



# PhiC31 Reprogrammer & miPSCs, User Manual

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# **PhiC31 Reprogrammer & miPSCs**

## **Catalog#s FC300A-1, SC211A-1**

### *User Manual*

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ver. 1-11-14-12

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## I. Introduction

### A. Background of $\phi$ C31 Integrase Technology

The  $\phi$ C31 integrase is a site-specific recombinase encoded within the genome of the bacteriophage  $\phi$ C31. The  $\phi$ C31 integrase mediates recombination between two ~34 base pair sequences termed attachment sites (*att*), one found in the phage and the other in the bacterial host. This serine integrase has been shown to function efficiently in many different cell types including mammalian cells. In the presence of  $\phi$ C31 integrase, an *attB* containing donor plasmid can be unidirectionally integrated into a target genome through recombination at sites with sequence similarity to the native *attP* site. These sites are termed *pseudo-attP* sites.  $\phi$ C31 integrase can integrate a donor plasmid of any size, as a single copy, and requires no cofactors. The integrated transgenes are stably expressed and heritable.

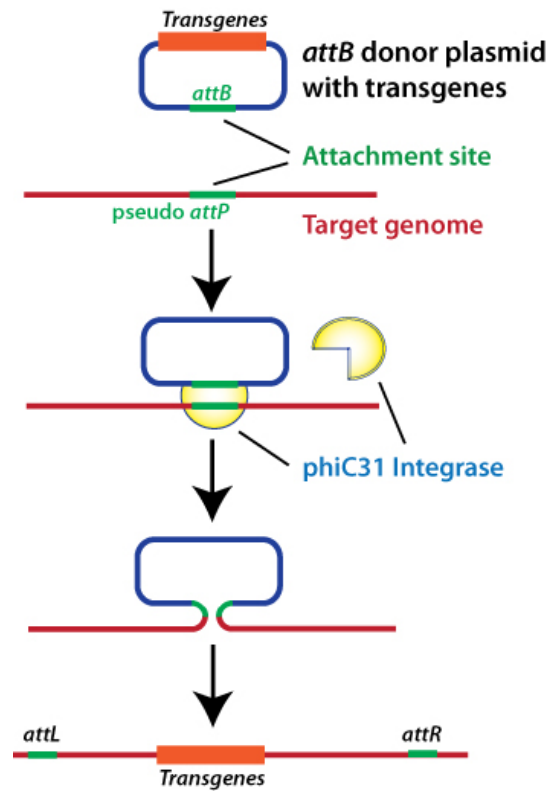
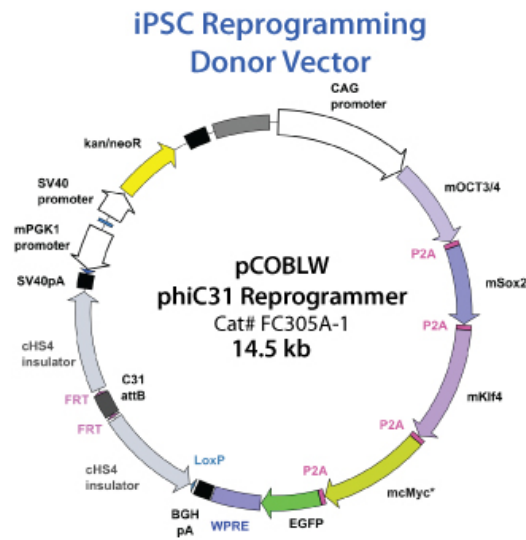


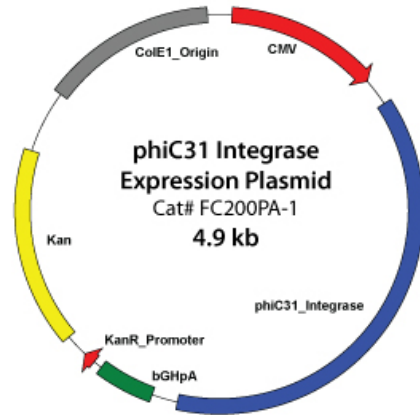
Fig. 1. Schematic of the  $\phi$ C31 Integrase mediated recombination of donor plasmid sequence into pseudo-*attP* sites in host genome

## B. $\phi$ C31 Reprogramming Vector Maps & Details

The classic Oct3/4, Sox2, Klf4 and cMyc factors have been adapted to the integrase system. The pCOBLW phiC31 reprogramming vector (catalog# FC305A-1) features the iPSC factors driven by a potent CAGs promoter with the factors separated by P2A elements. HS4 insulators flank the expression cassette and a functional attB site directs the integration of the entire plasmid into pseudo attP sites in any genome. The vector is engineered with FRT sites for later excision using standard FLP recombinase methods. After co-transfection of the reprogramming vector with the phiC31 expression plasmid (catalog# FC200PA-1) and integration, you can monitor for successful transfection with GFP fluorescence and select for positive colonies using the built-in Neo marker. The reprogramming iPSC factor donor vector and the phiC31 expression plasmid maps are shown below.



