

TECHNICAL MANUAL

Reversion and Maintenance of Naïve-Like Human Pluripotent Stem Cells with NaïveCult™ Induction Kit and NaïveCult™ Expansion Medium

Critical Parameters for Successful Cell Culture with NaïveCult™ Media

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 the NaïveCult™ protocol. For suggestions on measuring pluripotency of primed cultures, refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1, available at www.stemcell.com or contact us to request a copy.

Choosing Appropriate Mouse Embryonic Fibroblasts (MEFs) for Feeder Layers

Achieving successful reversion and maintenance of naïve-like hPSCs requires a suitable and ES-qualified source of MEFs for feeder layers. Unsuitable MEFs will lead to unsuccessful reversion of primed hPSCs and increased differentiation. Each unique source of ES-qualified MEFs should be mitotically inactivated (i.e. iMEFs) and tested to determine the optimum plating density for supporting culture of naïve-like cells. iMEFs from a single source should also be monitored for batch-to-batch variation. iMEFs should be plated 1 - 2 days before co-culture with hPSCs. It is recommended to use day E12.5 iMEFs derived from either the CF-1 or DR4 mouse strains.

Hypoxic Culture Conditions

The protocols outlined in this manual for reversion and maintenance of naïve-like hPSCs using NaïveCult™ Induction Kit and NaïveCult™ Expansion Medium require the cultures to be incubated at 37°C under **hypoxic conditions (5% O₂, 5% CO₂)**. Culturing naïve-like hPSCs in NaïveCult™ media under normoxic conditions (20% O₂, 5% CO₂) 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, increased spontaneous differentiation may occur as shown in Figure 4. To maintain good-quality undifferentiated naïve-like hPSCs, it is important to use appropriate split ratios (see section 7.4). Naïve-like hPSCs should be passaged when colonies are typically 50 - 150 µm in diameter (Figure 5). If allowed to grow too large, colonies will tend to lose their naïve-like morphology.

Medium Changes

Daily medium changes are typically required to maintain high-quality naïve-like hPSCs. Media should be protected from light and warmed to room temperature (15 - 25°C) before use.

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1.0 Introduction

PSCs 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 ES cells are dependent on culture media that contain leukemia inhibitory factor (LIF), while conventional primed 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

NaïveCult™ was developed in collaboration with the Cambridge Stem Cell Institute at the University of Cambridge, UK. NaïveCult™ Induction Kit (Catalog #05580), developed based on Guo et al. (2017), is composed of defined cell culture media used sequentially for the transgene-independent or chemical reversion of primed hPSCs to a naïve-like, or reset, state.⁸ NaïveCult™ Induction Kit requires the use of a histone deacetylase inhibitor (HDACi), typically sodium butyrate or valproic acid, during the initial stage of naïve state induction. NaïveCult™ Expansion Medium (Catalog #05590) has been developed for the maintenance and expansion of transgene-independent, transgene-dependent, and embryo-derived naïve-like hPSCs in t2iL + Gö conditions.^{4,7,8} NaïveCult™ media contain pre-screened high-quality components and are compatible with human ES and iPS cells.

hPSCs reverted using NaïveCult™ Induction Kit and expanded in NaïveCult™ Expansion 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, KLF17, and TFCP2L1 show increased expression in hPSCs cultured in NaïveCult™ Expansion Medium and demonstrate global gene expression profiles consistent with published results.^{4,7,8} hPSCs maintained in NaïveCult™ Expansion Medium can be converted back to a primed state by culture in mTeSR™ 1 (Catalog #85850) or TeSR™-E8™ (Catalog #05990) and can then be differentiated using STEMdiff™ products such as STEMdiff™ SMADi Neural Induction Kit (Catalog #08581), STEMdiff™ Mesoderm Induction Medium (Catalog #05220), or STEMdiff™ Trilineage Differentiation Kit (Catalog #05230).

2.0 Glossary of Terms

ABBREVIATION	DESCRIPTION
bFGF	Basic fibroblast growth factor
D-PBS	Dulbecco's phosphate-buffered saline
ES cells	Embryonic stem cells
FBS	Fetal bovine serum
HDACi	Histone deacetylase inhibitor
hPSCs	Human pluripotent stem cells
iMEF	Inactivated mouse embryonic fibroblast
iPS cells	Induced pluripotent stem cells
LIF	Leukemia inhibitory factor
PSCs	Pluripotent stem cells

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3.0 Materials, Reagents, and Equipment

3.1 NaïveCult™ Induction Kit (Catalog #05580)

For component storage and stability information, refer to the Product Information Sheet (PIS) for NaïveCult™ Induction Kit, available at www.stemcell.com or contact us to request a copy.

COMPONENT NAME	COMPONENT #	QUANTITY
NaïveCult™ Induction Basal Medium	05581	76 mL
NaïveCult™ 20X Induction Supplement A*	05582	4 mL
NaïveCult™ 1000X Induction Supplement B**	05584	80 µL
NaïveCult™ 5X Induction Supplement C*	05583	200 mL
NaïveCult™ 1000X Induction Supplement D**	05585	500 µL
NaïveCult™ Expansion Basal Medium	05591	2 x 400 mL
NaïveCult™ 1000X Expansion Supplement**	05593	500 µL

*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.

**Please refer to the Safety Data Sheet (SDS) for hazard information. This product contains components dissolved in dimethyl sulfoxide (DMSO). DMSO is a strong solvent and skin penetrant, and can transport many substances through the skin. DMSO can also penetrate some protective glove materials including latex and silicone. Extra caution should be utilized when handling this product.

3.2 NaïveCult™ Expansion Medium (Catalog #05590)

For component storage and stability information, refer to the PIS for NaïveCult™ Expansion Medium, available at www.stemcell.com or contact us to request a copy.

COMPONENT NAME	COMPONENT #	QUANTITY
NaïveCult™ Expansion Basal Medium	05591	400 mL
NaïveCult™ 5X Expansion Supplement*	05592	100 mL
NaïveCult™ 1000X Expansion Supplement**	05593	500 µL

*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.

**Please refer to the Safety Data Sheet (SDS) for hazard information. This product contains components dissolved in dimethyl sulfoxide (DMSO). DMSO is a strong solvent and skin penetrant, and can transport many substances through the skin. DMSO can also penetrate some protective glove materials including latex and silicone. Extra caution should be utilized when handling this product.

