# PluriSTEM™ Human ES/iPS Medium

Product Manual for Catalog No.

SCM130

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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## Introduction

EMD Millipore has developed a complete, defined and serum-free medium formulation for the feeder-free culture of human ES and iPS cells. PluriSTEM™ Human ES/iPS Medium (Cat. No. SCM130) is a small molecule based medium that enables weekend-free culture of human pluripotent stem cells and allows for media exchanges every other day without compromising the morphology or long term functionality of pluripotent stem cells. Pluripotent cells maintained in this moderate feeding regiment exhibited high cell health with minimal spontaneous differentiation, expressed high levels of pluripotency markers (NANOG, OCT3/4, SOX-2, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81), retained differentiation potential and possessed a normal karyotype. PluriSTEM™ Human ES/iPS Medium is provided as a stand-alone 500 mL bottle that is ready-to-use and does not require additional supplementation. Cells proliferate faster in PluriSTEM™ as compared to the same cells plated in other serum-free and feeder-free media systems; a typical 4-6 day passage is expected for most pluripotent cell lines.

PluriSTEM™ has been extensively tested and proven to possess the following characteristics:

- Supports culture of pluripotent human ES/iPS cells for >30 passages.
- Eliminates the requirement to feed cells over the weekend. Take the weekend off.
- Eliminates the requirement of daily feeding. While daily feeding is optional, cells may also be transitioned to every other day (i.e. Monday, Wednesday, Friday) media exchanges.
- Simple and easy transition (i.e. no period of low cell yield) of pluripotent cells to PluriSTEM™ from feeder-based and feeder-free culture systems.
- Enables superior single cell culture, passage and selection of human pluripotent clones.
- Superior freeze-thaw cell viability and recovery
- Supports suspension culture

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

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## Materials Provided

| PluriSTEM™ Human ES/iPS Medium (Cat. No. SCM130)<br>Store at -20°C | | |
|---|---|---|
| **Component** | **Item No.** | **Volume** |
| PluriSTEM™ Human ES/iPS Medium | SCM130 | 500 mL |

## Materials Required But Not Supplied

1. 6-well tissue culture plates
2. BD Matrigel™ hESC-qualified Matrix, 5 mL (BD Cat. No. 354277)
3. DMEM/F12, with HEPES, L-Glutamine, 500 mL (Cat. No. DF-041-B)
4. PluriSTEM™ Freezing Medium (1X) (Cat. No. SCM134)
5. Accumax™ Cell Detachment Solution (Cat. No. SCR006)
6. PluriSTEM™ Dispase II Solution, 100 mL (Cat. No. SCM133)
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)
9. Steriflip-GP, 0.22 μm, gamma irradiated, 25/pk (Cat. No. SCGP00525)

## Storage and Stability

PluriSTEM™ 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™ at room temperature (15 – 25°C) or overnight at 2 – 8°C. **Do not thaw at 37°C.** If desired, PluriSTEM™ may be aseptically dispensed into working aliquots and stored at -20°C. Use aliquots within expiration date as indicated on 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.**

## Preparation of Coated Plates

**Table 1: Volumes recommended for coating 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 |

**MATRIGEL COATING:**

Expansion of pluripotent human ES and iPS cells with PluriSTEM™ medium requires culturewares that are coated with Matrigel. Below are general guidelines for the coating of 6 – well plates and culture flasks with Matrigel.

1. Thaw Matrigel on ice. Keep on ice and use pre-cooled medium and pipettes to avoid gelling of the ECM gel. **IMPORTANT: Do not thaw Matrigel at temperatures higher than 15ºC to avoid gelling.**

2. Dilute the Matrigel 1:20 with cold DMEM/F12 medium. For example, to every 0.5 mL Matrigel, add 9.5 mL cold DMEM/F12 medium for a total volume of 10 mL. Scale according to the volumes required.

3. Cover the cultureware with the recommended volumes (see Table 1). Swirl the culture plates to spread the Matrigel evenly across the surface of the plate. Incubate at room temperature for at least 1 hour or 2 – 8°C overnight. If not used immediately, store coated cultureware at 2-8°C until ready to use.

