



HUMAN EMBRYONIC STEM CELL PROTOCOLS

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Human Embryonic Stem Cell Methods

1.1 Introduction

Human embryonic stem cells (hESCs) are pluripotent cells capable of differentiation to representatives of all three germ layers. The maintenance and differentiation of hESCs will form the basis for significant research in human cell and developmental biology, and in the potential clinical application of cell replacement therapies. However, due to their inherent capacity for differentiation, the maintenance of undifferentiated cultures of hESCs is not as simple as growing other types of mammalian cells. While the development and optimization of hESC culture techniques will be an ongoing concern of this research field, the following collection of protocols represent the current culture conditions used in our laboratories.

1.2 BresaGen's hESC lines

In 2001, BresaGen isolated four hESC lines that qualified for NIH funding under the criteria outlined by President Bush. Basic characterization of these lines has been published (Mitalipova et al. *Stem Cells*. 2003;21(5):521-6, Brimble et al, *Stem Cells and Development* 2004;13(6): in press). The lines BG01, BG02 and BG03 have been recovered, expanded and are listed on the NIH registry (<http://stemcells.nih.gov/index.asp>). A summary of the karyotype and gene expression characteristics of these cells is listed in Table 1. Short tandem repeat (STR) polymorphic markers were used to define the unique genotype of each line (Table 2). The lines were HLA isotyped (Appendix). The BG04 cell line was not recoverable and was removed from the NIH registry.

Table 1. BresaGen hESC Lines

	BG01	BG02	BG03
Karyotype	46,XY	46,XY	46,XX
Alk. Phosphatase	+	+	+
Immunostaining			
SSEA1	-	-	-
SSEA3	+	+	+
SSEA4	+	+	+
TRA-1-60	+	+	+
TRA-1-81	+	+	+
OCT4	+	+	+
RT-PCR			
OCT4	+	+	+
NANOG	+	+	+
REX1	+	+	+
SOX2	+	+	+
ABCG2	+	+	+
CRIPTO	+	+	+
TERT	+	+	+
DPPA5	+	+	+
FOXD3	+	+	+
UTF1	+	+	+

Table 2: Polymorphic Markers Used for Genotyping

	D3S1358	vWA	FGA	D8S1179	D2S11	D18S51
BG01	15,17	16,17	22	10,12	28,29	19
BG02	15,17	18,19	20,25	13,14	27,30	14,16
BG03	14,15	16	20,22	12,13	29	17,19

	D16S539	TH01	D2S1338	D19S433
BG01	9,11	7,9.3	17,24	13,15
BG02	12	8,9	17,24	13,14.2
BG03	11,13	6,7	21,23	13,14

Numbers of repeats in STR loci (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>) are indicated.

These cell lines were tested for infectious agents and were negative for hepatitis B and C, HIV-1 and -2, HTLV I/II, HSV-1 and -2 and CMV, as well as mycoplasma.

1.3 General Notes on hESC Culture

- hESCs can be maintained on a layer of mouse embryonic fibroblasts (MEF), in media consisting of either:
 - 20% KSR hESC medium: DMEM:F12, 20% KSR, 4 ng/ml bFGF, 2 mM glutamine, 0.1 mM non-essential amino acids, 50 units/ml penicillin and 50 µg/ml streptomycin, 0.1 mM β-Mercaptoethanol
 - Or, 20% KSR hESC medium conditioned by MEFs for 24 hours (MEF-CM) (section 2.3).
- We have found that the use of MEF-CM minimizes, but does not eliminate, the spontaneous differentiation of pluripotent stem cells, although we have successfully maintained hESC in both these media.
- hESC cultures are split primarily using microdissection passaging (section 2.4), using a dissecting microscope within a laminar flow cabinet. This remains the most effective approach for maintenance of undifferentiated cultures, due to the capacity to use morphological criteria to select undifferentiated cells.
- Cultures may be maintained on a high or low density of MEFs (Table 4), which results in different colony morphologies (Figure 1). hESC grow as domed colonies on high density MEFs, but colonies are flat and individual cells exhibit prominent nucleoli on low density MEFs. Both these colony types express pluripotent markers and differentiate effectively as embryoid bodies.
- hESC can also be expanded using bulk passaging with EDTA-free trypsin (section 2.5). Overtly differentiated colonies should still be removed prior to each passage, to maintain the general undifferentiated state of the culture. This requires competency in recognizing the morphology of differentiated cells/colonies and the capacity to remove them from the culture. Ideally, this is performed by mechanically excising the differentiated regions from the dish, using a dissecting microscope within a laminar flow cabinet. (sections 2.4 and 2.5). In these bulk passaging methods, the MEF feeder layer is also removed by peeling it away from the hESC colonies. To make this possible, MEFs are plated onto dishes that have not been coated with gelatin.
- MEFs and MEF-CM must be prepared several days in advance. MEFs can be prepared using standard procedures (sections 2.1 and 2.2), or purchased from companies such as Specialty Media (www.specialtymedia.com), or Stem Cell Technologies (<http://www.stemcell.com/default.asp>).
- Cryopreservation and thawing of hESCs is not an efficient process, with poor survival being a major issue. It is not uncommon for only a few colonies to proliferate after a thaw. In this situation, a dissection microscope and needle are used to break up colonies by microdissection and the pieces are transferred to a new dish (section 2.5). A similar approach is used to recover undifferentiated cells from a culture with a high level of spontaneous differentiation.
- We prefer to culture hESCs at high density, with ~200-250 colonies per 35 mm dish and ~500 colonies per 60 mm dish (when high density MEFs are used). In our hands, spontaneous differentiation appears to be reduced under these conditions. Alternatively, when low density MEFs are used, the hESC colonies exhibit flatter

