Episomal iPSC Reprogramming Plasmids

Cat. # SC900A-1

User Manual

Store kit at -20°C on receipt

Contents

I. Introduction

A. iPSC Reprogramming

In the past several years, the use of induced pluripotent stem cells (iPSCs) has gained attention from many researchers around the world as a potentially important resource for many applications, ranging from basic research to drug discovery. The use of four reprogramming factors delivered via retroviral transduction of cells was initially established as a powerful way to reprogram any somatic cell into an ES-like state (Takahashi et al. 2007). These reprogrammed cells are capable of differentiating into any cells representing the three germlines, presenting a system that mirrors human ES cells. While such systems are quite useful for *in vitro* studies of differentiation, the challenge of adapting these virally-derived iPSCs for *in vivo* applications (e.g. cell therapy) is quite high, owing to the potential for random integration and subsequent risk of mutagenesis from viral-mediated delivery of reprogramming factors.

Recently, the development of non-viral, non-integrating, plasmid-based reprogramming systems have gained popularity as an alternative to traditional retroviral-based reprogramming of cells (Fusaki et al. 2009, Yu et al. 2009). Notably, one of the systems, based on the Epstein-Barr Nuclear Antigen-1 (*oriP*/*EBNA-1*) has been proven to generate iPSCs very efficiently without the risk of transgenic sequences inserted into the target cell genome. Unlike traditional plasmid systems, the *oriP*/*EBNA-1* system replicates in synchrony with the host genome by anchoring itself to the host chromatin and replicating during the cell cycle. The reprogramming factors are contained in the same plasmid as *oriP*/*EBNA-1* and thus are expressed in a similar manner to other types of plasmids. Due to the potential silencing of the promoter driving *EBNA-1*, along with errors in episomal vector synthesis and plasmid dilution, the plasmids are preferentially lost at a rate of ~5% per cell division. After ~10-15 cell cycles, the bulk of the episomal plasmid is lost, leading to the generation of reprogrammed cells free of genomic integration or genetic alterations. These transgene-free iPSCs have the capability to be utilized for a broad range of applications, including pre-clinical research and human gene therapy, thus further delivering on the promise of iPS cells.

B. Product Description

Episomal iPSC Reprogramming Plasmids (catalog# SC900A-1) are a non-viral, non-integrating system that can reprogram somatic cells into induced pluripotent stem cells (iPSCs) in both feeder-dependent and feeder-free conditions. The system consists of an optimized mixture of episomal plasmids expressing a combination of reprogramming factors: Oct4, Sox2, Lin28, Klf4, L-Myc, p53shRNA, and miR-302/367 cluster. A GFP marker is also included for monitoring plasmid delivery and loss during real time. The plasmids are built in oriP/EBNA-1 (Epstein-Barr Nuclear Antigen-1) containing vectors. The presence of *oriP*/*EBNA-1* leads to the replication and partition of the plasmids into daughter cells during the cell cycle, and the robust expression of the factors allows the generation of human iPS cells in a single transfection. The plasmids are lost over the course of time due to loss of EBNA-1 expression, plasmid dilution, and errors in vector synthesis, allowing for transgene-free reprogrammed cell lines.

Package Contents

SC900A-1	Episomal iPSC Reprogramming Plasmids	5 rxn

C. Storage and Stability

Episomal iPSC Reprogramming Plasmids are stored at -20°C.

D. Additional Materials and Instruments Needed

Fibroblast Growth Medium (e.g. FibroLife® Serum-Free Cell Culture Medium supplemented with 2%FBS)

Human ESC/iPSC Growth Medium *(for feeder-dependent conditions)* (System Biosciences, Cat. No. SC100M-1)

PSGro® Human ESC/iPSC Growth Medium *(for feeder-free conditions)* (System Biosciences, Cat. No. SC500M-1)

PSGen™ Reprogramming Supplement (1000×) (System Biosciences, Cat. No. SC551M-1)

Gentle-Stem™ Enzyme-Free Human ESC/iPSC Dissociation Solution (System Biosciences, Cat. No. SC300M-1)

CRYO-GOLD™ Human ESC/iPSC Cryopreservation Medium (System Biosciences, Cat. No. SC150M-1)

Matrigel (BD Biosciences, Cat. No. 354277)

EmbryoMax® 0.1% Gelatin Solution (Millipore, Cat. No. ES-006-B)

Electroporation Instrument (e.g. Neon Transfection System)

Complete Antibody and AP staining Kit (System Biosciences, Cat. No. SAB-KIT-1)

Maxima SYBR Green 2X mix (Cat. No. K0223, MBI Fermentas)

II. Protocol for Reprogramming with Episomal Plasmids

* Episomal iPSC Reprogramming Plasmids (SC900A-1) can generate human iPS cells under both feeder-dependent and feeder-free conditions.

