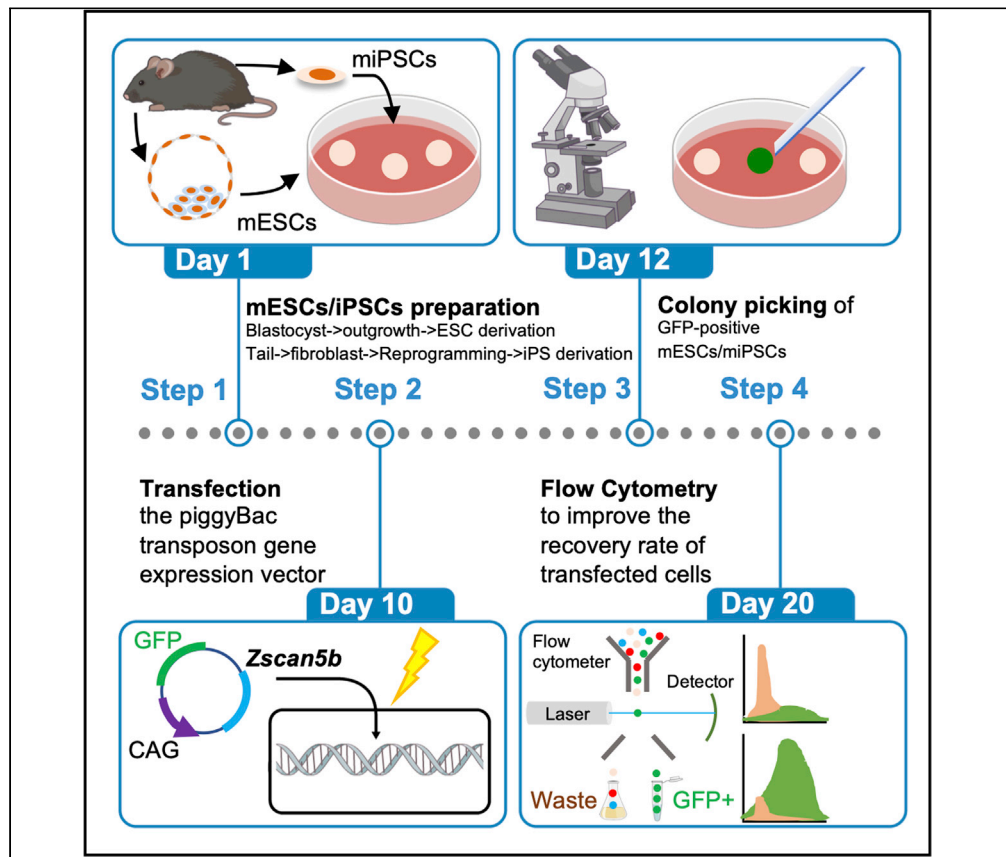


## Protocol

# Using piggyBac transposon gene expression vectors to transfect *Zscan5b* gene into mouse pluripotent stem cells



We recently demonstrated that the zygotic gene *Zscan5b* is involved in maintaining the genomic integrity of pluripotent stem cells (PSCs) during mitosis and that *Zscan5b* gene expression can prevent chromosomal abnormalities. Here, we provide a detailed protocol for the transfection of mouse embryonic stem cells and mouse-induced PSCs with a *Zscan5b* constitutive expression plasmid, using the piggyBac transposon gene expression vector system.

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### Highlights

Transfection of mESCs and miPSCs with a *Zscan5b* constitutive expression plasmid

A piggyBac transposon gene expression vector is used for transfection

CAG-promoter is recommended for mouse mESCs/ miPSCs transfection

## Protocol

Using piggyBac transposon gene expression vectors to transfect *Zscan5b* gene into mouse pluripotent stem cellsMitsutoshi Yamada,<sup>1,3,5,\*</sup> Tohru Sugawara,<sup>2,3,4</sup> Shinju Usami,<sup>1</sup> Rina Nakanishi,<sup>2</sup> and Hidenori Akustu<sup>2</sup><sup>1</sup>Department of Obstetrics and Gynecology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan<sup>2</sup>Center for Regenerative Medicine, National Center for Child Health and Development, 2-10-1 Ohkura, Setagaya-ku, Tokyo 157-8535, Japan<sup>3</sup>These authors contributed equally<sup>4</sup>Technical contact<sup>5</sup>Lead contact\*Correspondence: [mitsutoshi.yamada@gmail.com](mailto:mitsutoshi.yamada@gmail.com)  
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## SUMMARY

We recently demonstrated that the zygotic gene *Zscan5b* is involved in maintaining the genomic integrity of pluripotent stem cells (PSCs) during mitosis and that *Zscan5b* gene expression can prevent chromosomal abnormalities. Here, we provide a detailed protocol for the transfection of mouse embryonic stem cells (mESCs) and mouse-induced PSCs (miPSCs) with a *Zscan5b* constitutive expression plasmid, using the piggyBac transposon gene expression vector system. For complete details on the use and execution of this protocol, please refer to Ogawa et al. (2019).

## BEFORE YOU BEGIN

## Medium set-up

⌚ Timing: 1–2 h

1. To prepare mouse embryonic fibroblasts (MEF) medium (FM10 medium), you can use the 500 mL bottles in which Dulbecco Modified Eagle's Medium (DMEM) comes in.
2. Prepare 500 mL DMEM (high glucose) with 56.8 mL fetal bovine serum (FBS), 5.6 mL GlutaMAX, 5.6 mL penicillin-streptomycin (P/S), and 990  $\mu$ L 2-Mercaptoethanol (2ME).
3. Filter the FM10 medium using a 0.22  $\mu$ m polyethersulfone filter for sterilization.
4. For mESC/miPSC medium (t2i/L medium), prepare 125 mL DMEM/F12 medium.
5. Add 125 mL Neurobasal, 1.3 mL N2 (100 $\times$ ), 2.6 mL B27 (50 $\times$ ), 2.6 mL non-essential amino acids solution (NEAA), 2.6 mL GlutaMAX, 266  $\mu$ L 2ME, 2.6 mL P/S, 266  $\mu$ L human recombinant leukemia inhibitory factor (hrLIF; final concentration 1,000 U/mL), CHIR99021 (final concentration 3  $\mu$ M), and PD0325901 (final concentration 0.2  $\mu$ M).
6. Filter the t2i/L medium using a 0.22  $\mu$ m polyethersulfone filter (Iwaki) for sterilization.

