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Excision of a viral reprogramming cassette by delivery of synthetic Cre mRNA

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Abstract

The generation of patient-specific induced pluripotent stem (iPS) cells provides an invaluable resource for cell therapy, *in vitro* modeling of human disease, and drug screening. To date, most human iPS cells have been generated with integrating retro- and lenti-viruses and are limited in their potential utility because residual transgene expression may alter their differentiation potential or induce malignant transformation. Alternatively, transgene-free methods using adenovirus and protein transduction are limited by low efficiency. This report describes a protocol for the generation of transgene-free human induced pluripotent stem cells using retroviral transfection of a single vector, which includes the coding sequences of human *OCT4*, *SOX2*, *KLF4*, and *cMYC* linked with picornaviral 2A plasmids. Moreover, after reprogramming has been achieved, this cassette can be removed using mRNA transfection of Cre recombinase. The method described herein to excise reprogramming factors with ease and efficiency facilitates the experimental generation and use of transgene-free human iPS cells.

Keywords

Reprogramming; Induced pluripotent stem cells; Synthetic mRNA; Cre-excision; Transgene-free iPSCs

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INTRODUCTION

The basic protocol in this report describes a method to generate transgene-free human iPS cells using a retroviral polycistronic reprogramming cassette encoding the Yamanaka reprogramming factors *OCT4*, *SOX2*, *KLF4*, *cMYC* linked by picornaviral 2A peptides. LoxP sites flanking the polycistronic cassette enable Cre recombinase-mediated excision. Transfection of Cre mRNA allowed for efficient recovery of factor-free human iPS cells as compared with viral delivery of Cre. The efficient generation of transgene-free human iPS cells, which with their closer resemblance to human embryonic stem cells, promise improved performance *in vitro* and represent invaluable tools for medical research.

NOTE: All protocols in this unit require standard tissue culture and sterilization facilities. Cells should be handled under sterile conditions in a Class II Biological biosafety cabinet.

NOTE: All human- and mouse-derived cells are incubated at 37 °C in a humidified atmosphere of 5% CO₂.

NOTE: All equipment and reagents that come into contact with live cells must be sterile, and proper aseptic technique should be used.

BASIC PROTOCOL 1: Direct Reprogramming of Human Fibroblasts Using a Cre-Excisable Retroviral Reprogramming Monovector

This protocol is used to generate concentrate VSVG-pseudotyped Cre-excisable polycistronic retrovirus using a protocol similar to one previously described (Park and Daley, 2009). The virus is then used to infect human fibroblasts to generate iPS cells similarly to as previously described (Ohnuki *et al.*, 2009; Park *et al.*, 2009).

Materials

293T cells for generating retrovirus (ATCC, Cat No. CRL11268)

293T medium (see recipe)

Fugene 6 (Roche Applied Science, Cat No. 1181509001)

pE4-OSKM vector

VSV-G (Addgene, clone 8454)

Gag-Pol (Addgene, clone 8455)

Human Fibroblasts

Human Fibroblast Medium (see 10% FBS medium recipe)

Protamine Sulfate (Sigma Aldrich, Cat. No. P4380)

Retroviral supernatant containing monovector

Irradiated CF-1 mouse embryonic fibroblast (MEFs) (GlobalStem, GSC-6001G)

CF1 MEF Feeder Medium (see 10% FBS medium recipe)

hESC growth medium (see recipe)

hiPSC freezing medium (see recipe)

Y-27632 (Sigma-Aldrich, Cat. No. Y0503)

0.25% Trypsin (Invitrogen, Cat. No. 25300120)

0.05% Trypsin (Invitrogen, Cat. No. 2530054)

Collagenase, Type IV (Invitrogen Cat. No. 17104-019)

Gelatin (Sigma-Aldrich, Cat No. G1890)

Low protein-binding 0.45 μ m pore size cellulose acetate filter (Pall Life Sciences, Cat No. PN4184)

10 ml disposable syringe (BD, Cat No. 301030)

Cell lifter (Corning, Cat No. 3008)

2 ml cryovials

–80 °C freezer

Beckman XL-90 Ultracentrifuge

1 $\times$ 3-1/2 polyallomer centrifuge tube (Beckman, Cat No. 326823)

