



Induced Pluripotent Stem Cell Culture Protocol

HGPS and Control iPS Cell Culture Media Preparation

iPSC and ESCs need to be fed with mTeSR Plus from Stem Cell Technologies (cat# 5825). Please follow the supplier's recommendations for storage.

Preparing Matrigel Plates

Note: All steps involving matrigel should be done as quickly as possible and stay as cold as possible.

1. Thaw matrigel bottle at 4°C. Check the certificate of analysis on that lot to find its protein concentration.
2. Add enough cold DMEM/F12 media to the thawed matrigel to reach a final concentration of 5mg/mL.
3. Make 1mL aliquots of the prepared matrigel from step 2 in pre-chilled 15mL falcon tubes.
4. Freeze and store all aliquots at -20°C.
5. To make matrigel plates, remove one aliquot of matrigel (1mL) from -20°C and add 10mL of cold DMEM/F12. Mix well until pellet thaws (without creating bubbles, and always keep solution cold).
6. Transfer to a 50mL tube, then add 20mL of cold DMEM/F12 (1mL of matrigel aliquot is diluted in 30mL of DMEM/F12), mix well.
7. Plate (1mL/well for 6 well plate, 0.5mL/well for 12 well plate, 0.25mL/well for 24 well plate). Make sure that the solution is covering the entire surface area by shaking the plate gently. Do not re-freeze any leftover matrigel.
8. If using the plate immediately, let the plate sit at room temperature for 1 hour (or 30 minutes at 37°C), observe matrigel under the microscope. Matrigel should be well dispersed and not "clumpy".
9. If using plates at another time, wrap the edge of the plate with parafilm and store at 4°C for up to 2 weeks.

Matrigel – BD/Fisher, cat#CB-40230

DMEM/F12 – Life Technologies, cat#11330-057

Thawing ES or iPS cells (per cryo-vial)

1. Take a matrigel plate out of 4°C and warm at room temperature for one hour, or make a fresh matrigel plate (see preparing matrigel plate protocol).
2. Warm 4mL of mTeSR Plus in a 15mL falcon tube.
3. Remove cells from the liquid nitrogen tank and swirl in a 37°C bath until only a small ice chunk is left. Vial should thaw in 1-2 minutes. This step must be done quickly.
4. Ethanol cell tube and falcon media tube and place in the hood.
5. Use a 1mL wide mouth tip to slowly add cells to 4mL of pre-warmed media (avoid mixing cell suspension).
6. Spin at 130 rcf for 5 minutes.
7. Remove supernatant.
8. Add 2mL of PSC media, and with a wide mouth tip break up clumps gently. Transfer media to one well of a 6 well plate add 2uL of ROCK inhibitor (Y27632, final concentration of 10uM). Plate into one matrigel coated well (of a 6 well plate).
9. Rock cells gently to evenly distribute cells, and place in a hypoxic incubator (5%O₂, 10% CO₂). Avoid plate disturbance for 24 hours post seeding. **NOTE:** It is very important to avoid excessive breaking down of clumps or aggressive pipetting. This can significantly reduce survival rate. The cells should remain in chunks of 100-300 cells big at the time of seeding. While being gentle, try working quickly once cells are thawed to minimize time they are in contact with cryoprotectant.
10. Remove media after 24 hours and add 2mL of PSC media (for a 6 well, 1mL for 12 well and 0.5mL for 24 well plate). See Harvesting and Caring for hESC/iPSC protocol.

PSC media

mTeSR Plus from Stem Cell Technologies (cat# 5825). Please follow the supplier's recommendations for storage.

What is ROCK Inhibitor Y27632?

ROCK Inhibitor Y27632 is a selective inhibitor of the Rho associated kinase p160 ROCK. Treatment with ROCK Inhibitor Y27632 prevents dissociation induced apoptosis of human embryonic stem cells (hESC) and human induced pluripotent stem cells (iPSC), increasing the survival rate and maintaining pluripotency during subcultivation and thawing of hESCs and hiPSCs. ROCK Inhibitor Y27632 also has been shown to enhance the survival rate of stem cells during cryopreservation. Note that Rock inhibitor

aliquots are sensitive to light and repetitive freeze thaw cycles. Make sure to use these aliquots within the supplier's recommended shelf life.

