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

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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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PROTOCOL integer ID: 97721

MATERIALS

- mTeSR Plus:

Catalog # 100-0276,mTeSR™ 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&searchType=RAPID&matchedCatNo=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

4 On day of thawing, cool plates at RT >1hr before use but do not add media

5 Label 15ml tubes for each cryovial that is being thawed

6 Take cryovials out of liquid nitrogen

7 Thaw cryovials in dry bath until only one ice crystal is left

8 Use a glass pipette to remove cells from cryovial and transfer to labeled 15ml tube

9 Slowly add 5ml mTeSR Plus dropwise into 15ml tube to avoid shocking the cells – rotate the tube while adding media to ensure even distribution

10 Centrifuge 3 mins at 300g

11 Aspirate supernatant – okay to leave a little film to avoid disturbing the pellet

12 10. Aspirate coating matrix from prepared plate

13 Use glass pipette to add 2ml mTeSR Plus with 5μM ROCK-Inhibitor and forcefully mix to break up pellet

14 Add 2ml/well cell suspension to coated plate

15 Incubate at 37C and change media with regular mTeSR Plus the next day/24 hours after to remove ROCK-I

Freezing Cells

16 Determine which wells on 6w plate are ready to be cryopreserved

17 Prepare labels and vials (2 vials per clone) – use label machine to create labels, ex. “g1b1c1A1”

18 Put label on vial and scan barcode on bottom of vial in excel to connect it to the right clone

19 Aspirate media from wells and wash with DPBS

20 Add 1ml ReLeSR per well and aspirate within 1 minute

21 Incubate at RT for 4-5 minutes

22 Spray wells with media (1ml/well for 6 well) then hold the plate in one hand and use other hand to firmly tap the side of the plate to detach cells

23 Use P1000 to wash/collect the detached cells – gently pipet the cells 3-4 times, being careful not to break cell aggregates into single cells – and transfer to labeled 15 ml tube

24 Centrifuge for 3 min at 300g

25 Gently aspirate the supernatant without disturbing the pellet

26 Use 5ml glass pipette to resuspend pellet in 2 ml mFreSR

27 10. Transfer 1ml of cell aggregate mixture into each labeled cryovial

28 11. Transfer cryovials into isopropanol container then put it in -80C freezer for 48 hrs

29 12. Transfer cryovials from isopropanol containers to boxes from LN2 on dry ice

30 Need three people – one checks which box the vial should go in, one scans it into the excel spreadsheet, one physically moves vial from isopropanol container to box on dry ice; Transfer all vials at same time to ensure correct placement and ease for future shipment

Prepare Cells for Transfection *all procedures in media with 5uM ROCK-I*

31 Cell are ready to be plated for transfection when they are 70-80% confluent. Use 3-5 wells from a 6w plate depending on confluency

32 Coat 2-24w plates 1 day before experiment – incubate at 37C overnight
Plate two different densities and determine which to use on day of transfection based on confluency and cell survival

33 On day of experiment, cool plates >1hr and set out appropriate amounts of media and accutase

34 Aspirate coating matrix and add 0.5ml media to each well – return plates to incubator until ready to use

35 Aspirate media and wash cells with DPBS

36 Add 1ml/well accutase and incubate at 37C for 5 min; if cells are not easily detaching incubate 7-10 mins

37 While incubating, add 2ml media to 15ml tube

38 Following incubation, gently pipet cells twice and transfer to the prepared 15ml tube

39 Centrifuge 300g for 3 min

40 Aspirate supernatant – okay to leave a little film to not disturb pellet

41 Resuspend the pellet with a glass pipette in 4ml media – take 10µl for counting

42 Count cells using hemocytometer or countess machine

43 Calculate 2e5 and 1.5e5 cells per well

44 Resuspend then aliquot with glass pipette for 25 wells into a new 50ml tube; repeat for both densities

45 Centrifuge 300g for 3 min

46 Remove plates from incubator and label for each condition

47 Aspirate supernatant – okay to leave a little film to not disturb pellet

48 Resuspend cells in 12.5ml media

49 Add 0.5ml cell suspension for each well in 24w plate

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

54 Change media for selected plate with antibiotics free mTeSR Plus with 5μM ROCK-I (700ul per well)

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)

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

66 24 hours post transfection, refresh media with regular mTeSR Plus

67 48 hours post transfection, refresh media with regular mTeSR Plus

68 48 hours post transfection, coat 24 – 96w plates for sorting

69 72 hours post transfection, prepare for FACS

Prepare for FACS *all procedures use mTeSR plus with CEPT cocktail*

70 Check 24w plate with confocal microscope before preparing for FACS – determine that there is enough GFP+ cells to sort

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.

