Human Pluripotent Stem Cell Protocols

This package provides a variety of protocols for both the routine culturing and maintenance of human pluripotent stem cells including embryonic stem (ES) cells and induced pluripotent stem cells (iPSC). Culture techniques for both, feeder and feeder-free conditions are provided. In addition, protocols for feeder depletion, gene targeting (through electroporation) and differentiation of hESCs are included.

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Feeder culture (using hESC media on inactivated feeder-plates)

Mouse Embryonic Fibroblasts:

Human ES cells are routinely co-cultured on a layer of inactive mouse embryonic fibroblasts (MEFs or “feeders”) derived from E12.5 day embryos. This feeder layer secretes a variety of factors that maintain the pluripotency of hES cells. Different feeder lines, such as DR4, CF1 or Swiss Websters, can be used following inactivation by either mitomycin C (MMC) or gamma irradiation. The density of this feeder layer is important and MEFs should be plated with the following densities:

<table>
<thead>
<tr>
<th>Plate</th>
<th>Total Culture Area</th>
<th>M12.5 feeders (cells per plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 well plate</td>
<td>7.60</td>
<td>2.5 x 10^5</td>
</tr>
<tr>
<td>6 well plate</td>
<td>57.60</td>
<td>1.4 x 10^6</td>
</tr>
<tr>
<td>12 well plate</td>
<td>45.60</td>
<td>1.4 x 10^6</td>
</tr>
<tr>
<td>24 well plate</td>
<td>48.00</td>
<td>1.4 x 10^6</td>
</tr>
<tr>
<td>48 well plate</td>
<td>36.00</td>
<td>1.0 x 10^6</td>
</tr>
<tr>
<td>96 well plate</td>
<td>30.72</td>
<td>9.0 x 10^5</td>
</tr>
<tr>
<td>15 cm plate</td>
<td>170.10</td>
<td>4.25 x 10^6</td>
</tr>
<tr>
<td>10 cm plate</td>
<td>56.70</td>
<td>1.6 x 10^6</td>
</tr>
<tr>
<td>6 cm plate</td>
<td>19.5</td>
<td>5.0 x 10^5</td>
</tr>
</tbody>
</table>

Human ES colonies attach to the surface of the plate and push apart the feeder layer as they grow. For this reason, if the feeder density is too high, hES cell colonies will not be able to push the feeders apart to sit down in a flat manner and will begin to differentiate by growing on top of the bordering feeder layer. Feeders should be prepared fresh whenever possible however frozen stocks can be used if necessary.

MEFs media:

*Media can be prepared and used for up to 1 month.*

- DMEM: 500 mL
- FBS: 90 mL
- GlutaMax: 6 mL
- Pen/Strep: 3 mL
Human Embryonic Stem Cell (hESC) Culture Media:

For routine culture of CA1 and CA2 hES cells (on feeders):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knockout DMEM</td>
<td>250 mL</td>
</tr>
<tr>
<td>Knockout Serum Replacement (KOSR)</td>
<td>37.5 mL</td>
</tr>
<tr>
<td>GlutaMax (100X)</td>
<td>3 mL</td>
</tr>
<tr>
<td>Non-Essential Amino Acids (100X)</td>
<td>3 mL</td>
</tr>
<tr>
<td>Pen/Strep (100X)</td>
<td>3 mL (optional)</td>
</tr>
<tr>
<td>B-mercaptoethanol</td>
<td>300 µL (final concentration 0.5mM)</td>
</tr>
<tr>
<td>Basic human FGF (10 µg/mL stock)</td>
<td>8 ng/mL</td>
</tr>
</tbody>
</table>

For routine culture of Hes2, H9 and ESO hES cells (on feeders):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F12</td>
<td>200 mL</td>
</tr>
<tr>
<td>Knockout Serum Replacement (KOSR)</td>
<td>50 mL</td>
</tr>
<tr>
<td>Pen/Strep (100X)</td>
<td>2.5 mL (optional)</td>
</tr>
<tr>
<td>Non-Essential Amino Acids (100X)</td>
<td>2.5mL</td>
</tr>
<tr>
<td>GlutaMax (100X)</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>B-mercaptoethanol</td>
<td>2.5 mL (final concentration 0.1 mM)</td>
</tr>
<tr>
<td>Basic human FGF (10 µg/mL stock)</td>
<td>4 ng/mL</td>
</tr>
</tbody>
</table>

Base media minus bFGF can be prepared and used for up to 1 month. bFGF should be supplemented to media for up to 1 week maximum. Additional bFGF (up to 20 ng/mL) can be added to media for cells that exhibit high levels of spontaneous differentiation.

