TECHNICAL MANUAL

Reversion and Maintenance of Human Naïve-Like Pluripotent Stem Cells with RSeT™ Medium

STEMCELL™ TECHNOLOGIES

Critical Parameters for Successful Cell Culture with RSeT™ Medium

Quality of Primed Human Pluripotent Stem Cells (hPSCs) Influences Reversion to Naïve-Like State

Reversion of human embryonic stem (ES) cells or induced pluripotent stem (iPS) cells to a naïve-like state requires high-quality cultures of primed cells. Primed ES or iPS cultures containing a high proportion of differentiated cells may not revert to a naïve-like state and could further increase rates of differentiation during reversion in RSeT™ Medium. For suggestions on measuring pluripotency of primed cultures, refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1 (Document #28315) available at www.stemcell.com or contact us to request a copy.

Choosing Appropriate Mouse Embryonic Fibroblast (MEF) Feeders

Achieving successful reversion and maintenance of naïve-like human pluripotent stem cells (hPSCs) in RSeT™ Medium requires a suitable and ES-qualified source of MEFs. Unsuitable MEFs will lead to unsuccessful reversion of primed hPSCs and increased differentiation. Each unique source of ES-qualified MEFs should be tested to determine the optimum plating density for supporting culture of naïve-like cells. MEFs from a single source should also be monitored for batch-to-batch variation. MEFs should be plated no more than 2 days before co-culture with hPSCs. It is recommended to use day E12.5 MEFs derived from the CF-1 mouse strain.

Hypoxic Culture Conditions

The protocols outlined in this manual for the reversion and maintenance of naïve-like hPSCs in RSeT™ Medium require the cultures to be incubated at 37°C under **hypoxic conditions (5% O_2, 5% CO_2)**. Culturing naïve-like hPSCs in RSeT™ Medium under normoxic conditions (20% O_2, 5% CO_2) may result in increased spontaneous differentiation and loss of naïve-like pluripotency characteristics.

Colony Density and Passaging of Naïve-like hPSCs

Plating density is an important parameter in maintaining good-quality naïve-like hPSCs. As colonies become too large and begin to merge with one another, increased spontaneous differentiation may occur (shown in Figure 4). To maintain good-quality undifferentiated naïve-like hPSCs, it is important to use appropriate split ratios (see section 6.5). Naïve-like hPSCs should be passaged when colonies are typically between 100 - 250 µm in diameter (Figure 5). If allowed to grow too large, colonies will tend to lose their naïve-like characteristics.

Medium Changes

Daily medium changes are typically required to maintain high-quality naïve-like hPSCs. Extending the time between medium changes to 2 or 3 days is not recommended. Medium should be protected from light and warmed to room temperature (15 - 25°C) before use.

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1.0 Introduction

Human pluripotent stem cells (hPSCs) can be maintained along a spectrum of different pluripotent states. In vitro, different cell culture media with specific combinations of cytokines or small molecules have been shown to maintain cells in either a naïve or primed pluripotent state.[1,2] Specifically, mouse embryonic stem (ES) cells are dependent on culture media that contain leukemia inhibitory factor (LIF), while conventional hPSCs and mouse epiblast-like stem cells are cultured in media containing bFGF and Activin A. Multiple research groups have recently identified different culture conditions capable of shifting and maintaining hPSCs toward the "ground" or "naïve" state and away from the traditional primed state of hPSCs.[3-6]

RSeT™ Medium is a defined cell culture medium used for the reversion of primed hPSCs to a naïve-like state and for maintenance of naïve-like hPSCs under feeder-dependent and hypoxic conditions. RSeT™ Medium contains pre-screened quality components and does not contain bFGF or TGFβ. It is compatible with human ES and induced pluripotent stem (iPS) cells.

hPSCs cultured in RSeT™ Medium exhibit features of a naïve-like state such as small, tightly packed, domed colonies with refractive edges. Key transcripts associated with naïve-like hPSCs such as KLF2, KLF4, and TFCP2L1 show increased expression in hPSCs cultured in RSeT™ Medium. RSeT™ hPSCs can be converted back to a primed state by culture in mTeSR™1 or TeSR™-E8™ and can then be differentiated with STEMdiff™ products such as STEMdiff™ Definitive Endoderm Kit (Catalog #05110), STEMdiff™ SMADi Neural Induction Kit (Catalog #08581), or STEMdiff™ Mesoderm Induction Medium (Catalog #05220).

2.0 Materials, Reagents, and Equipment

2.1 RSeT™ Medium (Catalog #05978)

For component storage and stability information, refer to the Product Information Sheet (PIS) for RSeT™ Medium (Document #DX22485); the PIS is also available at www.stemcell.com, or contact us to request a copy.

COMPONENT NAME	COMPONENT #	SIZE
RSeT™ Basal Medium	05969	400 mL
RSeT™ 5X Supplement*	05979	100 mL

*This product contains material derived from human plasma. Donors have been tested and found negative for HIV-1 and -2, hepatitis B, and hepatitis C prior to donation. However, this product should be considered potentially infectious and treated in accordance with universal handling precautions.

2.2 Additional Required Materials and Reagents

PRODUCT	CATALOG #
D-PBS (Without Ca++ and Mg++) (D-PBS)	37350
mTeSR™1	85850
DMEM/F-12	36254
Trypan Blue	07050
0.1% Gelatin in Water	07903
Inactivated CF-1 mouse embryonic fibroblasts (iMEFs) E12.5	Qualified source
TrypLE™	Gibco 12605028
Fetal bovine serum (FBS)	Sigma Aldrich 12103C
CryoStor® CS10	07930
Y-27632	72304
Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
Gentle Cell Dissociation Reagent	07174
2 mL serological pipette	38002
15 mL conical tubes	38009
12-Well Flat-Bottom Plate, Tissue Culture-Treated	38052
Cryogenic vials	38048