* Neon Transfection System (Invitrogen) is recommended in the protocol for electroporation. Other electroporation systems or transfection systems can also be used with the Episomal iPSC Reprogramming Plasmids.

* Human ESC/iPSC Growth Medium (SC100M-1) and PSGro® Human ESC/iPSC Growth Medium (SC500M-1) are recommended in the protocol for reprogramming and maintenance of iPS cells. Episomal iPSC Reprogramming Plasmids (SC900A-1) has also been tested with other commercially available media, (e.g. mTeSR1 Medium, Essential 8 Medium)

* PSGen^{TM} Reprogramming Supplement (1000×) (SC551M-1) is a small molecule mixture that greatly enhances the reprogramming efficiency under both feeder-dependent and feeder-free conditions. It has been tested for reprogramming in combination with Human ESC/iPSC Growth Medium (SC100M-1), PSGro® Human ESC/iPSC Growth Medium (SC500M-1), mTeSR1 Medium and KOSR-Based Medium.

A. Reprogramming with Episomal Plasmids under Feeder-Dependent Conditions

Day -4 to -2

1. Seed frozen human fibroblasts in a gelatin-coated T75 flask with fibroblast growth medium.

Day 0

2. Cells should be 70-90% confluent at the time of transfection.
3. Coat the wells of a 6-well plate with 0.1% gelatin solution (1.5 mL per well), let it sit at 37°C incubator for 1 hr.
4. Aspirate the gelatin solution from the gelatin-coated plate. Add 2 mL fibroblast growth medium (without antibiotics) per well. Place the 6-well plate with medium in 37°C incubator until needed.
5. Aspirate the medium from the fibroblasts in T75 flask. Wash the cells once with DPBS.
6. Add 2 mL 0.05% trypsin to the flask. Incubate the flask in 37°C incubator until the cells are completely detached.
7. Inactivate the trypsin with 5 mL fibroblast growth medium. Transfer the dislodged cells to a 15 mL conical tube.
8. Centrifuge the cells at 200 x g for 5 min. Aspirate the supernatant and resuspend the cells in 3 mL DPBS. Count the cells with a hemocytometer.
9. Centrifuge the cells at 200 x g for 5 min. Aspirate most of the supernatant and remove the remaining supernatant with a 200 µL pipette.
10. Resuspend cells in Resuspension Buffer R (provided with Neon Electroporation System) at a final concentration of 1 million cells/0.1mL.
11. Transfer 5-9 µL* of episomal iPSC reprogramming mixture to a sterile eppendorf tube. Add 100 µL of cell mixture.

    * The optimal amount of episomal plasmids for transfection can be different with different primary cell lines and different electroporation instruments

12. Electroporate the cells with the following program: 1650V, 10ms, 3 pulses. (100 µL DNA and cells mixture per electroporation with 100 µL electroporation tip from Neon Transfection System)
13. Plate 20% to 100%* from one reaction of the electroporated cells per well of 6-well plate to the gelatin-coated 6-well plate containing antibiotic-free fibroblast growth medium.

    * The optimal amount of cells plated depends on the proliferation speed of individual patient fibroblasts.

14. Incubate the plate at 37°C incubator overnight.

Day 1 - day 4~6

15. Replace the fibroblast growth medium every other day until cells reach 80% confluency.

Day 5~7 (one day before replating)

16. Coat the wells of a 6-well plate with 0.1% gelatin solution (1.5 mL per well) and let it sit at 37°C incubator for 1 hr.
17. Plate feeder cells in fibroblast growth medium at ~0.5 × 10^6 cells per well.

