Pluripotent stem cell maintenance

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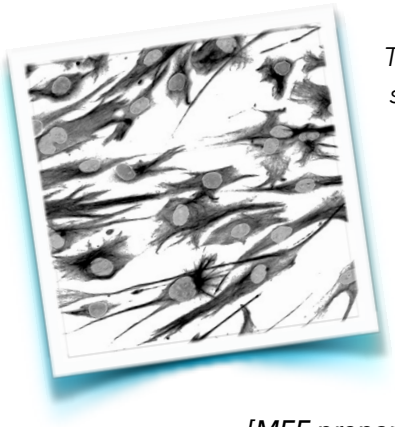

This protocol is a variation of the original published by Jamie Thomson in 1998. I still feel that it is the most robust, reproducible method to culture hPSCs. Care needs to be taken in the quality and density of the feeders. Quality should be assessed by plating feeders and assessing their health over time. Good cells should flatten out and look more dense over the course of a week. Bad cells will have lots of processes and look less dense over time. Test lots from reputable suppliers. These protocols work for hESCs and hiPSCs, although we've found that the addition of XAV939 (10 µM) helps keep many lines from differentiating, particularly hiPSCs.

[MEF preparation]

1) The day before passage, coat tissue-culture treated dishes with 0.1% gelatin/ PBS for at least 15 minutes.
2) Plate primary mouse embryonic fibroblasts in DMEM+10% FBS at a density of ~12,000-27,000 cells/cm2 (usually 12,500 for us depending on the lot). Manufacturers will often call for much higher densities but this causes premature differentiation of hPSCs (mouse ESCs require higher densities). Unfortunately, different batches of MEFs have different plating efficiencies, so each lot will vary in how many cells need to be plated to achieve a ~60-70% confluence. You should be able to see lots of bare plastic. Too many and too few feeders can cause differentiation.

[hESC thawing]

3) Preparing MEFs: Aspirate DMEM+10%FBS media used to plate MEFs, and wash with PBS to eliminate traces of serum.Aspirate PBS and add fresh hESC media. Place dish in the incubator to prewarm and correctly pH the media. *(This should preferably be done at least 30 minutes prior to thawing the tube.)*
4) Remove hPSCs from liquid nitrogen tank and move it rapidly to a 37˚C water bath. Move the tube quickly side to side to rapidly thaw. Do not submerge lid. Spray tube with ethanol and wipe off before bringing into the hood.
5) Move 1 ml cells into a 15 ml conical tube using a 2 ml pipette: do not use a p1000 or other pipette with a small orifice.The shear forces can kill the hPSCs. Slowly drop 4 ml of hESC media into the tube. Gently pipette once with 5 ml pipette after addition of media.
6) Spin at 200xg for 5 minutes. Aspirate media, then resuspend pellet in prewarmed media that was already on the MEF plate. Add media back to MEF plate.Take care not to break up the clumps.
7) Feed cells daily after thawing.You might not see anything for days or even weeks (up to 3 weeks in bad cases). Do not panic: continue to feed the dish everyday.
   For desperate situations,you can add 10 µMY-27632 to the media during the wake. Some believe that this can help suboptimal cultures survive the thaw, but it should not be necessary. Some labs also do not feed the day after waking - I do not believe that this matters, but I have not done carefully controlled tests to verify my opinion.What is clear is

that most of your success in freezing and thawing cells happens during freezing: you cannot revive dead cells no matter what technique you use after thawing.Always test a tube of cells after you freeze them to verify that they are viable after waking - until this point, you should not consider the line to be successfully frozen down.

[hESC passage]

8) Aspirate human ESC media and add 2 ml dispase (per 6 cm dish). Place in incubator (37˚C) for ~7 minutes. Check colonies on the microscope after 7 minutes. If the edges have started to detach, you are ready to proceed. If not, you can place them back in the incubator for another minute before checking again. I would not let them go more than 9 minutes if your enzyme is working correctly (use specified Dispase).
9) Aspire the dispase. Add 3 ml (per 6 cm dish) of hESC media and carefully aspirate to save a wash. Add another 2 ml hESC media and carefully pipette to dislodge the colonies. Do not worry about the MEFs: most of them will die during passage since they are mitotically inactive. Try to leave colonies as large as possible since you will perform further pipetting in the next step. Remove the colonies|media to a 15 ml conical containing 10 ml hESC media (to wash and help dilute out the dispase).
10) Spin at 200xg for 5 minutes. Aspirate media and resuspend in appropriate volume human ES media as above. Split accordingly. Depending on the growth characteristics of the cell line and plate density, cells can be split between ~1:3-1:12. I typically resuspend in 12 ml hESC media and move 1 ml to a new dish (1:12).
11) Feed cells daily. If you absolutely cannot feed your cells, we use StemBeads. They are a timed-released FGF2 preparation [see reagents].
12) Cells are ready to be split in 6-9 days. I usually split at day 7. It is best to split on a Friday if possible; cells are less metabolically active after the split, so they will exhaust media more slowly. We generally use StemBeads over the weekend but feed daily during the week.

