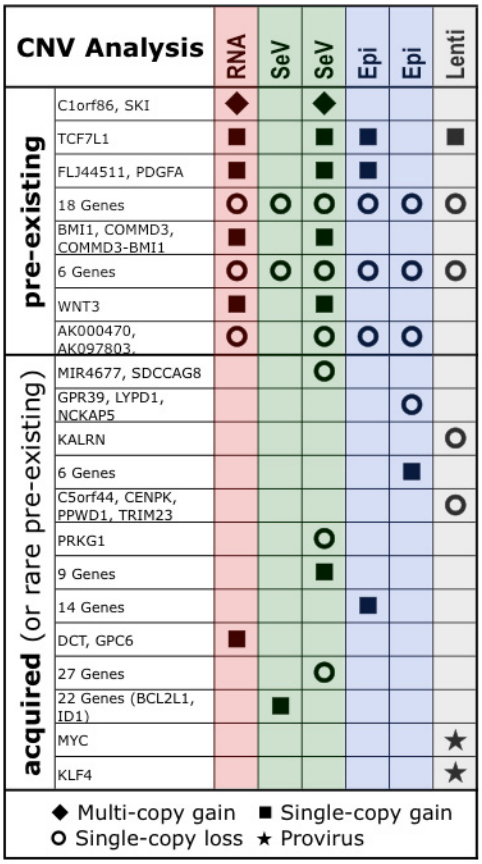
Supplementary Figure 1:

Aneuploidies and Copy Number Variations

A Karyotype Abnormalities

P#	Method	Karyotype change(s) [# of cells]
13	mRNA	add(12)(q13q15)[7]/–[15]
6	SeV	t(8;11)(q24.3;q13.5)[7]/–[13]
7	SeV	t(1;12)(q21;q22)[4]/–[15]
6	SeV	+X[19]
3	SeV	t(X;6)(q22.1;q21)[15]/t(X;6)(q22.1;q21),der(14)t(12;14)(q13.3;p12)[3]
5	SeV	add(17)(p11.2),add(17)(q25.3)[20]
11	SeV	inv(7)(p21.2p22.3)[20]
11	SeV	der(16)t(1;16)(q42.1;p13.3)[20]
5	Epi	t(1;14)(p31.2;q32.1)[20]
5	Epi	t(5;5)(p15.1;q22)[20]
8	Epi	t(1;10)(p13.1;q21.2)[2]/–[17]
8	Epi	der(1)t(1;1)(p36.3;q21)[20]
12	Epi	del(2)(q23q31)[20]
7	Epi	+1[2]/–[18]
9	Epi	+12[19]

B Copy Number Variations



Nature Biotechnology: doi:10.1038/nbt.3070

Supplementary Figure 1: Aneuploidies and Copy Number Variations

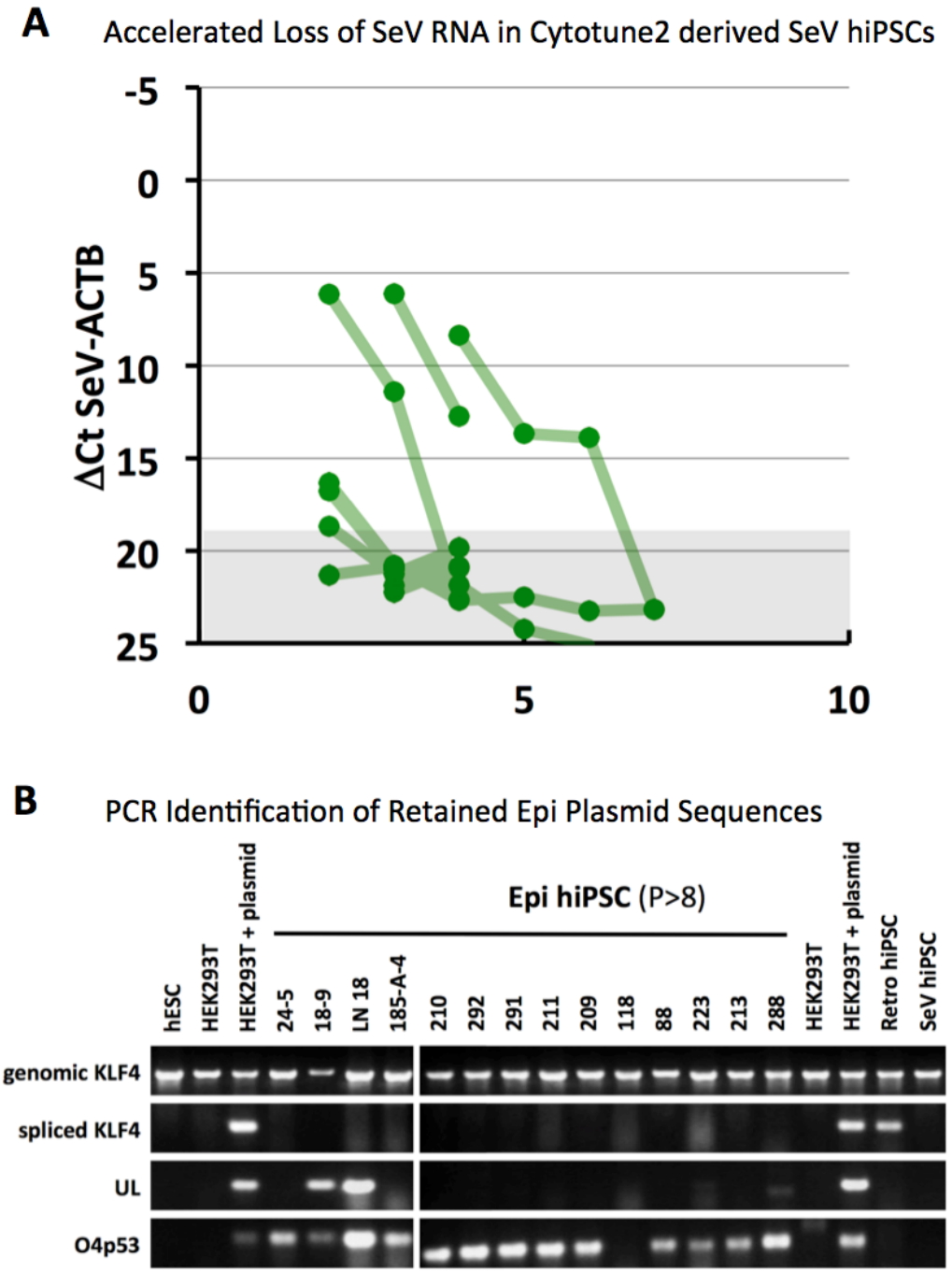
(A) Summary table with the precise aneuploidies, as demonstrated by G-banding cytogenetic analysis (Cell Line Genetics).

(B) Patient-derived primary dermal fibroblasts were reprogrammed using standard reprogramming protocols and DNA from resulting hiPSC lines (passage 15-16) and from parental fibroblasts were analyzed for CNVs using the StemArray^{TM} platform that covers the entire genome at an average resolution of 15kb, with increased probe coverage for 60 pluripotency-associated and >200 cancer-associated genes (Cell Line Genetics). Copy-number losses, low-copy gains, and high-copy gains were identified for the indicated loci. Two of the reprogramming factor genes were detected as gains exclusively in the (non-excised) Lenti hiPSC sample, suggesting that the platform is both sensitive and specific.