   ***Note****: If not used immediately, Matrigel coated culturewares should be sealed with parafilm to prevent evaporation and can be stored at 2 – 8°C for up to one week.*

4. Prior to seeding the cells, bring the plate back to room temperature, remove the coating solution and add an appropriate volume of PluriSTEM™ Human ES/iPS Medium. **IMPORTANT: Do not allow the flask to dry out.**

## Preparation of Dispase II

Pluripotent human ES and iPS cells maintained in PluriSTEM™ may be enzymatically passaged using Dispase II. EMD Millipore provides Dispase II as a ready to use 1 mg/mL stock solution (Cat. No. SCM133). We recommend that Dispase II (1 mg/mL) be aliquoted into smaller working volumes and store 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™ using Dispase II (Applicable for both Feeder-Based and Feeder-Free Media Systems)

Human ES and iPS cell that have been maintained in feeder-based or other feeder-free culture systems may be easily transitioned to PluriSTEM™ with minimal cell loss. Once cells have been transitioned to PluriSTEM™, subsequent passaging may be performed using Dispase II.

The following procedure describes the use of Dispase II to enzymatically passage pluripotent human ES and iPS cells in feeder-free or feeder-based media systems. Enzymatic passaging with Dispase II is recommended as there is a significant reduction in the amount of time and labor involved. For pluripotent cells that are maintained on feeder cultures, some carry-over of mouse embryonic fibroblast feeders (MEFs) may be present during the first passage to PluriSTEM™. However by the 2nd enzymatic passage, MEFs are no longer present in the culture.

If other culturewares are used, adjust the volumes according to the surface area. The following guidelines are based on the growth characteristics of H9 human ES cells and an in-house human iPS cell line and may vary between different cell lines and laboratories.

***Note: For feeder-free systems, start with step 1. For feeder-based systems, start from step 2.***

1. One to two days prior to passaging, exchange the media with 3 mL PluriSTEM™ per well of the 6 - well plates containing human pluripotent ES or iPS cells. Exchange with fresh PluriSTEM™ the next day. There should be minimal cell loss observed with the transition to PluriSTEM™ medium.

2. Coat the 6-well plates with 1:20 dilution of Matrigel (1.5 mL per well) (see “Preparation of Coated Plates”, pg. 3). Swirl the culture plates to spread the Matrigel evenly across the surface of the plate. Incubate at 2 – 8°C overnight or at room temperature for 1 – 2 hours before use.

3. On the day of passaging, acclimate matrigel coated plates for 1 hour at room temperature. After 1 hour, remove the matrigel coating. Add 2 mL PluriSTEM™ media to each well. Set plate aside until cells are ready to be passaged.

4. Aliquot sufficient PluriSTEM™, Dispase II (1 mg/mL, Cat. No. SCM133) and DMEM/F12 (Cat. No. DF-041-B) to passage the cells. Warm reagents at room temperature (15 – 25°C) for 5 – 10 minutes.

5. 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.*

6. 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 of differentiation.

   ***Note:*** *It is critical to start with high quality undifferentiated human ES and iPS culture. When maintained correctly, spontaneous differentiation should be <1 – 5% in PluriSTEM™ culture. However, in the event that the starting culture contains large areas of differentiation, the colonies may still be rescued using PluriSTEM™. Be discriminating and scrape away any areas that harbor a hint of differentiation even if it means sacrificing the majority of the colonies. So long as the remaining small colony pieces are of high quality and undifferentiated, PluriSTEM™ will still*

*rescue these low density cultures. Remaining undifferentiated colonies will recover and proliferate in PluriSTEM™ to repopulate into high quality pluripotent colonies.*

7. Aspirate the medium containing the scrapped areas from the well. Rinse with 2mL per well of DMEM/F-12 medium or 1X PBS (Cat. No. BSS-1006-B).

8. 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.

9. 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.

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

11. Add 1.5 – 2 mL PluriSTEM™ medium to each well. Gently detach the colonies using a cell scraper (Sarstedt Cat No. 83.1832).

12. 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.

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

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

15. Aspirate the supernatant. Resuspend the cell aggregates in an appropriate volume of PluriSTEM™ for passaging. 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 PluriSTEM™. For a 1:3 split ratio, resuspend the cell aggregates in 3 mL total PluriSTEM™.

