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MAINTENANCE CULTURE OF iPSCs (Basic Protocol 1)

In 1 collection

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Neurodegeneration Method Development Community

ABSTRACT

Human iPSCs are an ideal system for studying human biology due to their rapid proliferation, genomic stability, and ability to differentiate into many somatic cell types. Historically, specialized culture practices and costly reagents have hindered widespread adoption of iPSCs by the cell biology community. In recent years, however, development of new culture techniques and improved media formulations have dramatically simplified iPSC culture and reduced costs.

The protocols described in this unit are adapted from a collection of publications that establish optimal practices for the maintenance of human iPSC cultures (Beers et al., 2012; Ludwig et al., 2006; Chen et al., 2011). While these publications provide useful guidelines for the stem cell novice, here we distill the fundamental procedures necessary for maintaining iPSCs in a pluripotent state and highlight critical steps that may need to be optimized for individual applications. In practice, iPSC lines of interest are usually maintained in an undifferentiated state in small cultures (1 to 3 wells of a 6-well plate) to reduce reagent use before being expanded as needed for experimentation.

Essential 8 (E8) is the simplest defined medium for hiPSC culture. E8 may be prepared from its components by the consumer (Table 1; Chen et al., 2011) or purchased as a preformulated kit. Other commercially available media may be substituted, such as mTeSR1 or StemFlex. A volume of 12 ml of medium should be added to each 10-cm tissue culture dish or distributed evenly across each standard multiwell plate (i.e., 2 ml/well for a 6-well plate). E8 should be aspirated and replaced with fresh medium daily, although a double volume may be added at low confluency to permit an extra day of culture without medium changes. StemFlex and E8 Flex contain components that stabilize the recombinant growth factors present in the medium, permitting medium exchange every other day as a general practice. Some iPSC lines (e.g., WTC11) tolerate every-other-day medium changes of standard E8 medium without loss of pluripotency or cell death, further reducing costs of medium and consumables. Finally, mTeSR1 may promote cell survival in stressful conditions better than E8, especially for finicky iPSC lines, although supplementation with a ROCK inhibitor (RI) is also recommended in such scenarios. Use of standard E8 will be assumed throughout this basic protocol.

Additionally, this protocol uses Matrigel-coated tissue culture plates. Matrigel works well for iPSC culture and has been widely adopted for research applications. However, since Matrigel is derived from murine sarcoma cells, it is not chemically defined and exhibits batch-to-batch variability. Alternative defined coatings include recombinant laminin or vitronectin, although these substrates are typically more costly. Notably, downstream neural differentiation described in these protocols occurs in fully defined conditions (see Basic Protocols 5 to 8), so the choice of iPSC substrate is of minimal scientific importance to all but clinical-grade applications.

EXTERNAL LINK

https://doi.org/10.1002/cpcb.51

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Fernandopulle, M. S., Prestil, R., Grunseich, C., Wang, C., Gan, L., & Ward, M. E. (2018). Transcription-factor mediated differentiation of human iPSCs into neurons. Current Protocols in Cell Biology, e51. doi:https://doi.org/10.1002/cpcb.51

fernandopulle2018.pdf

MATERIALS TEXT

- Matrigel, hESC-qualified (Corning, cat. no. 354277)

Corning® Matrigel® hESC-Qualified Matrix
by Corning
Catalog #: 354277

- DMEM/F12 medium (Gibco, cat. no. 11320033)

DMEM/F-12
by Gibco, ThermoFisher
Catalog #: 11320033

- Human induced pluripotent stem cells (hiPSCs; e.g., WTC11, Corriell Institute, cat. no. GM25256)

hiPSCs
by Coriell Institute
Catalog #: GM25256

- E8 medium (may be user-formulated per Table 1 or purchased pre-formulated as Gibco, cat. no. A1517001; may also be substituted with E8 Flex, Gibco, cat. no. A2858501; StemFlex, Gibco, cat. no. A3349401; or mTeSR1, STEMCELL Technologies, cat. no. 85850; or similar)

