Published in final edited form as:

Methods Mol Biol. 2011 ; 767: 137–146. doi:10.1007/978-1-61779-201-4_10.

Adaptation of Human Pluripotent Stem Cells to Feeder-Free Conditions in Chemically Defined Medium with Enzymatic Single-Cell Passaging

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Abstract

This protocol describes the culture of human pluripotent stem cells (PSCs) under feeder-free conditions in a commercially available, chemically defined, growth medium, using Matrigel as a substrate and the enzyme solution Accutase for single-cell passaging. This system is strikingly different from traditional PSC culture, where the cells are co-cultured with feeder cells and in medium containing serum replacement. PSCs cultured in this new system have a different morphology than those cultured on feeder cells but retain their characteristic pluripotency. This feeder-free PSC culture system is conceptually similar to feeder-free systems that use mouse embryonic fibroblast (MEF)-conditioned medium (MEF-CM) and Matrigel substratum. Instead of MEF-CM, a very complex and undefined medium, this new system uses StemPro SFM, a chemically defined medium that permits enzymatic passaging with Accutase to disaggregate the colonies into single cells. Accutase passaging has been used in conjunction with Stempro in our hands for 20+ passages without detectable karyotypic abnormalities. We will also review techniques for adapting cultures previously grown on MEFs, routine passaging of the cells, and cryopreservation.

Keywords

feeder-free; stemPro; matrigel; single-cell passaging; accutase; cryopreservation; adapting to feeder-free culture

1. Introduction

Feeder cells support the growth of pluripotent stem cells (PSCs) in culture by contributing an as yet undefined and complex mixture of extracellular matrix components and growth factors. Feeder cells used for the co-culture of PSCs are usually fibroblasts and are usually mitotically inactivated so that they remain viable but cannot replicate and overgrow the PSC culture. Mouse embryonic fibroblasts (MEFs) are the most commonly used feeder-cell type and have reliably served as feeder cells for co-culture with mouse embryonic stem cells (1, 2) and human embryonic stem cells (3). The search for methods to develop nonxenogenic culture systems has led to the use of human-derived feeder cells, extracellular matrix components, and growth factors. The development of fully defined, nonxenogenic, culture systems is an important milestone for the PSC field and will greatly improve the usefulness of PSCs in basic science programs and, over the longer term, in human therapeutic applications. The use of defined culture systems will eliminate much of the inherent variability in culture media whose components are sourced from animals and are likely to improve our ability to pre-dictably and reliably direct differentiation. One such defined

medium is StemPro SFM, sold by Invitrogen. Its exact formulation is proprietary, but it is based on a system known to contain IGF1, heregulin1, and activin A, which act through different tyrosine kinase pathways to maintain pluripotency (4). Like traditional PSC media and other defined media, it also contains FGF2 (bFGF).

Simultaneously, a need has arisen for efficient expansion of PSCs using single-cell passaging. The traditional mechanical passaging used to separate clumps of PSCs from feeder layers is laborious and slow, and does not easily result in the large number of cells that would be desired for extensive experiments or ondemand clinical use. Not only is mechanical passaging a highly qualitative process as the cells cannot be counted when they are in clump form, dissociating the clumps into single cells under traditional culture conditions results in extensive cell death. Enzymatic passaging methods employing 0.05% trypsin, TrypLE Express, and nonenzymatic cell dissociation buffer have been used by some laboratories, but their use has not been for single-cell dissociation and has been frequently shown to generate populations of PSCs with abnormal karyotypes (5). A recent report has shown that Accutase can be used to passage human embryonic stem cells (6) in very small clumps and, therefore, we have begun using Accutase to support single-cell passaging of PSCs. When used in conjunction with defined media, single-cell passaging forms the basis of a modernized PSC culture system.