⚠ **CRITICAL:** Inhibitors (CHIR99021 and PD0325901) and hrLIF should be added immediately before use.

**Note:** CHIR99021 requires 3  $\mu$ M in t2i/L medium (final concentration). Accordingly, the product (1 mg) dissolves in 716.3  $\mu$ L DMSO for stock solution (3 mM), and 266  $\mu$ L of this solution is



used in t2i/L medium. The remaining samples are frozen at  $-20^{\circ}\text{C}$  (the calculated total volume is 266 mL).

**Note:** PD0325901 requires  $0.2\ \mu\text{M}$  in t2i/L medium (final concentration). The product (5 mg) dissolves in 10.369 mL DMSO media for stock solution (1 mM), and 53  $\mu\text{L}$  of this solution is used in t2i/L medium. The rest of the stock solution is split into 1 mL (1.7 mL Eppendorf tube) and stored at  $-20^{\circ}\text{C}$  (the calculated total volume is 262 mL).

**Note:** The reduced PD0325901 concentration enables to retain gamete-derived DNA methylation and autonomous developmental potential (Yagi et al., 2017).

**Note:** Use filter tip to prevent bacterial contamination.

**Note:** Media and buffers must be prepared under sterile conditions. Sanitize all exterior services of bottles and tubes with 70% Ethanol before placing inside laminar flow hood.

**▣▣ Pause point:** The medium can be stored before the addition of CHIR99021 and PD0325901 at  $4^{\circ}\text{C}$  for no more than two weeks. After adding CHIR99021 or PD0325901, the medium can be stored at  $4^{\circ}\text{C}$  for 2–3 days.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
DMEM (high glucose)	Gibco	11965-084
Fetal bovine serum (FBS), qualified, New Zealand origin	Gibco	10091-148
GlutaMAX	Gibco	35050-061
Penicillin-streptomycin (P/S)	Gibco	15140-122
2-Mercaptoethanol (2ME)	Gibco	21985-023
DMEM/F12	Gibco	11320-033
Neurobasal	Gibco	21103-049
N2 (100 $\times$ )	Gibco	17502-048
B27 (50 $\times$ )	Gibco	12587-010
NEAA	Gibco	11140-050
GlutaMAX	Gibco	35050-061
2ME	Gibco	21985-023
P/S	Gibco	15140-122
hrLIF	Wako	125-05603
CultureSure® CHIR99021	FUJIFILM Wako	038034-23103101 (5 mg)
PD0325901	FUJIFILM Wako	162-25291 (5 mg)
DPBS, no calcium, no magnesium	Gibco	14190-144
Opti-MEM® I Reduced Serum Media	Thermo Fisher Scientific	31985070
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific	25200-056
<b>Experimental models: cell lines</b>		
Mouse embryonic fibroblasts	Reprocell	RCHEFC003
Mus musculus: C57BL/6 ESC lines (from 6–8 weeks old female)	Keio University	N/A
Mus musculus: C57BL/6 iPSC lines (from 6–8 weeks old female)	Keio University	N/A
<b>Recombinant DNA</b>		
piggyBac transposon gene expression vector (pPB-CAG-mZscan5b-T2A-EGFP)	VectorBuilder	VB181015-1188bhq

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Other</i>		
TC20™ automated cell counter	Bio-Rad	1450101J1
Counting slide	Bio-Rad	1450011
Trypan Blue Stain 0.4%	Gibco	15250-061
NEPA21 Super Electroporator	NEPA GENE	N/A
Electroporation Cuvettes 2 mm gap Sterile package of 50 (green)	NEPA GENE	EC-002S
Borosilicate glass capillaries	World Precision Instruments, Inc.	TW150-4
Falcon® 5 mL Round Bottom Polystyrene Test Tube	Thermo Fisher Scientific	352052
Falcon® 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap	Thermo Fisher Scientific	352235
Falcon® 40 µm Cell Strainer, Blue, Sterile	Thermo Fisher Scientific	352340
Cell Sorter SH800	SONY	SH800
Falcon 50 mL Conical Centrifuge Tubes	Thermo Fisher Scientific	352098
Falcon 15 mL Conical Centrifuge Tubes	Thermo Fisher Scientific	13352097352340
Thermo Scientific™ Nalgene™ Unwire™ Polypropylene Test Tube Racks	Thermo Fisher Scientific	597603
Fisherbrand™ Disposable Graduated Transfer Pipettes	Thermo Fisher Scientific	1371120
0.22 µm polyethersulfone filter	Iwaki	11-0357

**MATERIALS AND EQUIPMENT**

**Recipe for MEF Media (FM10)**

REAGENT	Final concentration	Amount
DMEM (high glucose)	–	500 mL
Fetal bovine serum (FBS), qualified, New Zealand origin	10 %	56.8 mL
GlutaMAX	2 mM	5.6 mL
Penicillin-streptomycin (P/S)	100 U/mL–0.1 mg/mL	5.6 mL
2-Mercaptoethanol (2ME)	0.1 mM	990 µL

**Recipe for mESCs/miPSCs culture media (titrated two inhibitors of the Erk pathway and glycogen synthase kinase-3 with LIF: t2i/L)**

REAGENT	Final concentration	Amount
DMEM/F12	–	125 mL
Neurobasal	–	125 mL
N2 (100×)	0.5 X	1.3 mL
B27 (50×)	0.5 X	2.6 mL
NEAA	100 X	2.6 mL
GlutaMAX	2 mM	2.6 mL
2ME	0.05 mM	262 µL
P/S	100 U/mL–0.1 mg/mL	2.6 mL
hrLIF	1000 U/mL	262 µL
CultureSure® CHIR99021	3 µM	262 µL (3 mM stock)
PD0325901	0.2 µM	53 µL (1 mM stock)

**Note:** A stock of FM10 and mESCs/miPSCs culture media can be stored at 4°C for no more than two weeks.

## STEP-BY-STEP METHOD DETAILS

This protocol is aimed at isolating mouse pluripotent stem cells (mESCs and miPSCs) transfected with a target gene constitutive expression plasmid. Following mESCs/miPSC isolation, the cells are transfected with green fluorescent protein (GFP)-tagged plasmid constitutively expressing *Zscan5b*. Subsequently, GFP-positive cells are picked using a glass pipette. As an alternative approach, fluorescence-activated cell sorting (FACS) can be used to enrich for GFP<sup>+</sup> cells. Once an optimal stable cell clone showing high GFP signals was identified, these cells were expanded.

