PluriSTEM-XF™ Human ES/iPS Medium

Product Manual for Catalog No.

SCM132

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

PluriSTEM-XF™ Human ES/iPS Medium is a complete xeno-free medium formulation for the feeder-free culture of human ES and iPS cells. Pluripotent cells maintained in this small molecule based medium exhibit high cell health, express high levels of pluripotency markers (NANOG, OCT3/4, SOX-2, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81), retain differentiation potential and possess a normal karyotype. PluriSTEM-XF™ Human ES/iPS Medium is provided as a stand-alone 500 mL bottle that is ready-to-use and does not require additional supplementation.

For a complete xeno-free culture system, PluriSTEM-XF™ Human ES/iPS Medium should be used with PluriSTEM-XF™ Recombinant Vitronectin (Cat. No. CC130).

Each lot of PluriSTEM-XF™ Human ES/iPS Medium is rigorously quality control tested for the ability to sustain undifferentiated human ES/iPS cells for $\geq$ 3 passages.

Note: Before starting PluriSTEM-XF™, users are recommended to read the manual carefully and completely. Colony morphology and growth rates may differ from other serum-free, feeder-free media culture conditions.

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Materials Provided

PluriSTEM-XF™ Human ES/iPS Medium (Cat. No. SCM132) Store at -20°C		
Component	**Item No.**	**Volume**
PluriSTEM-XF™ Human ES/iPS Medium	SCM132	500 mL

Materials Required But Not Supplied

1. 6-well tissue culture plates
2. PluriSTEM-XF™ Recombinant Vitronectin, 500 μg (Cat. No. CC130)
3. DMEM/F12, with HEPES, L-Glutamine, 500 mL (Cat. No. DF-041-B)
4. PluriSTEM-XF™ Freeze Medium (1X), 50 mL (Cat. No. SCM135)
5. PluriSTEM™ Dispase-II Solution, 100 mL (Cat. No. SCM133)
6. Enzyme Free Cell Dissociation Solution Hank's Based (1X), 100 mL (Cat. No. S-004-C)
7. EmbyroMax® 1X Dulbecco's Phosphate-Buffered Saline w/o Ca^{++} or Mg^{++}, 500 mL (Cat. No. BSS-1006-B).
8. Cell Scrapers (Sarstedt Cat No. 83.1832)

Storage and Stability

PluriSTEM-XF™ Human ES/iPS Medium is provided frozen and can be stored at -20°C until the expiration date on the product label. Before use, thaw PluriSTEM-XF™ medium at room temperature (15 – 25°C) or overnight at 2 – 8°C. **Do not thaw at 37°C.** Upon thawing, PluriSTEM-XF™ medium may be aseptically dispensed into working aliquots and stored at -20°C until the expiration date as indicated on the label. **Thawed aliquots may be stored at 2 – 8°C for up to 2 weeks.** Do not refreeze aliquots after thawing. Before use, warm working aliquots to room temperature. **Do not warm the medium in a 37°C water bath.**

Critical Success Factors in PluriSTEM-XF™ culture

The following guidelines are critical to ensuring success in culturing human pluripotent stem cells in PluriSTEM-XF™ Medium.

1. Use tissue-culture treated plates. Non-tissue culture treated plates is not compatible with PluriSTEM-XF™ medium.

2. Always start with high quality undifferentiated human ES and iPS culture. Before passaging, scrape away any areas that harbor a hint of differentiation even if it means sacrificing the majority of the colonies.

2. Pluripotent cells cultured in PluriSTEM-XF™ are maintained on recombinant human vitronectin rather than on matrigel, which contains animal-derived products. As human pluripotent cells are highly sensitive to environmental perturbation, it is critical to perform the following 2-step transition process. A more detailed step-by step protocol is detailed later in the section titled “Transition to PluriSTEM-XF™ on vitronectin-coated plates using Dispase II” on page 6.

   Step 1: Transition cells to the new ECM matrix (i.e. human vitronectin)

   When cells are ready to be passage, use Dispase II to passage the cells to freshly coated vitronectin plates in the **same media** that the cells had been cultured in before the passage. Do **not** use PluriSTEM-XF™ medium at this time. Feed cells daily with the same media that the cells had been cultured in before the passage (i.e. for example this may be mTeSR1 or StemPRO if cells had been cultured in mTeSR1 or StemPRO, respectively).

   Step 2: Transition cells to the new media (i.e. PluriSTEM-XF™ Medium)

   At the next passage, use Dispase II to passage the cells to freshly-coated vitronectin plates in the **same expansion media** as in Step 1 (i.e. for example, this may be mTeSR1 or StemPRO if cells had been cultured in mTeSR1 or StemPRO, respectively). Do **not use** PluriSTEM-XF™ medium at this time.

   Two to three days after passaging, exchange the media with PluriSTEM-XF™ Human ES/iPS Medium. Feed cells daily with PluriSTEM-XF™ Medium.

3. Exchange with fresh PluriSTEM-XF™ medium daily. Cultures should be fed with 4 – 5 mL per well PluriSTEM-XF™ medium on Friday to ensure sufficient medium to sustain the cells over the weekend. One medium change is required over the weekend.

