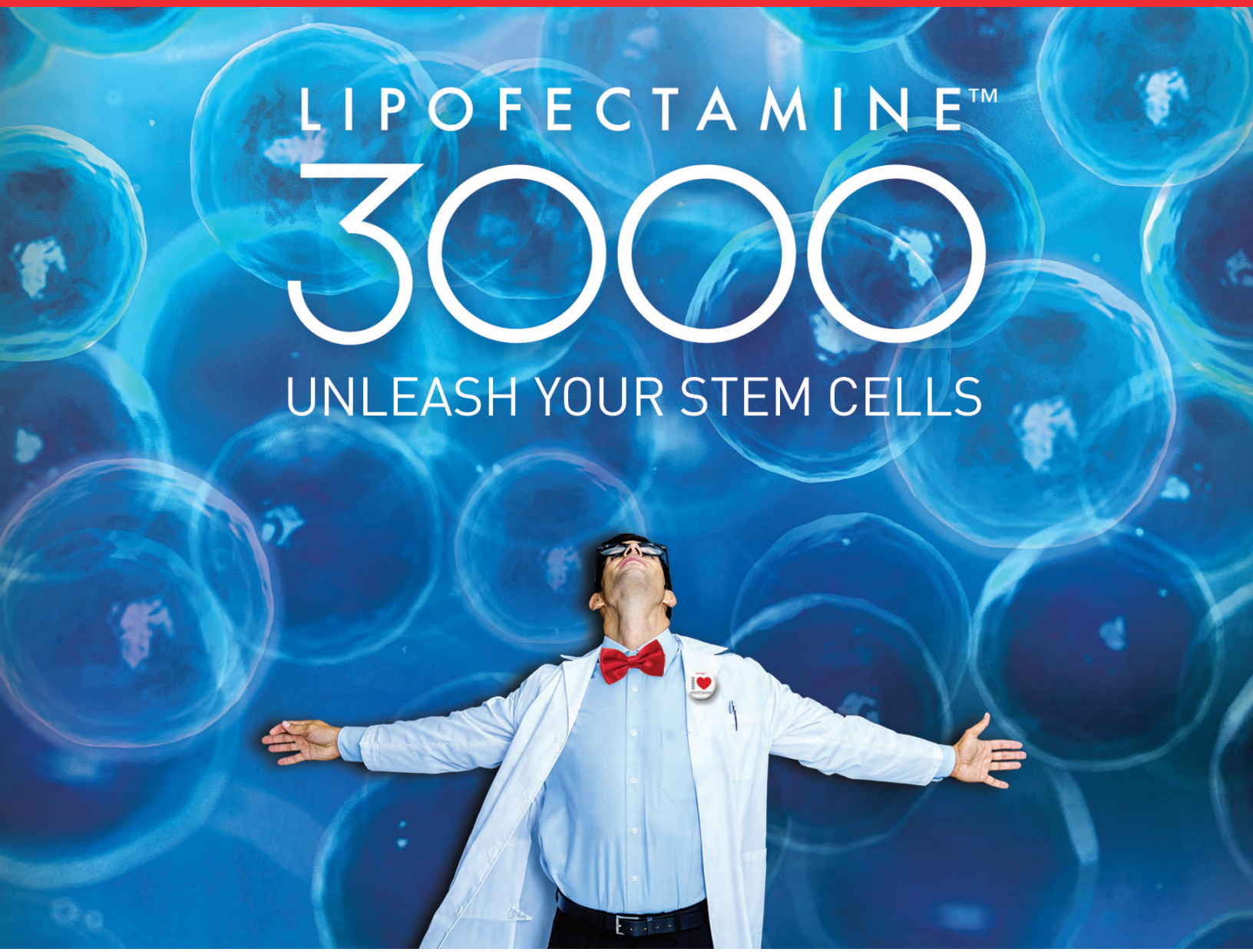
invitrogen



Lipofectamine 3000 Reagent

Generation and transfection of induced pluripotent stem cells (iPSCs)

ThermoFisher
SCIENTIFIC

Induced pluripotent stem cells (iPSCs) hold immense promise for the future of regenerative medicine and personalized therapeutic treatments for a myriad of diseases and conditions. Invitrogen™ Lipofectamine™ 3000 Transfection Reagent was developed to unleash the power of stem cells by providing a highly efficient, cost-effective nucleic acid delivery alternative to electroporation. This advanced lipid nanoparticle technology minimizes the stress on cells caused by electroporation, simplifies the reprogramming workflow, and enables advanced gene editing technologies.