### Preparation of MEF plates: Gelatinization of dishes

⌚ Timing: 20 min

**Note:** All steps should be performed in sterile conditions in a biological hood (i.e., biosafety cabinet). Prepare cell culture plates; 12-well, 6-well, and 10 cm plates are suitable for mESCs/miPSC plating.

**Note:** Gelatin-coated plates can be prepared days in advance, provided they are stored under sterile conditions in the incubator to prevent drying for up to a week (Conner, 2001).

1. Add 2 mL 0.1% gelatin (Sigma G1890) into the well of 6-well plate, and swirl to cover the entire surface with the gelatin solution.

**Note:** The plate should be of cell attachment type. Please ensure that the surface is completely covered with gelatin.

**Note:** 0.1% gelatin solution was made with cell culture grade water and sterilize by autoclaving. Filter the gelatin solution using a 0.22 μm polyethersulfone filter for sterilization after cooling. Gelatin solution can be stored up to 1 year at 4°C.

2. Leave the plate for at least 20 min in the incubator.

**Note:** Gelatin-coated plates can be stored in the incubator for up to a week.

3. Just before plating the MEF containing media, aspirate the gelatin in a laminar flow hood.

**Note:** It is imperative to observe sterile techniques and to work only in a laminar flow hood.

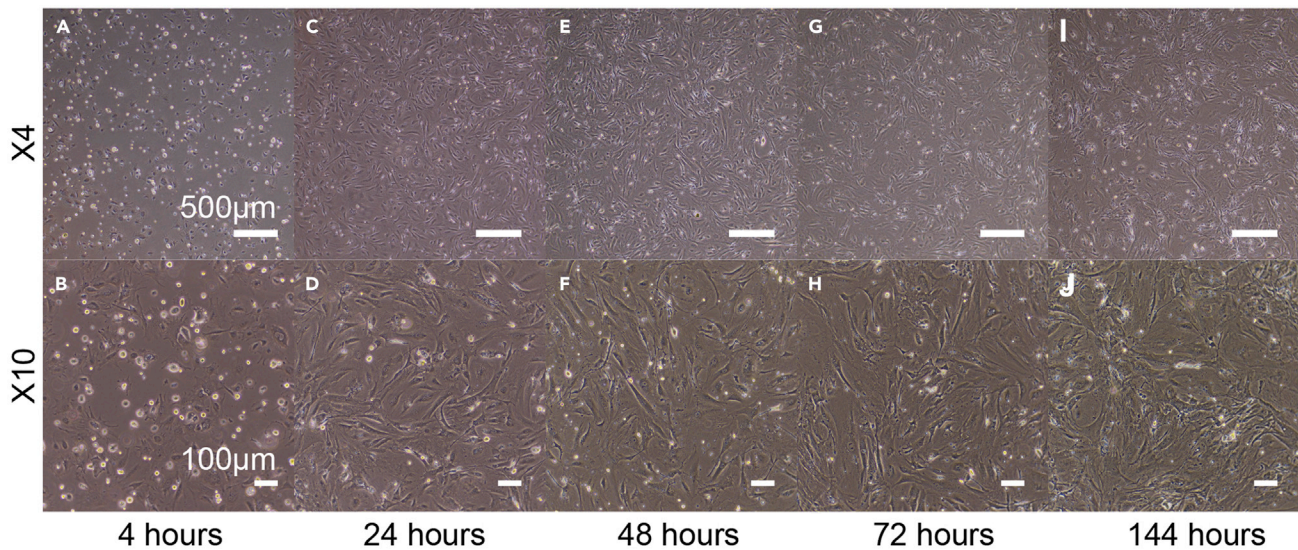
### Preparation of MEF plates: MEF preparation

⌚ Timing: 4 h (plus incubation overnight [12–24 h])

4. Take the frozen inactivated MEF vial ( $3 \times 10^6$  cells/vial) from the liquid nitrogen tank.

**Note:** MEFs were mitotically inactivated prior to the addition of mESCs/miPSCs by treatment with mitomycin C.

5. Immediately after that, put the vial on dry ice and bring it to the culture room.
6. Thaw the vial in the 37°C thermo heater.
7. Before completely dissolving MEF (with a small ice block), remove the vial from the water bath, spray the outside of the vial with 70% ethanol to sterilize and place it in hood.
8. Add the pre-warmed 1 mL FM10 media (the formulation is shown in the [key resources table](#)) into the MEF and pipet the thawed cells gently.



**Figure 1. Mouse embryonic fibroblast (MEF) plating**

Inactivated MEFs should be plated at a density of  $0.3 \times 10^5/\text{cm}^2$  for at least 4 h (A and B), preferably 24 h (C and D) to 48 h (E and F) till 72 h (G and H) before plating embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). MEFs should not be used 4 days after plating (I and J).

9. Transfer the mixture into a 15 mL Falcon tube.

**Note:** You can use a 10 mL pipette here and a 15 mL Falcon tube.

10. Subsequently, add another 8 mL FM10 medium (total 10 mL) and mix well.

11. Remove 100 μL of cell suspension for counting the cell number.

12. Centrifuge at  $200 \times g$  for 4 min.

13. Count the cell number during centrifuge.

**Note:** Add an equal volume (100 μL) of Trypan Blue (dilution factor = 2) and mix by gentle pipetting. Fill both sides of the counting slide with cell suspension (approximately 10 μL) and count cells using automated cell counter, which can calculate the total cell number and concentration of viable and non-viable cells.