4. Human ES and iPS cells cultured in PluriSTEM-XF™ medium may exhibit a more flat morphology compared to those cells cultured in other feeder-free system.

5. Colonies may grow slower in PluriSTEM-XF™ with some indications of peripheral differentiation between colonies. Cell lines may vary in the amount of peripheral differentiation observed. Allow colonies to grow until Day 7-8 by which time the majority of the peripheral differentiation will have lifted off, and are removed during media changes.

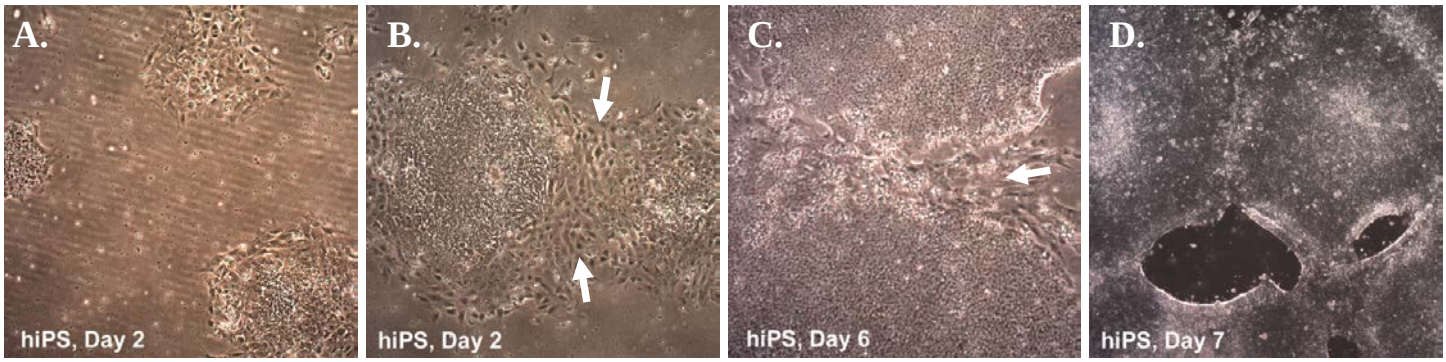

Figure 1. Human iPS cells cultured in PluriSTEM-XF™ medium on vitronectin-coated plates. Two days after passage, a variety of small colony morphologies may be observed; some colonies may consist of loose-packed cells (**A**) while others will have the tightly packed morphologies with high nucleus to cytoplasm ratio in the colony centre (**B**). By Day 6, the small colonies observed in (**A**) will have grown into the characteristic pluripotent morphology characterized by tightly packed colonies with high nucleus to cytoplasm ratio (**C**). Peripheral differentiation (white arrows) primarily occurs along the edges of the colonies and may be observed as early as day 2 to day 6 (**B, C**). By Day 7 -8, most of the peripheral differentiation will have lifted off during the daily media changes, leaving behind undifferentiated colonies (**D**).

6. Colonies are ready to be passaged when the colonies start to merge, covering 80 – 90% of the surface area (see Figure 1D), typically by Days 7 – 8. Right before passaging, scrape away any remaining areas of differentiation. As colonies adapt to PluriSTEM-XF™ medium, the degree of peripheral differentiation will diminish significantly with subsequent passaging (approximately after 5-6 passages in PluriSTEM-XF™ medium or earlier depending upon the cell line).

7. Do not wait beyond Day 7 – 8 to passage colonies as the edges of colonies may start to lift at the corners.

8. For enzymatic passsaging with Dispase II, reduce the incubation time to 3- 5 minutes rather than the usual 6-7 minutes normally used in cultures maintained on matrigel.

9. Do not use medium that had been stored at 2 – 8°C for longer than 2 weeks.

10. After coating with vitronectin, plates may be stored at 2 – 8°Cfor up to 2 days before use. It is not recommended to use vitronectin coated plates that have been stored longer than a week as cell attachment may be compromised.

11. Right after passaging, colony morphology may not appear as expected at early timeframes (up to day 5). Small colonies may be more loosely packed and may have a differentiated appearance with irregular borders. By days 5 – 8, colonies will take on the more characteristic pluripotent morphology of tightly packed colonies with defined borders and high nucleus-to-cytoplasm ratio.