**phiC31 Integrase  
Expression Vector**



**C. List of Components**

**List of Components for FC300A-1: Reprogramming Kit**

Each Non-Viral PhiC31 Integrase Mouse iPSC Reprogramming Kit includes 10ug of plasmid DNA for FC200PA-1 and FC305A-1.

FC300A-1	PhiC31 Integrase Plasmid (FC200PA-1) & PhiC31 Reprogrammer (FC305A-1)	10ug each
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**List of Components for SC211A-1: PhiC31 Mouse iPS Cells**

Each iPS cell line set comes as one vial of MEF-derived PhiC31-generated Mouse iPS cells and one vial of MEF at  $2 \times 10^5$  cells each.

SC211A-1	Mouse iPS Cell Line Set [MEF & PhiC31 miPSCs]	2x10 <sup>5</sup> cells each
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The product is shipped on dry ice and should be immediately stored in the **gas phase** of liquid nitrogen.

In general, iPS cells are challenging to culture and should only be operated by researchers experienced in the intricacies of mouse embryonic stem (mES) cell culture. The methods for culture are nearly identical to mES cell culture, although more careful maintenance will be required.

The MEF cells provided in the iPSC kit are meant to be used as **source cells only** and not as feeder cells for culturing iPS cells. Appropriate feeder cells for mouse iPS cell culture can be obtained from commercial sources, or generated through Mitomycin C treatment of mouse embryonic fibroblasts (MEFs). See protocol on Pgs. 9-12 for more details on generating feeder cells.

## II. Protocol for Deriving Mouse-Induced Pluripotent Stem Cells using the $\phi$ C31 Reprogrammer on MEF source cells

### A. Mouse Induced Pluripotent Stem Cell Generation

The following protocol has been optimized for mouse embryonic fibroblasts (MEF) cells as described in Karow M. *et al.* Other source cells may require transfection optimization for efficient reprogramming. In general, reprogramming requires a single transfection of approximately 3  $\mu$ g per well in a six-well plate.

- 1) Use the MEF nucleofector kit I (Lonza) and the program T-20 according to the manufacturer's instructions.
- 2) Nucleofect with 3  $\mu$ g **total DNA** using the PhiC31 Integrase Plasmid (FC200PA-1) & PhiC31 Reprogrammer Donor Plasmid (FC305A-1) at a 1:1 ratio by mass. The Neon transfection system from Life Technologies has also been demonstrated to work with similar efficiency.
- 3) After nucleofection, plate the cells in a well of a six-well plate using MEF media. Change media each day with MEF media.
- 4) On day two, transfer the cells to a 0.1% gelatin coated 10 cm dish containing irradiated or mitomycin C-treated MEF feeder cells. Switch to Mouse iPSC growth media (cat. no. #SC200M-1). Change the media every other day.

- 5) GFP positive colonies will be visible starting from 8 to 12 days and should be picked between 20 and 26 days for further expansion.

**See Section III for additional protocol details on MEF feeder cell generation and iPSC culture techniques.**

### III. Non-Viral $\phi$ C31 Integrase-derived Mouse iPS Cells

#### A. $\phi$ C31 Generated Mouse Induced Pluripotent Cell Line

Mouse induced pluripotent stem cells (iPSCs, Cat# SC211A-iPSC) were generated by nucleofecting genetically unmodified C57BL/6 mouse embryonic fibroblasts with the pCOBLW  $\phi$ C31 Integrase Mouse Reprogramming Donor plasmid (Cat# FC305A-1) and the  $\phi$ C31 Integrase expression plasmid (Cat# FC200PA-1) for stable integration into the mouse genome (Karow 2011). The Reprogramming Donor plasmid encodes the four murine transcription factors (Oct4, Sox2, Klf4, and c-Myc) that have been shown to induce the reprogramming of somatic cells to a pluripotent state. See Section II of this User Manual for more details on the reprogramming protocol used to make this cell line. Due to the specificity of recombination using the  $\phi$ C31Integrase system, the iPSCs are expected to have, on average, one copy of the OSKM plasmid per cell, which reduces the risk of insertional mutagenesis effects [integration analysis data available on Pg. 18]. The cells were derived using morphological selection criteria and GFP expression without drug selection. When cultured under standard mouse ES cell culture conditions, the morphology of SBI mouse iPSCs are identical to that of mouse ES cells. The cells also express the pluripotency markers SSEA-1, Nanog, Sox2, and Oct4 and demonstrate strong endogenous AP activity.