14. Aspirate the supernatant.

15. Add 1 mL pre-warmed FM10 medium.

16. Transfer the mixture into a 50 mL Falcon tube.

17. Add another pre-warmed FM10 medium (usually 23 mL: final cell concentration is  $1.2 \times 10^5$  cells/mL).

**Note:** You can use a 10 mL and/or 25 mL pipette here and a 50 mL Falcon tube.

18. Aspirate the gelatin from the 6-well plate (prepared as described above), and add 2 mL MEF containing FM10 media ( $2.5 \times 10^5$  cells/well).

19. Incubate overnight to attach the cells (leave the plate for at least 4 h up to 24 h) in the incubator (Figures 1A–1D).

**Note:** A density of  $0.25 \times 10^5$  cells/ $\text{cm}^2$  is a good density for MEF plating (Figure 1). This number should be optimized to provide full coverage without being too dense. If there are too many MEFs, there will be no foothold for mESCs/miPSCs to adhere to. Conversely, too little MEF will not provide adequate conditioning of the medium.

**Table 1. Plating density of mouse embryonic fibroblast cells (MEFs)**

Vessel	Surface area (per well)	Number of MEFs (per well)	Optimum volume
35 mm dish	9 cm <sup>2</sup>	2.5 × 10 <sup>5</sup> cells	2 mL
60 mm dish	19.5 cm <sup>2</sup>	5.0 × 10 <sup>5</sup> cells	5 mL
4-well plate	2 cm <sup>2</sup>	0.75–1.2 × 10 <sup>5</sup> cells	750 μL
6-well plate	9.6 cm <sup>2</sup>	2.5 × 10 <sup>5</sup> cells	2 mL

**Note:** Plating densities of MEFs are listed in [Table 1](#). Inactivated MEF feeder cells should be plated for at least 4 h ([Figures 1A and 1B](#)) or 1 day ([Figures 1C and 1D](#)) before the mESCs/miPSC plating to allow for attachment and spread of the MEF layer to completely cover the dish surface. The MEF feeder layer should not be used after 4 days of seeding ([Figures 1I and 1J](#)).

### Thaw and plate mESCs/miPSC frozen vials

⌚ Timing: 20 min for step 20

⌚ Timing: 24 h for step 29

**Note:** mESCs and miPSCs are isolated from C57BL6 strain.

20. Gelatinize the plate with 0.1% gelatin made with cell culture grade for at least 20 min in the incubator.
21. Take the frozen vial from the liquid nitrogen tank.
22. Immediately after that, put the vial on dry ice and bring it to the culture room.
23. Thaw the vial in the thermo heater.
24. Before completely dissolving (with a small ice block), add the pre-warmed 1 mL FM10 media (the formulation is shown in the [key resources table](#)) into the mESCs/miPSCs and pipet the thawed cells gently.

**Note:** You can use a 5 mL pipette here and a 15 mL Falcon tube.

**Note:** Usually, naive mouse ESCs can be efficiently cultured on gelatin coated plates without feeders.

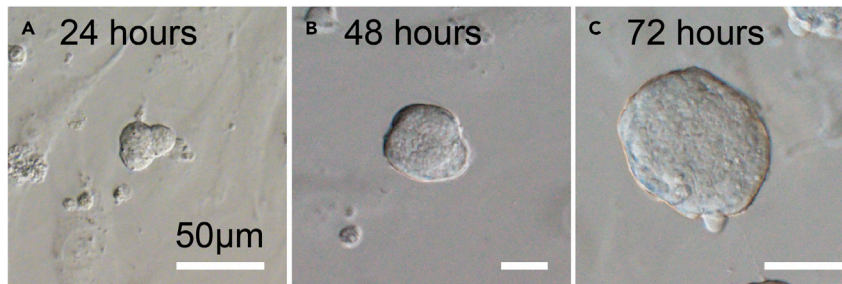
25. Centrifuge at 200 × g for 4 min.
26. Aspirate the supernatant.
27. Add 2–3 mL pre-warmed t2i/L media.
28. Aspirate the MEF media from the prepared 6-well plate.

**Note:** You need to leave the MEF plate for at least 4 h in the 37°C, 5% CO<sub>2</sub> incubator.

**Note:** Please ensure that the MEFs are 50%–60% confluent ([Figures 1C and 1D](#)). If it is not confluent, you need to thaw another vial.

**Note:** For mESCs/miPSCs derivation, 2i medium containing 15% FBS and feeders were used at p0 for better attachment of blastocysts. After derivation, naive mESCs/miPSCs can be efficiently cultured in serum-free 2i medium on gelatin coated plates without feeders.

29. Add the t2i/L media containing mESCs/miPSCs.
30. Leave it in a 37°C, 5% CO<sub>2</sub>, and 95% humidity incubator for 24 h.



**Figure 2. Mouse induced pluripotent stem cells (miPSC) plating**  
miPSCs are plated and observed at 24 h (A), 48 h (B) and 72 h (C) after passage.

### Culture mESCs/miPSCs

⌚ Timing: 2–3 days

31. After 24 h of incubation, observe the status of mESCs/miPSCs under a microscope (Figure 2A).

**Note:** Check to see if mESCs/miPSCs are viable. Small, evenly sized colonies should be evenly attached with good spacing.

32. Aspirate the t2i/L media.

33. Add 2–3 mL t2i/L media.

34. Every 24 h, it is necessary to check the cell conditions and change the t2i/L medium with 2–3 mL fresh t2i/L medium (Figures 2B and 2C).

35. When the cells reach 80%–90% confluence, you can passage the cells into another well (Figure 2C).

**Note:** Usually it takes about 2–3 days to reach confluency.

**Note:** If the cell concentration of mESCs/miPSCs is too high and the colonies contact each other, or if the cells are cultured for a long period, they will begin to differentiate. In that case, the edges of the colony become steep, and the center of the colony appears to turn brown.

### Passage mESCs/miPSCs

⌚ Timing: 1–2 h for step 36

⌚ Timing: 24 h for step 52

**Note:** Usually, frozen mESCs/miPSCs need to be passaged for 2–3 times for recovery.

36. Aspirate the t2i/L media.

37. Add 2 mL pre-warmed PBS and washout the culture medium completely in one step. Aspirate the PBS.

38. Add 1 mL pre-warmed 0.25% trypsin.

**Note:** Most protocols for culturing and splitting naive mESCs/miPSCs mention the use of Accutase (Gibco A1110501). Accutase is a mixture of natural enzymes with proteolytic and collagenolytic activity, free of mammalian and bacterial components. Accutase is more effective



than the combined treatment of trypsin and collagenase in dispersing cells in a less toxic and more gentle manner.