Nature Biotechnology: doi:10.1038/nbt.3070

Supplementary Figure 2:

Retention of Reprogramming Agents



Nature Biotechnology: doi:10.1038/nbt.3070

Supplementary Figure 2: Retention of Reprogramming Agents

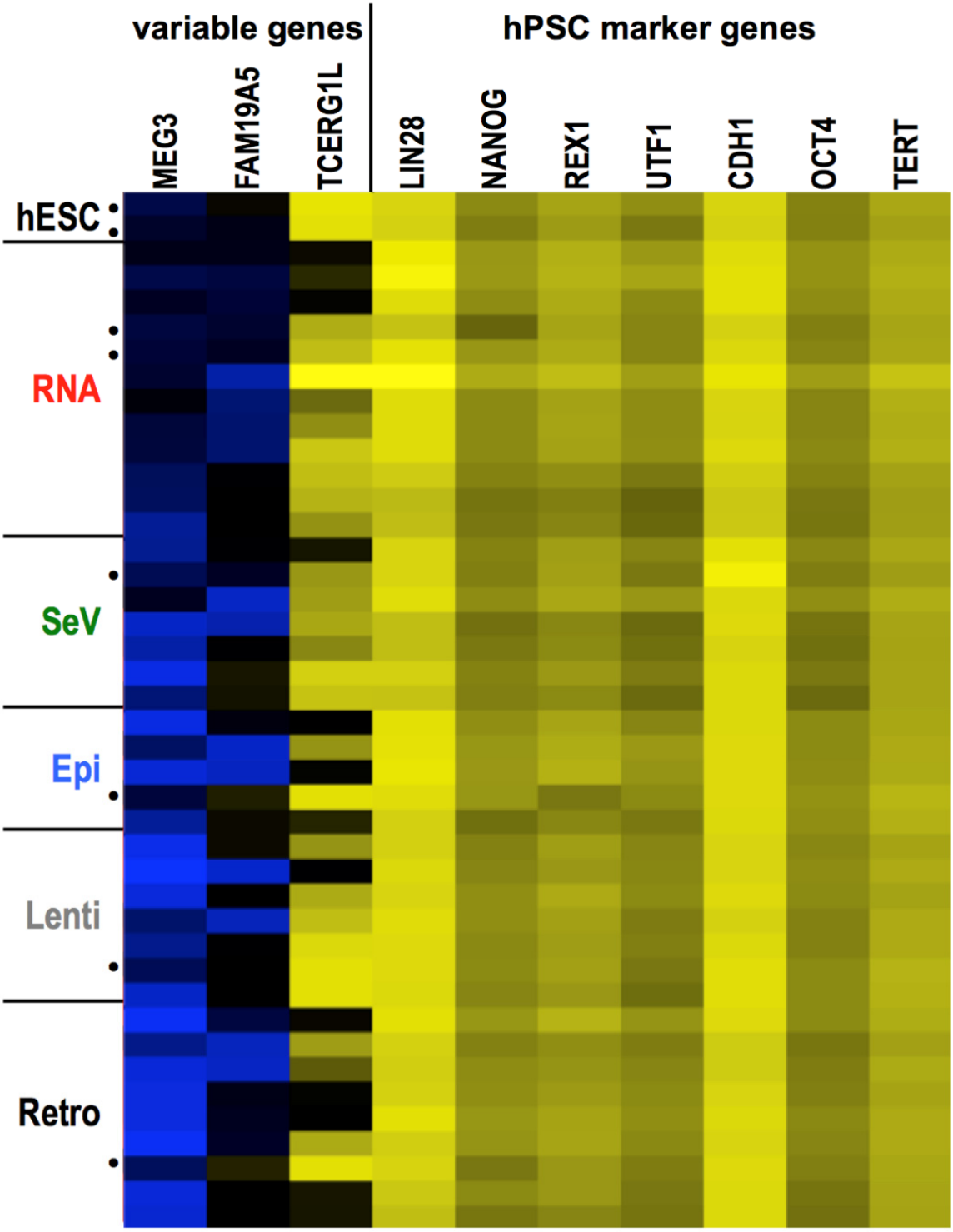
(A) Accelerated loss of SeV RNA in SeV hiPSCs derived using the Cytotune2^{TM} (Life Technologies) reprogramming kit. In contrast to the original Cytotune SeV reprogramming kit (Figure 2B), a majority of Cytotune2 SeV hiPSCs are SeV RNA PCR-negative by passage 5.

(B) Identification of the plasmids in higher-passage (P>8) Epi DNA^{high} hiPSC lines. DNA harvested from 293T cells transfected with the three plasmids served as positive controls. Retroviral hiPSC contain the spliced KLF4 gene as an integrated provirus whereas the O4p53 and the UL PCRs are specific for the episomal plasmids. Sample D118 was EBNA1 PCR-positive but does not appear to contain the PCR target sequences used here, indicating that these cells did not retain complete plasmid sequences.

Nature Biotechnology: doi:10.1038/nbt.3070

Supplementary Figure 3:

TaqMan Gene Expression Analysis



Nature Biotechnology: doi:10.1038/nbt.3070

Supplementary Figure 3: TaqMan Gene Expression Analysis

TaqMan gene expression analysis of known human pluripotent stem cell marker genes and of genes previously described as divergently expressed between hESC and hiPSCs. Expression levels are normalized to the mean of the expression levels of three control genes (TBP, GAPDH, and ACTB) and color-coded on logarithmic color intensity scale relative to fibroblasts (black = unchanged, yellow = induced in stem cells, blue = repressed in stem cells). Dots mark stem cell lines with a hESC-like expression pattern.

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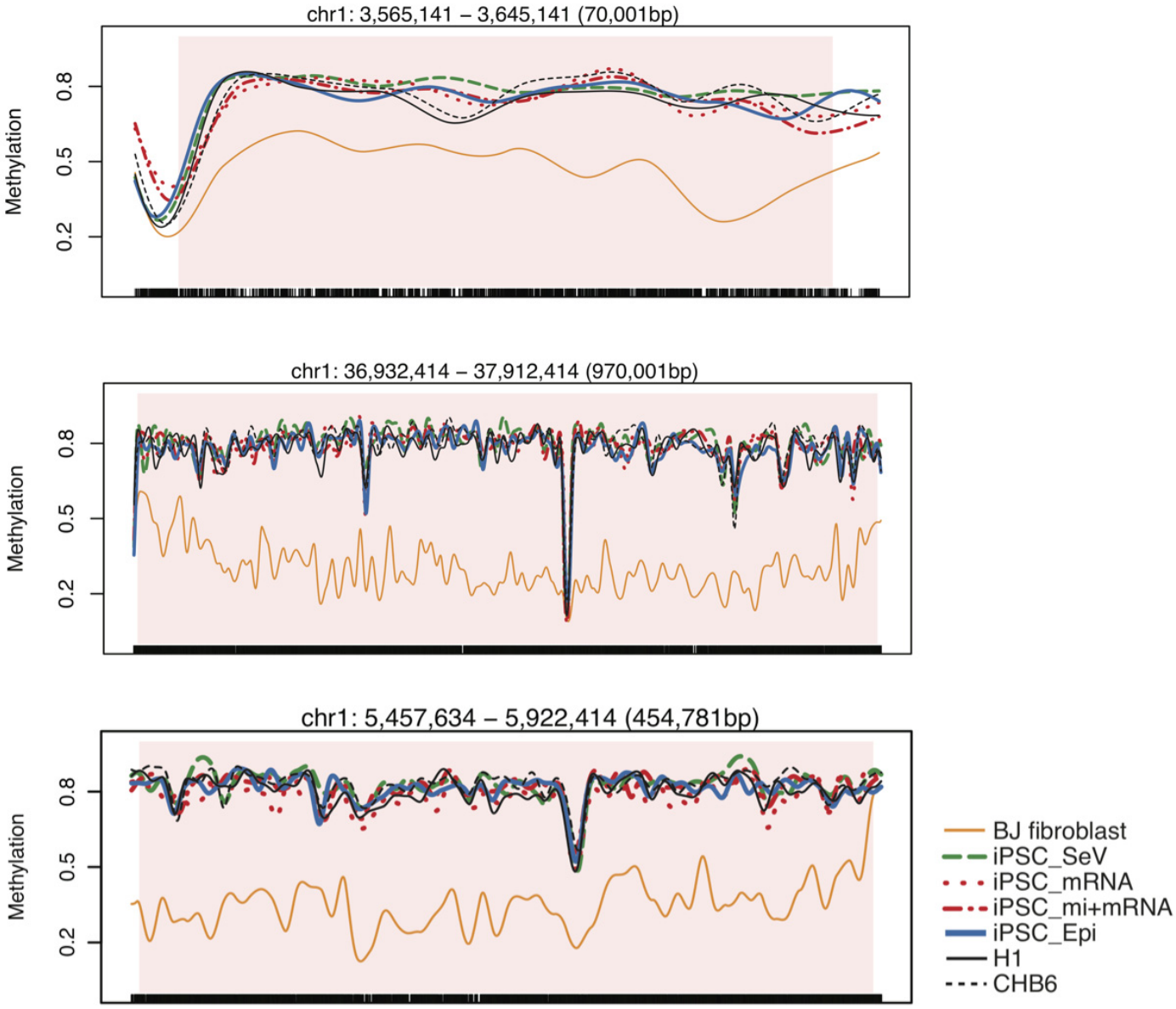
Supplementary Figure 4:

