TECHNICAL MANUAL
VERSION 1.0.0

Maintenance of hPSCs on StemAdhere™ Defined Matrix for hPSC

PRIMORIGEN BIOSCIENCES®

STEMCELL™ TECHNOLOGIES

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1.0 Introduction

1.1 Defined Systems for the Culture of Human Pluripotent Stem Cells

In the years since the earliest methods of human embryonic stem cell culture were published[1], the stem cell field has been moving towards better definition and control over the conditions used to grow human pluripotent stem cells (hPSCs). Replacement of undefined components and animal products from the culture system is a major step towards the realisation of the clinical potential of hPSC. Another issue is that undefined conditions are also vital for the achievement of standardization and reproducibility of research within the field. Great strides in the definition of the culture environment have been made with the development of defined and serum-free media to allow hPSC culture without the need for a supporting layer of feeder cells[2,3]. However, the culture of hPSCs in feeder-independent media typically necessitates the use of crude preparations of extracellular matrices to support cell attachment, such as the undefined, mouse tumour-derived surface coating, Matrigel™ (BD Biosciences). Understanding the specific requirements needed to create defined surfaces that are capable of supporting hPSC attachment and growth has proven to be technically challenging[4]: defined matrices often suffer from poor cell attachment, inability to support long term passaging, or prohibitive production costs. StemAdhere™ Defined Matrix for hPSC is a matrix for use with defined media such as mTeSR™1 and TeSR™2. StemAdhere™ Defined Matrix for hPSC, developed and manufactured by Primorigen Biosciences, is an effective alternative to Matrigel™ and allows for consistent long-term culture while maintaining hPSC pluripotency. When used with mTeSR™1 or TeSR™2, StemAdhere™ Defined Matrix for hPSC provides a completely defined culture system for the maintenance of hPSCs under feeder-independent conditions[5]. This system allows complete control over the culture environment, resulting in more consistent cell populations and more reproducible results in downstream applications.

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1.2 Morphology of Undifferentiated hPSCs grown on StemAdhere™ Defined Matrix for hPSC

Undifferentiated hPSC have a distinct cellular morphology that is easily recognisable to the trained eye. They exhibit a high nuclear to cytoplasmic ratio and prominent nucleoli. hPSC grown in either mTeSR™1 or TeSR™2 on StemAdhere™ Defined Matrix for hPSC retain these morphological characteristics (Figure 1). Unlike hPSC grown on feeders, which tend to grow in very dense, compact colonies, hPSC cultured in feeder-independent conditions, including on StemAdhere™ Defined Matrix for hPSC, tend to form colonies that are more loosely packed, with noticeable cellular spreading at the edges (Figures 2a and 3a). As the culture matures in the days post-passaging, the colonies become large, round and multilayered, eventually acquiring a phase bright center of the colony when viewed under phase contrast (Figures 2b and 3b). Differentiation within the culture is recognisable by the emergence of morphologically distinct cells in or around the colony, that create a pattern of non-uniformity (Figure 4 a,b,c, circled areas).

hPSCs grown in mTeSR™1 or TeSR™2 on StemAdhere™ Defined Matrix for hPSC are ready to passage when the colonies are large, beginning to merge, and have centers that are dense and phase-bright compared to their edges (see Figure 2b, 3b). Depending on the size and density of seeded aggregates, cultures are usually passaged 4 - 7 days after seeding in mTeSR™1 or TeSR™2. There is an approximate 24 hour window that is optimal for passaging. If there are large colonies, with dense centers, and the colonies are beginning to merge, cells should be passaged within 24 hours.

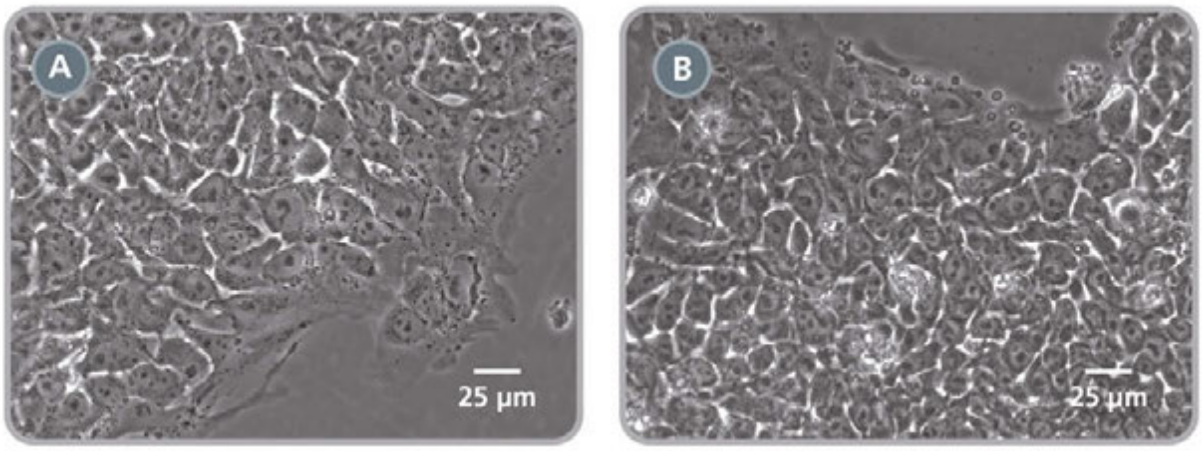

Figure 1: Undifferentiated hPSCs cultured in mTeSR™1 or TeSR™2 on StemAdhere™ Defined Matrix for hPSC exhibit high nuclear to cytoplasmic ratio and prominent nucleoli.

