Defined, Feeder-Independent Medium for Human Embryonic Stem Cell Culture

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ABSTRACT

The developmental potential of human ES cells makes them an important tool in developmental, pharmacological, and clinical research. For human ES cell technology to be fully exploited, however, culture efficiency must be improved, large-scale culture enabled, and safety ensured. Traditional human ES cell culture systems have relied on serum products and mouse feeder layers, which limit the scale, present biological variability, and expose the cells to potential contaminants. Defined, feeder-independent culture systems improve the safety and efficiency of ES cell technology, enabling translational research. The protocols herein are designed with the standard research laboratory in mind. They contain recipes for the formulation of mTeSR (a defined medium for human ES cell culture) and detailed protocols for the culture, transfer, and passage of cells grown in these feeder-independent conditions. They provide a basis for routine feeder-independent culture, and a starting point for additional optimization of culture conditions. *Curr. Protoc. Stem Cell Biol.* 2:1C.2.1-1C.2.16. © 2007 by John Wiley & Sons, Inc.

Keywords: feeder-independent culture • human ES cells • defined medium • bFGF

INTRODUCTION

The developmental potential of human ES cells means that they have tremendous potential to be a useful tool in elucidating the early stages of development, advancing pharmacological research, and improving human health. For human ES cell technology to be fully exploited, however, culture efficiency must be improved and large-scale culture enabled. Translating human ES cell technology to clinical applications will also make safety of paramount importance. Traditional human ES cell culture systems rely on poorly defined serum products and mouse embryonic feeder layers (MEFs). The inclusion of these ill-defined components in the culture system significantly reduces the efficiency of human ES cell culture and exposes the cells to potential contaminants from animal-sourced proteins. Development and refinement of defined culture systems that eliminate the need for feeder layers, while maintaining undifferentiated proliferation, will improve the safety and efficiency of human EC cell culture and enable translational research.

The protocols in this unit are designed with the standard research laboratory in mind, and contain complete recipes for the formulation of mTeSR (see Reagents and Solutions), a defined medium for the feeder-independent propagation of human ES cells. Also included are detailed protocols for the transfer and culture (see Basic Protocol 1), and passage (see Basic Protocol 2, Alternate Protocols 1 and 2) of cells grown in these feeder-independent conditions. These recipes and protocols are intended to provide a basis for routine feeder-independent culture, and a starting point for additional optimization of culture conditions.

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NOTE: For all procedures described in this unit, standard tissue culture, reagent preparation, and sterilization facilities are required. All cell handling should be performed under sterile conditions in a Class II Biological Hazard Flow Hood, and all biologically contaminated material should be disposed of properly.

NOTE: All cell cultures should be maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

NOTE: The use of human embryonic stem cells as described in these protocols usually requires specific MTA approval from the appropriate institutional research office, and may require ethics approval from the appropriate institutional committee.

NOTE: The authors recommend glass serological pipets for all measured media transfers, unless specifically indicated otherwise.

BASIC PROTOCOL 1

TRANSFERRING HUMAN EMBRYONIC STEM CELLS TO AND CULTURING IN FEEDER-INDEPENDENT CONDITIONS

The move to defined, feeder-independent culture systems can significantly increase the overall efficiency of the research laboratory. First and foremost, it eliminates the need for the labor-intensive derivation, maintenance, and routine preparation of MEFs otherwise necessary for culture or conditioning of media. Furthermore, the removal of highly variable components (including serum and MEFs) from the culture system results in more consistent, reliable, repeatable results, speeding the progress of research. The basic protocols for the feeder-independent culture of human ES cells outlined below are modifications of those originally published in Ludwig et al. (2006a).

Materials

Human ES cells in standard (MEF or feeder-free) culture, in 6-well plates
mTeSR culture medium (see recipe)
Matrigel-coated 6-well plates (see recipe)

Transfer to feeder-independent conditions

1. At a time point 3 days prior to normal passage time, replace current culture medium with 2 ml/well warmed mTeSR culture medium.

   While the authors recommend only warming aliquots of the medium, all of their testing was done while repeatedly warming the entire bottle. Since this is the method employed by the general scientific community, they wanted to ensure that the medium would perform under these circumstances.

2. Feed cultures daily with 2 ml mTeSR medium until ready to passage.

   Spent culture medium should be completely removed at each feeding and replaced with 2 ml/well warmed (37°C) mTeSR culture medium.

3. Passage cells using either Alternate Protocol 1 or Alternate Protocol 2, as appropriate. Plate cells directly onto Matrigel-coated 6-well plates with 2 ml of mTeSR medium at a density of $\sim 5 \times 10^5$ per well.

   On occasion, the authors have used both 35- and 60-mm dishes. This protocol is designed for 6-well plates, but could easily be adapted by adjusting media volumes to an alternate format.

   Cells should be passaged more densely than normal for this transfer passage.

If passaging from feeder-free (conditioned medium) cultures on Matrigel, the authors recommend transfer passaging as outlined in Alternate Protocol 1. If passaging from MEF-containing cultures, the authors recommend transfer passaging as outlined in Alternate Protocol 2. While the authors routinely transfer MEF cultures to mTeSR culture systems, some users have preferred first establishing cultures in a MEF-conditioned medium system before moving to feeder-independent culture systems.

Feeder-independent culture

4. Feed and examine cultures daily until ready to passage (~5 days). Each day, aspirate all spent medium and replace with 2 ml/well warmed (37°C) mTeSR culture medium.

   Medium volume/well is based on a single well of a standard 6-well plate with a surface area of 9.6 cm^2. If alternate size surface area is being used, adjust medium volume accordingly.

PASSAGING HUMAN EMBRYONIC STEM CELLS IN FEEDER-INDEPENDENT CONDITIONS

BASIC PROTOCOL 2

There is some evidence to suggest that manual passaging may result in more stable cultures than those enzymatic techniques that individualize cells at passage (Mitalipova et al., 2005). For this reason it has been recommended to reduce the accumulation of karyotypic abnormalities (Buzzard et al., 2004; Mitalipova et al., 2005). While manual passaging results in more consistent clump size at passage, it is labor intensive and incompatible with large-scale culture systems necessary for the industrial and clinical use of human ES cells. Alternatives to manual passaging include those bulk passaging systems (both enzymatic and non-enzymatic) that allow cultures to be primarily passaged in clumps, with limited individualization of cells. If appropriate care is taken to ensure that colonies are passaged on time and are not excessively disrupted, these bulk passaging methods described below can be used routinely with success. The authors have cultured cells for >100 passages using enzymatic passaging and maintained a normal karyotype, and routinely propagate cultures for >30 passages using all of these systems with no adverse effect.