    ***Note:*** *For a confluent culture, a split ratio of 1:5 – 1:6 is 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 the users set up a titration of split ratio ranging from 1:3 to 1:6 to determine the optimal split density.*

16. Aliquot 1 mL of the appropriately diluted cell aggregates into the matrigel coated plates containing 2 mL PluriSTEM™ medium that had been set aside from step 2. Total volume per well = 3 mL.

17. 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.

18. 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 forward and backwards for a longer extended time.

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

20. Monitor and exchange with 3 mL fresh PluriSTEM™ media daily. Depending upon the split ratio used, cells are typically ready for enzymatic passaging in 4 – 6 days. For example, for 1:5 – 1:6 split ratio, cells may be ready to be passaged within 5 – 6 days whereas for a more conservative split ratio of 1:3, cells may require passaging within 3 – 4 days time.

21. Cultures should be fed with 4 mL PluriSTEM™ on Friday to allow sufficient media to sustain the cells over the weekend. Medium exchanges during the weekend are not necessary.

22. When pluripotent cultures have been maintained in PluriSTEM™ for at least 3 passages, media exchanges may be transitioned to every other day. Monitor cell health daily to ensure that every other day exchange does not affect the cell health and quality of the colonies.

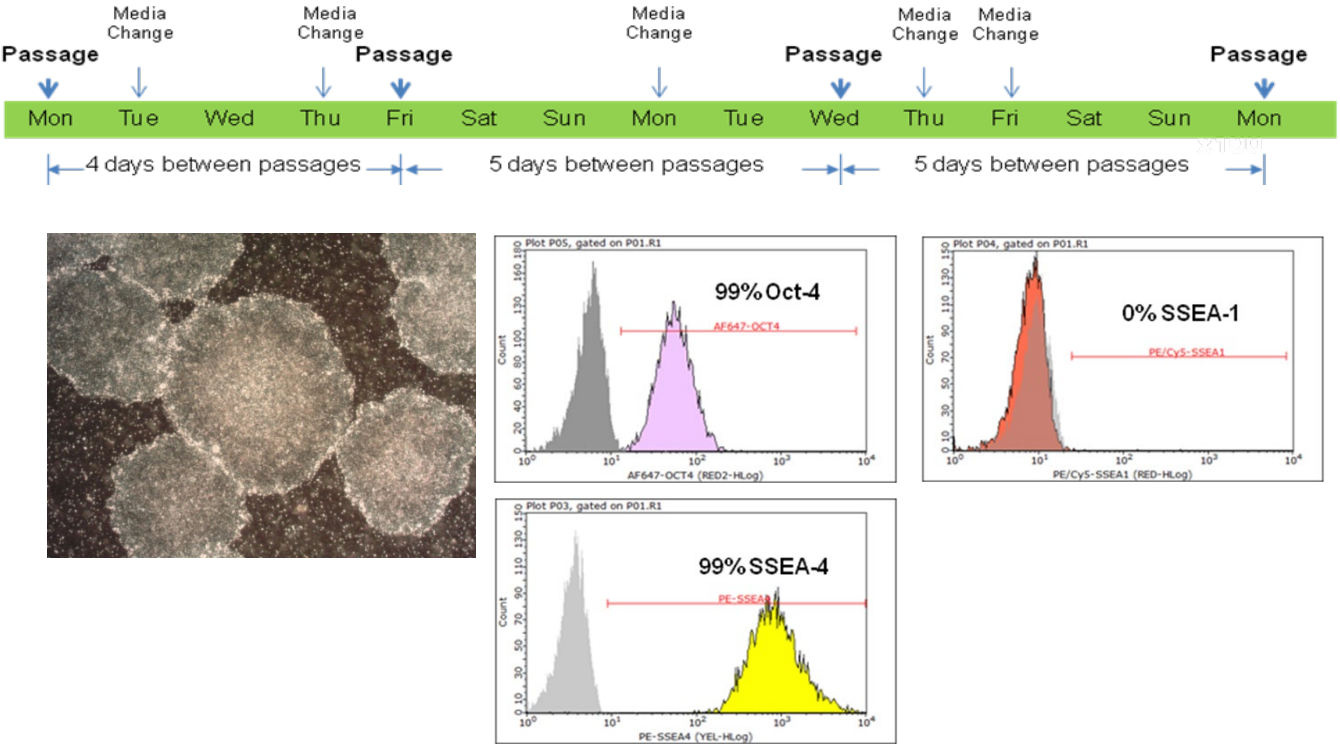



**Figure 1: Every Other Day and Weekend Free Media Exchanges with PluriSTEM™.**
H9 cells were maintained in PluriSTEM™ for 12 passages during which time media was exchanged every other day and not performed on the weekend. Cells retained characteristic pluripotent morphology (i.e. homogeneous round colonies with defined borders). Guava flow analyses indicate high expression levels of pluripotent markers OCT-4 and SSEA-4 and an absence of staining for SSEA-1.

23. Human ES and iPS cells cultured in PluriSTEM™ are ready to be passaged when the colonies are large with centers that are dense and phase bright compared to their edges. Colonies may also start to merge, covering 80 – 90% of the surface area (see Figure 1 and 2).