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3.3 Additional Required Materials

PRODUCT	CATALOG #
0.1% Gelatin in Water	07903
CryoStor® CS10 OR FreSR™-S	07930 OR 05859
DMEM/F-12 with 15 mM HEPES	36254
D-PBS (Without Ca++ and Mg++)	37350
Falcon® Conical Tubes, 15 mL	38009
Falcon® 6-Well Flat-Bottom Plate, Tissue Culture-Treated	38016
Fetal bovine serum (FBS)	e.g. Sigma Aldrich 12103C
Gentle Cell Dissociation Reagent	07174
Inactivated CF-1 or DR4 mouse embryonic fibroblasts (iMEFs) E12.5	Qualified source
mTeSR™ 1 OR TeSR™-E8™	85850 OR 05990
Nunc™ Biobanking and Cell Culture Cryogenic Tubes	Fisher Scientific 377267
Trypan Blue	07050
TrypLE™ OR ACCUTASE™	Gibco 12605028 OR 07920
Valproic Acid OR Sodium Butyrate	72292 OR 72242
Y-27632	72304

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

3.4 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% CO₂ 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% O₂ and 5% CO₂
- Low-speed centrifuge with a swinging bucket rotor
- Pipette-aid with appropriate serological pipettes (e.g. 2 mL, Catalog #38002)
- 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 (LN₂) vapor tank
- Refrigerator (2 - 8°C)

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4.0 Procedure Diagram

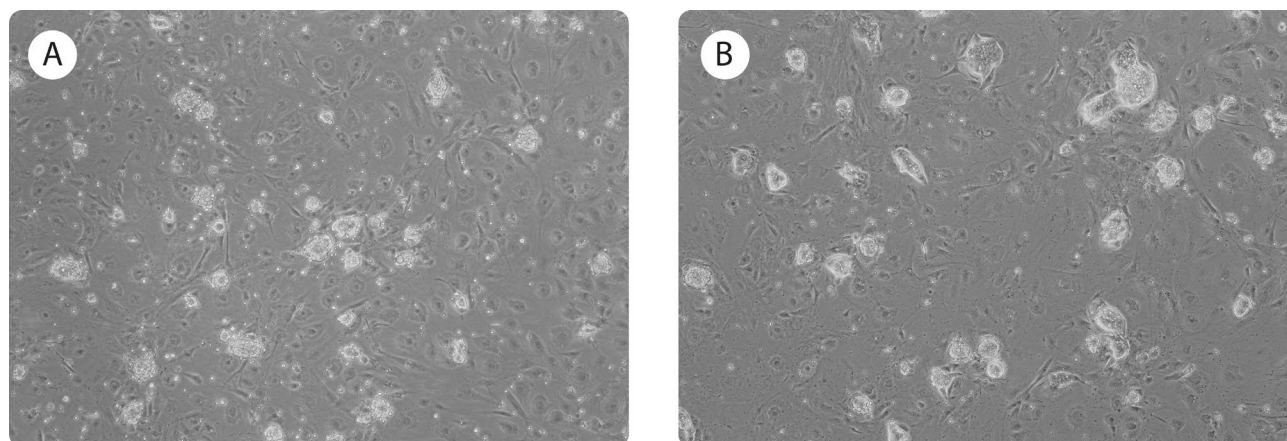
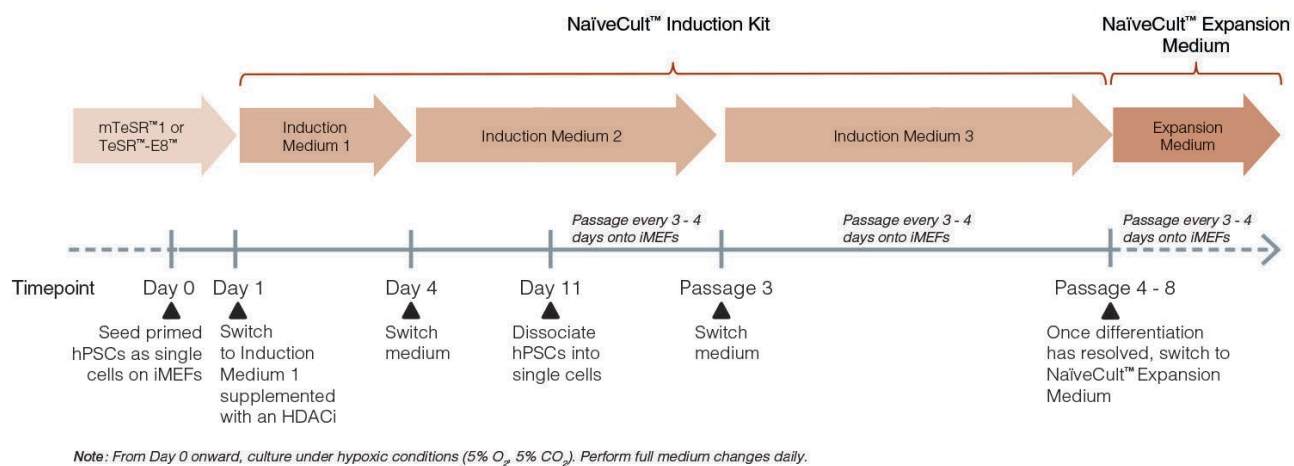


Figure 1. Morphology of Naïve-Like Reset hPSCs Following Reversion

mTeSR™ 1-cultured **(A)** H9 human ES cells and **(B)** WLS-1C human iPS cells following reversion in NaïveCult™ Induction Kit and maintained in NaïveCult™ Expansion Medium exhibit a tightly packed, domed morphology with refractive edges.

5.0 Preparation of Reagents and Materials

The reagents and materials in this section are required in the protocols in section 7.0. Prepare as needed.

5.1 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 6-well tissue culture-treated plate. If using other cultureware, adjust volumes accordingly. For instructions on preparation of iMEFs, refer to the Technical Manual: In Vitro Hematopoietic Differentiation of Mouse ES and iPS Cells Using ES-Cult™, available at www.stemcell.com or contact us to request a copy.

1. Prepare MEF medium (DMEM/F-12 + 10% FBS). Aliquot sufficient MEF medium and warm to room temperature (15 - 25°C).
2. Prepare a gelatin-coated plate by adding 1 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 (e.g. Catalog #38002) 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 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 and a hemocytometer, or an alternative cell counting method.
10. Aspirate gelatin solution from the plate prepared in step 2 and seed approximately 1.5 - 2.5 x 10⁵ iMEFs in 2 mL of MEF medium per well of a 6-well plate.