A: H9 hESCs at day 5 of culture in TeSR™2 on StemAdhere™ Defined Matrix for hPSC. Magnification: 400X

B: H9 hESCs at day 6 of culture in mTeSR™1 on StemAdhere™ Defined Matrix for hPSC. Magnification: 400X

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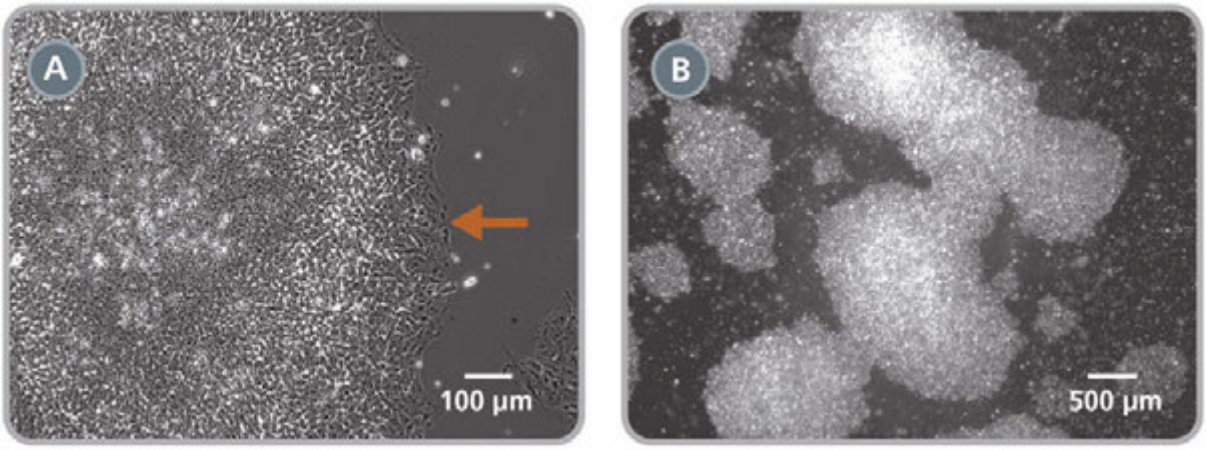

Figure 2: Undifferentiated hPSCs grown in TeSR™2 on StemAdhere™ Defined Matrix for hPSC have a slightly different morphology compared to hPSCs grown on other matrices.

A: H9 hESCs at day 5 of culture in TeSR™2 on StemAdhere™ Defined Matrix for hPSC tend to form colonies that are more loosely packed, with noticeable cellular spreading at the edges of the colonies. Magnification: 100X.

B: H9 hESCs at day 5 of culture in TeSR™2 on StemAdhere™ Defined Matrix for hPSC are ready to passage when the colonies are large, beginning to merge, and have centers that are dense and phase-bright compared to their edges. Magnification: 20X.

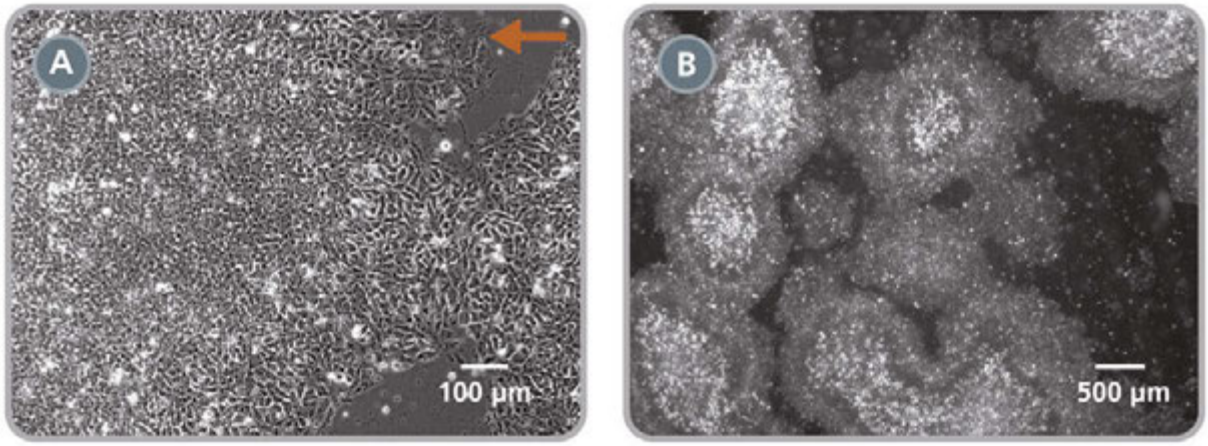

Figure 3: Undifferentiated hPSCs grown in mTeSR™1 on StemAdhere™ Defined Matrix for hPSC have a slightly different morphology compared to hPSCs grown on other matrices.

A: H9 hESCs shown at day 5 of culture in mTeSR1 on StemAdhere™ Defined Matrix for hPSC. Magnification: 100X.

B: H9 hESCs at day 5 of culture in mTeSR1 on StemAdhere™ Defined Matrix for hPSC are ready to passage when the colonies are large, beginning to merge, and have centers that are dense and phase-bright compared to their edges. Note that these cultures have a different pattern to phase brightness that is not indicative of culture quality or pluripotency. Magnification: 20X.

