Preparing Matrigel

Matrigel BD#356230 5ml or 10ml

-Let Matrigel thaw overnight at 4^{o}C (keep the frozen bottle on ice preferentially and keep the ice bucket in the fridge or in the cold room)

-Next day add an equal volume of cold DMEM to thawed Matrigel and mix well

-Incubate overnight on fresh ice at 4^{o}C

- the next day, mix well again

- prepare 50ml tubes and make aliquots: 1ml of diluted Matrigel per 50 ml tube

-freeze at –80C

*or use the diluted Matrigel to coat plates, see below for details

- thaw a tube of 1:1 diluted Matrigel on ice (or let it thaw over-night at +4C)

-dilute the Matrigel 1:40 or 1:50 in DMEM media (39ml media + 1ml Matrigel 1:1, final dilution 1:80) OR (49ml media + 1ml Matrigel 1:1, final dilution 1:100)

NOW WE USE CULTREX

3433-005-01 (5mL packaging)

R&D Systems™ Cultrex RGF Basement Membrane Extract with Phenol Red

https://www.rndsystems.com/products/cultrex-reduced-growth-factor-basement-membrane-extract-pathclear_3433-010-01

Preparing Cultrex

use the same protocol as described above for Matrigel (final dilution 1:100, 49ml DMEM medium + 1ml Cultrex 1:1)

- We use Cultrex without Phenol Red but the same can be done with Cultrex with Phenol Red, just add the aliquot of Phenol red to the tube of Cultrex as soon as the Cultrex is thawed

ALTERNATIVELY to thawing a tube of 1:1 diluted Matrigel/Cultrex on ice (or let it thaw over-night at +4C)

-thaw a tube of 1:1 diluted Matrigel/Cultrex by adding 10ml of cold DMEM

-invert the tube and warm up with your hands until you still see floating ice pieces

-add to 40ml DMEM

-invert the tube until ice disappears

Note 1: Matrigel needs to be tested as it contains endotoxin, which negatively affects hESC growth.

Note 2: Keep the aliquots on ice while working with them.

Coating with Matrigel/Cultrex

- to coat a tissue culture plate, layer the Matrigel/Cultrex in the wells (for example 1.5ml of Matrigel/Cultrex per well of a 6-well plate)
-let the plate sit at room temperature for 1h
-If you don't use the plates immediately, wrap them in parafilm and keep at +4C for 1-2 days (max 1 week)
- if you are ready to use the coated plates, aspirate the Matrigel/Cultrex and plate the cells

Note: Keep the aliquot of Matrigel/Cultrex on ice while working, as well as the plate(s) to coat

If u don't put them on ice, try to operate as fast as possible to avoid the Matrigel/Cultrex to solidify

Thawing hESC/iPSC in mTeSR Plus (same for mTeSR1)

1 vial=1 well (except if you know there are many more cells/vial)

1. thaw 1 vial of hESC/hiPSC at 37ºC (use the water bath) for 1 to 2 minutes or until there is a small amount of ice left (avoid leaving the thawed cells in freezing media for prolonged time, as this would highly increase the chance of cell death)
2. transfer to a 15ml tube and add 10mL DMEM/F12+10%FBS dropwise to the cells
3. spin at 1200rpm, discard the supernatant and resuspend the pellet in 1ml mTeSR Plus + ROCK inhibitor with a P1000

	STOCK	**per 1ml of media**
mTeSR Plus		1ml
Rock inhibitor (RI)	10mM	1ul

Rock inhibitor (RI) is also called Y-27362

4. add 1ml of mTeSR Plus + ROCK inhibitor (2ml total, this is for 1 well of a 6-well plate)
   *if u are plating cells onto more than 1 well, calculate how much medium to add, we usually use 2ml medium/well of a 6-well plate
5. aspirate the Matrigel/Cultrex from one well of a 6-well plate
6. add the 2ml of cells resuspended in mTeSR plus + RI
7. in incubator over-night, 5% CO2, 37ºC
8. the day after, cells should be sparse and should recover in about 5 days
   ***you should see really small colonies the day after thawing. If u don't see anything or if u are not sure, don't aspirate media, just add 1ml of fresh media with RI. U can repeat the day after if u are still not seeing anything. Before trashing cells, contact Barbara or the core**

9. feed cells every day (change media) with mTeSR Plus **(Rock inh is added only on the 1st day if u can see colonies, otherwise see above)**
10. when cells are confluent, split them into more Matrigel/Cultrex plates for maintenance or for differentiation

Using EDTA to split iPSC

Preparation of EDTA
Stock 0.5M EDTA (1000X)
Life Technologies cat #15575-020
To dilute to 1X (0.5mM) in 1X PBS Ca2+ Mg2+ free
= 1ml EDTA + 1000 mL 1X PBS Ca2+ Mg2+ free

Make sure to prepare the 0.5mM EDTA solution using 1X Dulbecco's phosphate-buffered saline (DPBS) without Calcium and Magnesium to dilute the 0.5M EDTA. In the presence of Calcium and Magnesium, the EDTA solution won't work! Keep the 0.5mM EDTA solution at RT.

