Subcloning of genotype-confirmed hPSCs clones

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In 1 collection

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ABSTRACT

This protocol describes a standard procedure for subcloning of genotype-confirmed human pluripotent stem cells (hPSCs).

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.

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PROTOCOL CITATION

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COLLECTIONS

Standard operating procedure for the isolation of genetically engineered hPSCs clones in a high-throughput way

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MATERIALS TEXT

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
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
Y-27632	Chemdea	CD0141
2-Mercaptoethanol	Sigma	M3148
0.25% Trypsin with EDTA (Trypsin)	Thermo Fisher	25200114
Proteinase K	Sigma	P6556
DMSO	Fisher Scientific	BP231-100

1 Change medium to hPSCs medium + Rock inhibitor one day before subcloning

1.1 hPSC medium

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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

Y-27632 (1,000X)

Y-27632	5 mg
DMSO	1.56 ml

hPSC medium + Rock inhibitor

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

Final volume: 500ml

2 Wash the genotype-confirmed wells with DPBS

3 Add 25 µl Trypsin to those wells

4 Incubate **00:05:00** **37 °C** 5m

5 Add 75 µl hPSCs medium + Rock inhibitor, mix well by pipetting

6 Transfer dissociated cells to a 15 conical tube

7 Prepare three MEF wells of a 6-well plate. Seed 1/100, 1/1,1000 and the rest of the cells respectively to those wells

8 Change medium daily for the high density well starting from day 3. Change medium every 3 days for the low density wells in the first week, then every other day in the second week

9 Once the high density wells grow to 50-70% confluency, freeze.

For a detailed protocol on freezing hPSCs, refer to "Freezing of hPSCs grown on MEFs" in the "Thawing, Passaging and Freezing of hPSCs on MEFs" collection; dx.doi.org/10.17504/protocols.io.b4msqu6e

For a detailed protocol on freezing feeder-free hPSCs, refer to "Freezing of feeder-free hPSCs" in the "Feeder-free culturing of hPSCs" collection; dx.doi.org/10.17504/protocols.io.b4mcqu2w

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10 When big hPSCs colonies form in the low density wells, change medium to hPSCs medium + Rock inhibitor

11 The day after, proceed with manual colony picking

12 For each original clone, prepare six microcentrifuge tubes pre-added with 20 µl Trypsin

13 Aspirate medium and add 2 ml DMEM/F12

14 Change medium to DPBS for the well where colonies will be picked

15 Under the dissecting microscope, use mouth pipet or a fine 10 µl tip to pick one undifferentiated colony which is fully separated from other colonies.

16 Transfer the colony to one microcentrifuge tube from step 12.

17 Repeat step 15-16 to pick another five colonies

18 Incubate the microcentrifuge tubes at **37 °C** **00:05:00** 5m

19 Add 70 µl hPSCs medium + Rock inhibitor to each tube, pipet to mix

20 Seed all cells into one well of a 12-well MEF plate

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21 Shake plates to distribute cells evenly

22 Culture for 7-10 days with medium change daily from day 3

23 When the subclones grow to 50-70% confluence, passage and prepare crude cell lysis for NGS genotyping

23.1 Crude lysis buffer (2x)

A	B
KCl	100 mM
MgCl2	4 mM
NP-40	0.9%
Tween-20	0.9%
Tris	20 mM
Proteinase K (add before use)	100 µg/ml

pH: 8

24 Once genotype confirmed, expand and freeze the one subclone, which shows the best hPSCs morphology and proliferates normally.

25 Test for mycroplasma, stain for pluripotent markers, and karyotyping

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