SOP culture and expansion of reprogrammed cells by Cytotun2.0 kit (Thermo Fisher Scientifics Cat. Nr. A3454) in Stemflex Medium

Requirements:
An aliquot of pre-warmed Stemflex Medium (Thermo Scientific, Cat. no: A3349401)
Pre-warmed Geltrex™ coated 12-well plate (Gibco, Cat. No. A1413302)
10 mM Rock inhibitor (Y-27632 dihydrochloride) (Biothechne/Tocris Cat. No. 1254)
P20 pipette
Shaped Pasture glass pipette
Biological safety cabinet with microscope

Selecting and expansion of clones

1. When colonies reach correct size, passage **P5 to P6**
   From passage 5, perform bulk passaging using enzyme-free reagent, 0.5mM EDTA as in protocol below.

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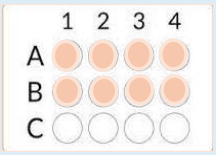

Requirements:
An aliquot of pre-warmed Stemflex Medium (Thermo Scientific, Cat. no: A3349401)
Pre-warmed Geltrex™ coated 12-well plate (Gibco, Cat. No. A1413302)

Note: Do not warm up the Stemflex medium in a 37°C water bath. Just place the bottle in RT for 30 minutes.
D-PBS (without Mg2+ and Ca2+)
0.5mM EDTA (Life Technologies, Cat. no: 15575-020)
5ml serological pipette

Method:

1. Aspirate the spent medium from the well of a 12-well plate and rinse once with 1 ml DPBS, no calcium, no magnesium.
2. Add 0.5mM EDTA solution to the 12-well, then swirl the vessel to cover the entire well surface.
3. Incubate at 37°C for 5 minutes or on a warm plate.
4. Aspirate EDTA and flash well with 0.5 ml pre-warmed complete Stemflex using a 5 ml serological pipette.
5. Pipet up & down once to detach the colonies.
   Transfer a drop (approximately 50 to 100 µl) of the detached cell aggregates using a 5 ml serological pipette into a well of a pre-warmed (30 minutes at room temperature) coated 12-well plate.

Commented [MG2]: To prevent clumps become too small

Commented [MG3]: It's necessary to calibrate the number of pipetting up-and-down cycles to generate the correct clump size.

Note: The split ratio can vary, though it is generally between 1:2 and 1:4 for newly derived

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iPSCs and between 1:3 and 1:12 for established cultures. Occasionally, cells may recover at a different rate and the split ratio will need to be adjusted.

6. Check directly the amount and size of cell aggregates in the well and adjust it by either adding more iPSC aggregate or removing some using 5 ml serological pipette to achive desirable density.

Note: In case that size of cell aggregates is bigger than approximately 50 - 200 µm, pipette one more time and check the size of cell aggregates. If needed, repeat it once more.

7. If the colonies are at an optimal density, the cultures can be split every 4 - 5 days.
8. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to side motions to evenly distribute the cell aggregates. Do not disturb the plate for 24 hours.

Note: Uneven distribution of aggregates may result in merging colonies and formation of super colonies which leads to increased differentiation of human PSCs.

9. Perform daily visual assess of all the wells to monitor growth until the next passaging time. Refresh medium every other day.

2. Bulk passaging from **P6** to **P7** in a coated 12-well plate.

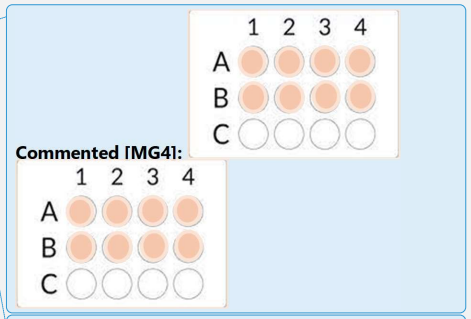
Are the size of colonies big enough to passage?

Yes
↓

Seed ~1 drop of cell clamps using a 5 ml serological pipette after EDTA
divide the rest of cell clamps to 1 well of a 12-well for Sendai Virus detection
and 1 well of 12-well for RNA isolation of SeV expression detection.

- Description (this step explain the duplication of each line in two separate plates:
  A. Check density of receiver well is satisfactory, mark this well as "keep" plate.
  B. Seed in a well of 12-well (separate) 12-well plate and mark for SeV RNA at day 3 post seeding.

*****Harvest the cells marked with SeV once they are confluent (day 3), and proceed with cell



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Commented [MG5R4]: Each plate is dulicated. One "keep"plate en one "RNA"plate

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collection as outlined below. Refer to the RNA protocol for RNA isolation using the ReliaPrep™ RNA Cell Miniprep System.

