Application Note

Reprogramming Human Fibroblasts using the Dox Inducible Reprogramming Polycistronic Lentivirus Set: Human 4F2A LoxP

Overview

The following protocol describes the reprogramming of one well of BJ Human Fibroblasts (BJ cells) into induced pluripotent stem (iPS) cells in a 6-well format. Transduction efficiency can vary depending on such factors as the amount of virus used, the type of cell transduced, the use of cationic polymers, and the type of medium used. If using cell types or conditions different from what is described, the amounts of virus used may need to be optimized for your experiment.

Additional Materials Required

- BJ Human Fibroblasts
- EMEM (Eagle’s Minimal Essential Medium)
- FBS (Fetal Bovine Serum)
- HEPES (1 M)
- 0.2% gelatin in water, tissue culture grade, sterile
- DMEM (Dulbecco’s Modified Eagle’s Medium)
- Non-essential amino acids (100x)
- l-glutamine (200 mM)
- 2-mercaptoethanol (55 mM)
- Fibroblast Growth Factor-basic
- 10 mM Tris, pH 7.6
- Knockout™ DMEM (Invitrogen)
- Knockout Serum Replacement (Invitrogen)
- Plasmanate® (Talecris Biotherapeutics NDC)
- Dox hyclate
- PBS (Phosphate Buffered Saline)
- 0.05% Trypsin/EDTA solution
- 6-well tissue culture plates
- 4-well tissue culture plates
- 24-well tissue culture plates
- 15 ml conical tubes

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**Material Preparation**

**BJ Medium**
- 435 ml EMEM
- 50 ml FBS
- 15 ml HEPES (1M)

Filter-sterilize using a 0.22 µm pore size, low protein-binding filter. Store BJ Medium at 4 °C.

**Gelatin Coated Plates**
- 0.2% gelatin in water

6-well or 4-well tissue culture plate.

Add 1.5 ml of the gelatin to each well of a 6-well plate or add 0.5 ml of gelatin solution to each well of a 4-well plate. Incubate at 37 °C for a minimum of 1 hour. Gelatin Coated Plates can be stored at 37 °C for up to 1 week.

**Transduction Medium (per well to be transduced)**
- 1 vial Stemgent Dox Lentivirus h4F2A LoxP
- 25 µl Lentivirus rtTA
- 2 ml BJ Medium

Add 2 ml of BJ Medium to a 15 ml conical tube. Thaw the vial of human 4F2A virus on ice and add the contents to the tube. Refer to the lot-specific certificate of analysis for the precise volume. Thaw one vial of Lentivirus rtTA and add half of the vial (25 µl) to the tube. Viral stock solutions have been validated to maintain their titer after one additional freeze/thaw cycle. Aliquot and store any remaining viral stock solution at -70 °C. Transduction Medium should be used immediately.

**MEF Medium**
- 450 ml DMEM
- 50 ml FBS
- 5 ml Non-essential amino acids (100x)
- 5 ml l-glutamine (200 mM)
- 0.5 ml 2-mercaptoethanol (55 mM)

Filter-sterilize using a 0.22 µm pore size, low protein-binding filter. Store MEF Medium at 4 °C for up to 2 weeks.

**MEF Feeder Plates**

The day before MEF Feeder Plates are needed, thaw γ-irradiated feeder layer MEFs and dilute to a concentration of 1 x 10^5 cells/ml in MEF Medium. Add 2.5 ml of cell suspension to each well of a 6-well Gelatin Coated Plate for a final concentration of 2.5 x 10^4 cells per well and 500 µl of cell suspension to each well of a 24-well tissue culture plate for a final concentration of 5 x 10^4 cells per well. Incubate overnight at 37 °C and 5% CO₂.

**bFGF Solution**
- 50 µg Fibroblast Growth Factor-basic

Briefly centrifuge the lyophilized vial of bFGF and reconstitute in 1 ml of 10 mM Tris, pH 7.6, to yield a 50 µg/ml solution. Aliquot and store at -20 °C for up to 6 months.

**hiPSC Culture Medium**
- 450 ml Knockout DMEM
- 45 ml Knockout Serum Replacement
- 45 ml Plasmanate
- 80 µl bFGF Solution
- 5 ml Non-essential amino acids (100x)
- 5 ml l-glutamine (200 mM)
- 500 µl 2-mercaptoethanol (55 mM)

Filter-sterilize using a 0.22 µm pore size, low protein-binding filter. Store hiPSC Culture Medium at 4 °C for up to 2 weeks.

