Chapter 3

Direct Cell Reprogramming of Mouse Fibroblasts into Functional Astrocytes Using Lentiviral Overexpression of the Transcription Factors NFIA, NFIB, and SOX9

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Abstract

Astrocytes play an important role in maintaining brain homeostasis and their dysfunction is involved in a number of neurological disorders. An accessible source of astrocytes is essential to model neurological diseases and potential cell therapy approaches. Cell reprogramming techniques offer possibilities to reprogram terminally differentiated cells into other cell types. By overexpressing the three astrocytic transcription factors NFIA, NFIB, and SOX9, we showed that it is possible to directly transdifferentiate fibroblasts into functional astrocytes. These induced astrocytes (iAstrocytes) express glial fibrillary acidic protein (GFAP) and S100 calcium binding protein B (S100B), as well as other astrocytic markers. Moreover, electrophysiological properties indicate that iAstrocytes are functionally comparable to native brain astrocytes. Here we describe an optimized protocol to generate iAstrocytes starting from skin fibroblasts and this approach can be adapted for a wide range of somatic cell types.

Key words Cell reprogramming, Astrocytes, Fibroblasts, Lentivirus, Transcription factors

1 Introduction

Astrocytes are the most abundant cell type in the central nervous system (CNS) and are essential for maintaining homeostasis of the brain. They are involved in many key processes which include coordinating synaptogenesis and neuronal firing [1, 2], nutrient and ion metabolism [3, 4], forming and maintaining the blood-brain barrier [5–7], regulating neurotransmitter transport and degradation [8, 9], and protection against oxidative stress [10]. Breakdown of these fundamental functions has been connected to multiple neurological disorders, such as Alzheimer's disease [11], Parkinson's disease [12], Huntington's disease [13], lysosomal storage disorders [14], and Rett's syndrome [15].

Easy accessibility to a source of astrocytes is a key point to carry out any astrocyte-related research. Current protocols for the generation of astrocytes use pluripotent cell (ESC/IPSC)-derived

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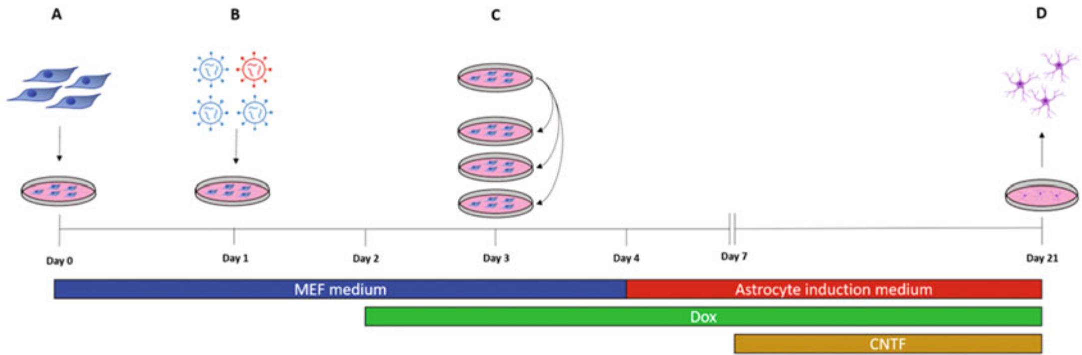

Fig. 1 Schematic overview of the cell reprogramming protocol. (**a**) On day 0, 2×10^5 MEF cells are seeded per well of a 6-well plate. (**b**) On day 1, MEFs are infected with the TetO-FUW NfiA/NfiB/SOX9 and M2rtTA viruses. (**c**) On day 3, cells are re-plated in new Matrigel-coated plates at 1.0×10^4 cells/cm^2. (**d**) After 3 weeks of differentiation, iAstrocytes can be directly characterized or be re-plated again for other applications

neural stem cells as a starting point [16]. A number of growth factors need to be added in the medium to drive astrocyte differentiation. These methods require precise manipulation of various growth factors at different stages during differentiation which is labor-intensive and can also be time-consuming, with some taking up to 6 months [17].