Materials

Human ES cell culture in Matrigel coated 6-well plates (Basic Protocol 1)
mTeSR culture medium (see recipe)
Washing medium (see recipe)
EDTA splitting medium (see recipe)

Inverted microscope with marking objective (Nikon)
Pasteur pipets (Fisher Scientific)
15-ml conical tube (optional)
Glass serological pipets (Fisher Scientific)

Prepare Matrigel plate

1. Prepare Matrigel plate for use by aspirating excess Matrigel and plating 2 ml/well of mTeSR culture medium into each well. Label plate appropriately, and set aside.
2. Observe cultures using phase contrast microscopy. Mark any small area of differentiation to be removed prior to passage with the marking objective (Fig. 1C.2.1).
3. Using a Pasteur pipet, aspirate spent medium. During aspiration, touch pipet to marked area to remove differentiating cells (Fig. 1C.2.1).
4. Wash culture twice with 1 ml/well of washing medium.
5. Aspirate washing medium and replace with 1 ml/well EDTA splitting medium.

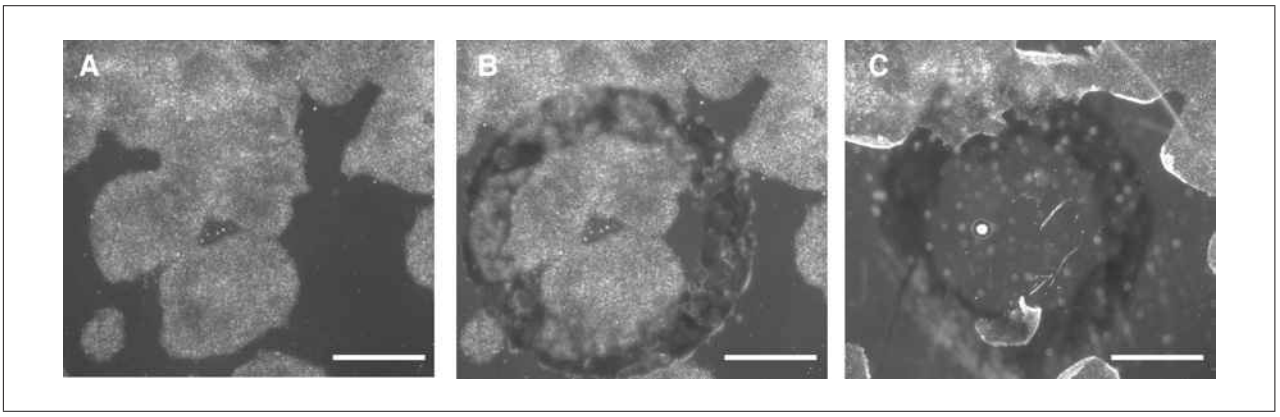

Figure 1C.2.1 Removal of areas of differentiation by aspiration. Using phase contrast microscopy, view cultures prior to passage (**A**) If small areas of differentiation are noted, they should be marked using a marking objective. (**B**) After aspirating medium, touch the Pasteur pipet to the marked area to remove differentiated area. (**C**) Scale bar = 100 μm.

6. Incubate 1 to 2 min at room temperature.

   Be cautious not to over-incubate, as cells will detach prematurely. Incubation for >3 min will make it impossible to split the cells without centrifugation.

7. Aspirate splitting medium and replace immediately with mTeSR culture medium.

8. Using a serological pipet, remove colonies from the plate by *gently* pipetting the medium against the bottom of the plate, releasing the colonies.

   In general, scraping is not necessary, but if cells do not dislodge immediately, use the serological pipet and gently scrape the bottom while simultaneously expelling medium.

9. If multiple wells are being passaged, pool the cell suspensions into a 15-ml conical tube.

10. Gently mix by pipetting the colonies to ensure even distribution, being careful not to disrupt the colonies more than necessary.

    Average colony size should be no smaller than 50 to 100 cells.

Passage cells

11. Passage cells so that roughly 2×10^5 cells are seeded into each fresh well.

    Generally this translates into a 1:8 to 1:15 split every 7 days.

12. Gently shake the plate to evenly distribute the colonies, and return to the incubator.

Culture cells

13. Culture as per Basic Protocol 1 until cells are again ready to passage.

    Cultures must be passaged at the appropriate time to ensure continued quality. While other culture methods allow for some flexibility in split timing, in this feeder-independent system there is only a 12 to 24 hr window in which to passage cells and achieve optimum attachment and continued undifferentiated proliferation. Cells will easily differentiate if allowed to overgrow, and cannot be rescued. Passaging too early, however, results in poor attachment and limited growth. Cultures should be passaged when colony centers become dense, appearing brighter than the edges when viewed using phase contrast microscopy (Fig. 1C.2.2). Some users may find it helpful to split sister wells of a single culture on successive days when initially working with the system. Observation of the individual cultures in the days immediately following passage may assist in accurately identifying the appropriate passage time.