DMEM/F-12, HEPES
by Thermo Fisher Scientific
Catalog #: 11330032

L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate
by Sigma Aldrich
Catalog #: A8960
CAS Number: 113170-55-1

Sodium selenite
by Sigma Aldrich
Catalog #: 214485
CAS Number: 10102-18-8

Sodium bicarbonate
by Sigma Aldrich
Catalog #: S3817
CAS Number: 144-55-8

Sodium chloride
by Sigma Aldrich
Catalog #: S7653

Sodium hydroxide
by Sigma
Catalog #: 71463

Hydrochloric acid solution
by Sigma Aldrich
Catalog #: H9892
CAS Number: 7647-01-0

Insulin solution human
by Sigma Aldrich
Catalog #: I9278
CAS Number: 11061-68-0

Recombinant Human TGF-β1 (HEK293 derived)
by peprotech
Catalog #: 100-21

Recombinant Human FGF-basic (154 a.a.)
by peprotech
Catalog #: 100-18B

holo-Transferrin human
by Sigma Aldrich
Catalog #: T0665
CAS Number: 11096-37-0

E8 may be made as described here and filter-sterilized before use (can be formulated in bulk and stored at -80°C) or may be purchased commercially.

Component	Amount
DMEM/F12 with HEPES	500 ml
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	32 mg (per 500 ml)
Sodium selenite (dissolve 0.1 mg/ml in PBS; handle in fume hood)	7 µg (70 µl) (per 500 ml)
Sodium bicarbonate	271.5 mg (per 500 ml)
Sodium chloride	As needed to adjust osmolarity to 340 mOsm
Sodium hydroxide (1 M)	As needed to adjust pH to 7.4
Hydrochloric acid (1 M)	As needed to adjust pH to 7.4
Aliquot and add fresh to each bottle:	
Insulin (supplied at 1000×, store at 4°C)	500 µl (per 500 ml)
TGF-β1 (2 µg/ml in PBS; 1000×, store at −80°C)	500 µl (per 500 ml)
FGF-basic (100 µg/ml in PBS; 1000×, store at −80°C)	500 µl (per 500 ml)
Holo-transferrin (10.7 mg/ml in PBS; 1000×, store at −80°C)	500 µl (per 500 ml)

Table 1: Essential 8 Medium

- 70% ethanol
- Rho-associated protein kinase (ROCK) inhibitor Y-27632 (e.g., Tocris Bioscience, cat. no. 1254 or Selleck Chemicals, cat. no. S1049), reconstituted to 10 mM in PBS

Y-27632 dihydrochloride
by Tocris
Catalog #: 1254
CAS Number: 129830-38-2

- Phosphate-buffered saline (PBS) without calcium or magnesium (e.g., Gibco, cat. no. 10010049)

Phosphate-buffered saline (PBS) without calcium or magnesium
by Gibco - Thermo Fischer
Catalog #: 10010049

- 0.5 mM EDTA in PBS (diluted from Gibco, cat. no. AM9260G or Sigma, cat. no. E6758; may also be purchased as Versene, Gibco, cat. no. 15040066)

EDTA (0.5 M, pH 8.0, nuclease-free)
by Thermo Fisher Scientific
Catalog #: AM9260G

- Accutase (Gibco, cat. no. A1110501 or StemCell Technologies, cat. no. 07920)

StemPro™ Accutase™ Cell Dissociation Reagent
by Thermo Fisher Scientific
Catalog #: A1110501

ACCUTASE™
by Stemcell Technologies
Catalog #: 07922

- Dimethylsulfoxide (DMSO; Sigma, cat. no. 472301)

DMSO
by Sigma Aldrich
Catalog #: 472301

- Fetal bovine serum (FBS), qualified, heat inactivated (Gibco, cat. no. 16140071)

Fetal Bovine Serum, qualified, heat inactivated, United States
by Thermo Fisher Scientific
Catalog #: 16140071

- Liquid nitrogen
- CoolRack M30 (BioCision BCS-108)

