PinPoint™ Targeted Integration System

Cat#s PINxxxA-1

User Manual

Store Kits at-20°C upon receipt

Contents

I. Introduction

A. Overview of the PinPoint™ Targeted Integration System

The PinPoint Targeted Integration system allows users to easily and efficiently create isogenic stable cell lines in mammalian and other cell types. Custom gene expression cassettes can be engineered into target genomes using the unique PinPoint integrase with site-specific control. This technology enables the generation of platform cell lines which can be used to routinely knock-in different transgenes and reporters at the same genetic locus in cells with the same genetic background. This level of targeting control allows for the study of phenotypic effects free from context and positional variations, which results in more accurate genotype to phenotype correlations. The following are some examples of when the PinPoint technology can be particularly useful:

- Gene function studies
- Disease modeling (e.g. cancer, differentiation)
- Gene therapy (e.g. derivation of patient-specific lines)

The PinPoint system relies on a two-step approach for engineering of target cells. The first step involves insertion of a plasmid bearing the PinPoint placement site via transfection into the target cell genome (Fig. 1). This can be done using two distinct approaches: i) the PinPoint-FC™ system that uses the well-characterized ϕC31 integrase system (Calos 2006, Karow *et al.* 2011) or ii) the PinPoint-HR™ system that uses either TALE-Nuclease or Cas9 genome engineering tools to induce a double-stranded DNA break in the genome and insertion of the PinPoint placement site by homology-directed recombination (HDR) in a site-specific manner (Hockemeyer *et al.* 2012).

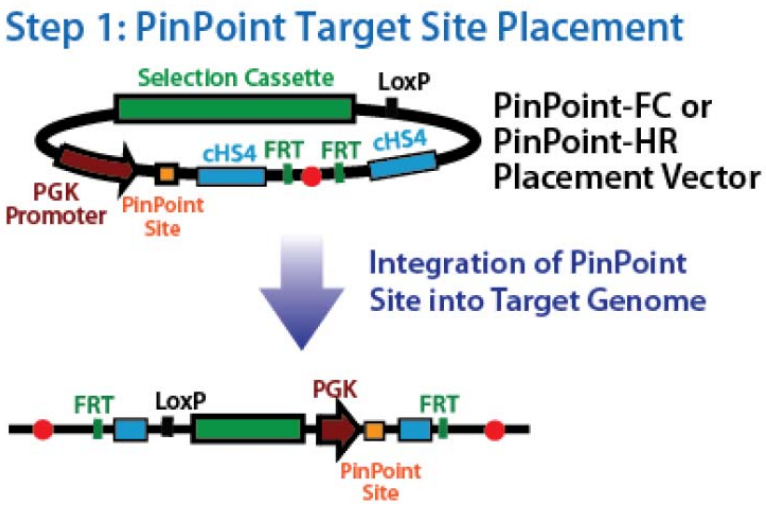

Fig. 1. Schematic Representation of Placement of PinPoint *attP* Vector into Target Genome

The second part of the PinPoint system relies on the introduction of a donor vector containing a desired gene cassette insert, which is integrated into the placed PinPoint site using a hyperspecific and efficient PinPoint integrase which only recognizes the placed PinPoint site (Fig. 2). The PinPoint integrase catalyzes the *attB* x *attP* reaction between the placed site (*attP)* and the *attB* site in the donor vector to insert the donor vector at the placed site each and every targeting event. Additional specificity of the system is conferred by the presence of a promoterless puromycin-resistance cassette in the donor vector, which uses a PGK promoter

introduced at the PinPoint site (by the placement vector in Step 1) to allow only those cells with a correctly-targeted donor plasmid to survive selection with puromycin.

