phiC31 Integrase Vector System

Catalog#s FC200PA-1, FC500A-1 FC501A-1, FC550A-1, FC551A-1, FC600A-1

User Manual

Please see product certificate for proper storage conditions

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I. Introduction

A. Background of ϕC31 Integrase Technology

The ϕC31 integrase is a sequence-specific recombinase encoded within the genome of the bacteriophage ϕC31. The ϕC31 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 (Calos, 2006). In the presence of ϕC31 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. ϕC31 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.

The ϕC31 integrase system is an attractive and safer alternative to traditional viral-mediated transgene delivery in mammalian cells. The system has been widely utilized in gene therapy and regenerative medicine applications. To date, it has found use for *in vivo* and *ex vivo* applications, including correction of hemophilia A and B (Keravala *et al.* 2011, Chavez *et al.* 2012), Duchenne muscular dystrophy (Quenneville *et al.* 2007), and inherited skin disorders in transgenic mouse disease models(Ortiz-Urda *et al.* 2002). Due to the tractability of the ϕC31 integrase system, it is widely considered to be one of the leading technologies for gene delivery in mammalian cells.

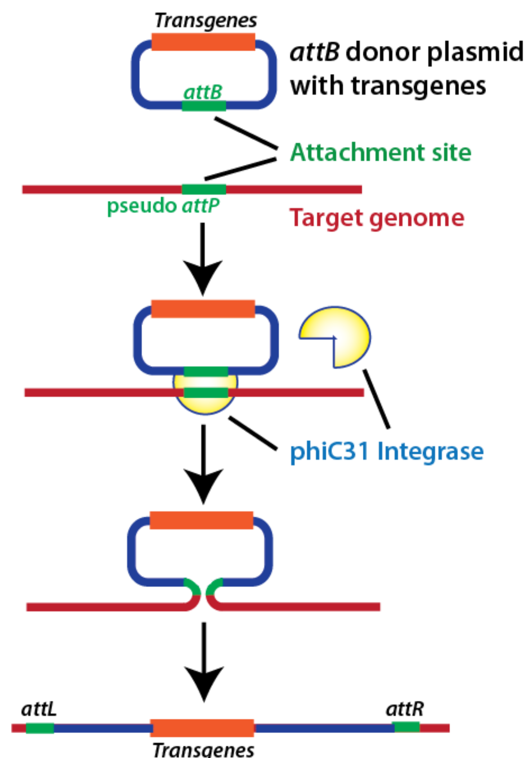


Fig. 1. Schematic of the ϕC31 Integrase mediated recombination of donor plasmid sequence into pseudo-*attP* sites in host genome

B. ϕC31 Donor Cloning Vector Maps & Details

We have generated a suite of *attB*-bearing donor cloning vectors as well as a positive control vector based on the ϕC31 Integrase system for cloning of any cDNA or microRNA insert of choice into each vector for insertion into target cells of interest. These cloning vectors offer the user maximum flexibility in the choice of the promoter to drive expression of the insert as well as fluorescent and antibiotic selection markers (Table 1).

Table 1. List of vectors for phiC31 Cloning System (#FC5xxA-1)

CAT. NO	DESCRIPTION	Amount of DNA	Promoter Driving GOI	Built-In Markers
FC500A-1	pFC-MCS-SV40-Neo	10 ug	Per user construct	Neomycin
FC501A-1	pFC-CMV-MCS-SV40-Neo	10 ug	CMV	Neomycin
FC520A-1	pFC-CMV-GFP-SV40-Neo positive control	10 ug	CMV	GFP/Neomycin
FC550A-1	pFC-Puro-T2A-RFP<=PGK/EF1=>MCS	10 ug	EF1a	RFP/Puromycin
FC551A-1	pFC-Puro-T2A-GFP<=EF1/PGK=>MCS	10 ug	PGK	GFP/Puromycin

When co-transfected with the plasmid expressing the ϕC31 integrase (catalog # FC200PA-1), the cloning vector will be integrated into pseudo-*attP* sites in the host genome through an *attB x attP* recombination. By using an excess of the ϕC31 integrase vector relative to the cloning vector (e.g. 50:1), random integration of the donor vector is minimized and most integration events will have been mediated by ϕC31 integrase. Cells that have the donor plasmid successfully integrated can be selected using puromycin (e.g. FC550A-1, FC551A-1), neomycin (FC500A-1, FC501A-1, FC520A-1) or sorted by flow cytometry for GFP or RFP positive cells (FC550A-1, FC551A-1) for downstream applications. Plasmids can also be directly injected into animals for *in vivo* transgene studies. The vector maps for each of the four donor cloning and ϕC31 integrase plasmids is shown below (Fig. 2)

