Supplemental Information

Reprogramming of T Cells from Human Peripheral Blood

Yuin-Han Loh, Odelya Hartung, Hu Li, Chunguang Guo, Julie M. Sahalie, Philip D. Manos, Achia Urbach, Garrett C. Heffner, Marica Grskovic, Francois Vigneault, M. William Lensch, In-Hyun Park, Suneet Agarwal, George M. Church, James J. Collins, Stefan Irion, George Q. Daley

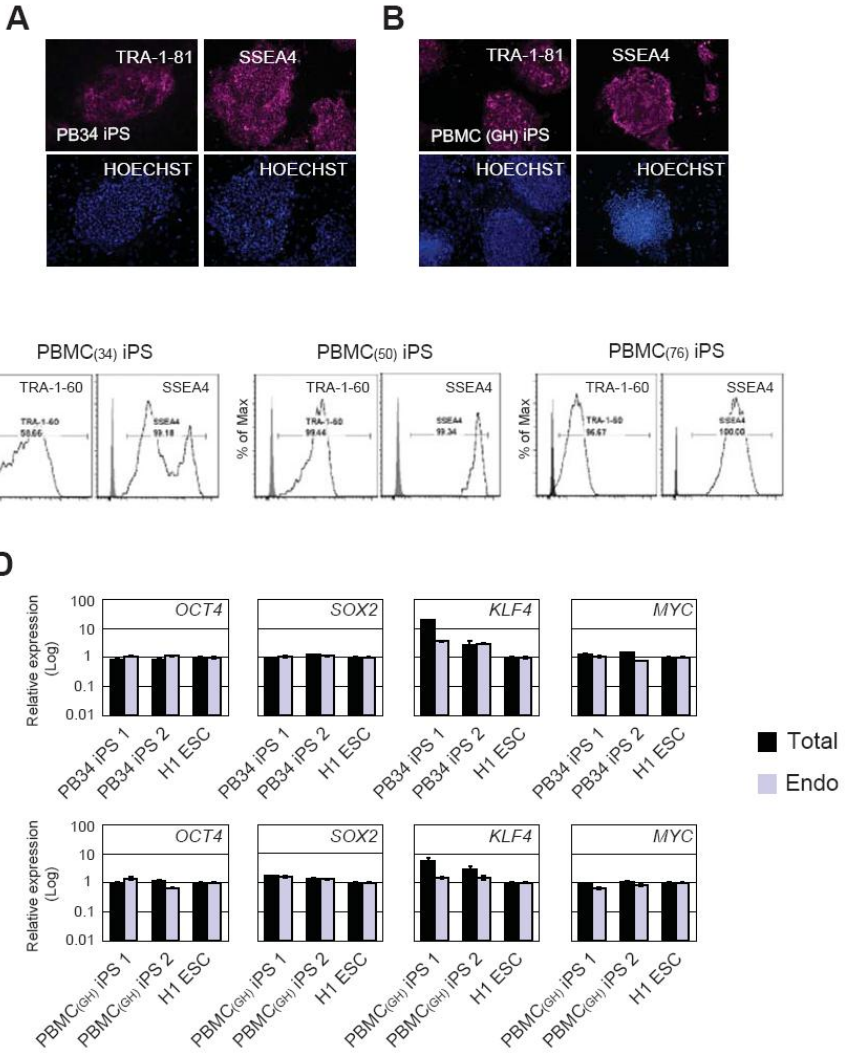
Inventory of Supplemental Information

Supplemental Information

Reprogramming of T Cells from Human Peripheral Blood

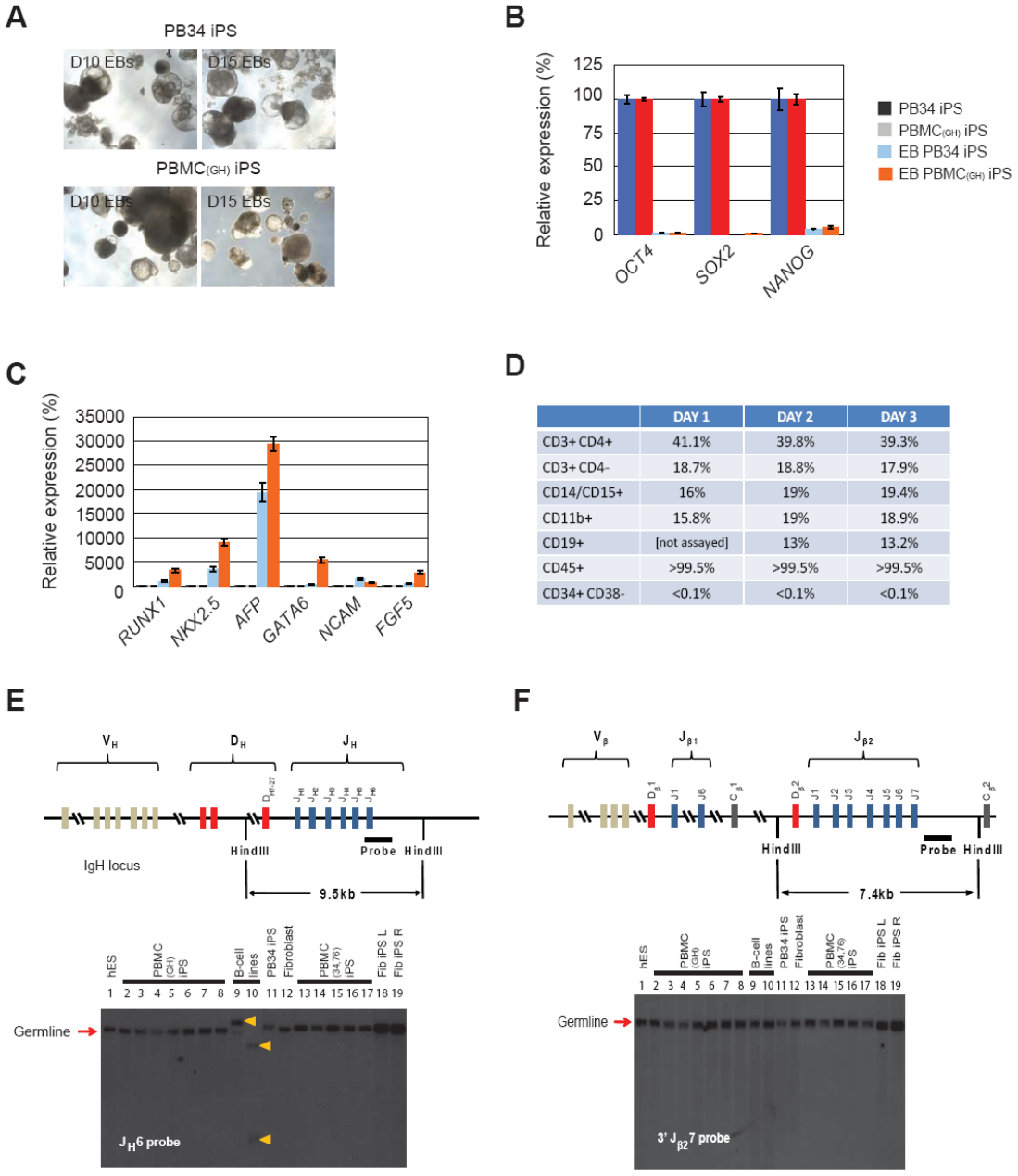
Yuin-Han Loh, Odelya Hartung, Hu Li, Chunguang Guo, Julie M. Sahalie, Philip D. Manos, Achia Urbach, Garrett C. Heffner, Marica Grskovic, Francois Vigneault, M. William Lensch, In-Hyun Park, Suneet Agarwal, George M. Church, James J. Collins, Stefan Irion, George Q. Daley

SUPPLEMENTAL FIGURES AND LEGENDS



Suppl figure 1

Figure S1. Characterization of the peripheral blood cell derived iPS cells.
(A, B) Immunohistochemistry of PB CD34+ and PBMCs (GH) derived iPS cell colonies expressing markers for Tra-1-81 and SSEA4. Hoechst staining indicates the total cell content per field. Fibroblasts surrounding human iPS colonies serve as internal negative controls for immunohistochemistry staining. Images were acquired with a standard microscope (Nikon, Japan) with a 10x objective.
(C) PBMC iPS (34, 50, 76) cells were analyzed by FACS for expression of pluripotency markers SSEA-4 and TRA-1-60.
(D) Expression of *OCT4*, *SOX2*, *KLF4* and *c-MYC* was analysed by quantitative real-time PCR in PB34 iPS (Top) and PBMC iPS cells (Bottom) with human H1 ES cells. Individual PCR reactions were normalized against *β-ACTIN* and plotted (Log_{10} scale) relative to the expression level in the H1 ES cells which was set to 1. Black and grey bars represent total (endogenous and exogenous) and endogenous gene levels, respectively.
(E) Euclidean distance was calculated and hierarchical clustering was performed, with the *Ward's* minimum variance method, for gene expression profiles from: H1 ES, H9 ES, dH1F, dH1F iPS, PB CD34+, PB34 iPS, PBMCs, and PBMC iPS cells. The distance metric for comparisons between different cell lines is indicated as the height of the tree.
(F) PB CD34+ (Top) and PBMC (Bottom) derived iPS lines maintain normal karyotype. High-resolution, G-banded karyotypes indicate a normal, diploid, male chromosomal content. Human iPS cells were passaged four times prior to karyotype analysis.
(See also Figure 1 and Table S1).