growth and colonies can be left to grow to a larger diameter. Therefore fewer colony pieces can be transferred to each new dish at passaging, for a density of ~150 colonies per 35 mm dish.

- Feeder free culture of hESCs has been described previously and detailed methods are available elsewhere (section 3.5).

2.1 Isolation of Primary Mouse Embryo Fibroblasts

This method is derived from standard laboratory protocols. Published versions are available in “Teratocarcinomas and Embryonic Stem Cells: A Practical Approach” (Robertson, 1987) and “Manipulating The Mouse Embryo” (Hogan, Beddington, Constantini, Lacy. 1994).

1. Asphyxiate pregnant (day 13.5) ICR mice with CO₂.
2. Place the mouse on its back and spray with 70% ethanol. Using scissors, make a cut through the belly skin. Carefully dissect out the uterus with sterile forceps and scissors and place it into a Petri dish containing sterile PBS.
3. Isolate the embryos from the uterus, release the embryos from the embryonic sacs, and transfer embryos to a second Petri dish containing sterile PBS.
4. Under a dissecting microscope, remove the embryo heads and livers, intestines, heart and all viscera using two watch-makers forceps.
5. Transfer the embryo carcasses to a fresh Petri dish. Carefully mince the embryos with a sterile scalpel blade or scissors (approximately 1x1 mm). Add 5.0 ml of 0.25% trypsin/EDTA per 10 fetuses, and triturate through a 10 ml pipette.
6. Transfer the tissue/trypsin solution with a 10 ml pipette from the Petri dish to the barrel of a 10 ml syringe with an attached 18 g needle. Replace the syringe plunger, invert and expel air, and then slowly, gently push the tissue/trypsin solution through the needle, and collect in a 50 ml conical tube. Gently pass the tissue/trypsin suspension through the needle a second time.
7. Incubate the tissue suspension for 15 minutes at 37°C, triturating 3 or 4 times using a 10 ml pipette to dissociate the tissue. Add an equal volume of complete MEF medium to inactivate the trypsin.
8. Triturate the suspension vigorously with a 10 ml pipette.
9. Plate 1.0 embryo equivalent per T175 flask, and add complete MEF medium to make up a final volume of 30 ml per flask. This density allows the cells to adhere but not become overly confluent before harvest at day 3-4. Incubate at 37°C with 5% CO₂.
10. Normally, the dissociated MEFs will attach to the flask and begin to divide overnight. Replace the medium the next day with an equal volume of fresh complete MEF medium. In 3-4 days, when the cells are nearly confluent, the MEF p0 cells are ready to be frozen. Rinse the cells once with Ca²⁺/Mg²⁺-free PBS. Loosen cells using 3 ml

0.05% trypsin/EDTA solution per flask. Inactivate the trypsin solution by adding 5 ml complete MEF medium.

11. Pool cells from the various flasks and count the number of viable cells using trypan blue exclusion and a hemocytometer. Viability should be between 90-95%. Centrifuge the cell suspension at room temperature for 4 minutes at 200 g. Aspirate medium and resuspend the cells in complete MEF medium at 2x the desired final freezing concentration (i.e., 2.4×10^7 /ml). Add an equal volume of Fetal Bovine Serum with 20% DMSO to get 1x final stocks. Dispense 1 ml per cryovial and freeze. Each vial contains 1.2×10^7 MEFs.
12. We freeze cells using the NALGENE™ Cryo 1°C Freezing Container. The container is stored at room temperature and filled with 250 ml of Isopropanol. Cryovials prepared as described above are placed in the Freezing Container, and the container is then placed in a -70°C Freezer overnight. This procedure ensures that a minus 1°C/minute rate of cooling is achieved, a step critical for retaining cell viability. After an overnight incubation, cryovials are removed from the Freezing Container and placed in Liquid Nitrogen (-196°C) for long-term storage.

2.2 Thawing and preparing p1 MEF feeder plates

- Thaw as many vials as appropriate for the number and density of feeder layer plates required (Tables 3 and 4).
- Gelatin coated plates can be used for plating MEFs, however we do not use gelatin coated plates when the MEFs are to be removed by peeling (bulk passaging, section 2.5). Generally we only use gelatin coating when MEFs are prepared for making MEF-CM, or the hESC are to be examined by immunostaining.

