CSNA: Standard Operating Procedure for Molecular Rhythms in Primary Fibroblasts

Primary Fibroblast Culture from Mouse Ear

1. Collect a whole ear in a 1.5ml tube with 1ml DMEM (stable at RT up to 10 days).

2. Preparation of enzyme solutions.
   a. Dissolve 10mg of collagenase D in 4ml DMEM.
   b. Pronase solution:
      1) Add 10mg of pronase in 494ul of culture grade water.
      2) Add 6ul TE buffer [5ul of 1M Tris buffer (pH 8.0) + 1ul of 0.5M EDTA (pH 8.0)].
      3) Incubate the pronase solution in a water bath at 37°C for 30 mins.
   c. Add 250ul of pronase solution to 4ml of collagenase D solution.
   d. Pass the prepared enzyme solution through a 0.2um syringe filter.

3. Discard medium from the tube containing the ear and add 1ml of 70% ethanol for 5 mins.

4. Remove the ear from the tube with tweezers and air-dry it on a clean paper towel.

5. Cut the ear into smaller pieces using scissors and then transfer them in a new 1.5ml tube including 1ml of enzyme solution (collagenase D + pronase).

6. Incubate the tube on a shaker at 200rpm at 37°C for 90 mins.

7. Place the enzyme-digested ear pieces in the center of a 60mm culture dish by sterile tweezers and incubate the culture dish at 37°C for 10 mins.
   - It allows the tissues attach on the bottom of the culture dish.

8. Take the culture dish and overlay the ear pieces with autoclaved 22 mm square cover glass.

9. Add ~500ul of the DMEM containing 10% FBS, 1% Amphotericin B and 0.1% Gentamycin at the edge of the cover glass and then carefully place the culture dish in an incubator at 37°C overnight.

10. Add 4.5ml DMEM containing 10% FBS, 1% Amphotericin B and 0.1% Gentamycin to the culture dish.

11. When the culture reaches approximately 50% confluence, transfer fibroblasts to a new 60mm dish.
    a. Aspirate medium and wash fibroblasts once with 1 X PBS.
    b. Add 2ml TrypLE and incubate the dish at 37°C for 2 mins.

c. Optional process: Deactivate TrypLE by adding 10% FBS.
d. Transfer fibroblasts to a new 60mm culture dish.
e. Add the DMEM containing 10% FBS, 1% Penicillin/Streptomycin/Glutamine (growth medium) up to 5ml.

12. When the culture reaches 80-100% confluence (takes 2-4 days), split fibroblasts into new culture dishes with 1:2-3 ratio by applying TrypLE.
    - Fibroblasts are cultured in 60mm culture dishes until lumicycle experiments.
    - Freeze extra fibroblasts in the growth medium containing 5% DMSO and then store in liquid nitrogen.
    - Low density of cells can induce morphological changes of fibroblasts such as large cell bodies and long projections, which do not generate good rhythms (i.e., number of cycles and amplitude).

Infection of Primary Fibroblasts by Bmal1-dLuc Lentivirus

1. Seed 1 x 10^5 cells in a 35mm culture dish with 2ml growth medium and place the dish in an incubator at 37°C for 24hrs (or overnight).
    - The culture reaches approximately 50% confluence by next day.
    - Cell density lower than 30-40% causes fewer cycles of clock gene rhythms.

2. Aspirate the medium from the culture and add 1ml growth medium containing Bmal1-dLuc lentiviral particles (~1 × 10^7 infectious units/plate). Place the dish in an incubator at 37°C for 24 hrs.

3. At 48 hrs post-infection, aspirate the medium containing virus and wash fibroblasts once with 1 X PBS.

4. Add 2ml growth medium and place the dish in an incubator at 37°C for 24 hrs for recovery.

Bioluminescence Recording from Primary Fibroblasts

1. Aspirate the medium and add 2ml growth medium containing 15uM forskolin in order to synchronize molecular rhythms in fibroblasts. Incubate the dish at 37°C for 2 hrs.

2. Aspirate the medium and wash once with 1X PBS.

3. Add 1.2ml DMEM containing 25mM HEPES, 15uM forskolin, 292 ug/ml L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin and 100uM luciferin (Recording medium).

4. Cover the 35mm culture dish with a 40mm sterile coverslip and seal with vacuum greases.

5. Record bioluminescence for 5-7 days in the Lumicycle luminometer.

Reagents:

Dulbecco's Modified Eagles Medium (DMEM)	HyClone SH3024302
Penicllin Streptomycin Glutamine solution	HyClone SV3008201
HEPES (1M)	Gibco 15-630-080
Beetle Luciferin, Potassium Salts	Promega PR-E1602
Bmal1-dLuc Lentivirus	VectorBuilder
Forskolin	Sigma F6886
60mm TC-Treated Culture Dishes	Corning 08-772-21
35mm TC-Treated Culture Dishes	Corning 08-772-20
TrypLE Express Enzyme (1X), No Phenol Red	Gibco 12-604-021
DPBS without Calcuium and Magnesium	Gibco 14-190-144
40mm Cover Glass Circle	Thermo Scientific 22-038-999
Collagenase, Type D, Powder	Gibco 17-104-019
Pronase Protease, Streptomyces griseus	Millipore 53-702-25KU
High-Vacuum Grease	Corning 14-635-5D
Cell Culture Grade Water	Hyclone SH3052901
Micro Cover Glasses, Square, No. 2	VWR 48368-062
Amphotericin B solution	Sigma A2942
Gentamycin Sulfate	Millipore 345815