	DAY 1	DAY 2	DAY 3
CD3+ CD4+	41.1%	39.8%	39.3%
CD3+ CD4-	18.7%	18.8%	17.9%
CD14/CD15+	16%	19%	19.4%
CD11b+	15.8%	19%	18.9%
CD19+	[not assayed]	13%	13.2%
CD45+	>99.5%	>99.5%	>99.5%
CD34+ CD38-	<0.1%	<0.1%	<0.1%

Suppl figure 2

Figure S2. *In vitro* differentiation and V(D)J recombination assay.
(A) Cystic embryoid bodies (EB) consisting of clusters of differentiating cells on day 10 and 15. Images were acquired with a standard microscope (Nikon) with a 10x objective.
(B,C) Quantitative RT-PCR performed on undifferentiated and embryoid body-mediated differentiated iPS cells shows down-regulation of pluripotency genes *OCT4*, *SOX2*, *NANOG* (B) and up-regulated expression of lineage markers (C) from the three embryonic germ layers (Mesoderm – *RUNX1* and *NKX2.5*, Endoderm – *AFP and GATA6* and, and Ectoderm – *N-CAM* and *FGF5*). Individual PCR reactions were normalized against *β-ACTIN* and plotted relative to the expression level in the undifferentiated iPS cells which is set to 100%.
(D) PBMC were cultured in cytokines (FLT-3L, SCF and IL-3) containing medium. At 24, 48, and 72 hours of culture, cells were collected and stained with a panel of fluorophore-conjugated antibodies to determine cell surface phenotype by flow cytometry. For each time-point, the percentage of propidium iodide negative cells with the indicated cell surface phenotype present in a representative cell culture is shown. Reprogramming factors were introduced by viral infection at day 2.
(E) Genomic DNA from peripheral blood-derived iPS lines grown was digested with Hind III and analyzed for V(D)J rearrangements at the IgH locus by Southern blotting using a J_H6 probe (Top). No indication of IgH V(D)J recombination was observed for PBMC-derived iPS lines (Bottom). Lanes 2-8 and lanes 13-17 are PBMC-iPS lines. Lanes 8 and 9 are LTR228 and ST486, B-cell lines used as positive controls. Lanes 1, 11, 12, 18, 19 are H1 hES cells, PB34 iPS cells, fibroblast cells, fibroblast derived iPS using retrovirus and lentivirus, respectively. The red arrow indicates expected size of the germline band. Orange arrows indicate re-arranged bands.
(F) Genomic DNA from peripheral blood-derived iPS lines grown was digested with Hind III and analyzed for V(D)J rearrangements at the TCRβ (T-cell receptor Beta) locus by Southern blotting using a 3'Jβ2 7 probe (Top). No indication of TCRβ2 V(D)J recombination was observed in blood-derived iPS cell clones (Bottom). Lanes 2-8 and lanes 13-17 are PBMCS iPS lines. B-cell lines on lanes 8 and 9 showed no rearrangement. Lanes 1, 11, 12, 18, 19 are H1 hES cells, PB34 iPS cells, fibroblast cells, fibroblast derived iPS using retrovirus and lentivirus, respectively. The red arrow indicates expected size of the germline band.
(See also Figure 2 and Table S2).

