Usage Protocol

To initiate a culture, irradiated J2 feeder cells and epithelial cells are thawed, counted, mixed together and plated into tissue culture vessels. The medium is changed every 3 – 4 days until the epithelial cells approach confluence. During this time, the feeder cells may slough off. Upon each sub-culturing, new irradiated feeder cells are used, and the process continues until the desired number of population doublings (PDs) or the desired amount of biomass has been achieved.

Reagents required

1. Irradiated J2 feeder cells (Propagenix cat # PF-1100).
2. CR Technology medium, CRM (Propagenix cat # 276-101). The medium should be stored at -20°C for long-term storage. After thawing, the medium is stable for up to one month at 4°C. Store protected from light.
3. 1 mg/mL Cholera toxin (Sigma cat # C8052 or Enzo cat # BML-G117-0001).
4. (Optional) Desired antibiotics.
5. 0.05% Trypsin-EDTA, e.g., Gibco Cat# 25300-120.
6. PBS (phosphate buffered saline), e.g., Gibco Cat# 10010-023.
7. DMSO for cell cryopreservation, e.g., Sigma Cat# D2650.
8. CoolCell® Cryopreservation Alcohol-Free Cell Freezing Containers, BioCision®.

Procedure

I. Supplement CRM medium

Prior to use, the CRM culture medium must be completed with Cholera toxin.

1. Aseptically rehydrate Cholera toxin in CRM to a final stock concentration of 1 mg/ml.
2. Aseptically add 1 mg/ml Cholera toxin stock solution to 250 ml of CRM to a final concentration of 8.6-20 ng/ml.
    a. To make 8.6 ng/ml final concentration, add 2.2 µl.
    b. To make 20 ng/ml final concentration, add 5 µl.
3. The stock solution of Cholera toxin should be aliquoted and stored at 2° to 8°C for up to six months.
4. If desired, antibiotics and antifungals may be added.

II. Thawing frozen irradiated J2 feeder cells

1. Transfer frozen irradiated J2 feeder cells to the vapor phase of liquid nitrogen for storage if they will not immediately be used.
2. Calculate the number of irradiated J2 feeder cells required for the vessels to be seeded. The optimal seeding density of irradiated J2 feeder cells are 30,000 cells/cm^2, with a range of 25,000 – 40,000

viable cells/cm^2. The irradiated J2 feeder cells are provided at a concentration of approximately 6 x 10^6 cells/ml, 0.5 ml/vial. For example, one frozen vial of irradiated J2 feeder cells will usually be sufficient to seed three T-25 flasks.

3. Immediately prior to use, thaw the number of vials of irradiated J2 feeder cells required by gentle agitation in a 37 °C water bath. Thawing should be complete within approximately 2 minutes.
4. Remove the vials from the water bath as soon as the contents are thawed, and decontaminate by spraying the outside of the vial with 70% ethanol.
5. Transfer the vial contents to a conical centrifuge tube containing 0.5 ml of warm CRM and mix thoroughly by pipetting. If more than one vial of irradiate J2 feeder cells are needed, increase the amount of CRM accordingly, for example, use 1 ml warm CRM for two (2) vials of irradiated J2 feeder cells, 1.5 ml CRM for three (3) vials of irradiated J2 feeder cells, and so on.
6. Count the viable irradiated J2 feeder cells/ml using a hemocytometer or automatic cell counter.
7. Use a suitable amount of irradiated J2 feeder cells based on the surface area of culture vessels as described below. The cells are ready to use for supporting epithelial cell growth.
8. Volume of irradiate feeders = $\frac{(25{,}000 - 40{,}000)\ x \quad Surface\ area\ of\ culture\ vessel\ (in\ cm2)}{Viable\ irradiated\ feeder\ cells/ml}$

III. Thawing frozen epithelial cells

1. Thaw the epithelial cells and initiate cell culture as soon as possible upon receiving the frozen cells. If not to be used right way, the cells should be stored in liquid nitrogen vapor phase.
2. Immediately prior to use, thaw the vial of epithelial cells by gentle agitation in a 37 °C water bath. Thawing should be complete within approximately 2 minutes.
3. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by spraying with 70% ethanol.
4. Transfer the vial contents to a 15 ml conical centrifuge tube containing 9 ml of warm CRM. Centrifuge the cell suspension at approximately 120 x g for 5 minutes.
5. Aspirate most of the supernatant from the conical centrifuge tube containing the cell pellet. Flick the tube briefly to loosen the pellet, resuspend the cells in 1 – 2 ml warm CRM.
6. Determine the viable cell number using a hemocytometer or automatic cell counter using standard Trypan Blue exclusion assay.
7. The optimal initial seeding density of epithelial cells is between 4,000 – 10,000 viable cells/cm^2. Mix the desired numbers of epithelial cells with freshly-thawed irradiated J2 feeder cells (25,000 – 40,000 viable J2 feeder cells/cm^2) to ensure a uniform suspension, and dispense the calculated volume into the culture flask. Use 1 ml CRM for every 5 cm^2 surface area, i.e., use 5 ml CRM for a 25 cm^2 flask.
8. Incubate the cultures at 37°C in a 5% CO2 humidified incubator.