Passaging of hES cells (on feeders):

Human ES cells require routine passing to maintain pluripotency and to increase cell numbers. Cells should be passaged every 5-7 days either when colonies appear large and are visible by eye or when the density of colonies reaches approximately 80%. Cells should be monitored daily for cell morphology and differentiated colonies removed by cell picking as described below.

Generally, cells can be passed by one of two methods:

A) Enzymatic passage by collagenase (pipette method)
B) Enzymatic passage by collagenase (scraper method)

Human ES cells are generally passaged in small clumps (clump passaging). When colonies are large enough, they are manually dissociated into clumps of approximately 15-20 cells and plated onto a new feeder plate. It is important to obtain clumps of consistent size for ease of maintenance following passage. Here, we describe manual dissociation through collagenase using two different methods of dissociation.
**A) Enzymatic passage by collagenase (pipette method):**

1) Dilute collagenase IV to 1 mg/mL (add 100 µL of 10 mg/mL stock of collagenase IV to 900 µL of pre-warmed DMEM/F12).
2) Remove culture media from well and add 1 mL of diluted collagenase per well.
3) Incubate at 37°C for ~ 5 minutes (until the edges of the colonies start to lift off).
4) Remove diluted collagenase and wash the well once with DMEM/F12 to remove residual collagenase (note: collagenase cannot be inactivated and must be removed/diluted from the culture through 1-2 washes).
5) Add 1 mL of pre-warmed hESC media and scrape well with pipette to dislodge colonies (scrape a grid-like pattern against the plate to break apart colonies into small clumps but not single cells):

![Diagram](image)

6) Collect the clumps (now in suspension) and pipette up and down 2-5 times with a P1000 pipette until clumps consist of approximately 15-20 cells. Cells should be monitored under the microscope throughout this process to ensure the correct clump size is obtained.
7) Transfer cell suspension to a 15 mL conical tube and add media to make correct split ratio, typically cells are split at a 1:4-1:8 ratio.
8) Prepare a new plate by removing media from pre-cultured feeders and adding 1 mL of hESC media per well. Feeders should be plated at least 1 day before use.
9) Add appropriate volume of cell suspension per well.
10) Cells should be fed daily with 1.5 mL hESC media per well until splitting is required.

**B) Enzymatic passage by collagenase (scraper method):**

1) Dilute collagenase IV to 1 mg/mL (add 100 µL of 10 mg/mL stock of collagenase IV to 900 µL of pre-warmed DMEM/F12).
2) Remove culture media from well and add 1 mL of diluted collagenase per well.
3) Incubate at 37°C for ~ 5 minutes (until the edges of the colonies start to lift off).
4) Remove diluted collagenase and wash the well 1-2 times with DMEM/F12.
5) Add 1 mL of pre-warmed hESC media to the well.
6) Using a cell scraper, section the well into a small, “grid-like” pattern and scrape well to dislodge colonies (make into small clump but not single celled).
7) Collect the clumps (now in suspension) and pipette up and down 2-5 times within the well with a P1000 pipette until clumps consist of approximately 15-20 cells. Cells should be monitored under the microscope throughout this process to ensure the correct clump size is obtained.

8) Transfer cell suspension to a 15 mL conical tube and add media to make correct split ratio.

9) Prepare the new plate by removing media from pre-cultured feeders and adding 1 mL of hESC media per well.

10) Add 0.5 mL of cell suspension per well.

11) Cells should be fed daily with 1.5 mL hESC media per well until splitting is required.