Production of VSVG-pseudotyped Cre-Excisable Retrovirus in 293T cells

- 1** On the day of transfection for viral production (day 0), aspirate the medium from a 70–80% confluent culture of 293T cells growing in a 100 mm tissue culture dish.
- 2** For each 100 mm dish, add 20 μ l of Fugene to 300 μ l DMEM and incubate the mixture for 15 minutes at room temperature. Add 2.5 μ g of the pE4-OSKM vector, 2.25 μ g of Gag-pol and 0.25 μ g of VSVG vector and incubate for 15 minutes to allow formation of FuGene/DNA complex.
- 3** Replace the medium on 293T cells with fresh 10% FBS media, and add 350 μ l of Fugene/DNA complex (from step 2) to the dish of 293T cells and rock it back and forth gently to allow for equal distribution. Incubate the dish overnight in a 37 °C, 5% CO₂ incubator.
- 4** On the morning of day 1, replace the 293T medium containing the FuGene/DNA complexes with fresh 10% FBS medium. Incubate in a BL2+ incubator.
- 5** On the morning of day 2, plate 1 $\times$ 10⁶ human fibroblasts per well of a 6-well plate for each desired infection.
- 6** 48 hours after transfection (day 2), under BL2+ conditions, collect virus-containing supernatant from the 100 mm dish with a 10 ml disposable syringe, and filter the supernatant using a 0.45 μ m pore size cellulose acetate filter.
- 7** Prepare the viral supernatant for ultracentrifugation by transferring the viral supernatant into a polyallomer ultracentrifuge tube. Balance the tubes with DMEM.
- 8** Use the Beckman XL-90 Ultracentrifuge to concentrate the retroviral supernatant. Spin the virus at 23,000 RPM at 4 °C for 90 minutes.

- 9 After carefully retrieving the ultracentrifuge tubes containing the virus from the ultracentrifuge, remove the supernatant while avoiding disturbance of the viral pellet.
- 10 Add 1 ml DMEM/F12 to the tubes to dissolve the pellet and leave the dissolved pellet undisturbed overnight.
- 11 Determine the titer of the virus following published protocols (Tiscornia *et al.*, 2007).

Retroviral Infection of Human Fibroblasts

- 12 On day 3, remove the human fibroblasts from the incubator and change media. Add aliquots of the retroviral supernatants at a multiplicity of infection (MOI) of 5 for the pE4-OSKM virus. Add protamine sulfate at a concentration of 5 µg/ml.
- 13 One day after infection (day 4), remove the viral supernatant, wash three times with PBS, then add 3 ml of human fibroblast medium.

Reseed fibroblasts onto CF1 feeders

- 14 Five days after infection (day 8), seed $1 \times 10^4/\text{cm}^2$ MEFs in MEF medium on a 100 mm dish coated with 0.1 % gelatin. Incubate until the next day.
- 15 On day 9, aspirate the medium and wash the infected human fibroblasts once with PBS.
- 16 Add 1 ml of 0.05% trypsin and incubate the dish for 5 – 10 minutes at 37 °C for 10 minutes.
- 17 Add 9 ml of 10% FBS medium to the dish and dissociate the masses of cells by pipetting.
- 18 After counting cells with a hemocytometer, transfer approximately 5×10^4 cells into a 100 mm dish containing irradiated CF1 MEFs (prepared in Step 14). Incubate overnight.
- 19 The next day and every following day, change the medium to 10 ml of hESC media (KOSR-bFGF).

Pick colonies

- 20 At least 3 weeks after retroviral infection, examine the dishes for ES-cell like colonies that grow slowly and have defined, smooth edges.
- 21 If colonies are observed, seed a gelatinized 12-well with CF1 MEFs in 10% FBS medium (1 ml per well). This plate will be used to expand nascent human iPSC colonies. Incubate overnight at 37 °C.
- 22 The next day, prior to picking the nascent colonies, refresh the 12-well plate with hES medium.
- 23 Wash the 100 mm tissue culture dish containing the candidate human iPS cells twice with 5 ml of PBS. Add 5 ml of F12 medium to the dish.
- 24 With 5 ml of DMEM-F12 medium still in the dish, cut out an iPS colony and separate it from feeder cells under the stereo microscope with a 2- or 10ul pipette tip and pipettor. Transfer the colony to an individual well of the 12 well plate and add the ROCK inhibitor Y-27632 (10 µM from 10 mM stock) to promote survival of human iPS cells.

- 25 After one week, the colony will become large enough for passaging. Mechanically passage the colony into a well of a 12-well plate with fresh irradiated MEFs.
- 26 After another week, the cells can be subsequently passaged into a 6-well plate.
- 27 Following subsequent passaging, iPS cells should be treated like human ES cells. Further instruction on how to passage, freeze, and characterize these cells can be found in Lerou *et al.*, 2008.

BASIC PROTOCOL 2: Synthesis of Modified Cre mRNA

This protocol describes the synthesis of Cre recombinase mRNA from a DNA template containing the Cre gene. Briefly, generic 5' and 3' UTRs are ligated to the Cre open reading frame (ORF). Ligation is facilitated by the use of splint oligonucleotides that bridge the junctions between the UTRs and the ORF. To this newly synthesized product, a tract of 120 Ts is appended by PCR. The final construct is amplified and used to template *in vitro* transcription of mRNA.