Mouse iPS cells from SBI are provided at passage 10 and can be passaged 50 times before differentiation.

#### B. Culture Conditions for MEF Feeder Cells

**Mouse iPS cells should be grown on a feeder cell layer.** Appropriate feeder cells for mouse iPS cell culture can be obtained from commercial sources, or generated through Mitomycin C treatment of mouse embryonic fibroblasts (MEFs).



**1. Required media and reagents**

Reagent	Information
MEF Medium	DMEM containing 10% FBS, 2 mM glutamine, $1 \times 10^{-4}$ M nonessential amino acids and 50 U and 50 $\mu$ g/ml penicillin and streptomycin.
2x Cold Freezing Media	20% DMSO and 80% FBS
Mitomycin C solution	1 mg/ ml

**2. Gelatin treatment of plates for MEF feeder cells**

1) Add enough sterile/ autoclaved 0.1% gelatin to cover the bottom of the wells.

Approximate amounts:

- 10cm plate – 5 ml
- 6 well – 1.5 ml/ well
- 24 well – 0.5 ml/ well
- 96 well – 100  $\mu$ l/ well

2) Incubate the gelatin-coated dishes for at least 15 min at 37°C.

3) Aspirate excess gelatin solution before use.

**3. Thawing MEF cells**

To insure the highest level of viability, be sure to warm medium to 37°C before using it on the cells. Cells should be plated at a minimum cell density of  $10^4$  cells/  $\text{cm}^2$ .

1) Remove the vial from liquid nitrogen and thaw quickly in a 37°C water bath.

2) Remove the vial from the water bath as soon as the cells are half-thawed, and sterilize the tube by spraying with 70% ethanol.

3) Transfer the cells with 10 ml of MEF medium to a 15 mL conical tube and pellet the cells by centrifugation at 200 *g* for 5 min.

4) Discard the supernatant and resuspend the cells with 10 ml fresh MEF medium and plate the cells on gelatin-coated plates at seed density of  $10^4$  cells/  $\text{cm}^2$ .

5) Incubate at 37°C with 5% CO<sub>2</sub>, until the cells reach 80-90% confluency.

- 6) Change medium twice a week or when pH decreases.

#### 4. Passaging MEF cells

Cells should be split when they reach confluency. We recommend splitting the cells based on  $0.5 \times 10^4$  cells/  $\text{cm}^2$ .

- 1) Discard the medium and wash the cells once with PBS.
- 2) Aspirate PBS, and add 2 ml per T75 flask of 0.25% trypsin-EDTA, and incubate for 2 min.
- 3) Add 5 ml of MEF medium, and break up the cell clumps by gently pipetting up and down several times.
- 4) Transfer cells into a conical tube and centrifuge at 200 *g* for 5 min.
- 5) Discard the supernatant and resuspend the cell pellet in 10 ml MEF medium.
- 6) Count the number of cells, plate cells at  $0.5 \times 10^4$  cells/  $\text{cm}^2$  and incubate at 37°C with 5%  $\text{CO}_2$ .

#### 5. Freezing MEF cells

- 1) Follow steps 1-4 from the Passaging MEF cells protocol (above).
- 2) Discard the supernatant, and resuspend the pellet in MEF medium.
- 3) Count the number of cells and adjust the cell suspension to  $4 \times 10^6$  cells/ ml.
- 4) Add equal volume of cold 2X Freezing Media to the cell suspension.
- 5) Aliquot 1 ml of suspension into each cryovial ( $2 \times 10^6$  cells/ vial).
- 6) Place the vials in a cell-freezing container and keep it at  $-80^\circ\text{C}$  overnight.
- 7) Transfer the vials to a liquid nitrogen tank for long-term storage.

## 6. Mitomycin C treatment of MEF

Mitomycin C acts to halt the division of MEF cells so that they can be used as a feeder layer for miPS cells. MEF cells should be at confluence when treated with mitomycin C.