Day 6~8

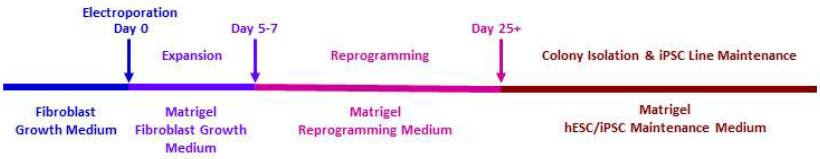
18. Change to fresh fibroblast growth medium for the feeder cells that were plated the day before. Put the plate back into the 37°C incubator.
19. Add 0.5 mL trypsin to each well of 6-well plate. Incubate the plate in 37°C incubator until the cells are completely detached.
20. Inactivate the trypsin with 2 mL of fibroblast growth medium per well. Transfer the dislodged cells to a 15 mL conical tube.
21. Centrifuge the cells at 200 x g for 5 min. Aspirate the supernatant and resuspend the cells in 2 mL fibroblast growth medium. Count the cells with a hemocytometer.
22. Seed 1-3 × 10^4 cells* in each well of 6-well plate with feeder cells. Incubate the plate in 37°C incubator overnight.

    * The optimal amount of cells plated depends on the proliferation speed of individual patient fibroblasts.

Day 9+

23. Prepare reprogramming medium by adding PSGen Reprogramming Supplement (1000×) to Human iPSC Growth Medium *(for Feeder-Dependent conditions)*. The reprogramming medium is stable at 4°C for a month.
24. Switch to reprogramming medium *(for Feeder-Dependent conditions)*. Change medium every other day.
25. Colonies start to appear after day 15-20.
26. Colonies can be picked day 20+.
27. Picked colonies can be maintained in Human ESC/iPSC Growth Medium. Change medium every day.

B. Reprogramming with Episomal Plasmids under Feeder-Free Conditions



Day -4 to -2

1. Seed frozen human fibroblasts in a gelatin-coated T75 flask with fibroblast growth medium.

Day 0

2. Cells should be 70-90% confluent at the time of transfection.
3. Coat the wells of a 6-well plate with matrigel according to manufacturer's instructions and dilution (1 mL per well) and let it sit at 37°C incubator for 1 hr (plates with matrigel solution can be stored at 4°C for up to one week)
4. Aspirate the matrigel solution from the matrigel-coated plate. Add 2 mL fibroblast growth medium (without antibiotics) per well. Place the 6-well plate with medium in 37°C incubator until needed.
5. Aspirate the medium from the fibroblasts in the T75 flask. Wash the cells once with DPBS.
6. Add 2 mL 0.05% trypsin to the flask. Incubate the flask in 37°C incubator until the cells are completely detached.
7. Inactivate the trypsin with 5 mL fibroblast growth medium. Transfer the dislodged cells to a 15 mL conical tube.
8. Centrifuge the cells at 200 x g for 5 min. Aspirate the supernatant and resuspend the cells in 3 mL DPBS. Count the cells with a hemocytometer.
9. Centrifuge the cells at 200 x g for 5 min. Aspirate most of the supernatant and remove the remaining supernatant with a 200 µL pipette.
10. Resuspend cells in Resuspension Buffer R (provided with Neon Electroporation System) at a final concentration of 1 million cells/0.1mL.
11. Transfer 5-9 µL* of episomal iPSC reprogramming mixture to a sterile eppendorf tube. Add 100 µL of cell mixture.

    * The optimal amount of episomal plasmids for transfection can be different with different primary cell lines and different electroporation instruments.

12. Electroporate the cells with the following program: 1650V, 10ms, 3 pulses. (100 µL DNA and cells mixture per electroporation with 100 µL electroporation tip from Neon Transfection System)
13. Plate one twelfth to one third* of the electroporated cells per well of 6-well plate to the matrigel-coated 6-well plate containing antibiotic-free fibroblast growth medium.

    * The exact amount of cells plated depends on the proliferation speed of individual patient fibroblasts.
14. Incubate the plate at 37°C incubator overnight.

Day 1 - day 5~7

15. Replace the fibroblasts growth medium every other day until cells reach 30% confluency.

Day 6~8+

16. Prepare reprogramming medium by adding PSGen Reprogramming Supplement (1000×) to PSGro Human ESC/iPSC Growth Medium. The reprogramming medium is stable at 4°C for a month.
17. Switch to reprogramming medium. Change medium every other day.
18. Colonies start to appear after day 15-20.
19. Colonies can be picked day 25+.
20. Picked colonies can be maintained in PSGro Human ESC/iPSC Growth Medium. Change medium every day.

C. Picking iPSC colonies

* Feeder-free condition is used as an example here for picking colonies, passaging, freezing and thawing iPS cells. Picking colonies, passaging, freezing and thawing iPS cells under feeder-dependent conditions can also follow the protocol below. Plates coated with feeder cells and Human iPSC Growth Medium (for Feeder-Dependent conditions) should be used for feeder-dependent conditions.