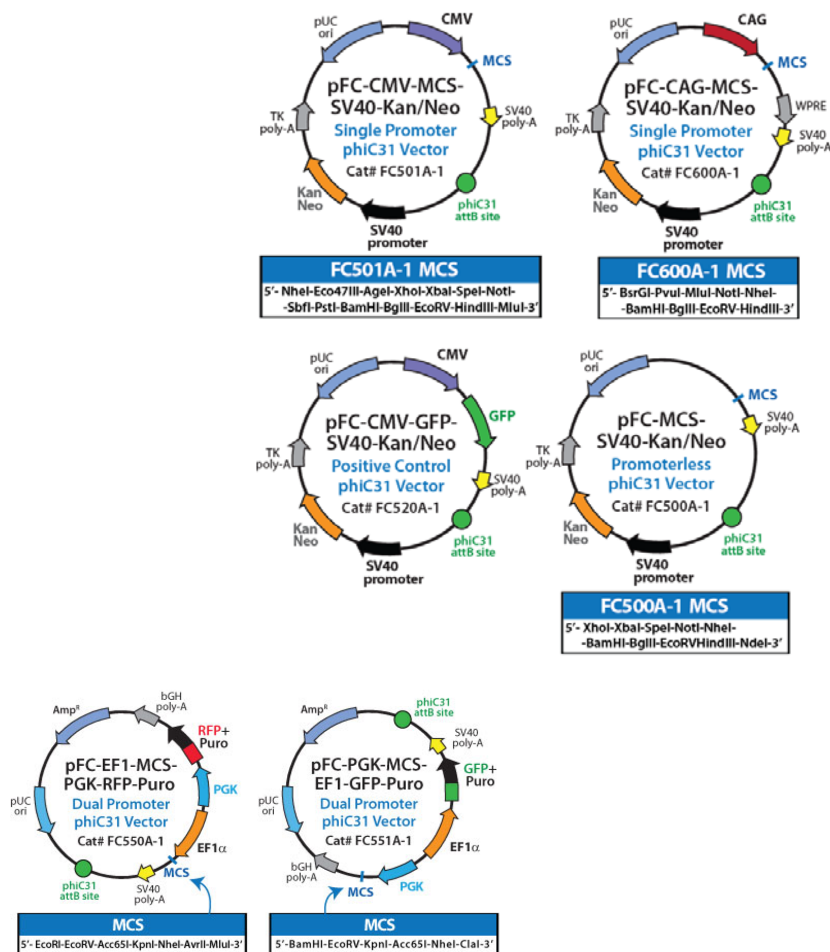


Fig. 2. Schematic of the available vectors in the ϕC31 Integrase Cloning System

C. Additional Materials Required

a) LB Agar and Broth containing 50 µg/ml kanamycin or ampicillin

b) Any high-transformation efficiency RecA- and EndA- *E.coli* competent cells

c) Dulbecco's Modified Eagle's Medium (D-MEM) high glucose with sodium pyruvate and glutamine (Invitrogen, Cat. # 11995073)

d) Lipofectamine 2000 transfection reagent (Invitrogen, Cat. # 11668019)

e) Qiagen EndoFree Plasmid Maxi Kit (Qiagen, Cat. # 12362)

f) Qiagen QiaQuick PCR Purification Kit (Qiagen, Cat. # 28104)

g) Qiagen DNeasy Blood and Tissue Kit (Qiagen, Cat. # 69504)

h) Fetal Bovine Serum (Invitrogen, Cat. # 16000036)

i) Penicillin/Streptomycin (Invitrogen, Cat. # 15070063)