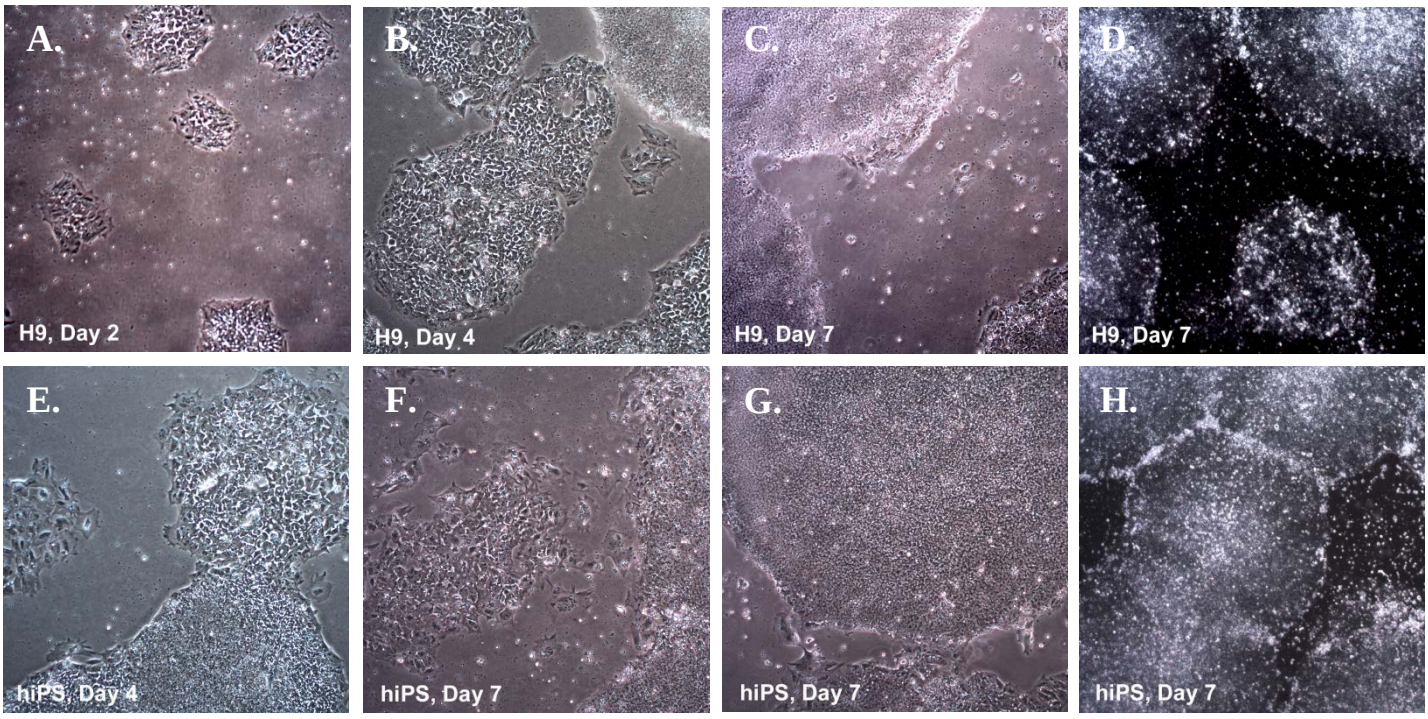

Figure 2. Human ES (H9) and hiPS cells cultured on vitronectin coated plates in PluriSTEM-XF™ medium at days 2 -7 after passaging. Colonies may appear loosely packed with irregular borders from days 1 – 5. By Days 5 – 8, the majority of the colonies will take on the more characteristic pluripotent morphology defined by tightly packed colonies with defined borders and high nucleus-to-cytoplasm ratio. Smaller colonies may still have the loosely-packed differentiated morphology even by day 7 (**F**) during the early transition to PluriSTEM-XF™ medium. These cells may be scraped off right before passaging with Dispase II.

Preparation of Vitronectin Coated Plates

Table 1: Volumes recommended for coating tissue culture treated cultureware:

CultureWare	Coating Volume (mL)	Surface Area (cm^2)
12 well plate	0.5 mL/well	2.0
6 well plate	1.5 mL/well	9.6
T25 flask	3 mL	25
T75 flask	8 mL	75

Expansion of pluripotent human ES and iPS cells with PluriSTEM-XF™ medium requires culturewares that are coated with recombinant vitronectin (Cat. No. CC130). **Normal tissue culture-**

treated vessels are used to coat with vitronectin. Below are general guidelines for the coating of 6 – well plates and culture flasks with vitronectin.

Note: All procedure should be performed under aseptic conditions in a biological safety cabinet

1. Thaw recombinant vitronectin at room temperature.
2. Dilute sufficient amounts of recombinant vitronectin to a final concentration of 10 µg/mL with 1X PBS (Cat. No. BSS-1006-B) (see Table 1). For example, add 180 µL vitronectin to every 9 mL 1X PBS. Scale according to the volumes required. Dilutions may be made in a 15 mL polypropylene conical tube. Mix gently. Do not vortex.
3. Cover the cultureware with the recommended volumes (see Table 1). Swirl the culture plates to spread the vitronectin solution evenly across the surface of the plate. Incubate at room temperature for 2 hours or at 2-8°C overnight. If not used immediately, store coated cultureware at 2-8°C for 1-2 days.
4. Before use, allow coated plates to come to room temperature for 30 minutes. Remove the coating solution and add an appropriate volume of media. IMPORTANT: Do not allow the wells to dry out.

Preparation of Dispase II

Pluripotent human ES and iPS cells maintained in PluriSTEM-XF™ may be enzymatically passaged using Dispase II (Cat. No. SCM133). Dispase II (1 mg/mL) may be aliquoted into smaller working volumes and stored at -20°C for up to 4 months from date of receipt. Frozen aliquots may be thawed and stored at 2-8°C for up to 2 weeks. Avoid multiple freeze thaw cycles to maintain proper enzymatic activity.

