Culturing and maintaining human ESCs and iPSCs on tissue culture plates coated with **Vivogel ® StemCoat**

Application Note

Catalog #: VM003-10

Package size: 10 mL

Application Introduction:

Human pluripotent stem cell (hPSC)-based models hold tremendous potential for the study of human development and disease. Maintaining and expanding human pluripotent stem cells (human embryonic stem [ES] cells and human induced pluripotent stem [iPS] cells) in feeder-free conditions eliminates the inherent biological variability of feeder cells, an undefined component of the culture system and improves the reproducibility. An ideal environment for hPS cell research consists of both a cell culture surface specifically qualified for hPS cells, and a serum-free, defined medium. **Vivogel ® StemCoat**, a high-quality surface and medium combination, create a complete environment to support feeder-independent expansion of hPSCs.

Vivogel ® Matrix is a solubilized basement membrane extracted from the Engelbreth-Holm-Swarm (EHS) mouse tumor that comprises extracellular matrix proteins including laminin (glycoprotein), collagen IV, nidogen (glycoprotein), perlecan (heparan sulfate proteoglycan), and many other essential growth factors. **Vivogel ® Matrix** has empowered applications such as stem cell culture, angiogenesis assays, and tissue engineering. **Vivogel ® StemCoat** is an optimized product specifically developed to support the attachment and expansion of hPSCs. Each lot of **Vivogel ® StemCoat** is qualified to be capable of maintaining high-quality human ES and iPS cells when used with.

This note demonstrates that **Vivogel ® StemCoat** has the ability to sustain culture of hPSC for more than 20 passages together with mTeSR™1 (Catalog #85850, STEMCELL Technologies). Cells maintained in **Vivogel ® StemCoat** coated culturewares express high levels of pluripotency markers such as Oct-3/4 and SSEA-3, and pluripotency of cells maintained in **Vivogel ® StemCoat** coated culturewares has also been demonstrated by the ability of these cells to differentiate into all three germ layers in the teratoma assay.

Product specifications:

Concentration: 8 - 12 mg/mL.

Source: Murine Engelbreth-Holm-Swarm (EHS) tumor.

Buffer: DMEM (Phenol red free, PRF), with 10 μg/mL gentamicin.

Storage: -80 ℃ for long-term storage. Do not use **Vivogel ® StemCoat** that has been stored at 4 ℃ for more than 24 h. Please aliquot upon receival of the product. Avoid multiple freeze-thaw cycles.

Procedure guidelines:

A.a. GENERAL HANDLING OF **Vivogel ® StemCoat**

Aliquots of **Vivogel ® StemCoat** are thawed as needed from -20/-80 ℃. All steps involving **Vivogel ® StemCoat** are to be finished on ice and require uses of pre-chilled tips and tubes. Freeze thaws should be minimized by aliquoting into one time use aliquots. It is extremely important that **Vivogel ® StemCoat** and all cultureware or media coming in contact with **Vivogel ® StemCoat** should be pre-chilled/ice-cold since **Vivogel ® StemCoat** will start to gel above 10 ℃

A.b. MATERIALS AND REAGENTS

PRODUCT NAME	SUPPLIER	CATALOG #
mTeSR™1 complete medium	STEMCELL Technologies	85850
Vivogel ® StemCoat	**Vivomatter**	VM003
Gentle cell dissociation reagent	STEMCELL Technologies	100-0485
TrypLE™ select	Thermo Fisher Scientific	12563011
CTS™ PSC Cryomedium	Thermo Fisher Scientific	A4239301
Y-27632 (ROCK inhibitor)	Sigma-Aldrich	Y0503
Gentamicin	Thermo Fisher Scientific	15750060
DPBS		
Tissue culture-treated culturewares (multi-well plate, flask)		
Cell scrapers		
50/15 mL conical tubes		
Trypan Blue		

B.a. COATING CLUTUREWARE WITH **Vivogel ® StemCoat**

To use with complete stem cell culture culture medium (i.e., mTeSR™1 complete medium), prepare aliquots of **Vivogel ® StemCoat** according to the dilution factor provided on the Certificate of Analysis. The volume of the aliquots is typically between 270-350 µL.