Methylation levels of PMDs in hESCs, hiPSC, and parental fibroblasts



Nature Biotechnology: doi:10.1038/nbt.3070

Supplementary Figure 4: Methylation levels of PMDs in hESCs, hiPSC, and parental fibroblasts

Comparison of genomic DNA CpG methylation levels over partially methylated domains (PMDs) that are hypo-methylated in parental fibroblasts. Ticks at the bottom of the figures represent CpGs. Shown are smoothed methylation values from whole genome bisulfite sequencing for fibroblasts, hiPSC, and hESC lines. Genomic DNA methylation patterns of hiPSCs are indistinguishable from each other, and very similar to methylation patterns of hESCs (hierarchical cluster analysis is provided in **Figure 2G**).

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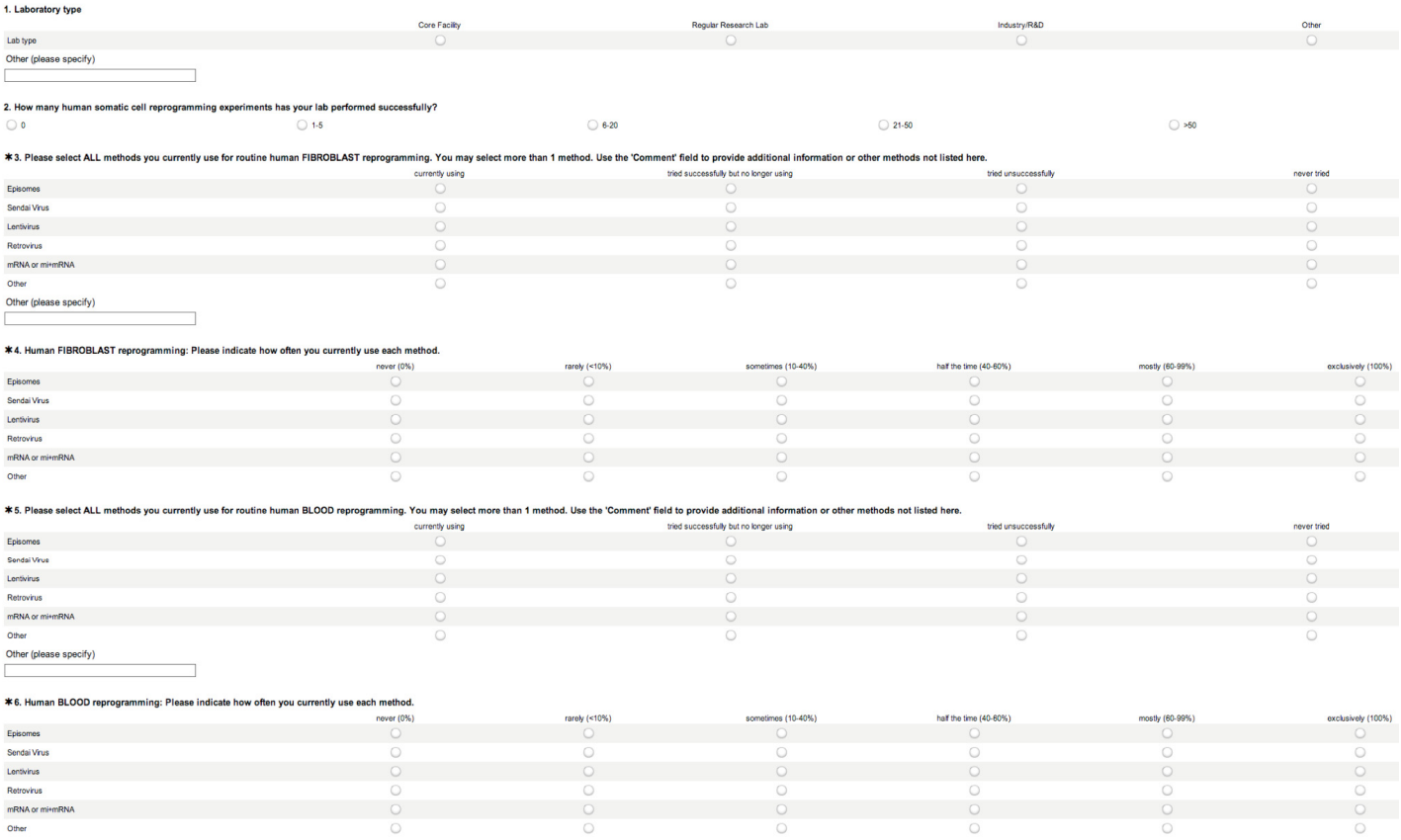
Supplementary Figure 5:

Online Survey questions



1. Laboratory type

	Core Facility	Regular Research Lab	Industry/R&D	Other
Lab type	○	○	○	○

Other (please specify)

2. How many human somatic cell reprogramming experiments has your lab performed successfully?

○ 0 ○ 1-5 ○ 6-20 ○ 21-50 ○ >50

***3. Please select ALL methods you currently use for routine human FIBROBLAST reprogramming. You may select more than 1 method. Use the 'Comment' field to provide additional information or other methods not listed here.**

	currently using	tried successfully but no longer using	tried unsuccessfully	never tried
Episomes	○	○	○	○
Sendai Virus	○	○	○	○
Lentivirus	○	○	○	○
Retrovirus	○	○	○	○
mRNA or miRNA	○	○	○	○
Other	○	○	○	○

Other (please specify)

***4. Human FIBROBLAST reprogramming: Please indicate how often you currently use each method.**

	never (0%)	rarely (<10%)	sometimes (10-40%)	half the time (40-60%)	mostly (60-99%)	exclusively (100%)
Episomes	○	○	○	○	○	○
Sendai Virus	○	○	○	○	○	○
Lentivirus	○	○	○	○	○	○
Retrovirus	○	○	○	○	○	○
mRNA or miRNA	○	○	○	○	○	○
Other	○	○	○	○	○	○

***5. Please select ALL methods you currently use for routine human BLOOD reprogramming. You may select more than 1 method. Use the 'Comment' field to provide additional information or other methods not listed here.**

	currently using	tried successfully but no longer using	tried unsuccessfully	never tried
Episomes	○	○	○	○
Sendai Virus	○	○	○	○
Lentivirus	○	○	○	○
Retrovirus	○	○	○	○
mRNA or miRNA	○	○	○	○
Other	○	○	○	○

Other (please specify)

***6. Human BLOOD reprogramming: Please indicate how often you currently use each method.**

	never (0%)	rarely (<10%)	sometimes (10-40%)	half the time (40-60%)	mostly (60-99%)	exclusively (100%)
Episomes	○	○	○	○	○	○
Sendai Virus	○	○	○	○	○	○
Lentivirus	○	○	○	○	○	○
Retrovirus	○	○	○	○	○	○
mRNA or miRNA	○	○	○	○	○	○
Other	○	○	○	○	○	○

Supplementary Figure 5: Online Survey questions.