Requirements:
Cold PBS (RNA designated bottle at fridge)
An aliquot Promega RNA lysis buffer
1-Thioglycerol (TG)
Aliquot of BL Buffer (1x 250 µl)

Note: Work fast, cold, RNAse free! Do not collect more than 2 RNA samples at the same time.

Procedure:
1. Add 2.5 µl of 1-Thioglycerol to an aliquot of (250 µl) BL Buffer.
2. Wash cells once in the well with cold PBS.
3. Add 250 µl of BL+TG to the well and pipette 10 times up & down in all the corners of the well.
4. Transfer the viscous cell lysis into a 1.5 ml safe lock Eppendorf tube and place the tube quickly in the RNA box at -20°C.

Conduct RNA isolation and RT-PCR to ensure the clearance of Oct4, Sox2, Klf4, c-Myc, and SeV backbone before the next passage.

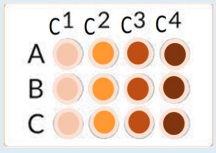
3. Bulk passaging from **P7** to **P8** **(If the clones are clear from Sendai expression)**.

#Is the clone clear of the Sendai expression?

Yes	No
↓	↓
Bulk passage each clone into 1well of a 12-well plate and 3 wells of a separat 12-well plate with proper density to expand.	Bulk passage clone into 1 well of a 12-well plate (P8) P8 to P9, duplicate well by passaging into 2 wells of a 12-well plate, mark one for keep and one for RNA isolation. Repeat RNA isolation and RT-PCR

Commented [MG6]:
1 well of a 12-well plate as backup and 3 wells of a 12-well plate for further expansion of 4 selected clones

Keep plate



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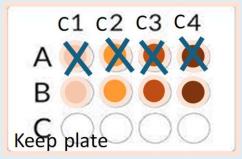
4. Bulk passage either **P8** to **P9**

Passage 1well of each clone on the back up 12 well plate to a new well on the same (keep) plate by seeding ~1 drop cell (50 µl to 100 µl) aggregate using a 5 ml serological pipette to 1 well of 12-well plate.



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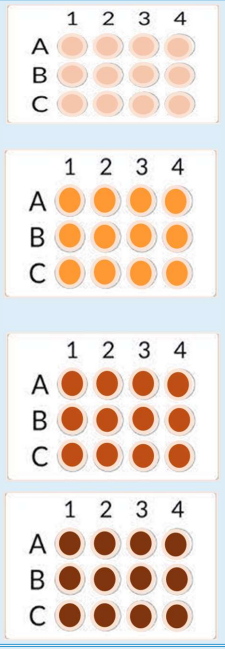
3 wells of the second 12-well plate: Passage 3 wells of each clone per line into 12 wells of a new 12-well plate. These wells will be annotated as freezing, mycoplasma, sterility, DNA, RNA and tri-lineage differentiation.



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5. Collecting material for mycoplasma, sterility, DNA and RNA and freezing and Tri-lineage differentiation of each clone of one iPS line.

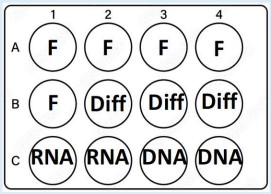
Commented [MG9]: For each clone of each line, collect the designated wells as shown in the figure.

<u>Freezing 7 vials of each iPS clone</u>

Requirements:
Pre-warm Stemflex Medium
DPBS, no calcium, no magnesium
0.5mM EDTA solution
5ml serological pipette
1x 50 ml tube
1 x 15 ml tube
2 Eppendorf tubes
Bambanker Nippon Genetics, cat.nr. BB02
10mM Rock inhibitor Y27632 (1000X)
Cryopreservation vials
Cell freezing container

Method:

Collect 8 ml of the spent medium from 5 wells of 12-well plate per each clone containing iPSCs in a separate 15 ml tube labeled for the sterility test and 1 ml for the Mycoplasma test in a labeled Eppendorf tube.



***Proceed without delay with the detachment of iPS cells from these 5 wells for the purpose of freezing the cells.**

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1. Rinse the vessel 2 times with DPBS, no calcium, no magnesium.
2. Add 0.5 ml of cold 0.5 mM EDTA solution into each well of the 12-well plate containing iPSCs and spread (no hard shaking; movement to warm plate is enough to spread it).
3. Incubate the vessel on a 37°C warm plate for 5 minutes.
4. Aspirate 0.5 mM EDTA and remove the cells from the wells by gently flashing 1 ml Stemflex medium over the surface of each well and pipette up and down 1 time using a 5 ml serological pipette.