Cell reprogramming techniques based on the forced overexpression of certain cell lineage transcription factors (TFs) have shown to directly convert somatic/pluripotent cells into a variety of cell types [18–24].

Here we describe a fast (2–3 weeks) and direct reprogramming protocol to differentiate induced astrocytes (iAstrocytes) from fibroblasts (Fig. 1). Fibroblasts are seeded and subsequently infected with lentivirus carrying three astrocytic TFs (NFIA, NFIB, SOX9) along with a reverse tetracycline-controlled transactivator (rtTA) virus. Overexpression of these TFs is introduced by doxycycline (DOX) addition in the medium. Two weeks later, the reprogramed cells begin to display astrocyte-like morphology and show expression of the two main astrocytic markers glial fibrillary acidic protein (GFAP) and S100 calcium binding protein B (S100B). Genetic profiling reveals that 3 week's iAstrocytes show similar gene expression levels compared to real brain astrocytes in terms of astrocyte-related genes. Additionally, calcium imaging reveals that iAstrocytes respond to ATP/KCL/glutamate stimulation in a similar pattern as native brain astrocytes. These results demonstrate that our protocol can generate induced astrocytes by overexpressing astrocytic transcription factors NFIA, NFIB, and SOX9 in fibroblasts. This fast source of astrocytes can be instrumental for the progress of neurological disease modelling and cell-based therapies.

2 Materials

2.1 Lentivirus Production and Validation

1. HEK 293T cells (ATCC, CRL11268).
2. HEK 293T medium: 10%(v/v) fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM). Store up to 4 weeks at 4 °C.
3. Plasmids:
   (a) pMDLg/pRRE (Addgene, clone 12251).
   (b) pRSV-Rev (Addgene, clone 12253).
   (c) pMD2.G (Addgene, clone 12259).
   (d) FUW-M2rtTA (Addgene, clone 20342).
   (e) TetO-FUW-NfiA (Addgene, clone 64901).
   (f) TetO-FUW-NfiB (Addgene, clone 64900).
   (g) FUW-TetO-SOX9 (Addgene, clone 41080).
4. Plasmid maxiprep kit.
5. 3 M sodium acetate (pH 5.5) in Milli-Q H_2O, filtered through 0.22μm filter. Store at room temperature (RT).
6. 95% and 75% ethanol. Store at −20 °C.
7. Milli-Q H_2O, filtered through 0.22μm Minisart® syringe filter.
8. 2 M $CaCl_2$ in Milli-Q H_2O, filtered through 0.22μm filter; store at 4 °C.
9. 2× HBS buffer (pH 7.4): 281 mM NaCl, 100 mM HEPES, 1.5 mM Na_2HPO_4 in Milli-Q H_2O, filtered through 0.22μm filter; store aliquots at −20 °C.
10. 145 mm cell culture dishes, PS, Cellstar®.
11. MS 3 basic vortex.
12. 0.45μm filter units.
13. Ultra-Clear™ centrifuge tubes.
14. 8 mg/ml polybrene in sterile Milli-Q H_2O.
15. TRIzol™ reagent.
16. Chloroform.
17. Isopropanol.
18. Nuclease-free water.
19. iScript™ cDNA Synthesis Kit.
20. iTaq™ Universal SYBR Green Supermix RT-qPCR Kit.
21. CFX96™ Real-Time PCR System C1000™ Thermal Cycler.
22. QPCR primers:
    (a) NfiA, **Forward**: CAGCCAAGTGAAGCTGACAT **Reverse**: CCTGATGTGACAAAGCTGTCC.

(b) NfiB, **Forward**: AGCTGCTGGAAGTCGAACAT **Reverse**: TCTGGGGAAGAATCCTGTG.

(c) SOX9, **Forward**: GTGCAGCACAAGAAAGACCA **Reverse**: CAGCGCCTTGAAGATAGCAT.