Transition to PluriSTEM-XF™ Medium on vitronectin coated plates using Dispase II

Pluripotent cells cultured in PluriSTEM-XF™ are maintained on recombinant human vitronectin rather than on matrigel, which contains animal-derived products. As human pluripotent cells are highly sensitive to environmental perturbation, it is critical to perform the following 2-stage transition process. Once cells have been transitioned to PluriSTEM-XF™ medium, subsequent passaging may be performed using either enzymatic (Dispase II) or non-enzymatic (Cell Dissociation Solution) methods. The procedures described below are based on the growth characteristics of H9 human ES cells and an internal human iPS cell line and thus should be used only as a guidance. Cell line differences may occur which may necessitate further optimization.

Stage 1: Transition cells to the new ECM matrix (i.e. human vitronectin). Use media that had been used to maintain pluripotent cells. Do **not** use PluriSTEM-XF™ medium at this time

1. Coat the 6-well plates with 10 µg/mL vitronectin (1.5 mL per well) (see “Preparation of Vitronectin Coated Plates”, pg. 5). Swirl the culture plates to spread the vitronectin evenly across the surface of the plate. Incubate at 2-8°C overnight or at room temperature for 2 hours before use.

2. On the day of passaging, acclimate vitronectin coated plates for 30 minutes to 1 hour at room temperature. After 1 hour, remove the coating. Add 2 mL of fresh media that had been used to maintain pluripotent cells to each well. Set the plate aside until the cells are ready to be passaged.

   Note: *Do **not** use PluriSTEM-XF™ medium at this time. As pluripotent cells are highly sensitive, it is important to minimize the amount of change introduced to the culture system. Transitioning to a new ECM matrix (i.e. vitronectin) should be the only change at this time.*

3. It is critical to start with high quality undifferentiated human ES and iPS culture. Use a dissection microscope to visually inspect the plate containing human pluripotent cells to be passaged. Inspect the colonies for areas of spontaneous differentiation.

   Note: *Areas of spontaneous differentiation are characterized as phase-bright, highly dense areas with irregular borders, non-uniform cell morphologies and cell types and are typically localized either in the center of the colonies or along the edges between colonies. Differentiated cells may also have the appearance of flat phase-dark epithelial-like cells occurring along the edges of colonies.*

4. Use a sterile p200 pipette tip attached to a p200 pipetman to scrape away areas of spontaneous differentiation. Be discriminating and scrape away any areas that harbor a hint a differentiation even if it means sacrificing the majority of the colonies.

5. Aspirate the medium containing the scrapped areas from the wells. Wash once with 1X PBS or DMEM/F12 medium. Aspirate after the wash.

6. Add 1 mL Dispase II (1 mg/mL) per well of the 6-well plate containing pluripotent human ES or iPS cells to be passaged. If the colonies are currently on matrigel, incubate at 37°C for 6-7 minutes. After incubation, visually inspect the colonies under a microscope. The edges of the colonies may appear slightly rounded up and folded back but the overall colony should still be attached to the plate.

7. Aspirate the Dispase II and gently rinse each well twice with 2 mL 1X PBS or DMEM/F12 medium to remove any residual Dispase II solution. Aspirate after each rinse.

8. Add 2 mL of the fresh medium that had been used to maintain the cells. Do **not** replace with PluriSTEM-XF™ medium at this time. Gently detach the colonies using a cell scraper (Sarstedt Cat. No. 83.1832).

9. Use a 5 mL serological pipette to collect the cell aggregates to a 15 mL conical tube. Minimize pipetting up and down as this may break up the colonies to suboptimal small pieces. The process of transferring the cell aggregates to the 15 mL conical tube should be sufficient to break the colonies to sufficient size.

10. Centrifuge the 15 mL conical tube containing the cell aggregates at 300 x g for 5 minutes at room temperature (15-25°C).

11. Aspirate the supernatant. Resuspend the cell aggregates in an appropriate volume of media that had been used to maintain the cells. Do **not** replace with PluriSTEM-XF™ medium at this time. Do not pipette the cell aggregates more than 1 – 2 times with a 5 mL serological pipette, taking care not to break the aggregates into single cell suspensions. For example, for a 1:5 split ratio, resuspend the cell aggregates in 5 mL total medium. For a 1:3 split ratio, resuspend the cell aggregates in 3 mL total medium.

Note: *For a confluent culture, a split ratio of 1:5 – 1:6 may be recommended. For less confluent cultures, a 1:3 – 1:4 split ratio may be more optimal. However as culture techniques and cell lines may vary, it is recommended that users set up a titration of split ratio ranging from 1:3 to 1:6 to determine the optimal split density. For subsequent passages, adjust the split ratio based on the colony morphology at the current passage and the last split ratio. If the colonies are too dense, increase the split ratio. If the colonies are too sparse, decrease the split ratio.*

12. Aliquot 1 mL of the appropriately diluted cell aggregates into the vitronectin coated plates containing 2 mL media that had been set aside from step 2. Total volume per well = 3 mL.

13. Place the plate in a 37°C incubator. Agitate the plate **gently** from side to side and forward and backwards to ensure that the cell aggregates are evenly distributed across the surface of the well. Incubate in a 37°C incubator.