2. Materials

2.1. Reagents and Supplies

1. Matrigel, reduced growth factor (BD Biosciences, #354231).
2. Dulbecco's phosphate buffered saline without Mg_2 and Ca_2 (DPBS).
3. StemPro SFM kit (includes 50 $\times$ supplement, DMEM-F12 with GlutaMax, and 25% BSA Solution) (Invitrogen, #A1000701).
4. 2-Mercaptoethanol (2-ME, Invitrogen, #21985-023).
5. Accutase (Millipore, #SCR005). Store in frozen at $-20^{\circ}C$ in 10 mL aliquots. Once thawed at $4^{\circ}C$, use within 7 days. Take care when thawing, as the enzyme can separate and settle at the bottom of the bottle or tube; mix the freshly thawed Accutase thoroughly prior to aliquotting or adding to cells (see Note 1).
6. Human bFGF/FGF2 (such as Stemgent, #03-0002). Dilute in DPBS, 1% BSA to a concentration of 20 $\mu g/mL$, or 1,000 $\times$, and freeze in small aliquots at $-20^{\circ}C$.
7. Hybri-Max dimethyl sulfoxide (DMSO, Sigma-Aldrich, #D2650).
8. 6-Well vacuum gas plasma-treated tissue culture dishes (such as BD Falcon, #353046).
9. Nylon membrane syringe filter (Pall Life Sciences, #PN 4433).
10. Mr. Frosty Nunc #5100-0001.

¹ Accutase: The exact formulation of Accutase is proprietary, but it is known to contain proteases and collagenases of non-mammalian and nonbacterial origin. It was originally developed by Innovative Cell Technologies of San Diego, CA and is distributed by several vendors, including Thermo Fisher-Hyclone, Sigma, and Millipore. We have found no functional differences between the different brands of Accutase.

2.2. Media

1. StemPro basal medium: Add 25% BSA solution to DMEM/ F12/GlutaMax to make a final BSA concentration of 1.8%. Add 2-ME to a final concentration of 55 μ M. Sterile filter, and store at 4°C. Discard unused medium after 2 weeks.
2. StemPro complete medium: Add 50 $\times$ cytokine supplement to StemPro basal medium to a final concentration of 1 $\times$ (e.g., 1 mL of supplement to 49 mL of basal medium), followed by bFGF to a final concentration of 20 ng/mL. This complete medium is only stable for 24 h, so it is recommended to pre-prepare small volumes and use it immediately.
3. StemPro cryopreservation medium: Combine equal volumes of fresh complete StemPro medium, and 1-day-old “conditioned” medium from the cells themselves. Add DMSO to a concentration of 10%, and sterile filter with a syringe filter approved for use with DMSO (e.g., nylon membrane). Keep cold and use immediately.

3. Methods

3.1. Preparing Matrigel

1. Thaw a 10 mL bottle of Matrigel at 4°C overnight. Do not place it in a water bath or attempt to thaw it rapidly (see Note 2).¹⁴⁰ A.E. Stover and P.H. Schwartz
2. Prepare 1.5-mL screw-top tubes for receiving 200 μ L aliquots of Matrigel. Have the caps loose and easily opened.
3. Chill several 2 mL serological pipets in a –20°C freezer for a few minutes.
4. Remove the Matrigel bottle from 4°C, and quickly spray the entire bottle with 70% alcohol. The rubber stopper caps that BD uses for its bottles are easily contaminated, so make sure the bottle is fully bathed in 70% alcohol.
5. Using a chilled 2 mL serological pipette, distribute 0.2 mL (200 μ L) of Matrigel in each screw cap tube. Change pipettes if the Matrigel starts to gel inside the pipette. Matrigel gels at 15°C, so this process must be done quickly. If necessary, take a break, and re-chill the pipettes and the stock Matrigel bottle. A repeater pipette may also be used for fast and efficient aliquotting.
6. Store the aliquots of Matrigel at –20°C. 200 μ L, when diluted 1:30 into 6 mL of cold medium, is sufficient for one 6-well dish. Different-sized aliquots can be made according to each user’s individual needs.

3.2. Plating Matrigel

This protocol describes a 1:30 Matrigel dilution. Less concentrated solutions can also be used, but the optimal concentration depends on the cell type.

1. Take a 200 μ L aliquot of stock concentration Matrigel (in a 1.5-mL screw-top tube), and thaw overnight at 4°C.
2. In the morning, add 6 mL of ice-cold DMEM to a 15-mL conical tube.

²Matrigel: Matrigel is an undefined mixture of extracellular matrix proteins isolated from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma cell line. It is a solid when stored at –20°C and a liquid when thawed and stored at 4°C, but at 15°C it turns to a colloid that is difficult to manipulate. For this reason, when diluting, pipetting, and coating plates with Matrigel, it must be worked with very quickly and it must be kept cold.