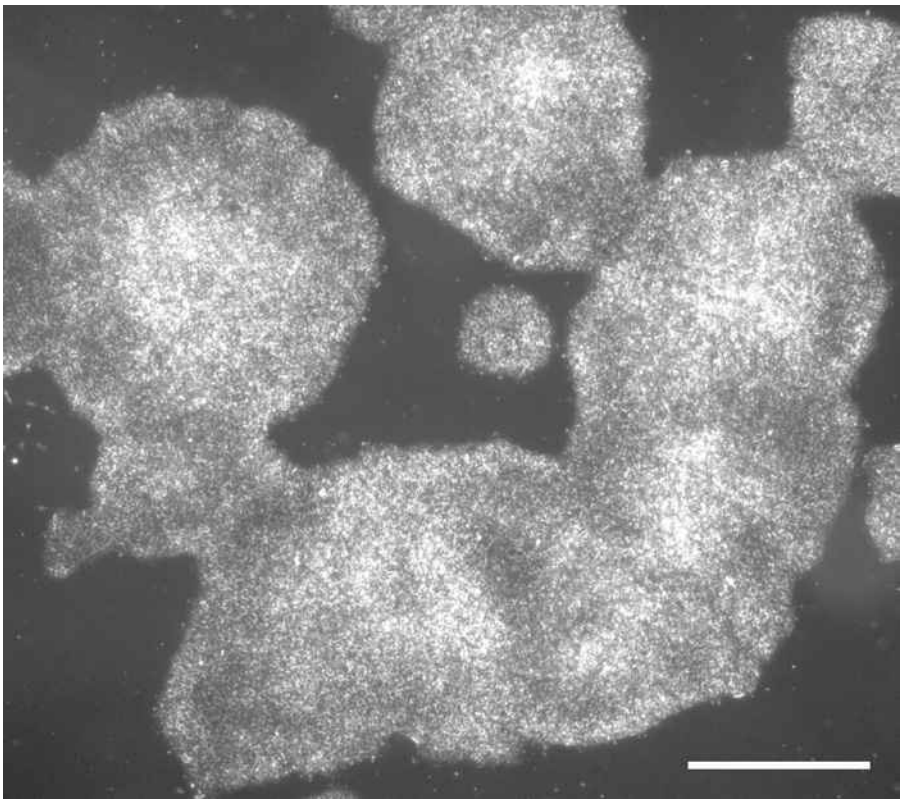

Figure 1C.2.2 Identifying appropriate passage timing. Cultures should be passaged just as the centers of the colonies become dense, appearing brighter than the edges using phase-contrast microscopy. If split too early, attachment will be reduced. If allowed to overgrow, cells will differentiate, and cannot be rescued. Scale bar = 100 μm.

PASSAGING WITH DISPASE

ALTERNATE PROTOCOL 1

In addition to EDTA (Basic Protocol 2), dispase may also be used to enzymatically passage cells in bulk while maintaining appropriate karyotypes. The authors have done extensive screening in their laboratory, and in their hands, and while collagenase works best for passaging cells in co-culture, they achieve the best results using dispase or EDTA for passaging cells on Matrigel. Better initial attachment and expansion was seen using these methods. The dispase-based technique may also be used to routinely passage feeder-independent cultures, although scale-up can be achieved more rapidly using Basic Protocol 2.

Materials

Human ES cell culture in Matrigel-coated 6-well plates (for transfer or from Basic Protocol 1)
mTeSR culture medium (see recipe)
Dispase splitting medium (see recipe)
Warmed DMEM/F-12 (Invitrogen)

Inverted microscope with marking objective (Nikon)
Pasteur pipets (Fisher Scientific)
37°C incubator
15-ml conical tube (optional)
Glass serological pipets (Fisher Scientific)

Prepare Matrigel plate and remove cells

1. Prepare Matrigel plate for use by aspirating excess Matrigel and plating 2 ml/well of mTeSR culture medium into each well. Label plate appropriately, and set aside.
2. Observe cultures using phase contrast microscopy. Mark any small area of differentiation to be removed prior to passage with the marking objective (Fig.1C.2.1).
3. Using a Pasteur pipet, aspirate spent medium. During aspiration, touch pipet to marked area to remove differentiating cells (Fig. 1C.2.1).
4. Add 1 ml/well dispase splitting medium and incubate plate 7 min at 37°C.
5. Following incubation, aspirate splitting medium and gently rinse cells on the plate a minimum of three times using 1 ml warmed DMEM/F-12 medium.

   Adequate rinsing at this step is critical, and reducing the number of washes will dramatically reduce or prevent colony plating.
6. Aspirate rinse medium and gently remove colonies from the plate by rinsing with 1 to 2 ml mTeSR culture medium, gently scraping the bottom of the plate as necessary.

   In general, scraping is not necessary, but if cells do not dislodge immediately, use the serological pipet and gently scrape the bottom while simultaneously expelling medium.
7. If multiple wells are being passaged, pool the cell suspensions into a 15-ml conical tube.
8. Gently mix by pipetting the colonies to ensure even distribution, being careful not to disrupt the colonies more than necessary.

Passage cells

9. Passage cells so that roughly 3×10^5 cells are seeded into each fresh well.

   This generally translates into a 1:3 to 1:6 split every 4 to 5 days.
10. Gently shake the plate to evenly distribute the colonies, and return to the incubator.

Culture cells

11. Culture as per Basic Protocol 1 until cells are again ready to passage.

ALTERNATE PROTOCOL 2

PASSAGING WITH COLLAGENASE

The best results the authors have obtained to date with enzymatically passaging human ES cell cultures on MEFs have been achieved using collagenase. The authors do not recommend this technique for passaging human ES cells grown on Matrigel or other extracellular matrices: it is used exclusively in their laboratory for the passaging of human ES cell cultures in direct contact with MEFs. The authors recommend this technique when transferring cultures from MEFs to feeder-independent culture systems. Once cultures have been established in feeder-independent systems, the authors recommend one of the above protocols (Basic Protocol 2 or Alternate Protocol 1) for continued passaging.

Materials

Human ES cell culture on MEFs (for transfer to feeder-independent systems)
mTeSR culture medium (see recipe)
Collagenase splitting medium (see recipe)
Warmed DMEM/F-12 (Invitrogen)

Inverted microscope with marking objective
Pasteur pipets (Fisher Scientific)
15-ml centrifuge tube (optional)
Glass serological pipets (Fisher Scientific)

Prepare Matrigel plate and remove cells

1. Prepare Matrigel plate for use by aspirating excess Matrigel and plating 2 ml/well of mTeSR culture medium into each well. Label plate appropriately, and set aside.
2. Observe cultures using phase contrast microscopy. Mark any small area of differentiation to be removed prior to passage with the marking objective (Fig. 1C.2.1).
3. Using a Pasteur pipet, aspirate spent medium. During aspiration, touch pipet to marked area to remove differentiation (Fig. 1C.2.1).
4. Add 1 ml/well collagenase splitting medium and incubate plate at 37°C for 5 min. To confirm colony separation from the plate, view surface under a microscope. Look for the perimeter of the colony to appear folded back. If necessary, keep collagenase on cells for another minute or two.
5. Following incubation, aspirate splitting medium and gently rinse cells on the plate using 1 ml/well warmed DMEM/F-12 medium.
6. Aspirate rinse medium and gently remove colonies from the plate by rinsing with 1 to 2 ml mTeSR culture medium.