24. Adjust the split ratios to achieve a balance between having too much space between colonies and having a confluent culture. We recommend a conservative 1:3 or 1:4 split ratio when colonies have just been transitioned to PluriSTEM™ from either feeder-based or other feeder-free culture systems. Once established, cultures may be passaged using 1:6 to 1:8 split ratio. Adjust the split ratio depending upon the cell growth characteristics of the specific cell line.

25. Later passages should be performed enzymatically using Dispase II (1 mg/mL)

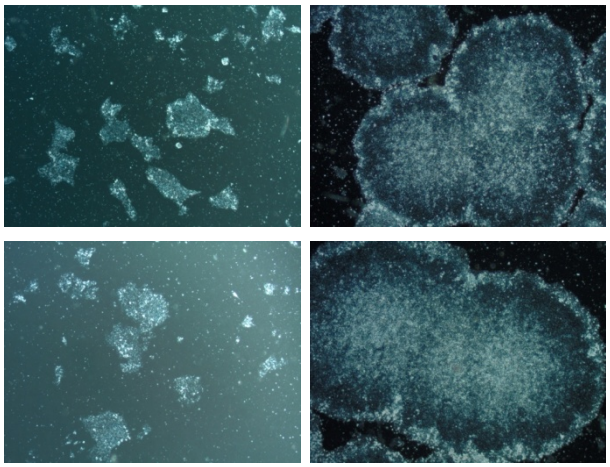


**Figure 2:** H9 (**top panels**) and human iPS (**bottom panels**) cells cultured long-term in PluriSTEM™ medium exhibited the characteristic pluripotent morphology defined by tightly packed colonies with defined borders and high nucleus-to-cytoplasm ratio. Cells were passaged using Dispase II. Representative images of cell attachment 24 hours after passaging (**left panels**). After 5 – 6 days, colonies are ready to be passaged (**right panels**).

## Transition to PluriSTEM™ from Feeder-Based Culture Systems using Manual Passaging

Human ES and iPS cells cultured on feeder-based systems may be transitioned to PluriSTEM™ by enzymatically passaging with Dispase II or by manual passaging. Manual passaging is significantly more labor intensive but does result in less carry-over of mouse embryonic fibroblast feeders (MEFs) during the first passage. Once transitioned to PluriSTEM™ subsequent passaging should be performed enzymatically using Dispase II.

1. Coat new 6-well plates with 1:20 dilution of Matrigel (1.5 mL per well) (see "Preparation of Coated Plates", pg. 3). Swirl the culture plates to spread the Matrigel 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 passaging, acclimate matrigel coated plates for 1 hour at room temperature. After 1 hour, remove the matrigel coating. Add 2 mL PluriSTEM™ media to each well. Set plate aside until cells are ready to be passaged.

3. On the day of passaging to PluriSTEM™, aspirate the medium from the 6-well plate containing human pluripotent ES/iPS cells on inactivated MEFs. Replace with 3 mL fresh PluriSTEM™ media per well.