1. Thaw one aliquot of **Vivogel ® StemCoat** on ice.

2. Dispense 25 mL of cold DMEM/F-12 with 15 mM HEPES into a 50 mL conical tube and keep on ice.

3. Add thawed **Vivogel ® StemCoat** to the cold DMEM/F-12 (in the 50 mL tube) and mix well. The vial may be washed with cold medium if desired.

4. Immediately use the diluted **Vivogel ® StemCoat** solution to coat tissue culture-treated cultureware. See Table 1 for recommended coating volumes.

5. Swirl the cultureware to spread the **Vivogel ® StemCoat** solution evenly across the surface.

*NOTE: If the cultureware surface is not fully coated by the **Vivogel ® StemCoat** solution, it should not be used for human ES or iPS cell culture.*

6. Incubate at room temperature (15 – 25 °C) for at least 1 hour before use. Do not let the **Vivogel ® StemCoat** solution evaporate.

*NOTE: If not used immediately, cultureware must be sealed to prevent evaporation of the **Vivogel ® StemCoat** solution (e.g. with Parafilm®) and can be stored at 2 – 8 °C for up to 1 week after coating. Allow stored coated cultureware to come to room temperature (15 – 25 °C) for 30 minutes before proceeding to step 7.*

7. Gently tilt the cultureware onto one side and allow the excess **Vivogel ® StemCoat** solution to collect at the edge. Remove the excess solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.

8. Immediately add complete culture medium (e.g. 2 mL/well if using a 6-well plate).

Table 1. Recommended Volumes for Coating Cultureware

CULTUREWARE	VOLUME OF DILUTED MATRIX
6-well plate	1 mL/well
100 mm dish	6 mL/dish
T-25 cm^2 flask	3 mL/flask
T-75 cm^2 flask	8 mL/flask

C. PASSAGING HUMAN ES AND iPS CELLS

Human ES and iPS cells maintained in **Vivogel ® StemCoat** coated culturewares can be passaged using a number of different methods, both enzymatic and non-enzymatic, that are described in this section. Gentle Cell Dissociation Reagent (STEMCELL Technologies) and TrypLE™ Select (Thermo Fisher Scientific) are both compatible with **Vivogel ® StemCoat**. Gentle Cell Dissociation Reagent are used for enzyme-free dissociation methods. The cell aggregates generated using these methods may be fragile, and they should be replaced as quickly as possible. Enzyme-free methods are recommended for their ease of use, high cell recovery, and for preserving the integrity of cell surface proteins that aid in the reattachment of cells to the matrix.

C.a. Gentle Cell Dissociation Reagent (non-enzymatic)

Gentle Cell Dissociation Reagent (GCDR) is an enzyme-free reagent for passaging of human ES and iPS cells as aggregates with manual scraping to generate cell aggregates. Once familiar with the following protocol, it is possible to adjust the time at which cells are ready to be passaged by altering the cell aggregate size or plating density. The following are instructions for passaging cells from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly.

1. At least 1 hour before passaging, coat new plates with **Vivogel ® StemCoat** (section **B.b.**).

2. Aliquot sufficient complete growth medium and warm to room temperature (15 – 25 °C).

3. Use a microscope to visually identify regions of differentiation. Mark these using a felt tip or lens marker on the bottom of the plate.

4. Remove regions of differentiation by scraping with a pipette tip or by aspiration. Avoid having the culture plate out of the incubator for more than 15 minutes at a time.

NOTE: Selection may not be required if differentiation is < 5%. Selection should not exceed 20% of the well if the culture is of high quality.

5. Aspirate medium from the well and add 1 mL of GCDR.

6. Incubate at room temperature for 6 - 8 minutes.

7. Aspirate the GCDR, then add 1 mL of complete growth medium. Gently detach the colonies by scraping with a serological glass pipette or a cell scraper.

NOTE: Take care to minimize the breakup of colonies.

8. Transfer the detached cell aggregates to a 15 mL conical tube. Optional: Rinse the well with an additional 1 mL of complete growth medium to collect remaining cell aggregates.