2.2 Cell Culture and Reprogramming

1. Mouse embryonic fibroblasts (MEFs) are derived from E14.5 mouse embryos [18].
2. MEF medium: 10% (v/v) FBS, 0.1 mM Non-essential Amino Acid (NEAA), 1 mM sodium pyruvate in high glucose DMEM. Store up to 4 weeks at 4 °C.
3. Matrigel® Growth Factor Reduced Basement Membrane Matrix. Thaw on ice overnight and store aliquots at −20 °C. For plate coating concentration, dilute Matrigel in F12/DMEM medium based on corresponding dilution factor provided by the company for each batch. Diluted Matrigel solution can be stored for 2 weeks at 4 °C (*see* **Note 1**).
4. Astrocyte induction medium: 2%(v/v) FBS, 2 mM L-glutamine, 1× N2 supplement, 1× Antibiotic-Antimycotic solution in F12/DMEM. Store up to 2 weeks at 4 °C (*see* **Note 2**).
5. 10μg/ml ciliary neurotrophic factor (CNTF) in PBS containing 0.1% BSA. Store aliquots at −20 °C.
6. Accutase. Once thawed from −20 °C, store at 4 °C for up to 2 months.
7. 1 mg/ml doxycycline hyclate (DOX) in Milli-Q H_2O, filtered through 0.22μm filter. Store aliquots at −20 °C (*see* **Note 3**).
8. Cell culture multiwell plates, PS clear, Cellstar®.

2.3 Immunofluorescence Staining

1. Fixation solution: 4% paraformaldehyde (PFA) in PBS. Store at 4 °C.
2. Permeabilization solution: 0.1% Triton X-100 in PBS. Store at 4 °C.
3. Blocking solution: 1% (m/v) BSA in PBS. Store at 4 °C.
4. Washing solution: 0.1% Tween-20 in PBS. Store at 4 °C.
5. Nucleus staining stock solution: 2 mg/ml Hoechst 33342 in PBS. Store aliquots at −20 °C, and protect from light. Dilute this stock solution at 1:1000 in PBS to make working solution.
6. Primary antibodies:
   (a) GFAP monoclonal antibody (ASTRO6) (Thermo Fisher Scientific, MA5-12023). Use at 1:200 in the experiment.
   (b) S100 polyclonal antibody (Dako, Z0311). Use at 1:400 in the experiment.

7. Secondary antibodies:
   (a) Goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488. Use at 1:400 in the experiment.
   (b) Goat anti-mouse IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 555. Use at 1:400 in the experiment.
8. Fluorescence microscope.

2.4 Calcium Imaging

1. Fluo-4 AM fluorescent labeling reagent in DMSO.
2. Standard bath saline solution (pH 7.3): 2 mM $CaCl_2$, 140 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, 4 mM KCl, 10 mM glucose in Milli-Q H_2O, filtered through 0.22μm filter. Store at 4 °C.
3. ATP assay solution: 50μM ATP in standard bath saline solution. Store at 4 °C for on longer than a week.
4. KCl assay solution: 65 mM KCl in standard bath saline solution. Filtered through 0.22μm filter. Store at 4 °C for on longer than a week.
5. Glutamate assay solution: 100μM glutamate in standard bath saline solution. Filtered through 0.22μm filter. Store at 4 °C for on longer than a week.
6. Confocal microscope.

3 Methods

3.1 Virus Production

BL-2 safety practices should be followed when preparing and handling lentiviral particles. Personal protective clothing should be worn at all times. Liquid waste should be decontaminated with at least 10% bleach. Laboratory materials that come in contact with viral particles should be treated as biohazardous waste and autoclaved. Please follow all safety guidelines from your BL-2 working facility.

Below, all procedures are performed at room temperature unless specified otherwise.