Table 3. Typical cell numbers during the MEF preparation process

STAGE	# of cells
p0 vial of MEFs	~1.2x10 ⁷
plated to each T175	2.5 x 10 ⁶
Cells per T175 at harvest	~9.6 x10 ⁶ to 1.0 x10 ⁷

Table 4. High (H) and Low (L) Densities for MEFs on Different Culture Dishes

Cell culture vessel	Actual Surface area in cm ²	# of MEF for hESCs	Optimum Volume For Plating (ml)
Dishes			
35 mm	10	H 2 X 10 ⁶ L 2 X 10 ⁵	2
60 mm	20	H 4 X 10 ⁶ L 4 X 10 ⁵	5
100 mm	60	H 1.2 X 10 ⁷ L 1.2 X 10 ⁶	10
Flasks			
T25	25	H 5.0 X 10 ⁶ L 5.0 X 10 ⁵	5
Multiwell plates			
24	2	H 4 X 10 ⁵ L 4 X 10 ⁴	1
6	10	H 2 X 10 ⁶ L 2 X 10 ⁵	3
Chamber slides			
4	1.75	H 3.5x 10 ⁵ L 3.5 x 10 ⁴	0.9

1. Frozen stocks of MEF p0 are stored in liquid nitrogen. Thaw cells quickly in a 37°C water bath, with gently shaking by hand.
2. Work in a laminar flow cabinet. Transfer the cells gently to a 50 ml conical tube. Add 10 ml of complete MEF medium dropwise, slowly with swirling. Add another 10 ml of medium and centrifuge at 200g for 4 minutes.

3. Resuspend the cells in 50 ml complete MEF medium.
4. Count viable cells using trypan blue exclusion. Viability is usually >95%.
5. Plate cells into T175 flasks at 2.5×10^6 per flask with 30 ml medium per flask. Flasks are incubated at 37°C, 5% CO₂.
6. Three days after thawing, the cells should be 90-95% confluent in T175 flasks.
7. Check each flask microscopically to assure cell growth and sterility.
8. Aspirate the medium and treat the cells with 10 µg/ml mitomycin C, 16 ml per T175 in complete MEF medium for 2.5 hours at 37°C, 5% CO₂.
9. After treatment, aspirate the mitomycin C. The cells receive a total of 5 washes after mitomycin C treatment.
10. WASH 1: Wash the cells with 20 ml Ca²⁺/Mg²⁺-free PBS. Lay flask down and rock gently to wash the cells. Aspirate the PBS.
11. Add 3 ml of 0.05% trypsin/EDTA. Disperse the trypsin by tilting and tapping the flask.
12. When the cells detach from the flask, typically after 1-2 minutes, add 5 ml complete MEF medium to each flask to neutralize the trypsin. Pipette the medium 3-4 times across the flask to wash off remaining adherent cells.
13. Pool cell suspensions into a 50 ml conical (up to 6 flasks can be pooled into one 50 ml conical).
14. Use 10 ml complete MEF medium to rinse all the flasks, and use this rinse to bring the pooled cell suspensions to 50 ml per tube.
15. WASH 2: Centrifuge cells at 200g for 4 minutes.
16. WASH 3: Aspirate and resuspend the cell pellets with 10 ml complete MEF medium. Cell pellets can be pooled at this time. After pooling, rinse the empty tubes with 5 ml complete MEF medium and add to the pooled tubes. Bring each tube to 50 ml with complete MEF medium. Centrifuge cells at 200g for 4 minutes.
17. WASH 4 and WASH 5: Aspirate and resuspend the cell pellets with 10 ml, triturate to resuspend cells, then bring each tube to 50 ml with complete MEF medium. Centrifuge cells at 200g for 4 minutes.
18. Resuspend in complete MEF medium. Count with trypan blue exclusion using a hemocytometer. If the cell suspension is too concentrated to count accurately, dilute a sample appropriately and recount.
19. Adjust the cell suspension to the desired concentration with complete MEF medium and plate out to generate inactivated MEF feeder layers. The MEF plates are cultured at 37°C, 5% CO₂ at least 24 hrs prior to use.
20. Currently we use high or low density MEFs for hESC cultures (2×10^6 or 2×10^5 MEF cells per 35 mm tissue culture dish, respectively). There are advantages to using each of these different densities: High density MEFs result in tightly packed, domed hESC colonies, and the MEF layer can be removed by peeling it off with watchmakers forceps. Low density MEFs result in hESC colonies that grow in a single layer, and are therefore easier to assess morphologically. However, it is not possible to remove the MEFs by peeling when plated at this lower density.

2.3 Preparation of MEF- Conditioned Medium (MEF-CM)

1. Plate 4×10^6 mitomycin C treated MEFs in a T75 Flask coated with 0.5% gelatin, in complete MEF medium.
2. The following day, replace the MEF medium with 37.5 ml 20% KSR hESC medium containing 4 ng/ml bFGF, and incubate for 24 hrs at 37°C, 5% CO₂.
3. Collect MEF-CM from the flasks after 24 hrs and 0.22 μM filter sterilize. MEF-CM can be used fresh or can be frozen.
4. Add fresh 20% KSR hESC medium containing 4 ng/ml bFGF to the flasks.
5. Collect MEF-CM for up to seven days using this procedure.
6. Depletion of L-Glutamine and bFGF from the MEF-CM is assumed, and L-Glutamine (to 2 mM final), and bFGF (to 4 ng/ml final) are therefore added back prior to using MEF-CM with hESCs. Freshly thawed β-Mercaptoethanol (β-Me) is added to 0.1 mM final fresh each day of use.