Donor's profiles					Reprogramming efficiency (%)	Clone	Marker expression			Characterizations			Pluripotency	
Cells	ID	Race	Gender	Age			RT-PCR	Immunostaining	FACS	Bisulfite sequencing	Karyotyping	VDJ	*In vitro* differentiation	Teratoma
PB34+ cells	A1818	Black	Male	49	0.002	1	√	√		√	√	√	√	√
						2	√	√		√	√		√	√
						3	√							
						4	√							
						5	√							
						6	√							
PBMCs	GH (Dad61)	White	Male	28	0.0008	1	√	√	√	√	√	√	√	√
						2	√	√		√	√	√	√	√
						3	√					√		
						4	√					√		
						5	√					√		
						6	√					√		
						7	√					√		
						8	√							
PBMCs	34	Black	Female	30	0.001	1	√	√	√			√		√
						2	√	√	√			√		
PBMCs	50	Black	Female	31	0.001	1	√	√	√					
PBMCs	76	Black	Male	49	0.0005	1	√	√	√			√		
						2	√	√	√			√		
						3	√	√	√					
						4	√	√	√					√

Table S1. iPS lines generated in this study. (See also Figure 1 and Figure S1).

	PBCD34+		PB34 iPS		PBMCs		PBMC(GH) iPS	
Amelogenin	X	Y	X	Y	X	Y	X	Y
vWA	15	17	15	17	14	18	14	18
D8S1179	14		14		11	14	11	14
TPOX	8	9	8	9	8		8	
FGA	22	24	22	24	20	25	20	25
D3S1358	16	17	16	17	16	17	16	17
THO1	8		8		7	8	7	8
D21S11	28	29	28	29	28	30	28	30
D18S51	13.2	18	13.2	18	15	18	15	18
Penta E	7	11	7	11	7	12	7	12
D5S818	12		12		11	12	11	12
D13S317	13	14	13	14	12		12	
D7S820	11	12	11	12	9	10	9	10
D16S539	9	13	9	13	11	12	11	12
CSF1PO	10	12	10	12	11		11	
Penta D	2.2	9	2.2	9	9	12	9	12

Table S2. Fingerprint analysis of human peripheral blood iPS clones.
STR analysis of normal PB CD34+ cells and it's derivatives PB34 iPS, and PBMCs and it's derivatives PBMC(GH) iPS. (See also Figure 2 and Figure S2).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Production of virus. Lentiviral vectors FU-TET-O-h*OCT4*, FU-TET-O-h*SOX2*, FU-TET-O-h*KLF4*, FU-TET-O-h*C-MYC* and FUdeltaGW-rtTA were gifts from Chad Cowan and Konrad Hochedlinger. Retroviral vectors containing h*OCT4*, h*SOX2*, h*KLF4* and h*MYC* were based on murine leukemia viral vector pMX described previously (Dimos et al. 2008). The viruses were produced and concentrated according to standard protocol (Park et al. 2008b).

Cell culture and human iPS cell induction. Normal peripheral blood CD34+ (PB CD34+) and mononuclear cells (PBMCs) were either maintained in medium described previously (Loh et al. 2009) or grown in dendritic cell medium supplemented with SCF and GM-CSF. Cells grown in culture for 2 days were used for infection with FU-TET-O-h*OCT4*, FU-TET-O-h*SOX2*, FU-TET-O-h*KLF4*, FU-TET-O-h*C-MYC* and FUdeltaGW-rtTA for peripheral blood CD34+ cells and peripheral blood mononuclear cells from donor GH Dad61. PBMCs from donors 34, 50 and 76 were maintained in Stemline dendritic cell media (Sigma). The cells were infected with pMX retrovial *OCT4*, *SOX2*, *KLF4* and *MYC*. Transductions were carried out on 10^5 or 10^6 cells in 12-multiwell plates, coated with the fibronectin fragment CH-296 (Retronectin; Takara Shuzo, Otsu, Japan) using spin infection as previously described (Loh et al. 2009). For doxycycline inducible lentivirus, doxycycline was added one day after infection. Removal of doxycycline commenced progressively after iPS cells colonies appeared in the culture. Three days after infection, cells were split into plates pre-coated with matrigel (BD Biosciences) or pre-seeded with mouse embryonic fibroblasts (MEFs). Medium was changed to standard human ES culture medium 5 days after infection. For some experiments, medium was switched to mTeSR (Stemcell technologies) and cytokine supplemented medium in 1:1 ratio on day 5 before switching completely to mTeSR medium by day 8.

