Generating a Cost-Effective, Weekend-Free Chemically Defined Human Induced Pluripotent Stem Cell (hiPSC) Culture Medium

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We have previously developed a cost-effective chemically defined medium formula for weekend-free culture of human induced pluripotent stem cells (hiPSCs), costing ~3% of the price of commercial medium. This medium, which we termed B8, is specifically optimized for robust and fast growth of hiPSCs and for a weekend-free medium change regimen. We demonstrated that this medium is suitable for reprogramming of somatic cells into hiPSCs and for differentiation into a variety of lineages. Here, we provide a protocol for simple generation of the most cost-effective variant of this medium, along with a protocol for making Matrigel-coated plates and culturing, passaging, cryopreserving, and thawing hiPSCs. © 2020 Wiley Periodicals LLC.

Basic Protocol 1: Preparation of a highly optimized, robust, and cost-effective human induced pluripotent stem cell culture medium
Basic Protocol 2: Weekend-free maintenance and passaging of human induced pluripotent stem cells in B8 medium

Keywords: chemically defined • cost-effective • culture medium • human induced pluripotent stem cells • weekend-free

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INTRODUCTION

Human induced pluripotent stem cells (hiPSCs), with their unlimited expansion and differentiation potential, serve as a unique source of cells for disease modeling, precision medicine, drug assays, and pharmacogenomics. All these aforementioned applications require representation of the variability existing in the human population, and therefore, cohorts with a large number of hiPSC lines are required. The traditional approaches to culturing hiPSCs using mouse embryonic fibroblasts (MEFs) as a feeder layer and medium containing knockout serum replacement (KSR) result in variable outcomes that lack the consistency required for high-throughput assays (Dakhore, Nayer,

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& Hasegawa, 2018). To resolve this issue, efforts were made to generate more defined culture conditions. MEFs were replaced by extracellular matrices such as Matrigel, and complex medium components like KSR were replaced with defined components, such as in the TeSR formula, which is a mixture of 19 components (see Current Protocols article; Ludwig & Thomson, 2007; Ludwig, Bergendahl, et al., 2006; Ludwig, Levenstein, et al., 2006). Further efforts then led to generation of the first robust chemically defined medium, named E8, with only eight components (Beers et al., 2012; Chen et al., 2011). Recently, we developed a new, cost-effective chemically defined culture medium called B8 that supports the high growth rate of hiPSCs, yet with minimal cost (Kuo et al., 2020). Cells grown in this medium maintain the normal expression pattern of undifferentiated cell markers. Moreover, no chromosomal abnormality is observed after long-term culture of the cells in B8 medium, as shown by normal G-banding karyotype analysis (Kuo et al., 2020). In this article, we present a detailed guide for the generation of B8 medium (Basic Protocol 1). We also describe approaches for the preparation of Matrigel-coated plates and for passaging, cryopreservation, and thawing of hiPSCs in B8 medium (Basic Protocol 2).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly. To this end, a Class II, Type A2, or better biosafety cabinet, such as Labconco Purifier Cell Logic+, should be used.

BASIC PROTOCOL 1

PREPARATION OF A HIGHLY OPTIMIZED, ROBUST, AND COST-EFFECTIVE HUMAN INDUCED PLURIPOTENT STEM CELL CULTURE MEDIUM

In this approach, hiPSCs are grown in chemically defined B8 medium (Kuo et al., 2020). In B8, each component has been individually optimized for fast growth and reduced costs, with more L-ascorbic acid 2-phosphate; less transferrin and insulin; no additional sodium bicarbonate; in-house-generated, codon-optimized fibroblast growth factor 2-G3 (FGF2-G3) with multiple point mutations to improve thermostability (Chen, Gulbranson, Yu, Hou, & Thomson, 2012); replacement of transforming growth factor beta-1 (TGFß1) with TGFß3; and addition of neuregulin 1 (NRG1). The plasmids for generating these growth factors are available via Addgene. The specific variant of B8 described here has lower levels of insulin and transferrin, as with the optimizations described, these have become the most expensive components, and lowering their concentrations has only a minimal negative effect on hiPSC proliferation. In this variant, we also use commercial recombinant TGFß3 and NRG1, as these components are used at very low concentrations and the effort required to make them is not cost efficient. See Table 1 for the components of B8 medium and their concentrations.