2.4 Microdissection Passaging of hESCs

- Typically, microdissection passaging is used to maintain undifferentiated hESC cultures. Developing proficiency in this approach is essentially an unavoidable necessity. At a minimum, we recommend attendance at one of the multiple hESC culture workshops (<http://stemcells.nih.gov/research/training/>) to become familiar with these techniques.
- Microdissection passaging must be used the first few passages after cells are thawed, or where extensive spontaneous differentiation has occurred in a culture, and undifferentiated cells must be identified morphologically and specifically excised for passaging. While we have used bulk passaging with EDTA-free trypsin (section 2.5) and there are numerous reports of passaging using collagenase, these methods do not circumvent the requirement for microdissection passaging.
- For microdissection passaging, some form of magnification equipment, for example a small inverted or dissecting microscope, needs to be placed in a sterile environment, such that the culture can be viewed and manipulated. The choice of equipment and environment will be site specific. Biosafety cabinets with incorporated dissecting microscopes are available, but the use of open front laminar flow cabinets, or a designated clean room with dissecting microscopes may be appropriate. We have seen successful culture of hESCs in different labs using a wide range of infrastructure. We use Class A-B3 Type II Nuair biosafety cabinets (NU-425-600) with modified view screens that fit a dissection microscope (blueprint available upon request).
- We routinely culture hESCs in 35 or 60 mm dishes. We use either high or low density MEFs cultures (eg, 2×10^6 or 2×10^5 MEF cells per 35 mm tissue culture dish, respectively. See Table 4).
- hESCs grow best when plated at high density and we typically aim for 200-250 colonies per 35 mm dish and ~500 colonies per 60 mm dish, when high density MEFs are used. Fewer colony pieces can be plated when using low density MEFs (~150 colonies per 35 mm dish), as hESC generate flatter, larger colonies under these conditions. After 4-6 days of culture the colonies should be ~1 to 2 mm in diameter when grown on high density MEFs. When grown on low density MEFs,

colonies should be ~3 to 5 mm in diameter after 5-7 days. Ideally, colonies should be spread evenly over the dish, with minimal overlap.

- We typically split one plate to three at passaging.
- Microdissection passaging requires (a) the identification and discrimination of undifferentiated and differentiated hESC colonies and (b) the capacity to excise the undifferentiated cells and transfer them to a new plate.
- Undifferentiated colonies exhibit the following morphological characteristics.
 1. On high density MEFs: hESCs grow as uniform domed colonies (Figure 1A). Individual cells have phase bright edges. As the colonies get larger, a central depression can form, giving the colony a saucer-like shape.
 2. On low density MEFs: hESCs grow as uniform flat colonies (Figure 1B). The cells exhibit prominent nucleoli and cell-cell edges are not phase bright.
- Differentiation is often observed as visible structure or organization, or it exhibits a different shade, within a hESC colony (Figure 1C,D; Figure 3A). Colonies or regions of colonies with overt differentiation are avoided upon passaging.

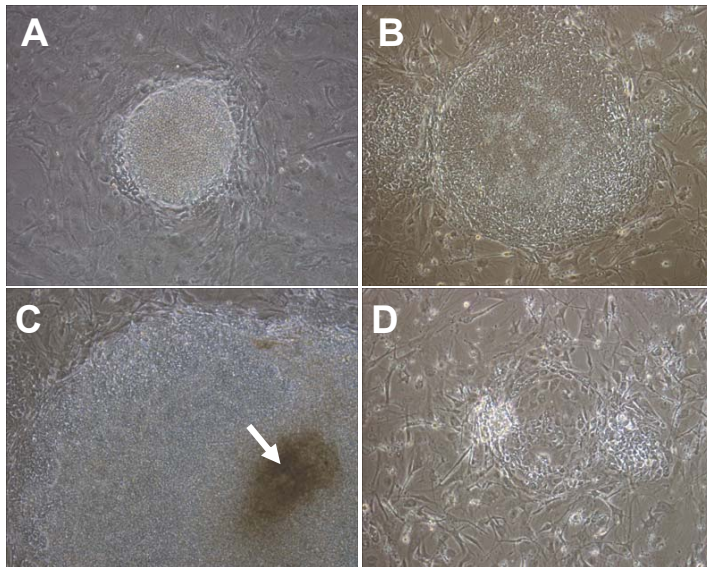


Figure 1. Morphology of hESC Cultures. (A) Undifferentiated domed hESC colony growing on high density feeders. Individual cells have phase-bright edges (B) Undifferentiated monolayer hESC colony growing on low density MEFs. Individual cells have prominent nucleoli. hESC colonies grow to a larger diameter than on low density MEFs. Undifferentiated colonies are uniform and do not exhibit structural organization on high or low density MEFs. (C) A large colony growing on low density MEFs with a central region undergoing differentiation (arrow). (D) A differentiated colony on low density MEFs. Images were taken under a 100x magnification