NOTE: Centrifugation of cell aggregates is not required.

9. Use 2 mL serological pipette and carefully pipette the cell aggregate mixture up and down 2 - 5 times to break up the aggregates as needed. A uniform suspension of aggregates approximately 50 - 200 µm in size is optimal; do not create a single-cell suspension.

10. Plate the cell aggregate mixture at the desired density onto coated wells containing complete growth medium. If the colonies are at an optimal density, the cultures can be split every 4 - 7 days using 1 in 10 to 1 in 50 splits (i.e. cell aggregates from 1 well can be plated in 10 to 50 wells). If the colonies are too dense or too sparse, at the next time of passaging adjust the split ratio accordingly.

NOTE: Work quickly to transfer cell aggregates into new cultureware to maximize viability and attachment.

11. Place the plate in a 37 °C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the cell aggregates. Do not disturb the plate for 24 hours.

NOTE: Uneven distribution of aggregates may result in increased differentiation of human ES and iPS cells.

12. Perform daily medium changes using complete growth medium and visually assess cultures to monitor growth until the next passaging time.

C.b. TrypLE™ Select (enzymatic)

TrypLE™ is designed as a direct substitute for trypsin in existing protocols. The following are instructions for passaging cells from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly.

1. At least 1 hour before passaging, coat new plates with **Vivogel ® StemCoat** (section **B.b.**).

2. Observe the cells by microscopy. If imaging is required, dead cells may be removed by replacing the medium. Culture medium is preferred over DPBS as cells tend to detach with extended incubation in DPBS.

3. Aspirate the medium.

4. Wash once with 1 mL of DPBS and aspirate.

5. Add 300 µL/well of 0.5X TrypLE™ Select and spread well over the surface.

6. Incubate the plates at 37 °C, CO_2 5%, for 1 min.

7. Remove the plates from the incubator after 1 min and gently re-distribute the TrypLE™ over the surface.

8. Incubate the plates for another 3 min at 37 °C, CO_2 5%, (total 4 min).

9. Remove the plates from the incubator and observe the cells by microscopy. (The cells should appear separate and rounded. During the 4 min incubation, cell-cell adhesion is destroyed, but the cells remain attached to the matrix. Caution: Longer incubation will also detach the cells.)

10. Aspirate the 0.5X TrypLE™ Select.

11. Wash once with 2 mL/well DPBS. (Add the DPBS gently as the cells tend to detach easily.)

12. Add 1 mL of culture medium per well.

13. Detach the cells with a cell scraper.

14. Observe the cells under the microscope to ensure they have detached.

15. Pipette the cells 10 times to fully dissociate. Transfer the detached cell aggregates to a 15 mL conical tube. (This prevents re-attachment of the cells to the plate surface).

16. Count the cells by trypan blue exclusion using the Countess automatic cell counter (iPSC program - sensitivity: 5, min size: 8, max size: 30, circularity: 75).

17. Plate 13,000 live cells in one well of a 6-well plate (10 cm^2) coated with **Vivogel ® StemCoat**. (Immediately distribute the cells evenly over the plate surface to avoid uneven attachment.)

18. Culture the plates at 37 °C, CO_2 5%.

NOTE: Medium change is performed on a daily basis. If the color of the medium turns orange or yellow, it should be changed every day. Should yield approximately 1 × 10^6 cells in 8 days (±1 day, 100 - fold expansion).

D. CRYOPRESERVING AND THAWING CELLS

Human pluripotent stem cells maintained in **Vivogel ® StemCoat** coated culturewares can be cryopreserved as aggregates or single cells using cryopreservation medium such as CTS™ PSC Cryomedium (Thermo Fisher Scientific). It is appropriate for use with cultures routinely passaged as aggregates using the methods described in this manual. The thawing protocols are for human ES and iPS cells that were maintained in mTeSR™1 prior to cryopreservation.

D.a. CRYOPRESERVING CELLS AS CELL AGGRAGATE

NOTE: Wipe down the outside of the bottle with 70% ethanol or isopropanol before opening.

