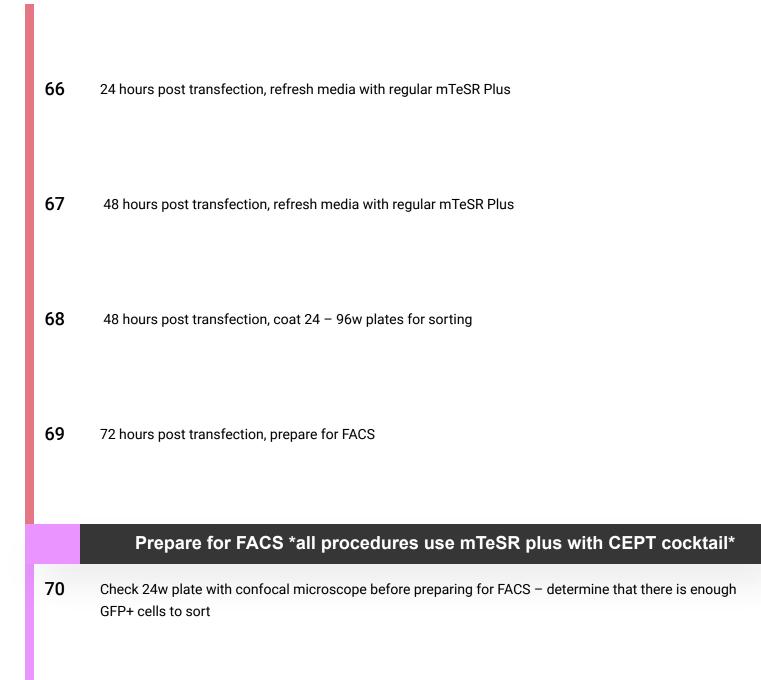
5) protocois.io		
50	After every 6 wells, move the plate in several quick, short, back-and-forth and side-to-side motions to evenly distribute the cells	
51	Incubate at 37C overnight	
52	Repeat steps 17-21 for other density	
	Transfection for iPSCs in 24w Plate	
53	Before transfection, decide which density has better cell survival and density	

- 55 Prepare working plasmid solution using endo-free TE buffer in hood: pEF-AncBE4max (from Brafman): 500ng/µl pEF-BFP: 200ng/µl pDT-sgRNA (with variant specific gRNA cloned): 300 ng/µl
- 56 Prepare tubes for each sgRNA, one for DNA master mix, and one for Lipo master mix (26 tubes)

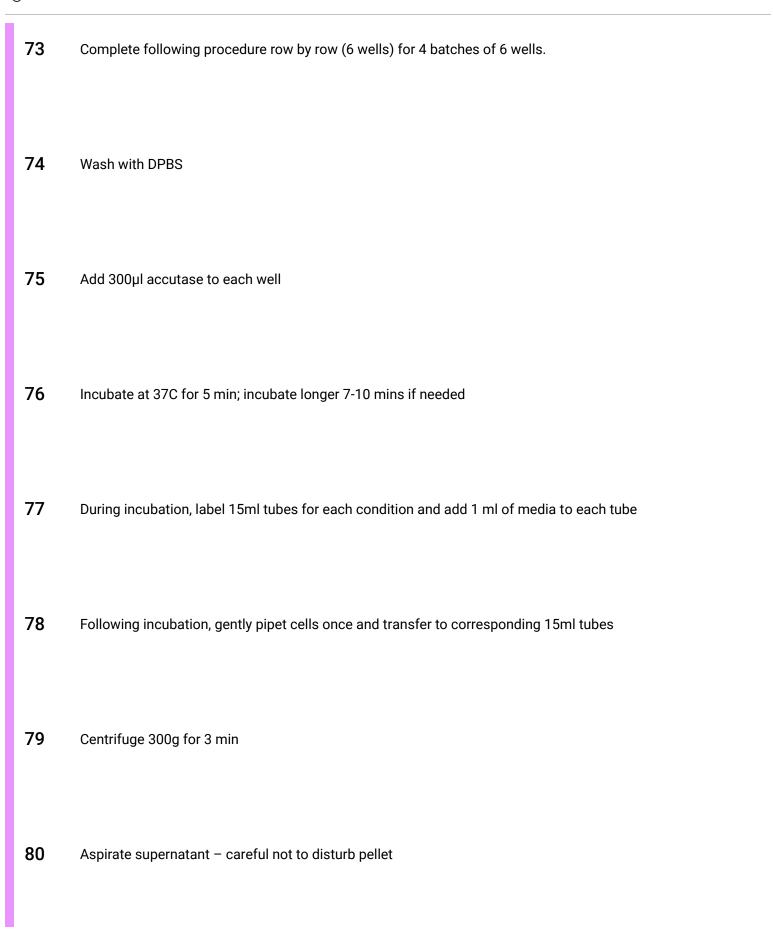
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57 Prepare DNA MM: 625ul Opti-MEM