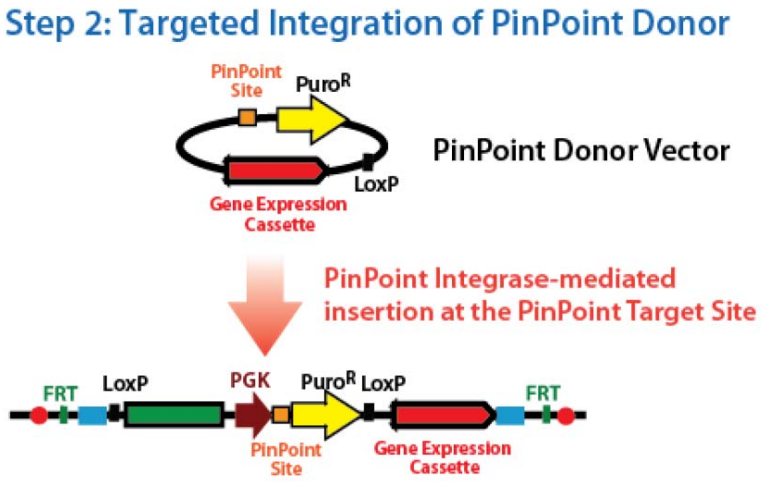

Fig. 2. Schematic Representation of Donor Vector Targeted to Placed PinPoint Site with PinPoint Integrase

Finally, the entire backbone (excluding the insert and its promoter) may be excised using the well-characterized *Cre/Lox*P reaction (Sauer *et al.* 1988) (Fig. 3), which allows for efficient excision of exogenous selection components, leaving only the promoter/insert combination (and a single *LoxP* site) in the genome.

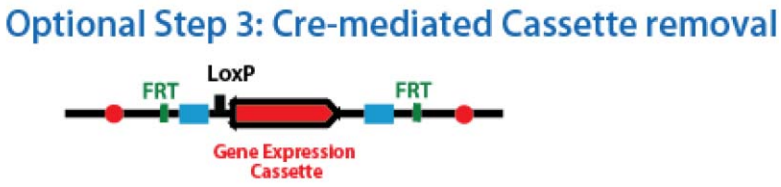

Fig. 3. Schematic Representation of Removal of Donor Vector Components using *Cre/LoxP* Reaction

As mentioned above, there are two different options for introducing the PinPoint site into a target genome, each with its own advantages. The PinPoint-FC system utilizes a ϕC31 integrase-based placement of the PinPoint site, while the PinPoint-HR system uses an HR-based approach for placement.

Choosing between the PinPoint-FC and PinPoint-HR methods to place the PinPoint site will depend on the application and preferences of the end-user. In general, the PinPoint-FC system is simpler, and is likely to be more efficient than HR-mediated approaches for most cell types being transfected. Use of PinPoint-FC is preferred when the end user wants to repeatedly target a single PinPoint site in the genome, but does not need sequence-specific control of the location being targeted. On the other hand, if precise control is required to achieve targeting of a specific locus (such as a 'safe harbor' site), or if the same locus needs to be targeted across multiple samples (e.g. isogenic lines from multiple patient-derived cell lines), then the PinPoint-HR system is the appropriate choice. This method requires use of a custom TALE nuclease pair or Cas9 guide RNA to induce a dsDNA break at a pre-determined location, along with an HR targeting vector to place the PinPoint site near the cleavage site. A table comparing both approaches is listed below:

	PinPoint-FC	PinPoint-HR
Ease of Protocol	Easy	More difficult
Efficiency of Placement	High (limited only by transfection efficiency)	Low (<1-10%)
Control of Placement	At pseudo-*attP* sites	At desired locus
Integration Efficiency	Single copy	Single copy

To save on hands-on time, we also offer pre-made PinPoint Platform Cell Lines with the PinPoint site already placed in the host genome, ready for retargeting with any donor vector of choice. This option bypasses the need for the initial placement step, and allows the end-user to quickly generate isogenic cell lines with the modifications of interest. Please **see Section V** for details.

B. List of Components in PinPoint Targeted Integration System

i) PinPoint-FC Vector Options

CAT. NO	DESCRIPTION	QTY
FC200PA-1	PhiC31 Integrase Expression Plasmid	10 µg
PIN200A-1	PinPoint Integrase Expression Plasmid	10 µg
PIN300A-1	PinPoint-FC *attP* Placement Vector	10 µg
PIN300A-KIT	PinPoint-FC System for Platform Cell Line Generation & Retargeting (includes PIN300A-1, FC200PA-1, PIN200A-1, PIN510A-1 & PIN600A-1)	1 kit

ii) PinPoint-HR Vector Options

CAT. NO	DESCRIPTION	QTY
PIN200A-1	PinPoint Integrase Expression Plasmid	10 µg
PIN400A-1	PinPoint-HR *attP* Placement Vector – Empty MCS for cloning HR Arms	10 µg
PIN400A-KIT	PinPoint-HR System for Platform Cell Line Generation & Retargeting (includes PIN400A-1, PIN200A-1, PIN510A-1 & PIN600A-1)	1 kit
PIN410A-1	PinPoint-HR *attP* Placement Vector	10 µg