Table 1 Components of B8 Medium and Their Concentrations

Reagent	Concentration
DMEM/F12	
L-Ascorbic acid 2-phosphate	200 µg/ml
Insulin	5 µg/ml
Transferrin	5 µg/ml
Sodium selenite	20 ng/ml
Fibroblast growth factor 2-G3 (FGF2-G3)	40 ng/ml
Neuregulin 1 (NRG1)	0.1 ng/ml
Transforming growth factor beta-3 (TGFß3)	0.1 ng/ml

Materials

Cell-culture water (Corning, 25-055-CV)
L-ascorbic acid 2-phosphate trisodium salt (Wako, 321-44823)
Insulin, recombinant human (Life Technologies, A11382IJ; store at −20°C)
1 N hydrochloric acid (HCl; Sigma, H9892)
1 N sodium hydroxide (NaOH; Sigma, S2770)
Recombinant human transferrin (Optiferrin, InVitria, 777TRF029-10G; store at −20°C)
2 mg/ml sodium selenite (see recipe)
4 mg/ml FGF2-G3, recombinant human (made by Recombinant Protein Production Core at Northwestern University, commonly resulting in 80 mg per 1 L of *Escherichia coli* at ∼5 mg/ml; store at −20°C)
250 µg/ml TGFß3, recombinant human (Cell Guidance Systems, GFH109; store at 4°C)
100 µg/ml NRG1 (see recipe)
DMEM/F12 medium (Corning, 10-092-CM; store at 4°C)

50-ml conical tubes (polystyrene; Corning Falcon, 352098)
Analytical balance (e.g., Mettler Toledo ML204)
pH meter (e.g., Mettler Toledo SevenCompact S220)
150-ml PES 0.2-µm bottle-top filters (Corning, 431153)
Sterile 1.7-ml microtubes (Axygen, MCT-175-C-S)

Generating B8 supplements

Tube A

1. Add 47 ml cell-culture water to a 50-ml conical tube.

   Here and below, all the mentioned values are for 100 L B8 medium. Smaller volumes could be obtained by reducing the weights/volumes of the components accordingly.

2. Weigh out 20 g L-ascorbic acid 2-phosphate trisodium salt.
3. Slowly add L-ascorbic acid 2-phosphate to water.
4. Mix by inverting until solution becomes clear.

Tube B

5. Add 49 ml cell-culture water to a 50-ml conical tube.
6. Add 0.5 g insulin.

   Insulin dissolves in acidic solutions (∼pH 3).

7. Using a pH meter, measure pH of the solution.
8. Slowly add 1 N HCl until pH reaches 3.

   Roughly 400 µl of 1 N HCl is needed.

9. Mix by inverting until solution becomes clear.
10. Slowly add 1 N NaOH until pH reaches 7.1.

    Roughly 500 µl of 1 N NaOH is needed.

    With the increase in pH, the solution becomes cloudy again.

11. Mix by inverting until solution becomes clear.

Tube C

12. Add 47 ml cell-culture water to a 50-ml conical tube.

13. Add 0.5 g recombinant human transferrin.
14. Add 1 ml of 2 mg/ml sodium selenite.
15. Add 1 ml of 4 mg/ml FGF2-G3.
16. Add 40 µl of 250 µg/ml TGFß3.
17. Add 100 µl of 100 µg/ml NRG1.
18. Mix by inverting until solution becomes clear.

Combining B8 supplements

19. In a cell culture hood, transfer contents of Tubes A, B, and C to the top of a 150-ml PES 0.2-µm bottle-top filter. Filter sterilize solution.
20. Mix by inverting until a homogenous solution is achieved.
21. Make 100 1.5-ml aliquots in sterile 1.7-ml microtubes.
22. Store aliquots ≤6 months at −20°C.

Preparing B8 medium

23. Thaw one aliquot of B8 supplements.
24. Add aliquot to 1 L cold DMEM/F12 medium aseptically.
25. Invert bottle to mix the reagents.
26. Store ≤4 weeks at 4°C and avoid exposure to light.