Screenshot of the online survey. The survey was developed and conducted using Surveymonkey (surveymonkey.com)

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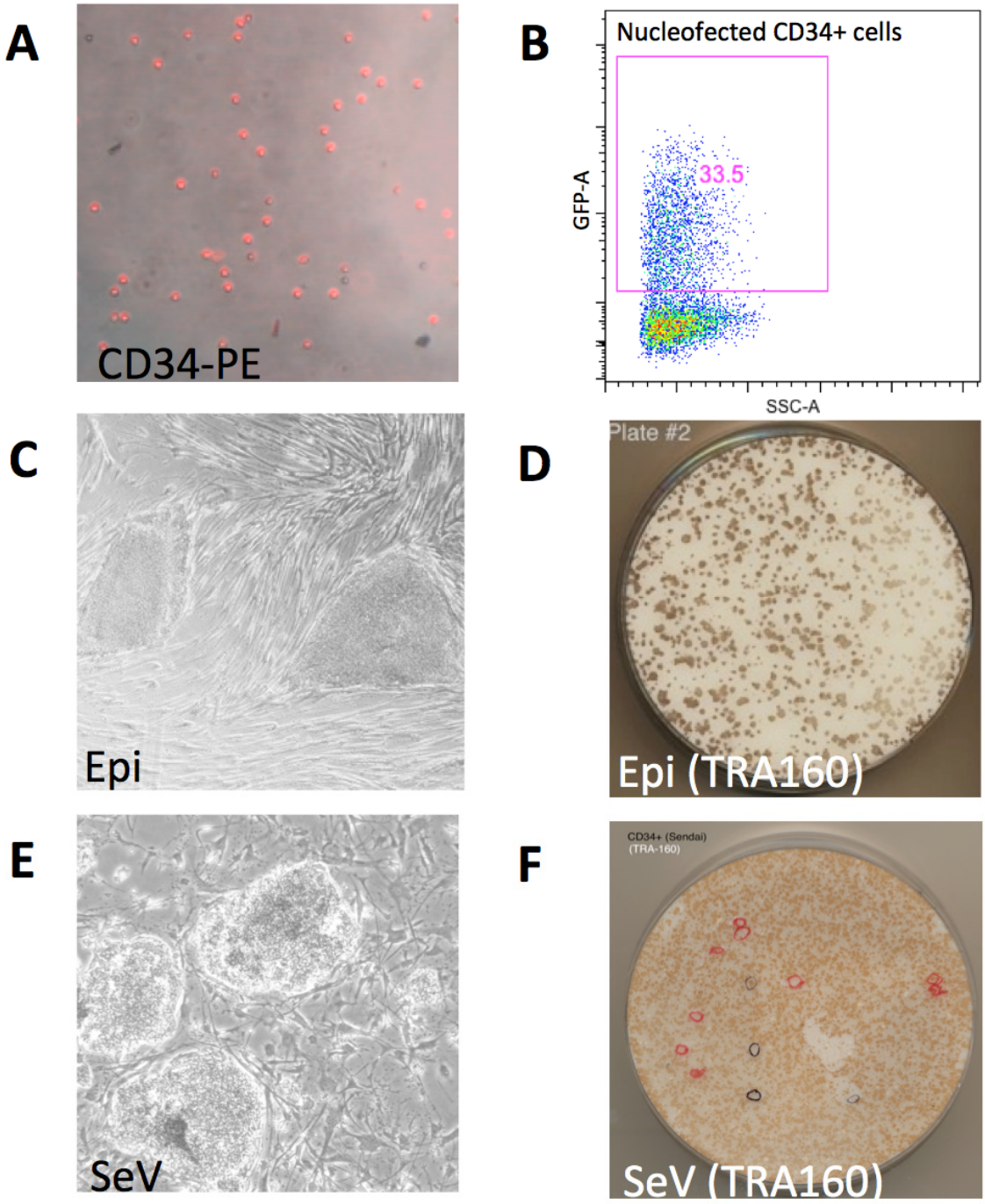
Supplementary Figure 6:

Human blood (CD34+) reprogramming.



Nature Biotechnology: doi:10.1038/nbt.3070

Supplementary Figure 6: Human blood (CD34+) reprogramming.

A) CD34+ cells were enriched from mononuclear cells using magnetic beads (Miltenyi; anti-CD34-PE immunofluorescence confirms enrichment of CD34+ cells)
B) CD34+ cells were nucleofected using a GFP construct using the Amaxa nucleofection kit for human CD34+ cells
C) hiPSC colonies emerging in a hCD34+ cell episomal reprogramming experiment (Phase contrast).
D) TRA160 immunocytochemistry was performed to determine the number of emerging hCD34 Epi hiPSC colonies.
E) Emerging hCD34+ derived SeV (Cytotune 2) hiPSC colonies (Phase contrast).
F) TRA160 immunocytochemistry was used to determine the number of emerging hCD34 SeV hiPSCs colonies.

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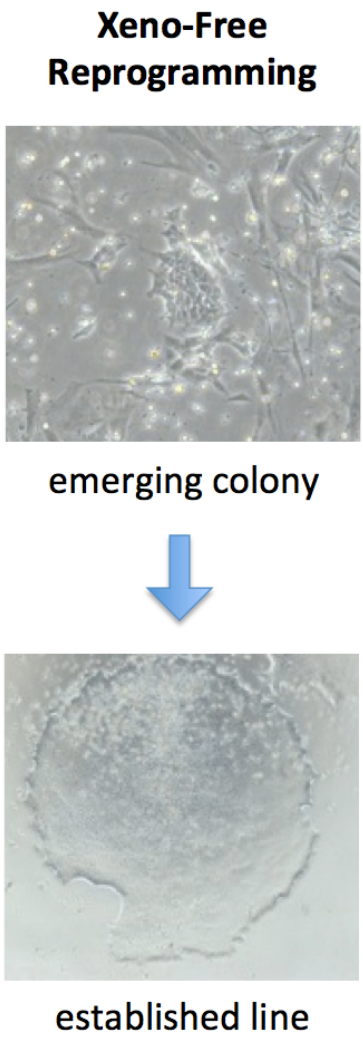
Supplementary Figure 7:

Comparison of conventional and xeno-free human blood reprogramming using episomal vectors.