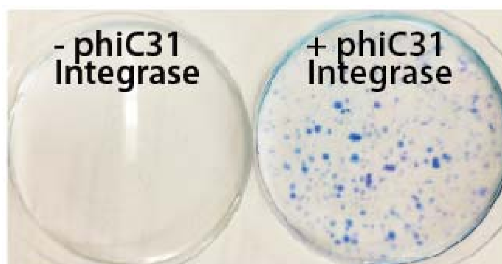
j) Trypsin-EDTA (Sigma, Cat. # T3924)

k) 6-well Tissue Culture Plates and Related Tissue Culture Supplies

l) Other specific media and additives specific for cell type of interest

m) **Optional** - For difficult-to-transfect cells, the use of an electroporation system (e.g. Lonza's NucleoFector or Invitrogen's Neon system) is highly recommended

II. Validation Data for ϕC31 Integrase Cloning System

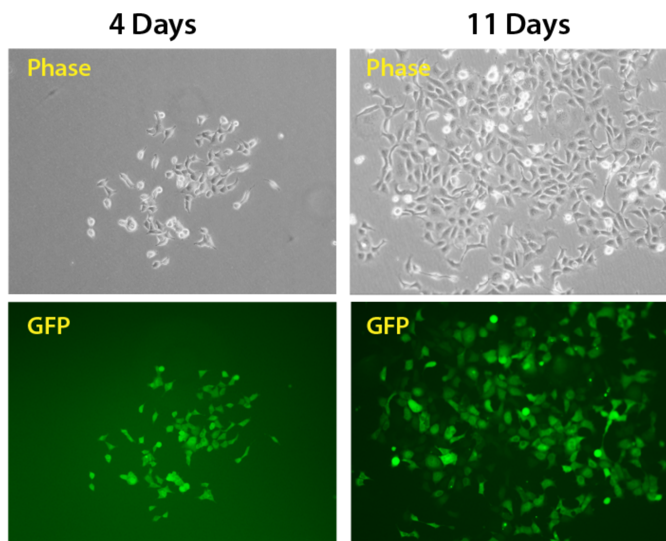


A. Colony Counting Assay

HEK293T cells seeded onto 6-well plates were transfected the next day in triplicate according to the manufacturer's instructions with Lipofectamine 2000 (Invitrogen) and 40 ng pFC-PGK-MCS-EF1-GFP-Puro Dual Promoter ϕC31 Donor Vector (Cat# FC551A-1) and either 1.96 µg carrier plasmid DNA or 1.96 µg ϕC31 integrase expression plasmid. The cells were trypsinized after 48 hours, resuspended in 1 ml of media, and split 1:20 onto 10 cm plates containing 10 ml of media. After 24 hours, puromycin 0.5 µg/ml was added to the medium to begin selection. After 11 days, the cells were fixed and stained with a solution of 50% methanol plus 1% methylene blue. The plates were washed twice with 1x PBS and allowed to air dry. The number of visible colonies was imaged and colony number counts were assessed. There were no visible colonies on the minus ϕC31 integrase negative control and >300 colonies on the ϕC31 integrase plate (colony assay data shown above).

B. ϕC31 Donor Cloning Vector Integration by GFP and Puromycin Marker Expression

Approximately 400,000 HEK293T cells were co-transfected with 1.96 µg ϕC31 Integrase expression vector with 40 ng pFC- PGK-MCS-EF1-GFP-Puro Dual Promoter ϕC31 Donor Vector (Cat# FC551A-1). The cells were split 1:10 24 hours post-transfection. Puromycin selection at 1 µg/ml was initiated after another 24 hours with continuous selection for 4 days. Cells were imaged at 4 and 11 days. Representative GFP fluorescence and phase contrast fields are shown below.



III. Protocol for Transfection and Generation of Stable Cell Lines using ϕC31 Cloning and Integrase Vectors

A. General Comments

We recommend propagation of the plasmids prior to starting the experiments. The plasmids can be transformed using standard conditions suitable in any high-quality RecA- and EndA- *E.coli* competent cell.

For cells transformed with Catalog # FC550A-1 or FC551A-1 vectors, we suggest plating 50-200 µl of transformed cells on fresh ampicillin plates (50 µg/ml). For all other vectors, including the ϕC31 integrase vector, cells should be plated on kanamycin plates (50 µg/ml). Incubate the plates at 37°C overnight. Colonies picked from the transformation can be grown at 37°C overnight in ~200 ml of LB media containing either 50 µg/ml of ampicillin (#FC550/551) or kanamycin (all other vectors). After overnight growth, plasmid DNA can be harvested from culture using an endotoxin-free DNA plasmid maxiprep kit.

For confirmation of the plasmid, we recommend performing restriction digestion analysis or direct sequencing to confirm integrity of the amplified plasmids.

