Standard Operating Procedure
for
Culturing hESCs on mouse embryonic fibroblasts (mEFs)

1.0 PURPOSE

This procedure describes the method of culturing hESCs on mEFs.

2.0 SCOPE (Should include which Cores this SOP applies to)

This procedure applies to all personnel who need to culture hESCs on mEF dishes.

3.0 PROCEDURE

3.1 Prepare inactivated mEF feeder cell layer.

1. Coat 6-cm dishes with 1 ml 0.1% gelatin solution for 1 hr in 37^{o}C incubator.
2. Aspirate the gelatin and passage mEFs (P1-P4) at a density of 2 x 10^4 cells/cm^2 onto the gelatin-coated dishes.
3. Incubate the cells for one night in 37^{o}C incubator
4. Add 10 μg/mL of mitomycin C to the dishes and incubate for 1.5 hrs in a 37^{o}C incubator.
5. Rinse cells once with 2 ml of PBS, and add 4 ml of MEF medium[1] to the dish, incubate at 37^{o}C for another 1.5 hrs.

3.2 Acquire hESC from ready-to-passage dish

1. Remove a 6-cm hESC dish from incubator and aspirate the spent hESC medium[2] from the dish.
2. Wash once with 2 ml PBS. Then add 2 ml of pre-warmed Collagenase solution[3] to 6-cm dish. Incubate at 37^{o}C for 5-10 min. Separation of colonies from the dish can be confirmed by checking highlighted or folded-back edges of the colonies under microscope.
3. Aspirate the Collagenase solution carefully. The hESC colonies should still attach to the dish.
4. Add 2 ml of hESC medium and scrape the colonies off the dish with a 5-mL glass pipette while keep pipetting the cell solution up and down.
5. Collect the 2 ml cell solution into a 15-mL conical tube. If there are still colonies left on the old dish, add another 1 ml fresh hESC medium to collect the colonies into the 15-mL conical tube.
6. Centrifuge hESC cell solution at 200xg for 5 minutes.
7. Aspirate the supernatant from the hESC pellet and re-suspend the pellet with 4 ml hESC medium or desired volume which depends on split ratio.

3.3 Plate hESC onto inactivated mEF feeder cell dish

1. Gently pipet cells up and down to break up pellet into small colonies.
2. Take inactivated mEF dish from the incubator prepared in 3.1 and aspirate mEF medium.
3. Wash 6-cm feeder dish with 2 ml of PBS and add 3 ml of hESC medium.
4. Take 1 ml of hESC cell solution from Step 7 and plate to feeder dish. To ensure even distribution of hESC colonies, add the cell solution drop-wise in a circular motion.
5. Move the dish in back-and-forth and side-to-side motions to further ensure even distribution.
6. Label the new hESC dish with cell line name, passage number and other necessary information.
7. Return the dish to the incubator and change 4 ml medium daily for 6-cm dish.

[1]mEF medium (500 ml)

Ingredients	Amount
DMEM	450 ml
Fetal Bovine Serum (FBS)	50 ml
Non-essential Amino Acids 100X Solution	2.5 ml
Penicillin/Streptomycin 100X Solution	2.5 ml

[2]hESC medium (250 ml)

Ingredients	Amount
DMEM-F12 Basal Media	200 ml
Knockout Serum Replacer (KSR)	50 ml
200 mM L-Glutamine + 2-Mercaptoethanol (1.25 ml+1.75µl)	1.25 ml
Non-essential Amino Acids 100X Solution	2.5 ml
Penicillin/Streptomycin 100X Solution	2.5 ml
bFGF solution (2 mg/ml)	0.5 ml

Filter and store at 4 ^{o}C for up to 20 days.

[3]Collagenase solution, 1 mg/ml (500 ml)

Ingredients	Amount
DMEM/F12	40 ml
Collagenase, Type IV	40 mg

Filter and store at 4 ^{o}C.

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