CoolRack® M30
Sample Cooling Rack
biocision BCS-108

- P2, P20, P200, and P1000 micropipettors and tips (e.g., Gilson)
- Sterile 5, 10, and 25-ml serological pipets (e.g., Corning, cat. no. 356543, 356551, and 357535, respectively)

Falcon® 10 mL Serological Pipet
Serological Pipet
Falcon 356551

Falcon® 25 mL Serological Pipet
Serological Pipet
Falcon 357535

- Sterile 15- and 50-ml polypropylene conical tubes (e.g., Corning, cat. nos. 352096 and 352070, respectively)

Falcon® 15 mL High Clarity PP Centrifuge Tube
Centrifuge Tube
Falcon 352096

Falcon® 50 mL High Clarity PP Centrifuge Tube
Centrifuge Tube
Falcon 352070

- Sterile polystyrene 10-cm tissue-culture dishes and 6-well, 12-well, and 24-well plates (e.g., Corning, cat. no. 353003, 353046, 353043, and 353047, respectively)

Falcon® 100 mm TC-treated Cell Culture Dish
Cell Culture Dish
Falcon 353003

Falcon® 6-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate
Cell Culture Plate
Falcon 353046

Falcon® 12-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate
Cell Culture Plate
Falcon 353043

Falcon® 24-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate
Cell Culture Plate
Falcon 353047

- Laminar flow biological safety cabinet (BSC)
- Vacuum aspirator and aspirating pipets (Fisher, cat. no. 1367820) or reusable Corning vacuum aspirator (cat. no. 4930) with disposable tips (e.g., Pure XLG pipet tips, Andwin Scientific, cat. no. 46600-020)

Fisherbrand™ Disposable Borosilicate Glass Pasteur Pipets
Pasteur Pipet
Fisherbrand 1367820

Corning® Vacuum Aspirator
Vacuum Aspirator
Corning 4930

MBP PURE XLG 200 UL PIPET TIP
Pipet Tip
Andwin Scientific 46600-020

- Phase-contrast and fluorescent microscope with 4×, 10×, 20×, and Object Marker objectives (e.g., Nikon Eclipse Ti)
- Cell counting apparatus [hemacytometer or automated cell counter; also see Phelan & May (2015)]
- 50-ml, 250-ml, and 500-ml sterile filters, 0.2-µm pore (Millipore, cat. no. SCGP00525; Thermo, cat. no. 568-0020 and cat. no. 566-0020, respectively)

Steriflip-GP
Sterile Centrifuge Tube Top Filter Unit
Millipore SCGP00525

Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with PES Membrane
Filter
Thermo Scientific™ 568-0020

Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with PES Membrane
Filter
Thermo Scientific™ 566-0020

- 1.5-ml cryogenic tubes (Thermo, cat. no. 5000-1020)

Nalgene™ General Long-Term Storage Cryogenic Tubes
Storage Tubes
Thermo Scientific™ 5000-1020

- Microcentrifuge for 1.5-ml tubes
- Cryovial freezing container (e.g., CoolCell LX, BioCision, cat. no. BCS-405; or Mr. Frosty, Thermo, cat. no. 5100-0001)

Cell freezing container
Freezing Container
CoolCell LX BCS-405

Mr. Frosty Freezing Container
freezing container
ThermoFisher Scientific 5100-0001

- Picking microscope inside sterile laminar flow enclosure (e.g., Etaluma LS620)

SAFETY WARNINGS

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

Matrigel Coating

1 Aliquotting concentrated Matrigel:

Matrigel polymerizes rapidly at room temperature when concentrated, so it is imperative to aliquot stocks with pre-chilled tips and tubes and to thaw the concentrated stock solution on ice.

1.1 Gradually thaw a **5 ml** bottle of Matrigel stock solution overnight at **0 °C** by burying in ice in a Styrofoam container placed within a refrigerator. Additionally, pre-chill microcentrifuge tubes by placing in an aluminum cool rack on ice before use.