B. Cloning of Inserts into ϕC31 Donor Vectors

For rapid and efficient cloning of any insert into donor vectors, we recommend SBI's Cold Fusion Cloning Kit as a ligase and restriction enzyme-free cloning method. More details can be found here: (http://www.systembio.com/molecular-tools/cold-fusion-cloning/overview)

For standard cloning strategies, please refer to the following protocol for more details:

1. Ligation of insert into vector

a) Dilute gel-purified, digested vector to 10 ng/µl
b) Set up 10 µl ligation reactions for each control and test samples as below:

Volume	Item
1.0 µl	ϕC31 empty donor vector
7.0 µl	DNA insert (~30-50 ng) or water control
1.0 µl	10X T4 DNA ligase buffer
1.0 µl	T4 DNA Ligase (40 U/µl)
10.0 µl	**Total Reaction Volume**

c) Incubate reactions at 25°C for 1-2 hours (sticky-end ligation) or O/N at 16°C (for blunt-end ligation)

2. Transform *E. coli* with the ligation product

Transform competent cells (with a transformation efficiency of at least $1x10^9$ colonies/µg pUC19) with the whole ligation reaction (10 µl) following the protocol provided with the competent cells. Plate the transformed bacteria on 50 µg/ml ampicillin or kanamycin agar plates depending on the vector(s) being ligated.

3. Identify clones with the correct insert

a) Depending on the ratio of colony numbers for the cDNA sample vs. the negative control sample, randomly pick 5 or more well-isolated colonies and grow each clone in 100 µl of LB Broth with 100 µg/ml ampicillin or kanamycin at 37°C for 2 hours with shaking.

b) Use 1 µl of each bacterial culture for screening DNA inserts by PCR and continue to grow the culture for another 4 hours. Store the culture at 4°C.

c) Prepare a PCR Master Mix with PCR primers flanking the insert:

1 rxn		10 rxn		Composition
0.5	µl	5	µl	PCR primer 1 (10 µM)
0.5	µl	5	µl	PCR primer 2 (10 µM)
0.5	µl	5	µl	50X dNTP mix (10 mM of each)
2.5	µl	25	µl	10X PCR Reaction Buffer
19.5	µl	195	µl	Nuclease-free water
0.5	µl	5	µl	Taq DNA polymerase (approx. 5 U/µl)
24.0	**µl**	**240**	**µl**	**Total volume**

d) Mix the master mix very well and aliquot 24 µl into each well of 96-well PCR plate or individual tubes.

e) Add 1 µl of each bacterial culture from step (b) into each well (or tube).

f) Proceed with PCR using the following program:

94°C, 4 min	1 cycle
94°C, 0.5 min, then 68°C, 1 min/1 kb*	25 cycles
68°C, 3 min	1 cycle

* Depending on the size of final PCR product, use a shorter or longer time.

g) Take 5 µl of the PCR reaction and run it on a 1.2% agarose/EtBr gel in 1X TAE buffer to identify clones with correct insert.

h) Grow a positive clone containing insert in an appropriate amount of LB-ampicillin or kanamycin broth, and purify the construct using an endotoxin-free plasmid purification kit. Sequence verification of the insert is optional.

C. Stable Cell Line Generation

Notes:

1) Depending on the cell type being transfected, please choose a transfection protocol which results in maximal transfection efficiencies. For adherent cell lines such as HEK293T, passive transfection methods using cationic lipid-based methods (e.g. Lipofectamine 2000, FuGene HD) work very well in transfection of donor and integrase vectors. For other types of cells such as primary, stem, or suspension cells, we suggest transfection using electroporation methods (NucleoFection or Neon) for optimal results.

2) The plasmids should be mixed well in minimal serum/no antibiotic media + cationic lipid transfection reagent or electroporation buffer to maximize efficiency of delivery.

3) For selection of target cells, we strongly recommend testing different concentrations of Puromycin or Neomycin (G418) on untransfected cells to determine the optimal concentration of selection agent – which is kills ~90-100% of cells within 48-72 hours after drug administration.

Day 0

1. In order to limit the number of colonies resulting from random integration of the donor plasmid, it is recommend to use the ϕC31 integrase plasmid (cat. #FC200PA-1) in a 50:1 ratio (w/w) over the donor. For example, for transfection of HEK293T cells using Lipofectamine 2000, a 50:1 ratio of ϕC31 integrase: donor plasmid successfully integrated the donor vector in a single-copy fashion with a very low incidence of random integration.