39. Leave it for 4 min in the incubator.

**Note:** Check trypsin digestion by tapping the plate every 4 min and observing the mESCs/miPSCs break apart the cells under microscope. If the cells do not detach, return the plate to the incubator and tap again after another minute.

- a. While waiting, perform the following steps
  - i. Take the MEF-plated well (not yet plated with mESCs/miPSCs).
  - ii. Aspirate the FM10 media.
  - iii. Add 2–3 mL pre-warmed t2i/L media.

40. After 4 min, take the trypsinized mESCs/miPSCs well from the incubator.
41. Add 1 mL t2i/L media.
42. Pipette with a 1000  $\mu$ L Pipetman thoroughly and separate each cell.
43. Transfer the cell mixture into a 15 mL Falcon tube.
44. Add another 8 mL FM10 medium (total 10 mL) and mix well with a 10 mL pipette.
45. Centrifuge at  $200 \times g$  for 4 min.
46. Aspirate the supernatant, leaving a small amount of medium.
47. Pipette with a 200  $\mu$ L Pipetman thoroughly and separate each cell.
48. Add 1 mL pre-warmed t2i/L medium.
49. Pipette with a 1000  $\mu$ L Pipetman thoroughly.
50. Count the cell number.
51. Take the cell-containing media ( $1 \times 10^5$  cells) and transfer into the MEF-plated 6-well plate.

**Note:** To identify how each cell line proliferates, we usually try to passage with different cell concentrations of 1:10 to 1:100 (thus, at least 2–3  $\times$  MEF-plated wells are needed for each cell line).

52. Gently move the wells approximately twice in forward-backward and left-right while maintaining a horizontal position to allow the mESCs/miPSCs to be evenly distributed.
53. Leave the well in the incubator overnight (12–24 h).
54. Replace the medium with t2i/L medium.

### Transfection (electroporation)

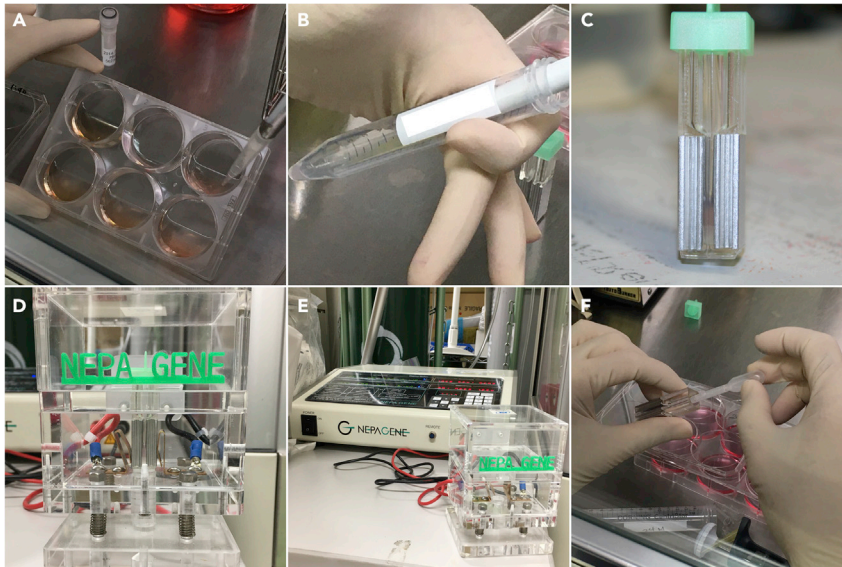
**Note:** You may use other alternative electroporators such as Nucleofector® (Lonza), Neon® Transfection System (Thermo Fisher Scientific) or Gene Pulser Xcell™ complete system (Bio-Rad).

### Dissociation

⌚ Timing: 10–20 min

**Note:** After 2–3 passages after thawing, mESCs/miPSC usually recover to a good status. mESCs/miPSCs grow as a three-dimensional colony, not a single layer. Undifferentiated colonies have a rounded appearance with sharp fractal edges. It is difficult to identify the boundaries of individual cells in the colonies. These cells can then be used for transfection.

55. Add 2 mL pre-warmed PBS and washout the culture medium in one step. Aspirate the PBS.
56. Add 1 mL pre-warmed 0.25% trypsin.



**Figure 3. Transfecting transposon-expressing vector with mouse induced pluripotent stem cells (miPSCs) by electroporation**

(A) miPSCs are dissociated into pieces by trypsin.

(B) Pellets are mixed with transposase-expressing vector and the piggyBac transposon gene expression vector (pPB-CAG-mZscan5b-P2A-EGFP) (Niwa et al., 1991) in 100  $\mu$ L Opti-MEM.

(C–E) The vectors are transfected to miPSCs by electroporation using NEPA21 (NepaGene) under the following conditions: Poring pulse: 125 V, 5ms-pulse width, 50-ms pulse interval, two pulses, 10% attenuation rate, +; Transfer pulse: 20 V, 50-ms pulse width, 50-ms pulse interval, five pulses, 40% attenuation rate, +/- . The piggyBac transposon gene expression vector in t2i/L medium is then moved into the cytoplasm with a few transfer pulses with a low voltage and long duration after the poring pulse.

(F) The electroporated cells are plated onto a 6-well MEF plate in mouse t2i/L medium.

57. Leave it for 4 min in the incubator.
58. Dissociate cells into a single-cell suspension by vigorous pipetting.
59. Add 1 mL t2i/L media.
60. Count the cell number.
61. Take the cell-containing media ( $1 \times 10^6$  cells) for transfection.