1. Examine the 6-well plate with cells under reprogramming under microscope, and mark the colonies to be picked at the bottom of the plate. (10-20 single colonies are recommended for establishing 3-6 independent and well-characterized iPS cell lines without integration)
2. In sterile cell culture hood, wash the well once with Gentle-Stem™ Enzyme-Free Human ESC/iPSC Dissociation Solution, and cover the well with 1mL Gentle-Stem™ Enzyme-Free Human ESC/iPSC Dissociation Solution.
3. Transfer the plate to an EVOS stereomicroscope placed in a sterile cell culture hood.
4. Using a 20 µL pipette, cut the colonies into small pieces and transfer the pieces to a 12-well matrigel coated plate with pre-warmed PSGro medium.
5. Aspirate the Gentle-Stem™ Enzyme-Free Human ESC/iPSC Dissociation Solution from the original plate and replace with pre-warmed PSGro medium.
6. Incubate the matrigel-coated 12-well plate containing the newly picked colonies in 37°C incubator. Allow the colonies to attach to the plate for 48 hours before changing medium.
7. Replace the PSGro medium every day.

D. Passaging iPSC colonies with Gentle-Stem™ Enzyme-Free Human ESC/iPSC Dissociation Solution

1. At least one hour before passaging, coat the plates with Matrigel, and incubate the plates in 37°C incubator.
2. Warm up 2 mL per well (after passaging) PSGro medium and 3 mL per well (before passaging) Gentle-Stem™ Enzyme-Free Solution to room temperature.
3. Use a microscope to mark the region of differentiation. Remove those regions with a pipette tip or an aspirator right before passaging.
4. Remove and discard the spent medium.
5. Add 2 mL/well of Gentle-Stem™ Enzyme-Free Solution to wash the well once.
6. Remove and discard the washing solution.
7. Add 1 mL/well Gentle-Stem™ Enzyme-Free Solution. Incubate at 37°C incubator for 3 min*.

   * The optimal incubation time could vary depending on the cell line, surface coating and medium.

8. Carefully remove and discard Gentle-Stem™ Enzyme-Free Solution. (Make sure the colonies are NOT detached from the plate.)
9. Add 2 mL/well PSGro medium. Wash colonies off the plate by pipetting the medium. (This step should be performed quickly, and take care not to break colonies too much by excess pipetting.)

10. Plate the appropriate amount of cells to newly prepared plate. If the colonies are at optimal density, the culture can be split every 4-6 days using 1:10 to 1:20 ratio.
11. Move the plate back and forth to ensure even distribution of the colonies. Put the plate back into 37°C incubator.

E. Freezing and Thawing iPS Cells with CRYO-GOLD™ Human ESC/iPSC Cryopreservation Medium

Freezing Cells with CRYO-GOLD™

1. Before mixing CRYO-GOLD™ Human ESC/iPSC Cryopreservation Medium with cells, centrifuge the cells first. After centrifuging, remove the supernatant as much as possible from the centrifuge tube (the remaining supernatant will affect the ability of CRYO-GOLD™ solution to maintain cell viability).
2. After removal of supernatant, slowly add cold (2-8°C) CRYO-GOLD™ solution to the cells and gently mix. Resuspend the cells at the concentration of 0.2-10×10^6 cells/mL.
3. Transfer the suspension to cryovials. Freeze the cryovials in controlled cooling container (~1 °C/min) and store the container at -80°C overnight. Transfer the cryovials to liquid N_2 tank the next day for long-term storage.

Thawing of Cells Stored in CRYO-GOLD™

1. Thaw samples quickly in 37°C water bath. Samples should be thawed with gentle swirling until all visible ice has melted (Do not allow sample to warm above chilled temperature (0-10°C). The cryovials should be cool to touch when removed from the water bath.).
2. Dilute the cells/CRYO-GOLD™ mixture immediately with appropriate culture medium for the cells. The culture medium should be at 20-37°C. A dilution ratio of 1:10 (cells/CRYO-GOLD™ mixture: medium) or greater is recommended. After diluting cells with warm culture medium, gently invert the centrifuge tube 5~10 times.
3. Centrifuge the cells for 10min at 1000rpm.
4. After centrifuging, completely remove the supernatant and add fresh warm culture medium.
5. Seed the cells in the tissue culture plate (coating the plate with appropriate material if needed) immediately.