   In general, scraping is not necessary, but if cells do not dislodge immediately, use the serological pipet and gently scrape the bottom while simultaneously expelling medium.
7. If multiple wells are being passaged, pool the cell suspensions into a 15-ml conical tube.
8. Using a serological pipet, break up colonies by gently pipetting up and down a few times, and gently mix the colonies to ensure even distribution.

Passage cells

9. Passage cells so that roughly 3×10^5 cells are seeded into each fresh well.

   This generally translates into a 1:3 to 1:6 split every 4 to 5 days.
10. Gently shake the plate to evenly distribute the colonies, and return to the incubator.

Culture cells

11. Culture as per Basic Protocol 1 until cells are again ready to passage.

REAGENTS AND SOLUTIONS

*For suppliers, see **SUPPLIERS APPENDIX**.*

Whenever possible, cell culture–tested reagents have been used, and are recommended by the authors. Water quality is critical, and only Type 1 reagent-grade water should be used in the recipes and protocols presented here. Good quality, Type 1 reagent-grade water can be obtained from a Milli-Q Ultrapure Water System (Millipore), and is best used immediately after drawing. Alternatively, if reasonable in-house purification systems are not available, Type 1 reagent-grade water is available for purchase from a few select vendors.

bFGF stock (50 µg/ml)

Reconstitute 500 µg bFGF [either zebrafish (Ludwig et al., 2006a) or human (Peprotech)] in 10 ml diluent solution (see recipe). Use immediately or freeze in 10-ml aliquots for up to 6 months at −80°C.

Collagenase splitting medium (1 mg/ml)

Add 25 mg collagenase IV (Sigma) to 25 ml DMEM/F-12 medium (Invitrogen). Mix until dissolved. Filter sterilize the resulting medium and store up to 2 weeks at 4°C. Warm aliquot to 37°C before use.

Diluent solution (0.1% w/v BSA in PBS)

Dissolve 100 mg BSA (Sigma) in 100 ml Ca/Mg-free PBS (Invitrogen). Filter sterilize the resulting solution, and store in 10-ml aliquots up to 6 months at 4°C.

Dispase splitting medium (2 mg/ml)

Add 50 mg dispase (Sigma) to 25 ml DMEM/F-12 medium (Invitrogen). Mix until dissolved. Filter sterilize the resulting medium and store up to 2 weeks at 4°C. Warm an aliquot to 37°C before use.

EDTA splitting medium

Add 1.48 g calcium- and magnesium-free DMEM/F-12 powder (Invitrogen) and 15 mg EDTA acid (anhydrous, crystalline, cell culture tested; Sigma) to 100 ml Type I water. Stir until dissolved, adding low heat as necessary. Once dissolved, adjust the pH of the medium to 7.2 and adjust the osmolarity to 340 ±5 mOsM using sodium chloride (Sigma). Filter sterilize and store up to 2 weeks at 4°C. Warm aliquot to 37°C before use.

As the DMEM/F-12 base medium is currently only available in quantities sufficient to make 10-liter volumes, the authors recommend that it be distributed into 1.48 g aliquots for ease of use. These aliquots should be stored desiccated at −80°C for no more than 6 months.

L-glutamine solution

Dissolve 146 mg L-glutamine (Sigma) into 10 ml Type 1 water. Add 7 µl 2-mercaptoethanol solution (Sigma). Use immediately.

Matrigel-coated plates

Thaw 0.5 mg (one vial) Matrigel from Matrigel stock (see recipe) by diluting into 6 ml of cold DMEM/F-12 (this should take no more than a minute). Mix well, and plate 1 ml of the resulting solution into each well of a 6-well culture plate. Allow Matrigel to settle at room temperature for at least 1 hr before use. Aspirate excess Matrigel from the plate immediately before use (plates need not be rinsed). Plates not used on the day plated can be wrapped with Parafilm or foil and stored at 4°C for up to 1 week. Warm plates to room temperature before use.

The authors have noted significant reductions in plating efficiency if the Matrigel solution is allowed to dry prior to plating cells. Therefore, discard any stored plates that have dried prior to use.

Matrigel stock

Thaw one bottle of Growth Factor Reduced (GFR) Matrigel (Becton Dickinson) on ice overnight at 4°C. Keeping the Matrigel on ice at all times and using chilled tips, aliquot 0.5 mg of Matrigel into prelabeled, prefrozen (−80°C) 1.5-ml tubes on ice. Immediately freeze tubes at −80°C.

The concentration of Matrigel varies from lot to lot, so the volume of Matrigel needed to obtain 0.5 mg will vary accordingly. Aliquoted as above, each tube will yield one coated 6-well plate. If multiple plates are desired, increase the volume of the aliquot accordingly. Do not allow Matrigel to warm at any point during this procedure. Doing so will cause the product to gel prior to use, resulting in uneven plating and reduced performance.

mTeSR Culture medium

Combine 800 ml DMEM/F-12 (Invitrogen) with 200 ml stock B (see recipe). Supplement with:

1% (v/v) non-essential amino acids (Invitrogen)
1% (v/v) L-glutamine solution (see recipe)
Adjust pH to 7.4 using 10 N sodium hydroxide (Sigma) and adjust the osmolarity to 340 ±5 mOsM using sodium chloride (Sigma). Filter sterilize the resulting medium (mTeSR) and store for up to 2 weeks at 4°C. If culture medium has been prepared using fresh (not frozen/thawed) stock B, then whole medium may be frozen in aliquots and stored up to 6 months at −80°C. Warm aliquot to 37°C before use.

There is no significant impact on culture performance when using medium that has been through a single freeze/thaw cycle (Fig. 1C.2.3).

Pipecolic acid stock (100 mg/ml)

Dissolve 1 g L-pipecolic acid (MP Biomedicals) in 10 ml diluent solution (see recipe). Store 1- to 2-ml aliquots up to 6 months at −80°C.