14. After 10 – 15 minutes, visually inspect the plate to ensure that newly passaged cell aggregates are evenly distributed across the surface of the well. Plates that have not been properly agitated may have cell clumps aggregating toward the center of the wells. This uneven distribution at the center may later cause spontaneous differentiation of human ES/iPS cells. In the event clumps are not evenly distributed, agitate the plate gently from side to side and forwards and backwards for a longer extended time.

15. The next day, replace with 3 mL per well of fresh media that had been used to maintain the pluripotent cells. Do **not** replace with PluriSTEM-XF™ medium at this time.

16. Monitor and exchange daily with 3 mL fresh media that had been used to maintain the pluripotent cells. Do **not** replace with PluriSTEM-XF™ Medium at this time.

Stage 2: Transitioning cells to the PluriSTEM-XF™ medium.

17. When cells are ready to be passaged, repeat steps 1 – 14 using 1 mg/mL Dispase II to passage the cells to freshly-coated vitronectin plates in the same media that cells had cultured in before the passage. Do **not** use PluriSTEM-XF™ medium on the day of passage. Change medium daily.

18. Two to three days after the passage, exchange the media with PluriSTEM-XF™ Human ES/iPS Medium. Monitor and feed cells daily with PluriSTEM-XF™ medium.

19. Once cells have been transitioned to PluriSTEM-XF™ medium, colony morphologies may not appear as expected at early timeframes (up to day 5). Smaller colonies may be more loosely packed and may have a flat differentiated appearance with irregular borders (see Figure 2). By days 5 – 8, colonies will take on the more characteristic pluripotent morphology of tightly packed colonies with defined borders and high nucleus-to-cytoplasm ratio.

20. Colonies may grow slower in PluriSTEM-XF™ with some indications of peripheral differentiation between colonies (refer to Figure 1B, C). Cell lines may vary in the amount of peripheral differentiation observed. Allow colonies to grow until Day 7-8 by which time the majority of the peripheral differentiated cells will have lifted off, and are removed during the daily media changes.

21. Colonies are ready to be passaged when the colonies start to merge, covering 80-90% of the surface area (see Figure 1E), typically by Days 7 – 8. Before passaging, scrape away any remaining areas of differentiation. As colonies adapt to PluriSTEM-XF™ medium, the degree of

peripheral differentiation will diminish significantly with subsequent passaging (approximately after 5 – 6 passages in PluriSTEM-XF™ medium or earlier depending upon the cell line).

22. For all subsequent passaging with Dispase II, use 3-5 minutes incubation rather than the usual 7-8 minutes on matrigel-coated cultures. In general it is best to wait until Day 7-8 when colonies are larger and have the characteristic pluripotent morphology before passaging. Right before passaging, remove any areas of spontaneous differentiation.

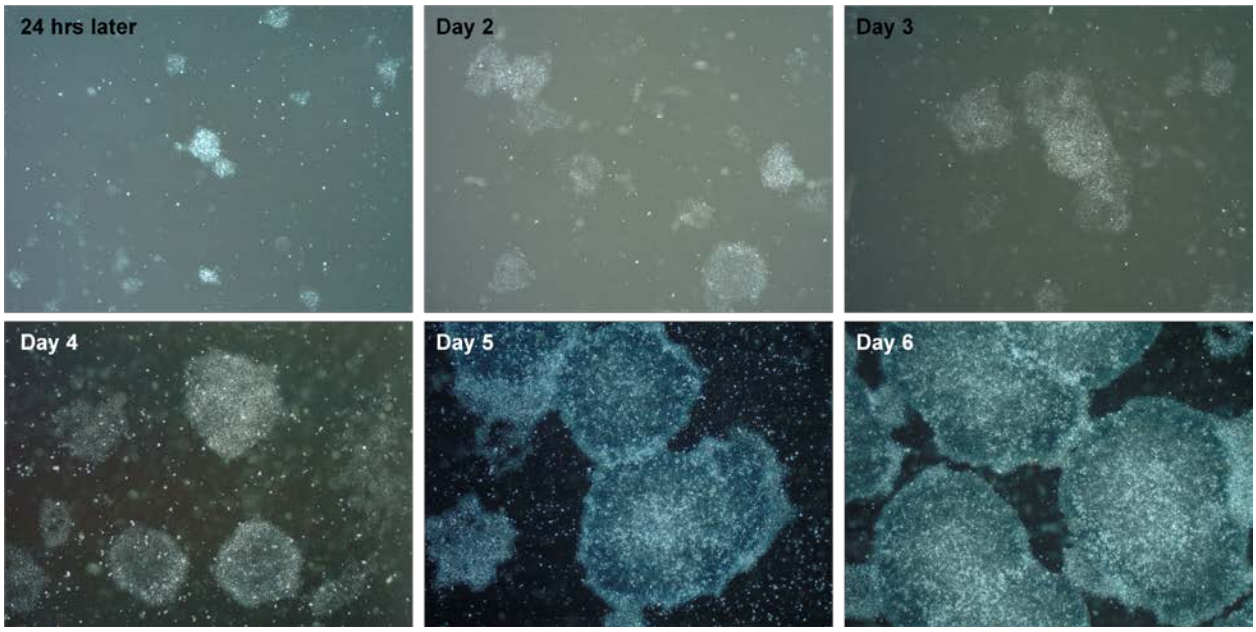

Figure 3: Representative images of colony proliferation in PluriSTEM-XF™ medium on vitronectin at 1-6 days. H9 hESCs cultured long-term (P25) in PluriSTEM™-XF medium exhibited the characteristic pluripotent morphology defined by tightly packed colonies with defined borders and high nucleus-to-cytoplasm ratio. Cells were passaged using Dispase II. Representative images of cell attachment 24 hours after passaging and a day by day morphological change are shown. . After 6-8 days, colonies are ready to be passaged .

