Sep 06, 2022

Freezing of hPSCs grown on MEFs

In 1 collection

DOI

dx.doi.org/10.17504/protocols.io.b4mqqu5w

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DOI: dx.doi.org/10.17504/protocols.io.b4mqqu5w

Protocol Citation: Hanqin Li, Oriol Busquets, Steven Poser, Dirk Hockemeyer, Frank Soldner 2022. Freezing of hPSCs grown on MEFs. **protocols.io https://dx.doi.org/10.17504/protocols.io.b4mqqu5w**

Manuscript citation:
Hanqin Li, Oriol Busquets, Yogendra Verma, Khaja Mohieddin Syed, Nitzan Kutnowski, Gabriella R Pangilinan, Luke A Gilbert, Helen S Bateup, Donald C Rio, Dirk Hockemeyer, Frank Soldner (2022) Highly efficient generation of isogenic pluripotent stem cell models using prime editing eLife 11:e79208

https://doi.org/10.7554/eLife.79208

Protocol status: Working

Created: February 03, 2022

Last Modified: May 31, 2024

Protocol Integer ID: 57744

Keywords: ASAPCRN

Funders Acknowledgement:
Aligning Science Across Parkinson's
Grant ID: ASAP-000486

Abstract

This protocol describes the standard procedure of freezing human pluripotent stem cells (hPSCs), which were grown on inactivated mouse embryonic fibroblasts (MEFs).

General notes

1. Throughout this protocol, the term hPSC is used to collectively refer to both hiPSCs and hESCs. All described procedures have been tested and work equally well for hiPSCs and hESCs.
2. Until otherwise indicated, hPSCs are routinely grown in a humidified cell culture incubator under “low” oxygen conditions. We have successfully maintained hPSCs using either 3% O2 (3% O2, 5% CO2) or 5% O2 (5% O2, 5% CO2) conditions.
3. While freezing hPSCs as single cell solution (using Rock Inhibitor) results in better cell recovery, some laboratories prefer freezing of hPSCs as cell clusters. We have used both approaches and do not observe obvious differences.

Materials

A	B	C
Item	**Vendor**	**Catalog #**
DMEM/F12	Thermo Fisher	11320082
DPBS w/o Calcium and magnesium (DPBS)	Corning	MT21031CV
Fetal Bovine Serum (FBS)	Corning	35-011-CV
Knockout Serum Replacement	Thermo Fisher	10828-028
FB Essence	Avantor	10803-034
FBS	Corning	35-011-CV
Newborn Calf Serum	Sigma	N4762
L-Glutamine	Sigma	G8540
Penicillin & Streptomycin (100X)	Thermo Fisher	15140163
MEM Non-Essential Amino Acids (100X)	Thermo Fisher	11140050
Heat Stable Recombinant Human FGF2	Thermo Fisher	PHG0360
Collagenase type IV	Thermo Fisher	17104019
DMSO	Fisher Scientific	BP231-100
BSA	Sigma	A4503
Y-27632	Chemdea	CD0141
2-Mercaptoethanol	Sigma	M3148
0.25% Trypsin with EDTA (Trypsin)	Thermo Fisher	25200114
Styrofoam microtube freezer box	Labnet	R8000
Nalgene® Mr. Frosty® Cryo 1°C Freezing Containers	Thermo Fisher	

A. Freezing of hPSCs as single cell solution using trypsin

10m

1 When hPSCs reach 50% confluency (usually on day 6 from last passage), change medium to 3 ml hPSCs medium + Rock inhibitor, to prepare for freezing the next day.

1.1 **hPSCs Medium**

A	B
DMEM/F12	385 ml
Fetal Bovine Serum (FBS)	75 ml
Knockout Serum Replacement	25 ml
L-Glutamine (100X)	5 ml
Penicillin & Streptomycin (100X)	5 ml
MEM Non-Essential Amino Acids (100X)	5 ml
2-Mercaptoethanol (10,000X)	50 µl
Heat Stable Recombinant Human FGF2 (25ug/ml)*	80 µl

*While we prefer Heat Stable Recombinant Human FGF2, we also have used regular FGF2.
Final volume: 500ml

L-Glutamine (100X)

L-Glutamine, powder	14.6 g
MilliQ H2O	500 ml

2-Mercaptoethanol (10,000X)

2-Mercaptoethanol	0.78 ml
MilliQ H2O	9.22 ml

Heat Stable Recombinant Human FGF2 (25µg/ml)