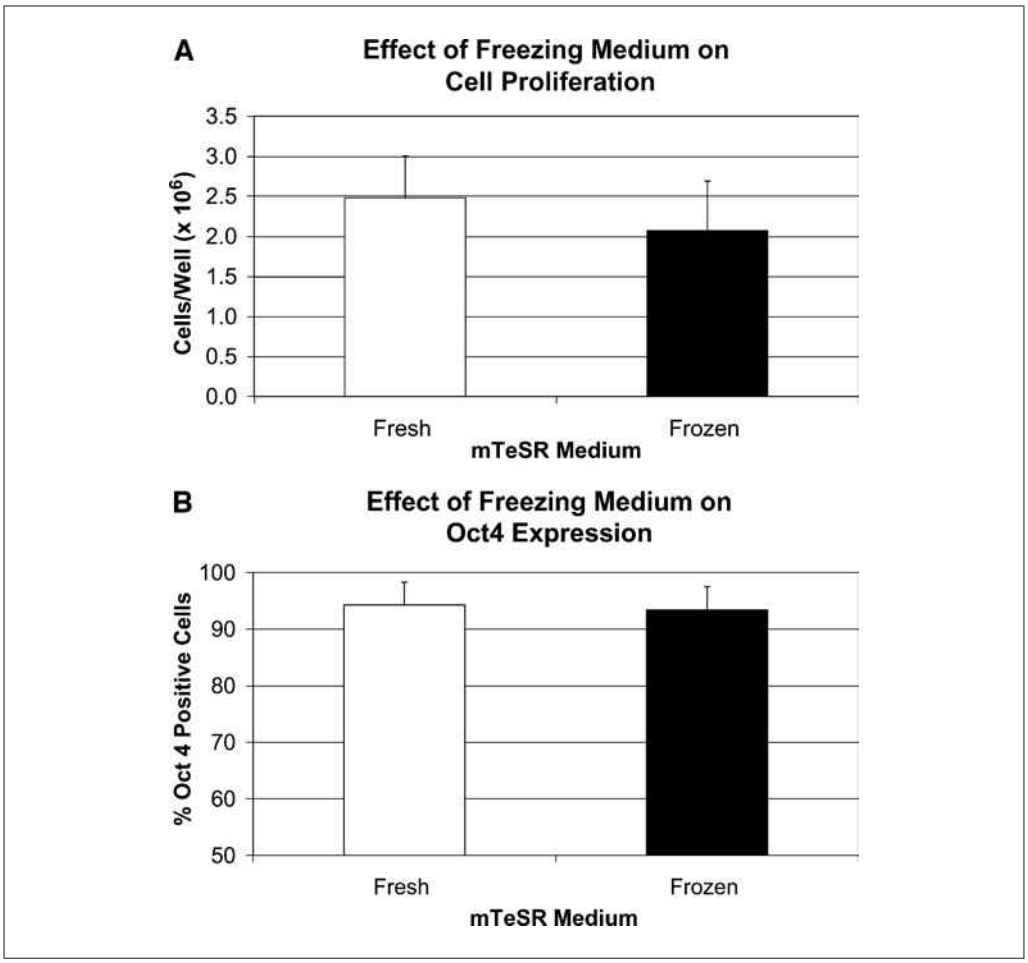

Figure 1C.2.3 Effect of freezing medium on cell competence. Human ES cells were cultured in either fresh or lot-matched frozen medium for three passages. At the end of the culture period, Oct4 expression was assessed by FACS analysis (**B**), and individual cell counts were obtained (**A**) as per Ludwig et al. (2006b). No significant differences ($P < 0.01$, *t*-test) were noted in Oct4 expression or cell proliferation in frozen versus fresh medium. Data represents 3 replicates in triplicate. The medium can be frozen up to 1 year with no decrease in performance. The authors have used frozen media to expand cultures for at least 6 months.

Selenium stock (0.07 mg/ml)

Dissolve 7 mg sodium selenite (Sigma) into 100 ml Type I water. Filter sterilize and store the resulting solution in 1-ml aliquots at 4°C up to 6 months.

CAUTION: Sodium selenite is highly toxic by inhalation. Appropriate safety precautions should be taken when handling, including the use of a respirator. If possible, all work with this compound in its powdered state should be performed in the fume hood.

Stock B

Slowly dissolve 67 g of BSA (Sigma; cat. no. A2153) into 500 ml Type I water while gently stirring at room temperature (this step should take up to several hours). To this solution, add:

2.8 g sodium bicarbonate (Sigma)
33 mg thiamine hydrochloride (Sigma)
10 mg reduced glutathione (Sigma)
330 mg L-ascorbic acid 2-phosphate Mg-salt (Sigma)
516 mg γ-aminobutyric acid (GABA, Sigma)
212 mg lithium chloride (LiCl, Sigma)
6.5 μl pipecolic acid stock (see recipe)
1 ml selenium stock (see recipe)
10 ml Trace Mineral Stock B (MediaTech/Cellgro)
5 ml Trace Mineral Stock C (MediaTech/Cellgro)
10 ml human insulin solution (Sigma)
10 ml bFGF stock (see recipe)
10 ml TGFβ stock (see recipe)
10 ml Defined Lipid Concentrate (Invitrogen)

Using a Class A volumetric flask (Fisher), bring volume up to 1 liter with Type 1 water. Filter sterilize the resulting solution and store in 200-ml aliquots for up to 2 weeks at 4°C, or up to 6 months at −80°C.

BSA must be added prior to the incorporation of any other protein components. Insulin, in particular, readily adheres to glass, and significant amounts will be lost to the glassware if added prior to BSA. BSA purchased from Sigma has traditionally been used in the authors' laboratory with good results. Variations have been seen from lot to lot on occasion, however, and therefore batch testing of all serum-derived products is imperative. Be cautious to avoid the formation of bubbles when dissolving, mixing, and filtering this solution. Loss of proteins will result if bubble formation is not adequately controlled. Use only low-protein binding filters, PES membrane, or equivalent.

TGFβ stock (300 ng/ml)

Dissolve 30 μg human TGFβ (R&D Systems) into 600 μl 4 mM HCl (Sigma). Add 99.4 ml diluent solution (see recipe). Store 20-ml aliquots up to 6 months at −80°C.

Washing medium

Add 1.48 g calcium- and magnesium-free DMEM/F-12 (Invitrogen) to 100 ml Type I water and stir until dissolved, adding low heat as necessary. Adjust the pH to 7.2 and the osmolarity to 340 ±5 mOsM using sodium bicarbonate. Filter sterilize the medium, and store in 12- to 60-ml aliquots up to one month at 4°C. Warm an aliquot to 37°C before use.