Materials

Cre ORF PCR

pBABE-puro-Cre (kindly provided by Dr. Zhe Li, Harvard Medical School)

KAPA HiFi Hotstart ReadyMix (Harvard Biopolymers – Catalog #KK2601)

5' ORF Oligo

- 5'TCCAATTTACTGACCGTACACC3'

3' ORF Oligo

- 5'CTAATCGCCATCTTCCAGCAGG3'

ORF Forward Primer Kinase Treatment

T4 Polynucleotide Kinase (PNK) with Buffer (New England Biolabs – Catalog #M0201)

Adenosine-5'-Triphosphate (ATP) (100 mM, USB (Affymetrix) – Catalog #77241)

5' UTR and 3' UTR Ligation

Cre Amplicon

5' UTR Oligo

-
5'TTGGACCCTCGTACAGAAGCTAATACGACTCACTATAGGGAAATAAGAGAGA
AAAGAAGAGTAAGAAGAAATATAAGAGCCACCATG3'

3' UTR Oligo, Phosphorylated

-5'TTGGACCCTCGTACAGAAGCTAATACGACTCACTATAGGGAAATAAGAGAGA
AAAGAAGAGTAAGAAGAAATATAAGAGCCACCATG3'

5' Splint Oligos

- 5'GGTGTACGGTCAGTAAATTGGACATGGTGGCTCTTATATTTCTT3'

3' Splint Oligos

- 5'CCCGCAGAAGGCAGCGATTAGCGGTAGAAGGTCGT3'

Ampligase Enzyme with Buffer (Epicentre Biotechnologies – Cat. No. A32750)

PCR to add poly-T tail (Tail PCR)

KAPA HiFi Hotstart Readymix (Harvard Biopolymers – Cat. No. KK2601)

Tail Forward Primer (Generic)

- 5'TTGGACCCTCGTACAGAAGCTAATACG3'

Tail Reverse Primer (Generic) PAGE Purified

- 5'T₁₂₀CTTCCTACTCAGGCTTTATTCAAAGACCA3'

In Vitro Transcription

T7 MEGAScript Kit (Ambion – Catalog #AM1334M)

Adenosine-5'-Triphosphate (ATP) (100 mM, USB (Affymetrix) – Catalog #77241, 75 mM, included in T7 MegaScript Kit)

Guanosine-5'-Triphosphate (GRP) (100 mM, USB (Affymetrix) – Catalog #77243, 75 mM, included in T7 MegaScript Kit)

Pseudouridine-5'-Triphosphate (100 mM, TriLink Biotechnologies – Catalog #N-1019)

5-Methylcytidine-5'-Triphosphate (100 mM, TriLink Biotechnologies – Catalog #N-1014)

3'-O-Me-m⁷G(5')ppp(5')G Cap Analog (New England Biolabs – Catalog #S1411)

Tail PCR Products (100 ng/μL)

RNA Purification

MEGAclean Kit (Ambion – Catalog #AM1908M)

Phosphatase Treatment

Antarctic phosphatase (New England Biolabs – Catalog #M0289)

Cre Recombinase ORF PCR Amplification

1 Perform 5' phosphorylation of the Cre ORF Forward primer.

1. Combine the following:

300 pmol primer (3 μL of 100 μL stock)

1 μL (10U) PNK enzyme

5 μL 10x buffer

50 nmol ATP (0.5 μL of 100 μL stock)

2. Incubate at 37 °C for 30 minutes
3. Heat-inactivate at 65 °C for 20 minutes
4. Add 250 μ L TE to make 1 μ M stock (100 μ L to make 2 μ M stock)
- 2 Amplify the Cre Recombinase ORF by PCR amplification. Perform a Gradient PCR around the T_m of the primers.
 1. Perform 25 μ L reactions by combining the following:
 - 12.5 μ L 2x KAPA HiFi Hotstart ReadyMix
 - 3.75 μ L Phosphorylated ORF Forward Primer (2 μ M)
 - 3.75 μ L ORF Reverse Primer (2 μ M)
 - Template DNA – 10–20 ng
 - H₂O to bring final volume to 25 μ L
 2. Thermal cycler profile:

95 °C	5 min		
98 °C	20 sec	}	25 cycles
Gradient	15 sec		
72 °C	60 sec		
72 °C	5 min		
4 °C	Hold		

3. Analyze on agarose gel (can use 1 μ L of PCR reaction diluted 10x)
- 3 Perform a QIAQuick purification to purify the gel-excised DNA product (CRE recombinase). Use QIAQuick Spin Columns.
 1. Combine 5 volumes of PB Buffer to 1 volume of reaction (5:1). Mix with pipettor and apply to column. Centrifuge at 6,000 rpm for 1 minute, and discard the flow-through.
 2. Wash with 750 μ L PE. Centrifuge at 6,000 rpm for 1 minute, and discard the flow-through.
 3. Centrifuge again at 6,000 rpm for 1 minute and place filter on a clean Eppendorf tube.
 4. Elute with water (nuclease-free) or EB buffer. Wait 1 minute before centrifuging at 6,000 rpm for 1 minute.
- 4 Quantify and quality control PCR product using Nanodrop machine.

