Application Protocol

Maintenance of hPSC culture suitable for Quick-Tissue™ Differentiation Kits

Introduction

The Quick-Tissue™ Differentiation kits give the best differentiation results when source human pluripotent stem cells (hPSCs) have been maintained in StemFit® Basic04, StemFlex™ Medium, or other similar culture media which enable the maintenance of cultures by single-cell passaging. This Application Protocol describes the method of maintaining hPSCs under StemFit® Basic04 conditions and addresses common pitfalls to be avoided.

Required Consumables

Item	Vendor	Catalog Number
StemFit® Basic04 (Complete type)	Elixirgen Scientific	ASB04-C
iMatrix-511 silk	Elixirgen Scientific	NI511S
ROCK inhibitor Y27632	Selleckchem	S1049
TrypLE Select Enzyme (1X)*	ThermoFisher	12563011
0.02% EDTA in DPBS*	Sigma-Aldrich	E8008-100ML
Penicillin-Streptomycin	ThermoFisher	15140122
Phosphate-buffered saline (without Ca^{++} Mg^{++})	ThermoFisher	20012050
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418
(Optional) STEM-CELLBANKER**	AMSBIO	11890

* Can be substituted with our Cell Dissociation Reagent (Solution D1), Catalog Number: CDR.

** This is only required if you intend to cryopreserve the cells.

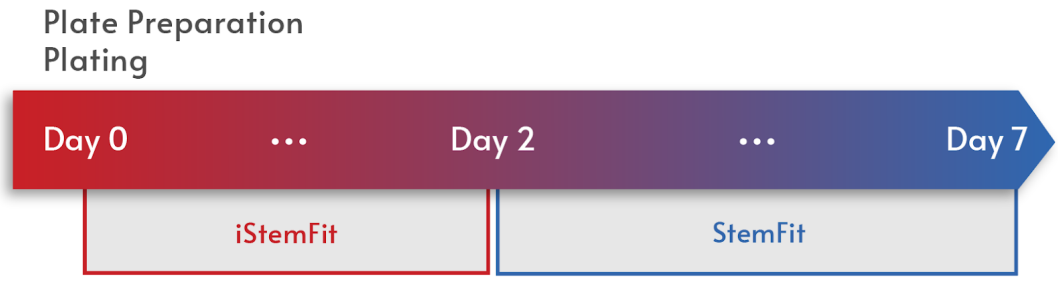
Condition of Use

This product is for research use only. It is not approved for use in humans or for therapeutic or diagnostic use.

Technical Support

For technical support, please contact us at cs@elixirgensci.com or call +1 (443) 869-5420 (M-F 9am-5pm EST).

Workflow



Media Preparation

StemFit

1. Thaw StemFit® Basic04 (Complete type) medium bottle at 4°C for approximately 48 hours until thawed.
   Note: DO NOT warm the medium at 37°C as it will result in degradation of medium components. See the manufacturer's instructions for more information.
2. Make aliquots of a convenient volume (e.g., 40 ml).
3. This solution, hereafter referred to as StemFit, can be stored at -20°C to -80°C. Once thawed, StemFit should be stored at 4°C for up to 2 weeks.

10 mM ROCK inhibitor Y27632 (iROCK)

1. Dissolve 10 mg ROCK inhibitor Y27632 in 3.12 ml DMSO.
2. Make aliquots of a convenient volume (e.g., 100 µl).
3. This solution, hereafter referred to as iROCK, can be stored at -20°C.

0.5X TrypLE Select with EDTA (Solution D1)*

1. Mix 1 ml TrypLE Select Enzyme (1X) with 1 ml 0.02% EDTA in DPBS.
2. This mixture, hereafter referred to as Solution D1, can be stored at 4°C for 2 weeks.

*Can be substituted with our Cell Dissociation Reagent (Solution D1), Catalog Number: CDR.

Notes

- In this protocol, instructions are described for one well of a 6-well plate. For other well sizes, multiply the protocol's consumables by the ratio between well surface area sizes.
- This protocol assumes starting on a Monday or Tuesday so as to allow weekend free culture.
- Do not allow the cultures to become fully confluent.
- Spontaneously differentiating colonies can be manually removed. If a culture has more than 20% spontaneously differentiating colonies we recommend discarding the culture and starting over.