The following are instructions for cryopreserving cultures grown in **Vivogel ® StemCoat** coated 6-well plates using CTS™ PSC Cryomedium. Cultures should be harvested and cryopreserved at the time they would normally be ready for passaging. Each vial should contain the cell aggregates from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly.

1. Thaw and pre-chill CTS™ PSC Cryomedium at 2 °C to 8 °C

2. Passage cells using an enzyme-free passaging protocol until step 8 (section **C.a.**) or the enzymatic passaging protocol until step 15 (section **C.b.**).

3. Centrifuge at 300 × *g* for 5 minutes at room temperature (15 – 25 ℃).

4. Gently aspirate the supernatant, taking care not to disrupt the cell pellet.

5. Gently resuspend the pellet with 1 mL per well harvested of cold (2 – 8 ℃) CTS™ PSC Cryomedium using a serological pipette. Minimize the break-up of cell aggregates when dislodging the pellet.

6. Transfer 1 mL of cell aggregates mixture into each labeled cryovial using a 2 mL serological pipette.

7. Cryopreserve cell aggregates using either:

- A standard slow rate-controlled cooling protocol that reduces temperatures at approximately -1 ℃/min, followed by long-term storage at -135 ℃ (liquid nitrogen) or colder. Long-term storage at -80 ℃ is not recommended.
- A multi-step protocol where cells are kept at -20 ℃ for 2 hours, followed by -80℃ for 2 hours, followed by long-term storage at -135 ℃ (liquid nitrogen) or colder.

D.b. CRYOPRESERVING CELLS AS SINGLE CELL

The following are instructions for cryopreserving cultures grown in **Vivogel ® StemCoat** coated 6-well plates. If using other cultureware, adjust volumes accordingly. Cultures should be harvested and cryopreserved at the time they would normally be ready for passaging.

NOTE: Wipe down the outside of the bottle with 70% ethanol or isopropanol before opening.

1. Warm medium, Gentle Cell Dissociation Reagent, and DPBS (Without Ca++ and Mg++) to room temperature (15 – 25 ℃) before use.

2. Wash the well to be passaged with 1 mL of DPBS (Without Ca++ and Mg++).

3. Aspirate the wash medium and add 1 mL of Gentle Cell Dissociation Reagent. Incubate at 37 ℃ for 8 - 10 minutes.

NOTE: The incubation time may vary when using different cell lines or other cell passaging reagents.

4. Harvest cells by pipetting up and down with either a serological pipette or a 1 mL pipettor to ensure a single-cell suspension and transfer cells to a 15 mL conical tube. Rinse wells with an additional 2 - 4 mL of medium and add the rinse to the tube containing the cells.

5. Perform a viable cell count using Trypan Blue.

6. Centrifuge cells at 300 × *g* for 5 minutes at room temperature (15 – 25 ℃).

7. Carefully remove the supernatant with a pipette, leaving a small amount of medium to ensure the cell pellet is not disturbed. Resuspend the cell pellet by gently flicking the tube.

8. Add cold (2 – 8 ℃) CTS™ PSC Cryomedium to obtain a cell suspension of 1×10^6 cells/mL and mix thoroughly.

9. Transfer 1 mL of the single-cell suspension to each cryovial.

D.c. THAWING CELLS AS CELL AGGRAGATE

Human ES and iPS cells should be thawed into **Vivogel ® StemCoat** coated cultureware (section **B.b.**). In general, one vial of cells cryopreserved as described above can be successfully thawed into 1 - 2 wells of a 6-well plate.

1. Have all tubes, warmed complete growth medium (15 – 25 ℃), and coated cultureware ready before starting the protocol to ensure that the thawing procedure is done as quickly as possible.

2. Wipe the outside of the vial of cells with 70% ethanol or isopropanol.

3. In a biosafety cabinet, twist the cap a quarter-turn to relieve internal pressure, then retighten.

4. Quickly thaw cells in a 37 ℃ water bath by gently shaking the vial. Remove the vial when a small frozen cell pellet remains. Do not vortex cells.