Non-Enyzmatic Passaging using Enzyme-Free Cell Dissociation Solution

Once human pluripotent cells have been adapted to PluriSTEM-XF™ medium (approximately 5-6 passages), colonies may be passaged using enzyme-free cell dissociation solution to attain high expansion (up to 1:80 split ratio). The following protocol is designed for 6-well plates; if other culturewares are used, adjust the volumes according to the surface area (Refer to Table 1).

1. Aspirate the medium from hES or iPS cell culture. Wash each well with 2 mL per well of 1X PBS buffer (Cat. No. BSS-1006-B). Aspirate after each wash.

2. Add 1 mL per well of Enzyme Free Cell Dissociation Solution (Cat. No. S-004-C) and incubate for 2 – 3 minutes at room temperature. Monitor the cells every 1-2 minutes and stop treatment accordingly. Do not over-dissociate.

   Note: *Duration of cell dissociation may be cell line dependent. Two minutes incubation time is specific to H9 cells. Treatment should be stopped when the cells begin to separate, or when holes start to appear in the colonies at higher microscope magnification (x10 objective).*

3. Gently aspirate the dissociation buffer. Wash with 2 mL per well of 1X PBS. Aspirate after the wash and add 2 mL per well of PluriSTEM-XF™ medium.

4. Gently detach the colonies from the well by gentle pipetting with a 5 mL serological pipette. Avoid creating bubbles. The split ratio may range from 1:10 to 1:50 based upon the cell density. Always adjust the split ratio based on the colony morphology at the current passage and the last split ratio. For example, if the colonies are too dense, increase the split ratio; if colonies are too sparse, decrease the split ratio. Start from a low split ratio and gradually increase the split ratio with subsequent passaging to attain higher rates of cell expansion.

   Note: *Do not try to scrape off all the colonies as this may lead to breaking colonies to suboptimal small pieces.*

5. Aliquot the appropriate amount of diluted cell aggregates to freshly coated vitronectin coated plates containing 2 mL PluriSTEM-XF™ medium that had been set aside. Total volume = 3 mL.

6. Place the plate in a 37°C incubator. Agitate the plate **gently** from side to side and forward and backwards to ensure that the cell aggregates are evenly distributed across the surface of the wells. Incubate in a 37°C incubator.

7. After 10-15 minutes, visually inspect the plate to ensure that the newly passaged cell aggregates are evenly distributed across the surface of the well. Plates that have not been properly agitated may have cell clumps aggregating toward the center of the wells. This uneven distribution at the center may later cause spontaneous differentiation of human ES/iPS cells. In the event clumps are not evenly distributed, agitate the plate gently from side to side and forwards and backwards for a longer extended time.

8. Change with fresh PluriSTEM-XF™ medium daily and monitor cell morphology and confluence before the next passage.

Cryopreservation Using PluriSTEM-XF™ Freeze Medium (Cat. No. SCM135)

Before cryopreservation, human ES/iPS cell cultures should be of high quality (primarily undifferentiated with less than 10% of the cells being differentiated). Cryopreservation should be performed when cells are ready to passaged. The following protocols are based on cultures in 6-well plates where wells are 70-80% confluent at the time of cryopreservation.

1. Thaw PluriSTEM-XF™ Freeze Medium (Cat. No. SCM135) on ice.

2. Aliquot sufficient PluriSTEM-XF™ medium, Dispase II (1 mg/mL) and DMEM/F12. Warm reagents at room temperature (15 – 25°C) for 5 – 10 minutes.

3. Prepared labeled cryovials. Estimate that 1 cryovial should contain colonies from 2-3 wells of a 6-well plate, depending upon the confluency of the plate.

4. Use a dissection microscope to visually inspect the plate containing human pluripotent cells to be passaged. Inspect the colonies for areas of spontaneous differentiation.

   Note: *Areas of spontaneous differentiation are characterized as highly phase-bright, dense areas with irregular borders, non-uniform cell morphologies and cell types and are typically localized either in the center of the colonies or along the edges between colonies. Differentiated cells may also have the appearance of flat phase-dark epithelial-like cells occurring between colonies.*

5. Use a sterile p200 pipette tip attached to a p200 pipetman to scrape away areas of spontaneous differentiation.

6. Aspirate the medium containing the scrapped areas from the wells. Rinse with 2 mL per well of DMEM/F12 medium or 1X PBS (Cat. No. BSS-1006-B).