**Dox Solution (2 mg/ml)**
- 10 mg Dox hyclate

Add 0.5 ml of tissue culture grade water to the vial of dox hyclate. Mix the suspension by inversion until all of the dox has dissolved and the solution is clear. Transfer the mixture to a 15 ml conical tube. Add another 0.5 ml of tissue culture grade water to the vial and mix to dissolve any additional dox. Transfer the mixture to the 15 ml conical tube. Bring the stock solution volume up to 5 ml with tissue culture grade water. Filter-sterilize using a 0.22 µm pore size, low protein-binding filter. Aliquot in 1.5 ml microcentrifuge tubes and store at -20 °C. Freeze/thaw cycles of Dox Solution (2 mg/ml) should be minimized.

**Dox Induction Medium**
- 10 ml hiPSC Culture Medium
- 10 µl Dox Solution (2 mg/ml)

Prepare Dox Induction Medium fresh daily. Thaw Dox Solution on ice and add to prewarmed hiPSC Culture Medium.
MEF Conditioned Medium

1. Plate γ-irradiated feeder MEFs at a density of 3 x 10^6 to 4 x 10^6 cells in 25 ml of MEF Medium in a T-75 flask and incubate overnight at 37 °C and 5% CO₂.

2. The next day, aspirate the medium and add 25 ml of fresh iPSC Culture Medium. Incubate overnight at 37 °C and 5% CO₂.

3. Twenty-four hours later, collect the MEF Conditioned Medium, filter, and store at -20 °C. Add 25 ml of fresh iPSC Culture Medium to the cells. Incubate overnight at 37 °C and 5% CO₂.

4. Repeat step 3 for a total of 7 collections over 7 days (175 ml total). Thaw the seven 25 ml aliquots, pool, filter-sterilize, and realiquot to appropriate volumes. Store at -20 °C for later use.

5. When thawing MEF Conditioned Medium, add an additional 16 ng/ml of bFGF to generate MEF Conditioned Medium with a final concentration of 20 ng/ml bFGF.

MEF Conditioned Dox Induction Medium

10 ml MEF Conditioned Medium
10 µl Dox Solution (2 mg/ml)

Prepare fresh MEF Conditioned Induction Medium daily. Thaw Dox Solution on ice and add to prewarmed MEF Conditioned Medium.

Reprogramming BJ Cells

Prepare Cells for Reprogramming (Day -3)

If using BJ cells, follow the plating protocol listed. If using another cell type, a different plating density may be required.

1. Plate BJ cells on a 6-well Gelatin Coated Plate at a final density of 1 x 10^5 cells per well.

2. Incubate overnight at 37 °C and 5% CO₂ to reach the desired density, around 50% confluency (see Figure 1 for reference).

Transduce BJ Cells (Day -2)

1. Prepare the Transduction Medium.

2. Aspirate the medium and add the Transduction Medium to the well of cells to be reprogrammed. Ensure the medium is evenly distributed by gentle rocking of the cell culture dish.

Replate Transduced Cells (Day -1)