4. Transfer the 6-well plate to a tissue culture hood containing a dissecting microscope. Using a 21 gauge needle attached to a 3 mL syringe, cut each ES/iPS colony into 9 – 16 pieces or more depending upon the colony size.

5. Use a sterile p200 pipette tip attached to a p200 pipetteman to nudge and transfer the pieces into the appropriate wells of the matrigel-coated plate containing 2 mL PluriSTEM™ media that had been set aside in step 2.

   ***Note: As culture techniques and cell lines may vary, it is recommended that users set up a titration of split ratio of 1:2, 1:3 and 1:4 to determine the optimal split density.***

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 well. Incubate in a 37°C incubator.

7. 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 towards the center of the wells. This uneven distribution in 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.

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

9. Monitor and exchange with 3 mL fresh PluriSTEM™ media daily. Cultures should be fed with 4 mL PluriSTEM™ on Friday to allow sufficient media to sustain the cells over the weekend. Medium exchanges during the weekend are not necessary.

   ***Note:*** *When pluripotent cultures have been maintained in PluriSTEM™ for at least 3 passages, media exchanges may be transitioned to every other day. Monitor cell health daily to ensure that every other day exchange does not affect the cell health and quality of the colonies.*

10. Subsequent passages should be performed enzymatically using Dispase II.

## Single Cell Passaging using Accumax

1. Coat new 6-well plates with 1:20 dilution of Matrigel (1.5 mL per well) (see "Preparation of Coated Plates", pg. 3). Swirl the culture plates to spread the Matrigel 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 passaging, acclimate matrigel coated plates for 1 hour at room temperature. After 1 hour, remove the matrigel coating. Add 2 mL PluriSTEM™ media to each well. Set plate aside until cells are ready to be passaged.

3. Aliquot sufficient PluriSTEM™, Accumax (Cat. No. SCR006) and DMEM/F12 (Cat. No. DF-041-B) to passage the cells. Warm reagents at room temperature (15 – 25°C) for 5 – 10 minutes.

4. One hour before the cells are to be passaged, add ROCK Inhibitor, Y-27632 (Cat. No. SCM075) to each well of the 6-well plate at a final concentration of 10 μM.

5. After 1 hour, use a dissection microscope to visually inspect the plate containing human pluripotent cells to be passaged. Inspect the colonies for areas of spontaneous differentiation.

6. Use a sterile p200 pipette tip attached to a p200 pipetman to scrape off areas of spontaneous differentiation. Be discriminating and scrape away any areas that harbor a hint of differentiation.

   ***Note:*** *It is critical to start with high quality undifferentiated pluripotent human ES and iPS culture. Pluripotent colonies cultured in PluriSTEM™ typically have <1% spontaneous differentiation when maintained correctly. However, in the event that the starting culture contains large areas of differentiation, the colonies may still be rescued using PluriSTEM™. Be discriminating and scrape away any areas that harbor a hint of differentiation even if it means sacrificing the majority of the areas within a colony. So long as the remaining small colony pieces are of high quality and undifferentiated, PluriSTEM™ will still rescue these low density cultures. Remaining undifferentiated pieces will recover and proliferate in PluriSTEM™ to repopulate into high quality undifferentiated pluripotent colonies.*

7. Aspirate the medium containing the scrapped areas from the well. Rinse with 2mL per well with DMEM/F-12 medium or 1X PBS (Cat. No. BSS-1006-B).

8. Aspirate and replace with 1 mL of Accumax (Cat. No. SCR006) per well of a 6-well-plate. Incubate at 37°C for 8-10 minutes.

   ***Note**: Different cell lines may require different incubation time. It is thus important to monitor the cell dissociation. Accumax treatment should be stopped when the cells start to dissociate and holes start to appear within colonies.*

9. Quench the Accumax reaction by adding 1 mL PluriSTEM™ for each mL of Accumax used. Gently detach cells using a sterile 1000-µL pipette tip. Cells should be easily dislodged.

10. Collect the dissociated cells to a 15 mL conical tube. Rinse the wells with an additional 2 mL of PluriSTEM™ medium to collect any remaining cells. Add the rinse to the 15 mL conical tube.