1. Prepare plasmid maxipreps of all the seven plasmids mentioned in the materials section according to manufacturer's instructions.
2. The eluted plasmid solution should be in a 50 ml Falcon tube for an additional ethanol precipitation step to concentrate the plasmids. First add 1/10th the volume equivalent to the plasmid solution of 3 M sodium acetate (pH 5.5), and mix well by inverting the tube for several times. Then add three times the

volumes equivalent to the plasmid solution of cold (stored at −20 °C) 95% ethanol, and mix well by inverting the tube or vortex. Store at −20 °C for 1 h.

3. Next, centrifuge at 5000 × *g* at 4 °C for 10–15 min. After the centrifuge, aspirate supernatant carefully without disturbing the pellet. Then add 10 ml cold 75% ethanol (stored at −20 °C) to wash the DNA pellet once while vortexing to detach the pellet. Centrifuge to pellet the plasmid again at 5000 × *g* at 4 °C for 5 min.
4. Next, aspirate supernatant, and dry pellet at room temperature for 1 h in a laminar flow hood. Finally resuspend plasmid DNA in small volume (300μl) sterile Milli-Q water, and measure the concentration using NanoDrop instrument.
5. The day before the transfection, seed 6.5 × 10^6 293T cells in 14.5 cm dishes in 20 ml 293T cell medium at 37 °C, 5% CO_2, and culture for 18 h.
6. 2 h before the transfection, change the medium for 22.5 ml of fresh high glucose HEK 293T medium in order to induce cell cycling (*see* **Note 4**).
7. Prepare in 15 ml Falcon tube the following DNA mix for each transfected dish (*see* **Note 5**).
8. For the TF-expressing virus, mix 32μg of TetO-FUW-NfiA/NfiB/SOX9, 6.25μg of pRSV-Rev, 9μg of pMD2.G, and 12.5μg of pMDLg/pRRE in sterile Milli-Q water to make 1.05 ml.
9. For the M2rtTA virus, add 32μg of FUW-M2rtTA to the DNA mix containing the same amount of pRSV-Rev, pMD2.G, and pMDLg/pRRE.
10. Add 150μl of 2 M $CaCl_2$ to the DNA mix and mix by pipetting 3–4 times and wait for 15 min.
11. Add 1.2 ml of the 2× HBS buffer dropwise to the calcium-DNA mix while vortexing at high speed (approximately 2000 rpm). At this point, the solution should appear slightly cloudy.
12. Immediately add the 2.4 ml mix solution to the cells drop by drop across the whole dish, and gently swirl to evenly distribute the mix content (*see* **Note 6**).
13. 18 h after the transfection, some virus is already present in the medium. Change the medium for 16 ml of fresh high glucose 293T cell medium with additional 1× Antibiotic-Antimycotic solution (*see* **Note 7**).
14. The day after (approximately 48 h after the transfection), collect the supernatants for each type of viral preparation in

50 ml Falcon tubes. Centrifuge at 850 × *g* for 5 min to pellet cells. Collect the supernatants again, and filter through 0.45μm filter unit (*see* **Note 8**).

15. Put up to 32 ml of viral preparation in an Ultra-Clear tube, and ultracentrifuge it at 20,000 × *g* for 2 h at 20 °C.
16. Carefully discard the supernatant and turn the tubes upside-down on absorbent paper and let dry the virus pellet for 10 min.
17. Add 80μl of PBS in each tube, and carefully pipette several times to resuspend the pellet (*see* **Note 9**).

3.2 Virus Validation

HEK 293T cells are infected with different TF-carrying viruses along with the M2rtTA virus. DOX is given in the medium to induce the overexpression. Total RNA is isolated and QPCR is performed to check the overexpression level of the TFs.