BASIC PROTOCOL 2

WEEKEND-FREE MAINTENANCE AND PASSAGING OF HUMAN INDUCED PLURIPOTENT STEM CELLS IN B8 MEDIUM

hiPSCs are grown on low-concentration (1:800) Matrigel, which we have optimized for cost effectiveness without sacrificing cell growth. We have shown that a dilution of 1:1000 is suitable for hiPSC culture, yet 1:800 is chosen to allow for variation in supplier concentrations. We have previously shown that Matrigel performs similarly to Geltrex or Cultrex at the same dilutions, irrelevant of the supplier's stated concentration (Kuo et al., 2020).

We recommend that cells are cultured in B8 using a 3.5-day splitting schedule at a 1:20 split ratio. This schedule avoids medium changes on weekends and reduces labor by ∼50% while maintaining differentiation capacity (Figure 1). We grow cells to about 70% to 80% confluence; critically, we avoid overgrowing cells (>90% confluence), which results in cells adopting a compact morphology and becoming contact inhibited, resulting in slower growth for subsequent passages and in poor differentiation efficiency.

Cells are passaged non-enzymatically using 0.5 mM EDTA in $DPBS^{-/-}$ to maintain small clumps of cells, rather than resulting in single cells, as is typically achieved using

AM Split 1:20	AM feed		PM Split 1:20	PM feed		
B8T	B8	no Δ	B8T	B8	no Δ	no Δ
Mon	Tue	Wed	Thu	Fri	Sat	Sun

Figure 1 Weekend-free hiPSC culture schedule, reflecting time course of pluripotent growth and indicating type of culture medium. Δ, medium change.

TrypLE. For 24 hr after splitting, B8 is supplemented with a Rho-associated protein kinase inhibitor (thiazovivin), forming B8T, to improve cell survival and split ratio reliability.

Materials

Growth factor–reduced Matrigel (Matrigel Reduced Growth Factor Basement Membrane Matrix, Corning, 354230)
DMEM, with L-glutamine and 4.5 g/L d-glucose (Corning, 10-017-CV), 4°C
B8T medium (see recipe), 4°C
hiPSCs (store in liquid nitrogen)
B8 medium (see Basic Protocol 1), 4°C
0.5 mM EDTA (see recipe)
Freezing solution (see recipe)

6-well tissue culture–treated plates (Greiner, 657165)
37°C water bath
15-ml conical tubes (polystyrene; Corning Falcon, 352097)
Standard tabletop centrifuge (Thermo Sorvall ST40)
Light microscope (e.g., Nikon Eclipse Ts2)
Cryovials (Greiner, 122261)
Cell-freezing container (CoolCell LX, Corning, 432002)

NOTE: All culture incubations are performed in a 37°C, 5% CO_2 incubator (preferably with 5% O_2; e.g., Thermo Scientific Heracell VIOS 160i) unless otherwise specified.

Preparing Matrigel-coated plates

1. Thaw a bottle of growth factor–reduced Matrigel overnight at 4°C.

   Matrigel can be stored at 4°C while being used. There is no need to aliquot or leave on ice if the bottle is kept at room temperature for >1 min. The bottle of thawed Matrigel will be stable at 4°C for ≥6 months, assuming that it is placed in a high-performance refrigerator that maintains a constant temperature, such as a Thermo TSG or TSX sliding-door laboratory refrigerator.

2. Set out 42 6-well plates in a cell culture hood.

3. Have a pipettor set to 625 µl with a P1000 tip ready.

4. Briefly take Matrigel stock out and add 625 µl Matrigel to 500 ml cold DMEM (1:800 dilution).

   While transporting the Matrigel stock, avoid keeping the bottle in your hand, as the heat from your hand could cause the Matrigel to gel.

   Matrigel is supplied at 8 to 12 mg/ml (see product insert); any concentration in this range is suitable for use at 1:800.

5. Return Matrigel bottle to 4°C very quickly to prevent gelling.

   Try to minimize the time that the Matrigel is outside the refrigerator. To keep Matrigel outside the refrigerator for >1 min, put it on ice.

6. Mix Matrigel/DMEM solution by inverting.

7. Add 2 ml solution per well of each 6-well plate.

   Addition of 2 ml Matrigel solution per well (rather than 1 ml) helps with long-term storage of coated plates in an incubator at 37°C (see step 8).