Note: Optimal feeder layer density may vary depending on the source and the embryonic developmental stage from which the iMEFs are derived. An iMEF confluency of approximately 85 - 90% is suitable for most cell lines (see Figure 2). It is recommended to use iMEFs derived from embryos at E12.5 from CF-1 or DR4 mouse strains.

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% O₂, 5% CO₂)** overnight to allow the iMEFs to adhere.

Note: The iMEFs will adhere within 4 - 5 hours, but ideally should be incubated for 18 - 48 hours before use. Plates may be used up to 4 days post seeding.

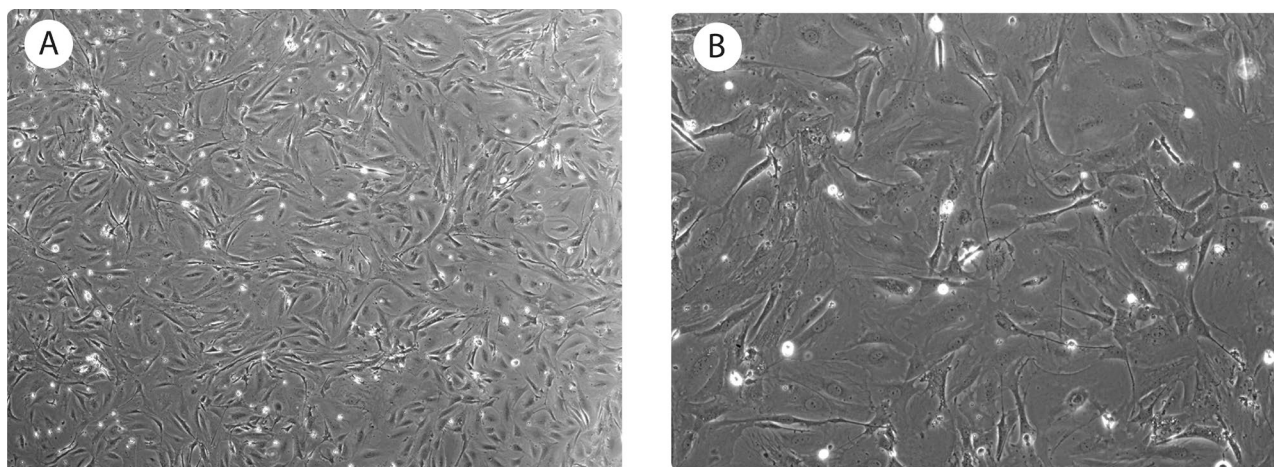


Figure 2. Mitomycin C-Inactivated CF-1 Mouse Embryonic Feeder Cells (iMEFs). Cultured for 24 hours on 0.1% gelatin in DMEM/F-12 + 10% FBS. Magnification 40X (A) and 100X (B).

5.2 Induction Medium 1

This medium is for use on Day 1 to Day 3 of the induction protocol.

Use sterile technique when preparing Induction Medium 1 (NaïveCult™ Induction Basal Medium + NaïveCult™ 20X Induction Supplement A + NaïveCult™ 1000X Induction Supplement B + [Valproic Acid or Sodium Butyrate]). Prepare sufficient medium for 3 days, allowing 2 mL of medium per well per day. The following example is for preparing 80 mL of medium. If preparing other volumes, adjust accordingly.

1. Thaw supplements at room temperature (15 - 25°C) or at 2 - 8°C overnight. Mix each supplement thoroughly.

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

2. Add 4 mL of Induction Supplement A and 80 µL of Induction Supplement B to 76 mL of NaïveCult™ Induction Basal Medium. Mix thoroughly.

Note: If not used immediately, store Induction Medium 1 (without HDACi) at 2 - 8°C for up to 7 days.

3. **Immediately before use**, supplement medium with an HDACi (either Valproic Acid or Sodium Butyrate).

Note: Most hPSC lines are induced at HDACi concentrations between 0.5 - 1.0 mM (0.75 mM works broadly across multiple hPSC lines). It is recommended that customers perform a titration for new hPSC cell lines, as cytotoxicity can be observed at higher concentrations.

4. Warm medium to room temperature (15 - 25°C) before use. Protect from exposure to direct light.

5.3 Induction Medium 2

This medium is for use on Day 4 to the end of Passage 2 of the protocol.

Use sterile technique when preparing Induction Medium 2 (NaïveCult™ Expansion Basal Medium + NaïveCult™ 5X Induction Supplement C + NaïveCult™ 1000X Induction Supplement D). Prepare sufficient medium for up to 7 days, allowing 2 mL of medium per well per day. The following example is for preparing 150 mL of medium. If preparing other volumes, adjust accordingly.

1. Thaw supplements at room temperature (15 - 25°C) or at 2 - 8°C overnight. Mix each supplement thoroughly.

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

2. Add 30 mL of NaïveCult™ 5X Induction Supplement C and 150 µL of NaïveCult™ 1000X Induction Supplement D to 120 mL of NaïveCult™ Expansion Basal Medium. Mix thoroughly.

Note: If not used immediately, store Induction Medium 2 at 2 - 8°C for up to 7 days. Warm medium to room temperature (15 - 25°C) before use. Protect from exposure to direct light.

5.4 Induction Medium 3

This medium is for use from Passage 3 onwards, prior to transfer of cultures to expansion medium (typically between Passage 4 and Passage 8).

Use sterile technique when preparing complete NaïveCult™ Induction Medium 3 (NaïveCult™ Expansion Basal Medium + NaïveCult™ 5X Induction Supplement C + NaïveCult™ 1000X Expansion Supplement). Prepare sufficient medium for up to 7 days, allowing 2 mL of medium per well per day. The following example is for preparing 150 mL of medium. If preparing other volumes, adjust accordingly.

1. Thaw supplements at room temperature (15 - 25°C) or at 2 - 8°C overnight. Mix each supplement thoroughly.

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

2. Add 30 mL of NaïveCult™ 5X Induction Supplement C and 150 µL of NaïveCult™ 1000X Expansion Supplement to 120 mL of NaïveCult™ Expansion Basal Medium. Mix thoroughly.

Note: If not used immediately, store Induction Medium 3 at 2 - 8°C for up to 7 days. Warm medium to room temperature (15-25°C) before use. Protect from exposure to direct light.