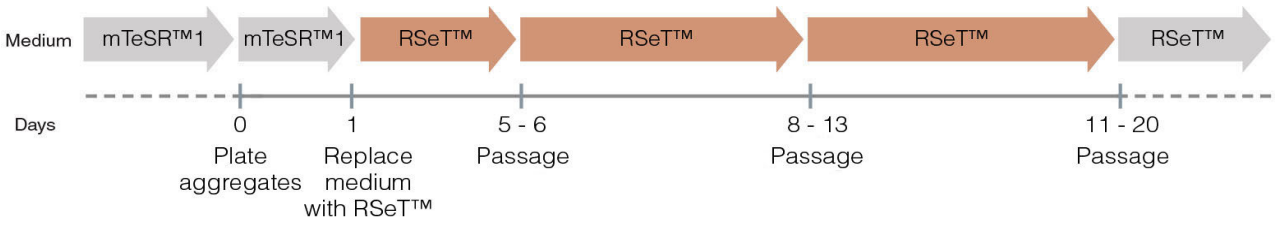
For a complete list of products available from STEMCELL Technologies Inc., visit www.stemcell.com.

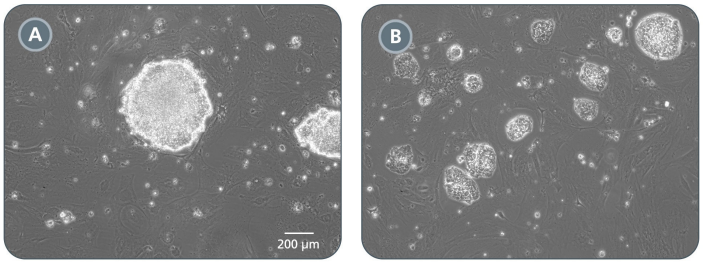
2.3 Equipment Required

- Biohazard safety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- **Hypoxic** incubator with humidity and gas control (e.g. Catalog #27310) to maintain 37°C and 95% humidity in an atmosphere of 5% O_2 and 5% CO_2
- Low-speed centrifuge with a swinging bucket rotor
- Pipette-aid with appropriate serological pipettes
- Pipettor with appropriate tips
- Inverted microscope with a total magnification of 20X to 100X
- -20°C freezer
- -80°C freezer
- -150°C freezer or liquid nitrogen (LN_2) vapor tank
- Refrigerator (2 - 8°C)

3.0 Procedure Diagram

Reversion of Primed hPSCs to a Naïve-Like State Using RSeT™ Medium





Morphology of Naïve-Like hPSCs During Reversion and Maintenance. (A) mTeSR™1-cultured hPSCs being reverted in RSeT™ Medium exhibit a tightly packed, domed morphology with refractive edges by day 5. (B) Naïve-like hPSCs maintained in RSeT™ Medium exhibit typical domed morphology and refractive edges.

4.0 Preparation of Reagents and Materials

The following reagents and materials are required in the protocols in section 6.0. Prepare as needed.

4.1 iMEF Feeder Layer on Gelatin-Coated Plates

The following instructions are for preparing an inactivated mouse embryonic fibroblast (iMEF) feeder layer for culturing naïve-like hPSCs in one well of a 12-well tissue culture-treated plate. If using other cultureware, adjust volumes accordingly. For instructions on preparation of iMEFs, contact us at techsupport@stemcell.com.

1. Prepare MEF medium (DMEM/F-12 with 10% FBS). Aliquot sufficient MEF medium and warm to room temperature (15 - 25°C).
2. Prepare a gelatin-coated plate by adding 0.5 mL of 0.1% Gelatin in Water into each well and incubate at room temperature (15 - 25°C) for at least 15 minutes.
3. Quickly thaw the iMEFs in a 37°C water bath by gently shaking the cryovial continuously until only a small frozen cell pellet remains.
4. Remove the cryovial from the water bath and wipe it with 70% ethanol or isopropanol to sterilize.
5. Use a 2 mL serological pipette to transfer the contents of the cryovial to a 15 mL conical tube.
6. Add 5 - 7 mL of MEF medium dropwise to the 15 mL tube, gently mixing as the medium is added.
7. Centrifuge cells at 300 x *g* for 5 minutes at room temperature (15 - 25°C).
8. Aspirate the medium, leaving the cell pellet intact. Gently resuspend the cell pellet in 1 mL of MEF medium using a 2 mL serological pipette.
9. Count viable iMEFs using Trypan Blue dye exclusion on a hemocytometer.
10. Aspirate gelatin solution from the plate prepared in step 2 and seed approximately 1.67 - 2.30 x 10^5 iMEFs in 1 mL of MEF medium per well of a 12-well plate.

    Note: Optimal feeder-layer density may vary depending on the source and the embryonic developmental stage from which the iMEFs are derived. It is recommended to use iMEFs derived from embryos at E12.5 from the CF-1 mouse strain.
11. Distribute iMEFs evenly across the well by moving the plate in several quick, short, back-and-forth and side-to-side motions. Incubate at 37°C under **normoxic conditions (20% O_2, 5% CO_2)** overnight to allow the iMEFs to adhere.

    Note: Although the iMEFs will adhere within 4 - 5 hours, ideally they should be incubated overnight before use.
    ***The iMEF-coated plates must be used within 48 hours of seeding**.*

4.2 Complete RSeT™ Medium

Use sterile techniques when preparing complete RSeT™ Medium (Basal Medium + 5X Supplement). The following example is for preparing 100 mL of complete medium. If preparing other volumes, adjust accordingly.

1. Thaw the 5X Supplement at room temperature (15 - 25°C) or at 2 - 8°C overnight. Ensure Supplement is evenly resuspended after thawing.

   Note: Once thawed, use immediately or aliquot and store at -20°C. Do not exceed the shelf life of the Supplement. After thawing the aliquots, use immediately. Do not re-freeze.

2. Add 20 mL of 5X Supplement to 80 mL of Basal Medium. Mix thoroughly.

   Note: If not used immediately, store complete RSeT™ Medium at 2 - 8°C for up to 1 week. Warm complete medium to room temperature (15 - 25°C) before use. Protect from exposure to direct light.