1.2 Before pipetting concentrated Matrigel into pre-chilled microcentrifuge tubes, chill a 1-ml pipet tip by pipetting ice-cold DMEM/F12 up and down several times, then immediately use this chilled tip to aliquot the Matrigel stock.

1.3 Aliquot **500 µl** concentrated Matrigel into each microcentrifuge tube, and refreeze aliquots at **-80 °C** .

2 Making Matrigel coating solution:

2.1 Aliquot **50 ml** of cold DMEM/F12 into a conical tube.

2.2 Using a P1000, pipet **1 ml** of cold DMEM/F12 from the conical tube into the microcentrifuge tube containing **500 µl** concentrated Matrigel stock. Pipet up and down several times, and then transfer what has thawed to the conical tube containing cold DMEM/F12.

2.3 Repeat until the frozen concentrated Matrigel has been completely transferred to the 50-ml conical tube containing DMEM/F12. Invert several times to mix.

3 Add one half of the normal culture volume of the Matrigel coating solution to the tissue culture surface.
Gently agitate plates to ensure full coverage.

For example, add **1 ml** per well of a 6-well plate.

4 Transfer plates to a **37 °C** incubator.

Plates may be used after 1 hr, but better long-term morphology typically results from overnight coating. It is also possible to prepare plates in bulk by adding additional DMEM/F12 to a full culture volume in order to prevent wells from drying. These plates may be stored in a **37 °C** incubator or wrapped in Saran wrap or Parafilm and stored at **4 °C** . Plates should be used within 2 weeks of preparation.

5 Aspirate Matrigel solution immediately before use and replace with culture medium and cells.

Thawing iPSCs

6

Often, iPSCs are stored and distributed as frozen stocks, so thawing is the first procedure performed. Routine use of antibiotics in stem cell culture medium is strongly discouraged, since these compounds can interfere with cell biochemistry and differentiation potential.
Consequently, proper sterile technique is critical to prevent contamination, and cells received from other environments should be quarantined for at least two passages and tested for mycoplasma.

Prepare biological safety cabinet (BSC) with tube racks, DMEM/F12, P1000 tips, conical tubes, and culture medium.

DMSO is toxic to cells at room temperature, so steps 2 to 5 should be completed as quickly as possible.

7 Transfer cryovial of hiPSCs from liquid nitrogen or dry ice and thaw in **37 °C** water bath or bead bath.

Thaw should be completed rapidly to limit exposure to DMSO. A small frozen core may remain, as pipetting and rinsing will complete thaw.

8 Sterilize cryovial by spraying with or dipping into 70 % ethanol and transfer into the BSC.

9 Pipet cell solution to new 15-ml conical tube, rinse cryovial twice with **1 ml** DMEM/F12, and add each rinse to the tube.

10 Centrifuge tube **00:05:00** at **300 x g** , **Room temperature** .

Speeds of 200 to 300 × g are well tolerated by iPSCs. For the purposes of this protocol, 300 × g is recommended to maximize capture of small cell numbers. For standard procedures, 200 × g is recommended.

11 Aspirate supernatant, resuspend in culture medium supplemented with **10 Micromolar (µM)** Y-27632 ROCK inhibitor, and transfer to Matrigel-coated plate.

Maintaining high cell density maximizes survival, so it is recommended to plate each vial (typically 1 × 106 cells) in one well of a 6-well plate. This may be modified depending on specific cell number or viability.

12 Return plate to **37 °C** incubator and evenly distribute cells by gently shaking platefront-to-back and side-to-side.

This procedure is critical any time cells are replated, and should be performed as soon as cells are transferred. Swirling or otherwise agitating culture medium before cells attach can cause higher cell densities in the middle of the well.

13 The next day, aspirate the medium and replace with fresh E8 culture medium (2 ml/well for 6-well dish). If colonies are small and/or if cell death is noted after the medium change, use of E8 with Y-27632 ROCK inhibitor may be necessary until colonies have expanded, after which inclusion of the ROCK inhibitor is not required.