5. Wipe the outside of the vial with 70% ethanol or isopropanol.

6. Use a 2 mL serological pipette to transfer the contents of the cryovial to a 15 mL conical tube.

NOTE: Using a 2 mL serological pipette instead of a 1 mL pipettor will minimize breakage of cell aggregates.

7. Add 5 - 7 mL of warm complete growth medium dropwise to the 15 mL tube, gently mixing as the medium is added.

8. Centrifuge cells at 300 × *g* for 5 minutes at room temperature (15 – 25 ºC).

9. Aspirate the medium, leaving the cell pellet intact. Gently resuspend the cell pellet in 1 mL of complete growth medium using a 2 mL serological pipette. Take care to maintain the cells as aggregates.

10. Transfer 0.5 mL of the cell mixture into one well of a coated 6-well plate containing mTeSR™1 (i.e. two wells can be plated from each cryovial).

Note: Number of wells plated may need to be adjusted depending on how many cell aggregates were cryopreserved. Typically more aggregates will need to be plated after thawing than during routine passaging.

11. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the cell aggregates. Do not disturb the plate for 24 hours.

Note: Uneven distribution of aggregates may result in increased differentiation of human ES and iPS cells.

12. Perform daily medium changes using mTeSR™1 and visually assess cultures to monitor growth until the next passaging time. Check for undifferentiated colonies that are ready to be passaged (dense-centered) approximately 6 - 7 days after thawing.

Note: If only a few undifferentiated colonies are observed after thawing, it may be necessary to select only these colonies for passaging and replate them in the same size well (i.e. without splitting) on a newly-coated plate.

D.d. THAWING CELLS AS SINGLE CELL

Human ES and iPS cells should be thawed into **Vivogel ® StemCoat** coated cultureware (section **B.b.**). In general, one vial of cells cryopreserved as described above can be successfully thawed into 1 - 2 wells of a 6-well plate.

1. Have all tubes, warmed complete growth medium (15 – 25 ºC), and coated cultureware ready before starting the protocol to ensure that the thawing procedure is done as quickly as possible.

2. Add Y-27632 to complete growth medium to reach a final concentration of 10 µM.

3. Wipe the outside of the vial of cells with 70% ethanol or isopropanol.

4. In a biosafety cabinet, twist the cap a quarter-turn to relieve internal pressure, then retighten.

5. Quickly thaw cells in a 37 ºC water bath by gently shaking the vial. Remove the vial when a small frozen cell pellet remains. Do not vortex cells.

6. Wipe the outside of the vial with 70% ethanol or isopropanol.

7. Use a 1 mL pipettor to slowly transfer the contents of the cryovial to a 15 mL conical tube containing 5 - 7 mL of DMEM/F-12 with 15 mM HEPES.

8. Centrifuge cells at 300 × *g* for 5 minutes at room temperature (15 – 25 ºC).

9. Carefully remove the supernatant with a pipette, leaving a small amount of medium to ensure the cell pellet is not disturbed.

10. Add 1 mL of complete growth medium containing 10 µM Y-27632 to the tube. Mix gently.

11. Plate cells onto coated cultureware.

NOTE: In general, one frozen cryovial containing 1 × 10^6 cells can be thawed and plated into 1 - 2 wells of a 6-well plate.

12. Place the cultureware in a 37 ºC incubator. Move the cultureware in several quick, short, back-and-forth and side-to-side motions to evenly distribute the cells across the surface.

13. Perform daily medium changes using complete growth medium (without Y-27632) and visually assess cultures to monitor growth until the next passaging time (i.e. 80 - 90% confluent). This takes approximately 2 - 5 days after thawing.

NOTE: The time to reach 80 - 90% confluency may vary when using different cell lines; cultures should be monitored under the microscope until the optimal passaging time is determined.

14. Passage cultures using standard techniques to generate cell aggregates.

NOTE: It is not recommended to perform serial single-cell passaging due to the increased risk of karyotype abnormalities.

Representative results:

Culture and passage of human iPS cells are tested in **Vivogel ® StemCoat** coated cultureware following the protocols above. Undifferentiated human iPS cells cultured in **Vivogel ® StemCoat** coated cultureware grow as compact, multicellular colonies characterized by distinct borders (Figure 1).