1. Seed 3 × 10^5 HEK 293T cells in 6-well plate in 2 ml HEK 293T medium at 37 °C, 5% CO_2, and culture overnight (*see* **Note 10**).
2. Infect the cells with either 0.5μl, 1μl, or 2μl of each of the TetO-FUW-NfiA/NfiB/SOX9 virus along with 2μl M2rtTA viruses in 1 ml fresh medium in the presence of 8μg/ml polybrene (*see* **Note 11**). Incubate for 18 h.
3. After the infection, discard the virus-containing medium, and wash the cells once with medium to get rid of any residual viruses. Add 2 ml fresh medium containing 1μg/ml DOX to induce overexpression of the TFs, and culture for 48 h.
4. 48 h later, total RNA is isolated with TRIzol™ reagent according to the manufacturer's instructions.
5. Following RNA isolation, 1μg total RNA is used for cDNA synthesis using iScript™ cDNA Synthesis Kit according to the manufacturer's instructions (*see* **Note 12**). Synthesized cDNA can be at least five times diluted with nuclease-free water in order to have enough material for QPCR assay.

Component	Amount per reaction (15μl)	Final concentration
iTaq™ Universal SYBR Green Supermix (2×)	7.5μl	1×
Primers mix (forward and reverse)	1.5μl	300 nM each
Nuclease-free H_2O	5μl	–
cDNA	1μl	–

6. Perform QPCR with cDNA template and desired primers as follows:

Cycle number	Denaturation at 95 °C	Annealing/extension at 60 °C	Melt curve analysis (65–95 °C)
1	30 s	–	–
2–41	5 s	30 s	–
42	–	–	0.05 s per 0.5 °C increment

7. Analyze the cycle threshold (Ct) values for gene expression fold change by using the $\Delta\Delta C_t$ method [25]. The amount of virus used to bring about a 1000× fold change in gene expression is suitable for cell reprogramming experiment (*see* **Note 13**).

3.3 Reprogramming of MEFs into Induced Astrocytes

1. Seed 2×10^5 MEFs in a single well of a 6-well plate in 2 ml MEF medium at 37 °C, 5% CO_2, and culture overnight (*see* **Note 14**).
2. Infect the cells with desired amount of TetO-FUW-NfiA/NfiB/SOX9 and M2rtTA viruses in 1 ml fresh MEF medium in the presence of 8μg/ml polybrene. Culture for 18 h.
3. After the infection, discard the virus-containing medium, and wash the cells once with medium. Add 2 ml fresh MEF medium containing 1μg/ml DOX to induce expression of the TFs, and culture overnight.
4. Coat new well plates with Matrigel for imminent re-plate procedure (step 5). Add 700μl or 300μl Matrigel to 6- and 24-well plates, respectively. Swirl to make whole area covered by Matrigel. Incubate for at least 30 min in the incubator. After Matrigel coating, discard the supernatant, and add 2 ml or 600μl fresh MEF medium to 6- and 24-well plates, respectively. 6-well or 24-well plates will be used, respectively, for transcriptional or immunocytochemical analyses.
5. Discard the medium and wash the cells once with PBS. Add 1 ml Accutase and put back to the incubator for 3–5 min to dissociate the cells. Guarantee 100% single cells by additional pipetting with 1 ml tips. Centrifuge at 200 × *g* for 3 min to pellet cells in a 15 ml Falcon tube. Discard the supernatant and resuspend the cells in 1 ml fresh MEF medium and count the cells. Re-plate the cells at low density in new Matrigel-coated well plate and culture overnight. A desired cell density is as follows: 1×10^4 cells/cm^2.
6. The next day, switch to astrocyte induction medium containing 1μg/ml DOX. Reprogramming lasts for 3 weeks, and 1μg/ml DOX is given in the medium throughout the whole process (*see*

Note 15). Fully refresh the medium three times a week for the first 2 weeks. Refresh half of the medium three times a week for the third week (*see* **Note 16**).

7. One week after DOX initiation, 10 ng/ml CNTF is given in the medium to promote astrocyte maturation.
8. After 3 week's reprogramming, DOX and CNTF are withdrawn from the medium. The resultant iAstrocytes are positive for GFAP and S100B. At the moment, these induced astrocytes should have gained stable phenotype and can be cultured for a few passages.