7. Add 1 mL Dispase II (1 mg/mL) per well of the 6-well plate containing pluripotent human ES/iPS cells.

8. Incubate at 37°C for 3 – 5 minutes. After incubation, visually inspect the colonies under a microscope. The edges of the colonies may appear slightly rounded up and folded back but the overall colony should still be attached to the plate.

9. Aspirate the Dispase II and gently rinse each well once with 2 mL per well of DMEM/F12 medium or 1X PBS to remove any residual Dispase II solution. Aspirate after each rinse.

10. Add 2 mL PluriSTEM-XF™ medium to each well. Gently detach the colonies using a cell scraper (Sarstedt Cat No. 83.1832).

11. Use a 5 mL serological pipette to collect the cell aggregates to a 15 mL conical tube. Minimize pipetting up and down as this may break up the colonies to suboptimal small pieces. The process of transferring the cell aggregates to the 15 mL conical tube should be sufficient to break the colonies to sufficient size.

12. Rinse the wells with an additional 2 mL of PluriSTEM-XF™ medium per well to collect any remaining cell aggregates. Add the rinse to the 15 mL conical tube.

13. Centrifuge the 15 mL conical tube containing the cell aggregates at 300 x *g* for 5 minutes at room temperature (15 – 25°C).

14. Aspirate the supernatant. Gently resuspend the cell pellet in the appropriate volume of cold (2 - 8°C) PluriSTEM-XF™ Freeze Medium using a 5 mL pipette. Take care to keep the cell aggregates as big clumps.

Note: *1 mL of PluriSTEM-XF™ Freeze Medium may be used to freeze colonies from 2 – 3 wells of a 6-well plate. However, if the colonies are greater than 80% confluent, 1 mL of PluriSTEM-XF™ Freeze Medium may be used to freeze 1 – 2 wells of a confluent 6-well plate.*

15. Transfer 1 mL of the cell suspension into a labeled cryovial using a 5 mL pipette.

16. Quickly place the cryovials into an isopropanol freezing container (e.g., Mr. Frosty) and place the container at -80°C overnight.

17. Next day, transfer frozen cryovials to a liquid nitrogen vapor tank for long-term storage.

Thawing Frozen Human ES and iPS Cells

Human ES and iPS cells should be thawed into tissue culture-treated plates coated with Vitronectin. Generally, one cryovial containing cells frozen in PluriSTEM-XF™ Freeze Medium may be successfully thawed into 1 well of a vitronectin-coated 6-well plate. If the cells have been cryopreserved using other methods, the viability of thawed cells may vary. Human ES/iPS cells grown on feeders or other feeder-free culture system prior to freezing should be thawed into the same culture condition used prior to cryopreservation. Once they have recovered from thaw, cells may be transitioned to PluriSTEM-XF™ medium following the protocol outlined in the section titled "Transition to PluriSTEM-XF™ medium on vitronectin coated plates using Dispase II" on page 6. The following protocol assumes that cells were cultured and frozen in PluriSTEM-XF™ Freeze Medium.

1. Coat new 6-well plates with 10 μg/mL vitronectin (1.5 mL per well) (see "Preparation of Coated Plates", pg. 5). Swirl the culture plates to spread the vitronectin evenly across the surface of the plate. Incubate 2 – 8°C overnight or at room temperature for 1 – 2 hours before use.

2. On the day of thawing, acclimate vitronectin coated plates for 30 minutes to 1 hour at room temperature. After 1 hour, remove the coating. Add 2 mL PluriSTEM-XF™ medium to each well. Set plate aside until cells are ready to be passaged.

3. Aliquot sufficient PluriSTEM-XF™ medium and DMEM/F12 to culture the thawed cells. Warm reagents at room temperature (15 – 25°C) for 5 – 10 minutes.

4. Remove the vial of cryopreserved cells from liquid nitrogen storage and quickly thaw the cells in a 37°C water bath. Closely monitor until only small ice crystals remain. Quickly remove the vial from the waterbath. **IMPORTANT: Do not vortex the cells or leave them in the water bath for too long.**

5. Disinfect the outside of the vial with 70% ethanol or isopropanol. Proceed immediately to the next step.

6. In a laminar flow hood, use a 1 or 2 mL pipette to transfer the cells to a sterile 15 mL conical tube. Be careful not to introduce any bubbles during the transfer process.

7. Using a 10 mL pipette, slowly add dropwise 9 mL of PluriSTEM-XF™ medium to the 15 mL conical tube. **IMPORTANT: Do not add the whole volume of media at once to the cells. This may result in decreased cell viability due to osmotic shock**.

8. Gently mix the cell suspension by slow pipeting up and down twice. Be careful not to introduce any bubbles. IMPORTANT: Do not vortex the cells.

9. Centrifuge the tube at 300 x g for 5 minutes at room temperature (15 – 25°C).

10. Aspirate the supernatant. Resuspend the cell pellet in 1 mL of PluriSTEM-XF™ medium by gently pipetting the cells up and down twice. Take care to maintain the cells as aggregates.

11. Transfer 1 mL of the thawed cell aggregates to one well of the vitronectin-coated 6-well plate containing 2 mL PluriSTEM-XF™ medium that had been set aside from step 2. Total volume per well = 3 mL.