NOTE:

- Some residual serum from the MEFs media left within the well will help with the attachment of colonies. However, if differentiation is a problem, feeders can be washed once with PBS before adding culture media.
- Human ES cells are density dependent. Therefore, if density is low, cells can be passed back down to a 12-well plate (from a 6-well plate) to allow rescue. Note cells at low density will grow at a reduced rate.
- All ES cultures are routinely grown in a humidified incubator at 37°C with 5% CO₂.
- Due to the high metabolism of hES cells, media must be changed daily to avoid toxic waste build-up and replenish unstable media components.
- Passaging with other enzymatic reagents such as accutase, gentle cell dissociation buffer, TrypLE etc. can be used in a similar manner, follow manufacturer’s direction and scrape/pipette cells to achieve cell clumps.
- If cells are over-pipetted and cells appear to be single celled addition of ROCK inhibitor can prevent complete cell death following single ceiling.
- DMEM/F12 or KnockOut DMEM base medias can be used for intermediate washing steps to save hESC media or mTeSR.
Feeder-free culture (using mTeSR media on Matrigel-coated plates):

Human ES cells can be cultured in feeder-free conditions with the use of mTeSR™1 (Stem Cell Technologies) on either Vitronectin or Matrigel (Corning) as the surface coating matrix.

For detailed reference, see:
http://www.stemcell.com/~media/Technical%20Resources/B/C/A/2/B/29106MAN.pdf?la=en

Preparation of Matrigel-coated plates:

A) Reconstitution & preparing 1:1 stock aliquots:
   1) Thaw frozen bottles of Matrigel on ice overnight in fridge (4°C). Store sterile 5 mL pipettes and microcentrifuge tubes in the freezer overnight to cool before aliquotting.
   2) The next day, make a 50% working stock by adding an equal volume of IMDM+P/S to each bottle (10 mL IMDM+P/S to 10 mL thawed Matrigel).
   3) Resuspend gently with a pre-chilled 5 mL pipette, careful not to warm bottle or pipette. Keep on ice.
   4) Transfer 0.5 mL into pre-chilled and pre-labeled 1 mL sterile microcentrifuge tubes on ice. Work quickly to avoid warming of pipette or Matrigel.
   5) Quickly snap close tubes, careful not to warm tubes with hand and transfer aliquots to -20°C.

B) Coating plates with Matrigel for use:
   1) Thaw a 1:1 frozen aliquot (from part A, see above) on ice or in the fridge overnight.
   2) Dilute 20 μL of thawed Matrigel aliquot in 1 mL pre-chilled KO DMEM or DMEM/F12; gently mix (this will coat 1 well of a 6-well plate—dilute appropriate stock for desired number of plates/wells).
   3) Transfer 1 mL to 1 well of a 6-well plate and ensure that the entire surface of the well is coated.
   4) Store at 4°C overnight to allow Matrigel to adhere and coat plate. Matrigel plates can be stored at 4°C for up to one week.
   5) Warm plates at room temperature or 37°C for up to an hour before use. Remove residual Matrigel and immediately add pre-warmed mTeSR media for routine use.

NOTE:
- Matrigel must be kept cool at all times when preparing aliquots or dilutions for coating plates to prevent it from gelling.
- Do not allow Matrigel to evaporate off of the well, sufficient liquid should remain within the well at all times prior to use.
- Different Matrigel dilutions can be used depending on the assay required.
Cells should be monitored daily for cell morphology and differentiated colonies removed by cell picking as described below.

**Human ESC media for feeder-free conditions (mTeSR® - Stem Cell Technologies)**

1) Thaw 5X supplement right before use at room temperature. Warm mTeSR basal media at room temperature
2) Pour 5X supplement into basal media bottle and mix thoroughly.
3) Store at 4°C for up to two weeks. Media may be aliquoted and frozen at -20°C and thawed as needed.