5.0 Culture of hPSCs in mTeSR™1

The protocols outlined in this manual describe the reversion of primed hPSCs cultured in mTeSR™1 under feeder-free conditions. For complete instructions on maintaining hPSCs in mTeSR™1, refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1 (Document #28315), available at www.stemcell.com or contact us to request a copy.

6.0 Culture of Naïve-Like hPSCs in RSeT™ Medium

6.1 Morphology of Naïve-Like hPSCs in RSeT™ Medium

Naïve-like hPSC colonies cultured in RSeT™ Medium typically exhibit a domed morphology with refractive edges (Figure 1) and are cultured on a feeder layer of inactivated mouse embryonic fibroblasts (iMEFs) (Figure 2). Naïve-like hPSCs are tightly packed clusters of cells (Figure 1C and Figure 1F, arrow) whereas iMEFs are flat mesenchymal-like cells (Figure 1C, arrowhead) surrounding the naïve-like hPSC colonies. As hPSCs can exist in a spectrum of pluripotent states, cultures of naïve-like hPSCs can also contain colonies that maintain a primed-like morphology. These colonies are tightly packed but flat and contain cells with prominent nucleoli and high nuclear-to-cytoplasmic ratios (Figure 1F, line).

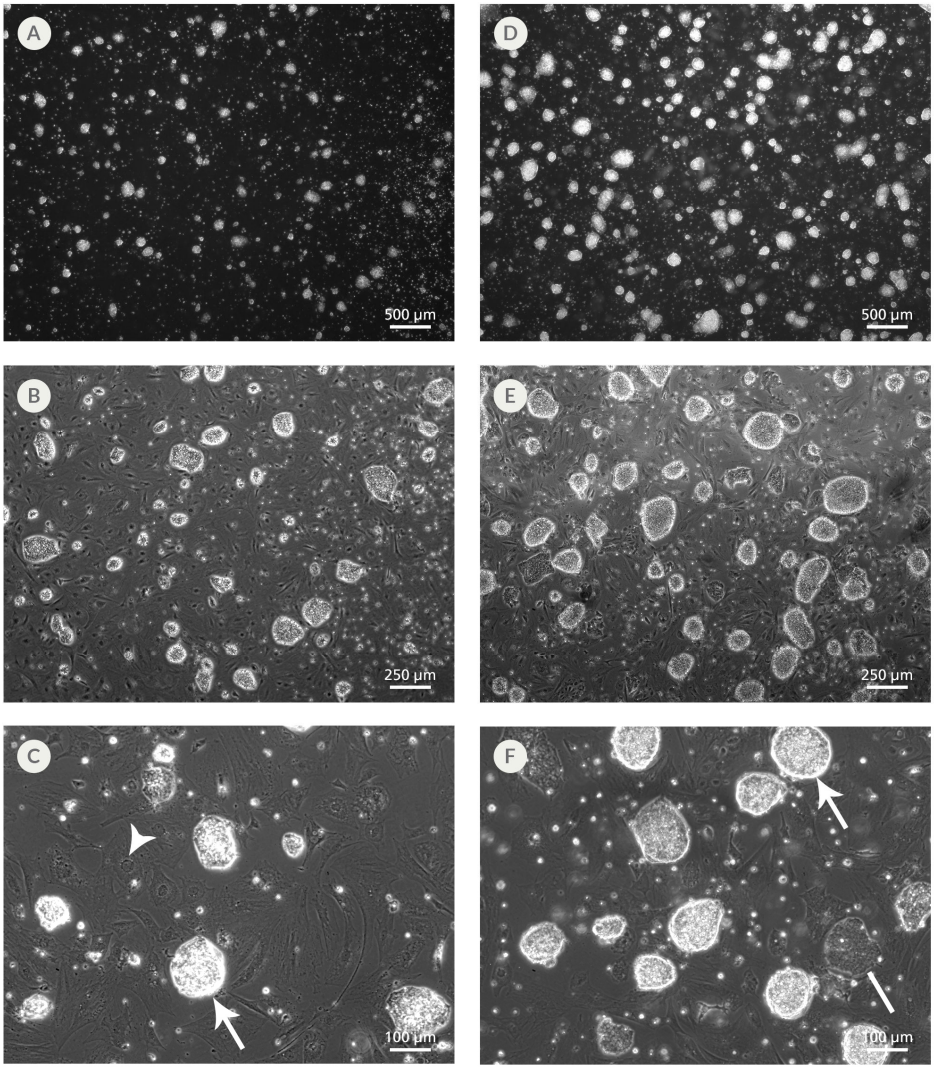


Figure 1. Characteristics and Morphology of Naïve-Like hPSC Colonies Cultured in RSeT™ Medium. Human ES (A - C) and iPS (D - F) cell lines were reverted and stably maintained as naïve-like hPSCs in RSeT™ Medium. Naïve-like hPSC colonies typically exhibit a domed morphology with refractive edges (C and F, arrows) when cultured on inactivated MEFs (C, arrowhead) which have a flat mesenchymal-like morphology. In addition, it is not uncommon to see colonies with features similar to primed hPSCs (compact and flat morphology) (F, line).