3.4 Immunofluorescence Staining

Immunofluorescence staining is carried out in 24-well plates. All procedures are performed at room temperature unless specified otherwise.

1. Cells are fixed for 25 min in 500μl 4% PFA solution followed by 1 ml PBS wash once.
2. Cells are permeabilized in 500μl permeabilization solution for 10 min followed by 1 ml PBS wash once.
3. Cells are incubated in 500μl blocking solution for 1.5 h. There is no need to wash the cells after this step.
4. Primary antibodies are diluted in blocking solution. Cells are incubated with 500μl primary antibody solution overnight at 4 °C.
5. The next day, cells are washed with 1 ml washing solution for three times on a shaker (speed 500 rpm), each wash lasting 5 min.
6. Secondary antibodies are diluted in blocking solution. Cells are incubated with 500μl secondary antibody solution for 2 h in the dark. There is no need to wash the cells after this step.
7. Continue to stain the cells with 500μl nucleus staining working solution for 10 min in the dark followed by the same washing procedure in **step 5**.
8. After the washing, submerge the cells with new PBS. Then the cells are ready for imaging. Representative imaging of iAstrocytes is shown in Fig. 2.

3.5 Calcium Imaging

1. Cells growing in 96-well plates are incubated with 100μl medium containing 5 mM Fluo-4 AM dye for 1 h in the incubator.
2. Fluo-4 AM dye is removed, and cells are washed once with standard bath saline solution to get rid of free dye. Add 100μl new standard bath saline solution to the cells, and place the 96-well plate in the confocal microscope with incubator function turned on.

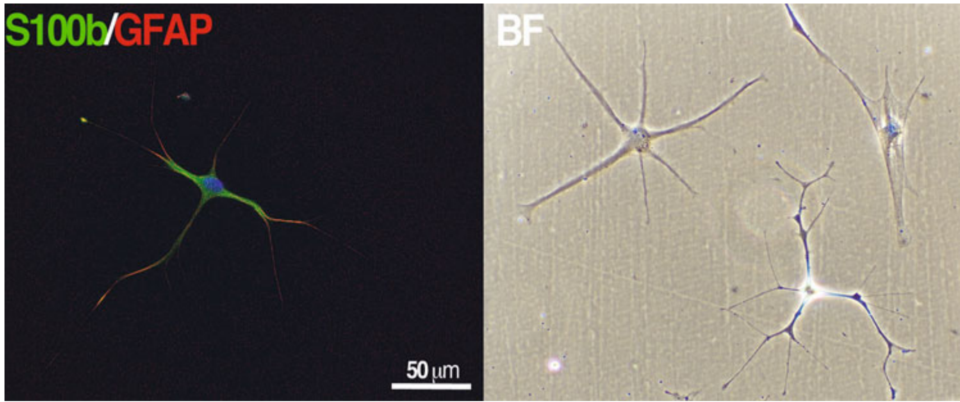

Fig. 2 Representative images of reprogrammed iAstrocytes. The images show iAstrocytes after 3 weeks of reprogramming. The left panel shows an immunocytochemical analysis for the astrocyte markers GFAP (red) and S100b (green) and the nuclear marker Hoechst (blue). The right panel shows the morphology of the iAstrocytes in bright-field