8. Place plates in an incubator at 37°C for ≥1 hr before use.

Plates may be stored in the incubator for up to 4 weeks without risk of the wells drying out and being unusable. Do not use the Matrigel-coated plates if they have dried out. Check the Matrigel plates frequently for significant reduction in the volume of the medium and top them up with 1 ml DMEM per well if necessary.

Thawing and initial plating of hiPSCs

9. Take one Matrigel-coated 6-well plate out of 37°C incubator. Aspirate Matrigel from three wells and add 2 ml B8T medium to each well.

   All the volumes here and below are for one well of a 6-well plate. For other types of plates, the volumes need to be changed accordingly.

10. Remove a vial of hiPSCs from liquid nitrogen and place it in a 37°C water bath immediately.

11. Warm cells until only a sliver of ice remains.

12. Fill a 10-ml pipet with B8T and use this to remove contents of the vial. Transfer ~5 ml to a 15-ml conical tube, wash out vial with ~1 ml medium, and transfer remaining medium to the conical tube.

    Addition of thiazovivin to B8 medium (see Basic Protocol 1), generating B8T, improves cell survival after dissociation, which enhances the consistency of plating.

    There is no need to warm up the medium to 37°C. No negative effect on the growth of the cells has been observed using 4°C medium in our hands.

13. Centrifuge 3 min at 200 × *g*. Aspirate supernatant.

14. Resuspend pellet in 2 ml B8T and transfer to the three medium-containing wells of a Matrigel-coated 6-well plate (see step 9) at a 1:2, 1:3, or 1:6 ratio.

    For the 1:2 ratio, add 1 ml of the cell suspension to one well of the 6-well plate; for the 1:3 ratio, add 670 μl; and for the 1:6 ratio, add 330 μl.

    Ideally, the cells should reach 70% to 80% confluence in 3.5 days. To achieve this, the cells may need to be plated at split ratios of 1:12, 1:15, 1:20, and 1:24 and assessed to see which ratio results in suitable confluence in 3.5 days. This can be repeated for multiple passages until the cells have adapted to a ≥1:20 split, which is the most efficient for differentiation.

15. Return plates to the incubator. Replace medium with B8 medium every 24 hr until wells become about 70% to 80% confluent, as assessed using a light microscope.

    We grow hiPSCs in 5% O_2 (hypoxic) incubators. Although 5% O_2 is not essential for the success of this protocol, it has been demonstrated to improve genome stability and maintenance of the pluripotent state.

    It is essential that the number of days of culture is kept consistent. In this protocol, the aim is to keep the pluripotent cells in the logarithmic growth phase. Cells should not be allowed to become >90% confluent (i.e., 90% of the culture surface would be covered with cells), which results in the cells becoming contact inhibited, in turn resulting in a slow lag phase of growth after passaging.

Passaging of hiPSCs with EDTA

16. Aspirate culture medium from cells at 70% to 80% confluence.

17. Add 1 ml of 0.5 mM EDTA per well and incubate for 6 min at room temperature in hood.

    All the volumes here and below are for one well of a 6-well plate. For other types of plates, the volumes need to be changed accordingly.

The length of incubation time may need to be optimized from ~3-8 min. Enough time should be used so that all cells in the well come off easily after ~5 times pipetting but not so long that cells begin to float freely.

18. Meanwhile, aspirate Matrigel from two new Matrigel-coated 6-well plates from step 8 and add 1 ml B8T per well.
19. Aspirate EDTA from the wells from step 17.
20. With a P1000 tip, add 1 ml B8T to each well, blasting the medium against the plate surface to dissociate the cells.

    The cells should come off easily after about five rounds of pipetting. Avoid pipetting cells too much, as it can decrease cell survival after passaging.

21. Top up wells with B8T according to the desired split ratio.

    For a 1:12 split ratio, top up the wells with B8T to 12 ml total. For 1:15, top up the wells to 15 ml. For 1:20, remove 250 μl and then top up the wells to 15 ml total. For 1:24, remove 400 μl and then top up the wells to 15 ml.

22. Plate cells at 1 ml per well into the two new Matrigel-coated 6-well plates from step 18, with a final volume of 2 ml per well.

Weekend-free culture of hiPSCs

23. On Monday morning, passage cells at 70% to 80% confluence at a 1:20 ratio in B8T.

    The ratio mentioned here could be optimized for the particular cell line in order to have cells ready for passage after 3.5 days.