**NOTE:**
- Do not warm mTeSR at 37°C; always warm at room temperature (15-25°C) and refrigerate when not using.

**Passaging hESCs on Matrigel:**

Human ES cells can be passaged using several methods. Three commonly used methods are explained here:

A) **Enzymatic passage by collagenase (pipette method) – same as above**
B) **Enzymatic passage by collagenase (scraper method) – same as above**
C) **Non-enzymatic passage by ReLeSR™** (Stem Cell Technologies)

Human ESCs can be passaged using the same collagenase methods as mentioned above for feeder culture (using mTeSR instead of hESC media and Matrigel plates instead of feeder plates). Either the pipette or scraper method can be used to dissociate colonies, similar to passage on feeders.

C) **Non-enzymatic passage by ReLeSR™** (Stem Cell Technologies)

1) Aspirate media from well. Wash well once with PBS (-/ Ca2+ and Mg2+).
2) Add 1 mL of ReLeSR™ to the well and remove within one minute, so that colonies are exposed to only a thin layer of liquid.
3) Incubate at 37°C for 5-7 minutes.
4) Add 1 mL of KO DMEM or DMEM/F12 or mTeSR™1.
5) Gently tap the side of the plate to dislodge pluripotent colonies from the plate (differentiated areas will remain attached to the plate).
6) Gently collect cell suspension and transfer cell suspension to a sterile 15 mL conical tube and pipette up and down 3-5 times to break down clumps to an appropriate size (~20 cells per colony). Avoid single-cell suspension.
7) Centrifuge tube with cells for 3-5 minutes at 1100 rpm.
8) Resuspend cell pellet in mTeSR™1 to an appropriate volume to split 1:5 to 1:8 (ideally consistently 1:6 each passage) into a new plate.
9) Place plate in 37°C—move plate gently back and forth and side-to-side to distribute colonies evenly throughout the plate (avoid circular motions as this will pool colonies in the middle of the well).
10) Change media everyday with pre-warmed mTeSR™1 with at least 1.5 mL per well. Cells should be split every 5-7 days.

NOTE:

- All ES cultures are routinely grown in a humidified incubator at 37°C with 5% CO₂.
- Due to the high metabolism of hES cells, media must be changed daily to avoid toxic waste build-up and replenish unstable media components.
- Passaging with other enzymatic reagents such as accutase, gentle cell dissociation buffer, TrypLE etc. can be used in a similar manner, follow manufacturer's direction and scrape/pipette cells to achieve cell clumps.
- If cells are over-pipetted and cells appear to be single celled, addition of ROCK inhibitor can prevent complete cell death following single celling.
- DMEM/F12 or KnockOut DMEM base medias can be used for intermediate washing steps to save hESC media or mTeSR.

Taking hESCs feeder-free:

Prior to setting up differentiations, cultures must be in feeder-free conditions. This can be done in several ways. Two methods are described here:

A) Feeder “peel” (light trypsinization and “peeling” off feeder layer)
   - This method takes advantage of the fact that feeders are attached less tightly to the plate than ESCs are. Thus, with light trypsinization, the feeders can be peeled off while ESCs stay attached.
B) Feeder dilution (three passages on Matrigel)
   - Since feeders are inactivated, they can be diluted with serial passage.

A) Feeder “peel” method:
   1) Aspirate to remove old media and wash well with 1-2 mL pre-warmed PBS (-/- Mg/Cl).
   2) Dilute pre-warmed 0.25% Trypsin-EDTA in PBS (-/-) 1:4 to 0.05% Trypsin.
   3) Wash well once with diluted trypsin then add 1 mL to well and incubate at 37°C for 1-3 minutes. (Continuous checking of the cells during this incubation is important to ensure hES cells to not detach from the plate)
   4) Add 1 mL MEFs media to inactivate trypsin.
   5) Carefully aspirate or “peel” off the feeder layer using vacuum suction. It is best to start near the edges of the well and start sucking the feeder layer off, trying to avoid removing hES cells.
6) Add KO DMEM or DMEM F/12 to wash and dissociate colonies into appropriate sized clumps using scraper/pipette methods as mentioned above.  
7) Collect clump suspension and centrifuge for 3-5 minutes at 1000 rpm.  
8) Resuspend in mTeSR or KOSR-hESC media (see Adaption vs no-adaption notes) and plate onto a pre-warmed Matrigel-coated plate:  
   a) **Adaption period**: plate cells in KOSR-hESC on the day of passage, but switch media to mTeSR on subsequent days or passages. The adaption period will differ between cell lines and cells should be monitored closely for morphological changes. Switching media to mTESR to quickly can result in complete cell death and/or differentiation of cells.  
   b) **No adaption period**: plate cells in directly in mTeSR and feed with mTeSR on following days.  
9) Incubate at 37°C and maintain as described above.  