Day 0

-4 hours

Note: Instead of precoating plates, many hPSC lines can be plated with iMatrix-511 silk mixed into the cell suspension, a method referred to as “direct plating”. However, not all hPSC lines can be plated in this manner. It is recommended to seed hPSCs on a precoated plate first and then test the direct plating method once the culture is maintained successfully (see Appendix B).

Plate preparation

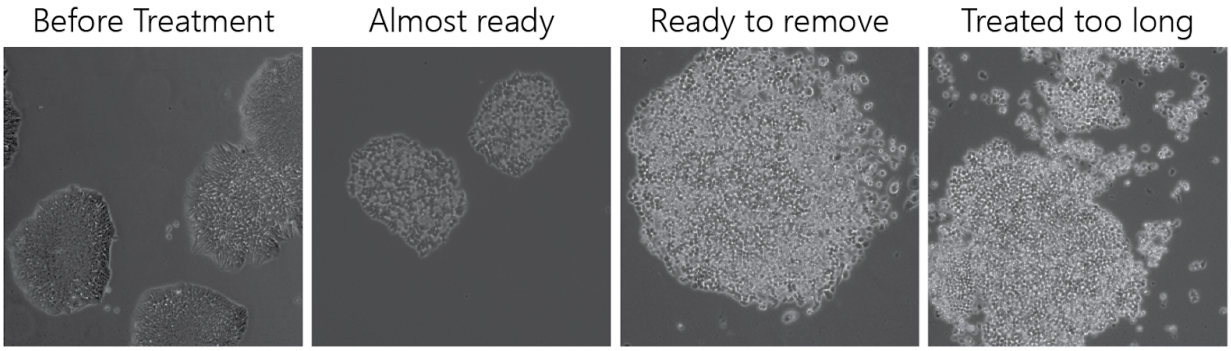
1. Prepare diluted iMatrix-511 silk by mixing together the following components in a 15 ml conical tube.
    - Make sure chilled PBS is used for this mixture.
    - The leftover iMatrix-511 silk can be stored at 4°C for later uses.

Diluted iMatrix-511 silk Reagents	Volume
iMatrix-511 silk	4.8 µl
Chilled PBS	2 ml

2. Add 2 ml diluted iMatrix-511 silk to 1 well of a 6-well plate.
3. Incubate the plate at 37°C, 5% CO_2 for 2 hours (or 4°C overnight one day before Day 1).
4. After incubation, aspirate the supernatant from the well and add 2 ml PBS.
5. Incubate the plate at 37°C, 5% CO_2 until hPSCs are ready for plating.

Passaging

1. Thaw an aliquot of StemFit at 4°C overnight or warm it at room temperature, protected from light, for 20-30 minutes.
2. Warm Solution D1 at room temperature for 20-30 minutes.
3. Transfer 4 ml StemFit into a tube and add 4 µl 10 mM iROCK. Mix well. The resulting medium is hereafter referred to as iStemFit.
4. Aspirate old medium from the culture and add 1.5 ml iStemFit.
5. Incubate the culture at 37°C, 5% CO_2 for 1 hour. This treatment enhances the survival of hPSCs after passaging.
6. Tap the Solution D1 tube 5 times with a finger and centrifuge at maximum speed for 1 minute.
7. Aspirate iStemFit from the hPSC culture and add 2 ml PBS.
8. Rock the plate 3 times, aspirate PBS from the culture, and add 300 µl Solution D1.
9. Incubate the culture at 37°C, 5% CO_2 for 5 minutes. If all the cells are not rounded under a microscope, continue to incubate at 37°C, 5% CO_2 in 1-2 minute increments (see images below).



10. Carefully pipet out Solution D1 from the culture using a P1000 pipettor and add 1 ml iStemFit.
11. Disperse the medium over the bottom surface of the well by pipetting 8-15 times to detach cells.
12. Using the same pipette tip, collect the cell suspension in a 1.5 ml tube.

13. Count cells to determine the volume of cell suspension needed.
    Note: The optimal seeding density will depend on the cell line being used and the desired time until next passage. We typically plate 0.5-1 x 10^4 cells per well for an 8-day culture schedule.
14. Take out the determined volume of the cell suspension from the previous step, place it in a new tube and bring the volume up to 1.5 ml with iStemFit.
15. Aspirate PBS from a new iMatrix-511 silk-coated well and add 1.5 ml cell suspension.
    Note: The rest of the cells can be used for assays or frozen down following the instructions in Appendix C.
16. Incubate the culture at 37°C, 5% CO_2 for 2 days.