COMMENTARY

Background Information

Several media have now been reported to support the feeder-independent culture of human ES cells (Table 1C.2.1), two of which have also been reported to support initial derivation (Klimanskaya et al., 2005; Ludwig et al., 2006a). FGF-signaling, and a balance between BMP-family members promoting differentiation and Activin/TGFβ family members inhibiting differentiation are common themes across these different media formulations. TeSR1 is one of the serum-free, animal product-free, defined media that supports both culture and derivation of human ES cells (Ludwig et al., 2006b). Although the initial formulation of TeSR1 contained only recombinant or human-sourced protein components, subsequent modifications include the use of animal sourced proteins (e.g., BSA, Matrigel) and cloned zebrafish bFGF that significantly reduced costs [mTeSR1: (Ludwig et al., 2006a)] and made the medium a practical alternative for most laboratories. The authors have now been using this defined medium for all of their routine human ES cell cultures for more than a year. Human ES cells cultured in mTeSR for >40 passages maintain a normal karyotype, expression of appropriate ES cell markers (>90% Oct4, SSEA4, Tra 1-60, Tra 1-81), and the ability to form all three germ layers in both embryoid bodies and teratomas.

Critical Parameters

Traditional serum- and MEF-based human ES cell culture can mask deficiencies within the culture system. In the absence of these components, quality control within the laboratory takes on an increased level of importance. Deficiencies in quality control are rapidly evident in feeder-independent culture systems. Vigilance in monitoring equipment and screening reagents is required to achieve the highest quality and most consistent cultures.

Maintenance of sterility is critical. All manipulations must be performed in a Class II Biological Hazard Flow Hood. Furthermore, all hoods should be certified at least yearly to ensure proper flow, and the UV bulbs should be replaced [this will depend on usage, per the manufacturers' recommendations (total use hours); at a minimum, the UV bulbs should be replaced yearly]. It is difficult to determine if UV lights are emitting the appropriate wavelength to ensure sterilization, and the bulb will continue to illuminate even when the UV is inadequate. Replacing the UV bulbs on a regular basis will help to ensure continued sterility of cultures.

Previous studies have demonstrated the impact of alterations in the physiochemical environment on human ES cell culture performance (Ludwig et al., 2006b). Minor alterations in pH and osmolarity can dramatically affect the proliferation and differentiation of cell cultures. Changes in CO_2 concentrations affect pH, and reductions in environmental humidity can affect osmolarity of media. Cells in culture, specifically undifferentiated and germ cells, can be exquisitely sensitive to temperature variations. Changes in temperature as small as 0.5°C can have a dramatic effect on embryo viability in culture (McKiernan and Bavister, 1990; Shi et al., 1998; Abeydeera et al., 2001). While no published studies have investigated the impact of temperature on human ES cell competence, ES cell sensitivity may be similar to that of embryos. Therefore, atmosphere, humidity levels, and temperature of incubators must be monitored daily.

Bovine serum albumin and Matrigel are biologically sourced products. While recombinant alternatives to BSA are available, they are beyond the financial reach of the common research laboratory. Studies have demonstrated significant inconsistencies between lots of serum albumin (McKiernan and Bavister, 1992). Individual lots of albumin, therefore, must be carefully screened because they vary considerably in their ability to sustain human ES cell growth. Likewise, in the authors' laboratory they have noticed variations between lots of Matrigel, and recommend screening each lot to ensure it is sufficient to adequately support undifferentiated human ES cell culture.

Water quality is perhaps the most critical factor in the success of any culture system. Only Type I reagent-grade water should be used in the preparation of solutions used for human ES cell culture. While "sterile water" is generally available for purchase, it is not manufactured to meet the appropriate specifications, and is not an adequate substitute for Type I reagent-grade water (Mather et al., 1986). As water quality is significantly affected by storage, even high-quality water should not be stored for an extended period of time (Gabler et al., 1983), but rather used directly from the source. The authors recommend using an in-house water purification system, such as the Milli-Q Ultrapure Water System (Millipore).

Table 1C.2.1 Feeder-Independent Human ES Cell Culture Systems

Medium	Basal medium	Supplement	Key medium additives	Matrix	Xeno-free	Medium defined	System defined	Formula dis-closed	Citation
TLF[a]	KO-DMEM	KOSR	4 ng/ml bFGF, TGFβ	Fibronectin	N	N	N	N	Amit et al. (2004)
UMFN	DMEM/F12	KOSR	40 ng/ml bFGF, noggin	Matrigel	N	N	N	N	Xu et al. (2005b)
E[a]	KO-DMEM	KOSR	40 ng/ml bFGF, Flt3L	Matrigel	N	N	N	N	Xu et al. (2005a)
DSR+ Activin	KO-DMEM	KOSR	Activin, KGF	Laminin	N	N	N	N	Beattie et al. (2005)
Un-named[b]	KO-DMEM	KOSR	10 ng/ml bFGF, 20 ng/ml hLIF	Murine cell extraction	N	N	N	N	Klimanskaya et al. (2005)
SR-bFGF	KO-DMEM	KOSR	36 ng/ml bFGF	Matrigel	N	N	N	N	Wang et al. (2005)
NC-SFM	X-Vivo 10	—	80 ng/ml bFGF	Laminin	Y	Y	Y	N	Li et al. (2005)
CDM	IMDM+F12	BSA	12 ng/ml FGF, Activin	FBS	N	Y	N	Y	Vallier et al. (2005)
UM100	DMEM/F12	KOSR	100 ng/ml bFGF	Matrigel	N	N	N	N	Levenstein et al. (2005)
TeSR1[b]	DMEM/F12	HSA	100 ng/ml bFGF, TGFβ, LiCl, PA, GABA	Matrigel or human matrix	Y	Y	Y	Y	Ludwig et al. (2006b)
HESCO	X-Vivo	HSA	4 ng/ml bFGF, Wnt3a, April/BAFF	Matrigel or fibronectin	Y	Y	Y	N	Lu et al. (2006)
N2-CDM	DMEM/F-12	BSA	20 ng/ml bFGF, N2, B27	Matrigel	N	Y	N	N	Yao et al. (2006)
NBF	DMEM/F-12	—	100 ng/ml bFGF, N2, B27	Fibronectin	Y	Y	Y	N	Liu et al. (2006)
mTeSR1	DMEM/F-12	BSA	100 ng/ml bFGF, TGFβ, LiCl, PA, GABA	Matrigel	N	Y	N	Y	Ludwig et al. (2006a)

[a] ~20% differentiation.
[b] Supports derivation.

