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Preparing MEF-cultured hPSCs for nucleofection V.2

In 1 collection

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We use this protocol and it's working

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Abstract

This protocol describes the procedure or preparing MEF-cultured human pluripotent stem cells (hPSCs) for the delivery of plasmids, mRNA or ribonucleoprotein (RNP) using nucleofection.

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. This protocol is to prepare cells for protocol nucleofection of hPSCs. Before starting, familiarize yourself with the protocol and the required preparations. A detailed protocol on maintaining MEF-cultured hPSCs can be found in the collection "Maintenance and inactivation of mouse embryonic fibroblasts (MEFs) as feeder cells for human pluripotent stem cell culture;" doi:
3. Detailed protocols for preparing plasmids, RNA, and RNP for nucleofection can be found in the collection "Nucleofection (Amaxa) and electroporation (Biorad) of hPSCs." A link to this collection can be found in the title section of this protocol, located above

Materials

	Item	Vendor	Catalog #
	DMEM/F12	Thermo Fisher	11320082
	DPBS w/o calcium and magnesium	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
	Collagenase type IV	Thermo Fisher	17104019
	2-Mercaptoethanol	Sigma	M3148
	mTeSR-plus	STEMCELL Technologies	100-0276
	StemFlex	Thermo Fisher	A3349401
	Vitronectin (VTN-N) Recombinant Human Protein, Truncated	Thermo Fisher	A14700
	Accutase	Thermo Fisher	SCR005
	Dispase	STEMCELL Technologies	NC9995391
	Y-27632	Chemdea	CD0141
	Cas9, purified protein, 40uM	Macrolab, QB3 UC Berkeley	
	Synthetic pegRNAs	IDT or Synthego	
	Synthetic sgRNAs	Synthego	
	P3 primary Cell 4D X kit S	Lonza	V4XP-3032

	Item	Vendor	Catalog #
	Countess™ Cell Counting Chamber Slides	Thermo Fisher	C10228
	pCMV-PE2	Addgene	132775
	4D-Nucleofector TM Core + X Unit	Lonza	AAF-1002B, AAF-1002X
	5 ml polystyrene round-bottom tube with cell-strainer cap	Corning	352235
	Cell-strainer (70 µm)	Fisher	07201431
	Gene Pulser Xcell Eukaryotic System	Bio-Rad	1652661
	Gene Pulser Electroporation Cuvettes, 0.4 cm gap	Bio-Rad	1652081
	Exact N Amp Blood PCR Kit	Sigma	XNAB2-1TK

Note: This protocol makes reference to other protocols. Please check for any materials found in those protocols, which might not be listed here

Troubleshooting

1 When MEF-cultured hPSCs reach 50% confluency, change medium to hPSCs medium + Rock inhibitor, preparing for nucleofection the next day. For each 20 µl nucleofection reaction, prepare half to 1 well of cells.

A detailed protocol on maintaining MEF-cultured hPSCs can be found in the collection "Maintenance and inactivation of mouse embryonic fibroblasts (MEFs) as feeder cells for human pluripotent stem cell culture;" dx.doi.org/10.17504/protocols.io.b4pbqvin

1.1 **hPSCs medium**

	Reagent	Volume
	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 (25µg/ml)*	80 µl

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

L-Glutamine (100X)

	L-Glutamine, powder	14.6 g
	MilliQ H2O	500 ml

Final volume: 500 ml

2-Mercaptoethanol (10,000X)

	2-Mercaptoethanol	0.78 ml
	MilliQ H2O	9.22 ml

Final volume: 10 ml

Heat Stable Recombinant Human FGF2 (25µg/ml)

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

Final volume: 20 ml

Y-27632 (1,000X)

	A	B
	Y-27632	5 mg
	DMSO	1.56 ml

hPSCs medium + Rock inhibitor, 500ml

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

Final volume: 500 ml

2 Prepare feeder plate at least 1 day earlier as depicted in the collection "Maintenance and inactivation of mouse embryonic fibroblasts (MEFs) as feeder cell for human pluripotent stem cell culture," dx.doi.org/10.17504/protocols.io.b4pbqvin

3 Wash MEF-cultured hPSCs with DPBS

4 Add 1 ml Dissociating Solution to each well

4.1 **Collagenase solution (10mg/ml)**

	A	B
	Collagenase type IV	100 mg
	KSR medium	10 ml

Final volume: 10 ml

4.2 **KSR medium**

	A	B
	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: 500 ml

4.3 **Dissociating solution, 10ml**

	A	B
	Collagenase solution (10mg/ml)	1 ml
	Dispase (1U/ml)	5 ml
	DMEM/F12	4 ml

Final volume: 10 ml

5 Incubate 00:30:00 37 °C . Watch for edge curling of the colonies as indication collagenase incubation is complete. 30m

6 Add 2 ml DMEM/F12 to each well

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

8 Collect into 15 ml conical tube.

9 Add 7 ml DMEM/F12.

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

11 Aspirate supernatant

12 Re-suspend cell pellet in 1 ml pre-warmed Accutase

13 Incubate 37 °C 00:05:00 5m

14 Add 9 ml DMEM/F12, invert to mix

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

16 Aspirate supernatant

17 Resuspend cell pellet in 1 ml DMEM/F12, triturate to single cells using P1000 tips

18 Take two 10 µl sets of the cell suspension. Mix each set with 10 µl trypan blue dye which comes with the Countess™ Cell Counting Chamber Slides

19 Count cells with Countess automated cell counter or hemocytometer, average the counts from the two sets. Continue with re-suspending the cell pellet in 20 µl nucleofection solution as described in the protocol "Nucleofection of hPSCs" (Step 2)

The protocol "Nucleofection of hPSCs" can be found in the collection "Nucleofection (Amaxa) and electroporation (Biorad) of hPSCs." A link to this collection can be found in the title section of this protocol, located above

20 Mix the cell suspension in the conical tube, take 500,000 cells per nucleofection reaction and transfer to a new conical tube

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

22 Aspirate supernatant

23 Resuspend cell pellet in 10 ml DPBS

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

25 Aspirate supernatant as much as possible, to minimize the interference to the nucleofection buffer system.

26 To proceed with the nucleofection process, refer to the protocol "Nucleofection of hPSCs;" **dx.doi.org/10.17504/protocols.io.b4pcqviw**