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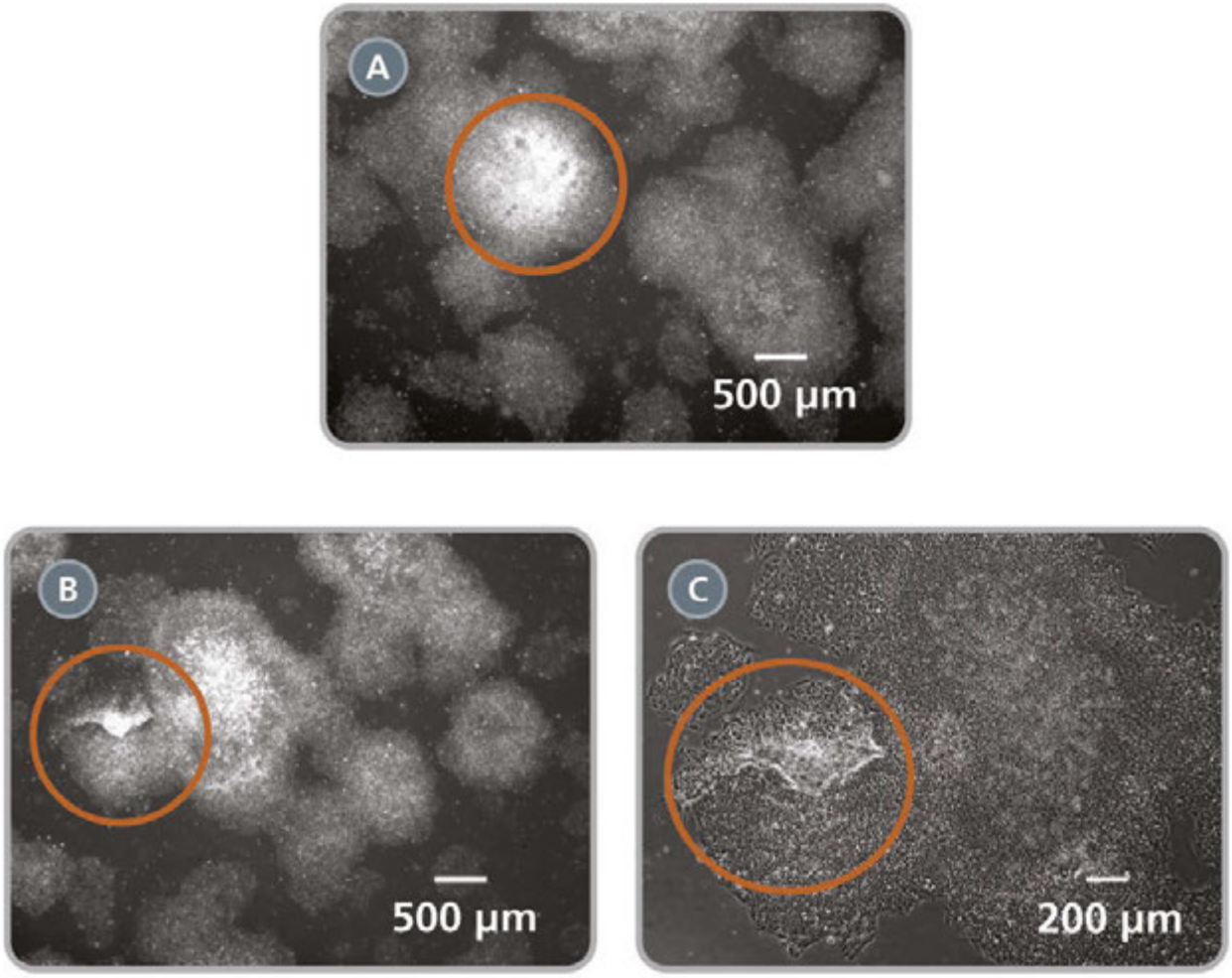

Figure 4: Differentiation within the culture is recognisable by the emergence of morphologically distinct cells in or around the colony.

Areas of differentiation (circled) in H9 hESCs cultured in TeSR™2 on StemAdhere™ Defined Matrix for hPSC. Cells shown have been in culture for 6 days at the time this image was taken. Note that it is normal to observe isolated areas of differentiation in hPSC cultures, even when high expression of pluripotency markers is maintained. Areas of differentiation should not exceed 20% of the colonies in the culture.

A: Magnification: 20X

B: Magnification: 20X

C: Magnification of the same colony pictured in B, but at 40X magnification

1.3 Critical Parameters for Successful Culture of hPSCs on StemAdhere™ Defined Matrix for hPSC

Culturing hPSCs is a very time-consuming process. It requires daily medium changes, use of the highest quality reagents and frequent morphologic observations of the cultures to ensure that they are maintained at the optimum density. For general tips regarding maintenance of hPSCs in mTeSR™1 and TeSR™2, please see the Technical Manual entitled "Maintenance of hPSCs in mTeSR™1 and TeSR™2", Catalog #29106, available on www.stemcell.com. The following tips will help ensure success with hPSC culture specifically on StemAdhere™ Defined Matrix for hPSC.

1.3.1 Use of Non Tissue Culture-Treated Plates

It is essential that ***non*** tissue culture-treated plates be used for the coating of StemAdhere™ Defined Matrix for hPSC. Use of standard tissue culture-treated plates will result in decreased performance and unsatisfactory cell attachment.

1.3.2 Enzyme Free Passaging

The protocols described in this manual are for the culture of hPSCs on StemAdhere™ Defined Matrix for hPSC. Ensure that exposure of cells to the enzyme free dissociation reagent does not exceed the suggested times described in this manual, as this may lead to dissociation of the aggregates to single cells. Significant amounts of single cells after dissociation may put unwanted selective pressure on cell populations, possibly leading to genetic aberrations.

1.3.3 Characterization of hPSCs

It is good practice to monitor your cell population frequently to ensure maintenance of pluripotency, and a normal karyotype. Flow cytometry protocols for assessing pluripotency are described in detail in the Technical Manual entitled "Maintenance of hPSCs in mTeSR™1 and TeSR™2", Catalog #29106, available on www.stemcell.com. hPSCs should retain a normal genetic makeup during routine culture, expansion and manipulation. Nonetheless, chromosomal and genetic aberrations may appear during long-term passaging. Accordingly, it is important to periodically check hPSC cultures to exclude the possibility of an abnormal karyotype.

1.3.4 Cryopreservation of hPSCs

For the cryopreservation of hPSCs cultured on StemAdhere™ Defined Matrix for hPSC, dissociate as described in Section 3.4 and resuspend in cryopreservation media such as CryoStor™ CS10 (Catalog #07930) or mFreSR™ (Catalog #05854/05855) prior to freezing.