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Harvesting and Caring for hESC/ iPSC

1. The day after cells were thawed take a look at them under the microscope to determine survival rate. NOTE: it is normal to observe a high number of unattached cells. As long as some cells are attached, colonies can arise from them within 3 – 7 days.
2. Remove media from the wells and pipette 2 mL (for a 6 well, 1mL for 12 well and 0.5mL for 24 wells plate) of fresh and warm PSC media per well. Return plate to incubator.
3. Cells are fed until 60-70% confluent (follow supplier recommendations).
4. As of day 2, cells should be observed and cleaned of any differentiated cells that might be growing.
5. To clean cells, use the picking hood and scrape off differentiated cells with a pipette tip.
6. Once cells are cleaned, change media as done in the above steps.

(See Freezing hESC/iPSC or Passing hESC/iPSC)

NOTE:

PSC media was determined to be more efficient in a hypoxic environment. We have also observed that less differentiation occurs when cells are grown in hypoxic incubators versus normoxic. Lastly, seeded cells have a better survival rate when using a hypoxic incubator.

Normoxic: 37°C, 21% O₂, 5% CO₂

Hypoxic: 37°C, 5%O₂, 10% CO₂

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Passing hESC/iPSC

1. Add 2mL of PSC media to a 6 well matrigel coated plate and set aside.
2. Take the plate to be passaged and remove the media from the well and wash once with 1mL of PBS(-/-).
3. Add 1mL of the EDTA solution to the well and leave for 3-4 minutes at room temperature. Don't move the plate around as cells can start detaching.
4. Remove EDTA solution and add 1mL of PSC media. Do not leave EDTA on the cells for more than 4 minutes as this will cause the cells to lift off.
5. Scrape cells using a cell scraper and divide cells amongst the 6 wells of your plate containing PSC media. Avoid excessive breaking up of the colony pieces,

and try to be gentle with scraping. Try to keep cells in large chunks. Use a wide mouth pipette tip to break up clumps if needed. Excessive breaking up of cells may cause cell death or excessive spontaneous differentiation following passaging.

6. Incubate at 37°C after evenly distributing cells in each well (8 figure or L shape shaking). Avoid plate disturbance for 24 hours post passaging.

NOTE: Once the cells have been scraped you want to transfer them to the new plate as soon as possible because the cells will reattach quickly (within 5 minutes).

If EDTA is left on the cells for more than 4 minutes, the cells can start to detach. If this happens, simply collect the cells in a 15mL falcon with 4mL of PSC media. Spin cells at 130 rcf for 5 minutes. Resuspend the pellet with 1mL of media and divide evenly among a 6 well matrigel coated plate (160uL per well).

EDTA solution: Add 500uL of 0.5M EDTA (pH 8.0) into 500mL of DPBS (-/-). Add 0.9g of NaCl. Filter the solution to sterilize and store it at 4°C for up to 6 months.

From the paper:

Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions

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Freezing hESC/iPSC

1. Turn on Bio-Cool (Controlled Rate Freezer) and adjust temperature to -7°C.
2. Remove cells from the incubator and observe confluency and morphology under the microscope.
3. If the wells are 70% confluent, remove old media and wash once with PBS(-/-) then add 1 mL of EDTA solution (see passing with EDTA solution) per well.
4. Incubate at room temperature for 3-4 minutes.
5. Aspirate the EDTA solution and add 1 mL of cold mFreSR media (cat#05855, Stem Cell Technologies).
6. Use a cell scraper to lift the cells gently. Keep cells in large chunks as much as possible and avoid pipetting up and down.
7. Transfer cells/mFreSR to a cryotube using a wide mouth tip. Keep vials on ice until ready for step 8.
8. Place tubes in Bio-Cool and incubate for 10 minutes.
9. Get liquid nitrogen.
10. After 10 minutes, seed the cells by dipping a spatula into liquid nitrogen and touching the side of the cryo-vial for approximately 10-30 seconds or until you see a crystal form on the side of the cryo-vial.

11. Start program 1 by pressing “PROG” button and going through the program by pressing the button again and you should see the rate of 0.5°C/min. , then press “RUN”.

12. Once temperature reaches -65°C the cryo-tubes can be transferred and stored in liquid nitrogen.

Alternatives

Another option is to place the cryo-tubes in a freezing container (Biocision-CoolCell) and store at -80°C overnight. Move the cryo-tubes to liquid nitrogen (liquid or vapor phase) on the following day.

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