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Protocol for Maintaining iPSC Cultures

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Petronio D. Zalamea[1], Jaime S. Messenger[1], Mia C. Zambrana[1], Ronald Hart[1]

[1]Department of Cell Biology & Neuroscience, Rutgers University, Piscataway, NJ 08854

Ronald P. Hart
Rutgers University

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Abstract

Protocols for passaging, maintaining, freezing, and thawing induced pluripotent stem cells (iPSC) have evolved. This version includes the use of CEPT instead of Y-compound as well as updated media and reagents.

Guidelines

Feeding schedule

Cells are fed with iPS-Brew medium every other day with “double” feeding (1.5x volume) on Friday to avoid weekend feeding. Medium is changed after 24-48 hours once cells are passaged to remove CEPT/polyamines. Use the following volumes of medium for desired cell culture format for normal (not double) feeding:
- 10 cm dish – 10 mL medium per dish
- 6 well plate – 2 mL medium per well
- 12 well plate – 1 mL medium per well
- 24 well plate – 500 µL medium per well
- 96 well plate – 200 µL medium per well

Materials

Reagents for iPS culture:
- StemMACS™ iPS-Brew XF (iPS-Brew, Milltenyi Biotec #130-104-368)
- DMEM (Gibco #11965092)
- Corning® Matrigel® hESC-Qualified Matrix (Corning® #354277)
- DPBS without Ca and Mg (Gibco #14190144)
- Accutase® Cell Detachment Solution (Stemcell Technologies #07922)
- ReLeSR™ (Stemcell Technologies #100-0483)
- CEPT/polyamines (10,000x or 1000x; see protocol)
- MFreSR (Stemcell Technologies #05855)
- 0.4% Trypan Blue Solution (Invitrogen #T10282)
- Countess cell counting chamber slides (Invitrogen #C10283)
- Zymo Quick-RNA Microprep Kit (Zymo #R1051)
- Invitrogen Qubit BR RNA Assay Kit (Invitrogen #Q33223)
- TapeStation High Sensitivity RNA ScreenTape (Agilent #5067-5579)
- TapeStation High Sensitivity RNA ScreenTape Ladder (Agilent #5067-5581)
- TapeStation High Sensitivity RNA ScreenTape Buffer (Agilent #5067-5580)
- Nanodrop spectrophotometer (for RNA concentration measurement)

Additional reagents for CEPT preparation (from pages shown):
- Chroman 1, MedChem Express HY-15392
- Emricasan, SelleckChem S7775
- Polyamine supplement (1000X), Sigma-Aldrich P8483
- Trans-ISRIB, R&D Systems 5284
- Dimethylsulfoxide (DMSO), Life Technologies A1516401

Safety warnings

- All work with human iPSC requires an approved biosafety protocol from your institution's Institutional Biosafety Committee (IBC). Some institutions require human subjects approval from your Institutional Review Board (IRB) and some may required that you file for an exemption.
- Always wear proper personal protective equipment (PPE) including but not limited to, a lab coat, gloves, and eye protection.
- Review Chemical Hygiene and Lab Safety protocols as well as the product's safety data sheets (SDS) before working with potentially hazardous materials.
- All cell culture work needs to be performed inside a certified class II biological safety cabinet, observing biosafety regulations and using sterile techniques.
- Do not let coating dry out.
- Caution: Accutase is inactivated after 45 minutes at 37°C.
- Wash iPSCs with DPBS to fully remove medium before adding dissociative reagent (to avoid interference).
- Osmotic pressure will lyse the cells if the cryoprotectant is diluted out of the cells too fast; add DMEM dropwise while swirling when diluting thawed cells.
- Cells should be about 75-80% confluent before freezing and have minimal cell differentiation.
- If cells are at least 95% iPSC, it's acceptable to continue with the freezing protocol.
- If cells appear to be spontaneously differentiating, manual removal of differentiation or passaging with reLeSR before freezing is recommended.
- If freezing multiple lines, keep cells on ice.
- Storage notes for CEPT stock solutions: store Chroman 1, Emricasan, and trans-ISRIB stocks at 4°C for up to one month or at -20°C for up to 1 year.
- For trans-ISRIB dissolution: gently warm at 45-60°C and vortex until completely dissolved.
- The Zymo Quick-RNA Microprep Kit recommends using less than 10^6 cells for the Microprep protocol.