1. Warm an appropriate amount of MEF-CM (or 20% KSR hESC medium) to 37°C in water bath.
2. Once media is warm, add freshly thawed 1M β -Mercaptoethanol to a final concentration of 0.1 mM.
 - 2.1. Unused media is discarded at the end of the day, so only add β -Me to the amount required for that day.
3. Set the hESC plate on a dissecting microscope in a biosafety cabinet or laminar flow so that it is comfortable to see the colonies.
4. Assess the culture to identify undifferentiated colonies or colony regions from differentiated colonies (Figure 1).

5. Use a fine pointed tool to grid the undifferentiated colonies (Figure 2). hESC colonies are typically ~1-2 mm diameter when grown on high density MEFs, and 3-5 mm when grown on low density MEFs. We use fire drawn Pasteur pipette needles (Figure 2), or a 21G 1½ needle (Figure 3). Score 4 to 5 times across a colony, and perpendicular to that another 4-5 times. This creates a grid that cuts the colony into pieces.
6. Use the needle or a P1000 pipette tip to lift the colony pieces from the dish or feeder layer.
7. Repeat this for all the undifferentiated colonies in the dish.
Use a pipette to transfer the pieces to another MEF plate (section 2.2), or split between plates as appropriate.

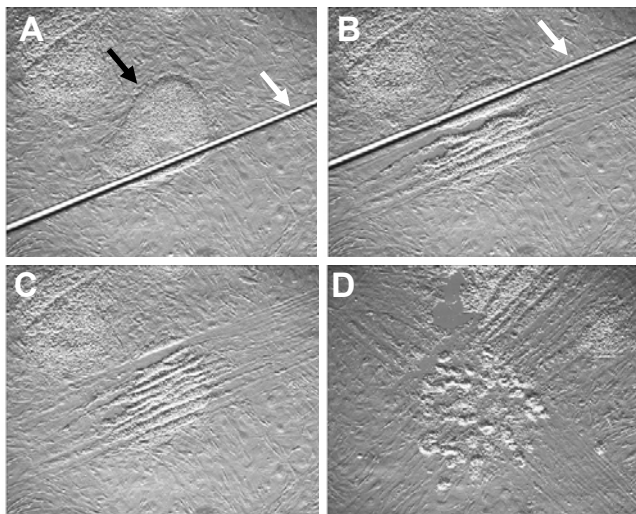


Figure 2. Microdissection passaging of hESC colonies. (A) An undifferentiated hESC colony growing on high density MEFs (black arrow). (A,B,C) A drawn out glass Pasteur pipette (white arrow), is pressed into the colony, to make a series of cuts. (D) An series of perpendicular cuts divides the colony into a grid of largely uniform pieces. The pieces are lifted from the plate with the glass needle. All the undifferentiated colonies in the dish are manipulated this way, and the pieces transferred to a new dish.

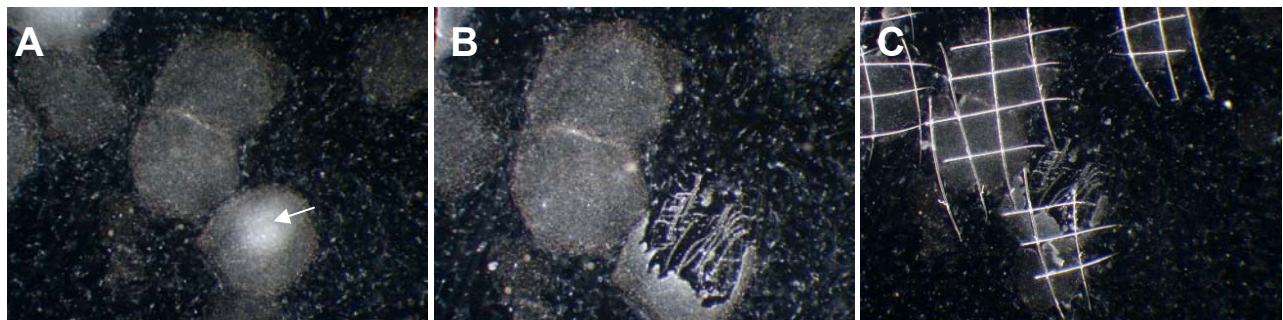


Figure 3. Microdissection passaging of hESC colonies on low density MEFs. (A) A hESC colonies viewed under a dissection microscope. Differentiated colony areas appear white (white arrow), while undifferentiated colonies are opaque. (B) Differentiated areas are excised and discarded. (C) The remaining undifferentiated colonies are scored with a needle (white lines), before lifting off and passaging.