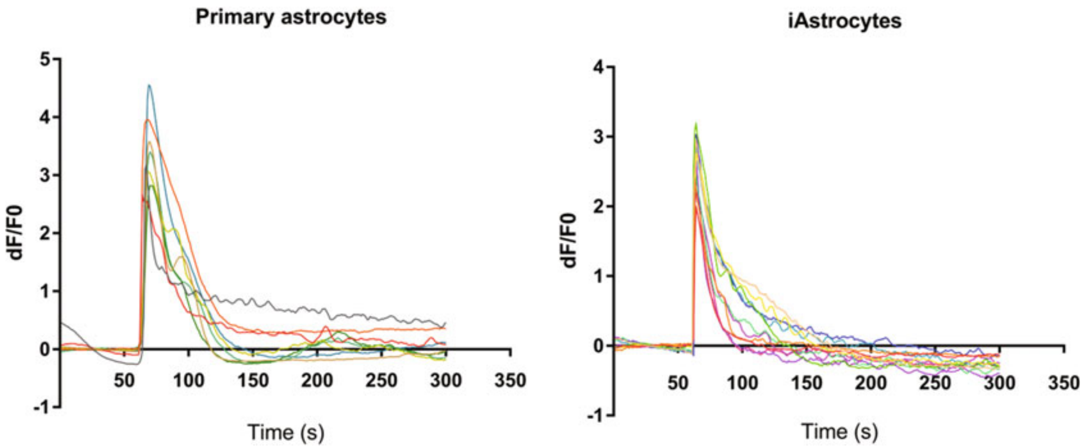

Fig. 3 Calcium imaging of iAstrocytes. The figure shows a comparison of the calcium waves analyzed after ATP stimulation in iAstrocytes (left panel) and primary mouse astrocytes. In both panels, every colored line corresponds to single analyzed cells

3. Cells are imaged with a 20× objective for 1 min at 1 Hz to check the baseline. Laser line (Ex/Em) = 494/506 nm.
4. With dispensing function of the microscope, refresh 100μl new standard bath saline solution containing either 50μM ATP, 65 mM KCl, or 100μM glutamate, and continue to image the cells for 5 min.
5. Fluorescence intensity of region of interest (ROI) is measured using ImageJ software, and data is present as ΔF/F0 [26]. Representative results of iAstrocyte calcium imaging are shown in Fig. 3.

4 Notes

1. Any culture vessels, equipment, or tools that get in direct contact with Matrigel should be pre-cooled.
2. N2 supplement is not stable at 4 °C for more than 2 weeks; thus, any medium containing N2 should be finished within 2 weeks. Also N2 stock should be made into aliquots and kept at −20 °C.
3. Protect doxycycline from light by switching off hood light and using aluminum foil on the aliquots. Used aliquots can be kept at 4 °C for few days; otherwise, keep them at −20 °C and reuse them only once.
4. When doing transfection, plated 293T cells should be around 40–60% confluence to have good transfection efficiency and allow them to grow for further 40–48 h.
5. It is suggested to prepare a plasmid DNA mix for each type of virus in a 1.5 ml Eppendorf tube. Then split the content of the mix in the number of 15 ml Falcon tubes equal to the number of 14.5 cm dishes to be transfected.
6. Small dark precipitates should be visible by bright-field optic microscope soon after adding the transfection mix to the cells.
7. There should be no antibiotic in the medium when doing the transfection in order to prevent any additional stress to the cells, whereas after transfection antibiotic should be added in the medium to prevent any possible contaminations.
8. Filtering with 0.22μm filters could cause loss of viral particles.
9. Avoid generation of bubbles during virus resuspension. If bubbles are present, spin down the virus solution at 100 × g for 5 min before freezing it. Make aliquots in 0.5 ml Eppendorf tubes that can be used in a single experiment, and store at −80 °C.
10. Prepare two to three wells of cells for counting the exact cell number when doing the infection.
11. The presence of polybrene and the use of lower volume of medium greatly increase infection efficiency.
12. Isolated RNA can be additionally treated with DNase to prevent genomic DNA contamination.
13. The amount of viruses used for reprogramming should be the minimum amount that allows the infection of 100% of the cells. Therefore, it is advised to perform a concentration curve with different amount of virus and assess the percentage of infected cells by immunocytochemical analysis.

14. Prepare two to three wells of cells for counting the exact cell number when doing the infection.
15. DOX is freshly added before medium changing.
16. When removing old medium during medium change, it is advisable to leave a thin layer of medium to prevent cells from drying, and some paracrine factors are released in the medium by cells undergoing differentiation.

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