UTR Ligation

- 5 Ligate the UTRs to the CRE recombinase ORF amplicon isolated above. Begin by combining the following for a 20 μ L reaction:
 - 4 μ L 5' UTR (1 μ M)
 - 4 μ L 3' UTR-Phosphorylated (1 μ M)

2 μ L 5' Splint (1 μ M)
 2 μ L 3' Splint (1 μ M)
 2 μ L 10x Buffer
 1 μ L Ampligase
 1 μ L CRE ORF amplicon
 4 μ L Water

6 Thermal profile for UTR Ligation:

95 °C	10 sec	}	5 cycles
45 °C	60 sec		
50 °C	60 sec		
55 °C	60 sec		
60 °C	60 sec		
4 °C	Hold		

Perform QIAQuick Purification as described above in the Cre Recombinase ORF PCR amplification protocol.

NOTE: It may be desirable to TA clone the newly generated UTR-flanked Cre amplicon into a plasmid to avoid the need to re-amplify from the Cre DNA plasmid. This can be accomplished by using the pcDNA3.3-TOPO TA Cloning Kit.

NOTE: Check and ensure the ligation worked by diagnostic PCR by using flanking primers and subsequently performing gel electrophoresis to double-check if the product is of the expected size. This is a critical juncture for troubleshooting—if the ligation is unsuccessful (it often requires multiple attempts) try either a wider/smaller gradient of temperatures or try a sequential ligation. The difficulty lies in that the two splints usually have different annealing conditions so both will not stick at once.

Tail PCR

- 7** This section will append 120 Ts to the above synthesized Cre ORF with newly ligated 5'UTR and 3'UTR. Begin by diluting the PCR product (Cre ORF with 5' UTR and 3'UTR) to 10 ng/ μ L.
- 8** Perform a 25 μ L PCR reaction by combining the following:
 - 12.5 μ L 2x KAPA HiFi Hotstart ReadyMix
 - 1 μ L Tail Forward Primer (5 μ M) – final concentration is 200 nM
 - 1 μ L Tail Reverse T₁₂₀ Primer (5 μ M) – final concentration is 200 nM
 - PCR product (10 ng/ μ L) – 10–20 ng
 - H₂O to bring final volume to 25 μ L
- 9** Thermal cycler profile for Tail PCR reaction:

95 °C	5 min	
98 °C	20 sec	} 25x
65 °C	15 sec	
72 °C	45 sec	
72 °C	10 min	
4 °C	Hold	

- 10 Analyze the PCR product via gel electrophoresis (can use 1 μ L of PCR reaction diluted 10x).
- 11 Perform QIAQuick Purification as described above in the Cre Recombinase ORF PCR amplification protocol.
- 12 Quantify and quality control the purification product by Nanodrop and dilute to 100 ng/ μ L

In Vitro Transcription of Modified Cre RNA

- 13 This section is for the in vitro transcription of modified Cre mRNA using the above stated protocol. Begin by preparing a nucleotide triphosphate (NTP) mix. This mix recipe is sufficient for 5.4 reactions and this proportion of Cap:GTP allows for ~80% of transcripts to be capped, though this can contribute to reduced yield. The cap powder should be dissolved in 35 μ L H₂O. The recipe is as follows:

	Mix Concentration	Desired Final Concentration
Cap	15 mM	6 mM
GTP	3.75 mM	1.5 mM
ATP	18.75 mM	7.5 mM
5methylCTP	18.75 mM	7.5 mM
PseudoUTP	18.75 mM	7.5 mM

- 14 For a 2x reaction (40 μ L), combine reagents in the following order in a PCR strip at room temperature. The NTP Mix and template should be thawed on ice, then moved to room temperature (but only for setting up the IVT, immediately return to ice afterward). The 10x Buffer can be pre-warmed to 37 °C. It is important to ensure there is no precipitate in the 10x Buffer. The template concentration should be pre-adjusted at 100 ng/ μ L.