12. Place the plate in a 37°C incubator. Agitate the plate **gently** from side to side and forward and backwards to ensure that the cell aggregates are evenly distributed across the surface of the well. Incubate in a 37°C, 5% CO_2 incubator.

13. After 10 – 15 minutes, visually inspect the plate to ensure that newly thawed cell aggregates are evenly distributed across the surface of the wells. Plates that have not been properly agitated may have cell clumps aggregating toward the center of the wells. This uneven distribution at the center may later cause spontaneous differentiation of human ES/iPS cells. In the event clumps are not evenly distributed, agitate the plate gently from side to side and forward and backwards for a longer extended time.

14. The next day, replace with 3 mL per well of fresh PluriSTEM-XF™ medium.

15. Monitor and exchange with 3 mL fresh PluriSTEM-XF™ medium daily.

16. Cultures should be fed with 4-5 mL PluriSTEM-XF™ medium per well on Friday to ensure sufficient medium to sustain the cells over the weekend. At least one medium change is required over the weekend.

17. It may take longer than 7 days for the cells to grow to confluence immediately after the thaw.

Data Analysis

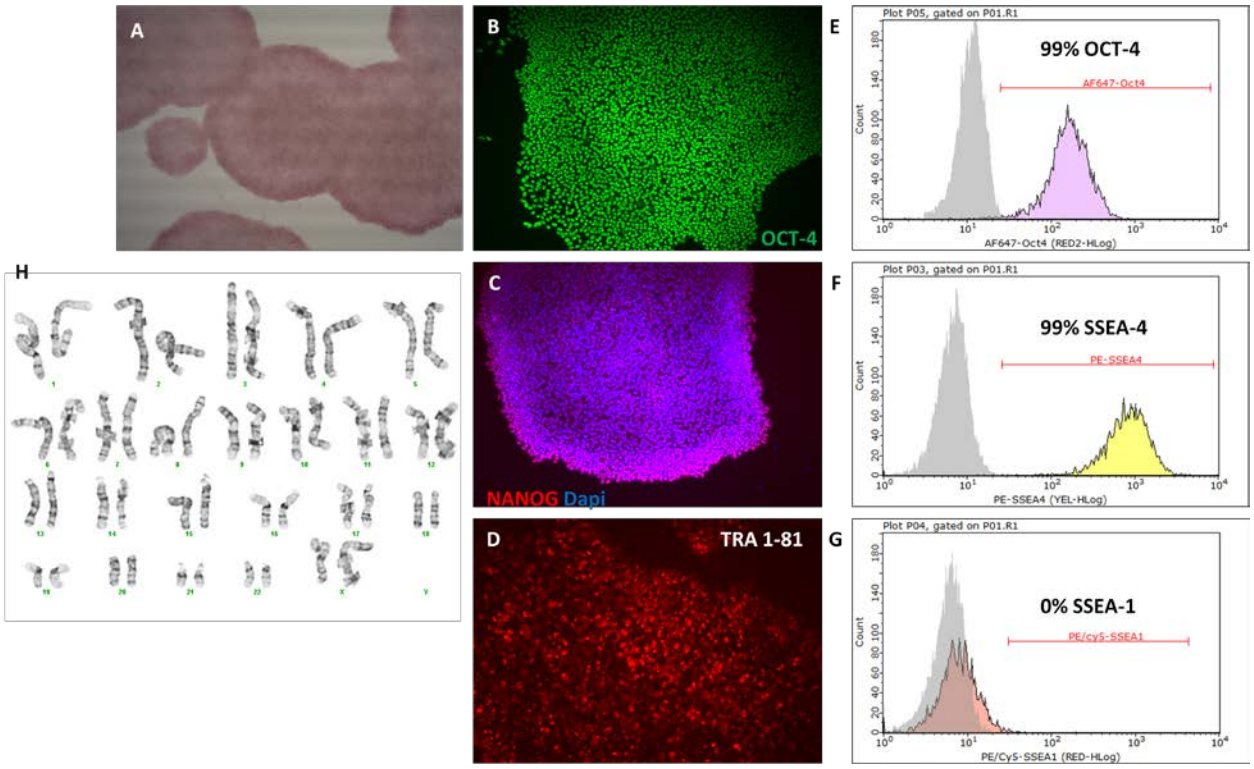

Figure 4. H9 cells cultured for 26 passages in PluriSTEM-XF™ medium express pluripotent markers, alkaline phoshatase (**A**), OCT-4 (**B**), NANOG (**C**) and TRA-1-81 (**D**). Guava flow analyses of H9 cells cultured in PluriSTEM-XF™ (P27 in PluriSTEM-XF™ medium) indicate high expression levels of pluripotent markers OCT-4 **(E)** and SSEA-4 **(F)** and an absence of staining for SSEA-1 **(G).** H9 cells cultured long term in PluriSTEM-XF™ (P25 in PluriSTEM-XF™) medium possessed a normal karyotype (46; XX). Cytogenetic analysis was performed by Cell Line Genetics on twenty G-banded metaphase cells. All twenty demonstrated an apparently normal female karyotype (**H**).

References

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