**B) Feeder dilution method:**  
1) Passage well/plate using a collagenase method as described above (either scraper or pipette).  
2) Plate final cell suspension on to Matrigel-coated plates in either mTeSR or KOSR-hESC media:  
   a) **Adaption period**: plate cells in KOSR-hESC on the day of passage, but switch media to mTeSR on subsequent days or passages. The adaption period will differ between cell lines, cells should be monitored closely for morphological changes. Switching media to mTESR to quickly can result in complete cell death and/or differentiation of cells.  
   b) **No adaption period**: plate cells in directly in mTeSR and feed with mTeSR on following days.  

**NOTE:**  
- Some cell lines prefer adaption period, others do not. When working with a new line, it is recommended to test both methods to see which conditions help hESC adapt more easily to feeder-free conditions.  
- Not all cell lines have the potential to go feeder free.  
- Cells require 3 passages (or 2 passages if feeder peeling is used) in order to be considered feeder free and can be used for differentiation experiments.  
- Split ratios should be adjusted during the initial feeder free passage as many cells will die or not attach to the new matrix.  

**Manual Removal of Differentiated Colonies**  
Cultures typically require regular maintenance between or prior to passaging by manual removal of regions of differentiation. If the culture has only a few regions of differentiation, the differentiated areas can be marked and removed from culture. If majority of the culture is differentiated, then the pluripotent colonies/regions can be “rescued” and replated on a fresh plate and expanded.
1) Assess the culture dish and mark regions to be removed under the plate with a marker.
2) Work in a dissecting microscope in a picking hood. Using a P20 pipette, carefully cut around the colonies to be removed. If on feeders, scrape around the circumference of the colony to be removed, and then scrape the rest of the colony to detach it from the plate surface.
3) If “rescuing” a pluripotent colony from culture, scrape (in a similar manner described above) the selected region to be rescued from culture off the plate. If needed, dissociate colony into appropriate sized clumps in a small droplet of collagenase or ReLeSR and wash in drops of DMEM/F12 or KO DMEM. Pipette up and down to achieve desired size and transfer cells to the new plate.

**Freezing/thawing of hES cells:**

For long-term storage, cells should be maintained in liquid nitrogen.

**Thawing:**

1) Remove vial of cells from liquid nitrogen.
2) Thaw cells quickly by warming vial between hands until only small ice crystals remain.
3) Collect thawed cells into a conical tube containing 5 mL MEFs media (to dilute the DMSO in the freezing media).
4) Centrifuge cells for 3 minutes at 1000 rpm.
5) Prepare plate by adding 1.5 mL of ES media supplemented with 10 μM Y27632 (ROCK inhibitor) per well. Cells can be thawed to the same matrix as they were frozen from, however thawing directly to feeders is advised for most cells as there is better recovery.
6) Resuspend cell pellet in 0.5 mL hESC media containing 10 μM ROCK inhibitor.
7) Plate ES cells into 1 well of a 6 well plate and place in incubator at 37°C.
8) Cells should be fed daily with 1.5 mL hESC media per well. Colonies will appear within 7-14 days following thaw.

**Freezing:**

1) Harvest cells normally as described above for passaging.
2) Centrifuge cells for 3 minutes at 1000 rpm to obtain a cell pellet.
3) Resuspend cell pellet into freezing media (make fresh prior to freezing and always keep cool on ice), 1 mL per pre-labeled vial:

<table>
<thead>
<tr>
<th>Medium</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>10%</td>
</tr>
<tr>
<td>FBS</td>
<td>40%</td>
</tr>
<tr>
<td>MEFs media</td>
<td>50%</td>
</tr>
</tbody>
</table>

*Each vial of cells should be frozen from 1 confluent well of a 6-well plate.*
4) Keep vials of cells on ice (to allow for gradual freeze).
5) Transfer vials to Mr.Freezy (Nalgene) containers with isopropanol and transfer to -80°C freezer.
6) After 48 hours, cells can be transferred to liquid nitrogen for long term storage.