Defined, Feeder-Independent Medium for hESC Culture

The system should be maintained regularly, and monitored daily. Total organic content in particular can have a dramatic and devastating effect on culture performance (Fig. 1C.2.4), and may not be detectable at the point of use. Because even carefully maintained systems can occasionally demonstrate substandard performance, water samples should be sent out regularly for independent testing of sterility, endotoxin, and total organic content.

Glassware used for preparation of media should be acid stripped before use, and washed

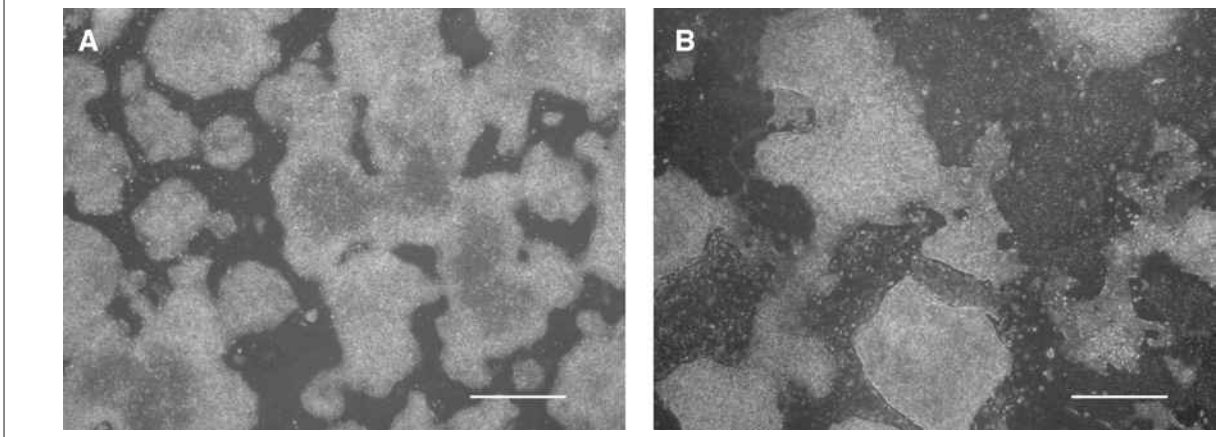

Figure 1C.2.4 Effect of water source on human ES cell cultures. Human ES cells were split from the same parental culture into medium made using water source A (**A**) or water source B (**B**) and cultured for three passages. All parameters with the exception of water source were identical for both cultures. Both water sources were Milli-Q Ultrapure Water Systems that had been well maintained and were not noticeably different upon observation. At the end of the culture period, cells exposed to water source B demonstrated increased levels of spontaneous differentiation and decreased proliferation. Testing of the water from each source by an outside agency revealed that water from source B contained elevated total organic counts (TOC). Scale bar = 100 μm.

between uses. Soap should *never* be used to wash glassware, as residues cannot be effectively removed, and will be toxic to human ES cell cultures. Instead, glassware should be rinsed a minimum of 10 times with high-quality (Type 1 reagent-grade) water immediately after every use. Glassware should be wrapped in foil and baked dry at a temperature not below 200°C. Acid stripping should be repeated every 3 to 5 uses.

Glutamine and sodium bicarbonate are highly labile, and are affected by changes in temperature, headspace in containers, and duration of storage. Because of this, media should be kept in an appropriate-sized container to reduce exposure to air, and should not be maintained longer than 2 weeks at 4°C.

Appropriate passage timing is absolutely critical to the success of this culture system. While other culture methods allow for some flexibility in split timing, in this feeder-independent system there is roughly a 24-hour window to passage cells and achieve optimum attachment and continued undifferentiated proliferation. Passage too early and cells will not attach, too late and cultures will easily differentiate. Cultures should be passaged when colony centers become dense, appearing brighter than the edges when viewed using phase contrast microscopy (Fig. 1C.2.2). When initially working with this system, splitting multiple wells of a single culture on successive days and observing the resulting cultures in the days immediately following passaging may identify appropriate timing.

Cells cultured in mTeSR, if maintained properly (i.e., high-quality water and media components, appropriate passage timing, adequate equipment maintenance) should continue to expand with <5% spontaneous differentiation routinely. Autologous feeder formation is not a feature of the mTeSR culture system. Presence of this type of spontaneous differentiation should be viewed as a sign of quality control issues. Cultures that express >10% spontaneous differentiation should not be maintained. "Pick to keep" is not recommended to rescue cultures that have differentiated in any system: co-culture, feeder-free, or feeder-independent. This technique places tremendous selection pressure on cultures and can drive them toward an abnormal karyotype. The only circumstances under which the authors would recommend these kinds of heroic measures are with modified or very low passage cultures that are irreplaceable.

Cells cultured in mTeSR medium can be frozen using mTeSR medium supplemented with 20% FBS and 10% DMSO. The authors recommend freezing at twice the standard density. The authors routinely freeze 2 confluent well/vials and thaw 1 vial into 1 well of a 6-well plate. The authors have not tested vitrification. Stem Cell Technologies (the commercial producer of mTeSR) is currently developing an improved freezing medium specifically

Table 1C.2.2 Troubleshooting Guide for Feeder-Independent Culture of Human ES Cells Protocols

Problem	Possible cause	Solution
Low or no attachment at passage	Dispase splitting: Dispase solution may not have been adequately rinsed away.	Increase number and volume of rinses to assure complete removal of dispase solution prior to plating cells.
	EDTA splitting: Cells may have been individualized prior to plating.	Decrease time of EDTA incubation and/or decrease disruption of colonies post-incubation.
	EDTA splitting: Cell may be damaged by inappropriate osmolarity or pH.	Check and adjust osmolarity and/or pH of solution as appropriate.
	Quality of Matrigel may not be appropriate for human ES cell culture.	Screen Matrigel.
	Inappropriate passage timing	Passage cells one day later to increase attachment at passage.
Good attachment, but limited proliferation	Inappropriate pH	Check pH of medium post equilibration and adjust accordingly.
	Inappropriate osmolarity	Check medium osmolarity and adjust accordingly.
	Poor water quality	Test TOC of water (should not exceed 30 ppb).
Increased spontaneous differentiation	Small, isolated areas of differentiation, not exceeding 10% of the culture, are normal and can be removed by aspiration with a Pasteur pipet at passaging. Increased differentiation may be due to the following causes: Poor quality BSA	Screen multiple lots of BSA to assure quality before use.
	Poor quality Matrigel	Screen prior to use.
	Inadequate water quality	Use only Type 1 reagent-grade water, measuring 18.2 mOhm. Routinely test total organic content and endotoxin levels to assure quality.
	Inappropriate passage timing	Passage cells one day earlier to reduce spontaneous differentiation following passage.

for mTeSR cells, and it will be available in October of 2007.