1. Twenty to twenty-four hours post-transduction aspirate the Transduction Medium.

2. Wash the well with 2 ml of PBS.

3. Aspirate the PBS and add 1 ml of 0.05% Trypsin/EDTA solution.

4. Incubate the plate for 2 to 3 minutes at 37 °C and 5% CO₂.

5. Add 2 ml of BJ Medium to neutralize the Trypsin/EDTA.

6. Pipette the medium across the surface of the well until the cells appear completely detached.

7. Transfer the cell solution to a 15 ml conical tube.

8. Centrifuge the cells for 5 minutes at 200 x g.

9. Aspirate the supernatant.

10. Resuspend the cell pellet in 2 ml of BJ Medium.

11. Mix the cell solution gently in order to create a uniform suspension of single cells.

12. Count the total number of cells in solution using a hemacytometer.

Reprogramming Timeline

<table>
<thead>
<tr>
<th>Day</th>
<th>Activity</th>
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<tbody>
<tr>
<td>-3</td>
<td>Plate BJ cells</td>
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<tr>
<td>-2</td>
<td>Transduce BJ cells and prepare MEF Feeder Plates</td>
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<tr>
<td>-1</td>
<td>Re-plate transduced BJ cells onto MEF Feeder Plates</td>
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<td>0</td>
<td>Induce cells by replacing medium with Dox Induction Medium</td>
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<td>2</td>
<td>Assess transduction efficiency on the 4-well plate</td>
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<td>1-7</td>
<td>Replace medium every 24 hours with Dox Induction Medium</td>
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<td>8-20</td>
<td>Replace medium every 24 hours with MEF Conditioned Dox Induction Medium</td>
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<tr>
<td>20-25</td>
<td>Remove MEF Conditioned Dox Induction Medium and replace with MEF Conditioned Medium</td>
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<td>25+</td>
<td>Begin picking colonies, expand in iPSC Culture Medium with 20 ng/ml of bFGF (see Figure 4 for iPSC cell colony examples). Continue to manually passage isolated colonies to expand for pluripotency analysis and characterization</td>
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13. Add the appropriate amount of BJ Medium to bring the cell suspension to 2.5 x 10^4 cells/ml.

14. Aspirate the medium from a 6-well MEF Feeder Plate and add 2 ml of cell suspension to each of the wells for a final cell density of 5 x 10^4 cells per well.

15. Aspirate the medium from a 4-well Gelatin Coated Plate and add 0.5 ml of the cell suspension to each of the 4 wells for a final plating density of 1.25 x 10^4 cells per well.

**Note:** The 4-well plate is used to assess the transduction efficiency by immunocytochemistry (ICC).

16. Incubate overnight at 37 °C and 5% CO₂.

**Note:** Depending on the ability of the targeted cell type to be reprogrammed, it may be necessary to plate more cells per well to increase the likelihood of obtaining an iPS cell colony that can be established as an independent cell line. Additionally, any transduced cells not re-plated can be frozen using standard cryopreservation techniques.

### Induce Reprogramming using Dox (Day 0)

1. Twenty to twenty-four post-replating, aspirate the BJ Medium from the wells in the 6-well plate.

2. Add 2 ml of Dox Induction Medium to 3 of the wells.

3. Add 2 ml of hiPSC Culture Medium to 1 well, which will serve as a negative control (See Figure 2 for reference).

4. Aspirate the BJ Medium from each well of the 4-well plate.

5. Add 0.5 ml of Dox Induction Medium to 2 of the wells.

6. Add 0.5 ml of hiPSC Culture Medium to 2 of the wells (See Figure 3 for reference).

7. Incubate plates at 37 °C and 5% CO₂.

### Determine Transduction Efficiency (4-well plate) (Day 2)

Forty-eight hours post induction, assess the transduction efficiency using the cells cultured in the 4-well plate. Perform ICC staining for 1 transcription factor per 4-well plate. OCT4 staining is often used to assess transduction efficiency in validation experiments with the h4F2A virus, as it is the first cDNA expressed once the polycistronic vector has integrated into the host genome. For each transcription factor to be tested, incubate a dox-induced and non-induced well with both the primary and secondary antibodies. The remaining dox-induced well should be used as a negative control by just incubating with the secondary antibody. See Figure 3 for the experimental configuration of each 4-well plate.

### Medium Changes (6-well plate)

1. Day 1 to day 7: Appropriately replace the hiPSC Culture Medium and Dox Induction Medium every 24 hours.

2. Day 8 to day 20: Appropriately replace the medium with MEF Conditioned Dox Induction Medium and MEF Conditioned Medium every 24 hours.

3. After day 20, replace MEF Conditioned Dox Induction Medium with MEF Conditioned Medium every 24 hours until all potential iPS cell colonies have been picked from the 6-well plate.

**Note:** It is necessary to remove dox from the growth medium once good colony morphologies are observed to ensure completion of the reprogramming process. Therefore we recommend removing dox from the growth medium at day 20. Removal of dox at day 20 ensures that the iPS cell colonies picked and passaged around day 25 are reliant on endogenous expression of pluripotency genes for colony survival and are not the result of sustained ectopic transcription factor expression. Human iPS cell colonies remaining 5 to 7 days after the removal of dox should expand and passage similarly to hES cell colonies.
Identify iPS Cell Colonies

Due to the stochastic nature of reprogramming, iPS cell clusters/colonies emerge at different timepoints once the culture is induced with dox. iPS morphologies can be noticeable as early as 10 to 12 days post-induction. Twenty days post-induction there is a distinct and noticeable set of iPS cell colonies. Some of these colonies are dox-dependent and may disappear once dox is removed. Around day 25, five days post-dox removal, remaining iPS cell colonies are dox-independent and ready to be picked for expansion. Manual isolation and passaging of iPS cell colonies from wells still being cultured with dox (<20 days) yields a lower percentage of colonies (10 to 15%) that are expandable and display typical pluripotency marker expression by immunocytochemistry (ICC). Larger, more established iPS cell colonies that have been cultured without dox (days 20 to 25) are easier to pick in 20 to 40 cell clusters. A higher percentage of these colonies (greater than 80%) are expandable and will display typical pluripotency marker expression by ICC. See Figure 4 for examples of emerging iPS cell colonies.