3. Remove the Matrigel from the 4°C fridge, and immediately mix the 200 µL of Matrigel with the 6 mL of DMEM. If not done immediately after removal from fridge, the Matrigel will polymerize too quickly, and a glob of Matrigel will form, which will be useless for coating plates. An extremely effective way to quickly and safely do this is to take 1 mL of the icecold DMEM, and use a 2-mL pipet to rinse all the Matrigel out of the tube. In addition, if the Matrigel prematurely solidifies in solution, the tube may be stored at 4°C until the pellet is no longer visible (i.e., it depolymerizes), and then used for coating.
4. Immediately coat your plate with the 6 mL of working concentration Matrigel that you have just created. If not done immediately, the Matrigel will coat the inside of the conical tube.
5. Place the Matrigel-coated plate at 37°C for at least 30 min to an hour. Longer incubations increase cell attachment. An overnight incubation is preferred for hard-to-stick cell types.
6. Aspirate excess Matrigel before adding cells. There is no need to rinse the plate.
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3.3. Transitioning PSCs from Co-culture with Feeder Cells to StemPro/Matrigel Culture

It is recommended that cultures be adapted to feeder-free conditions with defined medium before routinely subculturing by single-cell passaging. Although a sudden, complete transition is certainly possible (we have done it several times in our laboratories), some cell lines do not tolerate it well, so it is not recommended. A more gradual shift minimizes the shock to the cells and provides the best possible chance of a smooth transition with little cell death or differentiation.

1. 1. Start feeding a feeder-cell-grown culture with a mixture of 50% StemPro complete medium/50% standard growth medium daily, 2–3 days prior to the day when the culture would normally be passaged.
2. 24 h Prior to passaging, feed the PSC culture with 100% StemPro complete medium.
3. On the day of passaging, exchange the medium with fresh StemPro complete medium and mechanically passage colonies onto a fresh Matrigel plate. Simply pipette the lifted colonies onto the receiving Matrigel-coated plate (see Note 3).
4. Feed the cultures daily with StemPro complete medium until the colonies have grown so that an average colony on the plate completely fills a 10× objective view. Some moderate differentiation may appear during this adaptation phase. Remove differentiated cells and colonies mechanically (see Chapter 8).
5. When the colonies are large enough to be passaged, proceed with Accutase passaging.

3.4. Single-Cell Passaging of PSCs with Accutase

1. Aspirate the medium from the culture.
2. Rinse well using 1 mL of DPBS/well and aspirate.

³Do not worry about any MEFs that are carried over during the initial transition – they will die off and disappear. Use a higher passaging ratio than you would normally use (i.e., if you normally passage cells 1 well to 6 wells, passage to only 2 or 3 wells).¹⁴⁴
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3. Add 1–2 mL of 37°C Accutase (fresh from the water bath or incubator) to each well. Only very dense cultures should require 2 mL (see Note 4).
4. Immediately take the culture dish to a phase-contrast microscope, and observe the culture carefully. Watch for signs that the individual cells are starting to loosen from the dish. This is characterized by both an increase in phase brightness and a more rounded appearance. At the periphery of colonies, where you can commonly see cells that have membrane stretched across the culture dish, you will see this membrane detach and the cells visibly loosen from the Matrigel-coated dish. After 1–2 min, the vast majority of the cells should appear very phase bright and rounded. There is no need to wait until the cells float into suspension, nor to tap or shake the culture dish to induce this effect.
5. Return the cells to the tissue culture hood. Using a 5-mL serological pipet, add 5 mL of DPBS for each milliliter of Accutase 142 A.E. Stover and P.H. Schwartz to dilute the enzyme, and then forcefully pipet up and down to dislodge the cells from the well surface. Be careful do not splatter/splash any of the resulting cell suspension (the wells will be quite full). If passaging multiple wells, add the DPBS to each well before dislodging the cells from the individual wells. This helps guard against Accutase overexposure. In our experience, diluting 1 mL of Accutase with 5 mL of DPBS is more than sufficient to prevent Accutase overexposure.
6. Transfer the Accutase/PBS cell suspension to an appropriately sized conical tube.
7. Rinse the well with an additional 5 mL of DPBS to remove remaining cells, especially around the edges of the well.
8. Spin the cells at $100 \times g$ for 5 min at room temperature.
9. Aspirate the supernatant, and resuspend the cells in 2 mL of StemPro complete medium.
10. Count the cells using a hemacytometer, and determine the number of receiving wells or plates required. Cells should be plated at 5×10^4 – 1×10^5 cells/cm² (see Notes 5 and ⁶).

