Penn Medicine
University of Pennsylvania School of Medicine
Department of Medicine
Cardiovascular Division
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Reprogramming of Murine Somatic Cells Using miR302/367 lentivirus

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MATERIALS

Media

Sodium Pyruvate (Mediatech; Cat# MT25-000-CI)
Leukemia Inhibitory Factor (Millipore, Cat# ESG1107)
Knock Out Serum Replacement (Invitrogen, Cat#10828028)
Penicillin/Streptomycin (Invitrogen, Cat#15140122)
Antimycotic-antibiotic (Invitrogen, Cat#15240062)
MEM Non-Essential Amino Acids (Invitrogen, Cat#11140050)
L-Glutamine (Invitrogen, Cat#25030081)
β-mercaptoethanol (Biorad Cat#: 161-0710)
Valproic Acid (2-Propylpentanoic acid) (2mM final conc.; Sigma Aldrich Cat# P6273)
DMEM (1X High Glucose DMEM 4.5 gm/L; Invitrogen, Cat#11965084)
Fetal Bovine Serum (FBS; Hyclone, Cat#SH30071.03)

Culturing Reagents

0.05% Trypsin/EDTA (Invitrogen, Cat# 25300054)
1X DPBS (without Calcium/Magnesium; Mediatech, Cat# MT21-031-CV)
DMSO (Sigma, Cat#D2650-100ML)

Cell Lines

Primary Mouse Embryonic Fibroblast (MEF)
293T cell line (ATCC, Cat#CRL-11268)

Transfection Reagents

Fugene 6 HD Transfection Reagent (Roche, Cat# 4709705001)
Polybrene (10mg/mL; American Biolytical, Cat# AB01643-00001)
pMD.G (5μg; Addgene, Plasmid: 12259)
psPAX2 (5μg; Addgene, Plasmid: 12260)
Vector plasmid of interest, pLOVE-miR-302/367 (10ug)

Consumables

Steriflip GP Filter Unit (0.22μm filter; Millipore, Cat#SCGP00525)
Steriflip HV Filter Unit (0.45μm filter; Millipore, Cat# SE1M003M00)
Amicon Ultra-15 Centrifugal filter (Fisher, Cat# UFC9-100-24)
6-well Plates (Costar 6 well flat bottom tissue culture treated plates, Cat# 07-200-83)
10cm Dishes (BD Falcon 100mm tissue culture treated plates, Cat# 08-772E)

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MEDIA RECIPES

Fibroblast Growth Media (for feeders and human fibroblasts)

FBS = 50mL
DMEM= 440mL
Pen-Strep = 5mL
Antimycotic-Antibiotic = 5mL
Filter solution through a 0.22μm filter

293T Viral Media

FBS = 50mL
DMEM= 450mL
Filter solution through a 0.22μm filter
Note: *Do NOT add antibiotics to the media!*

Mouse ES Media

Knock Out Serum Replacement = 75ml
Pen-Strep = 5ml
Sodium pyruvate = 5ml
Antimycotic-antibiotic = 5ml
Non-essential amino acid = 5ml
100X L-Glutamine = 5ml
Leukemia Inhibitory Factor (LIF, 10^7 units/ml) = 50ml
β-mercaptoethanol = 4μL
*Valproic Acid = 160ul
Top with DMEM to 500mL and run through 0.22μm filter

*NOTE: Omit VPA when cells are fully reprogrammed (14 days post-infection, approx. day 21).

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PROCEDURE

Production of Virus

Day 1

1. In fibroblast growth media, plate 293T cells at $1.5x10^6$ cells on a 10cm plate.
2. Incubate at 37°C with 5% CO_2 O/N.

Day 2

Generation of virus

1. Aliquot 0.6mL DMEM into 1.5mL Eppendorf tube.
2. Add 54μL of Fugene directly into the DMEM without making any contact with the tube—contact will adversely affect viral production.
3. Finger tap and incubate at R/T for 10 minutes.
4. On the inner surface of the tube's cap, pipette 5μg of pMD.G, 5μg of psPAX2 and 10μg of pLOVE-miR302/367.
5. Carefully cap the tube (without spilling the DNA) and mix by finger tapping.
6. Incubate at R/T for 15 minutes.
7. Replace the media containing the 293T cells with fresh fibroblast growth media.
8. Add the DNA-Fugene mixture to the 293T cells drop-wise throughout the entire plate. Rock the plate back and forth, sideways to ensure even distribution of the DNA-Fugene mix.
9. Return the plate into the incubator and allow cells to grow for 24hrs.