Before start

Matrigel preparation

1. Thaw frozen 5 mL stock bottle on ice at 4°C overnight.
2. Dilute 1:1 with ice cold DMEM. Aliquot 1 mL stock solutions (x10) and store at −20°C until ready to use.
3. Dilute 1 mL stock solution 1:35 with cold DMEM to prepare 35 mL working stock solution. This may be stored up to 2 weeks at 4°C. Allow coated plate/dish to incubate for at least 1 hour at 37°C before using. Use the following volumes of Matrigel for desired cell culture format:
   a. 10 cm dish - 3 mL Matrigel per dish
   b. 6 well plate – 1 mL Matrigel per well
   c. 12 well plate – 500 µL Matrigel per well
   d. 24 well plate – 250 µL Matrigel per well
   e. 96 well plate – 50 µL Matrigel per well

Preparation of CEPT

Reagents:

- Chroman 1, MedChem Express HY-15392
- Emricasan, SelleckChem S7775
- Polyamine supplement (1000X), Sigma-Aldrich P8483
- Trans-ISRIB, R&D Systems 5284
- Dimethylsulfoxide (DMSO), Life Technologies A1516401

CEPT Stock Solutions

0.5 mM (10,000X stock) Chroman 1.

- Dissolve 5 mg of chroman 1 in 22.91 mL of DMSO. Vortex until completely dissolved.
- Store at 4°C for up to one month or -20°C for up to 1 year.

50 mM (10,000X stock) Emricasan

- Dissolve 5 mg of emricasan in 0.1756 mL of DMSO. Vortex until completely dissolved.
- Store at 4°C for up to one month or -20°C for up to 1 year.

7 mM (10,000X stock) trans-ISRIB

- Dissolve 10 mg of trans-ISRIB in 3.165 mL of DMSO. Gently warm at 45-60°C and vortex until completely dissoved.
- Store at 4°C for up to one month or -20°C for up to 1 year.

Dilute Chroman, Emricasan, and trans_ISRIB at 1:10,000 and polyamine supplement at 1:1,000 into desired medium.

Passaging

1 Dissociate iPSCs from 1 well – should be about 70-80% confluent. Will use the same volumes for Accutase or ReLeSR as listed in Matrigel preparation. For both methods, wash iPSCs with DPBS to fully remove medium before adding the dissociative reagent. Do not let coating dry out.

2 Coat desired wells or cell culture dish with Matrigel working stock solution and incubate at 37°C for at least 1 hour before plating cells. Can coat the day before or on Friday for use on Monday. Aspirate Matrigel before replating cell cultures. Passage using either Accutase or ReLeSR.

3 **Accutase for single cell suspension**, when counting cells or collecting cell pellets for DNA/RNA extractions.

3.1 Use 1X CEPT/polyamines for passaging single cell suspensions. Accutase is stored at 4°C. Before using, let warm to room temperature or can place in water bath to quickly warm. (Caution: Accutase is inactivated after 45 minutes at 37°C)

4 **ReLeSR for standard passaging** (reduces the need for physical removal of differentiated cells)

4.1 Add ReLeSR (same volume as listed for Matrigel) enough to cover cells and let sit 30-60s at room temperature.

4.2 Aspirate ReLeSR. Incubate @37°C for 5-7 min. Will vary based on cell line – typically, will sit in ReLeSR 1 min. and incubate 5 min.