[hESC freezing]

Keep everything cold after the cells have been detached. Make sure that your tubes have been prelabeled and precooled.

13) Treat as above for passage. Wash and detach the same way.
14) You might want to add an additional wash to eliminate Dispase since residual enzyme will inhibit attachment upon waking.
15) Aspirate, and gently resuspend colonies in hESC media. Use half of the total volume desired. I prefer to freeze one 6 cm dish in 1 ml (total) per vial (= 500 ul hESC media + 500 ul 2x freezing solution = 1 ml per vial/6 cm dish used)
16) Add 2x freezing media dropwise to colonies in hESC media slowly. Gently invert to mix, then dispense into the appropriate number of tubes.
17) Place in freezing container and store at -80 C overnight.The next day, transfer to liquid nitrogen. DO NOT LEAVE AT -80 FOR EXTENDED PERIODS OF TIME

***Notes**: if the colonies completely detach in dispase, I perform a second wash/spin step to remove the residual dispase. Dispase is not inactivated so it must be diluted out. Aspirating the dispase before detaching colonies acts as a first wash: if the colonies are floating in the dispase, then it is safest to perform the second wash.*

This protocol was largely derived from the Thomson Lab's protocol (http:// ink.primate.wisc.edu/ ~thomson/).Another wonderful resource is theWiCell Research Institute protocols (www.wicell.org).

I prefer to order MEFs from Applied Stem Cells.

Reagents

Human ES media

780 ml DMEM:F12
200 ml Knockout serum replacement (KSR)
10 ml MEM non-essential amino acids
1 ml 2-mercaptoethanol
5 ml L-glutamine
Sterile filter and cover bottle with aluminum foil. Add FGF2 to 6 ng/ml to an aliquot of media just before feeding cells (or, to the entire bottle if you use it within two weeks). If you warm it, also warm just an aliquot - not the whole bottle. KSR does not like to be warmed.

DMEM:F12

Life Technologies
11330-032

Knockout Serum Replacement

Life Technologies
10828-028
Read the instructions on how to thaw and aliquot - aliquot into 50 ml tubes and freeze once (-20˚C). It is wise to lot test this reagent too, particularly for differentiation protocols.

MEM Non-essential amino acids

Life Technologies
11140-050

2-mercaptoethanol

Life Technologies
21985-023

L-Glutamine (200mM)

Life Technologies

25030-081
L-glutamine does not go fully into solution. Keep it well suspended as you aliquot. Aliquot into 5 ml tubes and freeze once (-20˚C).

FGF2 (bFGF)
R&D Systems
233-FB-025
reconstitute (100µg/ml) in sterile PBS with 0.1% BSA, aliquot and store at -80˚C)

StemBeads *(time-release FGF2 for the weekends)*
StemBeads FGF2
StemCulture, LLC
[Use 7.5 ul/ml of hES media or as directed by manufacturer. Media can be left on feeder-based, pluripotent stem cell cultures for up to 4 days.]

DMEM+10% FBS for plating MEFs
DMEM 900 ml
FBS 100 ml
Sterile filter. No need to aluminum foil.

DMEM
Life Technologies
11965-118

Fetal bovine serum (for plating MEFs)
Life Technologies
26140-095

Dispase (Neural Protease)
Stem Cell Technologies
07913
100 ml
Thaw, mix, and store in working aliquots at -20˚C. Warm to 37˚C for 10 minutes prior to use. Do not leave at 37˚C for too long or you will inactivate the enzyme.
[Many of us now simply thaw the bottle and store it at 4˚C for at least 2 weeks. It does not lose appreciable activity.]

2x Freezing media
60% Hyclone FBS 20% DMSO
20% hESC media
[Use freshly made freezing media, and sterile filter before use. Keep freezing media and pre-labeled cryotubes on ice before use.]