2.5 Bulk passaging of hESC

- While we routinely rely on microdissection passaging, hESC cultures can also be expanded using several different bulk passaging methods. We have used EDTA-free trypsin to successfully passage hESC, as detailed below. This method uses MEF-CM.
 - Care should be taken to avoid disaggregation to single cells during passaging, as hESC have a low viability when split to single cells. Generating clumps of ~10-100 cells appears to be effective when passaging with this approach.
 - For this method of bulk passaging, we use hESC maintained on high density feeders (Table 4). The MEFs are removed during passaging by peeling them away from the hESC colonies. This does not work well if the plates have been gelatin coated.
 - hESCs grow best when plated at high density and we typically aim for 200-250 colonies per 35 mm dish and ~500 colonies per 60 mm dish. After 4-6 days of culture the colonies should be ~0.5 to 2 mm in diameter when grown on high density MEFs. Ideally, colonies should be spread evenly over the dish, with minimal overlap.
 - We typically split one plate to three at passaging.
1. Warm appropriate amount of MEF-CM to 37°C in water bath.
 2. Once media is warm, add freshly thawed 1M β -Me to a final concentration of 0.1 mM.
 - 2.1. Unused media is discarded at the end of the day, so only add β -Me to the amount required for that day.
 3. Set the hESC plate on a dissecting microscope in a biosafety cabinet or laminar flow so that it is comfortable to see the colonies.
 4. Assess the culture to identify overtly differentiated colonies within the plate of undifferentiated cells (Figure 1). Cut out any overtly differentiated colonies with a fire drawn pipette needle, or a 21G 1½ needle, and remove them with a P1000 pipette.
 5. Aspirate the plate and add 0.05% EDTA-free trypsin (1 ml for a 35 mm dish, 2 ml for a 60 mm dish) and leave for 30 seconds.
 6. Remove the MEF layer using flamed watchmakers forceps. Take hold of the side of the MEF layer and gradually peel the MEF layer from the dish. The MEFs will pull off as a mesh, taking some of the hESC colonies. Discard this layer, most of the hESC colonies are left attached to the dish.
 7. Large colonies should be scored with a needle as per microdissection passaging. Break up the undifferentiated hESC colonies using a P1000 tip. Pass the tip back and forth across the dish, scraping the entire surface. This will break the colonies into pieces.
 8. While viewing under a dissection microscope, gently triturate the clumps (3-4 strokes) using a pipetteman and a 1000 μ l tip until clumps consist of ~10-100 cells. Do not over triturate to single cells or cell survival will be poor.
 9. Transfer the cell suspension into a 15 ml centrifuge tube containing 9 ml 10% FCS in DMEM/F12.

10. Spin cells at 1000 rpm (200g) for 4 mins at room temperature.
11. Gently aspirate media and flick tube to loosen cells from the bottom.
12. Gently resuspend the cells in the appropriate amount of MEF-CM for as many plates as you are preparing, with a 5 ml or 10 ml serological pipette.
13. Aspirate the medium from the new plates of mitomycin C treated MEFs and dispense the hESC suspension.
14. Place the plate into an incubator set at 37°C with 5% CO₂, and mix gently in a figure 8 pattern to spread the clumps out evenly.
15. Feed cells the next day and every second day there after.
16. Observe cells every day and passage by the above protocol whenever required (3-6 days). Overgrowth of the culture will result in increased differentiation.

2.6 Cryopreservation of hESCs

- hESCs do not survive well when passaged as single cells and are therefore routinely passaged as small clusters or clumps of cells. Unfortunately, when these cell suspensions are frozen, their viability upon thawing is particularly low.
 - We typically freeze the cell suspension from one 35 mm dish to 3 cryovials, 500 µl per vial.
 - It is not uncommon to only have 5-20 colonies recover from a hESC thaw. Use microdissection passaging as described above to expand the culture.
1. Collect cell suspension as per routine hESC passaging
 2. Pellet the cells at 1000 rpm (200g) for 4 mins at room temperature and aspirate the supernatant. Pre-label the appropriate number of cryovials.
 3. During the spin, prepare the freezing solutions:
Solution I: 50% MEF-CM, 50% FCS
Solution II: 80% MEF-CM, 20% DMSO
 4. Be as gentle as possible after this point. Resuspend the pellet in 250 µl Solution I per vial to be frozen. Transfer the cell solution to a 50 ml tube so that the cells can be mixed by swirling during the procedure.
 5. Add 250 µl Solution II per vial to be frozen, dropwise, over 2 minutes with gentle swirling.
 6. Dispense 500 µl aliquots into pre-labelled cryovials.
 7. We freeze cells using the NALGENE™ Cryo 1°C Freezing Container. The container is stored at room temperature and filled with 250 ml of Isopropanol. Cryovials prepared as described above are placed in the Freezing Container, and the container is placed in a -70°C Freezer overnight. This procedure ensures that a -1°C/minute rate of cooling is achieved, a step critical for cell viability. After an overnight incubation, cryovials are removed from the Freezing Container and placed in Liquid Nitrogen (-196°C) for long-term storage.