1.3.5 Density of hPSC cultures

Culture density is a critical aspect of maintaining hPSCs in mTeSR™1 or TeSR™2 on StemAdhere™ Defined Matrix for hPSC. Cultures that are either too sparsely or too densely populated can lead to unexpected differentiation. Many colonies in the dish should be just beginning to touch each other at the time of passaging (i.e. plate is approximately 75% confluent). Adjust plating and/or split ratios to achieve a balance between having too much space between colonies and having a confluent culture (see Figure 5, for an example of an hPSC culture ready for passaging). As a general guideline, an acceptable density would be approximately 400 colonies per well of a 6-well plate. Note that this seeding density is somewhat higher than what is typically seeded when using Matrigel™ as a surface coating (which is approximately 150 colonies/plate).

For an alternative method of determining split ratios, see Section 5.0.

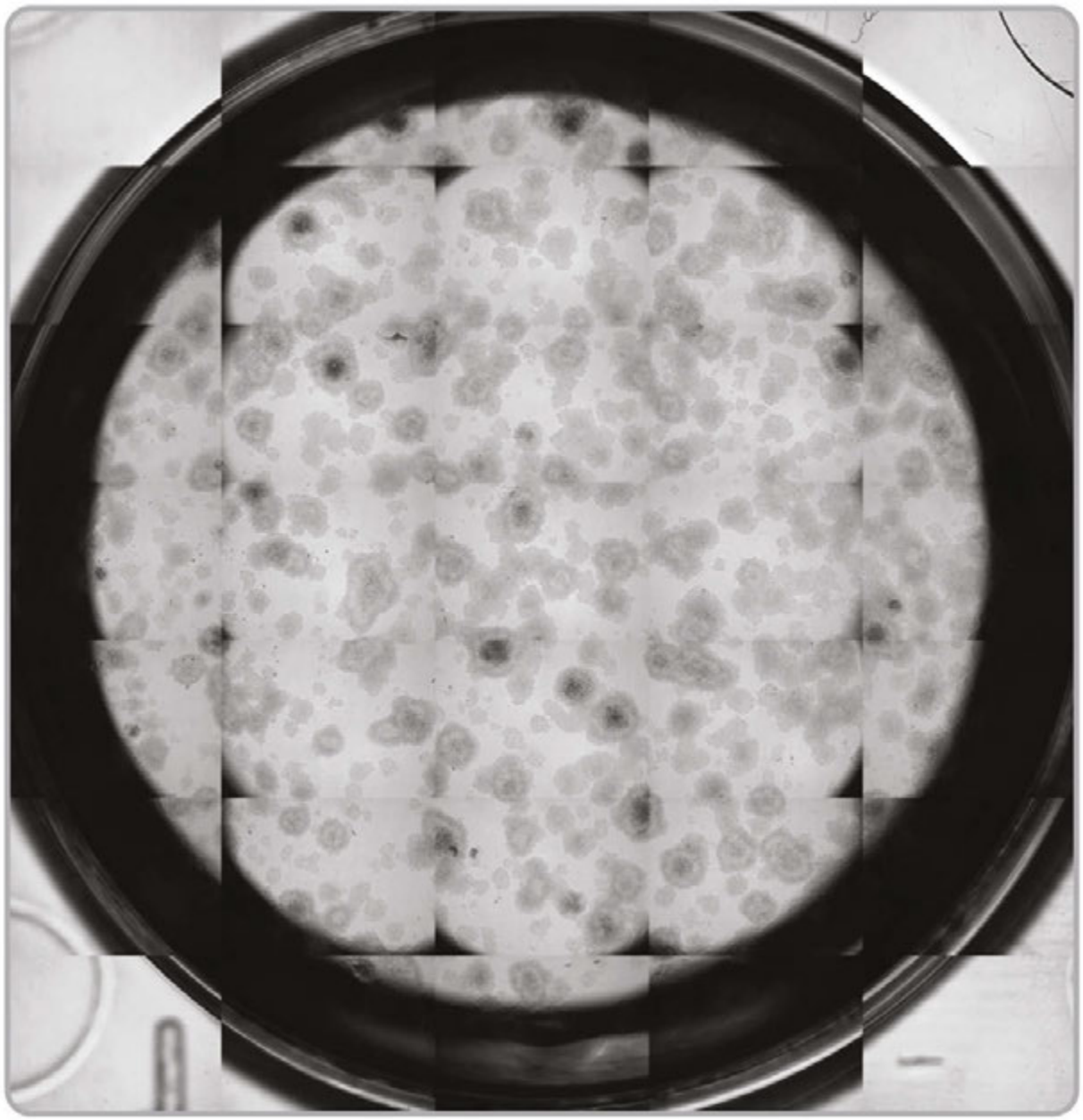

Figure 5: H9 hESCs at day 5 of culture in TeSR™2 on StemAdhere™ Defined Matrix for hPSC ready to passage.

Composite image of an entire well of a Non Tissue Culture-Treated 6-well Plate (Catalog #27147). This picture is a representation of optimal colony density immediately prior to passage.

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2.0 Materials and Reagents

2.1 Products for hPSC Culture using StemAdhere™ Defined Matrix for hPSC

Product	Quantity	Catalog #
StemAdhere™ Kit: - StemAdhere™ Defined Matrix for hPSC (Catalog #07160, 2 mL) - Gentle Cell Dissociation Reagent (Catalog #07174, 100 mL) - StemAdhere™ Dilution Buffer (Catalog #07163, 100 mL) - Non Tissue Culture-Treated 6-well Plates, (Catalog #27147, 8 plates/pk)	1	07170
StemAdhere™ Defined Matrix for hPSC	2 mL	07160
Gentle Cell Dissociation Reagent	100 mL	07174
StemAdhere™ Dilution Buffer	100 mL	07163
Non Tissue Culture-Treated 6-well Plates	8 plates/pk	27147

2.2 Additional Reagents Required for hPSC Culture Using StemAdhere™ Defined Matrix for hPSC

The protocols described in this Technical Manual were developed using mTeSR™1 or TeSR™2 feeder-independent media to maintain the starting population of undifferentiated hPSCs. We recommend the use of these media to get optimal results.