Lipofectamine 3000 reagent is designed to provide:

- **Superior efficiency**—for the broadest spectrum of difficult-to-transfect cells
- **Low toxicity**—gentle on cells for improved viability
- **Versatility**—single kit for DNA, RNA, and cotransfection

> "Successfully transfected GT1-1 cells. Never achieved with any other reagent except viruses and nucleofectors."
>
> —Julien Sebag, PhD
> Vanderbilt University

Contents

Generation of iPSCs from donor-derived fibroblasts

Reprogramming of fibroblasts

Patient-derived iPSCs offer exciting potential by enabling access to cell populations that are otherwise unavailable from living donors. Efficient reprogramming of donor somatic cells to iPSCs plays a key role in the realization of this potential. In this study, fibroblasts from skin biopsies of three donors were reprogrammed to iPSCs using the Invitrogen™ Epi5™ Episomal iPSC Reprogramming Kit* and Lipofectamine 3000 reagent (Figures 1 and 2). Reprograming efficiencies equivalent to those seen with electroporation were observed (Figure 3). This advancement in delivery helps to minimize stress on the cells caused by electroporation, simplifies the reprogramming workflow, and results in transgene-free, virus-free human iPSCs at efficiencies in the range of 0.04% to 0.3%.

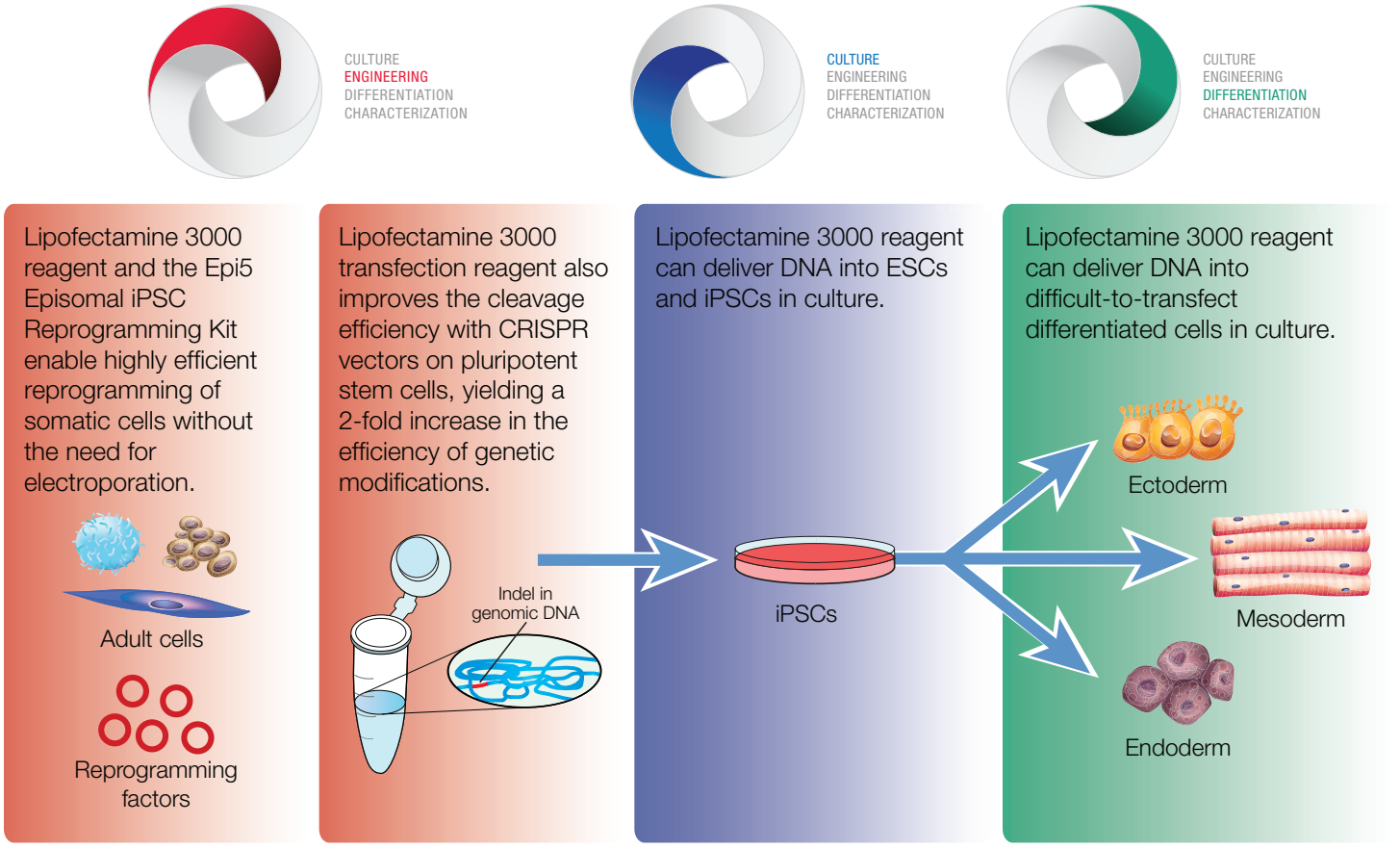


Figure 1. Lipofectamine 3000 reagent for pluripotent stem cell applications. High transfection efficiency enables Lipofectamine 3000 to be used for a variety of applications, including reprogramming of fibroblasts, gene editing, and transfection of difficult cells.