5.5 NaïveCult™ Expansion Medium

This medium is for the expansion of established naïve-like hPSC cultures.

Use sterile technique when preparing NaïveCult™ Expansion Medium (NaïveCult™ Expansion Basal Medium + NaïveCult™ 5X Expansion Supplement + NaïveCult™ 1000X Expansion Supplement). Prepare sufficient medium for up to 7 days, allowing 2 mL of medium per well per day. The following example is for preparing 150 mL of medium. If preparing other volumes, adjust accordingly.

1. Thaw supplements at room temperature (15 - 25°C) or at 2 - 8°C overnight. Mix each supplement thoroughly.

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

2. Add 30 mL of NaïveCult™ 5X Expansion Supplement and 150 µL of NaïveCult™ 1000X Expansion Supplement to 120 mL of NaïveCult™ Expansion Basal Medium. Mix thoroughly.

Note: If not used immediately, store NaïveCult™ Expansion Medium at 2 - 8°C for up to 7 days. Warm medium to room temperature (15 - 25°C) before use. Protect from exposure to direct light.

6.0 Culture of hPSCs in mTeSR™1 or TeSR™-E8™

The protocols outlined in this manual describe the reversion of primed hPSCs cultured in mTeSR™1 or TeSR™-E8™ under feeder-free conditions. For complete instructions on maintaining hPSCs in mTeSR™1 or TeSR™-E8™, refer to the Technical Manuals for mTeSR™1 or TeSR™-E8™, available at www.stemcell.com or contact us to request a copy.

7.0 Procedures for the Establishment of Reset Naïve hPSCs Using NaïveCult™ Induction Kit and Expansion of Reset Naïve hPSCs in NaïveCult™ Expansion Medium

7.1 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 starting primed ES or iPS culture. Primed cultures must not contain more than 20% differentiated cells. Thus, we recommend culturing primed human hPSCs in mTeSR™1 or TeSR™-E8™ 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, available at www.stemcell.com or contact us to request a copy.

Refer to section 7.3 for images of cells at various stages of the reversion process.

7.1.1 Medium Change for iMEF Feeder Layer (Day 0)

Note: Prepare iMEF feeder layer as per section 5.1. For optimal results, seed iMEFs 48 hours prior to use.

1. Aliquot sufficient mTeSR™1 or TeSR™-E8™. Warm to room temperature (15 - 25°C) before use.
2. Aspirate MEF medium from a 6-well iMEF-coated plate.
3. Wash each well with 1 mL of D-PBS. Aspirate D-PBS.
4. Repeat step 3.
5. Add 1 mL of mTeSR™1 or TeSR™-E8™ supplemented with 10 µM Y-27632 per well and incubate at 37°C under **normoxic conditions (20% O₂, 5% CO₂)** while completing section 7.1.2.

7.1.2 Passaging mTeSR™1-Cultured hPSCs as Single Cells (Day 0)

The following are instructions for passaging mTeSR™1- or TeSR™-E8™-cultured hPSCs from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly.

1. Aliquot sufficient mTeSR™1 or TeSR™-E8™. Warm to room temperature (15 - 25°C) before use. Supplement medium with 10 µM Y-27632.
2. Use a microscope to visually identify regions of differentiation in a plate of mTeSR™1- or TeSR™-E8™-cultured hPSCs. Mark these using a felt tip or lens marker on the bottom of the plate.
3. 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 of differentiation may not be required if differentiation is < 5%. Differentiation should not exceed 20% of the well if the culture is of high quality.

4. Aspirate medium from the well and add 1 mL of Gentle Cell Dissociation Reagent per well of a 6-well plate. Incubate at 37°C for 8 - 10 minutes.

Note: If using an alternative dissociation reagent, refer to the appropriate Product Information Sheet for instructions.

5. Carefully pipette the cell and aggregate mixture up and down to break up the aggregates and obtain a single-cell suspension.
6. Transfer the detached cells and aggregates to a 15 mL conical tube (e.g. Catalog #38009) containing an equal volume of mTeSR™1 or TeSR™-E8™.

7. Optional: Rinse the well with an additional 1 mL of mTeSR™1 or TeSR™-E8™ to collect remaining cells and any aggregates. Add the rinse to the 15 mL tube from step 6.
Note: Centrifugation of cells and aggregates is not required.
8. Perform a viable cell count using Trypan Blue and a hemocytometer, or an alternative cell counting method.
9. Adjust cell concentration to 5×10^4 to 2×10^5 cells per mL of mTeSR™1 or TeSR™-E8™.
10. Seed 1 mL of cell suspension per well of the 6-well plate prepared in section 7.1.1. If using other cultureware, adjust accordingly.
11. Incubate at 37°C under **hypoxic conditions (5% O₂, 5% CO₂)** for 24 hours.

Day 1 - 10

7.1.3 Reversion

1. **Day 1:** Prepare Induction Medium 1 (without HDACi) (section 5.2 steps 1 - 2). Aliquot sufficient medium (2 mL per well).
2. Supplement aliquoted medium with an HDACi (section 5.2 step 3).
3. Remove plate from incubator (from section 7.1.2) and aspirate medium. Add 2 mL of Induction Medium 1(+ HDACi) per well.
4. Incubate at 37°C under **hypoxic conditions (5% O₂, 5% CO₂)**.
5. **Day 2 and Day 3:** Perform a full-medium change daily with Induction Medium 1 (+ HDACi). Incubate at 37°C under **hypoxic conditions (5% O₂, 5% CO₂)**.
6. **Day 4:** Prepare Induction Medium 2 (see section 5.3). Aliquot sufficient Induction Medium 2 (2 mL per well). Perform a full-medium change with Induction Medium 2. Incubate at 37°C under **hypoxic conditions (5% O₂, 5% CO₂)** for 24 hours.
7. **Day 5 - 10:** Perform a full-medium change daily with Induction Medium 2. Incubate at 37°C under **hypoxic conditions (5% O₂, 5% CO₂)**.
8. **Day 9:** Prepare a fresh iMEF feeder layer on a gelatin-coated plate (see section 5.1), to be used for passaging on Day 11 (section 7.2).
9. **Day 11:** Proceed to section 7.2 for passaging.

7.2 Passing Reset Naïve hPSCs

Cells should be passaged for the first time on Day 11 by transferring all the cells to a new well of iMEFs (prepared on Day 9, section 7.1.3).