**NOTE:**
- Always pre-label vials with either printed labels or ethanol-proof markers prior to freezing.

**Gene targeting:**

Generation of genetically modified hES can be performed through a variety of techniques, described here is electroporation. Alternatively nucleofection can be used. These protocols can be used for cells on both MEFs or Matrigel and cells should be maintained in the same culture conditions as the parental lines were maintained.

**Electroporation:**

1) Pre-treat hES cells with hESC media with 10 μM Y27632 for at least 2 hours prior to electroporation to prevent cell death associated with dissociation of cell colonies.
2) Aspirate media from well. Wash well once with PBS (-/- Ca2+ and Mg2+).
3) Add 0.5 mL of 0.25% trypsin per well and incubate for 5 minutes. Add equal volume of MEFs media and dissociate into a single-cell suspension using a 5 mL pipette or P1000. Three confluent wells should be used per electroporation.
4) Transfer cells to a 15 mL conical tube and cells centrifuge for 3 minutes at 1100 rpm.
5) Prepare electroporation cuvette by adding DNA (15-20 μg per electroporation depending on construct size) to cuvette and placing on ice.
6) Gently resuspend ES cells in 400 μL pre-cooled electroporation buffer and add ES cell suspension to electroporation cuvette (careful not to exceed volume over the metal part of cuvette—cells over the metal part will not receive current for electroporation).
7) Place cuvette into electroporator pod so that the metal sides are in contact with metal sides on the cuvette. Pulse cells at 170V, 1050 μF.
8) Immediately transfer cells into 15 mL conical tube containing 9 mL hES media with ROCK inhibitor using a glass Pasteur pipette (be extremely gentle with cells from this point on – avoid trituration).
9) Plate cell suspension onto a 10 cm dish of MEFs or Matrigel, prepared as outlined above.
10) After 24 hours, change media to regular hESC media (with no ROCKi).
11) After 48 hours, start drug selection and feed daily.
12) Colonies of drug resistant cells should become apparent after approximately one week, and colonies should be large enough to pick following 10-12 days (at this point colonies should be visible when observing the 10 cm plate from below).
Note:

- Kill curves should be performed on all cell lines prior to electroporation to determine optimal drug concentration required.
- High quality DNA is required for electroporation, DNA should be prepared using an endotoxin free kit.
- For higher rates of integration DNA should be linearized prior to electroporation.
Gene Targeting Considerations:

1. The length of homology arms greatly influences the efficiency of homologous recombination. We recommend using homology arms between 3 and 5kb each, with the 3’ arm being at least 3kb. There may be a significant advantage in using substantially longer targeting arms; however, large constructs are often more difficult to manipulate. Total donor plasmid should be <25kb total.

2. Selection markers are essential to minimize background when performing gene targeting experiments. We recommend using the positive selection marker neomycin (neo). This cassette should be flanked using either the loxP or FRT system to allow for subsequent removal by Cre or FLPe recombinases. Removal of this selection cassette following line generation may be essential for proper expression of the targeting vector. Other selection markers such as puromycin and hygromycin can also be used for these experiments.

3. Negative selection cassettes such as thymidine kinase (TK) or diphtheria toxin A (DTA) can be employed to negatively select for non-homologous recombination and thus reduce the number of colonies screened following targeting.

4. Ideally, gene targeting vectors should be designed based on the genomic DNA that is isogenic to the ES cell line being utilized. Use of isogenic DNA can result in a higher targeting efficiency.

5. Targeted clones to identify positive hits will require screening by long range PCR. Proper placement and design of primers is essential for detection of these rare events. Positive controls through BAC recombineering can be generated to ensure proper PCR conditions can be determined prior to screening of clones.