* Designed by Dr. Okita in the laboratory of Professor Yamanaka at the Center for iPS Cell Research and Application (CiRA), Kyoto University.

Day –1	Day 0	Day 14	Day 21
• Day –1: Seed cells on Geltrex matrix–coated dishes	• Day 0: Transfect in Fibroblast Medium for 24 hours • Day 1–14: Culture in N2B27 Medium with 100 ng/mL bFGF (change daily)	• Day 15–20: Culture in E8 Medium (change daily) • Days 21+: Manually pick and expand colonies	
Fibroblast Medium	N2B27 Medium with bFGF (100 ng/mL)	Essential 8 Medium	

Geltrex matrix

Lipofectamine 3000

Epi5 Episomal iPSC Reprogramming Kit

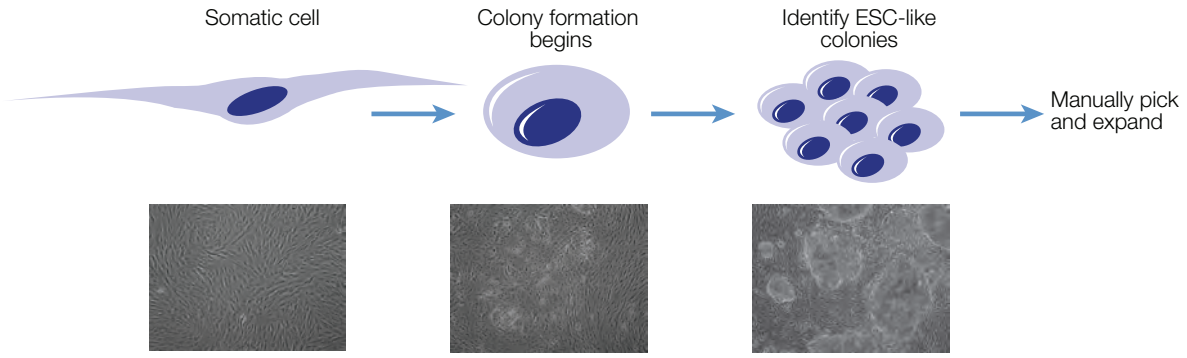


Figure 2. Protocol outline for generating iPSCs using the Epi5 reprogramming kit and Lipofectamine 3000 reagent. Colonies can be picked and expanded less than 21 days after fibroblast transfection.

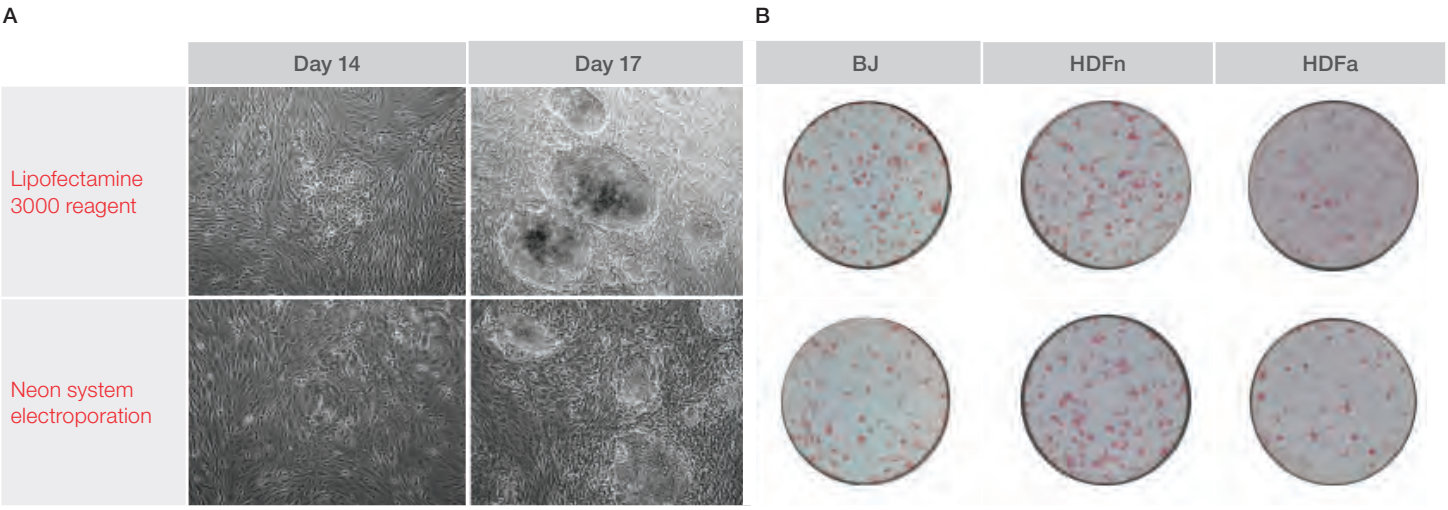
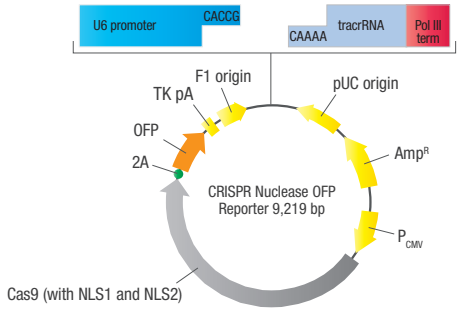