1. Aliquot Induction Medium 2, allowing 2.5 mL per well to be passaged.
2. Add Y-27632 to a final concentration of 10 µM. Warm to room temperature (15 - 25°C) before use.
3. Aspirate medium from wells to be passaged and rinse with 2 mL of D-PBS.
4. Aspirate D-PBS. Add 500 µL of TrypLE™ or ACCUTASE™ per well.
5. Incubate at 37°C for approximately 3 minutes.
Note: Use either normoxic (20% O₂, 5% CO₂) or hypoxic (5% O₂, 5% CO₂) conditions for this incubation.
6. Add 500 µL of Induction Medium 2 + Y-27632 per well to stop the enzymatic agent.
7. Using a 1000 µL pipette tip, gently pipette up and down twice to dislodge the naïve cells from the well.
8. Transfer the cells to a 15 mL conical tube, taking care to leave behind any large pieces of iMEF sheet.

9. Centrifuge the cells at 300 x g for 5 minutes.
10. Remove supernatant and resuspend the cells in 2 mL of Induction Medium 2 + 10 µM Y-27632 per well passaged.
11. Aspirate medium from a 6-well iMEF-coated plate (prepared on Day 9).
12. Wash each well twice with 2 mL of D-PBS. Aspirate D-PBS.
13. Add 2 mL of cell suspension to each well. Incubate at 37°C under **hypoxic conditions (5% O₂, 5% CO₂)** for 24 hours.
14. Perform a full-medium change daily with Induction Medium 2 (**without** Y-27632).
15. Cells can be passaged every 3 - 4 days depending on the growth of the cells (this varies with cell line).

Note: High levels of background differentiation are common between Passages 1 - 3. Cells are typically passaged at a 1:1 to 1:3 ratio for the first 3 passages. When assessing the appropriate time to passage hPSCs cultured in NaïveCult™ media, colony size is a good indicator. In general, when the majority of colonies have reached a diameter of 50 - 150 µm, the culture requires passaging. Small colonies passaged too early may result in decreased viability. Alternatively, larger colonies may start to exhibit increased spontaneous differentiation. In general, naïve-like hPSCs tend to require passaging after 3 - 4 days of culture in NaïveCult™ media; in extenuating circumstances, cells can be passaged as early as Day 2 or as late as Day 6. Passaging later than Day 6 is not recommended, due to the resulting decrease in feeder viability.

16. For Passage 3, proceed to section 7.2.1.

7.2.1 Maintenance of Reset Naïve hPSCs (Passage 3 - Passage 8) in Induction Medium 3

Cells can be passaged every 3 - 4 days depending on the growth of the cells (this varies with cell line).

Note: Prepare iMEF-coated plates one day prior to passaging cells (see section 5.1).

1. Prepare Induction Medium 3 (see section 5.4), allowing 2.5 mL per well to be passaged.
2. Add Y-27632 to a final concentration of 10 μ M. Warm to room temperature (15 - 25°C) before use.
3. Aspirate medium from wells to be passaged and rinse with 2 mL of D-PBS.
4. Aspirate D-PBS. Add 500 μ L of TrypLE™ or ACCUTASE™ per well.
5. Incubate at 37°C for approximately 3 minutes.
Note: Use either normoxic (20% O₂, 5% CO₂) or hypoxic (5% O₂, 5% CO₂) conditions for this incubation.
6. Add 500 μ L of Induction Medium 3 + Y-27632 per well to stop the enzymatic agent.
7. Using a 1000 μ L pipette tip, gently pipette up and down 2 - 3 times to dislodge the naïve cells from the well.
8. Transfer the cells to a 15 mL conical tube, taking care to leave behind any large pieces of iMEF sheet.
9. Centrifuge the cells at 300 x g for 5 minutes.
10. Remove supernatant and resuspend the cells in 2 mL of Induction Medium 3 + 10 μ M Y-27632 per well passaged.
11. Aspirate MEF medium from a 6-well iMEF-coated plate.
12. Wash each well twice with 2 mL of D-PBS. Aspirate D-PBS.
13. Add 2 mL of cell suspension to each well. Incubate at 37°C under **hypoxic conditions (5% O₂, 5% CO₂)** for 24 hours.
14. Perform a full-medium change daily with Induction Medium 3 (**without** Y-27632).
15. Cells can be passaged every 3 - 4 days depending on the growth of the cells (this varies with cell line).
16. Between Passages 3 and 7 the level of background differentiation will reduce and disappear. At this point the cultures may be transferred to NaïveCult™ Expansion Medium for maintenance and expansion (see section 7.4).

7.3 Morphology of Cells Cultured in NaïveCult™ Induction Kit: Reversion to Stable Naïve-Like hPSCs

7.3.1 Characteristics of Cultures During the Reversion Process

During the 10-day stepwise reversion process using NaïveCult™ Induction Kit, the morphology of hPSC colonies will begin to change. After passaging the primed hPSCs as single cells from feeder-free mTeSR™1 or TeSR™-E8™ cultures onto iMEF feeder layers, small colonies can be observed within 24 hours. These generally exhibit primed hPSC morphology. After transitioning the cells from mTeSR™1 or TeSR™-E8™ to Induction Medium 1, the colonies continue to grow and maintain their primed hPSC morphology. Some cell death may occur during this step. However, during culture with Induction Medium 2, areas of phase-bright hPSC colonies begin to appear and become increasingly prominent. By Day 11 the cultures should be ready to be passaged (Figure 3). Over subsequent passages, the cultures will become homogeneous populations of densely packed colonies with a raised 3-dimensional (dome-shaped) morphology. Cells that do not revert will differentiate and will be lost during passaging.