Splitting

14

An advantage of using iPSCs as a model system is their rapid rate of proliferation; however, culture health is superior if cultures are only split to modestly low densities. Furthermore, cells will rapidly differentiate and die if allowed to grow into a monolayer. When colonies grow too large and/or begin to converge (approximately 80% confluency), they must be dissociated in order to maintain proper growth and pluripotency. Dissociating cells with EDTA is gentler and faster, and typically results in improved survival over enzymatic dissociation methods, making it ideal for general culture maintenance. EDTA acts by chelating the calcium necessary for cell attachment and transfers cells as small clumps, which promotes colony formation and growth. Alternatively, Accutase provides gentle enzymatic dissociation for iPSCs and should be used for any applications that require single-cell suspensions, such as for clonal derivation, cell counting, or flow sorting. Supplementation of culture medium with ROCK inhibitor is optional following EDTA treatment, but is required after Accutase treatment to prevent apoptosis of single cells. Additionally, cultures of stem cells can often spontaneously differentiate, particularly after overgrowth or stressful procedures. Isolated loci of differentiated cells may be removed directly by aspirating areas of the well, and this is particularly effective during EDTA splitting. For highly differentiated cultures, however, several passages are often required to regain a healthy pluripotent population. Plating at high density following EDTA splitting (1:3 ratio) can promote iPSCs to outcompete differentiated cells. Alternatively, a modified version of the EDTA split is also included below to remove spontaneously differentiated cells, which takes advantage of higher adhesion of differentiated cells compared to iPSCs.

step case

EDTA

15 Aspirate culture medium and rinse with one-half culture volume of PBS.

Since EDTA works by chelating the calcium ions necessary for iPSC attachment, be sure the PBS does not contain calcium.

16 Aspirate PBS and add one-half culture volume of EDTA solution ([M] **0.5 Milimolar (mM)** in PBS).

17 Incubate for **00:05:00** to **00:10:00** at **Room temperature** .

Exact timing varies by cell line, but 7 min is typical for hiPSCs. Cell colonies should be opaque to the naked eye, and colony edges should be just starting to detach when viewed under a microscope. Single-cell dissociation may be achieved by incubating for up to 15 min, and time may be reduced by incubating plates at **37 °C** .

If colonies begin sloughing off, collect EDTA and cells in a conical tube, rinse with culture medium and add to the tube, centrifuge **00:05:00** at **200 x g** , aspirate supernatant, resuspend in culture medium supplemented with [M] **10 Micromolar (µM)** ROCK inhibitor, and plate cells.

Reduce EDTA incubation time in future passages.

18 While incubating, prepare recipient Matrigel-coated plates by aspirating Matrigel solution and loading with three-quarters volume of culture medium.

For example, **1.5 ml** medium/well of a 6-well dish. Supplementation of **5 Micromolar (µM)** ROCK inhibitor is optional to improve survival.

19 Aspirate EDTA solution, taking care not to disturb cell colonies, which should remain attached.

Small-scale differentiation may be removed at this step by directly aspirating areas of the well which have noticeable bumps (particularly in themiddle of large colonies) or by designating areas for removal beforehand with an object marker microscope objective.

20 Dissociate cells by pipetting two to three times with culture medium equivalent to half of the denominator of the splitting ratio.

Typical splitting ratios for 6-well plates are between 1:6 and 1:12, for which 3 and 6 ml medium should be used, respectively. Mix well, but avoid pipetting up and down more than three times in order to keep cell clumps intact. If colonies remain attached, dispense medium, gently scrape the bottom of the well with the end of the pipet, and pipet up and down twice to mix. Increase EDTA incubation time in future passages.

21 Add one-fourth of the volume of culture medium with cells to each recipient well, and discard any excess cells.

22 Return plate to **37 °C** incubator and evenly distribute cells by gently shaking platefront-to-back and side-to-side.