6. Proper promoter selection is essential to many gene targeting experiments. ES cells have the ability to silence many common promoters such as CMV. Utilization of other promoters such as PGK will prevent silencing of vectors.
Picking hESC colonies:

The goal of picking colonies is to establish and expand distinct clonal populations containing the gene of interest. Care must be taken to avoid contamination between populations. Individual colonies will be picked and dissociated in drops of trypsin and then plated into separate wells of a 4 well dish of MEFs/Matrigel.

4) Prepare trypsin by placing individual drops (approximately 30 µL) onto the lid of a 6 cm dish (make sure drops do not touch each other).
5) Prepare a 1.5 mL Eppendorf tube of MEFs media (to stop trypsin)
6) Prepare the 4 well dishes containing hES cell media.
7) Remove media from 10 cm dish of targeted colonies and rinse once with PBS and replace with 9 mL PBS.
8) Work with either a dissecting microscope in a picking hood or an inverted microscope. Using a P20 pipette and a new tip for each separate colony, pick colonies by first scraping around the circumference of the colony to detach cells from feeders, and then gently scrape colony off from bottom of the dish. (If cells are on Matrigel you are not required to scrape around the circumference.)
9) Pick up cells using pipette and transfer into drop of trypsin, trying to minimize the volume of PBS being transferred along with the colony
10) Change tip, and continue picking colonies.
11) The individual colonies should not remain in the trypsin for longer than 2-4 minutes, so depending on your speed, pick either 4 or 8 colonies at a time
12) Once you have transferred 4-8 colonies, with a new tip and P200 set to 150 µL, aspirate approximately 30 µL MEFs media from Eppendorf tube and while looking under the microscope, pipette up and down in the first drop to dissociate the colony within into clumps 15-20 cells big. Transfer all liquid to first well of dish to plate. Continue until all colonies have been transferred to culture dishes.
13) Selection media can be added following 24 hours and cultures are maintained until wells are ready to be passaged.
14) From here, each confluent well can be passaged into 3 wells of a 12 well dish – 1 well for screening (Southern/qPCR) and 2 wells to be frozen down. If only 1 colony forms in 4 well dishes, cells can be trypsinized and plated into fresh well to let cells recover prior to expansion.
**Spontaneous Differentiation:**

This protocol is a generic differentiation, which will allow generation of all three germ layers. Cells require feeder depletion prior to starting differentiation.

1) Count cells from one well of 6 well plate as a representative number of cells/well. Collect cells from remainder of dish, and spin down into a pellet at 1000 rpm for 5 minutes.

2) Resuspend in differentiation media:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Concentration</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mL</td>
<td>DMEM/F12</td>
<td></td>
</tr>
<tr>
<td>0.25 mL</td>
<td>N2</td>
<td></td>
</tr>
<tr>
<td>0.5 mL</td>
<td>B27</td>
<td></td>
</tr>
<tr>
<td>50 μg/mL</td>
<td>ascorbic acid</td>
<td></td>
</tr>
<tr>
<td>2 mM</td>
<td>glutamax</td>
<td></td>
</tr>
<tr>
<td>50 μL</td>
<td>MTG</td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>Y-27632</td>
<td></td>
</tr>
</tbody>
</table>

**Monolayer differentiation:**

3) Add cells at ~50K, 150K, and 300K cells per well, with 1.5 mL differentiation media in each well on a regular plate. Different cell densities, as well as the addition of different growth factors/medias will direct cells toward a specific lineage and/or cell type.

**Embryoid Bodies (EBs):**

4) Pipette ~250K cells onto non-adherent plates for EB formation and add 2 mL differentiation media. Incubate at 37°C.

5) Harvest EBs at 6 days by trypsinizing for 5 minutes, adding media to neutralize reaction and pipetting EBs using an 18G needle and syringe to dissociate clumps. Plate cells on adherent plates and add 1.5 mL differentiation media. Note emergence of all three germ layers over the next few days.

**NOTE:**

- **EBs can also be made by Aggrewells to get more uniformly-sized EBs**
- **Addition of various growth factors (ex. BMP4, retinoic acid) at various stages of the differentiation can push cells towards a specific cell type as required.**