	37.5ul AncBE4max 37.5ul BFP
58	Aliquot 28ul of DNA MM into each sgRNA tube
59	Add 1µl of each sgRNA to their corresponding tubes containing DNA MM
60	Prepare Lipo Master mix: 625ul Opti-MEM 82.5ul Lipofectamine STEM
61	Aliquot 28.3ul of LIPO MM to each sg RNA tube and mix well
62	Incubate at RT for 10 min
63	Towards end of incubation, label prepared 24w plate with corresponding gene numbers
64	Add 50µl complex dropwise into the correct well and gently swirl plate to ensure even distribution
65	Return plate to incubator



- 71 Set out media and accutase to warm to RT add CEPT components (1:10,000 chroman I, emricasan, and transISRIB; 1:1,000 polyamine supplement) to media then filter before use
- 72 Cool 96w plates >1hr at RT then aspirate coating matrix and add 120µl media. Return plates to incubator for later use.



Care after Sorting *All media changes with regular mTeSR Plus to dilute C... 89 24hr post sorting: do not disturb cells 90 48hr post sorting: use robot pipettor to add 50µl media 91 72hr post sorting: use robot pipettor to change 50µl media 92 96hr (Day 4) post sorting: use robot pipettor to change 120µl media 93 144hr (Day 6) post sorting: aspirate 120µl, add 100µl media 94 Afterwards, use robot pipettor to change 100µl media every other day

95 It takes 10-14 days for a single cell to grow into a colony with appropriate size for picking Approximately one week post sorting, determine which wells have colonies and start to mark/plan for colony picking

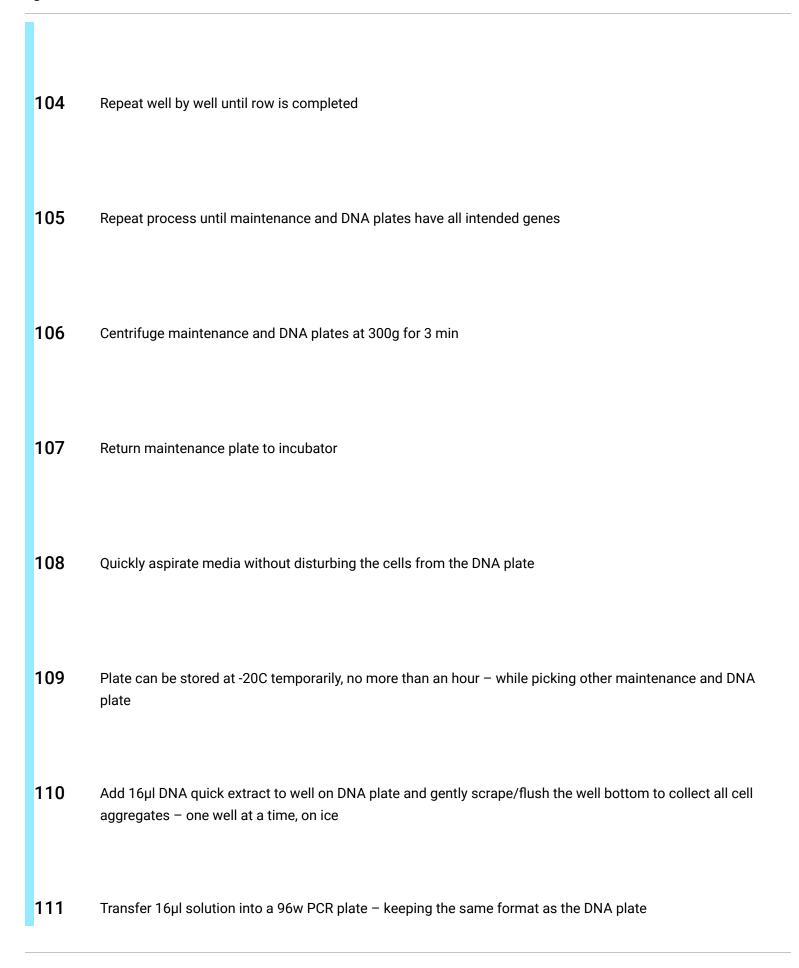
Colony Picking 96 Day before experiment: Determine which plates you will be picking from based on size and colony morphology - mark 12 best colonies with microscope; Each colony marked plate will go onto one row on two identical 96w plates (see example below) - one plate for maintaining cells and one plate for DNA extraction 97 Day before experiment: Coat one row on 96w plate for each plate that is ready to be picked-normally 2-3 plates for the first round of picking depending on growth rate, then another 2 plates for the second round Ex. 8 marked plates = one full 96 well plate 98 On day of the experiment, cool plates >1hr RT then aspirate coating matrix and add 80µl mTeSR Plus with 5μM ROCK-I to each well (maintenance plates); an uncoated empty 96w plate copy is needed for each coated plate for DNA extraction (DNA plates). Label plates to correspond to the correct genes and indicate whether they are the "DNA plate" or the "maintenance plate" 99 Pick colonies from one marked plate at a time following the below procedures 100 Aspirate 50-70µl media from marked wells so around 70-100µl left 101 Set p200 pipettor at 50µl and scrape in marked area of well ~30 seconds then collect as many aggregates as possible in 50µl volume