Freezing

23

Culturing over many passages may result in mutations and genetic drift, and stressful events can select for abnormal genotypes such as oncogene mutations or chromosomal deletions and rearrangements (Merkle et al., 2017). To circumvent these adverse outcomes and to provide backup in case of contamination, several vials of cells should be cryopreserved immediately after cell line isolation. Additionally, freezing cell clones during validation reduces reagent use, and expanding cultures and freezing a large, pooled batch of cells on the same date with the same passage number provides useful downstream reproducibility.

Prepare cells as for an EDTA split (see above). During EDTA incubation, label cryovials and formulate cryopreservation medium by combining culture medium with 10 % DMSO.

Optionally, the addition of 20 % fetal bovine serum or knockout serum replacement to cryopreservation medium may improve viability. 90 % fetal bovine serum/10 % DMSO solutions (i.e., no culture medium) have also been used successfully. Note that if doxycycline-inducible promoters are integrated into the iPSC lines, we recommend the use of validated, tetracycline-free FBS for such purposes (e.g., Tet System Approved FBS from Clontech). Cryopreservation medium may also be purchased commercially (e.g., CryoStor CS10, STEMCELL Technologies cat. no. 07930).

24 Aspirate EDTA and dissociate cells with cryopreservation medium.

It is optional to dissociate with culture medium and count cells (Phelan & May, 2015) in order to ensure that standard cell numbers are frozen across samples, typically 1 × 106 cells/vial. This is followed by centrifugation, aspiration of culture medium, and resuspension in an appropriate volume of cryopreservation medium. For routine applications, however, **1 ml** to **2 ml** cryopreservation medium per well of a 6-well plate at approximately 80 % confluency provides adequate cell density for a healthy thaw. Steps 3 to 4 should be completed as quickly as possible to reduce DMSO exposure.

25 Transfer **1 ml** cryopreservation cell suspension to each 1.5 ml cryovial and freeze in a CoolCell freezing container or Mr. Frosty isopropanol caddy at **-80 °C** for **02:00:00** to overnight.

Gradually reducing temperature by 1°C/minute improves viability.

26 Transfer cryovials to liquid nitrogen for long-term storage.

Cryovials should be placed on dry ice for transit from the freezer to the liquid nitrogen tank to prevent thawing.

Manual manipulation

27

Manual changes in the composition of an iPSC population may be accomplished by either isolating a desired colony into a separate culture (manual passage, or pick-to-keep) or by scraping away undesired cells for aspiration (pick-to-remove). In practice, manual passaging is an important part of the clonal isolation protocol below, and picking-to-remove is most commonly used to remove isolated areas of spontaneously differentiated cells from maintenance cultures or to provide more room for the desired cells to grow prior to picking-to-keep. Proper sterile technique is essential during both of these procedures,
as plates may be uncovered in the biosafety cabinet for extended periods of time.

step case

Pick-to-keep

Manual changes in the composition of an iPSC population accomplished by isolating a desired colony into a separate culture (manual passage, or pick-to-keep).

28 Prepare a recipient 24-well plate by aspirating Matrigel solution and adding 250 µl/well of culture medium supplemented with [M]**10 Micromolar (µM)** Y-27632 ROCK inhibitor.

Place the cell culture dish on a picking microscope in a sterile enclosure.

A microscope (e.g., Lumascope) connected to a screen or tablet computer may be sterilized and kept inside a BSC. Alternatively, a PCR enclosure with a stereoscope that can be sterilized by ethanol and/or UV treatment provides sufficient protection.

29 Remove the cover of the culture dish, center the colony desired to be picked, flush the P1000 tip with medium to avoid cell retention, and align the end of a P1000 pipet tip just above the colony.

Colonies are often pre-selected with a marking objective, which leaves a 1.8 mm ring on the bottom of the culture dish. Colonies approximately the size of the inside of the ring are of ideal size for picking, and a wide-diameter tip is desirable to reduce shear forces on the cells. P200 or smaller tips should not be used. Set the pipet to 250 µl. Balance the tip on your opposite index finger to improve stability and control.

30 Lower the pipet tip until it makes contact with the culture surface.

If necessary, raise the plunger of the pipettor slightly to remove the air bubble for better viewing.