- 1) Add 6 ml of fresh MEF medium contain 50  $\mu$ l of mitomycin C solution (1 mg/ ml) to one T75 flask of confluent MEF cells, and swirl it briefly. The final concentration of mitomycin C is 8  $\mu$ g/ml.
- 2) Incubate at 37°C for at least 3 hrs.
- 3) Aspirate the mitomycin C-containing medium off the cells and wash the cells twice with 10 ml PBS.
- 4) Aspirate PBS and add 1 ml of 0.25% trypsin-EDTA, swirl to cover the entire surface, and incubate for 2 min at room temperature.
- 5) Add 5 ml MEF medium and break up the cells to a single-cell suspension by pipetting up and down. Count the number of cells.
- 6) Seed the cells on gelatin-coated dishes (3 x 10<sup>6</sup> cells per 100-mm dish, or 5 x 10<sup>5</sup> cells per well of a 6-well plate).
- 7) Cells should be ready to use by the next day.

## C. Growth Conditions for PhiC31 Mouse iPS cells

### 1. Required Media and Reagents

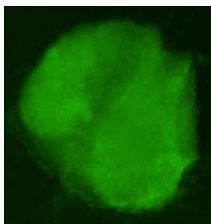
Reagent	Information
Mouse iPSC Growth Medium	SC200M-1
2x Cold Freezing Media	20% DMSO and 80% ES-FBS
Trypsin-EDTA	GIBCO

### 1. Thawing Mouse iPS cells

To insure the highest level of viability, be sure to warm medium to 37°C before using it on the cells.

- 1) Remove the vial from liquid nitrogen and thaw quickly in a 37°C water bath.
- 2) Remove the vial from the water bath as soon as the cells are half-thawed, and sterilize by spraying with 70% ethanol.
- 3) Transfer the cells with 10 ml of mouse iPSC medium to a 15 mL conical tube and pellet the cells by centrifugation at 200 *g* for 5 min.
- 4) While centrifuging, remove MEF medium from the feeder cell plates, and wash the wells twice with DMEM. Then add 1 ml of mouse iPSC Medium.
- 5) Discard the supernatant from the mouse iPS cells, and resuspend cells with 1 ml fresh mouse iPSC medium. Plate the cells on MEF feeder cells in a 6-well plate.
- 6) Incubate at 37°C with 5% CO<sub>2</sub> until the cells reach 80% confluency. The mouse iPSC media must be changed every day.

### Mouse iPS Cells Express GFP



## 2. Maintenance of mouse iPS cells

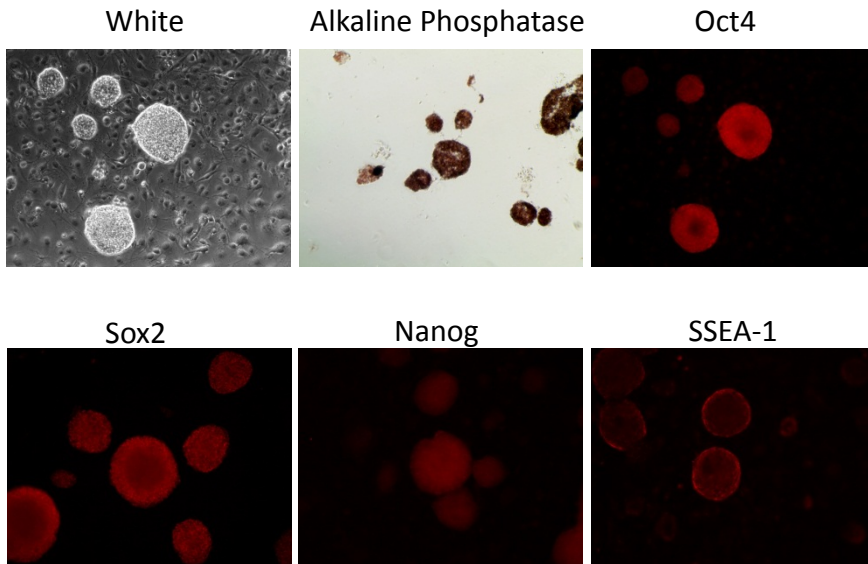
It is important not to keep mouse iPS cells in culture for long period of time without passaging, to maintain the pluripotency.