Reagent / Materials	Catalog #
DMEM/F-12	36254
mTeSR™1 Medium Kit	05850/05870/05875/05857
TeSR™2 Medium Kit	05860/05880
Trypan Blue	07050
70% Ethanol or Isopropanol	-
Polypropylene conical tubes (50 mL)	e.g. BD Biosciences, Catalog #352070
Polypropylene conical tubes (15 mL)	e.g. BD Biosciences, Catalog #352196
T-75 polystyrene flasks, non-tissue culture-treated	e.g. Greiner Catalog #658190
12-well polystyrene plates, non-tissue culture-treated	e.g. Greiner Catalog #665102
Cell scrapers	e.g. Corning Catalog #3010
Serological pipettes (2 mL, 5 mL, 10 mL)	e.g. BD Biosciences Catalog #357507, 357543, 357551

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2.3 Equipment Required for hPSC Culture using StemAdhere™ Defined Matrix for hPSC

- Vertical laminar flow hood certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and >95% humidity in an atmosphere of 5% CO_2 in air
- Low speed centrifuge (e.g. Beckman GS-6) with a swinging bucket rotor fitted with a plate holder

  All protocols described in this manual can be performed with the brake on.
- Pipette-aid (e.g. Drummond Scientific)
- Hemacytometer (e.g. Neubauer, Reichert)
- Micropipette (e.g. Eppendorf, Gilson) with appropriate tips
- Inverted microscope with 2x, 4x and 10x phase objectives (e.g. Olympus CKX31)
- Isopropanol freezing container (e.g. Nalgene, Fisher; Catalog #1535050)
- -150ºC freezer or liquid nitrogen vapour tank
- -80ºC freezer

3.0 Culture of hPSCs Using mTeSR™1 or TeSR™2 on StemAdhere™ Defined Matrix for hPSC

Please read the entire Technical Manual before beginning culture of hPSCs on StemAdhere™ Defined Matrix for hPSC, as it requires different techniques than those used with other matrices.

3.1 Preparation of mTeSR™1 and TeSR™2 Media

For complete instructions on the preparation of complete mTeSR™1 and TeSR™2 media, please see the Technical Manual "Maintenance of hPSCs in mTeSR™1 and TeSR™2" (Manual Catalog #29106) available on our website at www.stemcell.com.

3.2 Coating of Non Tissue Culture Treated Plates with StemAdhere™ Defined Matrix for hPSC

StemAdhere™ Defined Matrix for hPSC (Catalog #07160) may be divided into 240 µL aliquots (enough for one 6-well plate) and frozen down.

1. Dispense 6 mL of StemAdhere™ Dilution Buffer into a 15 mL polypropylene conical tube (BD Biosciences, Catalog #352196) at room temperature.

   Note: Use only polypropylene tubes. Do not use polystyrene or other tubes, because StemAdhere™ Defined Matrix for hPSC is strongly adherent to other surfaces and may be lost upon exposure.

2. Remove a 240 µL aliquot of frozen StemAdhere™ Defined Matrix for hPSC from -80°C. Thaw at room temperature until liquid, then add the thawed StemAdhere™ Defined Matrix for hPSC to the aliquoted StemAdhere™ Dilution Buffer and gently mix 2-3 times with a serological pipette. Do not vortex to mix. The vial may be washed with StemAdhere™ Dilution Buffer if desired.

3. Immediately use the diluted StemAdhere™ Defined Matrix for hPSC solution to coat a Non Tissue Culture-Treated 6-well plate. For each well, use 1 mL of diluted StemAdhere™ Defined Matrix for hPSC. Deposit the desired volume in the center of the well. Gently rock the plate back and forth to spread the diluted StemAdhere™ Defined Matrix for hPSC solution evenly across the surface.

   Only non tissue culture-treated plate(s) should be used for coating with StemAdhere™ Defined Matrix for hPSC. To coat other sizes of tissue cultureware, scale the volume of diluted StemAdhere™ Defined Matrix for hPSC solution by the surface area of the vessel to be coated.

4. Coated plate(s) should be placed in the incubator (37°C, 5% CO_2) for at least 1 hour before use. Do not allow the plate(s) to dehydrate.

5. If not used immediately, the plate(s) must be sealed to prevent dehydration (e.g. with Parafilm®) and can be stored at 2 - 8°C overnight after coating. Plate(s) are not optimal for hPSC culture if the StemAdhere™ Defined Matrix for hPSC solution does not completely cover the surface to be used for culture; therefore, plate(s) that have regions where the solution has evaporated are not recommended for use.

6. Aspirate excess StemAdhere™ Defined Matrix for hPSC solution from the plate. Rinse each well with 1 mL of StemAdhere™ Dilution Buffer, aspirate and add the appropriate volume of mTeSR™1 or TeSR™2 prior to seeding.

   Seed with hPSC aggregates within 1 hour of adding media to the coated plate.

3.3 Transfer of hPSCs from Matrigel™ to StemAdhere™ Defined Matrix for hPSC

The following steps can be taken to transition hPSC colonies from Matrigel™ or other feeder-free matrices to StemAdhere™ Defined Matrix for hPSC.

Volumes given in this section are for Non Tissue Culture-Treated 6-well Plates (Catalog #27147); scale volumes accordingly for different sized tissue cultureware. Note that StemAdhere™ Dilution Buffer is NOT used in this procedure, only in Section 3.2.