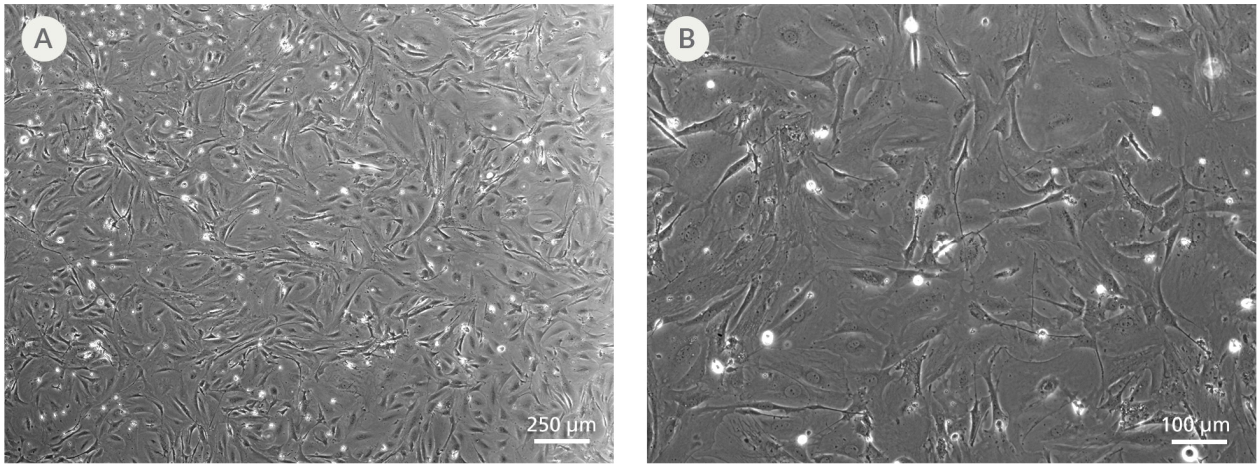


Figure 2. Mitomycin C-Inactivated CF-1 Mouse Embryonic Feeder Cells. MEFs cultured for 24 hours on 0.1% gelatin in DMEM/F-12 + 10% FBS medium. Magnification: 40X (A) and 100X (B).

6.2 Reversion of Primed hPSCs to a Naïve-Like State

Achieving successful reversion of primed hPSCs to a naïve-like hPSC state relies heavily on the quality of the primed ES or iPS culture. Primed cultures must contain a minimal amount of differentiated cells. Thus, we recommend culturing primed human hPSCs in mTeSR™1 to achieve the highest-quality source cultures for reversion. For information on maintaining high-quality hPSCs, refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1 (Document #28315), available at www.stemcell.com or contact us to request a copy.

Note: Prepare iMEF feeder layer one day prior to culturing cells (see section 4.1).

1. Aliquot sufficient mTeSR™1 and D-PBS. Warm to room temperature (15 - 25°C) before use.
2. Aspirate MEF medium from a 12-well iMEF-coated plate.
3. Wash each well twice with 1 mL of D-PBS. Aspirate D-PBS.
4. Add 1 mL of mTeSR™1 medium per well and incubate at 37°C under **normoxic conditions (20% O_2, 5% CO_2)**.

Passaging Aggregates of mTeSR™1-Cultured hPSCs

5. Aliquot sufficient mTeSR™1 and Gentle Cell Dissociation Reagent. Warm to room temperature (15 - 25°C) before use.
6. Use a microscope to visually identify regions of differentiation in a plate of mTeSR™1-cultured hPSCs. Mark these with a felt tip or lens marker on the bottom of the plate.
7. Remove regions of differentiation by scraping with a pipette tip or by aspiration. Avoid having the culture plate out of the incubator for more than 15 minutes at a time.

   Note: Selection may not be required if differentiation is < 5%. Selection should not exceed 20% of the well if the culture is of high quality.
8. Aspirate medium from the well and add 1 mL of Gentle Cell Dissociation Reagent per well of a 6-well plate. Incubate at room temperature (15 - 25°C) for 5 - 7 minutes.

   Note: If using an alternative dissociation reagent, refer to the appropriate Product Information Sheet.
9. Aspirate the Gentle Cell Dissociation Reagent and add 1 mL mTeSR™1.

10. Gently detach the colonies by scraping with a serological glass pipette or a cell scraper (e.g. Catalog #38065).
11. Transfer the detached cell aggregates to a 15 mL conical tube.

    Optional: Rinse the well with an additional 1 mL of mTeSR™1 to collect remaining cell aggregates. Add the rinse to the 15 mL tube.

    Note: Centrifugation of cell aggregates is not required.
12. Carefully pipette the cell aggregate mixture up and down to break up the aggregates as needed. A uniform suspension of aggregates approximately 50 - 200 µm in size is optimal; do not create a single-cell suspension.
13. Count cell aggregates > 50 µm in diameter. For a cell aggregate counting method, refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1 (Document #28315) available at www.stemcell.com or contact us to request a copy.
14. Plate 200 - 250 cell aggregates into each well of the iMEF-coated plate containing mTeSR™1 (prepared in steps 1 - 4). If using other cultureware, adjust accordingly.
15. Incubate at 37°C for 24 - 36 hours under **normoxic conditions (20% O_2, 5% CO_2)**.
16. Aspirate mTeSR™1 and replace with 1 mL RSeT™ Medium (warmed to room temperature) per well.
17. Incubate at 37°C under **hypoxic conditions (5% O_2, 5% CO_2).**
18. Perform a full medium change daily with RSeT™ Medium during the reversion to naïve-like hPSCs.
19. After 4 - 5 days in RSeT™ Medium, colonies are typically ready to be passaged. Colonies will begin to adopt a tightly packed, domed morphology with the edges of the colony becoming more refractive (Figure 3).

6.3 Morphology of Cells Cultured in RSeT™ Medium: Reversion to Stable Naïve-like hPSCs

Characteristics of Cultures During the First Week of Reversion

During the first week of reversion in RSeT™ Medium, the morphology of hPSC colonies will begin to change. After passaging the primed hPSC colonies from feeder-free mTeSR™1 cultures onto iMEF feeder layers, small colonies observed within 24 hours generally exhibit primed hPSC morphology. After replacing mTeSR™1 with RSeT™ Medium on day 2, colonies will begin to adopt a dome-shaped morphology as shown in Figure 3 (day 1) and will expand in size. The edges of these colonies tend to become more refractive, indicating a change from a flat, compact colony to a raised 3-dimensional colony. By day 5 in RSeT™ Medium, colonies should be ready to be passaged (Figure 3).