2.7 Thawing of hESCs

- Perform the following steps as gently as possible.
 - It is not uncommon to only have 5-20 colonies recover from a hESC thaw. Use microdissection passaging as described above to expand the culture.
1. Remove the vial of hESCs from the liquid nitrogen storage tank.
 2. Thaw the cells by swirling gently in a 37°C water bath, wipe the vial with 70% Ethanol.
 3. Pipette the cells gently to a 50 ml tube, and add 10 ml MEF-CM dropwise over 2 minutes with gentle swirling.
 4. Transfer the cells to a 15 ml tube and pellet the cells at 200g for 4 mins at room temperature.
 5. Aspirate the supernatant, resuspend the cells in 2 ml MEF-CM.
 6. Aspirate the medium on a 35 mm plate of 2×10^6 MEF feeders, and plate the 2 ml of hESCs.
 7. Feed the cells the following day, and every 2 days after that. Visible colonies can often be observed within several days (Figure 4), but may appear later and only be ready for passaging up to two weeks after thawing.

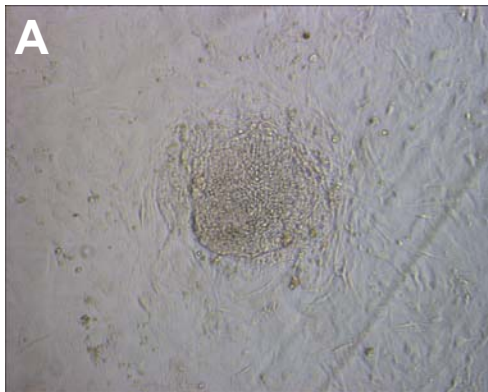


Figure 4. Thawed hESC colony. (A) A small colony, 3 days after thawing. Colony pieces should be visible when a thawed vial is plated out. It is not uncommon for these to appear to have died within the next few days. However, small colonies are often not possible to visualize, and may appear later, even after one week. hESC thaws should be monitored daily. Small colonies should be left to proliferate until at least 0.5 mm in diameter. However, colonies of any size should be passaged if there are morphological indicators of differentiation. The image was taken under 100x magnification.

2.8 Karyotyping

While we have determined that early passage BG01, BG02 and BG03 cells have a normal karyotype (Table 1), it has been reported that karyotypic instability can sometimes be observed with long term passage of hESCs (Draper et al, “Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells”. *Nat Biotech.* 2004 22(1):53-4). It is therefore prudent to periodically assess the karyotype of hESC cultures.

- The following method has been effective in generating nuclear preparations from hESCs in our laboratory. There are numerous cytogenetics facilities, usually in major hospitals, that offer diagnostic karyotyping services.
1. We routinely karyotype from 2x60 cm plates of hESCs grown on high density MEFs, although using fewer hESCs should be possible.
 2. Cell cultures should be fed with 5 ml of MEF-CM the day before the procedure.
 3. The following day, add 10 μ l KaryoMAX® Colcemid Solution directly to the plate (to make a final concentration of 0.02 μ g/ml). Colcemid is toxic, wear gloves.

4. Incubate 2 hours at 37°C, 5% CO₂.
5. Remove the culture medium containing colcemid.
6. Add 1 ml 0.05% trypsin (EDTA free) to the plate.
7. After 30 seconds, use watchmakers forceps to peel off the MEFs.
8. Use a P1000 tip to scrape all the hESCs off the plate, by passing it back and forth across the dish. Triturate the cell suspension and ensure cells have been split to primarily single cells
9. Transfer the cells to a 15 ml tube and add 10 ml 10% FCS in DMEM:F12.
10. Spin cells at 1000 rpm (200g) for 4 mins, at room temperature.
11. Resuspend the pellet in 2 ml of warm (37°C) Hypotonic Solution and vortex for 10 seconds.
12. Incubate in a water bath at 37°C for 30 minutes.
13. Add 0.5 ml of freshly prepared Fixative, dropwise with swirling.
14. Centrifuge cells at 1000rpm (200g) for 4 mins, at room temperature.
15. Carefully aspirate the supernatant.
16. Add 1 ml fixative dropwise, swirling the cells.
17. Repeat steps 14-16 two more times
18. Store at 4°C until cells are sent for analysis.

3.1 Cell Shipments

- BresaGen Inc. routinely ships frozen vials of hESC in N₂-cooled (-190°C) Dry shippers.
- On a case-by-case basis we are prepared to ship live cells in a T25 flask. This requires careful coordination with the receiving laboratory and depends on both the preparedness of the receiving lab and the availability of the appropriate cultures at BresaGen.
- There are different initial handling requirements for each format, but the receiving lab must prepare MEFs and MEF-CM in advance.
- The receiving laboratory should be prepared to place a high priority on the careful expansion of the hESCs and cryopreservation of stocks.
- To expect to succeed in maintenance of hESC cultures, the receiving laboratory should at a minimum have a member attend one of the hESC culture workshops (<http://stemcells.nih.gov/research/training/>).

Cryopreserved vials

1. Cryopreserved vials are prepared according to section 2.6 and can be thawed according to section 2.7. A minimum of 2 vials are supplied per shipment.