1. Aliquot sufficient mTeSR™1 or TeSR™2 (prepared in Section 3.1) to passage cells. Warm aliquoted mTeSR™1 or TeSR™2 to room temperature (15 - 25°C). Gentle Cell Dissociation Reagent (Catalog #07174) and DMEM/F-12 (Catalog #36254) should already be at room temperature.
2. Use a microscope to visually identify regions of differentiation. 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.

   This selection should not exceed 20% of the well if the hPSC culture is of high quality.
4. Aspirate medium from the hPSC culture and rinse with Gentle Cell Dissociation Reagent (1 mL/well). Aspirate the well.
5. Add 1 mL per well of Gentle Cell Dissociation Reagent. Leave at room temperature for **9 minutes.**
6. Remove Gentle Cell Dissociation Reagent, and add 2 mL of DMEM/F-12 or mTeSR™1/TeSR™2 per well.
7. Gently detach cell aggregates with gentle pipetting, or scrape colonies off with a cell scraper if necessary (e.g. Corning Catalog #3010). **Take care to minimize the breakup of colony clumps, ensuring that single cells are not generated**.
8. Transfer the detached cell aggregates to a 15 mL conical tube and rinse the well with an additional 2 mL of DMEM/F-12 or mTeSR™1/TeSR™2 to collect any remaining aggregates. Add the rinse to the 15 mL tube.

   Note: If cells are scraped in mTeSR™1 or TeSR™2, Steps 9 and 10 are not necessary. Adjust volume of medium for an appropriate split and proceed to Step 11.
9. Centrifuge the 15 mL tube containing the aggregates at 300 x *g* for 5 minutes at room temperature (15 - 25°C).
10. Aspirate the supernatant. For each well of hPSC aggregates collected in the 15 mL tube, add 1 - 2 mL of mTeSR™1 or TeSR™2. Resuspend pellet by gently pipetting up and down with a micropipette outfitted with a disposable 1 mL tip (1 - 2 times). Ensure that cells are maintained as aggregates (for more information, see Section 1.3.2).
11. Plate the hPSC aggregates with mTeSR™1 or TeSR™2 onto a new plate coated with StemAdhere™ Defined Matrix for hPSC (Section 3.2).
12. For this initial seeding step only, it is recommended to plate colonies either with a 1:1 or a 1:2 split to ensure high levels of attachment. For subsequent passages, higher split ratios are typically used to maintain optimal cell densities. For detailed discussions of seeding densities used with StemAdhere™ Defined Matrix for hPSC, please see Section 1.3.5.

13. Move the plate in several quick, short, back-and-forth and side-to-side motions to disperse aggregates across the surface of the wells. Place the plate in a 37°C incubator.

Ensure that newly seeded colonies are evenly dispersed across the entire surface of a StemAdhere™ Defined Matrix for hPSC-coated plate. Uneven distribution of cell clumps may result in differentiation of hPSCs.

3.4 Passaging of hPSCs on StemAdhere™ Defined Matrix for hPSC

Volumes given in this section are for Non Tissue Culture-Treated 6-well Plates (Catalog #27147); scale accordingly for different sized tissue cultureware.

Note that StemAdhere™ Dilution Buffer is NOT used in this procedure, only in Section 3.2.

1. Aliquot sufficient mTeSR™1 or TeSR™2 (prepared in Section 3.1) to passage cells. Warm aliquoted mTeSR™1 or TeSR™2 to room temperature (15 - 25°C). Gentle Cell Dissociation Reagent (Catalog #07174), and DMEM/F-12 (Catalog #36254) should already be at room temperature.
2. Use a microscope to visually identify regions of differentiation. Mark these using a felt tip or lens marker on the bottom of the plate.

   Selection of differentiated areas should not exceed 20% of the well if the culture is of high quality.
3. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
4. Aspirate medium from the hPSC culture and rinse with 1 mL/well of Gentle Cell Dissociation Reagent.
5. Aspirate the well and add 1 mL per well of Gentle Cell Dissociation Reagent. Leave at room temperature (15 - 25°C) for **3 minutes**.
6. Ensure that the cells are not exposed to Gentle Cell Dissociation Reagent for longer than the recommended times, as this may result in the undesired breakdown of clumps into single cells.
7. Remove the Gentle Cell Dissociation Reagent, and add 2 mL of DMEM/F-12 or mTeSR™1/TeSR™2 per well.
8. Gently detach aggregates with gentle pipetting, or scrape colonies off with a cell scraper if necessary (e.g. Corning Catalog #3010). **Take care to minimize the breakup of colony clumps, ensuring that single cells are not generated.**
9. Transfer the detached cell aggregates to a 15 mL conical tube and rinse the well with an additional 2 mL of DMEM/F-12 or mTeSR™1/TeSR™2 to collect any remaining aggregates. Add the rinse to the 15 mL tube.

   Note: If cells are scraped in mTeSR™1 or TeSR™2, Steps 10 and 11 are not necessary. Adjust volume of medium for an appropriate split and proceed to Step 12.
10. Centrifuge the 15 mL tube containing the aggregates at 300 x *g* for 5 minutes at room temperature (15 - 25°C).
11. Aspirate the supernatant. For each well of hPSC aggregates collected in the 15 mL tube, add 1 - 2 mL of mTeSR™1 or TeSR™2. Resuspend pellet by gently pipetting up and down with a micropipette outfitted with a disposable 1 mL tip (1 - 2 times). Ensure that cells are maintained as aggregates (for more information, see Section 1.3.5).
12. Plate the hPSC aggregates with mTeSR™1 or TeSR™2 onto a new plate coated with StemAdhere™ Defined Matrix for hPSC.

    If the colonies are at an optimal density, the cells can be split every 4 - 7 days using 1:6 to 1:12 splits (i.e. the aggregates from 1 well of a 6-well plate can be plated in 6 - 12 wells of a 6-well plate). If the

colonies are too dense or too sparse, adjust the split ratio accordingly. Note that these split ratios will result in a greater density of colonies compared to that typically seeded on Matrigel™ coated plates. For an alternative method of determining split ratios, see Section 5.0. Please note that these guidelines are based on the growth characteristics of the H1 and H9 hESC lines, and may vary between different lines and laboratories.