103

102

Transfer the 50µl aggregates into a well on the maintenance plate, mix well by pipetting 3 times

Withdraw 50ul cell suspension from maintenance plate and add to DNA plate



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112 Briefly spin PCR	plate
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- Run DNA extraction protocol on thermos cycler (1st step in Sanger sequencing workflow)
- 114 Follow Sanger sequencing protocol to get results in 2-3 days

Sanger Sequencing

115 DNA Extraction -> Amplification PCR -> SAP -> Sequence PCR -> Purification -> Sequencing

DNA Extraction (cell lysate in 16ul solution)

- 116 Run DNA extraction protocol on thermocycler:
 - a. 65C for 15 min
 - b. 68C for 15 min
 - c. 95C for 10 min
 - d. 4C for ever
- 117 Go directly to PCR Amp step, do not pause here!

PCR Amplification

118	Prepare working primer from stock solution (100uM to 10uM)
119	Prepare master mix for each gene and for appropriate number of wells, accounting for 20% extra: AmpPCR mix 1x (ul) Water 5.5 10X PCR Amplification Buffer 1 10X PCR Enhancer 1 50mM MgS04 0.3 10mM dNTP 0.2 Primer F 0.2 Primer R 0.2 Polymerase 0.1
120	Transfer prepared AmpPCR mixes into corresponding rows on a 96w PCR plate (8.5ul per well)
121	Add 1.5ul quick extracted DNA to plates and mix 3 times
122	Seal plates and briefly centrifuge 2000g ~5 seconds
123	Run PCR Amp protocol on thermocycler: a. 95C for 10 min b. 95C for 30 sec c. 53C for 1 min 30 sec d. 72C for 1 min e. Step b-d 40 times f. 72C for 10 min g. 4C for ever

Directly to SAP step or can pause here; leave plate at 4C <18 hrs

SAP Reaction (5ul mix + 5ul PCR product= 10ul total)

125 Prepare master mix:

SAP mix 1x(ul)

Water 3.9

10x SAP buffer 0.5

SAP 0.5

E. coli exonuclease I 0.1

126 Pipette 5ul SAP mix into each well on a 96w plate

Add 5ul PCR product to each well on SAP plate, pipetting 3 times to mix

128 Briefly centrifuge 2000g ~5 sec

129 Run SAP protocol on thermocycler:

- a. 37C for 50 min
- b. 95C for 15 min
- c. 4C for ever

May pause here, leave the plate at 4C <18hr, or go directly to Seq PCR step

Seq PCR (5ul mix + 5ul SAP product = 10ul total)

131	Prepare master mix following table above for each gene - only use either F primer or R primer, do not use		
	both! Be aware of primer records, some genes require more of less big dye		
	Seq PCR mix 1x (ul)		
	Water 2		
	Big dye 2		
	Primer F or R		
132	Pipette 5ul mix into a 96w PCR plate, being careful to be consistent with gene placement		
133	Add 5ul SAP product, pipetting 3 times to mix		
.00	And our of a product, processing of three to make		
101			
134	Seal the plate and briefly centrifuge 2000g ~5 sec		
135	Run Seq PCR protocol on thermocycler:		
	a. 96C for 1m in		
	b. 96C for 10 sec		
	c. 55C for 5 sec		
	d. 60C for 4 min		
	e. Step b-d for 25 times		
	f. 4C for ever		
105			
136	May pause here, leave the plate at 4C <18 hrs or go directly to purification step		
	Duvidication		
	Purification		