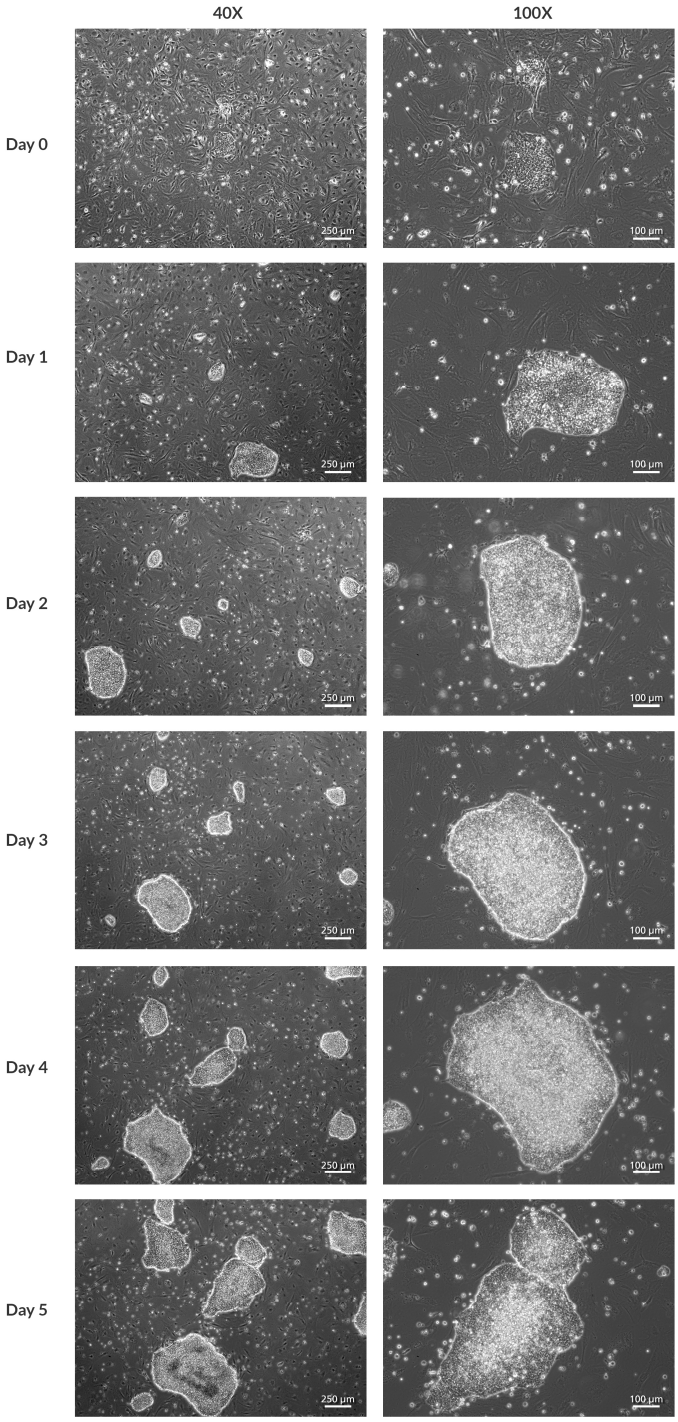


Figure 3. Time Course of Changes in hPSC Colony Morphology During Reversion in RSeT™ Medium. At day 0, hPSC aggregates cultured for 24 hours in mTeSR™1 on feeder cells maintain a primed hPSC morphology. The medium is replaced with RSeT™ Medium on day 1 and is exchanged daily until day 5. During the initial culture in RSeT™ Medium, colonies expand and begin to adopt naïve-like characteristics such as refractive edges and multi-layering suggestive of a dome-shaped colony. By day 5, these colonies are generally large enough to be passaged. Magnification: 40X and 100X.

Morphology of Naïve-Like hPSCs During the First Few Passages Following Reversion

The morphology of cultures in RSeT™ Medium may fluctuate during the initial passages, containing a mixture of colonies that resemble both primed and naïve-like hPSCs. In addition, spontaneously differentiated cells can be present in the cultures (Figure 4) during the early passages of naïve-like hPSCs. Morphology from passage to passage will be heavily influenced by split ratio; cultures which have been over- or under-seeded and have lost some degree of their naïve-like characteristics may be rescued by carefully considering the split ratio in future passages.