F. Detecting Episomal Plasmids

To determine if the episomal reprogramming vectors are still present in your iPS cells, you will need to isolate total DNA from each clone you wish to test. We recommend using a kit similar to Qiagen's DNeasy Blood & Tissue Kit (cat#69504). Follow the kit's protocol on how many cells to grow and how to prepare the DNA.

Episomal Plasmid Detection Primers:

Forward Primer 5'- AGG TCC CTC GAA GAG GTT CA -3'

Reverse Primer 5'- TTC CAA CGC GAG AAG GTG TT -3'

Amplicon size: 95 bp

Internal Control Primers:

UCR1-Fwd 5'- TTA ACA ATC CGC TTG GAC ATG A-3'

UCR1-Rev 5'- AAA ATC AGT TCG CCA GAA GCA-3'

Episomal iPSC Reprogramming Plasmids Cat# SC900A-1
Amplicon size: 62 bp

PCR Reaction	Amount (µL)
Genomic DNA (10 ng/µL)	1.0
2X SYBR Mix	5.0
5 uM Primer Mix	0.4
Water	3.6
Total	**10.0**

qPCR Program:

1. 50 C 2 min
2. 95 C 10 min
3. 95 C 15 sec
4. 60 C 1 min
5. Go to step 3, 39 more times (40 total cycles)
6. 95 C 15 sec
7. 60 C 15 sec

qPCR was carried out on the Applied Biosystems 7900HT Fast Real-Time PCR System.

III. Validation Results

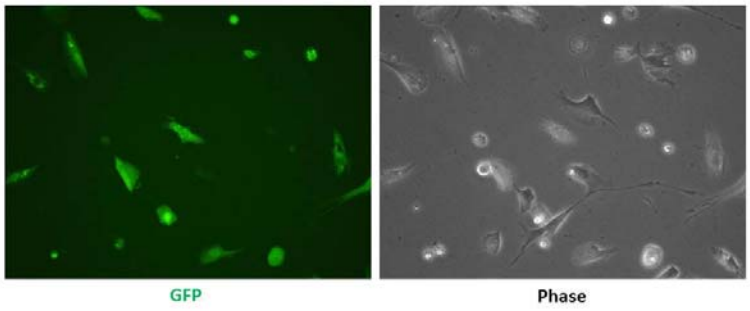


Figure 1. Human adult primary dermal fibroblasts one day after transfection. Cells were cultured in FibroLife® Serum-Free Cell Culture Medium supplemented with 2%FBS.

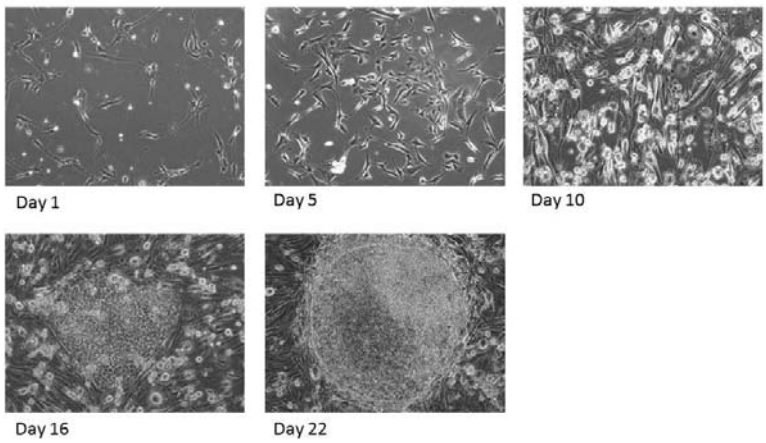


Figure 2. Derivation of iPS cells under feeder-dependent conditions. iPSC colonies were derived with PSGro® Human ESC/iPSC Growth Medium *(for feeder-dependent conditions)* plus PSGen™ Reprogramming Supplement (1000×)