   Transfer the medium containing detached cells to a 50 ml tube (pool all the detached cells from each clone into one 50 ml tube).

5. Using another 1 ml of medium per well, gently flash the dish and add into the same 50 ml tube from previous step.

Note: There may be obvious patches of cells that were not dislodged and left behind. Do not attempt to recover them.

6. Spin the tube at 200 x g for 4 minutes.
7. Aspirate the medium slowly without disrupting the cell pellet.
8. Crush the cell pellet by flashing 5 ml of cold Bambanker freezing medium, and transfer directly to 5 cryopreservation tubes (1 ml/tube). Transfer cryopreservation tubes to a freezing container and place it at -80°C. Transfer frozen vials after 24 hours to liquid nitrogen and add a record to the freezing database.

A. Collect RNA well:
Prepare 1.5 ml RNAse free labeled Eppendorf tube per each clone.

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Requirements:
Cold PBS (RNA designated bottle at fridge)
An aliquot Promega RNA lysis buffer
2.5 µl 1-Thioglycerol (TG)
500 µl aliquot of BL Buffer
Eppendorf tube

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iPS line	Stemflex	Geltrex

Note: Work fast, cold, RNAse free! Do not collect more than 2 RNA samples at the same time.

Procedure:

5. Add 5 µl 1-Thioglycerol (TG) to 500 µl BL Buffer.
6. Wash 1x cells in the well with cold PBS.
7. Add 250 µl of BL+TG to each of the 2 designated wells for RNA collection in the 12-well plate for each clone, and pipette 10 times up and down in all the corners of the well.
8. Transfer lysed cells to an 1.5 ml safe lock Eppendorf tube and place the tube quickly in the RNA box at -20°C.

B. Collect DNA well:

Prepare labeled safe lock Eppendorf tube per each clone:

Requirements:

DPBS, no calcium, no magnesium
Safe lock 1.5 Eppendorf tubes
Corning® cell lifter

Procedure:

1. Wash well with 1 ml PBS.
2. Add 1 ml DPBS to well and scrape cells.
3. Transfer scrape cells using a Corning cell lifter in DPBS to an 1.5 ml safe lock Eppendorf tube.
4. Add another 0.5 ml DPBS to well to wash the well and add to the tube.
5. Spin 5min @ 3500 rpm in an Eppendorf centrifuge.
6. Aspirate DPBS and store cell pellets till DNA isolation in the DNA box at -20°C.

Commented [MG11]: Should develop how we can detach the cells instead of scraping.

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C. Set up Tri-lineage direct differentiation using STEMdiff™ Trilineage Differentiation Kit from Stem Cell Technologies Cat. nr. 05230

Requirements:
3 confluent wells of the 12-well plate from each clone
Aliquot of pre-warmed Stemflex medium
Aliquot of STEMdiff™ Trilineage Ectoderm Medium
DMEM/F-12 with 15 mM HEPES (Invitrogen, Cat.no. 36254)
D-PBS (Without Ca++ and Mg++)
Gentle Cell Dissociation Reagent (Stemcell Technologies, Cat.no. 07174)
Pre-warmed 6 wells of coated 12-well plate with Geltrex
10 mM Rock inhibitor (1000x) (Tocris, Cat. No. 1254) aliquot (1000x)
Trypan Blue
Countess™ Cell Counting Chamber Slides(Thermo Scientific, Cat. no: C10228)
1x 1.5 ml tubes with 15 µl Trypan Blue
6 x 1.5 ml tubes labeled with Ectoderm
Endoderm
Mesoderm

Print two labels per each clone of iPS line, for 2 wells of a 12-well plate for each clone per iPS line per each embryonic germ layers.

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Method:

1. Use a microscope to visually identify regions of differentiation in the wells to be passaged. Mark these using a felt tip or lens marker on the bottom of the plate. Remove regions of differentiation by aspiration.

Note: Most of the time, there is no or very little differentiation and it is not needed to remove differentiated cells.

2. Wash each well to be passaged with 1 mL of D-PBS (Without Ca++ and Mg++).
3. Aspirate the wash medium and add 0.5 mL/well of Gentle Cell Dissociation Reagent.
4. Incubate on a 37°C warm plate for 5 minutes.
5. In each well, dislodge cells by pipetting up and down 1 - 3 times using a p1000 pipette. Ensure all remaining cell aggregates are broken up into single cells.
6. Immediately transfer cells to a tube containing 1 mL of DMEM/F-12 per well harvested. Wash each well once with 1 mL of DMEM/F-12 supplemented with 3 µl of 10 mM Rock inhibitor to collect any remaining cells and transfer to the tube.
7. Transfer 10 µl of cells to the tube already prepared with Trypan Blue and mix well. Transfer 10 to 11 µl of cells/Trypan Blue mix to a Countess slide and count live cells.
8. Calculate volume of cells needed for each lineage as it shown in table below and transfer that amount to each already marked Eppendorf tube.