Blood Reprogramming (Epi) – Overview			
Process	**Agents**	**Regular** (CD34+)	**Xeno-free** (PBMNC)
Isolation	Kit	Anti-CD34 Microbeads	Use cGMP-grade Ficoll (Ficoll-Paque PREMIUM, GE-Healthcare, #R8758)-purified PBMNCs
	Medium	StemSpan SFEM	StemSpan H3000 or CellGenix CellGro GMP SCGM
	Cytokines	CC100	cGMP-grade hFlt3, SCF, IL3, IL6
Transfection	Nucleofection-Solution	CD34 Kit (program U-008)	Kit V (program T-20)
Recovery	Recovery medium	RPMI, FCS	Fibrolife Xeno-Free fibroblast medium
Reprogramming (1)	Feeders	BM derived MSCs (irradiated)	cGMP-grade MRC5 (irradiated)
	Matrix	Retronectin	none
	Medium, CKs	SFEM, CC100	Feeder plating: E8-Fib H3000 or CellGenix CellGro
Splitting	Enzyme	Trypsin	TrypLE-Select
Reprogramming (2)	Feeders	MEFs	none
	Matrix	Gelatin	Cellstart
	Medium	SFEM, CC100; transitioning to D-F12/KOSR/FGF	H3000/CellGro, Cytokines; transitioning to E8
Passaging	Enzyme	Collagenase IV	EZ-Passage or Collagenase-NB6 (cGMP-grade)
Cryo-preservation	Cryo-protectants	DMSO+FCS	CryoStor CS10



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Supplementary Figure 7: Comparison of conventional and xeno-free human blood reprogramming using episomal vectors.

The table summarizes key differences between the conventional protocol and the xeno-free protocol. The main reason for using PBMNCs in the xeno-free protocol is the lack of a cGMP-grade CD34+ cell purification kit for small volumes of blood. cGMP-grade MRC5 cells were obtained for the Center for Human Cell Therapy (CHCT, Dr. Myriam Armant). A detailed description of the method is provided as a supplementary document.

Nature Biotechnology: doi:10.1038/nbt.3070

Supplementary Table 1:

Usage Restriction

	Cat. #	Vendor	Links to documents specifying kit usage restrictions	Contact for requesting additional rights	References
RNA	00-0071	Stemgent	http://assets.stemgent.com/files/1310/original/Stemgent_mRNAUserManual_2012.pdf http://assets.stemgent.com/files/1328/original/Limited%20Use%20License_MIT.pdf http://assets.stemgent.com/files/1329/original/Limited%20Use%20License_iPS%20Academia%20Japan.pdf	tlo@mit.edu techsupport@stemgent.com	1. Warren L, et al. (2010) Cell Stem Cell 7:618-630 2. Warren L, et al. (2012) Sci Rep 2:657. 3. Mandal PK, Rossi DJ (2013) Nat Protoc 8:568-82
SeV	A1378002	LifeTechn.	http://tools.lifetechnologies.com/content/sfs/manuals/CytoTune_iPS_Reprogramming_Kit_man.pdf https://tools.lifetechnologies.com/search/index.cfm?fuseaction=search.lullsearch&lulid=505 https://tools.lifetechnologies.com/search/index.cfm?fuseaction=search.lullsearch&lulid=518	cytotune@dnavec-corp.com outlicensing@lifetechnologies.com	1. Fusaki, N., et al. (2009) Proc Jpn Acad Ser B Phys Biol Sci 85:348-362 2. Macarthur CC, et al. (2012) Stem Cells Int 2012:564612
Epi	27077 27078 27080	Addgene	http://www.addgene.org/terms/1047/ http://www.addgene.org/terms/1041/	yamanaka-mta@addgene.org mta@addgene.org	1. Okita K, et al. (2011) Nat Methods 8:409-12 2. Okita K, et al. (2013) Stem Cells 31:458-466 3. Goh PA, et al. (2013) PLoS One 2013 8:e81622 4. Nakagawa, et al. (2014) Sci Reports 4:3594.
	A15960	LifeTechn.	http://tools.lifetechnologies.com/content/sfs/manuals/epi5_episomal_ipsc_reprogramming_man.pdf https://tools.lifetechnologies.com/search/index.cfm?fuseaction=search.lullsearch&lulid=465	outlicensing@lifetechnologies.com	
Lenti	SCR545	Millipore	http://www.emdmillipore.com/INTL/en/product/Human-STEMCCA-Cre-Excisable-Constitutive-Polycistronic-(OKSM)-Lentivirus-Reprogramming-Kit,MM_NF-SCR545#documentation	marie.azzaria@emdmillipore.com gmostosl@bu.edu	1. Sommer CA, et al. (2009) Stem Cells 27:543-549 2. Sommers CA, et al. (2010) Stem Cells. 28:64-74 3. Sommers A, et al. (2010) Stem Cells 28:1728-1740 4. Awe JP, et al. (2013) Stem Cell Res Ther. 26:87

Supplementary Table 1: Usage Restriction

The commercial reprogramming kits shown in this table are generally sold for exclusively non-commercial, non-clinical (therapeutic or diagnostic) *in vitro* research or educational use. In some cases, the reagents can be acquired under less-restrictive stipulations from the originating research group (e.g., the STEMCCA Lenti plasmid is available from Prof. Gustavo Mostoslavsky, Boston University; gmostosl@bu.edu). However, additional intellectual property rights must still be observed; in the hiPSC field, many patents are held by Academia Japan (AJ; license@ips-ac.co.jp).

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Supplementary Method: Xeno-free Episomal reprogramming of PBMNCs

Reagents
10mL peripheral blood, drawn into heparin-coated tubes
RPMI-1640 medium (Sigma, cat no. R8758)
Ficoll-Paque PREMIUM (GE Healthcare, cat no. 17-5442-02)
PBS+/+ (Gibco, cat no. 14040-133)
0.5M EDTA (Gibco, cat no. 15575-038)
StemSpan H3000 medium (Stemcell Tech, cat no. 09850)
StemSpan CC100 cytokine mix (Stemcell Tech, cat no. 02690)
Bleach
0.1% gelatin
Amaxa Nucleofector kit V (Lonza)
Fibrolife xeno-free complete medium (Lifeline)
Endo-free maxipreps (Qiagen) of 3x Yamanaka episomal reprogramming plasmids (Addgene)
TrypLE-Select (Lifetech)
cGMP-grade MRC5 cells (kindly provided by Dr. Myriam Armant, CHCT, Boston)

E8 Medium (Lifetech or home-made):
98 ml DMEM/F12 (StemCell Technologies 36254)
64 µl MG Ascorbate-2-PO (Sigma A8960; 100mg/ml in H2O; -80°C)
28 µl Selenium (Sigma S5261; 0.05mg/ml in H2O; -20°C)
20 µl hTransferrin (Sigma T0665-1G; 30mg/ml in IMDM; -80°C)
1 ml Insulin (Sigma I9278; 2mg/ml in H2O; -20°C)
10 µg hFGF2 (R&D 233-GMP-025)
200ng TGFβ1 (R&D 240-GMP-010)

E8Fib Medium is E8 with rh-EFG and hydrocortisone added:
hydrocortisone (100uL/100mL; 100uM stock; Sigma H0135-1MG)
rh-EGF (100uL/100mL; R&D 236-GMP-200)

Supplies
15mL and 50mL conical tubes
5mL glass serological pipettes
Benchtop swinging-bucket centrifuge (eg. Beckman-Coulter Allegra 25R Centrifuge)
40um cell strainer
6w ultra-low attachment tissue culture plates
6w and 10cm tissue culture plates
Biohazard waste collection bags

PPE *(check with your institutional safety office)*
Lab coat
Double gloves

Safety glasses

Day -20: Thaw MRC5 fibroblasts for expansion • TIMING 20m

1. *Pre-warm E8Fib medium aliquots.* In 15mL conical tubes, prepare 2 x 10mL aliquots of E8Fib medium. Place on a 37°C heat block.