⁴Accutase passaging of PSCs on Matrigel: Although Accutase is relatively gentle to cells compared to harsher enzymes such as trypsin, it reacts with cells extremely quickly, and exposure to the enzyme must be kept to a minimum. When performed properly, lifting cells with Accutase can take as little as 1 min (this can vary with culture density). Second, although the manufacturers of Accutase claim that the enzyme(s) self-digest and do not require inactivation, we have found that Accutase still needs to be either diluted or completely removed after it has served its purpose. In other words, although Accutase passaging is easy, it must be done carefully and quickly.

Temperature is another variable that affects Accutase performance. Some laboratories use room-temperature (25°C) Accutase to passage their cells. This has the overall effect of slowing down the reaction, meaning digestion can take up to 10 min. In addition, the cells will tend to come up in clusters rather than as single cells, perhaps due to incomplete digestion of the attachment proteins.

A further consideration is the appearance of the cells once they have been passaged with Accutase. The cultures may appear differentiated to researchers used to standard culture morphologies – they typically have large amounts of membranous material surrounding them, and the individual cells will appear abnormally large and phase dark. However, close observation of the cells should show that the cells are still grouping together, and rarely appear alone even when recently seeded as single cells. They will attain a high nucleus-to-cytoplasm ratio as they expand, and the nuclei should have prominent nucleoli. Several days after passaging (if not the next day), it should be obvious at low magnification that the cells are organized as colonies, and not as single cells (see Fig. 1). We typically passage cells when they first form a monolayer that covers the entire surface area of the well/ dish, or slightly beforehand.

⁵Seeding density: The cells migrate to form colonies and, if the seeding density is low, they will have difficulty doing this. Under-seeded plates can eventually recover, but will experience more differentiation and apoptosis than normal. Over-seeded plates, on the other hand, will simply need to be passaged sooner.

⁶Cell concentration: In our experience, one confluent well generates 3–6 million cells, so it is possible to perform routine 1:6 splits every 5–7 days. This should help predict how many Matrigel plates should be prepared in advance of passaging. In addition, it should help predict the rate of expansion, thus helping preparations for experiments involving large numbers of cells. For example, one plate initially containing 3×10^6 cells, passaged weekly, will generate 3.9×10^9 cells in 1 month (1,296 plates)!

3.5. Cryopreservation of Accutase-Passaged Cells

With traditional PSC cryopreservation, initial viability following a thaw tends to be very low and it may take up to 2 weeks for even a single colony to appear on the plate. When freezing PSCs as a dense single-cell suspension using StemPro and conditioned medium (see below), thawing viability is greatly improved, although there is still some noticeable cell death. We have found that the use of the apoptosis-blocking Y27632 ROCK inhibitor is not necessary for successful cryopreservation using the method described below (see Note 7).

1. Save spent culture medium (conditioned medium, CM) by removing it with a serological pipette and placing it into a sterile conical tube for later use in the procedure.
2. Rinse the cells with DPBS, and lift with Accutase as described above in Subheading 3.4.
3. While the cells are in the centrifuge, prepare the freezing medium containing 45% conditioned medium, 45% fresh StemPro complete medium, and 10% DMSO. Chill at 4°C.
4. Aspirate the supernatant from the pelleted cells, and resuspend the cell pellet in 1 mL of freezing medium for each well lifted ($\sim 3 \times 10^6$ cells/mL).
5. Dispense 1 mL of the cell suspension to a cryopreservation vial (1 vial for each well of 6-well plate).
6. Place the vials in isopropanol-jacketed freezing containers (Mr. Frosty) and place at -80°C overnight.
7. The next day, transfer the vials to long-term storage at cryogenic temperatures below -130°C .¹⁴³

3.6. Thawing Cells in StemPro Complete Medium

1. Remove a vial from the liquid nitrogen.
2. Rapidly thaw in a 37°C water bath. Gently swirl the vial in the water, being careful not to submerge the cap or get the water near the cap threads. Thawing should take about 60 s. Any unnecessary time the cells spend in DMSO-containing medium at room temperature will result in increased cell death.
3. Spray the vial with 70% alcohol, wipe with a tissue, and allow to air dry briefly in the tissue culture hood.
4. Transfer the contents of the tube to a 15-mL conical tube.
5. Slowly and drop wise, add 10 mL of fresh StemPro complete medium to the cells while swirling the tube to promote even mixing. Do not add the medium along the side of the tube – this will result in a medium gradient forming. Instead, have the drops of media fall directly vertical into the tube. Gently triturate once or twice before capping the tube.
6. Centrifuge the cells at $150 \times g$ for 5 min at room temperature, and aspirate the supernatant.