**Note:** Make sure the cells are dissociated into single cell completely. The 30–40  $\mu$ m strainer might be helpful.

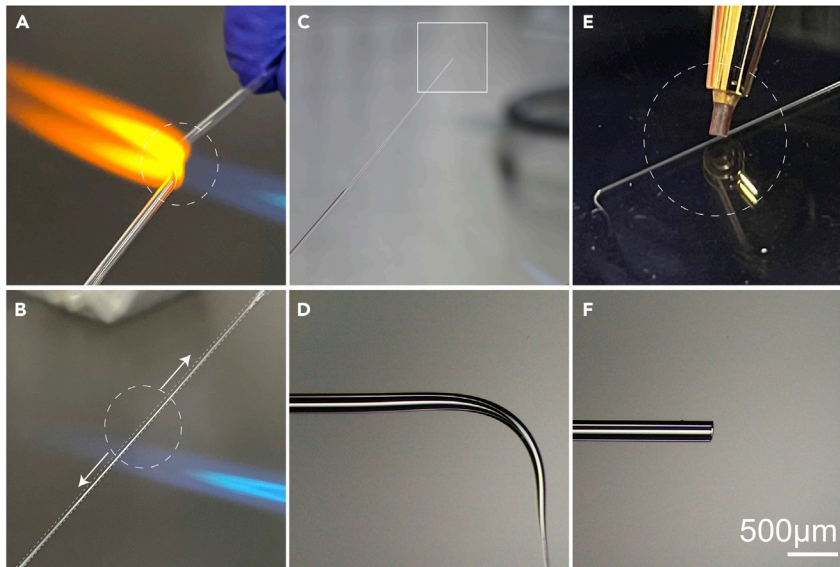
### Transfection

⌚ Timing: 20–30 min

62. Prepare the pellets by mixing with 3  $\mu$ g of transposase-expressing vector and 3  $\mu$ g of the piggyBac transposon gene expression vector (pPB-CAG-mZscan5b-P2A-EGFP) (Niwa et al., 1991) purchased from VectorBuilder (VB181015-1188bhq) in 100  $\mu$ L Opti-MEM media (NEPA Gene) (Figures 3A and 3B).

**Note:** For mESCs/miPSCs transfection, CAG-promoter is recommended. CAG-promoter is reported to be highly active and stable over the long term in the expression of transgenes in mESCs (Hong et al., 2007).

**Note:** Do not whisk the cell suspension.



**Figure 4. Procedure to prepare glass tool**

Grasp both ends of the glass capillary and scald it with fire (A). Pull it to both ends after leaving it for 1–2 s (B). When the capillary is stretched, heat it and cut the center (C). The tip of the needle to curl into a hook shape caused by the force of the rising heat (D). Cut it with a ruby pen by making a slit at the end (E). The final product after cut with a ruby pen (F).

**Note:** NEPA21 does not need special buffers for transfection.

63. Transfer the cell suspension to a cuvette. The electroporation conditions were as follows: Poring pulse: 125 V, 5 ms-pulse width, 50-ms pulse interval, two pulses, 10% attenuation rate, +. Transfer pulse: 20 V, 50-ms pulse width, 50-ms pulse interval, five pulses, 40% attenuation rate, +/- (Figures 3C–3E).
64. The electroporated cells were plated onto a 6-well MEF plate in a mouse t2i/L medium (Figure 3F).

### Colony picking of GFP-positive mESCs/miPSCs for single-cell cloning

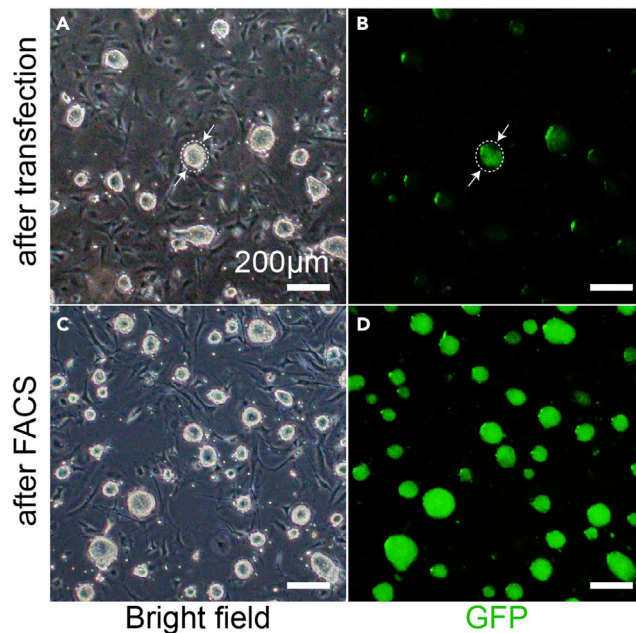
⌚ Timing: 24 h

**Purpose:** To perform initial colony picking of GFP-positive ESCs/iPSCs and maintain master stocks.

**Feeder cells:** Inactivated mouse embryonic fibroblast (MEF) feeder cells (Reprocell RCHEFC003).

**Tools:** Glass tools (World Precision Instruments Inc., TW150-4) or syringe needles can be used for microdissection of colonies for passaging. It is also possible to pick mESC/miPSC colonies using a pipette tip or cell lifter. However, for fine control of dissection when initially isolating good areas of ESC/iPSC colonies from undesirable regions of the colony, glass tools are optimal. For glass tools, Pasteur pipettes are pulled hair thin. Fine glass needles with hooked ends are forged in two steps over a micro burner, as follows:

65. While holding the two ends of a long Pasteur pipette, place the thin end of the pipette at a distance of about half to two-thirds of the distance away from the tip into the orange part of the flame until the glass melts into a solid constriction (Figure 4A).
66. In a single motion, upon removing the pipette from the flame, pull on each end of the pipette gently and quickly to draw out a thin filament before the glass hardens (Figure 4B). This is done without breaking the connection between the two ends of the pipette.



**Figure 5. Green fluorescent protein (GFP)-positive colony picking**

After transfecting transposon-expressing vector with mouse induced pluripotent stem cells (miPSCs) by electroporation, around 20% of colonies are GFP-positive (A and B). After colony picking and fluorescence-activated cell sorting (FACS), the ratio of GFP-positive colony gradually increases (C and D).