2. Retrieve 1 x 1M vial of MRC5 fibroblasts from LN_2 storage and thaw in a 37°C 70% isopropanol bath for approximately 2 minutes, or until a small frozen pellet remains in the tube.

- CRITICAL STEP Do not over-thaw the vial. Ensure that a small frozen pellet remains when the vial is removed from the isopropanol bath.

3. Using a P1000 pipetman, add 1mL E8Fib medium from one of the 10mL aliquots, dropwise, to the vial.

4. Add the cell suspension to the 9mL E8Fib remaining in the conical tube.

5. Centrifuge at 200 x g for 4 minutes.

6. Aspirate supernatant and resuspend the pellet in 10mL pre-warmed E8Fib medium.

7. Add the cell suspension to a tissue culture-treated 10cm dish.

8. Place the dish in a 37°C incubator and shake to evenly distribute cells. Do not move the dish for at least 12 hours while the cells adhere.

Day -18: Expand MRC5 fibroblasts for preparation of irradiated feeder cells
Timing: Approximately 2 week expansion; 5m on feeding days, 30m on splitting days

1. Feed fibroblasts every other day by aspirating spent medium and adding 10mL fresh, pre-warmed E8Fib medium to the dish.

2. When the 10cm dish reaches 80% confluency, split and expand by following steps 3-8, below.

3. *Pre-warm E8Fib medium.* Prepare 1 x 10mL aliquot of E8Fib medium in a 15mL conical tube, and 1 x 50mL aliquot in a 50mL conical tube. Place on a 37°C heat block.

4. Aspirate the medium from the dish containing the MRC5 culture, and add 4mL TrypLE Select to the dish. Place in a 37°C incubator for 2 minutes, or until the fibroblasts have begun to lift from the surface of the dish.
- CRITICAL STEP Do not over-trypsinize the fibroblasts. When the cells are visibly lifting from the plate (appear small and rounded instead of elongated and flat), immediately add E8Fib medium to dilute the enzyme.

5. Using a glass 5mL serological pipette, add 5mL E8Fib from the 10mL aliquot to the dish, and triturate to finish lifting the cells from the dish.

6. Transfer the cell suspension to a 15mL conical tube, and rinse the dish 2 x 2mL from the 10mL aliquot. Add rinses to the conical tube.

7. Centrifuge at 200 x g for 4 min.

8. Aspirate supernatant and resuspend the pellet in the 50mL E8Fib aliquot. Plate 25mL cell suspension in each of 2 x tissue culture-treated 15cm dishes.

9. Continue to feed the fibroblasts every other day by aspirating spent medium and adding 25mL fresh, pre-warmed E8Fib medium per dish.

10. When the 15cm dishes reach 80% confluency, split and expand by following steps 11-17, below.

11. *Pre-warm E8Fib medium.* Prepare 1 x 40mL aliquot of E8Fib medium in a 50mL conical tube, and 1 x 300mL aliquot in a 500mL bottle. Place on a 37°C heat block.

12. Aspirate the medium from the dishes containing the MRC5 culture, and add 10mL TrypLE Select to each dish. Place in a 37°C incubator for 2 minutes, or until the fibroblasts have begun to lift from the surface of the dish.
13. Using a 10mL serological pipette, add 10mL E8Fib from the 40mL aliquot to each dish, and triturate to finish lifting the cells from the dish.

14. Transfer the cell suspension from each dish to a 50mL, and rinse each dish with 2 x 5mL E8Fib from the 40mL aliquot. Add rinses to the conical tube.

15. Centrifuge at 200 x g for 4 min.

16. Aspirate supernatants. Combine and resuspend the pellets in the 300mL E8Fib aliquot. Plate 25mL cell suspension in each of 12 x tissue culture-treated 15cm dishes.

17. Continue to feed the fibroblasts every other day by aspirating spent medium and adding 25mL fresh, pre-warmed E8Fib per dish.

18. When the 12 x 15cm dishes reach 80% confluency, proceed to irradiation and preparation of frozen vials.

Day -5 (approx.): Prepare frozen vials of MRC5 fibroblast stock and irradiated MRC5 feeders
TIMING 1h + overnight hold at -80°C

1. *Pre-warm E8Fib medium.* Prepare 1 x 20mL E8Fib aliquot in a 50mL conical tube and 1 x 250mL E8Fib aliquot in a 250mL bottle. Place on a 37°C heat block.

2. *Pre-chill CryoStor CS10 and Mr. Frostys.* Prepare 1 x 15mL CryoStor CS10 aliquot in a 15mL conical tube. Place on ice. Place 3 x Mr Frostys on ice.

3. *Label cryovials.* Prepare 12 cryovials for non-irradiated MRC5 fibroblast freeze batch and 20 cryovials for irradiated MRC5 freeze batch.

4. Aspirate medium from 1 x 15cm MRC5 culture dish, and add 10mL TrypLE Select to the dish. Place in a 37°C incubator for 2 minutes, or until the fibroblasts have begun to lift from the surface of the dish.

5. Using a 10mL serological pipette, add 10mL E8Fib medium from the 250mL aliquot to the dish and triturate to finish lifting the cells from the dish.

6. Transfer the cell suspension from the dish to a 50mL conical tube, and rinse the dish with 2 x 5mL E8Fib from the 250mL aliquot. Add rinses to the conical tube.

7. Centrifuge at 200 x g for 4 minutes.

8. Aspirate supernatant and resuspend the pellet in 6mL CryoStor CS10.

9. Add 500µL cell suspension to each of the 12 cryovials, place in a chilled Mr. Frosty, and place in a -80°C freezer for overnight storage, and proceed immediately to step 10.

10. Aspirate medium from remaining 11 x 15cm MRC5 culture dishes and add 10mL TrypLE per dish. Place in a 37°C incubator for 2 minutes, or until the fibroblasts have begun to lift from the surface of the dish.

11. Using a 10mL serological pipette, add 10mL E8Fib medium from the 250mL aliquot to each dish and triturate to finish lifting the cells from the dish.

12. Transfer the cell suspension from each dish into a 50mL conical tube, and rinse each dish with 2 x 5mL E8Fib from the 250mL aliquot. Add rinses to the conical tubes.

13. Centrifuge at 200 x g for 4 minutes.

14. Aspirate supernatant and resuspend each pellet in 5mL PBS (+/+).
15. Combine cell suspensions and divide total volume into 2 x 27.5mL aliquots in 50mL conical tubes.

16. Centrifuge at 200 x g for 4 minutes.

17. Aspirate supernatants and resuspend each pellet in 10mL E8Fib medium from the 20mL aliquot.

18. Perform a live cell count. There should be ≤14M cells in each tube.

- CRITICAL STEP If there are >14M cells in a tube, combine suspensions, centrifuge, and resuspend in an appropriate volume to achieve ≤14M/10mL. Divide suspension into 10mL aliquots in 50mL conical tubes. It may be necessary to prepare more cryovials and Mr. Frostys.

19. Irradiate each 10mL aliquot of cell suspension with 4000rad.
! CAUTION Appropriate PPE when using an irradiator includes a labcoat, gloves, and dosimeter.

20. Combine 10mL cell suspension aliquots and perform another live cell count.

21. Centrifuge at 200 x g for 4 minutes.

22. Reususpend pellet in an appropriate volume of CryoStor CS10 to achieve 2M cells/mL.

23. Add 500µL cell suspension to each of the 20 cryovials (number of vials yielded may vary). Place vials in chilled Mr. Frostys and place in a -80°C freezer overnight.