73 Complete following procedure row by row (6 wells) for 4 batches of 6 wells.

74 Wash with DPBS

75 Add 300µl accutase to each well

76 Incubate at 37C for 5 min; incubate longer 7-10 mins if needed

77 During incubation, label 15ml tubes for each condition and add 1 ml of media to each tube

78 Following incubation, gently pipet cells once and transfer to corresponding 15ml tubes

79 Centrifuge 300g for 3 min

80 Aspirate supernatant – careful not to disturb pellet

81 Resuspend pellet in 700µl media; 500ul if low cell confluence and 1ml if cell confluence is high

82 Filter cell suspension twice using two different 5ml corning round bottom tubes with blue strainer cap

83 Replace the blue cap with a filterless white cap from a sample collection tube to avoid contamination

84 Put samples on ice immediately

85 Sorting with FACS Aria – 1 double positive cell (BFP + GFP) into one well on 96w plate

86 After sorting, centrifuge the plates at 300g for 1 min to help cells attach

87 Return plates to incubator ASAP

88 Repeat until all 4 batches are done

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

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

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

104 Repeat well by well until row is completed

105 Repeat process until maintenance and DNA plates have all intended genes

106 Centrifuge maintenance and DNA plates at 300g for 3 min

107 Return maintenance plate to incubator

108 Quickly aspirate media without disturbing the cells from the DNA plate

109 Plate can be stored at -20C temporarily, no more than an hour – while picking other maintenance and DNA plate

110 Add 16µl DNA quick extract to well on DNA plate and gently scrape/flush the well bottom to collect all cell aggregates – one well at a time, on ice

111 Transfer 16µl solution into a 96w PCR plate – keeping the same format as the DNA plate

112 Briefly spin PCR plate

113 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 MgSO4 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

124 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

127 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

130 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

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

136 May pause here, leave the plate at 4C <18 hrs or go directly to purification step

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

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

160 10. Put the PCR plate sandwich into the “In stack” drawer – make sure to close the door properly

161 11. When the system detects plates in the “In stack”, click “play” button – top left in the software – then click “yes” to confirm and start the program

162 12. Takes ~2 hours to run a full 96w plate

163 13. After run, copy data to a flashdrive

164 14. Exit the software, shutdown the computer, and return the polymer to 4C

165 Analyze data on computer with SeqScape

166 18. Check each sample for expected editing and record – use to decide which wells to expand

Expand Selected Clones

167

168 After viewing Sanger sequencing results, label 4 wells to keep on 96w plate – two to expand and two as backups; prioritize homozygous clones and clones with the best morphology

169 24hr post picking, add 50µl regular mTeSR Plus into selected wells

170 48hr post picking, change 80µl media for selected wells

171 Change 100µl media every other day

172 Once colonies have grown to proper size (typically 4-6 days), expand from 96w to 6w

173 Coat one well on 6w plate for each well on 96w plate that is ready to be expanded – incubate >4hr

174 Cool 6w plates >1hr at RT and label for each well being expanded – cell line, gene #, clone, gene name, passage #

175 Aspirate coating matrix and add 2ml media to each well

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

RNA isolation

183 Add 800ul buffer RLT Plus to expanded 6w plates with 70-80% confluence

184 Pipet 30-60 seconds, washing the well until the solution is homogenized and clear

185 Add 350ul each to two locking lid 1.5 ml microcentrifuge tubes

186 Store in -80C for temporary storage. Within a week, isolate RNA using the RNeasy Plus Mini Kit

187 Transfer cell lysate to QIA shredder and centrifuge 2 min at max speed

188 Transfer liquid to gDNA elimination column and centrifuge 15 sec at 11,000 rpm
Discard flow through and keep spin column and tube

189 Add 350ul 70% ethanol to lysate and mix until homogenized

190 Transfer to RNA easy spin column and centrifuge 15 sec at 11,000 rpm
Discard flow through and keep spin column and tube

191 Add 700ul buffer RW1 to RNA easy spin column and centrifuge for 1 min at full speed
Discard flow through and keep spin column and tube

192 Add 500ul buffer RPE to RNA easy spin column and centrifuge 15 sec at 11,000 rpm
Discard flow through and keep spin column and tube

193 Repeat previous step, centrifuge for 2 min rather than 15 sec

194 Transfer RNA easy spin column to collection tube and centrifuge 1 min at full speed

195 Transfer RNA easy spin column in labeled 1.5l tube and add 35ul RNAse free water directly to the membrane

196 Centrifuge for 1 min at 11,000 rpm

197 Discard RNA easy spin column and place 1.5ml tube directly on ice

198 Measure the concentrations of each sample using the NanoDrop

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

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

200.8 Wrap the 4w dish using parafilm and store the plate in 4C; you can pause her for a few days

201 Permeabilization

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 Antibody

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

204.1 Aspirate primary antibody

204.2 Add 1ml PBS or 0.1% PBST to each well - 3 times, incubating for 5 min at RT each time

205 **Secondary Antibody**

205.1 Prepare 1:1000 dilution in blocking buffer; make sure to se different hosts and colors and match the primary antibody

205.2 Aspirate wash

205.3 Add 350ul secondary antibody in blocking buffer to each well and incubate at RT for 1 hr. It is light sensitive at this point so be sure to incubate in the dark (cover in foil, keep in a drawer or cabinet)

206 **Wash**

206.1 Repeat same washs step as above but incubate in the dark

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