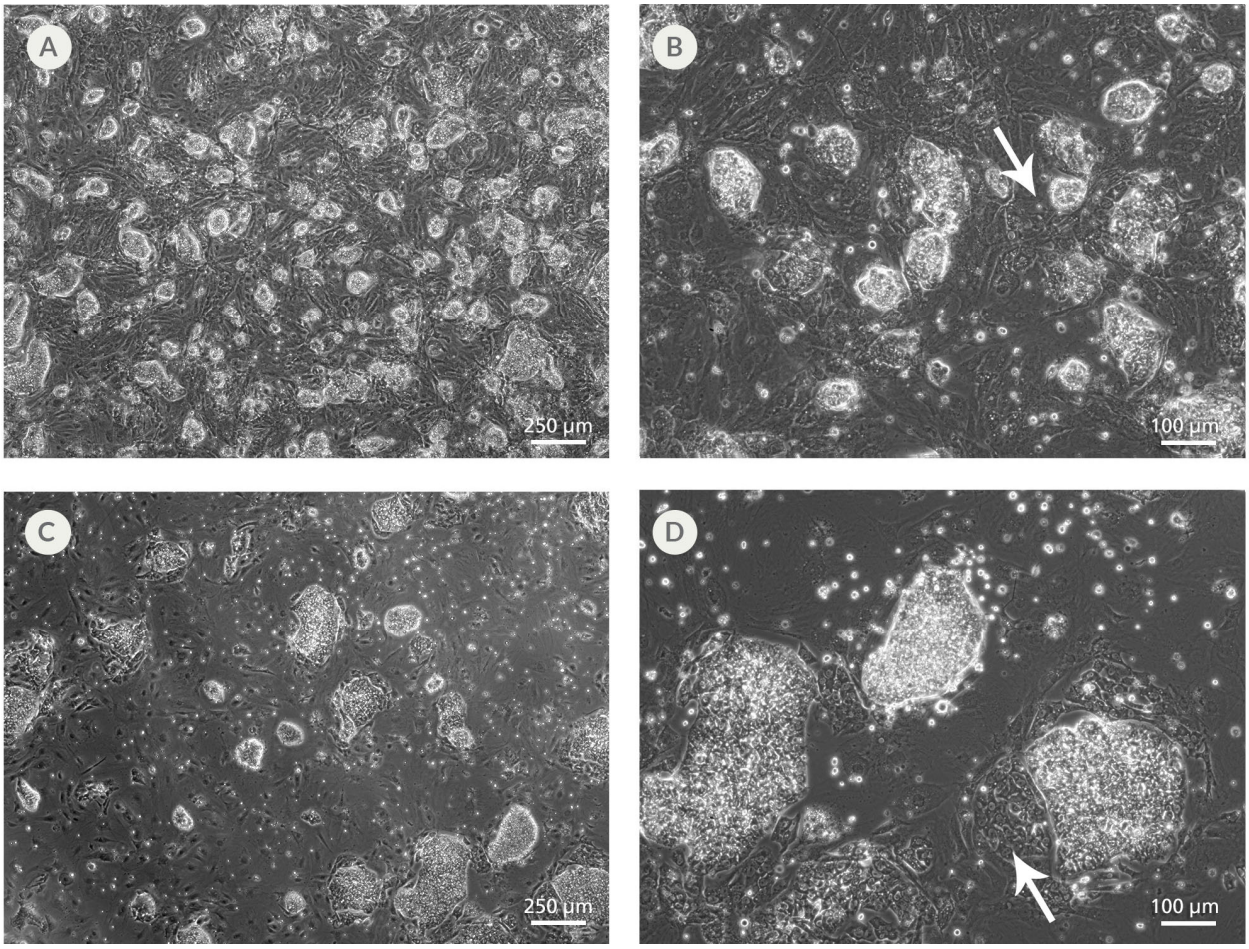


Figure 4. Spontaneous Differentiation in Human ES and iPS Cell RSeT™ Cultures. (A) and (B) A naïve-like human ES cell line at passage 1 maintained in RSeT™ Medium (Magnification: 40X and 100X). (C) and (D) An iPS cell line at passage 2 maintained in RSeT™ Medium (Magnification: 40X and 100X). During early passage of naïve-like hPSCs in RSeT™ Medium, spontaneous differentiation can occur; differentiated cells will exhibit a flat morphology (B and D, arrow) surrounding the dome-shaped colonies.

Morphology of Stable Naïve-Like hPSCs

Stable naïve-like hPSCs have been observed as early as passage 3; in some cases achieving stable morphology requires culturing to passage 7. In stable naïve-like hPSC cultures, more than 70% of colonies will exhibit dome-shaped morphology, with minimal contamination from differentiated cells or colonies exhibiting primed hPSC morphology (Figure 5). Further, Figure 5 shows examples of the recommended densities for culturing naïve-like hPSCs and the appropriate diameter for passaging (150 - 250 µm).