Plating of Target Fibroblasts to be reprogrammed

1. Thaw a vial of mouse embryonic fibroblasts.
2. Transfer the cell suspension into a 15mL tube containing 10mL of fibroblast growth media.
3. Spin at 1000rpm for 4min.
4. Discard supernatant and resuspend cells in fibroblast growth media.
5. Plate pre-determined number of fibroblasts in each well of a 6-well plate (approximately 20,000 cells).
6. Incubate at 37°C with 5% CO_2 O/N.

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Day 3

Virus Collection & Infection of Target Cells

1. Collect the media from 293T plate and filter through a 0.45μm filter.
2. Add fresh fibroblast growth media to the 293T cells and return to 37°C and 5% CO_2 to be used for virus collection on Day 4.
3. Transfer the collected media into the Amicon centrifugal tube and spin at 3600x*g* for 15min.
4. Collect the concentrated viral suspension into a fresh 50mL tube containing 10mL of fibroblast growth media. Rinse column with same media 1-2 times to ensure you have all of the viral suspension.
5. We use freshly made virus for reprogramming.
6. Add 5μL of polybrene (10mg/mL stock) to the viral suspension.
7. Invert the tube a couple of times.
8. Discard the fibroblast growth media on the target fibroblasts to be reprogrammed.
9. Pipette 2ml of viral suspension on to the plated cells in 6-well plate or 10ml in 10cm plate.
10. Incubate at 37°C with 5% CO_2 O/N.

Day 4

Second Infection of Target Cells

1. Repeat steps 1, 3-10 on Day 3.
2. Decontaminate 293T virus producing cells with 10% bleach.

Day 5

Culturing Infected Cells in ES Growth Media

1. Replace the viral media with fresh Mouse ES growth media (+ 2mM final conc. VPA) and incubate at 37°C with 5% CO_2 O/N.

Plating of Feeder Cells

1. Thaw a vial of Primary Mouse Embryo Fibroblast Feeder Cells.
2. Transfer the cell suspension into a 15mL tube containing 10mL fibroblast growth media.
3. Spin at 1000rpm for 4min.
4. Discard supernatant and resuspend cells in fibroblast growth media.
5. Plate out one vial of feeder cells onto ten 10cm dishes.
6. Incubate at 37°C with 5% CO_2 O/N.

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Day 6

Plating Infected Cells

1. Discard the fibroblast growth media on the infected target fibroblast cells and wash once with DPBS.
2. Discard DPBS, add 200-300ul 0.05% Trypsin per well of a 6-well plate or 2mL 0.05% Trypsin into 10cm plate (or just enough to cover the cells). Incubate at 37°C for 2min, tap lightly to loosen cells.
3. Inactivate trypsin/EDTA with 2mL ES media (+VPA) and spin at 1000rpm for 4 minutes.
4. Discard the media and add 10mL of ES media (+VPA).
5. Pipette up and down a few times to dissociate cells.
6. Replace the fibroblast growth media on the feeder cells with the suspended cells in the ES growth media (+VPA).
7. Return the plates to 37°C incubator

Day 7 and beyond-until colonies appear (approx. 6-8 days post-infection, Day 13-Day 15)

Change the ES growth media (+2mM final conc. VPA) daily until colonies appear. Continue culturing cells with VPA until clones are well-established (approx. 2 weeks post-infection/day 21).

Day Colonies Appear and beyond

Passaging iPS cells

1. Aspirate ES media off plates and gently wash with 1x DPBS and aspirate wash.
2. Add 2ml of 0.05% trypsin to 10cm plate of iPS cells. Incubate for 1-2 min at 37°C with 5% CO_2 O/N.
3. When colonies start to lift off, add 10ml ES growth media. Triturate the cell suspension to get medium-small fragments. Split confluent cells 1:5 and partially confluent plates 1:3.
4. Change ES media every day until confluent/cells begin to appear optically darker/colony size increases. Then repeat splitting procedures.

Thawing iPS cells

1. Thaw cells, add 1 ml of thawed cells to 10ml ES growth media.
2. Spin 1000x rpm for 4 minutes.
3. Aspirate and resuspend in 10ml of fresh ES media.
4. Pipette gently with a serological pipette before plating the entire contents onto a plate of feeder cells (*note:* feeder cells should be plated the day prior at a density of 1 vial of feeders to ten 10cm plates).