4.3 Add iPS Brew to well.

4.4 Detach colonies according to ReLeSR protocol.

4.5 Break up colonies by triturating carefully to not create a single cell suspension. See pics in ReLeSR protocol.

5 Passage at 1:20-1:40 ratio if using IPS Brew/CEPT/polyamines.

6 Plates/dishes should be clearly labeled with cell line name, passage number (+1 passage number from well taken), date, initials

7 Shake plate well 3 times up/down, 3 times left/right to evenly distribute cells among wells/dish.

8 Use 1X CEPT/polyamines for desired cell culture format/number of wells. Note: if the cell aggregates are too small, the CEPT/polyamines will keep the smaller aggregates from dying. If the aggregates are large enough, they will survive without the CEPT/polyamines. The morphology of the colonies will be different in the presence of the CEPT/polyamines. Change medium 24-48 hours later with no CEPT/polyamines.

9 Depending on colony size and confluency, iPSCs may need to be passaged after 5-7 days. When planning neural induction, I let the wells grow to over 90% confluency at day 7. For maintaining undifferentiated cells, I also have 2 wells in the same plate at about 70% at 5-7 days which I use to passage. Some cell lines seem to balance on a knife's edge to have enough for a neural induction experiment and to have cells in good condition for passaging.

Freezing

10 Wash desired wells or dish with DPBS. Use the same volumes as the feeding schedule.

11 Add Accutase (same volume as used for Matrigel) and incubate 37°C for 3-5 minutes or until cells can be seen detaching when viewed under the microscope. Can tap the sides of the plate/dish to help dissociate cells stuck to the edges. (will typically take about 5 minutes for a normal iPSC line)

12 Add equal volume of medium to the Accutase/cell mixture.

13 Break up colonies by triturating thoroughly with P1000 to create a single cell suspension and add to a prelabeled 15 mL conical tube.

14 If cells will not go into single cell suspension easily, 30 µm cell strainers may be used.

15 If the cell suspension appears very turbid, it may be diluted up to 10 mL with DMEM for counting. Mix cell solution well – inverting/pipette mixing/swirling.

16 Take 10 µL of cell solution and add to 10 µL 0.4% Trypan Blue Solution in a microcentrifuge tube. Mix very well by pipette mixing - 15-20x. Add 10 µL of the cell solution/Trypan Blue mix to each side of the Countess slide and obtain viability and cell

count per mL – may average two counts. If the numbers are more than 15% different, repeat the count, making sure there are no clumps and the cells are well mixed.

17 Determine the total amount of cells and how many vials to be frozen (typically 1-2 million per vial) and calculate how much of the cell mix to add to a new, labeled 15 mL conical tube. Bring volume up to 10 mL with DMEM.

18 Centrifuge 180 x g for 3 min.

19 Carefully aspirate supernatant and resuspend cell pellet in cold mFreSR – enough for 1 mL freezing solution per vial.

20 If freezing multiple lines, keep cells on ice.

21 Aliquot cells in mFreSR to labeled cryovials (cell line name, passage number-should be same as passage on plate, cell type, cell count, date, initials), store in slow freezing container like Mr. Frosty, place in -80°C freezer overnight, and then move to liquid nitrogen tank for long term storage (update LabCollector database with above information and box position location).

Preparation of RNA for QC

22 Harvest cells with Accutase and obtain a cell pellet between 100,000 and 1,000,000 cells. Wash with DPBS to remove medium before extracting RNA.

23 Use Zymo Quick-RNA Microprep Kit and follow the (III) Total RNA Purification protocol, along with the recommended DNase I treatment. (For more than 1,000,000 cells, Zymo Quick-RNA Miniprep Kit would be necessary.)

24 Use the Nanodrop to obtain the RNA concentration – 1 µL of eluted RNA sample or dilute 1:100 and use the Qubit RNA BR Assay Kit.

25 Dilute RNA 1:5 with TapeStation High Sensitivity RNA ScreenTape Buffer and run on TapeStation with TapeStation High Sensitivity RNA ScreenTape Ladder diluted 1:1 with High Sensitivity RNA ScreenTape Buffer to obtain average fragment lengths.

26 Submit for bulk RNAseq. Note: The Microprep kit recommends less than 10^6 cells.

Protocol references

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