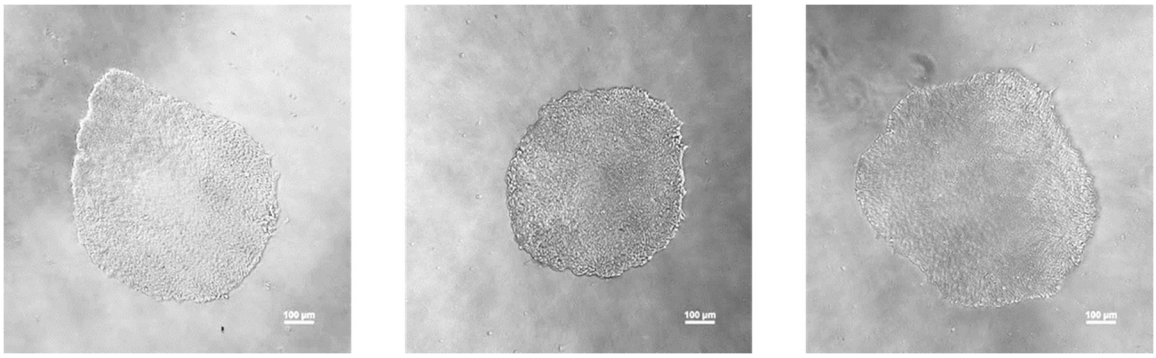

Figure 1. Undifferentiated human iPS cell culture on **Vivogel ® StemCoat** coated plate.

Undifferentiated human iPS cells can also be characterized through immunocytochemistry (ICC) staining of specific biomarkers (Figure 2).

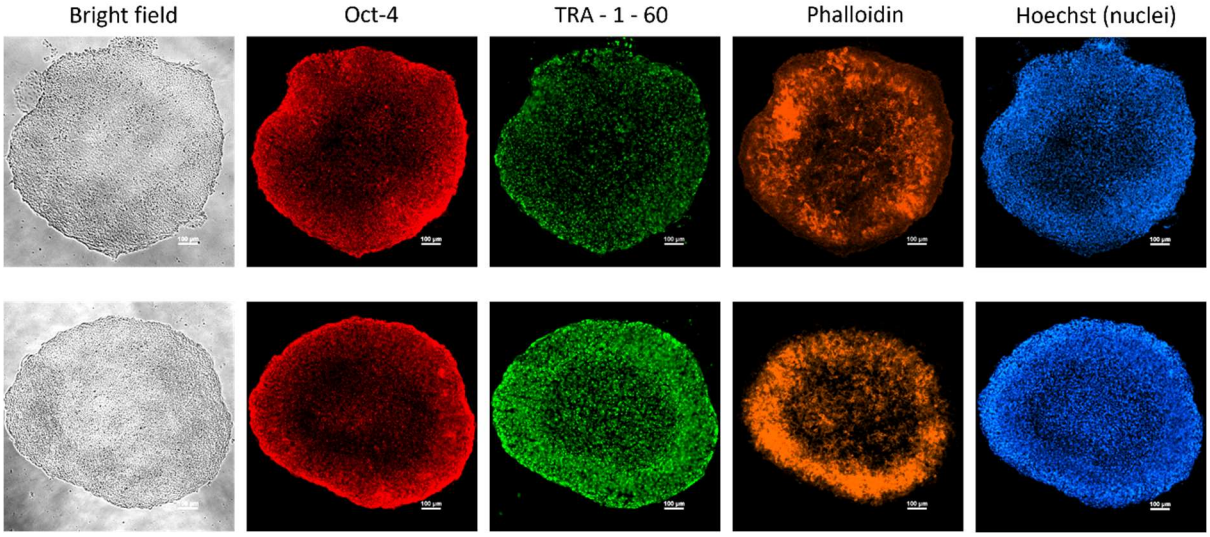

Figure 2. Immunostaining of human induced pluripotent stem cells (iPSCs) cultured on **Vivogel ® StemCoat**. iPSCs maintain expression of pluripotency markers Oct-4 and TRA-1-60 after six passages on **Vivogel ® StemCoat**.