

Improved Method for Generation of Mouse iPS Cells

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Since the first report of mouse induced pluripotent stem (iPS) cells in 2006, questions have been raised regarding the method of retroviral transduction and the necessity of antibiotic selection, and various refinements have been made to the method of induction. The pluripotency of Nanog-GFP⁺ mouse iPS cells has been demonstrated to be sufficient to contribute to the germline in chimeric mice. Modification of plating density has also made it possible to induce iPS cells from wildtype mouse cells in the absence of antibiotic selection as well as by using only three factors, without the oncogene *c-Myc*. In this protocol, we describe a method for the induction of pluripotency by gene transfection into mouse fibroblasts, as was previously detailed in ref. 1, with refinements to the previously published protocol shown in red letters.

1. Materials

OPTI-MEMI (Invitrogen 31985-062)

See ref. 1 for other necessary materials, such as cells, vectors and reagents.

2. Cell culture

See ref. 1 for culture methods for MEF and TTF cells and PLAT-E packaging cells.

3. Establishment of mouse iPS cells

3.1.1 Construction of retroviral vectors: preparation of PLAT-E cells (Day 1)

As described in ref. 1. Follow the table below when plating PLAT-E cells.

Dish size	Number of cells
100 mm dish	3.6×10^6
60 mm dish	1.5×10^6
35 mm (6-well plate)	6×10^5

Also note that it is necessary to prepare a sufficient number of PLAT-E cultures in order to be able to transfect each plate with a single plasmid in step 3.1.2 (below). For example, experiments using 4 genes (Oct4, Sox2, Klf4 and c-Myc) as well as Ds-Red require 5 separate dishes of PLAT-E cells.

3.1.2 Construction of retroviral vectors: Transfection into PLAT-E cells (Day 2)

The following procedures are for PLAT-E cells in 100mm dish. In the case of PLAT-E cells in 60mm and 35mm dishes, use 1/3 and 1/6 volume of the transfection reagent in 100 mm dish, respectively.

- Inject 0.3 ml of OPTI-MEMI into separate 1.5 ml tubes, one for each gene to be transfected.
- Add 27 μ l of Fugene 6 transfection reagent by gently tapping to mix, and incubate at room temperature for 5 minutes.
- Add 9 μ g of pMXs plasmid DNA (as a negative control for Oct4, Sox2, Klf4, c-Myc and DsRed) dropwise into separate solutions of Fugene 6/OPTI-MEMI, and mix by tapping with finger. Incubate at room temperature for 15 minutes.

IMPORTANT: Use appropriate controls. At CiRA, vectors encoding EGFP or DsRed are used to monitor transfection efficiency, and we routinely achieve efficiencies of 80% or higher. High-efficiency transfection is essential for the induction of iPS cells.

- On the next day (Day 3), aspirate the Fugene 6 transfection agent-containing medium, add 10 ml of 10% fresh fetal bovine serum (FBS), and return to the incubator.

3.2 Preparation of MEF, TTF cells (Day 3)

- See ref. 1.

3.3 Retroviral transfection (Day 4)

- See ref. 1. Make a mixture of equal parts of each of the retroviruses; however, when attempting to establish iPS cells without antibiotic selection, use a ratio of 1:1:1:3 for Oct4, Sox2, Klf4 and DsRed. Aspirate the medium from the culture dish and replace with the virus-containing medium and incubate overnight at 37°C, 5% CO₂.
- After 24 hours (Day 5), aspirate the medium and replace with fresh 10% FBS.
- After two more days (Day 7), aspirate the medium and replace with fresh 10% FBS.

3.4 Re-seed of MEFs to mitomycin C-treated SNL feeder cell culture

- Aspirate medium and wash with 10 ml PBS.
- Aspirate PBS, add 1 ml 0.05% trypsin/0.53 mM EDTA, and incubate at 37°C for 10 minutes.
- Add 9 ml medium, suspend the cells such all cells are separated (single cells), and collect in a 50 ml tube.
- Calculate the number of cells, and re-seed the cells to a 100 ml culture dish containing SNL feeder cells following the table below, and culture overnight at 37°C, 5% CO₂.

	4 factors	3 factors (minus c-Myc)
Cell number	0.5 ~ 5 X 10 ⁴	3.5 X 10 ⁵
Antibiotic selection	From Day 11	From Day 18

IMPORTANT: The frequency of iPS colony formation varies for number of experiments performed. There is a risk that no colonies will form if the number of re-seeded cells on MEFs is too low. Conversely, if too many cells are re-seeded, the growth of iPS cells will be affected, and it will be difficult to isolate Nanog-GFP-positive colonies. For these reasons it is recommended that re-plating be staged so as to allow the evaluation of optimal number of cells. When establishing iPS cells using only three factors (minus c-Myc), the number of

colonies will be approximately 10% that seen when using four factors, so it should not be a problem to re-seed all cells which were retrovirus-infected.

- The next day (Day 9), replace the medium with 10 ml mouse ES cell culture medium. Continue to replace with fresh mouse ES medium on alternating days thereafter.

3.5 Antibiotic selection

- See ref. 1. However, note that antibiotic selection should be performed at Day 11 when using 4 factors, but at Day 18 when using only 3 factors (minus c-Myc).

IMPORTANT: Delaying the antibiotic selection can yield a greater numbers of colonies. If you experience difficulties obtaining cultures using three factors, it may be possible to obtain more by selecting with puromycin at Day 25.

3.6 Isolation of mouse iPS colonies (from Day 25 on)

- See ref. 1. However, when attempting to derive iPS cells without antibiotic selection, pick cells that show silencing of DsRed.

IMPORTANT: Silencing of retroviruses serves as a good index of the degree (quality) of pluripotency in iPS cells. It is advisable to transfect DsRed etc. for visualization of gene expression at the time that the reprogramming factors are transfected. High-quality iPS cells may be found in DsRed-negative colonies.

3.7 iPS cell culture, passaging, freezing and thawing

- See ref. 1. Essentially similar to protocols used for mouse ES cells.

4. References

1. Takahashi, K., et al. Induction of pluripotent stem cells from fibroblast cultures. *Nat. Protoc.* 2, 3081-9 (2007)
2. Nakagawa, M., Koyanagi, M., et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat. Biotechnol.* 10.1038/nbt1374 (2007)

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