Donor blood cells collection and isolation. Frozen normal peripheral blood (PB) CD34+ cells (PB033F-1, Lot A1818) were obtained from Allcells. Briefly, peripheral blood was isolated from a 49 year-old male donor who had undergone apheresis (Cobe Spectra Apheresis machine). CD34+ cells were purified using Miltenyi's Indirect CD34+ Microbeads Kit (130-046-701) and AutoMACS Pro. Typically, 350ml of whole blood yield $1x10^9$ PB mononuclear cells from which 1 million CD34+ cells can be isolated. For the isolation of mononuclear cells, peripheral blood was collected by venipuncture from a healthy 28-year old male donor (GH Dad61). Ficoll-hypaque density-gradient centrifugation was used to separate the mononuclear cells with the red blood cells and granulocytes. In another experiment, whole blood was purchased from Zen-bio, Inc. (Research Triangle Park, NC). Whole blood samples were collected from a healthy 30-year old female donor (34), 31-year old female donor (50) and 49-year old male donor (76) by venipuncture in heparinized collection tubes and tested for human pathogens. The samples were shipped at 4^oC and processed 3 days later at iPierian, Inc. where PBMCs were isolated using a Ficoll-hypaque density-gradient centrifugation. Remaining red blood cells were lysed using red blood cell lysis buffer (Sigma-Aldrich). Isolations of samples from human subjects were performed according to the institutional guidelines and regulations.

Immunohistochemistry staining. Cells were fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 for 30 min, and blocked in 3% BSA in PBS for 2 hours. Cells were incubated with primary antibody overnight at 4 ^{o}C, washed, and incubated with Alexa Fluor (Invitrogen) secondary antibody for 2 hours. SSEA4, TRA-1-60 and TRA-1-81 antibodies were obtained from Millipore. OCT3/4 and NANOG antibodies were obtained from Abcam. Alkaline phosphatase staining was done according to the manufacturer's recommendations (Millipore).

qRT–PCR. RNA was isolated using an RNeasy kit (Qiagen) according to manufacturer's protocol. First-strand cDNA was primed via oligo-dT primers and RT–PCR was performed with primer sets described previously (Park et al. 2008a). Quantitative RT–PCR was performed using Brilliant SYBR green (Stratagene).

Global gene expression. Gene expression profiling was carried out using Human Genome HGU133 plus 2.0 Affymetrix GeneChip arrays. In brief, total RNA was extracted from cells with the RNAeasy kit including DNase digestion (Qiagen). Biotin-labeled cDNA was obtained from 1 microgram of total RNA with the GeneChip One-Cycle labeling kit (Affymetrix). One-and-a-half micrograms of cDNA were fragmented and hybridized to Affymetrix HGU133 plus 2.0 GeneChip arrays according to the manufacturer's instructions. DNA chips were washed, stained and scanned using an Affymetrix Fluidics device and a GCS3000 scanner, and the images obtained were analysed using GCOS software. The experiment was performed in duplicate for the dH1F, PB_CD34+, PBMC, hESC and all the iPS cells. Data normalization was calculated with the robust multichip average (RMA) algorithm (Rafael et al. 2003) implemented in BioConductor (http://www.bioconductor.org/). The cluster analysis was performed and scatter plots were generated in R packages (http://www.r-project.org/).

Bisulfite genomic sequencing. Bisulfite treatment of genomic DNA (gDNA) was carried out using a CpGenome DNA Modification Kit (Chemicon) according to the manufacturer's protocol. Briefly, converted gDNA was amplified by PCR NANOG primer set 3, as reported previously (Freberg et al. 2007). PCR products were gel-purified and cloned into bacteria using the TOPO TA cloning kit (Invitrogen). Bisulfite conversion efficiency of non-CpG cytosines ranged from 80% to 99% for all individual clones for each sample.

DNA fingerprinting and karyotyping. Analysis of the DNA fingerprinting was performed on parental PB CD34+ cells, PB MNCs and their derivative iPS cell lines. DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's protocol and sent to Cell Line Genetics (Wisconsin, USA) for analysis. For karyotyping of the chromosome, live iPS lines were sent to Cell Line Genetics (Wisconsin, USA) for analysis.

Embryoid body formation. To form embryoid bodies, confluent undifferentiated iPS cells were mechanically scraped into strips and transferred to 6-well, low attachment plates in differentiation medium consisting of knockout DMEM (Invitrogen)

supplemented with 20% fetal bovine serum (StemCell Technologies), 0.1 mM non-essential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen), 50ug ml^{-1} ascorbic acid (Sigma) and 2mg ml^{-1} human holo-transferrin (Sigma) and 0.1 mM β-mercaptoethanol (Sigma).