A	B
Heat Stable Recombinant Human FGF2	500 µg
0.1% BSA	20 ml

Final volume: 20ml

hPSCs Medium + Rock Inhibitor

	A	B
	hPSCs medium	500 ml
	Y-27632 (1,000X)	500 µl

Final volume: 500ml

Y-27632 (1,000X)

	Y-27632	5 mg
	DMSO	1.56 ml

2 Before starting:

a. Prepare Freezing Medium I and II and keep on ice.

b. Pre-label appropriate number of cryovials (freeze approx. 2 vials/well of a 6-well plate)

2.1 **Freezing Medium I**

	A	B
	hPSCs medium	5 ml
	FB essence*	5 ml

*We have successfully used FB essence and FBS to freeze hPSCs and have not observed obvious difference. Final volume: 10ml

Freezing Medium II

	A	B
	FB essence*	8 ml
	DMSO	2 ml

*We have successfully used FB essence and FBS to freeze hPSCs and have not observed obvious difference. Final volume: 10ml

3 Wash hPSCs 1x with DPBS

4 Add 0.5 ml Trypsin to each well

5 Incubate 00:05:00 37 °C in incubator 5m

6 Gently shake the plate

7 Check under microscope to confirm all cells have been lifted from the plate.

8 Add 2 ml Wash Medium to each well to inactivate trypsin

8.1 **Wash medium**

DMEM/F12	470 ml
Newborn Calf Serum	25 ml
Penicillin & Streptomycin (100X)	5 ml

Final volume: 500ml

9 Triturate using P1000 tips and collect all cell suspension to a 15 ml conical tube

10 Centrifuge at 200-300 x g, 00:05:00 5m

11 Aspirate supernatant

12 Gently re-suspend the pellet in 500 µl Freezing Medium I per vial to be frozen.

13 In each cryovial, add 500 µl pre-chilled Freezing Medium II.

14 Dispense 500 µl cell suspension into each cryovial using P1000, mix

15 Temporarily keep cryovials On ice until all cells dispensed

16 Place cryovials into Styrofoam microtube freezer box or pre-cooled (4°C on ice) NALGENE™ Cryo 1°C Freezing Container filled with 250 ml of Isopropanol.

17 Freeze at -80°C Overnight

18 For long term storage, store cryovials in liquid nitrogen (-196°C).

B. Freezing of hPSCs as as cell aggregates using collagenase 20m

19 Before starting:
a. Prepare Freezing Medium I and II and keep on ice.
b. Pre-label appropriate number of cryovials (freeze approx. 2 vials/well of a 6 well plate)

20 Wash hPSCs (on feeders) 1x with PBS.

21 Use 1 ml Collagenase Solution/well of a 6-well plate.

21.1 **Collagenase solution**

Collagenase type IV	10 mg
KSR medium	10 ml

Final volume: 10ml

KSR medium

DMEM/F12	385 ml
Knockout Serum Replacement	100 ml
L-Glutamine (200 mM)	5 ml
Penicillin & Streptomycin (100X)	5 ml
MEM Non-Essential Amino Acids (100X)	5 ml

Final volume: 500ml

22 Incubate 00:45:00 37 °C . Watch for edge curling of the colonies as this indicates that collagenase incubation is complete. 45m

23 Add 3 ml hPSC medium to quench collagenase

24 Pipette repeatedly with 5 ml pipette to lift colonies, careful not to carry over too many MEFs.

25 Collect into 15 ml conical tube.

26 Add 4-5 ml Wash Medium.

27 Gravity precipitate cells 00:10:00 10m

28 Reduce volume to 1-2 ml

29 Using a 10 ml strip pipette, triturate the colonies 5-10 times against the bottom of the tube to break up cell clusters

Note

a. The objective is to reduce cluster size, not to completely dissociate to single cells.
b. Avoid introducing air bubbles.

30 Gravity precipitate cells 00:10:00 10m

31 Resuspend cells in 10 ml hPSC medium

32 Pellet the cells at 200 x g, Room temperature, 00:05:00 aspirate the supernatant 5m

33 Gently re-suspend the pellet in 500 µl Freezing Medium I per vial to be frozen.

34 Carefully add 500 µl Freezing Medium II per vial to be frozen.

35 Dispense 1 ml aliquots in pre-labeled cryovials and keep On ice

36 Place cryovials into Styrofoam microtube freezer box or pre-cooled (4°C on ice) NALGENE™ Cryo 1°C Freezing Container filled with 250 ml of Isopropanol.

37 Place container in a -80°C Freezer Overnight

38 After an overnight incubation, cryovials are removed from the Freezing Container and placed in Liquid Nitrogen (-196°C) for long-term storage.