24. The following day, move the vials into LN_2 storage.

Day -1: Preparation of irradiated MRC5 fibroblast-coated 6-well tissue culture plates for 3-day expansion
TIMING 20 m

1. *Pre-warm E8Fib aliquots.* In 15mL conical tubes, prepare 1 x 10mL and 1 x 12mL aliquots of E8Fib medium. Place on a 37°C heat block.

2. Retrieve 1 x 1M vial of irradiated MRC5 from LN_2 storage and thaw in a 37°C 70% isopropanol bath for approximately 2 minutes, or until a small frozen pellet remains in the tube.

- CRITICAL STEP Do not over-thaw the vial. Ensure that a small frozen pellet remains when the vial is removed from the isopropanol bath.

3. Using a P1000 pipetman, add 1mL E8Fib medium from the 10mL aliquot, dropwise, to the vial.

4. Add the cell suspension to the 9mL E8Fib remaining in the conical tube.

5. Centrifuge at 200 x g for 4 minutes.

6. Aspirate supernatant and resuspend the pellet in 12mL pre-warmed E8Fib medium.

7. Add 2mL cell suspension per well of a tissue culture-treated 6-well plate.

8. Place the plate in a 37°C incubator and shake to evenly distribute cells over the surface of the wells. Do not move the plate for at least 12 hours while the cells adhere.

Day -1: Isolation of mononuclear cells from human peripheral blood by Ficoll density gradient centrifugation
TIMING 3.5 hr

1. *Prepare Ficoll buffer.* To a new 500mL bottle of PBS+/+, add 2.2mL 500mM EDTA. Mix well and set aside at room temperature.

2. *Prepare Ficoll-Paque aliquots.* For every 5mL blood, prepare a 50mL conical tube containing 15mL Ficoll-Paque. Set aside at room temperature.

4. *Prepare an RPMI aliquot for rinses.* Place 10mL RPMI for every 5mL blood in a 50mL conical tube. Set aside at room temperature.

5. Using a glass serological pipette, divide blood sample into 5mL aliquots in 50mL conical tubes. Without changing pipettes, rinse each tube with 2 x 5mL pre-aliquotted RPMI, adding rinses to the tubes.
! CAUTION Wear appropriate personal protective equipment, including lab coat, sterile sleeves, and double gloves, when handling human blood samples. Rinse all pipettes and tubes with bleach, discard into a biohazard waste collection bag, and autoclave. Centrifuge samples in sealed buckets.

- CRITICAL STEP Carry out all steps, except centrifugation, in a sterile tissue culture hood using proper aseptic technique to avoid contamination.

6. Raise the volume in each tube to 35mL using RPMI.

7. Very gently and carefully, add each 35mL blood dilution to a 50mL tube containing 15mL Ficoll-Paque, so that the Ficoll-Paque and the blood dilution form two distinct layers.

- CRITICAL STEP The blood dilution and the Ficoll-Paque must form discrete layers in order to successfully form a gradient upon centrifugation. This can be done by drawing the blood/RPMI dilution into a 10mL serological pipette, pressing the pipette tip against the inside of the 50mL tube containing the Ficoll-Paque, and ejecting very slowly so that the blood dilution trickles down in a thin stream.

8. Centrifuge at 400 x g for 30 minutes at 20°C with the centrifuge brake turned OFF.

9. 4 layers should now be visible in the conical tubes: 1) plasma, 2) mononuclear cells, 3) Ficoll-Paque, and 4) erythrocytes. Aspirate most of the plasma layer, leaving approximately 5mL above the mononuclear cell layer.

10. Collect the entire mononuclear cell layer using a glass 5mL serological pipette, and transfer to a new 50mL conical tube.

- CRITICAL STEP Depending on the volume of input blood, this layer may be very thin. In order to ensure 100% collection of mononuclear cells, collect the bottom 1mL of the plasma layer, the top 1mL of the Ficoll-Paque layer, and the entire volume in between.

11. Raise the volume in each tube to 50mL using Ficoll buffer.

12. Centrifuge at 400 x g for 30 minutes at 20°C with the centrifuge brake turned ON.

13. Aspirate supernatants, leaving approximately 3mL above each cell pellet.

14. Raise the volume in each tube to 50mL using Ficoll buffer.

15. Centrifuge at 400 x g for 30 minutes at 20°C with the centrifuge brake turned ON.

16. Aspirate supernatants, leaving approximately 3mL above each cell pellet.

17. Resuspend each pellet using a glass 5mL serological pipette, and combine the contents of all tubes into a new 15mL conical tube. Raise the volume in the tube to 15mL with Ficoll buffer.

18. Centrifuge at 400 x g for 10 minutes at 20°C with the centrifuge brake turned ON.

19. *Pre-warm H3000 + CC100 aliquot.* To 12mL H3000 medium, add 120μL CC100 cytokine mix. Mix well and place on a 37°C heat block.

20. Aspirate supernatant from pelleted mononuclear cells.

21. Resuspend pellet in 1mL H3000 + CC100 and count cells.

22. Adjust cell suspension concentration to 250k cells/mL using H3000 + CC100. If fewer than 500k cells were yielded, raise the suspension volume to 2mL with H3000 + CC100.

23. Add 2mL cell suspension per well of a 6-well ultra-low attachment tissue culture plate.

24. Place cells in a 37°C incubator overnight or up to 24h, then proceed to nucleofection.

Day 0: Nucleofection of human peripheral blood mononuclear cells with Yamanaka episomal reprogramming plasmids
TIMING 1 h + 5 h incubation + 24 h incubation

1. *Prepare nucleofector solution + plasmids.* Combine 82µL nucleofector solution + 18µL supplement from Amaxa Cell Line Nucleofection Kit V. To the resulting 100µL solution, add 1µg of each Yamanaka episomal plasmid (3µg DNA total). Place on ice.

2. *Pre-warm Fibrolife aliquot.* Add 1mL Fibrolife to a 15mL conical tube and place on a 37°C heat block to warm.

3. Collect mononuclear cells into a 15mL conical tube and centrifuge at 400 x g for 10 minutes at 20°C.

4. Resuspend pellet in 1mL PBS+/+. Count cells.

5. Collect 1M cells, or entire cell yield, in a 15mL conical tube and centrifuge at 400 x g for 10 minutes at 20°C.

6. Aspirate supernatant carefully, leaving behind the least volume possible.

7. Resuspend pellet in 100µL nucleofector solution + Yamanaka plasmid suspension, and immediately add to a nucleofection cuvette.

- CRITICAL STEP In order to avoid a nucleofector instrument error, add exactly 100µL cell suspension directly to the bottom of the cuvette. Avoid getting droplets on the walls of the cuvette or introducing air bubbles.

8. Place cuvette in the Amaxa Nucleofector II instrument and run program U-008.

9. Add 500µL Fibrolife from the 1mL pre-warmed aliquot to the cuvette.

10. Add the remaining 500µL Fibrolife to one well of a 12-well tissue culture plate.

11. Using the transfer pipette provided in the Amaxa Cell Line Nucleofector Kit V, transfer the 600µL cell suspension from the cuvette to the well containing 500µL Fibrolife.

12. Place cells in a 37°C incubator for 4 hours.

13. *Pre-warm H3000 and H3000 + CC100 aliquot.* In a 15mL conical tube, place 2mL H3000 + 20µL CC100 for every 200k cells nucleofected. In another 15mL conical tube,

place 2mL H3000. With 10 minutes remaining in the 4 hour incubation, place both aliquots on a 37°C heat block to warm.