24. On Tuesday morning, change medium to B8.
25. On Wednesday, skip medium change.
26. On Thursday afternoon, split cells at a 1:20 ratio in B8T.

    The ratio mentioned here should be optimized for the particular cell line in order to have cells ready for passage after 3.5 days.

27. On Friday afternoon, change medium to B8.
28. On Saturday and Sunday, skip medium changes.
29. On Monday morning, again passage cells at a 1:20 ratio in B8T.

Freezing of hiPSCs

30. Aspirate culture medium from cells at 70% to 80% confluence.
31. Add 1 ml of 0.5 mM EDTA per well and incubate for 6 min at room temperature in hood.

    All the volumes here and below are for one well of a 6-well plate. For other types of plates, the volumes need to be changed accordingly.

32. Aspirate EDTA from the wells.
33. With a P1000 tip, add 1 ml B8T medium to each well, blasting the medium against the plate surface to dissociate the cells. Transfer cells to a 15-ml conical tube.

    The cells should come off easily after about five rounds of pipetting. Avoid pipetting cells too much, as it can decrease cell survival after passaging.

34. Centrifuge 3 min at 200 × *g* and aspirate supernatant.
35. Resuspend one well of cells in 1 ml freezing solution and transfer to a cryovial.

36. Place cryovials in a cell-freezing container at −80°C.

After addition of the freezing solution to the cells, move the cells to the −80°C freezer quickly. Keeping the cells in freezing solution for a long time at room temperature decreases the viability of the cells.

It is important to reduce the temperature of the cells slowly; therefore, the reason that a cell-freezing container, such as a CoolCell, is used here is to avoid temperature shock to the cells.

37. After 1 day, transfer cells to liquid nitrogen storage.

Make sure not to keep the cells in the −80°C freezer for a long time, as this will decrease the viability of the cells after thawing.

REAGENTS AND SOLUTIONS

B8T medium

Add one thawed 200-µl aliquot of 10 mM thiazovivin (see recipe) to 1 L B8 medium (see Basic Protocol 1) to a final thiazovivin concentration of 2 µM and invert to mix. Store ≤4 weeks at 4°C.

EDTA, 0.5 mM

Add 500 µl of 0.5 M EDTA (Gibco, 15575020) to 500 ml Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} ($DPBS^{-/-}$; Corning, 21-031-CV) and invert to mix. Store indefinitely at room temperature.

Freezing solution

For 50 ml freezing solution, add 5 ml dimethylsulfoxide (DMSO; Fisher BioReagents, BP231-100) to 45 ml B8T medium (see recipe) to a final concentration of 10% (v/v) DMSO and mix well by inverting. Prepare fresh immediately before use.

NRG1, 100 µg/ml

Dissolve 250 µg recombinant human NRG1 (PeproTech, 100-03-250UG) in 2.5 ml cell-culture water (Corning, 25-055-CV). Make 100-µl aliquots and store ≤6 months at −20°C.

Sodium selenite, 2 mg/ml

Dissolve 100 mg sodium selenite (Sigma, S5261-10G) in 50 ml cell-culture water (Corning, 25-055-CV). Filter sterilize, make 1-ml aliquots, and store ≤6 months at −20°C.

Thiazovivin, 10 mM

Dissolve 10 mg thiazovivin (LC Labs, T9753) in 3.2 ml DMSO (Fisher BioReagents, BP231-100) to a final concentration of 10 mM. Make 200-µl aliquots. Store ≤6 months at −20°C.

COMMENTARY

Background Information

In this article, we present a simple, efficient, and cost-effective approach for high-quality culture of hiPSCs. Using these protocols (Basic Protocols 1 and 2), more than 100 hiPSC lines have been generated and maintained. To achieve the simplest approach, we have eliminated unnecessary procedures during hiPSC culture. This efficient and cost-effective approach could be used in high-throughput generation and maintenance of hiPSCs. Additionally, it could pave the way for the application of hiPSCs in personalized medicine as well as the pharmaceutical application of hiPSCs in drug toxicity assays.