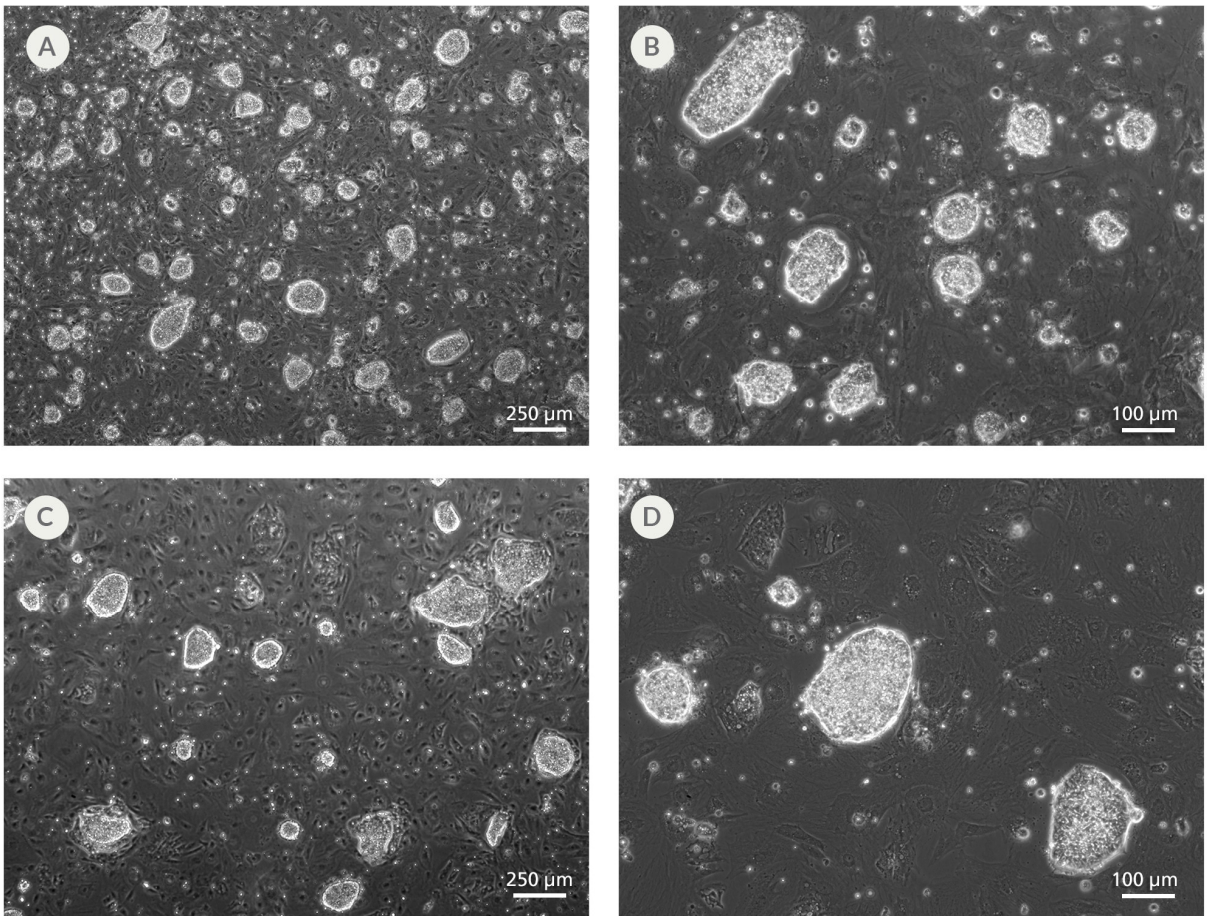


Figure 5. Stable RSeT™ Cell Lines can be Achieved by Passage 3 - 7. (A) and (B) Human iPS cell line maintains naïve-like morphology from passage 3 onward. (C) and (D) Human ES cell line (H1) maintains naïve-like morphology from passage 7 onward. Magnification: 40X and 100X.

6.4 Assessing RSeT™ Cultures to Determine Day of Passaging

Colony size is a good indicator for determining when to passage hPSCs cultured in RSeT™ Medium. In general, when the majority of colonies have reached a diameter of 100 - 250 µm, the culture typically requires passaging. Small colonies passaged too early may result in decreased viability (Figure 6A and 6B). Alternatively, larger colonies may start to exhibit increased spontaneous differentiation (Figure 6C and 6D). In general, naïve-like hPSCs tend to require passaging after 3 - 6 days of culture in RSeT™ Medium; in extenuating circumstances, cells can be passaged as early as day 2 or as late as day 7. Passaging later than day 7 is not recommended, due to the resulting decrease in feeder viability.

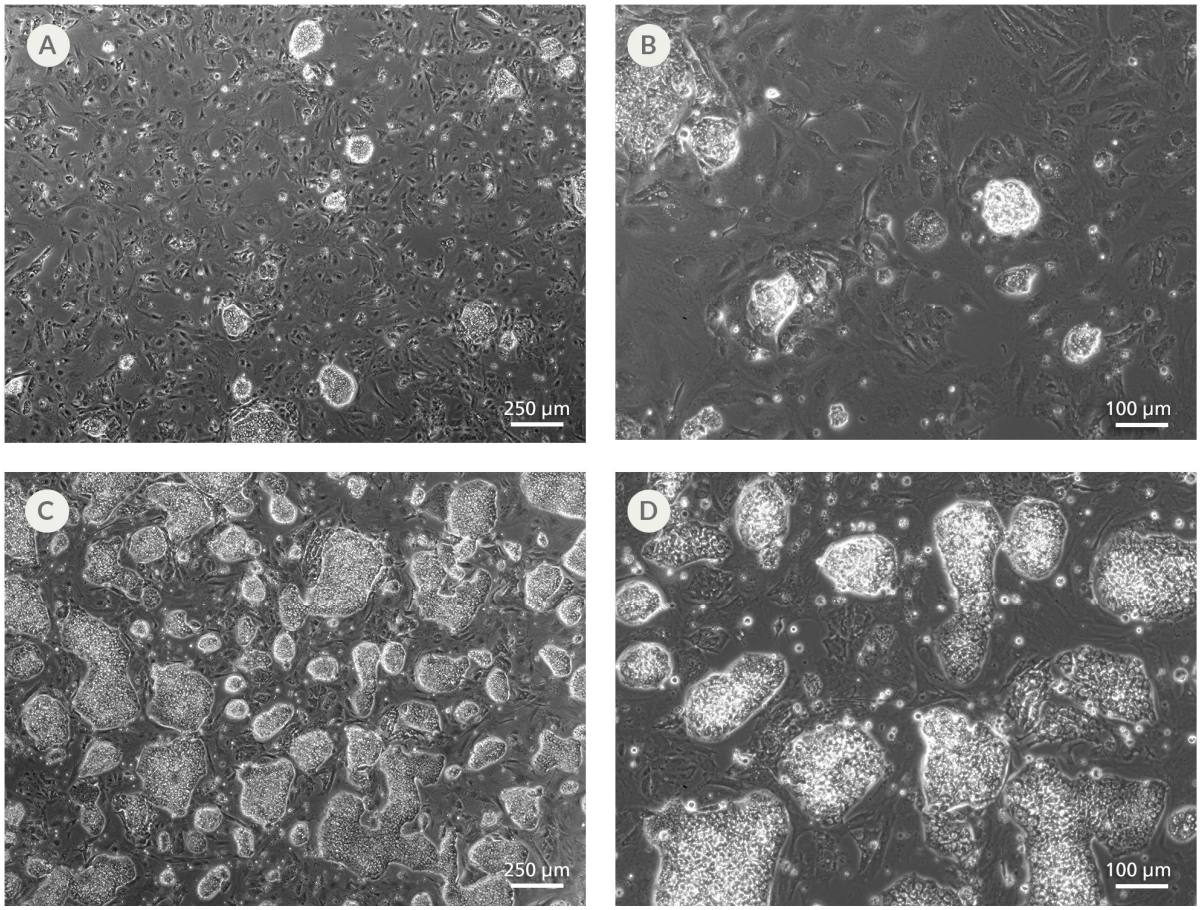


Figure 6. Passaging Ratio May Vary Between Cell Lines and Also From Passage to Passage Within Individual Cell Lines. The passaging ratio should be assessed based on growth rate and morphology, and may need to be adjusted to avoid excessively high- or low-density cultures. (A) and (B) Small colony size and low-density naïve-like hPSCs cultured in RSeT™ Medium may result in decreased rates of culture growth. (C) and (D) High-density naïve-like hPSCs cultured in RSeT™ Medium may lead to overlapping colonies and result in a loss of naïve-like morphology and/or increases in the rate of differentiation. Magnification: 40X and 100X.