Troubleshooting

See Table 1C.2.2 for troubleshooting tips.

Anticipated Results

Feeder-independent human ES cell culture is relatively simple and efficient provided that the medium is properly prepared. Transfer of cells from MEF-containing cultures to feeder-independent conditions may result in some MEF carryover for the first few passages. After the initial passages, however, cultures should remain clear of "feeder-like" cells. Morphologically, cells will appear as expected, exhibiting classic ES cell morphology (minimum of 2 nucleoli, large nucleus to cytoplasm ratio, distinct cell borders; Fig. 1C.2.5). Individual cells, however, will be smaller than in standard culture conditions, and users may easily underestimate the number of cells within a culture. If cell numbers are important, it is advisable to perform cell counts.

Cultures should proliferate well, with $<10\%$ spontaneous differentiation overall. On average, 2 to 4 $\times$ 10^6 cells/well can be expected at passage. Generally, cells can be passaged at split ratios between 1:6 and 1:10 every 7 days. The authors have not seen elevated karyotypic instability in this system compared to standard culture systems, and if cells are maintained properly (on-time passaging and limited disruption at passaging), normal karyotypes can be expected. Cells will remain pluripotent, expressing appropriate ES cell markers (Fig. 1C.2.5), and retain the ability to differentiate into all three germ layers. Cloning efficiency is reduced in this culture system however, and users should expect

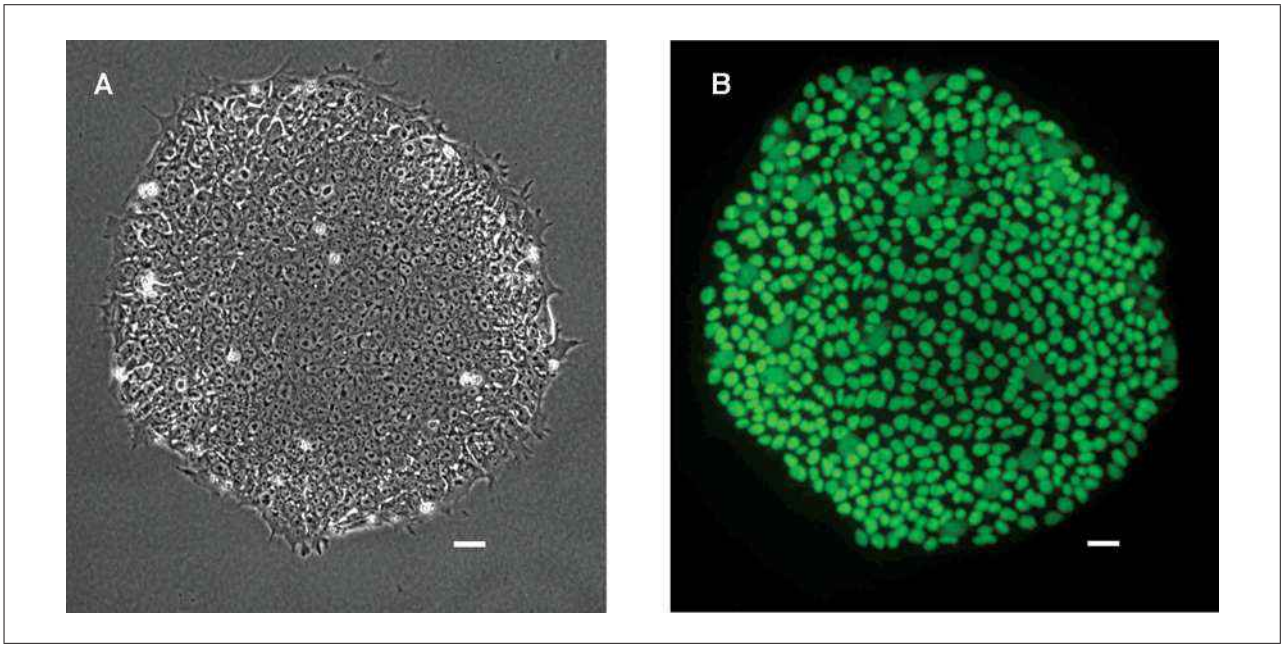

Figure 1C.2.5 Morphology and marker expression of human ES cells cultured in mTeSR. Following 15 passages of culture in mTeSR medium on Matrigel, H9 (WA09) human ES cells demonstrate classic ES cell morphology (**A**), and >95% of cells express Oct4 when stained (**B**). Scale bar = 10 μm.

<0.1% single cell survival rates using mTeSR medium as formulated. Increasing cloning efficiencies remains an important area of research in human ES cell culture overall, and is particularly important in feeder-independent culture systems.

Time Considerations

Media

Allow ~10 to 15 min for the preparation of the following stock solutions: diluent stock, pipecolic acid stock, bFGF stock, TGFβ stock, and L-glutamine stock. When preparing Stock B, allow at least 4 hr for BSA to dissolve. The remainder of the Stock B preparation should take ~30 min, proceeding slowly to prevent bubble formation. Approximately 30 min should be allowed for assembly of mTeSR culture medium, including pH and osmolarity adjustments.

Matrigel

Thawing bottles of Matrigel will take at least 12 hr and must be performed at 4°C on ice. It is most convenient to do this overnight. Allow at least 1 hour for tips and tubes to properly chill before aliquoting Matrigel. Aliquoting Matrigel stock will require ~1 hr/bottle. While concentrations vary, on average each bottle of Matrigel will cover between 150 and 200 6-well plates.

Passaging

Matrigel coating of tissue culture plates will take 3 to 5 min per plate, and must be done at least 1 hour prior to passaging cells. All reagents should be warmed for a minimum of 15 min prior to passaging cells. Allow 1 to 5 min per plate for observation and preparation (marking of differentiation). Once splitting and washing solutions are warmed, allow 7 to 10 min per plate for EDTA passaging, 12 to 15 min per plate for dispase passaging, and 10 to 12 min per plate for collagenase passaging.

Culture

Medium should be warmed for ~30 min before feeding cells. Once medium is properly warmed, completing the culture protocol should take no more than 3 to 5 min per plate daily.

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