Custom NTP Mix	16 μ L
10x Buffer (Kit)	4 μ L
Template (Tail PCR)	16 μ L (1.6 μ g)
T7 Enzyme Mix (Kit)	4 μ L
Total	40 μ L

- 15 Incubate the reaction at 37 °C for up to 12 hours.
- 16 Perform DNase treatment with 2 µL TURBO DNase (Kit) for each reaction. Gently mix and incubate at 37 °C for 15 min.
- 17 Perform purification with MEGAclean Kit as described in the manufacturer's instructions: Begin by pre-warming the 115 µL Elution Solution per reaction at 95 °C using PCR machine.
- 18 Bring the in vitro transcription (IVT) reaction volume to 100 µL by adding 58 µL of Elution Solution. Mix by pipetting and transfer the total volume to an RNase-free Eppendorf tube.
- 19 To the new RNase-free Eppendorf tube, add 350 µL Binding Solution Concentrate and mix by pipetting.
- 20 Add another 250 µL of 100% ethanol solution and mix by pipetting.
- 21 Transfer the sample to a filter cartridge (assembled on a collection tube).
- 22 Centrifuge for 1 min at 10,000 RPM. Take care to avoid the escape of glass fibers out of the column and into the collection tube. Discard the flow-through.
- 23 Wash each reaction with 500 µL Wash Solution and centrifuge for 1 min at 10,000 RPM. Discard the flow-through.
- 24 Repeat wash with 500 µL Wash Solution. Discard the flow-through.
- 25 Dry by centrifuging for 30 seconds at 10,000 RPM.
- 26 Place filter on a new collection tube and elute RNA by adding 50 µL of 95 °C Elution Buffer. Centrifuge for 1 min at 10,000 RPM.
- 27 Repeat the elution step with 50 µL of 95 °C Elution Buffer. Centrifuge for 1 minute at 10,000 RPM.
- 28 Quantify the RNA using a Nanodrop machine before proceeding to Antarctic phosphatase treatment and a second of purification by the MEGAclean Kit.
- 29 Perform Antarctic Phosphatase treatment by adding 10 µL 10x buffer to each reaction. Then add 3 µL Antarctic Phosphatase. Gently mix and incubate the reaction for 30 minutes at 37 °C. Optional: Inactivate reaction at for 5 minutes at 65 °C.
- 31 Perform RNA Purification with MEGAclean Kit as described above (Step 17 – 27).
- 32 Quantify the RNA using Nanodrop. Each 2x reaction will generally produce ~50 µg RNA (the expected yield of a 40 µL reaction is generally 40 – 60 µg).
- 33 Adjust the RNA concentration to 100 ng/µL using RNase-free TE buffer.
- 34 Aliquot RNA and store at –20 °C or –80 °C. –20 °C is preferable to reduce freeze/thaw cycles.

Note: To quality control RNA, load the RNA and run gel electrophoresis under native conditions. High quality RNA should result in the observation of a single band.

BASIC PROTOCOL 3: Efficient Excision of Reprogramming Factors Using mRNA Transfection of Cre Recombinase

This protocol describes the use of mRNA transfection of Cre recombinase to excise the reprogramming factor monovector from the primary human iPS cells generated in Protocol 1.

Materials

Matrigel hESC-Qualified Matrix (BD Biosciences Cat. No. 354277)

Growing hiPSCs generated with pE4-OSKM retrovirus (Basic Protocol 1) at 80% to 90% confluency in 60 mm dish

1X Dulbecco's PBS without calcium and magnesium (Mediatech, cat no. 21-040-cv)

mTeSR1 media (Stem Cell Technologies, Cat. No. 05850)

hESC media (see recipes)

Cre mRNA (Basic Protocol 2)

Opti-MEM reduced serum media (Invitrogen Cat. No. 31985-070)

B18R Interferon Inhibitor (Invitrogen Cat. No. 34-8185)

Accutase (Invitrogen Cat. No. A11105-01)

RNAiMax (Invitrogen Cat. No. 13778-100)

Coat 6-well plates with Matrigel

- 1 Please refer to Ludwig et al, 2006 for a more detailed description on appropriate storage and aliquoting of Matrigel. NOTE: Matrigel is frozen at -80°C , is liquid at 4°C , and gels rapidly at room temperature.
- 2 Place a sterile 15 ml conical tube and a cold, sterile bottle of DMEM/F12 into a sterile biosafety cabinet.
- 3 Remove a Matrigel aliquot (1 mg for two 6-well plates) from the -80°C freezer, and add 1 ml of cold DMEM/F12 to it.
- 4 Pipet up and down to dissolve to thaw and dissolve the Matrigel. Transfer to the 15 ml conical tube.
- 5 Dilute the Matrigel to 0.5 mg/6 well-plate. The final volume will be 1 ml/well. For a 1 mg aliquot, add an additional 11 ml of DMEM/F12 to the 15 ml conical.
- 6 For same-day use, allow one hour to coat. If plates are to be used more than one hour later, add an additional 1 ml of DMEM/F12 to each well to prevent drying.
- 7 If plates are to be stored, add an additional 1 ml of DMEM/F12 to each well, wrap in parafilm, and store it at $2-8^{\circ}\text{C}$ for 7–10 days.