Take an aliquot before starting your experiment and warm it up (calculate that for example, 2ml of 0.5mM EDTA solution are required to detach cells from one well of a 6-well plate).

- when PSC colonies are confluent, split using EDTA
- pre warmed an aliquot of 0.5mM EDTA in beads bath or water bath at 37C
- Aspirate media from each well
- Add 1mL of pre warmed 0.5mM EDTA for a fast rinse
- aspirate it off immediately
- Add 1 mL of pre warmed 0.5mM EDTA and incubate for 2-3 minutes
- You should watch your cells under the microscope to see when your cells start to round up and separate from each other. If the culture is confluent, then watch for the cells at the edge of the colony to begin to curl up.

*Colonies start getting loose after ~2 minutes, with cells starting to round up and separate from each other. Some iPSC lines may take more time than others, in which case more time may be needed to get the colonies loose. Cells won't be detaching from the surface of the plate.

If the cells detach from the surface of the plate (which happens very rarely), collect the cells in EDTA, then spin, remove supernatant and resuspend the pellet in 1 ml of mTeSR Plus media with 10 uM RI.

- Remove 0.5mM EDTA

- Add 1 mL of mTeSR Plus or mTeSR 1 media containing 1ul/ml of 10mM stock solution of RI (if splitting onto Matrigel) or 1 mL of hESC media (if splitting onto feeders)
- Using a p1000, gently dislodge the cells from the plate by pipetting a few times (u can see the cells detaching). Do this especially if trying to separate undifferentiated cells from differentiated cells. The differentiated cells are more adherent and will remain attached during this process.

  *for example if your cells are plated onto $\gamma\mathrm{MEF}$, the $\gamma\mathrm{MEF}$ will remain attached to the plastic while the iPSC will be dislodged and will be in suspension.

  *If dislodging cells from a 10cm plate OR if the cells do not come off easily when using a p1000 tip, then use a cell scraper to dislodge cells from the plate.

- Using a 10ml pipette, gently pick up the cells and dilute 1:3 or 1:6 or your wanted dilution in mTeSR Plus media + RI (or hESC or mTeSR1)

Freezing:

Freezing media recipe depends on which cells you are freezing

-make Freezing Media for hESC/iPSC in feeders:
(make up to 2 freezing vials from each well of a P6)
40% KO-SR
10% DMSO
50% hESC media
Add 1ul/ml ROCK inhibitor

-make Freezing Media for hESC/iPSC in matrigel:
(make 2 freezing vials from each well of a P6)
40% KO-SR
10% DMSO
50% mTeSR1
Add 1ul/ml ROCK inhibitor

-make Freezing Media for fibroblasts:
40% FBS
10% DMSO
50% DMEM/10% FBS

-make Freezing Media for any other cell lines:
40% FBS

10% DMSO
50% any media your cells are grown in

WE NOW USE CRYOSTOR CS10 (follow manufacturer instructions)

Keep it cold

-remove media from wells to freeze
-detach the cells with methods of choice (trypsin or EDTA)

-spin @ 1200rpm for 5 minutes (in the regular media with RI. If you detach with EDTA spinning cells is not necessary unless you have a lot of wells)
-remove supernatant
-resuspend with 1ml of Freezing Media using P1000 pipette
(-add amount of freezing media required per number of freezing vials)
-mix with pipette
-distribute 1ml/freezing vial
-put directly into –80 degrees.
-day after (or by one month), bring the vials to liquid nitrogen

Plating feeders (γMEF, primary embryonic fibroblasts, γ-irradiated)
We use Life Technologies cat# A34181 (MTI-Globalstem cat# GSC-6001G)