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

12. Aspirate the supernatant. Resuspend the cells in fresh PluriSTEM™ containing 10 µM ROCK Inhibitor, Y-27632.

13. Count the number of cells using a Scepter or hemacytometer. Ensure that the cells are in a single cell suspension. Determine the cell viability using Trypan Blue exclusion.

14. Set up a titration of different cell densities ranging from 0.5 – 1 x $10^4$ cells/$cm^2$. This corresponds to 50,000 – 100,000 cells per well of a matrigel-coated 6-well plate in PluriSTEM™ medium containing 10 µM ROCK Inhibitor, Y-27632.

15. The next day, replace with fresh PluriSTEM™ media. The ROCK Inhibitor, Y-27632 is no longer required from this step forward. Replace with fresh PluriSTEM™ medium daily (3 mL volume per well).

16. After 6 – 7 days, human ES/iPS cells should be ready for passaging. Human ES/iPS cells are ready for passaging when the colonies are large with centers that are dense and phase-bright compared to their edges. Colonies may also start to merge, covering 80-90% of the surface of the wells.

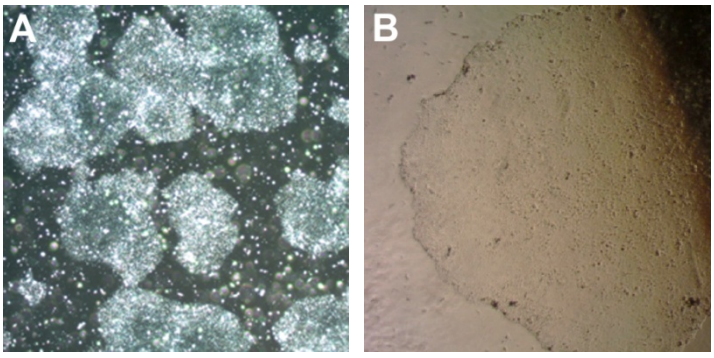



**Figure 3: Robust single cell passaging with PluriSTEM™.** H9 hESCs cultured in PluriSTEM™ for 22 passages were dissociated into single cells using Accumax (Cat. No. SCR006). One hour before dissociation, 10 µM ROCK Inhibitor (Cat. No. SCM075) was added. 100,000 singly dissociated cells were seeded into each well of a matrigel-coated 6-well-plate. After 6 days in PluriSTEM™, singe cells had formed distinct pluripotent colonies (**A**). Single cells continued to form distinct pluripotent colonies after undergoing four rounds of single cell passaging using Accumax (**B**).

## Cryopreservation Using PluriSTEM™ Freeze Medium (Cat. No. SCM134)

Before cryopreservation, human ES/iPS cell cultures should be of high quality (primarily undifferentiated with less than 5% 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™ Freezing Medium (Cat. No. SCM134) on ice.

2. Aliquot sufficient PluriSTEM™, 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 wells of a 6-well 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.*

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 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.

9. Aspirate the Dispase II and gently rinse each well two times 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 1.5 – 2 mL PluriSTEM™ 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™ 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™ Freezing Medium using a 5 mL pipette. Take care to keep the cell aggregates as big clumps.

    ***Note:*** *1 mL of PluriSTEM™ Freezing Medium may be used to freeze colonies from 2 wells of a 6-well plate. However, if the colonies are greater than 80% confluent, 1 mL of PluriSTEM™ Freezing Medium may be used to freeze 1 well 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 1:20 dilution of Matrigel™. Generally, one cryovial containing cells frozen in PluriSTEM™ Freezing Medium may be successfully thawed into 1 well of a matrigel-coated 6-well plate. If the cells have been cryopreserved using other methods, the viability of thawed cells may vary. Human ES/iPS cells frozen using mFreSR™ may be thawed directly into PluriSTEM™. For human ES/iPS cells grown on feeders prior to freezing, the morphology of hPSCs may appear unusual after being thawed directly into PluriSTEM™, but should look normal after 3 – 4 passages.

1. Coat new 6-well plates with 1:20 dilution of Matrigel (1.5 mL per well) (see “Preparation of Coated Plates”, pg. 3). Swirl the culture plates to spread the Matrigel 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 matrigel coated plates for 1 hour at room temperature. After 1 hour, remove the matrigel coating. Add 2 mL PluriSTEM™ medium to each well. Set plate aside until cells are ready to be passaged.