Figure 3. Reprogramming efficiency of Lipofectamine 3000 reagent compared to electroporation. BJ fibroblasts as well as neonatal (HDFn) and adult (HDFa) human dermal fibroblasts were reprogrammed to iPSCs by transfection of Epi5™ vectors using either Lipofectamine 3000 reagent or the Invitrogen™ Neon™ Transfection System. Colonies were **(A)** visualized by brightfield microscopy and **(B)** stained for alkaline phosphatase.

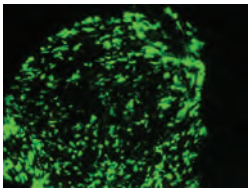
Genome editing of pluripotent stem cells

Genome editing technology derived from clustered regularly interspaced short palindromic repeats (CRISPRs) allows precise cleavage of DNA at specific loci. However, the effectiveness of genome editing is contingent upon the intrinsic properties of the locus of interest, efficiency of delivery, and the painstaking downstream processes of generating stable cell lines and knockout models to study the phenotypic effects of the genetic modifications. Here we demonstrate that Lipofectamine 3000 reagent can deliver plasmid DNA into various iPSC clones for genome engineering purposes using targeted CRISPR vectors. More than a 2-fold increase in gene modification efficiency compared to Invitrogen™ Lipofectamine™ 2000 reagent was observed using Lipofectamine 3000 reagent, and with a lower amount of lipid (Figure 4).

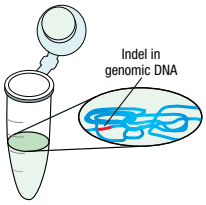
Design insert for GeneArt CRISPR vector

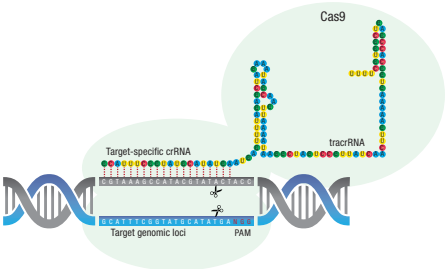


Deliver with Lipofectamine 3000 reagent

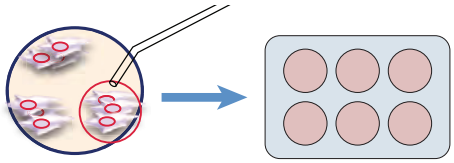


Measure cleavage efficiency using GeneArt Genomic Cleavage Detection (GCD) Kit

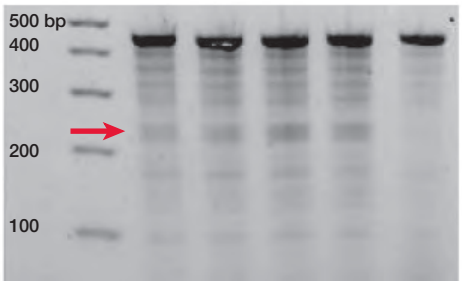




Colony screening and expansion



	1 kb ladder	Lipofectamine 2000 reagent		Lipofectamine 3000 reagent		GFP control vector
Dosage (µL)		0.3	0.5	0.15	0.3	0.3



Gene modification efficiency (%)	2.5	3.6	5.5	4.0	0

Figure 4. Lipofectamine 3000 reagent improves cleavage efficency of CRISPR vectors in iPSCs. An Invitrogen™ GeneArt™ CRISPR vector targeting a specific locus was transiently transfected using Lipofectamine 2000 or Lipofectamine 3000 reagent. Cleavage efficiency was assessed using the Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit.