Critical Parameters

One common misconception is that the hiPSC medium should be warmed up before

use. Not only is it important to avoid any heat shock to the cells, but it is also important to consider limiting or avoiding keeping the medium at 37°C for a long time due to the limited stability of the growth factors in the medium (Chen, Gulbranson, & Hou, 2011). Moreover, water baths can be a contamination source in tissue culture because it can provide an environment for the growth of bacteria and fungi if not cleaned regularly. Hence, in Basic Protocol 2, it is recommended to use the medium as soon as it is removed from the refrigerator. This protocol has been tested on several hiPSC lines in long-term culture, and thus far, no negative effect on the survival, growth, or differentiation of the cells has been observed.

One other approach to simplify hiPSC culture is our method for the preparation of Matrigel-coated plates (Basic Protocol 2). The Matrigel bottle is thawed overnight in the refrigerator. However, unlike the common procedure, it is not aliquoted and kept in a −20°C freezer; rather, the Matrigel bottle is kept at 4°C until use. This is particularly beneficial for laboratories that use Matrigel routinely and go through a bottle quickly. Extra care is required while handling the bottle here. Therefore, in Basic Protocol 2, we recommend preparing everything for the plate coating before bringing the bottle out of the refrigerator. Immediately after use, the bottle should be moved back to the refrigerator to avoid gelation. Moreover, we recommend preparation of Matrigel-coated plates for 2 to 3 weeks of use at once to save time. The coated plates can be kept at 37°C for up to 1 month and will keep their quality as long as they do not dry out.

Troubleshooting

If L-ascorbic acid 2-phosphate does not dissolve in water at the concentration mentioned in Basic Protocol 1:

• Make sure to use the L-ascorbic acid 2-phosphate trisodium salt, as only this form of L-ascorbic acid 2-phosphate can be used at this high concentration.

If the cells do not survive after thawing in Basic Protocol 2:

• Make sure that the frozen cells are of high quality before freezing. This will affect the survival rate after thawing of the cells.

• When freezing the cells, avoid keeping them in freezing solution at room temperature for a long time. Cells need to be transferred to −80°C as soon as possible after addition of freezing solution.

• When freezing the cells, avoid keeping them at −80°C for a long time. The survival rate decreases significantly after 1 week at −80°C. Cells should be transferred to liquid nitrogen storage as soon as possible.

• Check the quality of the Matrigel-coated plates. If the Matrigel is dried out, do not use the plates.

• Check the B8 and B8T media batches. If medium is >1 month old, do not use it.

If the cells do not attach after passaging in Basic Protocol 2:

• Avoid overgrowing the cells. Cells need to be around 70% to 80% confluent at the time of passaging. Overgrowing the cells causes contact inhibition and reduces the growth rate of the cells, which can lead to slow growth after passaging.

• Avoid leaving the cells in EDTA for >6 min, which leads some cells to start floating. These cells will be aspirated, reducing the number of cells plated after passaging.

• Check the quality of the Matrigel-coated plates. If the Matrigel is dried out, do not use the plates.

• Check the B8 and B8T medium batches. If medium is >1 month old, do not use it.

If the cells are growing slowly in Basic Protocol 2:

• Avoiding overgrowing the cells (to >90% confluent) due to late passaging (day 5) or plating them at too low of a split ratio (1:8 or 1:12) when the cells are growing well.

• Make sure that the cells are fed on the correct schedule.

• Make sure that the cells are not passaged too early (when <60% confluent; day 3) or plated at too high of a split ratio (1:15 or 1:20) when the cells are growing poorly.

• Avoid leaving the cells in EDTA for >6 min, which leads some cells to start floating. These cells will be aspirated, reducing the number of cells plated after passaging.

• Check the quality of the Matrigel-coated plates. If the Matrigel is dried out, do not use the plates.

• Check the B8 and B8T media batches. If the medium is >1 month old, do not use it.

Understanding Results

Following the protocols described here (Basic Protocols 1 and 2), hiPSCs could be generated and maintained at high quality. Moreover, this approach prevents spontaneous differentiation of hiPSCs. In an established cell line, 1 to 2 hr post-passaging, a significant number of the cells are attached and have formed small clusters (Figure 2A). After