Single cell dissociation and low-density plating of pE4 hiPSCs

- 8 Dissociate a pE4-human iPS cell line at 80% to 90% confluency maintained on CF1 feeders and standard KSR-bFGF conditions with Accutase into single cells in the presence of $10\text{ }\mu\text{m}$ Y-27632.

- 9 Plate the pE4-hiPSC line at clonal density (Define clonal density) in mTeSR1 media containing Y-27632 (10 μ m) to enable picking of Cre-excised clones.

Cre RNA Transfection

- 10 After allowing one or two days for recovery of pE4-hiPSCs from plating and single cell-dissociation, prepare to transfect with mRNA continuously for seven days.
- 11 Dilute 100 ng/ μ l of Cre RNA 5x in Opti-MEM basal media. Dilute 5 μ l of RNAiMAX per microgram of RNA 10x in Opti-MEM. Combine these two dilutions and incubate at room temperature for 15 minutes.
- 12 Take the six-well plate(s) of pE4 hiPSCs out of the 37 °C incubator. Feed each well with 2 ml of fresh mTeSR1 media and 50 – 100 ng/ml of B18R interferon inhibitor.
- 13 After changing the media, add RNA/RNAiMAX complex directly to the media.
- 14 After 4 – 6 hours, refresh each well with 2 ml of mTeSR1 media and 50 – 100 ng/ml of B18R interferon inhibitor. All media changes should contain B18R when RNA transfection is occurring.
- 15 Incubate the plates in the incubator overnight. and repeat this procedure (Step 11 – 14) every day for 7 days.
- 16 After the seventh day of RNA transfection, allow putative Cre-excised hiPSC clones to expand into colonies. After observing colony formation, seed a 6-well gelatinized plate with CF1 MEF feeder cells.

Expansion in KSR-bFGF/CF1 conditions

- 17 After observing colony formation, prepare to transfer Cre-mRNA transfected pE4-hiPSCs into standard hESC growth conditions (CF1 MEFs and KOSR-bFGF). Remove 6-well plate(s) of CF1 feeders from incubator and replace MEF media (10% FBS/DMEM) with standard human ES media (KOSR-bFGF) containing 10 μ m Y-27632.
- 18 Wash the 6-well plate containing the candidate Cre-excised human iPS cells twice with 2 ml of F12. Add 2 ml of F12 to the dish.
- 19 Pick Cre-mRNA transfected pE4-hiPSC colonies and transfer into CF1 plate. With 2 ml of F12 still in the dish, cut out an iPS colony and separate it from feeder cells under the stereo microscope with a 2- or 10ul pipette tip and pipettor. Transfer the colony to an individual well of the 6 well plate.
- 20 Expand candidate Cre-excised human iPS cell clones to at least 50% confluence to identify Cre-excised clones via genomic PCR and for additional characterization.

Screening for Transgene-Free hiPSC via Genomic PCR and Southern Blot

- 21 Collect genomic DNA using the Qiagen DNeasy Blood and Tissue Kit by following the manufacturer's instructions.
- 22 Run genomic DNA quantitative real-time PCR using a thermal cycler with primer sets forward 5' CATCGCAGCTTGGATACA 3' and reverse 5' CCGATGATTAATTGTCAA 3' that recognize pE4 retroviral vector.

- 23 Confirm transgene excision with Southern Blot. Make probe for southern blot by amplifying the pE4 retroviral vector with primer set forward 5' CAAGAGTTACTAACAGCC 3' and reverse 5' CCATGGTGAATTCCTAC 3' that recognize retroviral vector sequence. Digest genomic DNA with EcoRI and HindIII for Southern blot analysis. Please refer to (Sambrook et al., 1989) for Southern Blot protocol.
- 24 Multiple rounds of Cre-excision may need to be performed to obtain a transgene-free human iPS cell line. If there is failure of excision, perform a second round of single-cell seeding using Accutase and Y-27632 as described above.

REAGENTS AND SOLUTIONS

For supplier, see supplier's Appendix

Human fibroblast medium (10% FBS medium, also used for CF-1 feeders)

DMEM (e.g. Invitrogen) containing:

10% fetal bovine serum (FBS)

50 U penicillin/50 µg/ml streptomycin (Invitrogen, Cat No. 15070063)

293T medium

DMEM (e.g. Invitrogen) containing:

10% fetal bovine serum (FBS)

2 mM L-glutamine (Invitrogen, Cat No. 25030-081)

1×10^{-4} M nonessential amino acids (Invitrogen, Cat No. 11140-050)

1 mM sodium pyruvate (Sigma-Aldrich, Cat No. S8636)

50 U penicillin/50 µg/ml streptomycin (Invitrogen, Cat No. 15070063)

hESC growth medium (KSR-bFGF)