13. Move the plate in several quick, short, back-and-forth and side-to-side motions to disperse cells across the surface of the wells. Place the plate in a 37°C incubator.

Ensure that newly seeded colonies are evenly dispersed across the entire surface of a StemAdhere™ Defined Matrix for hPSC-coated plate. Uneven distribution of cell clumps may result in differentiation of hPSCs.

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4.0 Troubleshooting

Problem	Solution
Single cells are generated during colony dissociation	• Reduce exposure time of cells to the Gentle Cell Dissociation Reagent during passaging, as your cell line/culture may be more sensitive. • Minimize the manipulation of cell aggregates after dissociation.
Cells don't adhere to the StemAdhere™ Defined Matrix for hPSC	• Use Non Tissue Culture-Treated 6-well Plates (Catalog #27147) or other recommended cultureware. • Avoid using enzymatic dissociation reagents. • Ensure StemAdhere™ Dilution Buffer is used to dilute StemAdhere™ Defined Matrix for hPSC during plate coating. Do not use the Gentle Cell Dissociation Reagent for dilution of StemAdhere™ Defined Matrix for hPSC. • Ensure that polypropylene tubes are used when diluting the StemAdhere™ Defined Matrix for hPSC.
Excessive (>20%) differentiation in cultures	See the Technical Manual "Maintenance of hPSCs in mTeSR™1 and TeSR™2" (Manual Catalog #29106) available on our website at www.stemcell.com for suggestions. Differentiation is not generally caused by StemAdhere™ Defined Matrix for hPSC.
Low cell expansion	Allow cells to culture longer prior to passage. The majority of cell expansion occurs just prior to optimal passage points.
Non-uniform aggregate attachment	Ensure that well is completely covered with StemAdhere™ Defined Matrix for hPSC during coating step.
Significant scraping is required to dislodge cells	Ensure that the Gentle Cell Dissociation Reagent is being used in Section 3.2.

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5.0 Appendix 1: Plating hPSCs by the Clump Count Method

The seeding densities used with StemAdhere™ Defined Matrix for hPSC are higher than those typically used with Matrigel™. For this reason, it may be helpful to use a more quantitative approach to determine split ratios to ensure a smooth transition to StemAdhere™ Defined Matrix for hPSC. A more quantitative alternative to splitting clump suspensions into defined volumes is to perform clump counts on the hPSC suspension and to always plate a defined number of clumps according to the size of the well or dish that is being seeded. This can be a valuable learning tool for those new to hPSC culture because it aids in defining how much a suspension should be pipetted to achieve optimally-sized clumps. An eyepiece micrometer placed in the microscope eyepiece is required to enumerate clumps of appropriate size (~50 - 60 µm in diameter) that are likely to attach and grow. Eyepiece micrometers are available from most microscope manufacturers.

Performing a Clump Count*

1. Aliquot 40 µL of DMEM/F-12 (Catalog #36254) into 2 wells of a 96-well flat-bottom plate.
2. Draw a "+" centered on the bottom of these wells to serve as a counting grid.
3. Add 5 µL of a freshly mixed clump suspension to each well. Count clumps that are approximately 60 µm or greater in diameter (using a calibrated eyepiece micrometer). This corresponds to clumps with an area of approximately 3500 μm^2
4. Perform duplicate counts, then average the results and calculate the total number (x) of clumps.

$$\frac{\text{\# of clumps counted}}{5\ \mu L} = \frac{x\ \text{clumps}}{\text{total volume of suspension (µL)}}$$

5. Calculate the volume of clump suspension (y) required to seed new dishes using the following guide for appropriate seeding densities:

Plate or Well Size	Target # of Clumps/Plate or Well
Wells in a 6-well dish	500 -700 clumps

For example, to seed 1 well of a 6-well dish, the volume of clump suspension required for 500 clumps is calculated as follows:

$$\frac{\text{\# of clumps counted}}{5\ \mu L} = \frac{500\ \text{clumps}}{y\ \mu L}$$

6. Completely remove excess StemAdhere™ Defined Matrix for hPSC solution from a pre-coated 6-well plate (coated as described in Section 3.2). Rinse with 1 mL of StemAdhere™ Dilution Buffer, aspirate and add the appropriate volume of mTeSR™1 or TeSR™2 to each well.
7. Gently mix the clump suspension prior to plating to ensure a uniform suspension.
8. Add the required seeding volume of clumps to each well.
9. Move the plate in several quick, short, back-and-forth and side-to-side motions to disperse cells across the surface of the wells. Return the plate to the incubator.

Ensure that clumps are evenly dispersed across the entire surface of the plate. Uneven distribution of clumps may result in differentiation of hPSCs.

* Protocol kindly provided by the Vancouver Human Embryonic Stem Cell Core Facility.