Day 2-6

< 1 hour

Medium Change

1. Warm StemFit for 20-30 minutes at room temperature.
2. Aspirate the old medium from the well and add 1.5 - 4.5 ml StemFit (See Notes below).
3. Incubate the culture at 37°C, 5% CO_2.
4. Repeat steps 1-3 daily until Day 7 (See Notes below).
   Notes:
   - When at low confluence shortly after passage, wells can be fed with 1.5 ml of medium. The volume required will increase as the cultures become more confluent.
   - For weekend-free culturing, feed the well with 4.5 ml of medium on Friday afternoon. Cultures can then be left until Monday morning.
   - If the color of the culture medium becomes orange or yellow the medium should be changed daily with a larger volume of 2-3 ml.
   - Do not allow the cultures to become fully confluent. Passage as needed when approaching 80% confluency.

Day 7

< 1 hour

CONTINUOUS CULTURE OR FREEZING

On Day 7 cultures should be ready for passaging again or freezing.

Appendix A

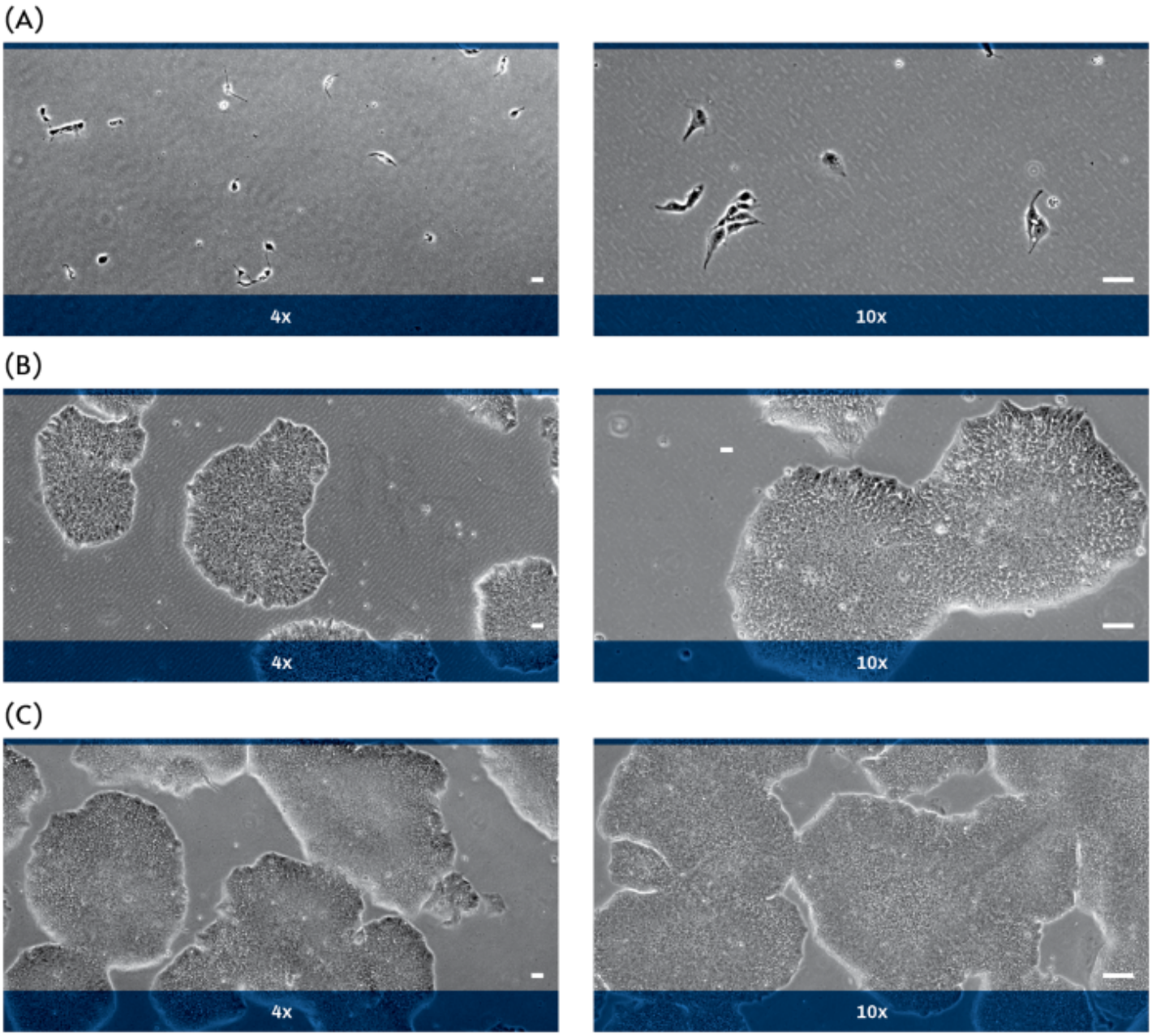


Figure 1. Representative 4x (left) and 10x (right) phase contrast images of human iPSC cultures (scale bars = 100 µm). (A) Images were taken on day 1 after plating and show that cultures contain single cells or very small clusters of less than 10 cells. (B) Images were taken on day 7 after plating and show that cultures contain large distinct colonies with smooth edges and dense centers. These cultures are ready for passaging or freezing. (C) Images of cultures that are over-confluent with many colonies touching and merging together.

Appendix B

Direct Coating Method

To culture hPSC using direct coating, skip precoating the wells with diluted iMatrix-511 silk as instructed in the "plate preparation" step. Instead, add 4 µl per well iMatrix-511 silk to the harvested cell suspension immediately before plating

during step 14 in the “passaging” section. Mix by pipetting up and down and dispense cells with iMatrix-511 silk into each well.

Note: Once iMatrix-511 silk is added to the master mix, work as quickly as possible otherwise the plating efficiency may decrease. Keep in mind that iMatrix-511 silk becomes solidified when it is left at 15°C or above for 5-10 minutes.

Appendix C

Freezing cells down

Note: After thawing frozen cells, over 60% of the cells will be viable.

1. Determine the volume of the cell suspension and number of 1.5 or 2.0 ml cryovials needed to freeze 2.5 x 10^5 cells per cryovial.
2. Centrifuge the cell suspension at 200 x g for 4 minutes.