Transfection of pluripotent stem cells using Lipofectamine 3000 reagent

Lipofectamine 3000 Transfection Reagent was used to deliver DNA into difficult-to-transfect stem cells (H9 ESCs and iPSCs) growing as colonies in Gibco™ Geltrex™ matrix–coated plates (Figure 5). Transfection efficiencies of 40–70% were observed, with high mean fluorescence intensity (Figure 6).

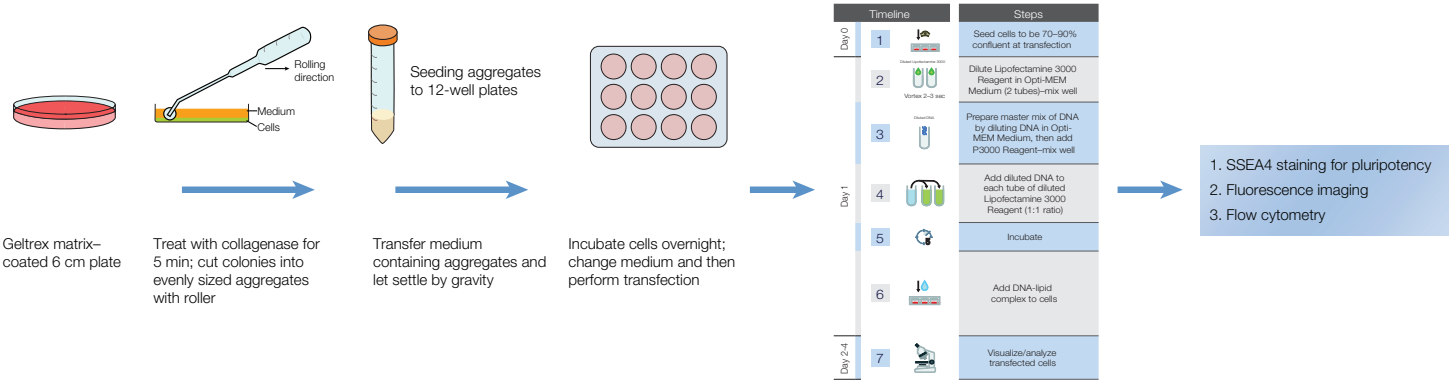
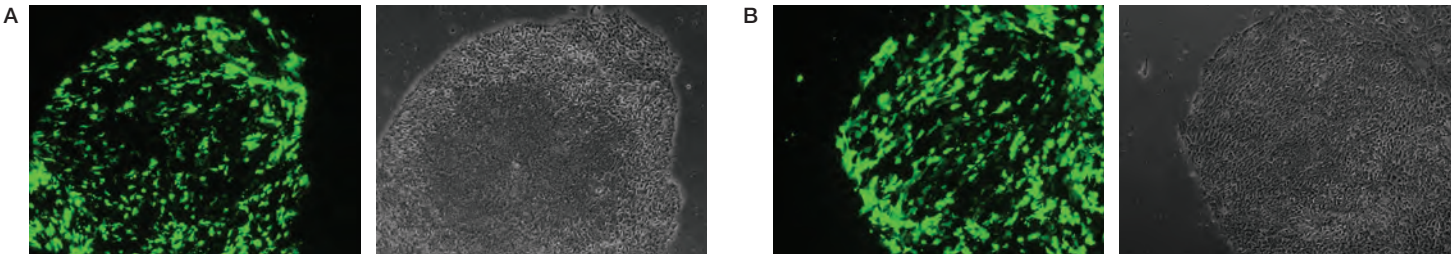


Figure 5. Protocol outline for transfection of pluripotent stem cells with Lipofectamine 3000 reagent. Cells were transferred into multiwell plates from Geltrex matrix–coated plates and transfected with Lipofectamine 3000 reagent. Transfection efficiency was assessed using flow cytometry and fluorescence imaging.



DNA: 1.0 µg
Lipofectamine 3000: 1.5 µL
SSEA+/GFP+: 42%
GFP MFI 247344

DNA: 1.3 µg
Lipofectamine 3000: 1.5 µL
SSEA+/GFP+: 69%
GFP MFI 456741

Figure 6. Transfection of stem cells. (A) H9 ESCs or **(B)** iPSCs were transfected using Lipofectamine 3000 reagent. Cells were visualized by fluorescence microscopy and processed using flow cytometry to determine transfection efficiency.

Summary

Lipofectamine 3000 Transfection Reagent was developed to improve transfection efficiency in difficult-to-transfect cells. In this study, we demonstrated that using a superior transfection reagent such as Lipofectamine 3000 reagent in conjunction with the Epi5 Episomal iPSC Reprogramming Kit enables highly efficient reprogramming of somatic cells without the need for electroporation.