2. Seed ~400,000 cells in a 6-well plate in suitable growth medium optimal for the cell type of interest and grow overnight at 37°C. Please include well(s) for a positive and negative control vectors, if desired.

Day 1

3. When cells are ~60-80% confluent, co-transfect target cells with ϕC31 integrase and donor vector at at a 50:1 ratio (w/w) using the transfection reagent of your choice.

Day 2

4. Split co-transfected cells at 1/10 and 1/20 ratios in 10cm plates, and replace transfection medium with complete growth medium including antibiotics.

Day 3

5. Add recommended amount of puromycin or neomycin (G418) suitable for optimal selection of the transfected cells.

Days 4-14

6. Untransfected cells will begin to die and colonies will begin to form from cells that were successfully transfected. When the colonies are large enough, transfer them to a single well of a 6-well plate. Keep cells under antibiotic selection at all times.

7. When colonies become confluent, isolate cells and split cells for seeding into a single well of a 6-well plate for the optional Plasmid Rescue assay (see Section D) and 10 cm plate/T-75 flask for continued propagation. Remaining cells can be frozen down for archival purposes.

8. Test the cell lines for expression of your transgene of interest by qPCR, western blot, immunofluorescence, or any specific assays designed to give a readout of transgene expression.

9. Select the cell line(s) that give the desired level of transgene expression for further characterization. Expression may vary from line to line depending on chromatin structure surrounding the integration site.

D. Verification of Insert Integration at Specific Genomic Loci using the Plasmid Rescue Assay

In order to ascertain the precise genomic location(s) of donor vector integration, a Plasmid Rescue Assay can be performed on genomic DNA isolated from cells which have been transfected with the donor plasmid and selected. The general idea for this assay is to determine the sequence of the genomic DNA flanking the integrated donor vector by using a series of blunt-cutting restriction enzymes that cut outside the donor vector, induce intramolecular ligation of the cut fragments, and sequencing the regions away from the insert with the provided *attB* sequencing primers. The results of the sequencing can be mapped to the genome by BLAT analysis (http://genome.ucsc.edu/cgi/bin/hgBlat?command=start) which confirms integration of the donor vector at a specified locus.

Detailed Protocol for Plasmid Rescue Assay

1. Isolate cells from one well of a 6-well plate which were previously plated for this assay and isolate genomic DNA from the cells using a suitable genomic DNA isolation kit. 1-5 µg of genomic DNA will be sufficient for this assay.

2. Digest between 1-5 µg of genomic DNA from each sample using 2-5 different blunt-cutting restriction enzymes that do not cut within the donor plasmid and have good activity (>50%) activity in the same reaction buffer.

3. Clean-up the restriction digest using a suitable column purification kit and elute the digested DNA in 20 µl of elution buffer.

4. Set up the following ligation reaction:

Volume	Item
10.0 µl	Digested DNA
40.0 µl	10X T4 Ligase Buffer
2.0 µl	T4 DNA Ligase (40 U/µl)
348.0 µl	ddH20
400.0 µl	**Total Reaction Volume**

5. Incubate the ligation reaction O/N at 16°C overnight

Performing ligations in a large volume minimizes intermolecular and favors intramolecular ligation events.

6. Purify the ligation reactions in a suitable purification column and elute in 10 µl of elution buffer.

7. Transform bacteria with 5 µl of the purified DNA and plate cells onto either kanamycin or **ampicillin** (50 µg/ml) antibiotic selection plates, depending on the donor vector being tested

8. Select 2-4 colonies from the plates and inoculate 3-5 ml LB + antibiotic for overnight growth at 37°C.

9. Isolate the plasmid DNA from the cultures using a suitable plasmid DNA purification kit.

10. Sequence the plasmids with the following primers to obtain the genomic sequences flanking the ligated donor vector:

attBR2 5'-actaccgccacctcgac-3'
attBF2 5'-atgtaggtcacggtctcgaag-3'

11. Analyze the sequence data and map the results to the genome using BLAT analysis to identify the integration locus.

IV. References

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V. Technical Support

For more information about SBI products or to download manuals in PDF format, please visit our website:

http://www.systembio.com

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

tech@systembio.com

650-968-2200

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