DMEM/F12 medium containing:

DMEM/F12 (Invitrogen, Cat No. 10565-018)

20% Knockout Serum Replacement (KSR) (Invitrogen, Cat No. 10828028)

2 mM L-glutamine (Invitrogen, Cat No. 25030-081)

1×10^{-4} M nonessential amino acids (Invitrogen, Cat No. 11140-050)

1×10^{-4} M 2-mercaptoethanol (Invitrogen, Cat No. 21985-023)

50 U penicillin/50 µg/ml streptomycin (Invitrogen, Cat No. 15070063)

5 ng/ml Recombinant bFGF (Invitrogen, cat. no. 13256-029)

- i. To prepare PBS containing 0.1% bovine serum albumin (BSA), add 50 µl of 10% BSA into 5 ml of PBS. Dissolve 50 µg of bFGF in 5 ml of PBS containing 0.1%

BSA (10 µg/ml). Aliquot and store at –20 °C. Add 0.2 ml of 10 µg/ml bFGF into 500 ml of hESC media before use.

mTeSR1 medium (mTeSR1) (Stem Cell Technologies, Cat. No. 05850)

hiPS Cell Freezing Medium—20% DMSO (Sigma Aldrich, Cat No. 494429)

20% FBS

60% hESC medium (see above)

Equipment (BL2+)

- Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Cat No. ND-1000)
- Inverted tissue culture microscope with phase contrast microscope (x4, x10, x20, x40 objectives)
- Stereomicroscope (Nikon; SMZ-1500)
- Biosafety cabinet with aspirator for tissue culture
- Biosafety cabinet with aspirator for tissue culture, fitted for stereomicroscope

Biosafety cabinet with aspirator for tissue culture

- CO2 incubator, 37 C, humidity
- Mini centrifuge (VWR, Galaxy Mini)
- Beckman XL-90 Ultracentrifuge
- 6-well tissue culture plates (VWR, 73520-906)
- Disposable sterile filter system, 0.2 micron, 500 ml (Corning 430758)
- 6-well and 12-well tissue culture plate
- 100 mm tissue culture plate
- 15 ml conical tube (Falcon)
- 1 ml, 5 ml, 10 ml, 25 ml plastic disposable pipette

COMMENTARY

Background Information

The ability to generate patient-specific pluripotent stem cells is anticipated to contribute significantly to regenerative medicine (Takahashi *et al.*, 2006; Takahashi *et al.*, 2007; Yu *et al.*, 2007; Park *et al.*, 2008a; Park *et al.*, 2008b). The protocol described here allows this important cell type to resemble human ES cells even more closely by eliminating the integrated transgenes that may predispose more traditional iPS lines towards residual transgene-mediated differentiation bias or malignant transformation (Soldner *et al.*, 2009). Although various methods have been devised to generate both murine and human iPS cells without integrated transgenes (Anokye-Danso, *et al.* 2011; Fusaki *et al.*, 2009; Kim *et al.*, 2009; Okita *et al.*, 2008; Stadtfeld *et al.*, 2008; Woltjen *et al.*, 2009; Yu *et al.*, 2009; Zhou *et al.*, 2009), most protocols to date are limited by extremely low efficiency and technical difficulties. One possible solution to generate research-grade transgene-free human iPS cells is to combine the high efficiency of viral transduction with minimal genetic perturbation. The protocol herein details the generation and characterization of transgene-free human iPS

cells using a retroviral construct that expresses four reprogramming factors in tandem on a single “monovector.” Flanking loxP sites in the viral LTRs facilitate proviral excision following transient mRNA expression of Cre-recombinase, and the resulting human iPS cells are pluripotent. The described method’s chief advantage is the ease and efficiency of generating transgene-free lines, which promise improved performance *in vitro* and represent invaluable tools for medical research. Although Cre-mediated excision of the provirus removes all transgenes, a single LoxP remains at each integration site, leaving the possibility that nearby genes could be disturbed. Analysis of the sequences flanking the integration sites and exhaustive characterization could mitigate safety concerns.

Critical Parameters

Several critical parameters have previously been reported to critically influence observation of reprogramming events in human cells (Ohnuki *et al.*, 2009; Park *et al.*, 2009). Briefly, first, the quality and titer of the retrovirus is critical; a multiplicity of infection (MOI) of 5 is used for reprogramming with the pE4 monovector. Second, it is desirable to use high-quality, low passage fibroblasts and to avoid the use of senescent fibroblasts for reprogramming.

When attempting to ligate the UTRs to the ORF, it may be difficult to find ligation conditions that allow for simultaneous annealing of both splints. A gradient temperature approach has been described here and in earlier literature (Warren *et al.*, 2010) in an attempt to avoid this difficulty, but if the double ligation proves too difficult a sequential ligation can be completed instead. This approach would add first the 3′ UTR, then in a separate reaction add the 5′ UTR. A sequential ligation allows for annealing conditions to be designed specifically for a particular splint.