3. Aliquot sufficient PluriSTEM™ 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™ 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™ by gently pipetting the cells up and down twice. Take care to maintain the cells as aggregates.

11. Transfer 1mL of the thawed cell aggregates to one well of the matrigel-coated 6-well plate containing 2 mL PluriSTEM™ 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™ medium.

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

16. Cultures should be fed with 4 mL PluriSTEM™ per well on Friday to ensure sufficient medium to sustain the cells over the weekend. Medium exchanges during the weekend are not necessary.

17. When pluripotent cultures have been maintained in PluriSTEM™ for at least 3 passages, media exchanges may be transitioned to every other day.

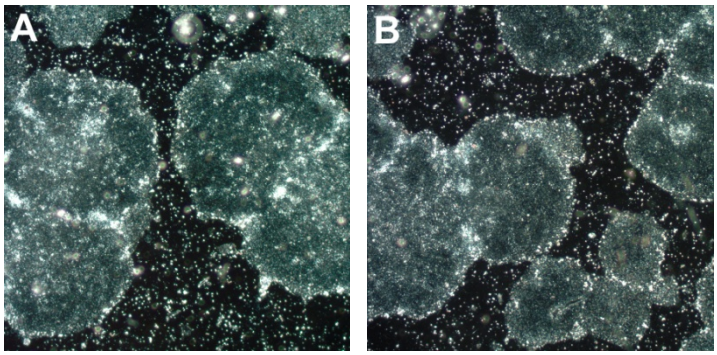



**Figure 4.** Representative images of 1 frozen vial of H9 cells in PluriSTEM™ Freezing Medium that were thawed into 1 well of a matrigel-coated 6-well plate in PluriSTEM™ medium. Six days post-thawed, colonies are large, undifferentiated with defined borders and are ready to be passaged.

## Data Analysis

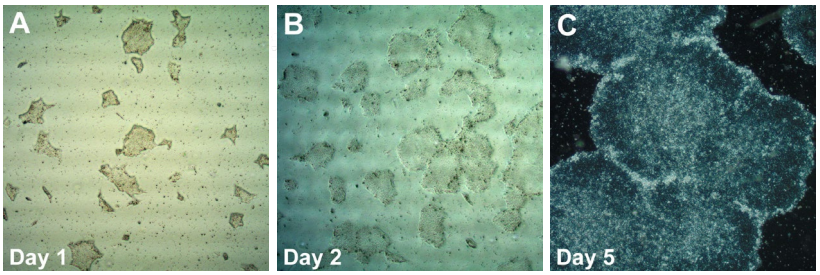



**Figure 5.** Representative images of colony proliferation in PluriSTEM™ at 1, 2, and 5 days.

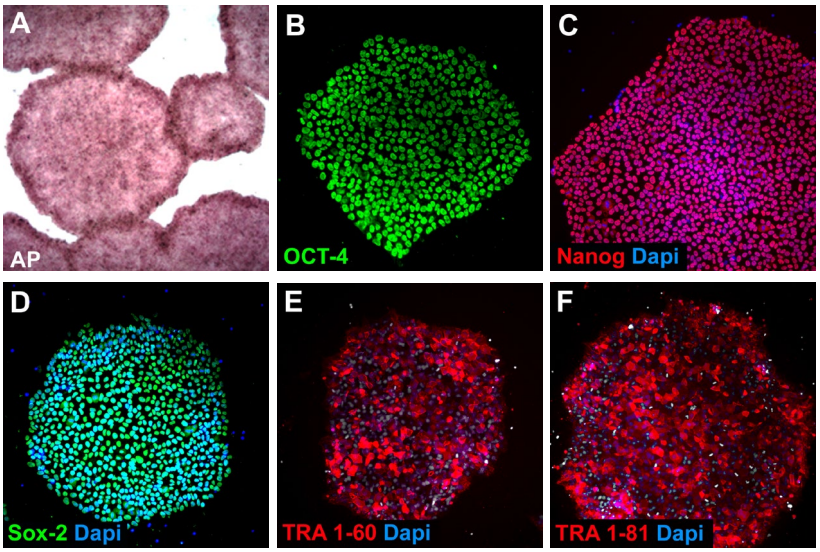
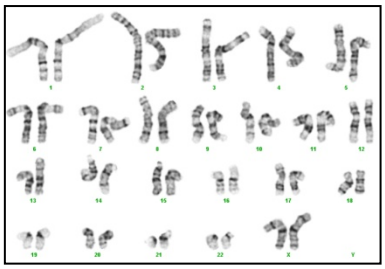