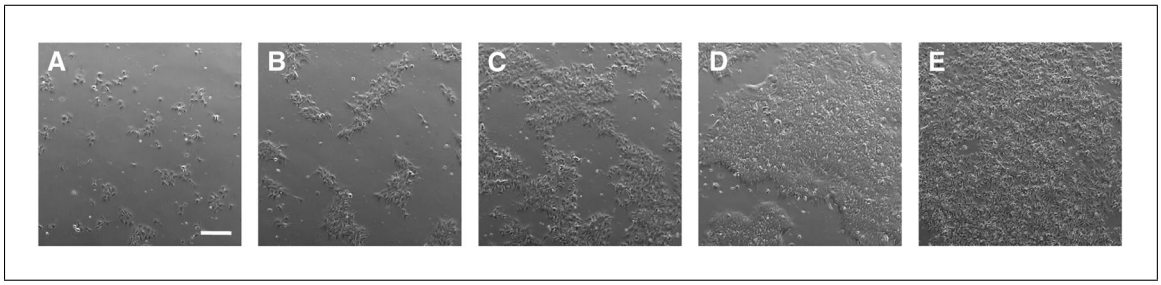

Figure 2 Time course of hiPSC proliferation post-seeding: (**A**) 1 hr, (**B**) 1 day, (**C**) 2 days, (**D**) 3 days, and (**E**) 4 days. Scale bar, 200 µm.

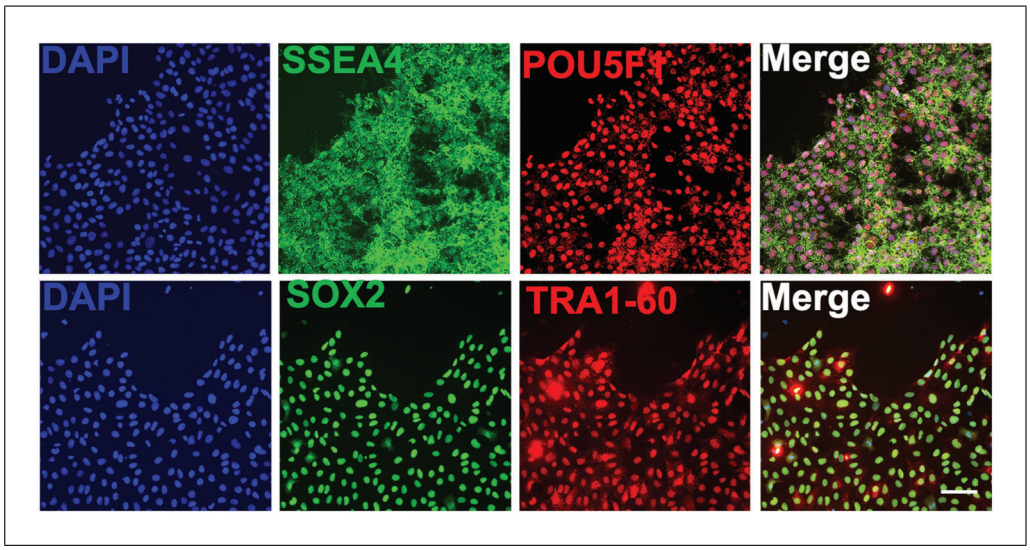

Figure 3 Example expression of undifferentiated cell markers in hiPSCs maintained in B8 medium. Scale bar, 100 µm.

24 hr, the clusters start to grow (Figure 2B). By day 2, the clusters start coming together (Figure 2C). By day 3, the cells have reached ~70% confluence and are ready for passaging (Figure 2D). If the cells are not passaged on time, they will reach ~90% confluence by day 4 (Figure 2E). It is highly recommended to avoid overgrowing the cells, as the quality of the culture will drop dramatically. Additionally, this will affect cell survival after passaging. Immunostaining of hiPSCs grown using our protocol demonstrates the quality of the cultured cells, as marked by the expression of pluripotency markers SSEA4, POU5F1/OCT4, SSEA4, SOX2, and TRA1-60 (Figure 3).

Time Considerations

Basic Protocol 1: B8 aliquot preparation takes ~2 hr.

Basic Protocol 2: Preparation of 42 Matrigel-coated 6-well plates takes 30 to 60 min. The plates need to be kept at 37°C for <1 hr prior to use. Thawing and initial plating of cells take ~15 min on the first day. Cells become 70% to 80% confluent after 3 to 5 days. Passaging of hiPSCs with EDTA takes ~15 min. Cells become 70% to 80% confluent after 3 to 4 days. Freezing of hiPSCs takes ~15 min.

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