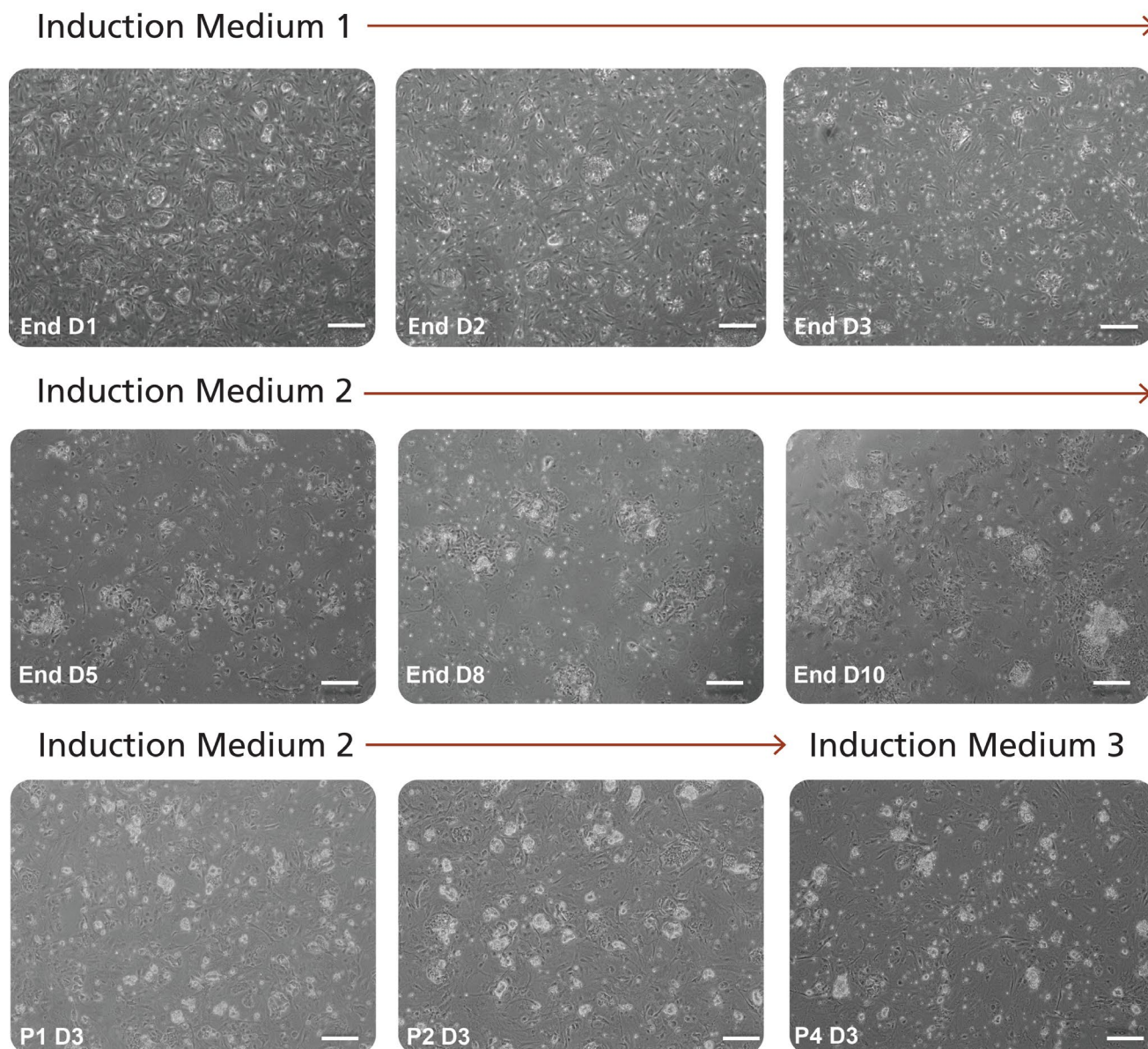


Figure 3. Time Course of Changes in hPSC Colony Morphology During Reversion Using NaïveCult™ Induction Kit. At Day 0, hPSCs cultured for 24 hours in mTeSR™1 on feeder cells maintain a primed hPSC morphology. The medium is replaced with Induction Medium 1 on Day 1 and is changed daily until Day 4 when cells are transitioned to Induction Medium 2. During this time, areas of phase-bright colonies will begin to appear and expand. By Day 11, the colonies are generally large enough to be passaged. Reset naïve hPSCs should be passaged every 3 - 4 days initially in Induction Medium 2 and then in Induction Medium 3 starting on Passage 3. Scale bars represent 250 µm.

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

The morphology of cultures in NaïveCult™ Induction Media 2 and 3 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 early-passage cultures (Figure 4). Morphology from passage to passage is 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.