**Figure 6**. H9 cells cultured for >21 passages in PluriSTEM™ medium express pluripotent markers, alkaline phoshatase (**A**), OCT-4 (**B**), NANOG (**C**), SOX-2 (**D**), TRA-1-60 (**E**), and TRA-1-81 (**F**) and possess normal karyotype (46; XX). Cytogenetic analysis was performed by Cell Line Genetics on twenty G-banded metaphase cells. All twenty demonstrated an apparently normal female karyotype (**G**)

G



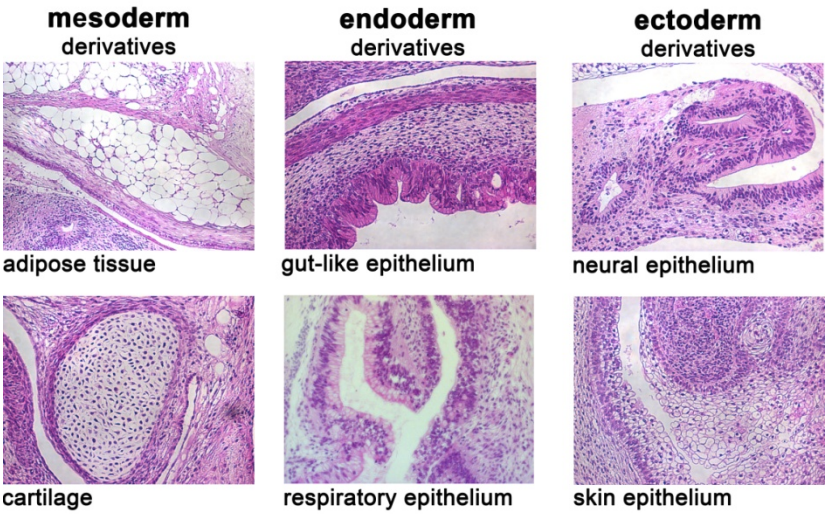



**Figure 7**. Human pluripotent stem cells cultured in PluriSTEM™ for >20 passages differentiate *in vivo* to the 3 germ layers. H&E stained teratoma sections. Photo courtesy of Dr. Boris Greber.

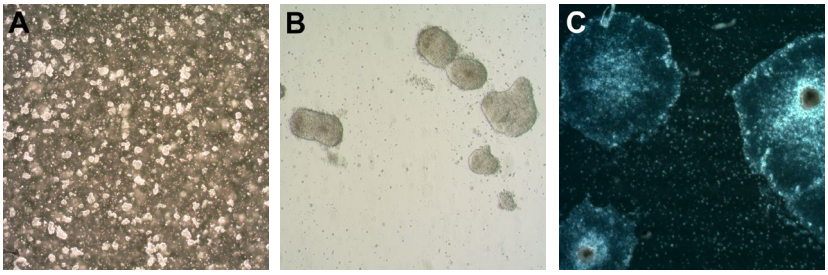



**Figure 8**. Human pluripotent cells can be maintained in PluriSTEM™ as suspension culture. H9 cells were passaged using Dispase II and cultured on uncoated T75 flasks with PluriSTEM™ (**A**). Higher magnification of H9 suspension cells (**B**). Additional PluriSTEM™ was added every other day. Cells were passaged once a week by mechanical pipetting several times to break up the suspension into smaller clumps. H9 suspension cells can be rapidly transitioned to 2D culture by plating suspension cells onto matrigel-coated plates (**C**).

## References

Frank, S., Zhang, M., Schöler, H. R., and Greber, B. (2012). Small molecule-assisted, line-independent maintenance of human pluripotent stem cells in defined conditions. *PLOS One* **7(7)**: e41958.

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