Madeline A. Lancaster

Culturing feeder-free human ES cells

Modified from WiCell protocols

**Materials**

* 6-well dishes from BD Falcon
* Low Growth Factor Matrigel: Corning #356230
* DMEM/F12: Invitrogen cat#11330-032
* mTESR1 media: Stem Cell Technologies # 05850
* Rock inhibitor Y27632, 5mg: VWR cat# 688000-5
  + Working solution prepared by reconstituting 5mg in 2.96 ml water
* 0.5M EDTA stock solution prepared in water
* Sterile PBS w/o Ca and MG
* ES-quality FBS: Tested batch from stem cell core
* DMSO
* mFresr1: Stem Cell Technologies # 05855
* Mr. Frosty

**Solutions**

EDTA working solution (0.5mM) – Store at 4C

* 50 ml PBS without Ca/Mg
* 50ul 0.5M EDTA stock solution

2X ES Freezing Media (10 ml) – Store at -20C up to 6 months

* 8 ml ES-quality FBS
* Filtered using 0.22 um filter
* 2 ml DMSO

Procedures

**Matrigel coating plates**

1. First prepare Matrigel aliquots:
   1. Calculate the volume of matrigel needed for one well of a 6-well plate (0.5 mg for 6 wells). Thaw Matrigel and freeze various aliquot sizes (for example: 3 wells, 6 wells, 8 wells). Store aliquots at -80.
   2. Be sure to use cold pipettes, tubes and tips.
2. Thaw Matrigel aliquot:
   1. Take aliquot directly from -80 and add 1 ml cold DMEM/F12. Pipette up and down, keeping the tube cold on ice, until Matrigel dissolves and can be pipetted into a larger 15 ml conical tube.
   2. Add additional DMEM/F12 to make a final volume so that you have 1 ml for each well (for example: 6 well aliquot should be dissolved in 6 ml DMEM/F12). Mix well.
   3. Pipette 1 well per well.
3. Place in the incubator for 1 hour before plating cells or store wells at 4C for up to 2 weeks wrapped in parafilm.

**Thawing human feeder-free ES cells**

1. Prewarm 13ml mTesr1 media.
2. Remove Matrigel solution from coated warm well.
3. Add 1.5 ml mTesr1 media containing Rock inhibitor to the well.
   1. Add Rock inhibitor to a final of 2ul per ml media (so 4ul per well)
4. Partially thaw hES vial of cells until sliver of ice remains
5. Transfer ES cells from vial to a 50ml conical tube.
6. Add 1 ml mTesr1 media dropwise, swirling between each drop until 9ml mTesr1 has been added.
7. Spin at 200xg for 5 min
8. Resuspend cell pellet in 0.5ml mTesr1 gently and pipette into prepared well.
9. Gently shake the plate back and forth and side to side to evenly distribute cells in the well. Avoid swirling.

**Routine maintenance and feeding of ES cells**

1. Feed cells daily by replacing media with 2 ml fresh mTesr1 media.
   1. When cells are more confluent they should be fed with 3ml media.
   2. Double feeding with 4 ml media can be performed right after splitting and the cells can then wait 2 days to be fed again.
2. Routine tests for mycoplasma should be performed using a combination of PCR and MycoAlert assays. This should be performed at least every month.

**Routine passaging of ES cells**

1. Once colonies are ~80% confluent or the colony sizes have reached maximal size, it’s time to split (usually every 5-7 days).
2. Prepare new Matrigel plates as above or warm pre-prepared Matrigel coated dishes in the incubator.
3. Suck off Matrigel solution and replace with 1.5 ml mTesr1 media per well.
4. Before splitting, remove differentiated cells by first marking on a tissue culture microscope, then use vacuum aspiration or pipet to scrape differentiated colonies off.
5. Wash cells quickly 2X with 1-2 ml warm EDTA working solution.
6. Add 600ul EDTA working solution and place back in incubator for 4-5 min depending on how confluent cells are.
7. Remove EDTA working solution and spray cells off plate with 0.5ml mTesr1 media at a time. Do not pipette this off the plate immediately, simply continue adding and spraying to reach a final volume that is appropriate for splitting:
   1. Reach a final volume 0.5ml per new well.
   2. For example: if splitting at 1:6, spray colonies 6 times with 0.5 ml mTesr1 media to reach a final volume 3 ml. If the final volume is too large to fit in the well, reach a final volume of 3ml and transfer this gently with a 5 ml Pasteur pipette to a 15 ml conical. Add additional media needed.
   3. Generally minimize pipetting!
8. Transfer 0.5 ml cells per new well and place back in the incubator, being careful to distribute cells evenly.
9. Typically, splitting 1:8 is adequate but can range from 1:4 to 1:10.

**Freezing ES cells using 2X hES freezing media**

1. Prepare cells as described in steps 4-7 of routine passaging, but resuspend in 1ml mTesr1 media total per well (spray two times 0.5 ml) to freeze ½ well per vial. If extra pipetting is needed to spray off cells, do this but try to minimize.
2. Add 500ul 2X hES freezing media and quickly transfer to vial by pipetting very gently.
3. Transfer to Mr. Frosty and place in -80 C within 3 minutes.
4. 24-48 hours later, transfer to liquid N2.

**Freezing ES cells using mFresr**

1. Prepare cells as described in steps 4-7 of routine passaging, but resuspend in 1ml mFresr total per well (spray two times 0.5 ml) to freeze ½ well per vial. If extra pipetting is needed to spray off cells, do this but try to minimize.
2. Quickly transfer 1 ml cells in mFresr to vial by pipetting very gently.
3. Transfer to Mr. Frosty and place in -80 C within 3 minutes.
4. 24-48 hours later, transfer to liquid N2.