6.5 Passaging Naïve-Like hPSCs onto iMEF-Coated Plates

Note: Prepare iMEF-coated plates one day prior to culturing cells (see section 4.1).

1. Aliquot sufficient RSeT™ Medium and D-PBS. Warm to room temperature (15 - 25°C) before use.
2. Add Y-27632 to RSeT™ Medium to a final concentration of 10 µM.
3. Aspirate MEF medium from a 12-well iMEF-coated plate.
4. Wash each well twice with 1 mL of D-PBS. Aspirate D-PBS.
5. Add 1 mL of RSeT™ Medium containing 10 µM of Y-27632 per well.

Passaging Naïve-Like hPSCs

6. Aliquot sufficient medium (RSeT™ Medium with or without 10 µM Y-27632), D-PBS, and TrypLE™. Warm to room temperature (15 - 25°C) before use.
7. Aspirate RSeT™ Medium from wells containing naïve-like hPSCs.
8. Wash with 1 mL of D-PBS. Aspirate D-PBS.
9. Add 250 µL of TrypLE™ to each well.
10. Incubate cells at 37°C for approximately 3 minutes under **hypoxic conditions (5% O_2, 5% CO_2)**.
11. Without aspirating TrypLE™, add 750 µL of RSeT™ Medium to the well.
12. Using a 1 mL pipette tip, gently pipette up and down 2 - 3 times to dislodge cells from well.

    Note: Do not excessively pipette up and down, as this may decrease viability. The cell suspension should contain a mixture of single cells and very small cell aggregates of approximately 2 - 10 cells, as well as iMEFs which may remain visibly clumped together.
13. Transfer to a 15 mL conical tube.
14. Centrifuge at 300 x *g* for 5 minutes.
15. Remove supernatant and gently resuspend the cell pellet in 100 µL of RSeT™ Medium.
16. Plate cells onto a freshly prepared iMEF-coated plate. Typically, a 1:4 to 1:5 split ratio (approximately 35,000 cells plated after passaging) is used after the first reversion step (see section 6.2).

    Note: Subsequent passaging of cultures can vary between 3 - 7 days using 1:3 to 1:10 split ratios, which will be dependent on the number of colonies and may also vary between different hPSC lines. Optimal density for passaging is shown in Figure 5, when colonies reach approximately 100 - 250 µm in diameter.

    Note: During the early stages of reversion, low-density cultures may arise and a 1:1 split ratio may be necessary. Alternatively, high-density cultures should be avoided because this may lead to spontaneous differentiation. Thus, split ratios should be adjusted according to the density and size of the colonies. Refer to Figure 5 for representative images.
17. Incubate at 37°C under **hypoxic conditions (5% O_2, 5% CO_2)**.
18. Perform a daily medium change with RSeT™ Medium and visually assess cultures to monitor growth until the next passage.

7.0 Cryopreservation of Naïve-Like hPSCs

1. Aspirate RSeT™ Medium from wells containing naïve-like hPSCs.
2. Wash each well with 1 mL of D-PBS. Aspirate D-PBS.
3. Add 250 µL of TrypLE™ to each well.
4. Incubate at 37°C for approximately 3 minutes under **hypoxic conditions (5% O_2, 5% CO_2)**.
5. Without aspirating TrypLE™, add 750 µL of RSeT™ Medium to the well.
6. Using a 1 mL pipette tip, gently pipette up and down 2 - 3 times to dislodge cells from well.

   Note: Do not excessively pipette up and down, as this may decrease viability. The cell suspension should contain a mixture of single cells and very small cell aggregates of approximately 2 - 10 cells, as well as iMEFs which may remain visibly clumped together.
7. Transfer to a 15 mL conical tube.
8. Centrifuge at 300 x *g* for 5 minutes at room temperature (15 - 25°C).
9. Gently aspirate the supernatant, taking care not to disrupt the cell pellet.
10. Using a serological pipette, gently resuspend the pellet with 1 mL of cold (2 - 8°C) CryoStor® CS10 per well harvested. Minimize the breakup of cell aggregates when resuspending the pellet.
11. Using a 2 mL serological pipette, transfer 1 mL of the cell aggregates mixture into a labeled cryovial.
12. Freeze cell aggregates using either:
    - A standard slow rate-controlled cooling protocol that reduces temperatures at approximately -1°C/min, followed by long-term storage at -135°C (liquid nitrogen) or colder. Long-term storage at -80°C is not recommended.
    - A multi-step protocol in which cells are kept at -20°C for 2 hours, followed by -80°C for 2 hours, followed by long-term storage at -135°C (liquid nitrogen) or colder.

8.0 Troubleshooting

When culturing naïve-like cells in RSeT™ Medium, most problems can be addressed by assessing a small number of key parameters. iMEF source and plating density have a strong effect on the quality of naïve-like hPSC cultures. iMEFs should be screened to be ES compatible and the optimum plating density from each unique source should be determined. When passaging cells, the split ratio being used will also have an effect on the quality of the cultures. It is important to note that if there is a decrease in cell growth or in naïve-like qualities of cells cultured in RSeT™ Medium, cultures can be rescued by subsequent careful passaging with the appropriate split ratios.

PROBLEM	SOLUTION
Poor reversion and high heterogeneity of colonies in the culture	• Re-examine quality of starting prime cultures; perform expression analysis of pluripotency markers on cultures • Incompatible or low-density iMEFs could contribute to this; screen an alternative MEF source
Increased differentiation during maintenance of naïve-like hPSCs	• Prime cultures may have been low-quality; start with high-quality prime cultures • Incompatible iMEFs could contribute to this; screen an alternative MEF source • Over-confluent cultures can lead to increased differentiation; passage naïve-like hPSCs earlier
Low viability during passaging/ Slow growth of naïve-like hPSCs	• Optimal passaging ratio is important to maintain good viability of the cultures; passaging the cultures at low split ratios (higher density) may improve the growth of the cells
Increased primed morphology in naïve cultures	• Possibly due to low MEF density; increase MEF density

9.0 References

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