⁷Cryopreservation: This frozen cell concentration is much greater than the concentration typically used with other cell types (about 1×10^6 cells). However, the high number of cells is necessary for PSC survival; these cells appear to depend on being able to group together shortly after being seeded, otherwise they differentiate or die. Also, it is not unusual to experience some cell death after any thaw. Increasing the frozen cell concentration tends to ensure a more successful thaw.

7. Resuspend the pellet in 2 mL of fresh StemPro complete medium and seed onto one well of a Matrigel-coated plate.
8. Feed and observe daily.
9. Begin passaging with Accutase when the cells reach confluence (see Notes 8 and 9).

Acknowledgments

This work has been funded by the National Institutes of Health (T15HL074286, R21MH087925, R01HD059967). The NCI Preclinical Repository supplied FGF-2.

References

1. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by tera tocarcinoma stem cells. *Proc Natl Acad Sci.* 1981; 78:7634–8. [PubMed: 6950406]
2. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature.* 1981; 292:154–6. [PubMed: 7242681]
3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science.* 1998; 282:1145–7. [PubMed: 9804556]
4. Wang L, Schultz TC, Sherrer ES, Dauphin DS, Shin S, Nelson AM, Ware CB, Zhan M, Song CZ, Chen X, Brimble SN, McLean A, Galeano MJ, Uhl EW, D'Amour KA, Chesnut JD, Rao MS, Blau A, Robins AJ. Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. *Blood.* 2007; 110:4111–119. [PubMed: 17761519]
5. Mitalipova MM, Rao RR, Hoyer D, Jones K, Johnson J, Dalton S, Meisner L, Stice SL. Preserving the genetic integrity of human embryonic stem cell lines. *Nature Biotechnology.* 2005; 23:19–20.
6. Bajpai R, Lesperance J, Kim M, Tersikh AV. Efficient propagation of single cells Accutase-dissociated human embryonic stem cells. *Mol Reprod Dev.* 2008; 75:818–27. [PubMed: 18157870]

⁸A note on aneuploidies: Although we have successfully grown cells in StemPro with accutase passaging for 20+ passages without seeing genetic abnormalities, we have observed some higher passages picking up common PSC trisomies, such as 12 and 17. It needs to be noted, however, that all culture systems (including traditional mechanical passaging) have had reported (either in the literature or in anecdote) incidents of aneuploidies, and some of this may be related to individual laboratories' handling of their cells. For example, Accutase overexposure and underfeeding may contribute to stress on the cultures, and, over time, select for a subpopulation of cells with both an abnormal karyotype and a proliferative advantage. The exact mechanisms are not yet fully understood. Therefore, it is extremely important to regularly karyotype PSCs grown in any culture system, and to keep reserves of earlier passages banked.

⁹You can easily seed Accutase-passaged cells onto a MEF layer in traditional PSC medium and obtain “normal”-looking colonies once again. They take on a “normal” appearance less than 24 h after seeding. Since the seeding densities prescribed in this chapter are for Matrigel-coated plates, we recommend reducing the density to take into account the surface area occupied by feeders. Otherwise, the culture will be very crowded soon after seeding.

Fig. 1.

Growth progression and morphology of hESCs when plated as single cells. (a) Despite being seeded as single cells, Accutase-passaged cells will quickly migrate to form small colonies with lots of obvious membranous material (shown with a 10 $\times$ objective at day 1 after seeding). (b) After 2 days, the colonies have gradually grown larger and begun to fuse. (c) After 4–5 days, the small colonies have expanded so that the entire area of the cell culture well is essentially one large, monolayer colony. This is when the cells should be passaged to avoid cell death from underfeeding the dense culture. (d) At 40 $\times$ magnification, dense Stempro cells appear very similar to traditional feeder-grown colonies. Note: The presence of small numbers of floating cells is normal. Larger numbers indicate an underfed culture.