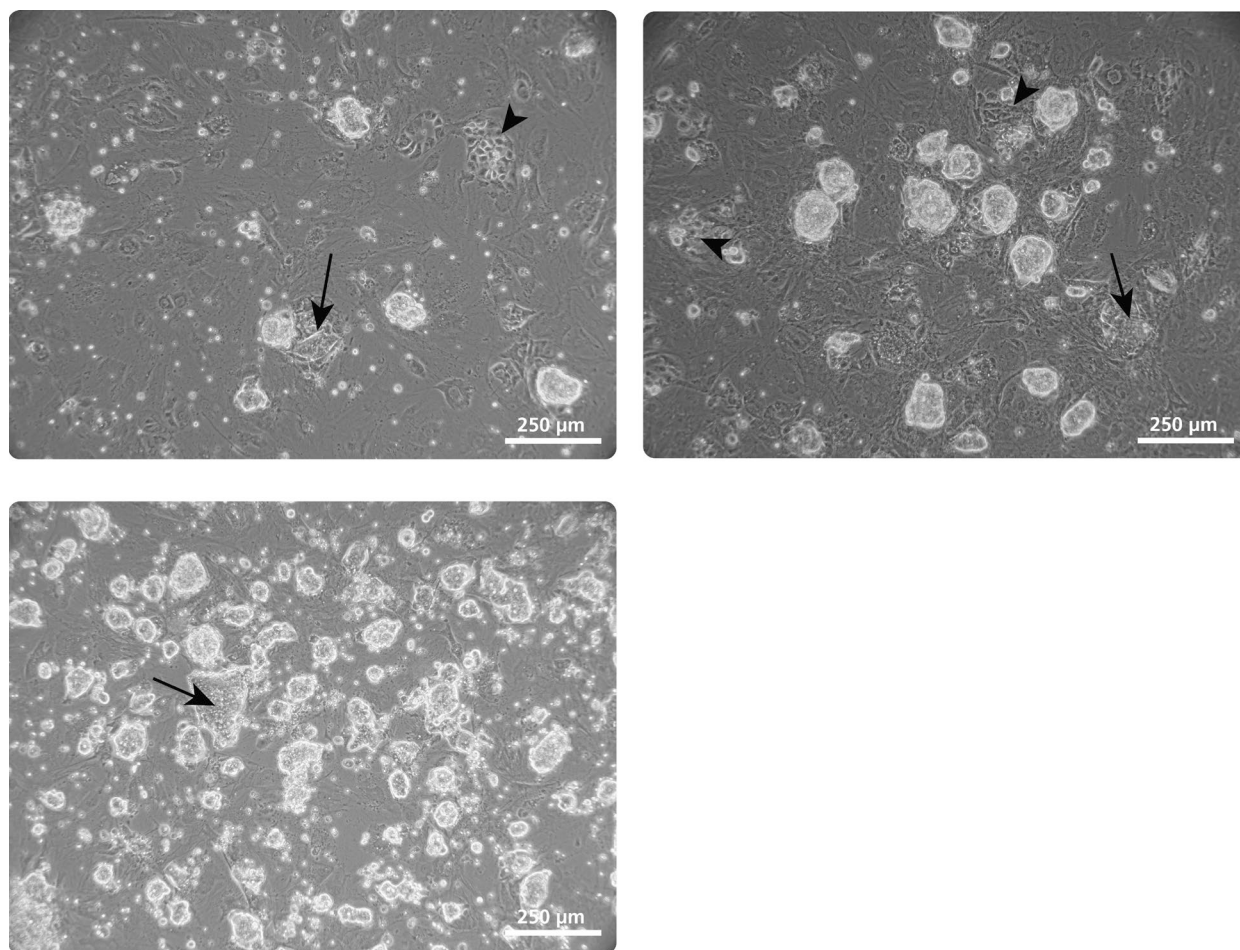


Figure 4. Areas of Differentiation and Flattened Morphology in NaïveCult™ Cultures

H9 human ES cells maintained in NaïveCult™ Induction Medium 3. During early passages of naïve-like hPSCs in NaïveCult™ media, colonies can appear flattened (arrows). Areas of differentiated cells can also be present, which exhibit a phase-dark morphology surrounding the dome-shaped colonies (examples indicated with arrowheads).

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7.3.3 Morphology of Stable Naïve-Like hPSCs

Stable naïve-like hPSCs in Induction Medium 3 have been observed as early as Passage 4; in some cases achieving stable morphology requires culturing to Passage 8. 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). Additionally, Figures 5A, 5B, and 5D show appropriate diameter for passaging (50 - 150 μm).

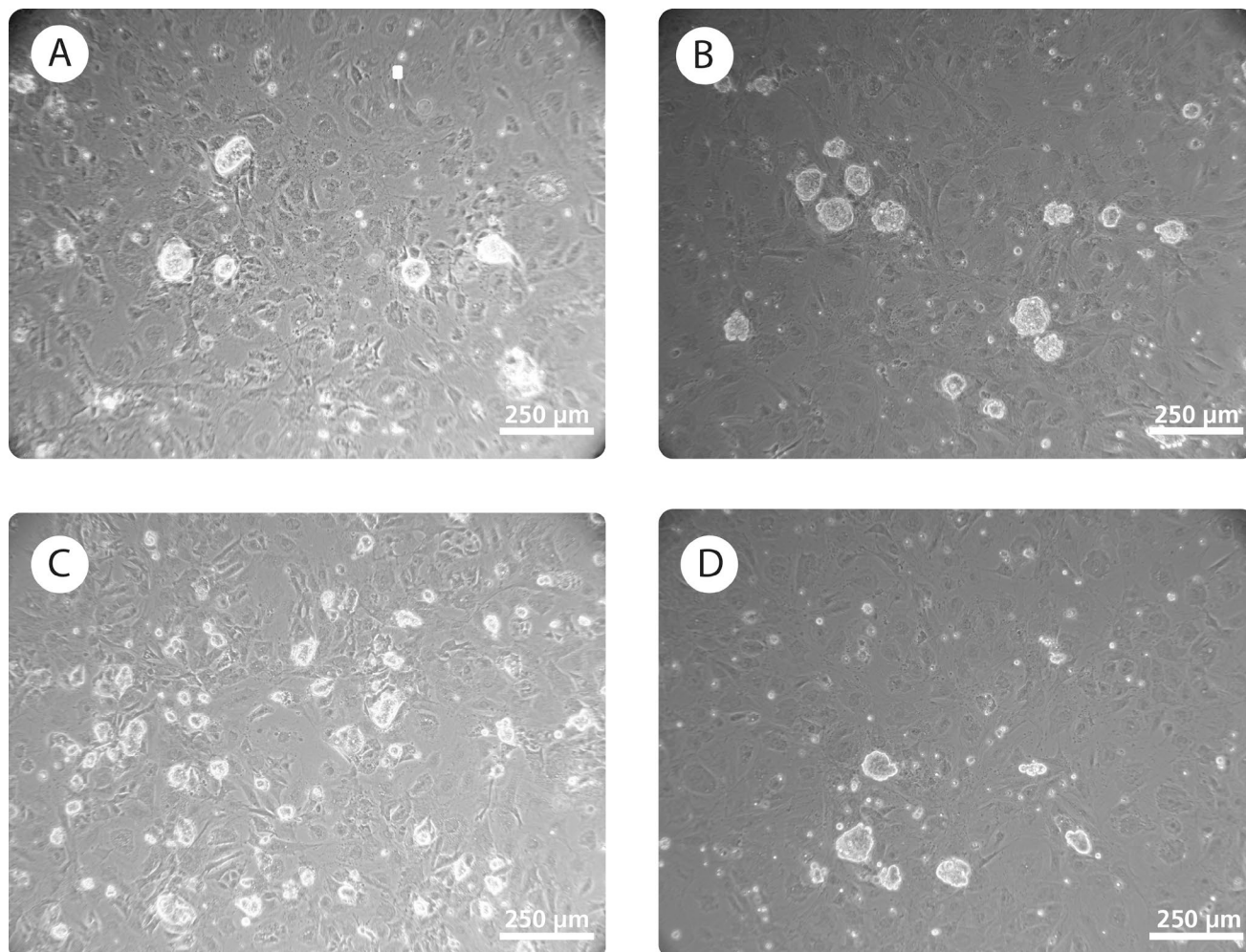


Figure 5. Stable NaïveCult™ Cell Lines Can be Achieved by Passage 4 - 8

Human ES cell lines (A) H1 and (B) H9 maintain a naïve-like morphology in NaïveCult™ Induction Medium 3. Human iPS cell lines (C) WLS-1C and (D) STiPS- F016 maintain a naïve-like morphology in NaïveCult™ Induction Medium 3.