Detailed protocol for reprogramming human fibroblasts

Lipofectamine 3000 Transfection Reagent, used with the Epi5 Episomal iPSC Reprogramming Kit, enables highly efficient reprogramming of somatic cells without electroporation.

Reprogramming timeline				
Day –1	**Day 0**	**Days 1–14**	**Days 15–20**	**Days 21+**
Seed cells on Geltrex matrix–coated 6-well dishes	Transfect in fibroblast growth medium for 24 hr	N2B27 Medium with 100 ng/mL FGF (change medium daily)	Essential 8 Medium (change medium daily)	Colonies ready for picking for further culture and expansion

Cells and reagents	Cat. No.	Equipment
Epi5 Episomal iPSC Reprogramming Kit	A15960	Sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereomicroscope
Lipofectamine 3000 Transfection Reagent	L3000015	Inverted microscope
Opti-MEM I Reduced Serum Medium	31985062	Incubator set at 37°C, 5% CO_2
Basic Fibroblast Growth Factor (bFGF), Recombinant Human Protein	PHG0261	Water bath set at 37°C
KnockOut Serum Replacement	10828010	Sterile serological pipettes (5 mL, 10 mL)
Essential 8 Medium	A1517001	Centrifuge
DMEM/F-12, GlutaMAX Supplement	10565018	15 mL centrifuge tubes
N-2 Supplement (100X)	17502048	6-well tissue culture treated plates
B-27 Supplement (50X), Serum Free	17504044	10 µL, 200 µL, and 1,000 µL micropipettors with tips
100X MEM Non-Essential Amino Acids (NEAA)	11140050	
β-Mercaptoethanol	21985023	
Fetal Bovine Serum, embryonic stem cell–qualified	16141061	
DMEM, High Glucose, GlutaMAX Supplement	10566016	
0.05% Trypsin-EDTA (1X), phenol red	25300054	
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	A1413301	
Dulbecco's PBS (DPBS) without Calcium and Magnesium	14190	

Preparation of cells and reagents

I. Media preparation

Fibroblast Medium

To prepare 500 mL of Fibroblast Medium, aseptically mix the following components. Fibroblast Medium can be stored at 4°C for up to 1 month.

DMEM	445 mL
Fetal bovine serum (FBS), ESC-qualified	50 mL
100X MEM NEAA (10 mM)	5 mL

N2B27 Medium

To prepare 500 mL of complete N2B27 Medium, aseptically mix the following components. N2B27 Medium (without bFGF) can be stored at 2–8°C for up to 1 week.

DMEM/F-12	479 mL
N-2 Supplement (100X)	5 mL
B-27™ Supplement (50X)	10 mL
100X MEM NEAA (10 mM)	5 mL
β-Mercaptoethanol (1,000X)	908 µL

bFGF (100 µg/mL)

1. bFGF stock solution: To prepare 1 mL of 100 µg/mL bFGF solution, aseptically mix the following components:

bFGF	100 µg
DPBS without Ca^{2+} and Mg^{2+}	999 µL
KnockOut Serum Replacement	1 µL

2. Aliquot and store the bFGF solution at –20°C for up to 6 months.
3. A total of 1.0 µL of bFGF is needed for every mL of N2B27 Medium to be used. Prepare the N2B27 Medium without bFGF, and then supplement with fresh bFGF to a final concentration of 100 ng/mL when the medium is used.

Essential 8 Medium

1. To prepare 500 mL of Gibco™ Essential 8™ Medium, thaw the frozen Essential 8 Supplement at 2–8°C overnight. Do not thaw the frozen supplement at 37°C.
2. Mix the thawed supplement by gently inverting the vial a couple of times, remove 10 mL from the bottle of Essential 8 Basal Medium, and then aseptically transfer the entire contents of the Essential 8 Supplement to the bottle of Essential 8 Basal Medium.
3. Swirl the bottle to mix and to obtain 500 mL of homogenous complete medium.
4. Complete Essential 8 Medium can be stored at 2–8°C for up to 2 weeks. Before use, warm complete medium required for that day at room temperature until the bottle is no longer cool to the touch. Do not warm the medium at 37°C.