31 Gently and slowly scrape the bottom of the well with the tip, slowly raise the plunger to detach cells in strips, and collect in the pipet tip.

Keep a shallow angle with the plate and avoid pressing down on the plate. Raise the plunger more quickly to provide more force if cells remain stuck or are close to the well wall.

32 Deposit the picked cells (in **250 µl** medium) into the destination well.

If picking has been slow, cells may be stuck to the inside of the tip, so check the well under the microscope before disposing of the tip to ensure that cells are present. Try to avoid pipetting multiple times in the well in order to keep cells clumped. If the clone was not completely picked, the same tip may be reused to acquire more cells; otherwise, change pipet tips between each clone.

Splitting

step case

Spontaneously differentiating cells

EDTA-mediated removal of spontaneously differentiating cells

15 Prepare cells as for an EDTA split.

16 Incubate cells at **Room temperature** and view by phase-contrast microscopy every **00:02:00** .

17 When colony edges begin to detach (typically **00:05:00** to **00:10:00**), gently tap the plate three to five times against your hand until most iPSC colonies are in suspension.

step case

Accutase

15 Aspirate culture medium and rinse with one-half culture volume of PBS.
Aspirate PBS and add up to one-half culture volume of Accutase.

To save reagent, only enough Accutase is required to cover the culture surface (i.e., 0.5 ml/well of a 6-well dish).

16 Transfer to **37 °C** incubator for **00:05:00** , or until most cells have detached.

If colonies remain attached, gently tap the plate against your hand three to five times or extend incubation to at most **00:15:00** in total.

17 While incubating, prepare recipient Matrigel-coated plates by aspirating Matrigel solution and loading with culture medium supplemented with **10 Micromolar (µM)** ROCK inhibitor.

Manual manipulation

step case

Pick-to-remove

Manual changes in the composition of an iPSC population accomplished by scraping away undesired cells for aspiration (pick-to-remove).

Depending on the scale of cells to be removed, picking may be accomplished with a pipet tip (without a pipettor) or with a more specialized cell scraper, either purchased commercially or made from a borosilicate Pasteur pipet (Kent, 2009).

28 Place the cell culture dish on a picking microscope in a sterile biological safety cabinet.

29 Remove the cover of the culture dish, center the cells to be removed, and align the end of the picking implement just above the cells.

Hold the pipet tip near the end or balance the picking implement on your opposite index finger to improve stability and control. Be careful not to hold your hand above the well or to otherwise compromise sterility.

Manual manipulation

step case

Pick-to-keep

Manual changes in the composition of an iPSC population accomplished by isolating a desired colony into a separate culture (manual passage, or pick-to-keep).

NaN Prepare a recipient 24-well plate by aspirating Matrigel solution and adding 250 µl/well of culture medium supplemented with [M]**10 Micromolar (µM)** Y-27632 ROCK inhibitor.

Place the cell culture dish on a picking microscope in a sterile enclosure.

A microscope (e.g., Lumascope) connected to a screen or tablet computer may be sterilized and kept inside a BSC. Alternatively, a PCR enclosure with a stereoscope that can be sterilized by ethanol and/or UV treatment provides sufficient protection.

NaN Remove the cover of the culture dish, center the colony desired to be picked, flush the P1000 tip with medium to avoid cell retention, and align the end of a P1000 pipet tip just above the colony.

Colonies are often pre-selected with a marking objective, which leaves a 1.8 mm ring on the bottom of the culture dish. Colonies approximately the size of the inside of the ring are of ideal size for picking, and a wide-diameter tip is desirable to reduce shear forces on the cells. P200 or smaller tips should not be used. Set the pipet to 250 µl. Balance the tip on your opposite index finger to improve stability and control.

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NaN Place the cell culture dish on a picking microscope in a sterile biological safety cabinet.

NaN Remove the cover of the culture dish, center the cells to be removed, and align the end of the picking implement just above the cells.

Hold the pipet tip near the end or balance the picking implement on your opposite index finger to improve stability and control. Be careful not to hold your hand above the well or to otherwise compromise sterility.