7.4 Passaging Naïve-Like hPSCs onto iMEF-Coated Plates in NaïveCult™ Expansion Medium

Between Passages 4 - 8 in Induction Medium 3, background differentiation will reduce and disappear. At this point the cultures may be passaged into NaïveCult™ Expansion Medium for maintenance and expansion. In NaïveCult™ Expansion Medium, passaging is usually done every 3 - 4 days using a 1:2 to 1:5 split ratio. Reset hPSCs derived using transgenes or from embryos can also be expanded in NaïveCult™ Expansion Medium.

Note: Prepare iMEF-coated plates 1 - 3 days prior to passaging (see section 5.1).

1. Prepare NaïveCult™ Expansion Medium (see section 5.5). Aliquot sufficient NaïveCult™ Expansion Medium and warm to room temperature (15 - 25°C) before use.
2. Add Y-27632 to NaïveCult™ Expansion Medium to a final concentration of 10 µM (prepare 2 mL per well to be passaged).
3. Aspirate MEF medium from a 6-well iMEF-coated plate.
4. Wash each well twice with 2 mL of D-PBS. Aspirate D-PBS.
5. Add 2 mL of NaïveCult™ Expansion Medium + 10 µM Y-27632 per well.

Passaging Naïve-Like hPSCs

6. Aspirate NaïveCult™ Expansion Medium from wells containing naïve-like hPSCs (from section 7.2.1).
7. Wash each well once with 1 mL of D-PBS. Aspirate D-PBS.
8. Add 500 µL of TrypLE™ or ACCUTASE™ to each well.
9. Incubate at 37°C for approximately 3 minutes.

Note: Use either normoxic (20% O₂, 5% CO₂) or hypoxic (5% O₂, 5% CO₂) conditions for this incubation.

10. Add 500 µL of NaïveCult™ Expansion Medium to each well to inactivate the passaging reagent.
11. Using a 1000 µL pipette tip, loosen the iMEF sheet and remove from the well. Gently pipette up and down 2 - 3 times to dislodge cells from the well.

Note: Do not excessively pipette up and down, as this may decrease cell viability. The cell suspension should contain a mixture of single cells and very small cell aggregates of approximately 2 - 5 cells, as well as iMEFs that may remain visibly clumped together.

12. Transfer cells to a 15 mL conical tube.
13. Rinse each well with an additional 1 mL of NaïveCult™ Expansion Medium and transfer to the conical tube containing the harvested cells.
14. Centrifuge tube(s) at 300 x g for 5 minutes.
15. Remove supernatant and gently resuspend the cell pellet in 2 mL of NaïveCult™ Expansion Medium.
16. Plate cells onto the iMEF-coated plate prepared in step 5. Typically, 1:2 to 1:5 split ratios can be used for cells cultured in NaïveCult™ Expansion Medium (seeding with 1 x 10⁵ - 2 x 10⁵ cells per well of a 6-well plate).

Note: Optimal density for passaging is shown in Figure 5, when colonies reach approximately 50 - 150 µm in diameter.

17. Incubate at 37°C under **hypoxic conditions (5% O₂, 5% CO₂)**.
18. Perform a daily full-medium change with NaïveCult™ Expansion Medium and visually assess cultures to monitor growth until the next passage.

Note: It is recommended that cells are not maintained beyond 30 passages, as karyotypic abnormalities can occur. Routine screening for karyotypic stability is recommended.

8.0 Cryopreservation of Naïve-Like hPSCs

1. Aspirate NaïveCult™ Expansion Medium from wells containing naïve-like hPSCs.
2. Wash each well once with 1 mL of D-PBS. Aspirate D-PBS.
3. Add 500 µL of TrypLE™ or ACCUTASE™ to each well.
4. Incubate at 37°C for approximately 3 minutes.

Note: Use either normoxic (20% O₂, 5% CO₂) or hypoxic (5% O₂, 5% CO₂) conditions for this incubation.

5. Add 500 µL of NaïveCult™ Expansion Medium to the well.
6. Using a 1000 µL 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 - 5 cells, as well as iMEFs that may remain visibly clumped together.

7. Transfer to a 15 mL conical tube.
8. Rinse the well with a further 1 mL of medium and transfer to the conical tube containing the harvested cells.
9. Centrifuge at 300 x g for 5 minutes at room temperature (15 - 25°C).
10. Gently aspirate the supernatant, taking care not to disrupt the cell pellet.
11. Using a serological pipette, gently add 1 mL per well harvested of cold (2 - 8°C) CryoStor® CS10 or FreSR™-S to resuspend the pellet.
12. Transfer 1 mL of cell aggregate mixture into a labeled cryovial using a 2 mL serological pipette (e.g. Catalog #38002).
13. 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 (gas phase of 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 (gas phase of liquid nitrogen) or colder.

9.0 Troubleshooting

When culturing naïve-like cells in NaïveCult™ media, 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 for ES compatibility and the optimum plating density from each source should be determined. When passaging cells, the split ratio 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 NaïveCult™ Expansion Medium, cultures can be rescued by subsequent careful passaging with the appropriate split ratios.

PROBLEM	SOLUTION
Poor reversion of colonies	<ul style="list-style-type: none"> Re-examine quality of starting primed cultures; perform expression analysis of pluripotency markers on cultures Incompatible or low-density iMEFs could contribute to this; screen alternative MEF source Titrate starting cell density and concentration of valproic acid/sodium butyrate (HDACi). If cultures fail to adopt a domed morphology, a higher concentration of HDACi may be required. If too much cell death is observed, a lower concentration of HDACi may be required. Starting with a higher seeding density may also be helpful when there is high cell death.
Increased differentiation during maintenance of naïve-like hPSCs	<ul style="list-style-type: none"> Incompatible iMEFs could contribute to this; screen alternative MEF sources Over-confluent cultures can lead to increased differentiation; passage naïve-like hPSCs earlier or at a higher split ratio Switching to NaïveCult™ Expansion Medium too early can also result in increased differentiation during maintenance
Low viability during passaging/ Slow growth of naïve-like hPSCs	<ul style="list-style-type: none"> 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 Cells may be sensitive to the passaging reagent; decreasing the incubation time with passaging reagent may improve viability
Increased primed morphology in naïve cultures	<ul style="list-style-type: none"> Possibly due to low MEF density; increase MEF density

10.0 References

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TECHNICAL MANUAL

Reversion and Maintenance of Naïve-Like Human Pluripotent Stem Cells with NaïveCult™ Induction Kit and NaïveCult™ Expansion Medium



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