II. Preparation of Geltrex matrix–coated dishes

1. Thaw Geltrex matrix at 4°C overnight.
2. Remove DMEM/F-12 from 4°C storage. Work asceptically on ice to prevent polymerization. Dilute the Geltrex matrix with an equal amount of DMEM/F-12 (1:1) and mix gently. Aliquot into microcentrifuge tubes and freeze immediately at –20°C.
3. Prepare a working stock solution on ice from the 1:1 diluted Geltrex matrix with additional DMEM/F-12. For general growth, a final dilution of 1:100 is recommended. Cover the whole surface of each well of a 6-well dish with 1.5 mL of the working stock of Geltrex matrix.
4. Incubate the coated culture vessels for 1 hour at 37°C. Transfer the plates or dishes to a laminar flow hood and allow them to equilibrate to room temperature (about 1 hour) before use. If the coated plates or dishes are not used right away, seal with Parafilm™ wrap and store at 4°C for up to 2 weeks. Care must be taken to prevent these stored dishes from drying out or being contaminated.

III. Fibroblast culturing

1. Prewarm Fibroblast Medium in a 37°C water bath.
2. Wear eye protection, as cryogenic vials stored in liquid nitrogen may explode when warmed.
3. Wear ultralow-temperature cryogenic gloves. Remove the cryogenic vial of BJ fibroblasts from the liquid nitrogen storage tank using metal forceps.
4. Roll the vial between your hands for about 10–15 seconds to remove frost.
5. Immerse vials in a 37°C water bath. Swirl gently.

   Note: Do not submerge the cap.
6. When only an ice crystal remains, remove the vial from the water bath.
7. Spray the outside of the vial with 70% ethanol and place in the hood.
8. Pipet cells into a 50 mL conical tube with a 1 mL micropipettor.
9. Using a 10 mL pipette, add 10 mL of prewarmed Fibroblast Medium to the 50 mL conical tube dropwise (to avoid osmotic shock to cells) while gently swirling the conical tube. Pipette up and down gently to mix.
10. Centrifuge at 200 x *g* for 4 minutes and aspirate the supernatant.
11. Resuspend the cell pellet in the appropriate amount of Fibroblast Medium. Typically seed the contents of the vial in 5–7 mL of medium in a T25 tissue culture treated flask.
12. Incubate the BJ fibroblast cultures in a 37°C, 5% CO_2 incubator overnight.
13. The following day, aspirate off spent medium and replace with fresh, prewarmed Fibroblast Medium. Change medium every other day until the flask is approximately 70% confluent.
14. When 70% confluency is reached, the cultures must be split and reseeded for creating cell banks, or seeded directly for Epi5 kit transfection. It is recommended to use a portion of early-passage cells for experimentation.
15. To split fibroblast cultures, aspirate the Fibroblast Medium from culture vessel.
16. To wash, add 5 mL of room-temperature DPBS without $CaCl_2$ and $MgCl_2$ (divalent cations must not be present for proper dissociation of the fibroblasts from the culture dish) and allow the wash solution to remain at room temperature for 5 minutes.
17. Aspirate the DPBS wash and add 2 mL of 0.05% trypsin-EDTA, at room temperature, to the culture vessel (adjust volume of trypsin up or down for appropriate culture vessel size). Incubate the culture in a 37°C, 5% CO_2 incubator for approximately 3 to 5 minutes. Observe the cells under a microscope and stop incubation when the cells start to round up.
18. Add 4 mL prewarmed Fibroblast Medium to the culture dish to stop trypsinization. Wash off all the cells from the vessel surface by flushing the suspension several times to harvest all the cells in a single cell suspension. Transfer the cell suspension to a 15 mL centrifuge tube.
19. Centrifuge at 200 x *g* for 4 minutes and aspirate the supernatant.
20. Resuspend the cell pellet with 2 to 3 mL of fresh prewarmed Fibroblast Medium and pipet up and down to create a single cell suspension.
21. Prepare a cell count and seed the appropriate amount of cells into a new tissue culture treated dish with the appropriate volume of medium. Typically a 1:6 split can be used for regular maintenance every 3 to 4 days.

IV. Epi5 episomal reprogramming

Seeding human fibroblasts on Geltrex matrix–coated dishes

Plate 50,000 to 100,000 cells per well into a 6-well plate at ~30–60% confluence (see Figure 7) in 2 mL Fibroblast Medium and culture overnight at 37°C and 5% CO_2.

Transfection of human fibroblasts in a 6-well dish using Lipofectamine 3000 reagent

1. Prewarm Gibco™ Opti-MEM™ I Reduced-Serum Medium to room temperature and prepare Tube A and Tube B as described below.
2. Add 1.2 µL each of the two Epi5™ Reprogramming Vector mixes (2.4 µL total) to 118 µL Opti-MEM medium in a 1.5 mL microcentrifuge tube labeled Tube A. Add 4.8 µL of P3000™ Reagent and mix well.
3. Dilute 3.6 µL Lipofectamine 3000 reagent in 121 µL prewarmed Opti-MEM medium in a 1.5 mL microcentrifuge tube labeled Tube B.