Hematopoietic colony-forming assays. Human iPS lines were differentiated for 14 days as embryoid bodies in culture media described above supplemented with SCF (300 ng ml^{-1}), Flt-3 ligand (300 ng ml^{-1}), IL-3 (10 ng ml^{-1}), IL-6 (10 ng ml^{-1}), G-CSF (50 ng ml^{-1}) and BMP4 (50 ng ml^{-1}). Embryoid bodies were dissociated and plated into methylcellulose colony-forming assay media containing SCF, GM-CSF, IL-3 and Epo (H4434, Stem Cell Technologies) at a density of 25,000 cells ml^{-1}.

Assay for teratoma formation. For teratoma formation, PB34 and PBMC (GH) iPS cells were suspended in a mixture of DMEM, Matrigel and collagen (ratio of 2:1:1) and injected intramuscularly into immune-compromised $Rag2^{-/-}$ $\gamma C^{-/-}$ mice. Xenografted masses formed within 4 to 7 weeks, and paraffin sections were stained with haematoxylin and eosin for all histological determinations.

Flow cytometry. Peripheral blood cells were stained with a panel of antibodies at the manufacturer's suggested dilution in PBS/2% serum on ice for 20 minutes. This panel included CD3 (UCHT1), CD4 (RPA-T4), CD11b (ICRF44), CD15 (HI98), CD19 (HIB19), CD34 (563), CD38 (HB7), and CD45 (HI30) from BD Bioscience, and CD14 (TUK4) from Caltag. Propidium iodide was used for live/dead discrimination. Flow cytometry was performed on a 5-laser LSR-II cell analyzer (BD Bioscience) or Accuri C6 Flow Cytometer (Accurisystems), and data were analyzed using FlowJo software (Treestar, Inc.).

Southern Blot analysis. DNA was isolated from iPS and control cell lines using DNeasy Blood and Tissue kit (Qiagen) per the manufacturer's protocol. 5-10 micrograms of DNA were digested with the appropriate restriction enzymes (New England Biolabs), size-separated in 8% agarose gels and transferred to nylon membranes (Bio-Rad) as previously described (Szczepanski et al. 2001). IgH gene rearrangements were detected with IgH J_H6 probe (772bp; forward 5'-GTGACCTCTCTCCGCTTCAC-3', reverse 5'-TATTTCACGATTCGCTGCTG-3') in HindIII digests. TCRδ gene rearrangements were studied by the use of 3' $J_\delta3$ probe (993bp; forward 5'-AATTTGAGTCCTGGCACTGG-3', reverse 5'-CCATTGTTTGGCATCTTGGT-3') in Nco1 digests. TCRβ gene rearrangements were detected with 3' $J_{\beta2}7$ probe (883bp; forward 5'-TCAGAGTCCGGAAAGCTGAG-3', reverse 5'-TAGCTGAGCCCCATTACACC-3') in HindIII digests.

SUPPLEMENTAL REFERENCES

Dimos, J.T., Rodolfa, K.T., Niakan, K.K., Weisenthal, L.M., Mitsumoto, H., Chung, W., Croft, G.F., Saphier, G., Leibel, R., Goland, R., et al. (2008). Science *321*, 1218-12121.

Freberg, C.T., Dahl, J.A., Timoskainen, S., and Collas, P. (2007). Mol Biol Cell. *18*, 1543-1553.

Loh, Y.H., Agarwal, S., Park, I.H., Urbach, A., Huo, H., Heffner, G.C., Kim, K., Miller, J.D., Ng, K., and Daley, G.Q. (2009). Blood *113*, 5476–5479.

Park, I.H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W., and Daley, G.Q. (2008a). Nature *451*, 141-146.

Park, I.H., Lerou, P.H., Zhao, R., Huo, H., and Daley, G.Q. (2008b). Nat Protoc. *3*, 1180-1186.

Rafael. A. Irizarry, Benjamin M. Bolstad, Francois Collin, Leslie M. Cope, Bridget Hobbs and Terence P. Speed (2003), Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Research* 31(4):e15

Relander, T., Brun, A., Hawley, R.G., Karlsson, S., and Richter, J. (2001). J. Gene Med. *3*, 207–218.

Szczepanski, T., Willemse, M.J., van Wering, E.R., van Weerden, J.F., Kamps, W.A. and van Dongen, J.J.M. (2001). Leukemia *15*, 1415–1423.