Single Colony Pick and Passage

1. Supplement hiPSC Culture Medium with an additional 16 ng/ml of bFGF to reach a final concentration of 20 ng/ml.

2. Manually pick colonies with iPS cell morphology to a single well of a 24-well MEF Feeder Plate.
   a. Aspirate the medium from a 24-well MEF Feeder Plate and wash with 0.5 ml of PBS.
   b. Aspirate the PBS and add 0.5 ml of hiPSC Culture Medium (supplemented to 20 ng/ml of bFGF) to each well.
   c. Aspirate the medium from the well of hiPS cells to be picked from the 6-well plate and add 2 ml of fresh hiPSC Culture Medium (supplemented to 20 ng/ml of bFGF).
   d. Using a sterile glass picking tool, gently separate the identified colony from surrounding cells.
   e. Using the glass picking tool, gently divide the colony into clusters that contain 30 to 40 cells.
   f. Using the glass picking tool, gently detach each colony cluster from the tissue culture well.
   g. Using a P10 or a P20 pipettor, pipette the detached colony pieces out of the 6-well plate and into an individual well of the 24-well MEF Feeder Plate.

3. Incubate the 24-well plate at 37 °C and 5% CO₂.

Note: Any reagent that can enhance the survival and cloning efficiency of human ES cells, such as the Y27632 molecule, a Rho-associated kinase ROCK inhibitor, can be used when picking and passaging human iPS colonies for expansion.

4. Replace medium daily with 500 µl of fresh hiPSC Culture Medium (supplemented to 20 ng/ml of bFGF). If there is no visible colony attachment or expansion by 8 days after picking, then those wells do not need to be maintained and can be discarded.

5. Manually passage individual wells from the 24-well plate that exhibit colony expansion and iPS cell morphology. All colonies from 1 well of a 24-well plate can be picked and plated into one well of a 6-well plate.

6. Monitor cultures for equivalent iPS cell colony growth and morphology.

7. For the first 3 to 5 passages, colonies should be manually picked and plated at a very low split ratio of 1:2 to 1:3. Colonies can then be adapted to passaging protocols using collagenase.

8. Continue to passage and culture iPS cell colonies for use in experimentation and banking.
Pluripotency Analysis

Expanded iPS cell colonies can be tested for pluripotency by using an Alkaline Phosphatase Staining Kit II and staining for typical pluripotency markers such as NANOG, OCT4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and Rex1.

Figure 1: BJ Cells before Transduction
Cells were plated and incubated overnight to achieve the optimal density for transduction (10×).

Figure 2: 6-Well Plate Layout
Typical set-up of a 6-well plate used for induction and culture of iPS cells.
Figure 3. 4-Well Plate Layout used to Assess Transduction Efficiency

Well 1 is used for transduction efficiency assessment. These dox(+) cells are stained with both primary and secondary antibodies as well as DAPI for nuclear identification.

Well 2 serves as a negative control. These dox(+) cells are stained with DAPI and the secondary antibody only to evaluate background signal from the antibody.

Well 3 is a negative control for dox-induction. These dox(-) cells are stained with primary and secondary antibodies as well as DAPI to assess both the specificity of the primary antibody and to monitor successful induction with dox. This well of dox(-) cells should have little to no expression of the transcription factors, but dox-inducible systems have demonstrated levels of basal expression in the absence of dox and should therefore be monitored.

Well 4 is a double negative control, and can be omitted from the plate if there is a shortage of transduced cells for replating. These dox(-) cells are stained with secondary antibody and DAPI.

Figure 4: Dox-independent iPS Cell Colonies

Two examples of iPS cell colonies post-dox removal. A) This colony exhibits compact cell morphology, a two-dimensional colony, and a clean border ideal for picking and expansion.

B) Smaller emergent iPS colony with more three-dimensional structure that requires additional growth before manual picking.
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