67. Beginning several inches above the flame and slowly moving the thin drawn part of the filament down towards the flame, pull a very fine filament, as previously described. The two ends of the pipette should separate, this time forming a fine needle end on the tip of the pipette (Figure 4C and 4D).
68. If the tip remained straight after the second pull, pass the fine end a few inches quickly over the top of the flame. The force of the rising heat will curl the tip of the needle into the hook. The hooked end should be thin enough for the microdissection of the colonies but thick enough to withstand some pressure during the dissection (Figures 4E and 4F).

△ **CRITICAL:** Glass tool safety precautions – Do not hold glass tool near the sharp (thinner) end.

△ **CRITICAL:** Be careful not to stick yourself with this end of the tool. To guide the tool, the opposite hand can be used to guide the thicker end of the tool.

**Note:** Be sure to make the appropriate number of tools. Limit to one or two tools per cell line.

**Note:** Avoid manual dissection for multiple lines at a time.

△ **CRITICAL:** Be sure to discard tools immediately after use in an appropriate glass tool waste receptacle.

**Note:** Set the pore size of the pipette to less than 100  $\mu\text{m}$  (50–70  $\mu\text{m}$ ).

69. **Colony picking:** For mESC/miPSC colony picking after transfection, identify GFP-positive colonies under a fluorescence microscope. Transfection efficiency of electroporation should be around 20% (Figures 5A and 5B).

70. Before microdissection, change the medium in the well to be passaged and on the feeders.
71. Wash the new feeders once, and add 2 mL t2i/L medium (6-well plate) on them.
72. Keep the feeders in the incubator during dissection.

**Note:** Please take care while maintaining the temperature, pH, and osmotic pressure of the culture medium by working quickly.

**Note:** Utilizing a humidified 5% CO<sub>2</sub>/20% O<sub>2</sub> mixed gas source and a warm plate will help maintain an optimal environment even when the cultured cells are outside the incubator.

73. Use fluorescence microscopy to find an ideal colony consisting of small, round, random organized cells with a high ratio of nucleus to cytoplasm that have not yet begun to form structures within the colony.
74. Circle the bottom of the dish with the colony to be labeled with a pen.
75. Bring a stereomicroscope into a laminar flow hood.
76. Peel off the whole colony, and incubate those cells in a small amount of 0.25% trypsin (100  $\mu$ L drop).
77. Pipet and microdissection of the chunks of approximately 100 cells using the glass hooks (Figures 5A and 5B).

**Note:** If you make a grid of cuts in the colony at the back of the hook, each piece can be easily pulled away from the colony.

78. After micro-dissection, the cell chunks are swirled into the center of the dish, and 20 to 50 chunks are transferred to the new feeder wells (a small-diameter 4-well dish) using 1 mL micropipettes.

**Note:** Pre-coat the micropipette tip with the t2i/L medium so that the cells do not stick (a regular sterile pipette or a Pasteur pipette can also be used).

79. Leave the dishes for 24 h to allow the chunks to attach to the dish.
80. Exchange medium 0.5 mL/well.

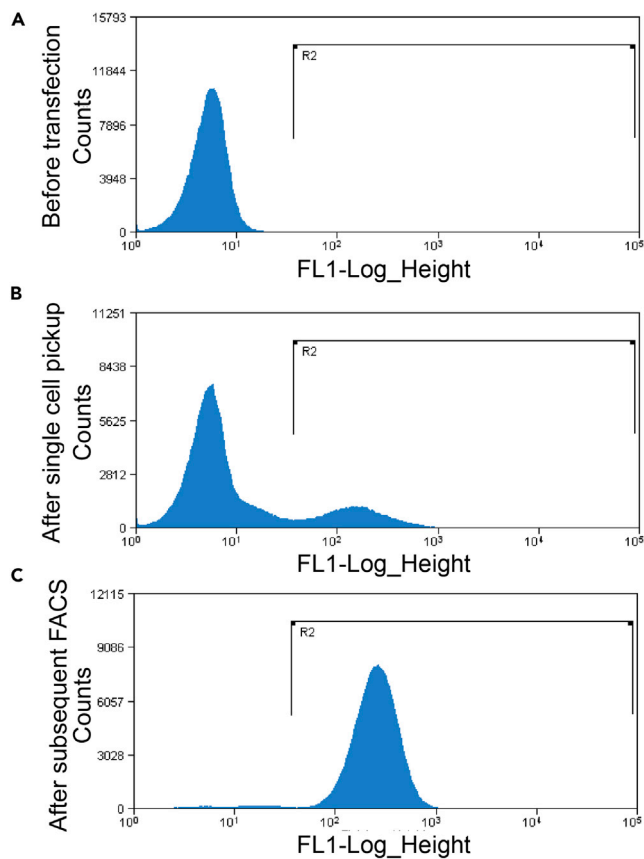
**Note:** The size of the piece should be sufficiently large to survive the cutting and adhere to the feeder layer. An excessively large piece will tend to form an embryoid body-like structure on the feeder layer as it takes too long for the entirety of a large colony to come into contact with the feeders. The resulting colony will have an area of differentiation in the center arising from the embryoid 24 body-like structure.

### Alternate protocol based on FACS sorting

*Prepare cells for FACS analysis*

⌚ Timing: 1–2 h

81. Aspirate t2i/L culture media from mESC/miPSC culture dishes.
82. Add 0.5 mL (12-well) or 1 mL (6-well) of 0.25% Trypsin.
83. Incubate for 5 min at 37°C.
84. Neutralize with an equal volume of FM10 media.
85. Transfer the dissociated cells to a 15 mL Falcon tube and bring up to 10 mL with FM10 media.
86. Centrifuge at 200  $\times$  g for 4 min.
87. Aspirate the supernatant carefully.
88. Resuspend the cell pellet in PBS medium (volume depends on the cell number).



**Figure 6. Sorting purified green fluorescent protein (GFP)-positive mouse induced pluripotent stem cells (miPSCs) by fluorescence-activated cell sorting (FACS)**

(A–C) (A) A representative negative control for FACS plot using miPSCs (A). Sorting GFP+ miPSCs after single-cell picking (B) and FACS (C).