6.0 Appendix 2: Representative Cultures of hPSCs grown in TeSR™2 on StemAdhere™ Defined Matrix for hPSC

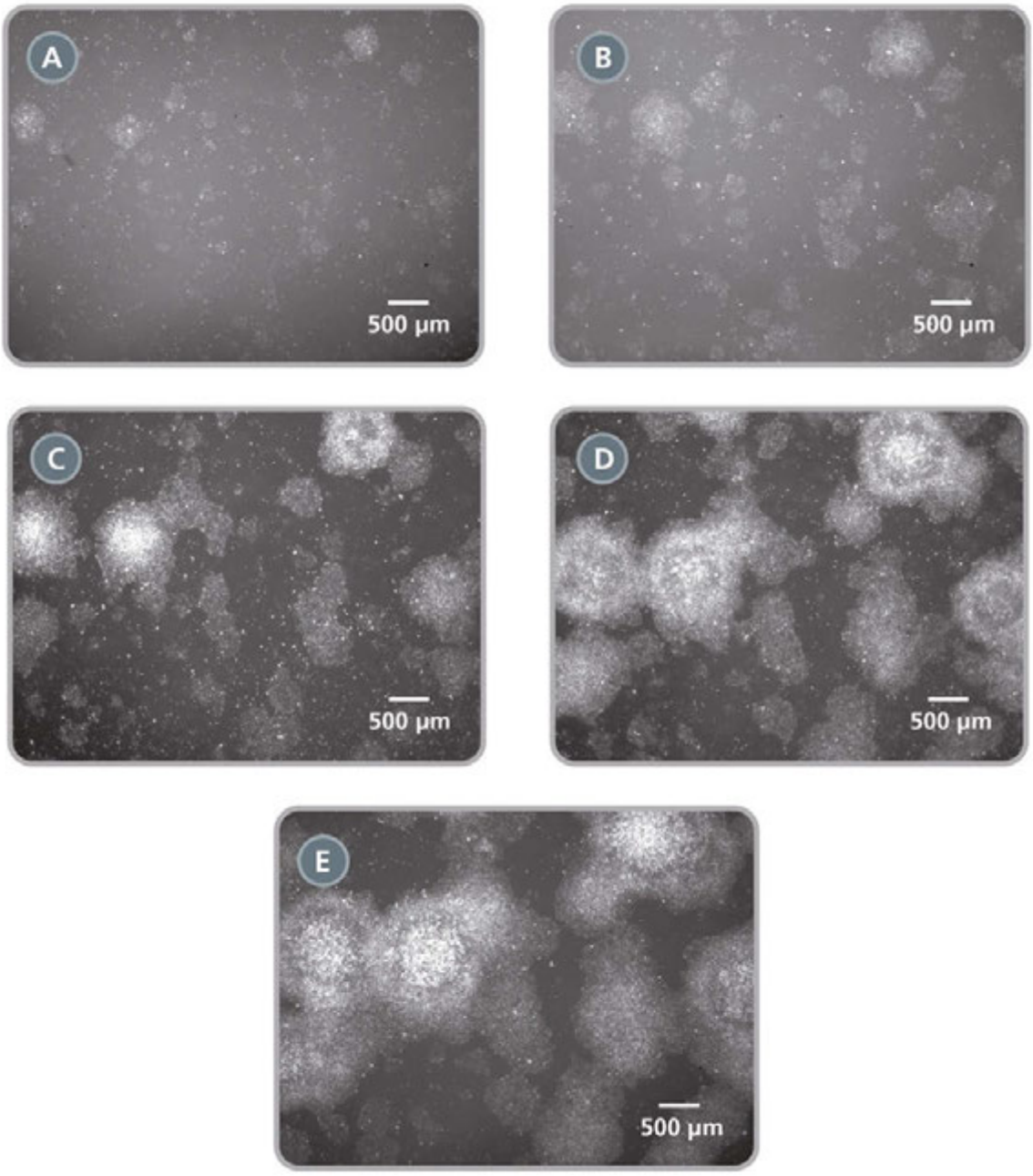

Figure 6: Time-lapse photos showing a fixed area of H9 hESCs in TeSR™2 on StemAdhere™ Defined Matrix for hPSC. Note that the phase-bright centers appear approximately by Day 3 and increase in brightness until Day 5. At Day 5 in this time-lapse, the cultures are ready for passage. Magnification: 20X.

A: Day 1, B: Day 2, C: Day 3, D: Day 4, E: Day 5

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7.0 Appendix 3: Products for hPSC Research Available from STEMCELL Technologies

CATALOG #	PRODUCT DESCRIPTION
AggreWell™ plates for hPSC Research	
27845 (1/pack) 27945 (5/pack)	AggreWell™400 (8 wells, each with approximately 1,200 microwells per well)
27865 (1/pack) 27965 (5/pack)	AggreWell™800 (8 wells, each with approximately 300 microwells per well)
27840 (1/pack) 27940 (5/pack)	AggreWell™400Ex (6 wells, each with approximately 4,700 microwells per well)
Antibodies for hPSC Research	
01550 / 01551	Anti-Oct-3/4 antibody
01552	Anti-SSEA-1 antibody
01553	Anti-SSEA-3 antibody
01554	Anti-SSEA-4 antibody
01555	Anti-TRA-1-60 antibody
01556	Anti-TRA-1-81 antibody
01557	Anti-TRA-2-49 antibody
01558	Anti-TRA-2-54 antibody
10210	FITC-conjugated goat anti-mouse IgG
10211	FITC-conjugated goat anti-mouse IgM
10215	APC-conjugated goat anti-rat IgM
Cytokines for hPSC Research	
02508	Recombinant human NT-3
02509	Recombinant human NT-4
02514	Recombinant human Activin A
02517	Recombinant human BAFF
02519	Recombinant human BDNF
02523	Recombinant human BMP2
02524	Recombinant human BMP4
02525	Recombinant human Noggin
02527	Recombinant mouse Wnt-3A
02634	Recombinant human Basic Fibroblast Growth Factor (bFGF)
02647	Recombinant human Transforming Growth Factor-β1 (TGF-β1)

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CATALOG #	PRODUCT DESCRIPTION
Complete Media for hPSC Research	
05850 / 05870	mTeSR™1 Medium Kit
05860 / 05880	TeSR™2 Medium Kit
05893	AggreWell™ Medium
Accessory Reagents for hPSC Research	
05854 / 05855	mFreSR™ defined cryopreservation medium
07930	Cryostor™ CS10
BD Biosciences, Catalog #354277	BD Matrigel™ hESC-qualified Matrix (qualified for hESC culture by STEMCELL Technologies Inc.)
27215 / 27250	37 µm Reversible Strainer, small and large
36254	DMEM/F-12
07600	Non-Essential Amino Acids
07100	L-glutamine

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