3. While waiting for the centrifugation, label each cryovial. We recommend writing the name of the PSC line used, passage number, date, and the number of cells in the vial.
4. Aspirate the supernatant as much as possible but not completely.The pellet should still be covered with a small volume of the supernatant.
5. Tap the tube 10 times to loosen the pellet.
6. Resuspend the pellet 0.2 ml / vial STEM CELLBANKER.
7. Distribute 0.2 ml of the suspension to each cryovial.
8. Make sure that the caps are closed tightly and transfer the cryovials into a Mr. Frosty Freezing Container. Make sure that Mr. Frosty contains 250 ml isopropanol.
9. Loosely close the lid of Mr. Frosty with cryovials, put it into a -80°C freezer and leave it overnight or up to a few days.
10. Transfer the cryovials into a liquid nitrogen storage tank.

Appendix D

Thawing cells

Note: After thawing frozen cells, we recommend users culture cells in medium supplemented with ROCK inhibitor (Y27632) for 48 hours.

1. Transfer 10 ml of iStemFit to a 15 ml conical tube and warm it in a 37°C water bath for 30 minutes until it reaches 37°C.
2. Get the cryovial of cells from LN2.
3. Spray a Kimwipe with 70% EtOH and wipe the cryovial. Transfer the cryovial to the hood.
4. Add 800 µl of warm iStemFit to the vial using a P1000 pipettor.
5. Gently pipet up and down with the P1000 until just thawed.
6. Using the same tip, increase the P1000 pipette volume setting from 800 µl to 1000 µl and transfer the cell suspension to a new 15 ml conical tube.
7. Using a P1000 pipettor, add 1 ml of warm iStemFit to the cryovial and pipet up and down, washing the sides of the vial, to obtain any cells remaining in the vial.
8. Transfer the cell suspension to the same 15 ml conical tube with the same P1000 pipette tip.
9. Bring the volume in the 15 ml conical up to 5 ml by adding additional iStemFit using a 5 ml pipette.
10. Centrifuge the cell suspension in the 15 ml tube at 200 x g for 4 minutes.
11. Visually confirm that there is a pellet. Use a pasteur pipet to aspirate most of the supernatant from the conical tube, leaving a small volume of supernatant (< 50 µl) to cover the cell pellet.
12. Tap the side of the conical tube up to 10 times to break up the cell pellet.
13. Add 1 ml iStemFit to the conical tube using a P1000 pipette and pipet up and down no more than 2-3 times to resuspend cells.
14. Plate the cells as described in step 13 of Day 0 “Passaging”.