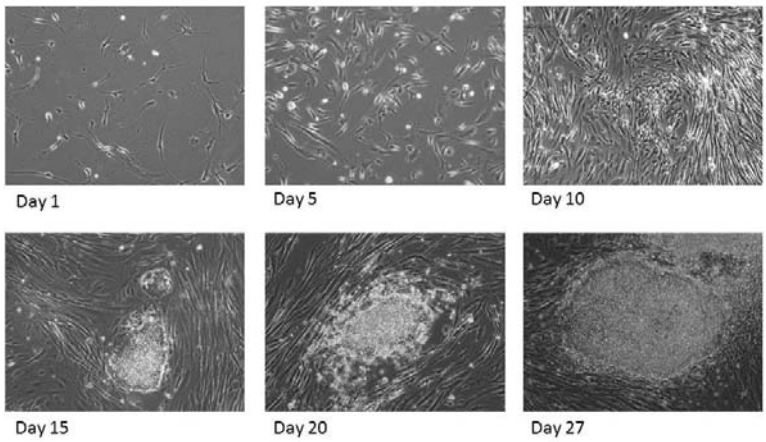


Figure 3. Derivation of iPS cells under feeder-free conditions. iPSC colonies were derived with PSGro® Human ESC/iPSC Growth Medium *(for feeder-free conditions)* plus PSGen™ Reprogramming Supplement (1000×).

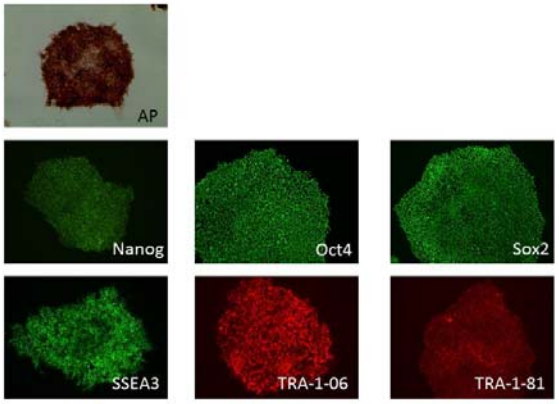


Figure 4. Characterization of iPS cell colonies by AP staining and immuno-staining of pluripotencies markers, Nanog, Oct4, Sox2, SSEA3, TRA-1-60 and TRA-1-81. (Complete Antibody and AP staining Kit, SAB-KIT-1)

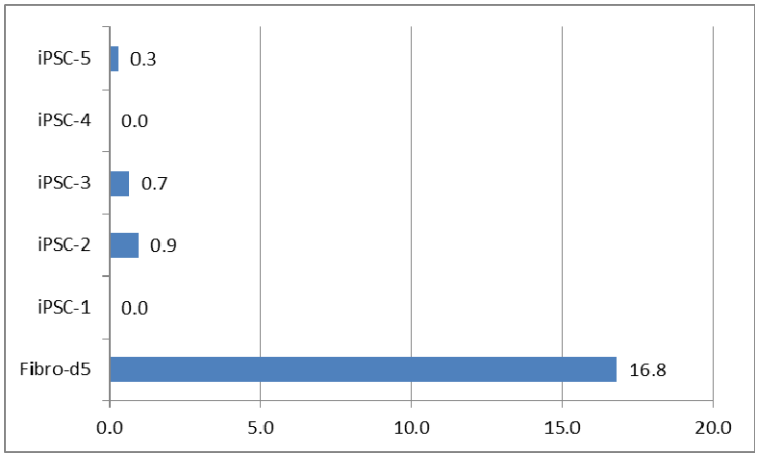


Figure 5. Average copy number of episomal plasmids from iPS cell lines derived from individual clones measured by qPCR. Genomic DNA of human dermal fibroblasts 5 days after electroporation was analyzed as positive control (Fibro-d5). The episomal plasmids were below detectable levels in iPSC lines 1 and 4 and <1 for several other characterized lines.

IV. References

Takahashi K et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007 Nov 30;131(5):861-72.

Fusaki N et al. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome.
Proc Jpn Acad Ser B Phys Biol Sci. 2009;85(8):348-62.

Yu J et al. Human induced pluripotent stem cells free of vector and transgene sequences. Science 2009 May 8;324(5928):797-801.

V. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

Phone: (650) 968-2200
(888) 266-5066 (Toll Free)
Fax: (650) 968-2277
E-mail:
General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

System Biosciences (SBI)
265 North Whisman Road
Mountain View, CA 94043

VI. Licensing and Warranty

Use of the Episomal iPSC Reprogramming Plasmids (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

The purchaser of the Product is granted a limited license to use the Product under the following terms and conditions:

The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use.

Limited Warranty

SBI warrants that the Product meets the specifications described in the accompanying Product Analysis Certificate. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

SBI's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

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