T25 flask

1. The flask will contain hESCs within 3 or 4 days of plating, and it is shipped overnight within the USA, in a warmed foam box.
2. When the flask arrives, it should be aspirated and the cells fed with 5 ml fresh medium. The hESC should be incubated at 37°C until ready for passaging.
3. To passage from the T25 flask, place the flask in a laminar flow cabinet and use a heated razor blade to cut a window in the top surface of the flask.
4. Use microdissection passaging to split the hESC to new dishes.

3.2 Media Recipes and Solutions

1. Complete MEF medium
DMEM with high glucose, 10% FBS, 2 mM glutamine, 0.1 mM non-essential amino acids, 50 units/ml penicillin and 50 µg/ml streptomycin.
2. 20% KSR hESC Medium.
DMEM:F12, 20% KSR, 4 ng/ml bFGF, 2 mM glutamine, 0.1 mM non-essential amino acids, 50 units/ml penicillin and 50 µg/ml streptomycin.
3. β-ME
Dilute β-Mercaptoethanol to a 1 M solution in water. Aliquot into small (10-50µl) aliquots and freeze at -20°C. Thaw a fresh vial each day and add to warmed medium at 1/10000 dilution.

4. Hypotonic Solution (0.075M KCl)

Add 5.6 gm of KCl to 1 L of distilled MQ water and stir until dissolved.

5. Fixative: Methanol:Acetic Acid 3:1. Use fresh.

6. Mitomycin C

Mitomycin C is highly toxic. Handle accordingly.

Dissolve 2 mg Mitomycin C in 200 ml complete MEF medium in a vial to generate a 10 µg/ml working stock. Store protected from light at 4°C, for up to 6 weeks or frozen at -20°C for longer storage. After inactivating MEFs, collect the used Mitomycin C solution add 15 ml bleach per 500 ml to neutralize it.

3.3 Tissue Culture Flasks and Plates

	Distributor	Cat. Numbers
35 mm Tissue Culture plates	Falcon	353001
60 mm Tissue Culture plates	Falcon	351007
100 mm Tissue Culture Plates	Falcon	353003
T25	Falcon	353009
T75	Falcon	353136
T175	Falcon	353112
24 well	Corning	2526
6 Well	Corning	3516
4 Well Permax Chamber Slides	Nalgene Nunc	177 437

3.4 Reagents and Suppliers

	Distributor	Cat. Numbers	Stock Solution
Distilled Water	Gibco	15230-162	1x
PBS	Mediatech	21-031-CM	1x
Ca ²⁺ /Mg ²⁺ -free PBS	Mediatech	21-031-CM	1x
DMEM/F12	Gibco/Invitrogen	11320-033	1:1 (1x)
DMEM High Glucose	Hyclone	SH30081.01	1x
DMEM/F12	Gibco/Invitrogen	11320-033	1:1 (1x)
Foetal Bovine Serum	Hyclone	SH30070.03	1x
L-Glutamine	Gibco/Invitrogen	25030-081	200 mM (100x)
bFGF	Sigma	F0291	25 µg/ml
L-Glutamine	Gibco/Invitrogen	25030-081	200 mM (100x)
bFGF	Sigma	F0291	25 µg/ml
β -mercaptoethanol	Sigma	M7522	1 M
0.25% Trypsin/EDTA	Gibco/Invitrogen	25200-056	0.25%
0.05% trypsin/EDTA	Gibco/Invitrogen	25300-054	0.05%
0.25% Trypsin (EDTA free)	Gibco/Invitrogen	15050-065	0.25% (5x)
Laminin	Sigma	L20-20	1 mg/ml
KaryoMAX®Colcemid Solution	Gibco/Invitrogen	15212-012	10 µg/ml (500x)
Gelatin	Sigma	G1393	2%
Potassium Chloride	Fisher Biotech	BP366	Salt
Methyl alcohol 99.93% ACS HPLC grade	Sigma - Aldrich	270474	1x
Glacial Acetic Acid	Fisher Scientific	A38 ST -212	1x
Trypan Blue	Gibco/Invitrogen	15250-061	2x
Mitomycin C	Sigma	M4287	
DMSO	Sigma	D2650	
Cryo 1°C Freezing Container	NALGENE	5100-0001	

3.5 Additional Resources

These additional publications contain variations of these methods and are a useful resource:

- “Protocols for the Maintenance of Human Embryonic Stem Cells in Feeder Free Conditions”. **Geron Corporation**.
<http://www.geron.com/>
- “Human Embryonic Stem (HUES) Cell Collection, Instruction Manual Version 1.0”. **Douglas A. Melton**
<http://mcb.harvard.edu/melton/HuES/>
- **WiCell Research Institute**, Technical Information
<http://www.wicell.org/forresearchers/index.jsp?catid=12&subcatid=20>

3.6 Appendix

HLA Isotyping

	A	Cw	B	Bw	DRB1	DRB3	DRB4	DRB5	DQB1
BG01	0201	0401	3503	6	301	xx			0201
	0301	0701	4901	4	405		xx		0302
BG02	2402	0304	3503	6	1502			xx	0601
	6801	0401	3511	6	1602				0301
BG03	0201	05AC	4402	4	0401		xx		0301
	3201								0302