Note: Lipofectamine 3000 reagent diluted in Opti-MEM medium should be used within 15 minutes of dilution. Longer times can result in a loss of transfection efficiency.

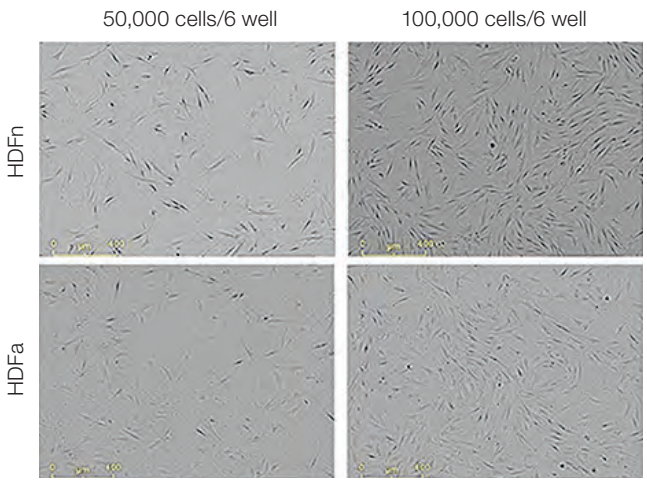


Figure 7. Seeding densities of neonatal (HDFn) and adult (HDFa) human dermal fibroblasts.

Preparation of contents of Tube A and Tube B on a per-well basis of a 6-well dish is outlined in the table below:

4. To prepare a transfection master mix, add the contents of Tube A to Tube B and mix well.
5. Incubate the transfection master mix for 5 minutes at room temperature.
6. Mix one more time and add the entire 250 µL of transfection master mix to each well of human fibroblasts seeded on Geltrex matrix–coated plates containing 2 mL fresh fibroblast growth medium.
7. Incubate the plates in a 37°C, 5% CO_2 incubator for 24 hours.

Component	Volume
Tube A	
Epi5 reprogramming vectors	1.2 µL
Epi5 p53 and EBNA vectors	1.2 µL
Opti-MEM medium	118 µL
P3000 reagent	4.8 µL
Tube B	
Lipofectamine 3000 reagent	3.6 µL
Opti-MEM medium	121 µL

Removal of transfection reagent and recovery of cells

1. 24 hours posttransfection, aspirate the medium from the plates. Add 2 mL N2B27 Medium supplemented with 100 ng/mL bFGF (added fresh prior to use) to each well.
2. Change the N2B27 Medium every day for a total of 14 days by replacing the spent medium with 2 mL N2B27 Medium supplemented with 100 ng/mL bFGF per well.

Transition to Essential 8 Medium

1. Aspirate the spent N2B27 Medium on Day 14 and replace it with complete Essential 8 Medium. Resume medium changes every day at 2 mL per well.
2. Observe the plates every other day under a microscope for the emergence of cell clumps, indicative of transformed cells. Within 15 to 21 days posttransfection, the iPSC colonies will grow to an appropriate size for transfer.
3. Colonies are distinct by Day 21 and can be picked for further culture and expansion. An example of results is shown in Figure 8.

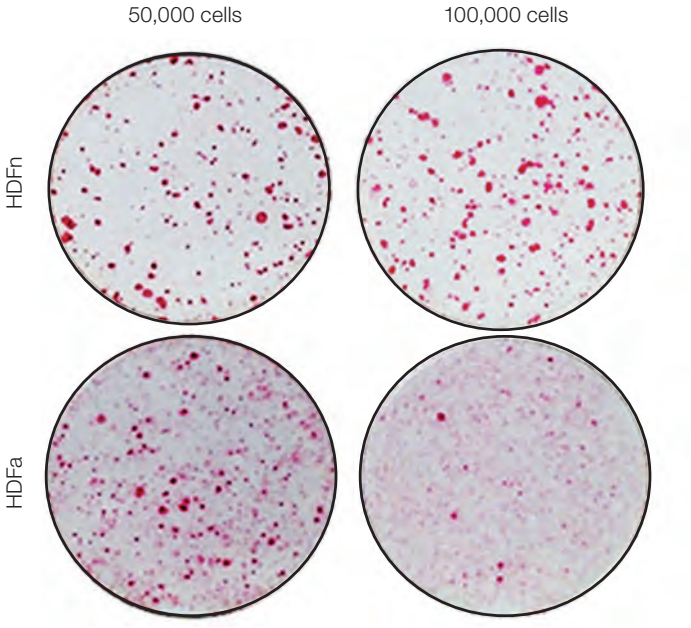


Figure 8. Alkaline phosphatase–positive colonies obtained after reprogramming of neonatal (HDFn) and adult (HDFa) human dermal fibroblasts.

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