9. Change the growth medium every 3 – 4 days. As the cells become more confluent, increase the volume of medium as follows: under 40% confluence feed 1 ml per 5 cm^2, over 40% confluence feed 1.5 ml per 5 cm^2.

IV. Sub-culture epithelial cell culture

The epithelial cell culture should be split when the cells are near confluence. Volumes used in this protocol are for a 25 cm^2 flask; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove spent medium.
2. Add 3 – 5 ml PBS to the flask, briefly rinse the cells and discard the rinse solution.
3. **Optional Step**. If considered necessary, most of the J2 feeders can be efficiently removed by differential trypsinization.
    a) Add 1 ml room temperature 0.05% Trypsin-EDTA to the flask, swirl to cover all surface area. Leave the Trypsin-EDTA on the cells for 30-60 seconds with continuous observation under a microscope.
    b) When most of the feeders detach but the epithelial cells remain adherent, gently tap the flask to loosen the feeder cells, quickly aspirate the Trypsin-EDTA solution.
4. Add 1 ml room temperature 0.05% Trypsin-EDTA to the flask. Incubate at 37°C for 5 min, or longer if necessary, until the cells have detached. Tap the flask several times in your palm to loosen the cells.
5. Neutralize the trypsin by adding 3-4 ml warm CRM, and pipette up and down several times to break any cell clumps into suspension.
6. Transfer the cell suspension to a sterile 15 ml centrifuge tube. Rinse the flask with an additional 1-2 ml CRM and pool into the centrifuge tube with cells.
7. Centrifuge cells at 120 x g for 5 min at room temperature.
8. Aspirate most of the supernatant from the conical centrifuge tube containing the cell pellet. Flick the tube briefly to loosen the pellet, resuspend the cells in 1 – 2 ml warm CRM.
9. Dilute the cells in 2 – 3 ml of warm CRM. Determine cell count and viability using a hemocytometer and Trypan blue.
10. Use the following equation to determine the population doubling.

$$PD = 3.32 \quad x \quad \log \frac{[total\ cell\ number\ at\ harvest]}{[total\ cell\ number\ seeded\ at\ the\ beginning]}$$

11. Seed between 3,000 – 10,000 viable cells/cm^2 to new subculture vessels. Mix desired number of epithelial cells and freshly-thawed irradiated J2 feeder cells (25,000 – 40,000 viable J2 feeder cells/cm^2) to ensure a uniform suspension, dispense the calculated volume in to the subculture flask. Use 5 ml CRM for a 25 cm^2 flask.
12. Incubate cultures at 37°C in a 5% CO_2 humidified incubator.

13. Change the growth medium every 3 – 4 days. As the cells become more confluent, increase the volume of media as follows: under 40% confluence feed 1 ml per 5 cm^2, over 40% confluence feed 1.5 ml per 5 cm^2.

V. Addition of fresh irradiated feeder cells

The addition of fresh irradiated feeder cells may be required if the epithelial cell culture is not ready for subculture within the second week after plating. Additional feeder cells may be supplemented to the cell culture vessel(s) by following steps 1-8 outlined in the section titled "Thawing irradiated J2 feeder cells".

Optional Step. If considered necessary, the previous J2 feeders can be efficiently removed by differential trypsinization, as outlined above in step 3 in the section titled "Sub-culture epithelial cell culture".

VI. Cryopreservation of epithelial cell culture

1. Cryopreservation medium: CRM 90% (v/v); DMSO 10% (v/v).
2. Follow the subculture protocol to dissociate epithelial cells when they are nearly confluent. Resuspend cells in appropriate amount of cryopreservation medium.
3. Determine cell count and viability using a hemocytometer and Trypan blue.
4. The cells can be frozen at a density of 0.5 – 5 x 10^6 cells/ml. Resuspend cells in an appropriate amount of cryopreservation medium, and aliquot into cryovials.
5. Put the cryovials into a CoolCell™ and leave it in a -80°C freezer for at least 2 hours or overnight.
6. Transfer the cells to the vapor phase of liquid nitrogen for long-term storage.

Quality Assurance

1. CRM Medium is tested prior to shipment to meet quality control specifications. Each lot is tested for sterility, pH, osmolality, endotoxin level, visually inspected for appearance, and functionally tested to support the growth of epithelial cells in CR Technology.
2. Additional information is available upon request.

Limited Use Label License

Conditional Reprogramming (CR) Technology, covered under US Patent No. 9,279,106 (issued March 8, 2016), and subsequent patent applications pending in the US and other Jurisdictions.

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