137

Prepare fresh 75% isopropanol

138	Add 40µl 75% isopropanol into each well, pipetting 3 times to mix
139	Gently attach a sealing film and incubate at RT for 20 min
140	Centrifuge plate at 2700g for 30 min
141	Gently invert the plate onto paper towel
142	Add 150µl 75% isopropanol in each well, do not mix!
143	Reseal the plate and centrifuge at 2700g for 12 min
144	Gently invert the plate onto a paper towel

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145	Keep the plate inverted and transfer to a new paper towel and centrifuge at 200g for 1 min to remove residual isopropanol	
146	11. Directly go to next step	
	Prepare for Loading	
147	Add 16µl HiDi formamide to each well, pipetting 3 times to mix	

- 148 Seal the plate and centrifuge at 2000g for ~5 sec
- 149 Run the forma protocol on thermocycler:
 - a. 95C for 3 min
 - b. 4C for 2 min
 - c. 4C for ever
- 150 Centrifuge at 2000g for 1 min
- **151** Ready to load

Setup 3730 for Sequencing

152	Start 3730 software
153	Select instrument status
154	Connect the polymer bottle – set waste bottle and cap on white tray
155	Run bubble remove wizard twice (check for bubbles) then fill array
156	In plate manager – import or build a new template for today's run
157	Go to run scheduler – search the plate ID and add plate in the queue
158	Make sure to remove any sealing film on PCR plate (will damage array if not)
159	Put the PCR plate in the black bottom, add a clean grey septa on it, then attach the white top – make sure it clicks closed and have angled corner of black bottom on top right of PCR plate, A12 on PCR plate goes in angled corner to have correct orientation



176	Then aspirate media from wells ready to be expanded with p200
177	Add 100µl ReLeSR to wells and immediately aspirate – add and aspirate from wells one by one
178	Incubate for 3 min at RT
179	Add 100μl media to each well
180	Scrape colonies one by one using p200 – scape entire area
181	Aspirate full volume from well and add to corresponding well on the 6w plate – move back and forth, right and left to gently disperse cells
182	Passage cells once to have 2 wells on 6w plates with appropriate confluence for each clone – one well is needed for cryopreservation and one well for RNA isolation

199

Aliquot 20-25ul into new labeled 1.5ml tubes and wrap well with parafilm to ship to Novogene

Immunohistochemistry: Staining for Quality Control of iPSCs

	initiation stochemistry. Stanning for Quanty Control of it 503		
200	Fixation		
	200.1	Coat coverslips the day before in 4w dish	
	200.2	Start from iPSCs culture on 96w plate- plate on 12mm 1.5 image-grade glass	
	200.3	Prepare 4% PFA solution in 1xPBS (16% stock in PBS, 400ul per well)	
	200.4	Aspirate old media and add 4% PFA into each well	
	200.5	Incubate at RT for 12-15 min	

	200.6	Aspirate PFA, wash cells 3 times with PBS
	200.7	After third wash, add 1ml PBS + 0.03% sodium azide to each well
	200.0	Wrap the 4w dish using parafilm and store the plate in 4C; you can pause her for a few days
201	Permeabil	ization
	201.1	Prepare blocking buffer (3% BSA in 0.1% PBST)
	201.2	Prepare 1% triton X-100 in PBS
	201.3	Add 500ul PBST to each well and incubate at RT for 15 min
202	Blocking	

	202.1	Aspirate PBST from permeabilization step
	202.2	Add 1ml blocking buffer to each well (3% BSA in 0.1% PBST (0.1% triton in 1x PBS))
	202.3	Incubate RT for 1 hr; can leave in 4C overnight
203	Primary A	ntibody
	203.1	Following vendors instructions, prepare antibodies in blocking buffer; can use 1ug/ml to start if no instructions are available
	203.2	Aspirate blocking buffer
	203.3	Add 350ul primary antibodies in blocking buffer to each well and incubate RT for 1.5 hr; can leave overnight if necessary but increase the volume of solution
204	Wash	



207	DAPI	
	207.1	Ready to use solution in 4C fridge (0.5ug/ml in PBS)
	207.2	Aspirate wash
	207.3	Add 400ul DAPI solution to each well and incubate at RT for 10 min
208	Wash	
	208.1	Aspirate DAPI
	208.2	Briefly wash each well with 1ml PBS
	208.3	Thaw diamond anti-fade solution

209	Mount	
	209.1	Add one drop of diamond anti-fade solution to slide
	209.2	Aspirate PBS and use tweezers to grab and invert the coverslip to make sure the cells contact the anti-fade reagent
	209.3	Slides can be stored in the dark, > overnight to let the anti-fade dry
	209.4	Ready for imaging