- 1) Aspirate the medium and wash the cells twice with 1 ml PBS.
- 2) Remove PBS completely and add 0.7 ml of 0.05% trypsin-EDTA solution, and incubate at 37°C for 10 min.
- 3) While incubating, remove a 6-well plate of feeder cells (mitomycin C-treated MEFs). Aspirate the medium and add 2 ml of mouse iPSC medium to each well.
- 4) Remove the plate containing mouse iPS cells from the incubator and swirl to dislodge the cells from the bottom of the plate.
- 5) Add 2 ml of mouse iPSC medium, and suspend the cells by pipetting up and down to single cell suspension.
- 6) Transfer the cell suspension to a 15 ml conical tube and spin the cells at 200 g for 5 min.
- 7) Add 2 ml of iPSC medium to the plate and suspend the cells by pipetting up and down to single cell suspension.
- 8) Distribute ~0.2 ml of the mouse iPS cell suspension to each well of the 6-well plate. Right after plating iPS cells, gently swirl the plate back-and-forth and side-to-side and incubate at 37°C with 5% CO<sub>2</sub> until the cells reach 80% confluency.
- 9) The mouse iPSC media must be changed every day and mouse iPS cells subcultured ~1:10 every 2-3 days. Track the passage number of the iPS cells

### 3. Freezing mouse iPSC cells

- 1) Grow cells to the exponential phase in a 6-well plate.
- 2) Aspirate the medium, and wash the cells twice with 2 ml of PBS.
- 3) Aspirate the medium, and wash the cells twice with 2 ml of PBS.
- 4) Add 0.7 ml 0.05% trypsin-EDTA and incubated for 10 min at 37°C.
- 5) Add 2 ml of mouse iPSC medium, and suspend the cells by pipetting up and down to single cell suspension.
- 6) Transfer the cell suspension to a 15 ml conical tube, count the number of cells and spin the cells at 200 g for 5 min.
- 7) Discard the supernatant, and resuspend the cells with mouse iPSC medium to the concentration of  $1 \times 10^6$  cells per ml.
- 8) Add equal volume of 2x freezing medium and aliquot it at 0.5 ml per vial.
- 9) Put the vials in a cell-freezing container, and store them at -80°C overnight.
- 10) Transfer the vials to a liquid nitrogen tank for long-term storage.

## D. Validation of PhiC31 Mouse iPS cells

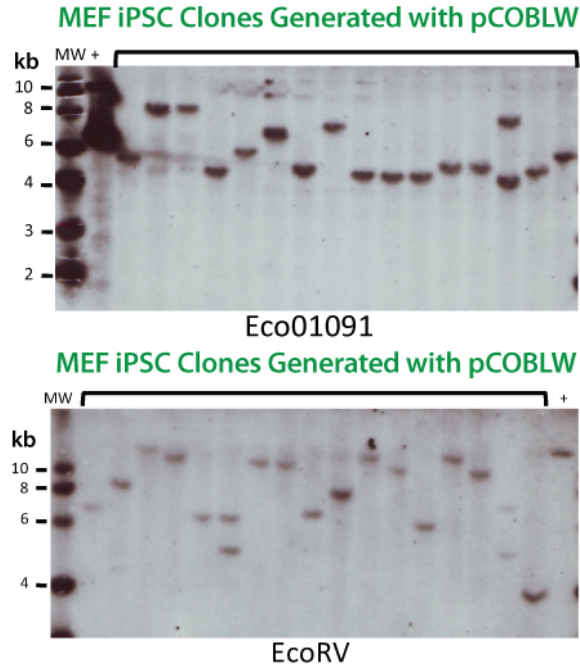


Stem cell markers for Oct4, Sox2, SSEA-1 and Nanog were determined by immunocytochemistry using primary antibodies for SSEA1 (Millipore), Oct4 (Abcam), Sox2 (Abcam), and Nanog (Abcam) followed by Alexa Fluor fluorescent-labeled secondary antibodies (Invitrogen). Detection of Alkaline Phosphatase activity was performed using the AP Detection Kit (Millipore).

### Integration Analysis

A major advantage of using the phiC31 integrase system is that copy number control is in your hands. The majority of clones that undergo integration events have single insertions. To verify the copy numbers in your targeted cells, you can perform Southern blot analysis on their genomic DNA. Shown below is an example of mouse genomic integration analyses using separate restriction digests with Eco01091 and EcoRV with 10ug gDNA. The reason for two separate enzyme digests in to assess the copy number status. The probe used in these Southern blot analyses was for GFP present in the

pCOBLW iPSC integrase vector. The data below show the copy number status for 16 different iPSC lines and demonstrate that 14 out of 16 lines show single insertions (88%).



## IV. References

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For additional information or technical assistance, please call or email us at:

[tech@systembio.com](mailto:tech@systembio.com)

650-968-2200

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- The Product may not be resold, modified for resale, or used to manufacture commercial products without prior written consent of SBI.
- This Product should be used in accordance with the NIH guidelines developed for stem cell research.

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