### Sort the transfected stem cells

⌚ Timing: 48–72 h

89. Check the resolution and sensitivity of the instrument using a standard particle.
90. If you have a control sample, measure them to check the reagents and process.
91. Analyze the cells.
92. Isolate and collect cell populations that emit relatively more fluorescence at a specific excitation wavelength (488 nm excitation) (Figures 6A–6C).
93. After the GFP-expressing cells have been sorted, plate the cells onto MEF coated plates.
94. Closely follow/watch the cells every day for 2–3 days (Figures 5C and 5D).

**Note:** To improve the recovery rate of transfected cells, we tried both colony picking with glass tools and then further sorting by FACS.

### MEF preparation

⌚ Timing: 4 h (plus incubation overnight)

95. See the MEF preparation step shown above (steps 4–19).

### Passage transfected mESCs/miPSCs

⌚ Timing: 4 h (plus incubation overnight)

96. Aspirate the t2i/L media.
97. Add 2 mL pre-warmed PBS and washout the culture medium in one step. Aspirate the PBS.
98. Add 1 mL pre-warmed 0.25% Trypsin.
99. Leave it for 4 min in the incubator.
  - a. While waiting, perform the following steps
    - i. Take the MEF-plated well (not yet mESCs/miPSCs plated).
    - ii. Aspirate media.
    - iii. Add 2–3 mL pre warmed t2i/L media.
100. After 4 min, take the trypsin-plated well from the incubator.
101. Add 1 mL FM10 media.
102. Pipette with a 1000  $\mu$ L Pipetman thoroughly and separate each cell.

**Note:** Use filter tip to prevent bacterial contamination.

103. Transfer the mixture into a 15 mL Falcon tube.
104. Add another 8 mL FM10 medium, and mix well with a 10 mL pipette.
105. Centrifuge at  $200 \times g$  for 4 min.
106. Aspirate the supernatant.
107. Add 1 mL pre-warmed FM10 medium.
108. Pipette with a 1000  $\mu$ L Pipetman thoroughly and separate each cell.
109. Count the cell number.
110. Take 100  $\mu$ L (1:10) cell-containing medium ( $1 \times 10^5$  cells) and transfer into the MEF-plated well of a 6-well plate.

**Note:** Usually, I performed 1:5, 1:10, and 1:20 passages into the different wells of a 6-well plate (accordingly, at least 2–3  $\times$  MEF-plated wells per cell line are needed).

111. Shake the mESCs/miPSC well so that cells can be plated into the whole well.
112. Incubate the well overnight (12–24 h).
113. Replace the medium with t2i/L medium.

### EXPECTED OUTCOMES

The transfection efficiency of electroporation was around 20%. By performing colony picking and FACS, it was possible to obtain GFP-positive lines for almost all colonies (Ogawa et al., 2019).

### LIMITATIONS

The aim of this protocol is to create a model cell line to investigate genome stability. These results are presented in the paper (Ogawa et al., 2019). Although the PiggyBac vector system is technically simpler than virus-based gene transfer, the efficiency of introducing designed foreign DNA into mESCs/miPSCs via the PiggyBac vector is lower than virus-based gene transfer. Although the PiggyBac vector system is more efficient for gene delivery than genome editing, it cannot control the copy number or location of foreign DNA incorporated into the genomic DNA of target cells.

### TROUBLESHOOTING

#### Problem 1

CHIR99021 is not fully dissolved in culture medium.

### Potential solution

DMSO can be used for dissolving CHIR99021 for stock solution (3 mM).

### Problem 2

Low viability of pluripotent stem cells after thawing (step 24).

### Potential solution

Low survival rate: Ensure that cryovials are thawed quickly. Before completely dissolving (with a small ice block), add the pre-warmed 1 mL FM10 media into the mESCs/miPSCs and pipet the thawed cells gently. Viability upon thawing also depends primarily on accurate freezing. If the cells do not survive thawing, try rechecking the freezing procedure and the condition of the frozen tubes.

In case of accidental aspiration of thawed cells after centrifugation: Aspirate the medium immediately after centrifugation to avoid disturbing the cell pellet. You can leave about 200  $\mu$ L of medium when aspirating. Pipette the pellet with the remaining medium and add 1 mL of FM10 media.

### Problem 3

Pluripotent stem cells spontaneous differentiation (step 34).

### Potential solution

Ensure that cells are cultured according to the recommendations listed here.

Reagents (medium): Maintain a sufficient amount of fresh culture medium on the cells. Reagents should be freshly prepared. The medium can be stored before the addition of CHIR99021 and PD0325901 at 4°C, and use within two weeks.

Plates: Do not leave the plate outside the incubator to avoid the effects of temperature changes and light on the cells.

Cell number: Reduce the number of cell aggregates per  $\text{cm}^2$  at cell passages to reduce colony density.

Colony picking: It is possible to remove differentiated cells by leaving the mESC/miPSC colonies in place and scraping off the differentiated areas with a pipette tip. Care should be taken not to disturb the mESC/miPSC colonies. If good mESC/iPSC colonies remain between the differentiated areas, the colonies with good mESC/iPSC morphology may be picked up manually using a pipette tip. Alternatively, you can use a glass capillary. Set the pore size of the pipette to less than 100  $\mu\text{m}$  (50–70  $\mu\text{m}$ ). Make sure that the cutting tips are sharp and free of jagged edges.

### Problem 4

Non-uniform distribution of colonies within plate (step 52).

### Potential solution

Gelatin coating: Make sure that the entire surface of the tissue culture vessel is properly coated with gelatin.

Plating: Gently move the wells after transfer cell-containing medium approximately twice in forward-backward and left-right while maintaining a horizontal position to allow the mESCs/miPSCs to be evenly distributed. Be careful not to shake the plate when placing it in the incubator, and leave it for 24 h.

### Problem 5

Colonies are too crowded together and difficult to pick (step 74).



### Potential solution

Reduce the number of cells to be seeded.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mitsutoshi Yamada ([mitsutoshi.yamada@gmail.com](mailto:mitsutoshi.yamada@gmail.com)).

#### Materials availability

We did not generate any new materials.

#### Data and code availability

We did not generate a data set or code.

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### AUTHOR CONTRIBUTIONS

Conceptualization, M.Y. and H.A.; investigation, M.Y., T.S., R.N., and S.U.; writing – original draft, M.Y.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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