Lineage	Number of cells /per 1 well	Calculated volume for two wells	Medium
Ectoderm	800,000	µl calculated volume	2 ml STEMdiff™ Trilineage Ectoderm Medium + 10 mM Y-27632
Endoderm	800,000	µl calculated volume	2 ml Stemflex + 10 mM Y-27632
mesoderm	200,000	µl calculated volume	2 ml Stemflex + 10 mM Y-27632

9. Centrifuge the Eppendorf tubes at 300 x g for 5 minutes.
10. Remove supernatant. Resuspend cells respectively in 2 mL of Stemflex + 10 µM Rock inhibitor for Mesoderm and Endoderm and seed them in 2 wells of 12-well plate per each clone of a line.
11. Mark on the top of slides date to collect RNA (Mesoderm/Endoderm 5 days & Ectoderm 7 days). Refresh the medium of wells and chamber slides daily.

Note: Shelf life of Endoderm medium is 1 week at 4°C. Shelf life of Ectoderm and Mesoderm medium is 2 weeks at 4°C.

RNA collection of Trilineage differentiation of 1 well of 12-well plate per each lineage

* At day 5 for Endoderm and Mesoderm differentiation. Prepare 1.5 ml RNAse free Eppendorf tubes with labels.

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*On day 7 for Ectoderm differentiation. Prepare 1.5 ml RNAse free Eppendorf tubes with labels.

Requirements:
Cold PBS (RNA designated bottle at fridge)
An aliquot Promega RNA lysis buffer
1-Thioglycerol (TG)
Aliquot of BL Buffer (1x 250 µl)

Note: Work fast, cold, RNAse free! Do not collect more than 2 RNA samples at the same time.

Procedure:
1. Add 2.5 µl 1-Thioglycerol (TG) to an aliquot of 250 µl BL Buffer or 2.5 µl of 1-Thioglycerol to an aliquot of 1x (250 µl) BL Buffer.
2. Wash 2x wells with cold PBS.
3. Add 250 µl of BL+TG to the wells and pipette up and down 10 times in each well designated for different lineages, Pipette well on the corners of the well.
4. Transfer lysed cells to an 1.5 ml safe lock Eppendorf tube and place tube quickly in RNA box at -20°C

Note: Due to lysis of the cells, the buffer will become viscous.

Preparation of single cells from trilineage differentiated cells derived from a single well of a 12-well plate for each lineage.

1. Wash the wells to with 1 mL of D-PBS (Without Ca++ and Mg++).
2. Aspirate the wash medium and add 0.5 mL/well of Gentle Cell Dissociation Reagent.
3. Incubate on a 37°C warm plate for 5 minutes.
4. In each well, dislodge cells by pipetting up and down 1 - 3 times using a p1000 pipette. Ensure all remaining cell aggregates are broken up into single cells.
5. Promptly move the cells to a tube that contains 1 mL of 2% FCS in PBS for each well harvested. Rinse each well once with 1 mL additional 2% FCS in PBS with to gather any residual cells and transfer them to the Eppendorf tubes.
6. Centrifuge the Eppendorf tubes at 300 x g for 5 minutes.
7. Aspirate supernatant without disrupting cell pellet.
8. Add 50 µl of proper antibody dilution.
9. Incubate for 30 minutes at RT for SSEA4 and TRA-1-80 antibodies.
10. Wash the cells 3 times with 2% FCS in PBS.
11. Resuspend the cell pellet in 50 µl of secondary antibody diluted in 2% FCS in PBS and incubate the cells in dark for 30 minutes.
12. Wash the cells 3 times with 2% FCS in PBS.

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13. Resuspend the cells in 300 µl of 2% FCS in PBS and perform FACS analysis.

6. Passage **P10 to P11** of 12-well plate

Are Trilineage direct differentiation successfully collect for all three lineages

Yes	No
↓	↓
Passage all the cells of 1x12-well (keep) well to a coated chamber slide for immunofluorescence staining of stem cell markers.	Depends of needed material passage cells to an extra well for repeat.

1x12: Seed all cell aggregate to a chamber slide