- Gelatin-coat a 6-well plate (or equivalent)-> let sit at 37ºC in incubator for 20-30min (can be done at RT for about 15-20 min)
- Thaw an irradiated vial of γMEF in 37ºC water bath
- Prepare DMEM+10%FBS media
- Add 9ml of DMEM+10%FBS media to a 15ml tube
- Use this media to collect thawed γMEF cells from vial
- Spin 1200 rpm, 3-4 min
- Discard supernatant and resuspend in 1ml DMEM+10%FBS media
- then add media needed for the wells (usually 1 vial of γMEF makes 4 or 5 6-well plates (or equivalent), it depends on the lot number)
- Add 1.5ml (can be 2mL) cells per well and mark plate as γMEF
- In incubator O/N in 5%CO2, 37ºC
- The day after, thaw hESC or hiPSC according to protocol

Thawing hESC/iPSC onto γMEF
1 vial=1 well (except if you know there are many more cells/vial)

1. thaw 1 vial of hESC/hiPSC at 37ºC for 1 to 2 minutes or until there is a small amount of ice left (avoid leaving the thawed cells in freezing media for prolonged time, as this would highly increase the chance of cell death)
2. transfer to a 15ml tube and add 10mL DMEM/F12+10%FBS dropwise to the cells

3. spin at 1200rpm, discard the supernatant and resuspend pellet in 1ml hES media+bFGF+ROCK inhibitor with a P1000

	STOCK	**per 1ml of media**
hESC media +bFGF		1ml
Rock inhibitor	10mM	1ul

4. add 1ml more of hES media+bFGF +ROCK inhibitor (2ml total, this is for 1 well)
5. add to 1 well of plated feeders after having removed the fibroblast media
6. in incubator over-night, 5% CO2, 37^{o}C
7. the day after, cells should be sparse and should recover in about 5 days ***you should see really small colonies the day after thawing. If u don't see anything or if u are not sure, don't aspirate media, just add 1ml of fresh media with RI. U can repeat the day after if u are still not seeing anything. Before trashing cells, contact Barbara or the core**
8. feed cells every day (change media) with hESC media contaning 4-10ng/ml bFGF (see media recipe) **(Rock inh is added only on the 1st day if u can see colonies, otherwise see above)**
9. when cells are confluent, split them into more γMEF for maintenance and onto Matrigel/Cultrex plates for differentiation or if u want them feeder-free onto Matrigel/Cultrex plates

SPLIT FIBROBLASTS from dermal biopsy
-remove media from wells to split
-add 1ml 0.25 % Trypsin-EDTA to each well
-place in 37^{o}C incubator for 3 minutes
-after 3 min, check to be sure cells are detached
-add 1ml DMEM+10% FBS to each well
-pipette several times with P1000 pipette, then add to tube containing 9ml DMEM+10% FBS
-spin @ 1200rpm for 5 minutes
-remove supernatant
-resuspend in media (amount depends on how you are splitting)
-mix with pipette

REAGENTS

Matrigel BD#356230 5ml or 10ml

CULTREX 3433-005-01 (5mL packaging)
R&D Systems™ Cultrex RGF Basement Membrane Extract with Phenol Red

DMEM+10%FBS media (500ml):

DMEM	435ml	Cat# 11960077 Life Technologies
Heat Inactivated-FBS (10%final)	50ml	Cat# 10082147 Life Technologies
Pen/Strepto 100X	5ml	Cat# 15140122 Life Technologies
Glutamine 100X	5ml	Cat# 25030081 Life Technologies
Na Pyruvate 100X	5ml	Cat# 11360070 Life Technologies

Filter media

hES Media (500ml)

DMEM-F12	to 500ml	Mediatech/CellGro cat#10-092-CV
Knock out Serum Replacement	100ml	Life Technologies #10828-028
Non-Essential Amino Acids	5ml	Life Technologies #11140-050
Pen/Strepto 100X	5ml	Cat# 15140122 Life Technologies
Glutamine 100X	5ml	Cat# 25030081 Life Technologies
b-Mercaptoethanol 1000x	500ul	Cat# 21985023 Life Technologies

Filter media. Cover with foil and add bFGF per bottle as needed (4-10ng/ml media) DON'T FORGET (we can make 250 ml media instead of 500 ml bottle)

bFGF

concentration of vials of bFGF 10ug/ml from R&D Systems #233-FB, use at **4ng/ml media but some lines may require more bFGF (10ng/ml or 20ng/ml)**

mTeSR1 media
from Stem Cell Technology cat # 05850, add supplement bottle to mTeSR1 bottle

mTeSR1 Plus media
Stem Cell Technology cat#100-0276
add supplement bottle to mTeSR Plus bottle

ROCK inhibitor Y-27632 dihydrochloride
Cat#1254/50, Tocris Fisher

Trypsin 0.25% Gibco Cat#25200-056

Scraper SARSTED Cat#83.1832

Gelatin SF008 Millipore