If necessary, the duration of the *in vitro* reaction may be adjusted somewhat from the 12 hours described in this protocol. The manufacturer’s instructions suggest a 2 – 4 hour reaction that can be extended up to 16 hours in order to increase yield. Increasing the amount of template included in the reaction may also enhance yield. Once the *in vitro* transcription reaction is completed, it is necessary to maintain strict RNase-free laboratory procedure in order to prevent degradation of the Cre mRNA.

The copy number of the monovector can influence the ease of Cre recombinase-mediated excision. Human iPS cell clones that contain multiple copies of the transgenes may require multiple rounds of Cre mRNA transfection to achieve removal of all integrated reprogramming factors. It is desirable to pre-select human iPS clones with a low transgene copy number (single copy is preferable) to reduce the number of rounds of Cre mRNA transfection and the number of LoxP scars left behind. Additionally, when inducing Cre-mediated proviral excision, chromosome rearrangement and/or translocations may form via recombination among multiple loxP sites (Yu and Bradley, 2001). We have not yet observed this in our experiments.

Troubleshooting

It is important to distinguish between partially and bona fide reprogrammed colonies. During primary reprogramming of human fibroblasts, early granulated colonies appear; clear-edged colonies that are more hESC-like in morphology appear later and these are more likely to represent human iPS cells. Additionally, our lab has found that expression of TRA-1-60, DNMT3B, and REX1 more specifically identifies faithfully reprogrammed colonies (Chan *et al.*, 2009).

Anticipated Results

We observed similar primary reprogramming efficiency between monovector reprogramming and multiple-retrovirus reprogramming. After isolation of transgene-free human iPS cells, most clones show hESC-like proliferation and morphology, including defined colonies with a tightly-packed epithelial morphology and cells with high nucleus-to-cytoplasm ratio, in hES cell culture condition. Most clones express hESC marker genes, such as OCT4, NANOG, SSEA-4 and TRA-1-60. When subcutaneously injected into immunodeficient Rag2^{-/-}GammaC^{-/-} mice, most iPSCs develop mature teratomas containing tissue derived from all three germ layers, demonstrating pluripotency.

Although Cre-mediated excision of the provirus removes all transgenes, a single LoxP remains at each integration site, leaving open the possibility that nearby genes could be disturbed. Considering the low efficiency and difficulty involved in techniques for reprogramming cells using entirely non-genetic means, analysis of the sequences flanking the integration sites and exhaustive characterization could mitigate safety concerns.

Time Considerations

It takes about 5 days to prepare the retrovirus for infection of human fibroblasts. Once plated on CF1 feeder cells, an additional 3 – 5 weeks are required for iPS cell colonies to appear and grow to a size where they can be passaged. It takes over 1 – 2 months to establish a human iPS cell line containing the transgenes. After expansion, basic characterization, and cryopreservation of the hiPS cell line occurs, it will take about 1 week to perform the mRNA-based Cre excision and another 1–2 months to screen, expand, characterize, and cryopreserve transgene-free hiPSCs.

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Figure 1. Generation of transgene-free human iPS cells using Cre-excisable 2A-linked 4-Factor retrovirus and Cre recombinase mRNA transfection

(A) General experimental scheme.

(B) Diagram of pE4-OSKM reprogramming monovector.

(C) Detailed experimental scheme.

Figure 2. Transgene-free human iPS cells after excising monovector via mRNA transfection of Cre

(A) Immunohistochemistry for Cre protein following RNA transfection.

(B) Genomic PCR analysis with primers recognizing monovector backbone shows the absence of monovector in Cre-excised human iPS cell line.

(C) Southern blot analysis with a probe specific to the monovector to confirm excision of reprogramming transgenes.

(D) Phase-contrast images of Cre-excised established human iPS cells growing on CF1 feeders. Immunohistochemistry for undifferentiated pluripotent cell markers: OCT4 (E), (F), SSEA3 (F), and TRA-1-60 (G). (H–K) *In vitro* differentiation of Cre-excised human iPS cell line into mesoderm, ectoderm, and endoderm. (H) Phase-contrast image of embryoid body formation of Cre-excised human iPS cell line after 13 days. (I) Brachyury immunoreactivity following spontaneous embryoid body differentiation in the presence of 10% FBS. (J) Keratin 14 immunoreactivity following spontaneous embryoid body differentiation in serum-free medium. (K) Alpha-feto protein immunoreactivity following directed

endodermal differentiation of hiPSC in WNT3A and ACTIVIN. (L–N) Teratoma formation of primary human iPS cell line into mesoderm (L), ectoderm (M), or endoderm (N).