14. After 4 hours, transfer the cell suspension to a 15mL conical tube. Wash the well 2 x 1mL with H3000 from the pre-warmed aliquot, and transfer washes to the tube.
40. Centrifuge at 400 x g for 10 minutes at 20°C.

15. Aspirate supernatant and resuspend pellet to a final concentration of 100k cells/mL in H3000 + CC100 using the pre-warmed aliquot. If fewer than 200k cells were nucleofected, resuspend the pellet in 2mL.

16. Aspirate the E8Fib medium from a prepared 6-well irradiated MRC5-coated tissue culture plate, wash wells with 1mL PBS (+/+) per well, and add 2mL nucleofected cell suspension per well.

17. Place cells in a 37°C incubator for 24 hours.

18. After 24 hours, move the cells to a 37°C incubator with 5% ambient O_2. Incubate 2 days, then proceed to replating.

Day 2: Preparation of irradiated MRC5 fibroblast-coated 10cm tissue culture dishes for reprogramming
TIMING 20 m

1. *Pre-warm E8Fib aliquots.* In 15mL conical tubes, prepare 2 x 10mL and 1 x 12mL aliquots of E8Fib medium. Place on a 37°C heat block.

2. Retrieve 2 x 1M vials of irradiated MRC5 from LN_2 storage and thaw in a 37°C 70% isopropanol bath for approximately 2 minutes, or until a small frozen pellets remain in the tubes.

- CRITICAL STEP Do not over-thaw the vials. Ensure that a small frozen pellet remains when the vial is removed from the isopropanol bath.

3. Using a P1000 pipetman, add 1mL E8Fib medium from one of the 10mL aliquots, dropwise, to the first vial.

4. Add the cell suspension to the 9mL E8Fib remaining in the conical tube.

5. Repeat steps 3-4 with the second E8Fib aliquot and the second MRC5 vial.

5. Centrifuge at 200 x g for 4 minutes.

6. Aspirate supernatants and resuspend the pellets in 10mL pre-warmed E8Fib medium each.

7. Add 10mL cell suspension to each 10cm tissue culture-treated dish.

8. Place the dishes in a 37°C incubator and shake to evenly distribute cells. Do not move the dishes for at least 12 hours while the cells adhere.

Day 3: Replating nucleofected mononuclear cells onto 10cm dishes
TIMING 20 m

1. *Pre-warm H3000 and H3000+CC100 media aliquots*. To a 50mL conical tube, add 5mL H3000 medium for every well of nucleofected cells plated on Day 0. To another 50mL conical tube, combine 20mL H3000 + 200μL CC100. Place on a 37°C heat block.

2. Collect the supernatants from each well of nucleofected cells. Combine in a 50mL conical tube and set aside.

3. Add 1mL TrypLE Select to each well. Place in a 37°C incubator for 2 minutes, or until the MRC5 layer, along with any adherent nucleofected cells, has begun to lift from the surface of the wells.

- CRITICAL STEP Do not over-trypsinize the fibroblasts. When the cells are visibly lifting from the plate (appear small and rounded instead of elongated and flat), immediately add H3000 medium to dilute the enzyme.

4. Using a p1000 pipetman set to 1mL, add 1mL H3000 medium to each well to dilute the TrypLE Select, and triturate to finish lifting the cells from the well.

5. Add the contents of each well to the 50mL conical tube containing the supernatants. Rinse each well with 2 x 2mL H3000, and add rinses to the tube.

6. Centrifuge at 200 x g for 4 min.

7. Aspirate supernatant and resuspend the pellet in 20mL H3000+CC100.

8. Aspirate the MEF medium from the prepared 10-cm irradiated MRC5-coated tissue culture dishes, wash dishes with 5mL PBS (+/+) each, and add 10mL nucleofected cell suspension per dish.

9. Return the cells to a 37°C with 5% ambient O_2.

Day 4: Add E8 medium
TIMING 5 m

1. *Pre-warm E8 medium.* To a 15mL conical tube, add 4mL E8 medium. Place on a 37°C heat block.

2. Add 2mL E8 medium to each reprogramming dish.

3. Return the cells to a 37°C with 5% ambient O_2.

Day 6: Transition to E8 medium
TIMING 10 m

1. *Pre-warm E8 medium.* To a 15mL conical tube, add 12mL E8 medium. Place on a 37°C heat block.

2. With a glass 5mL serological pipet, remove 5mL cell-containing supernatant from each reprogramming dish, and place in an individual 15mL conical tube.

3. Centrifuge at 200 x g for 4 min.

4. Aspirate supernatants and resuspend each pellet in 6mL E8 medium. Add each cell suspension back to its corresponding 10cm dish.

5. Return the cells to a 37°C with 5% ambient O_2.

Day 8: Transition to E8 medium
TIMING 10 m

1. Repeat media change procedure from Day 6

Day 10: Transition to E8 medium
TIMING 10 m

1. Repeat media change procedure from Day 6

Day 11: Complete media change with E8 medium and return cells to 20% O_2
TIMING 5 m

1. *Pre-warm E8 medium.* To a 50mL conical tube, add 20mL E8 medium. Place on a 37°C heat block.

2. Aspirate supernatants from each dish, and add 10mL E8 medium per dish.

3. Place the cells in a 37°C with 20% ambient O_2.

Day 12 – 20: Daily E8 medium changes until colonies emerge
TIMING 5 m

1. *Pre-warm E8 medium.* To a 50mL conical tube, add 20mL E8 medium. Place on a 37°C heat block.

2. Aspirate supernatants from each dish, and add 10mL E8 medium per dish.

3. Place the cells in a 37°C incubator with 20% ambient O_2.

4. Colonies should emerge around Day 12.

Day 15 (approx.): Plating CellStart and picking colonies
TIMING 45 m + 3 h incubation

1. To a 15mL conical tube, combine 6mL PBS (+/+) and 120µL CellStart. Mix well.

2. Add 250µL CellStart solution per well of a tissue culture-treated 24-well plate. Place in a 37°C incubator for ≥3 h.

3. *After 3 h, pre-warm E8 medium.* To a 15mL conical tube, add 12mL E8 medium. Place on a 37°C heat block.

4. Aspirate the CellStart solution from the 24-well plate and add 500µL E8 medium per well.

5. *Prepare picking hood.* In a tissue culture hood containing a stereoscope, place a reprogramming dish containing pickable iPSC colonies, the prepared 24-well plate, a sterile scalpel, a p20 pipetman set to 20µL, and 20µL pipet tips.

6. Using the stereoscope, zoom in and focus on an iPSC colony on the reprogramming plate.

7. Using the scalpel, score the colony 3x lengthwise and 3x crosswise, making a 3x3 grid.

8. Scrape up and collect the scored colony fragments with a p20 pipetman.

9. Eject the iPSC colony fragments into a single well of the 24-well plate.

10. Repeat steps 6-9 with as many colonies as desired, placing each clone in an individual well of the 24-well plate.

- CRITICAL STEP Ensure establishment of clonal iPSC lines by only placing fragments from a single scored colony into each well.

11. Place the plate in a 37°C incubator with 20% ambient O_2 and shake to evenly distribute colony fragments. Do not move the plate for at least 12 hours while the fragments adhere.

12. The 10-cm reprogramming dishes can now be discarded, maintained in culture with daily media changes for picking additional colonies, or fixed and stained with an anti-TRA-1-60 kit for efficiency analysis.

Day 16 (approx.): Feed iPSC lines with E8

1. *Pre-warm E8 medium.* To a 15mL conical tube, add 12mL E8 medium. Place on a 37°C heat block.

2. Add 1mL E8 medium per well of the 24-well plate.

3. Place the cells in a 37°C incubator with 20% ambient O_2.