	– for targeting the Human *AAVS1* Safe Harbor	
PIN410A-KIT	PinPoint-HR System for Platform Cell Line Generation & Retargeting of AAVS1 Safe Harbor Locus (includes PIN410A-1, GE601A-1, PIN200A-1, PIN510A-1, & PIN600A1)	1 kit

iii) PinPoint Retargeting Donor Vectors

CAT. NO	DESCRIPTION	QTY
PIN500A-1	EF1α PinPoint Donor Vector (pPP-EF1a-MCS-pA-attB-Puro-pA)	10 µg
PIN510A-1	CAG PinPoint Donor Vector (pPP-CAG-MCS-WPRE-pA-*attB*-Puro-pA)	10 µg
PIN520A-1	Empty PinPoint Donor Vector (pPP-MCS-WPRE-pA-*attB*-Puro-pA)	10 µg
PIN600A-1	PinPoint Donor Positive Control Vector: CAG->GFP+Luciferase (pPP-CAG-GFP-T2A-Luc-WPRE-pA-*attB*-Puro-pA)	10 µg
PIN610A-1	PinPoint Donor Positive Control Vector: CM->Luciferase (pPP-CMV-Luc-pA-*attB*-Puro-pA)	10 µg

iv) Other PinPoint-Related Vectors

CAT. NO	DESCRIPTION	QTY
CRE100A-1	Cre Recombinase Expression Plasmid (pCMV-CRE)	10 µg
GE601A-1	pZT-AAVS1-L1/R1 vectors (L+R TALEN constructs)	10 µg each

v) Pre-made Platform Cell Lines and Kits

CAT. NO	DESCRIPTION	QTY
PIN320A-1	PinPoint-FC HEK293T Platform Cell Line for Targeted Gene Insertion	>2x10^5 cells/vial
PIN320A-KIT	PinPoint-FC 293T Platform Kit for Targeted Gene Insertion (includes PIN320A-1, PIN200A-1, PIN510A-1 & PIN600A-1)	1 kit
PIN340iPS-1	PinPoint-FC Murine iPSC Platform Cell Line for Targeted Gene	>2x10^5 cells/vial

	Insertion	
PIN340iPS-KIT	PinPoint-FC Murine iPSC Platform Kit for Targeted Gene Insertion (includes PIN340iPS-1, PIN200A-1, PIN510A-1 & PIN600A-1)	1 kit

C. Additional Materials Required

a) LB Agar and Broth containing 50 µg/ml kanamycin
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 DNeasy Blood and Tissue Kit (Qiagen, Cat. # 69504)
g) iProof High-Fidelity DNA Polymerase (BioRad, Cat. # 172-5301)
h) Fetal Bovine Serum (Invitrogen, Cat. # 16000036)
i) Penicillin/Streptomycin (Invitrogen, Cat. # 15070063)
j) Trypsin-EDTA (Sigma, Cat. # T3924)
h) 6-well and 100mm Tissue Culture Plates and Related Tissue Culture Supplies
i) Other specific media and additives specific for cell type of interest
j) **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. Generation of PinPoint Platform Cell Lines with the PinPoint-FC™ System

A. Background for the PinPoint-FC System

The PinPoint-FC system uses the specificity of the ϕC31 integrase to place the PinPoint site in the genome of target cells. ϕC31 is a sequence-specific recombinase encoded within the genome of the bacteriophage ϕC31. 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, the *attB* containing PinPoint placement plasmid can be unidirectionally integrated into a target genome through recombination at genomic sites with sequence similarity to the native bacteriophage *attP* site, termed *pseudo-attP* sites.

B. Validation Data for PinPoint-FC System

In order to validate the PinPoint site placement, we have shown in HEK293T cells that upon co-transfection of ϕC31 integrase expression plasmid (Cat# FC200PA-1) and the PinPoint-FC *attP* placement vector (Cat# PIN300A-1), we can obtain G418-resistant colonies within 2 weeks of transfection, indicating functionality of the initial targeting (Fig. 4).

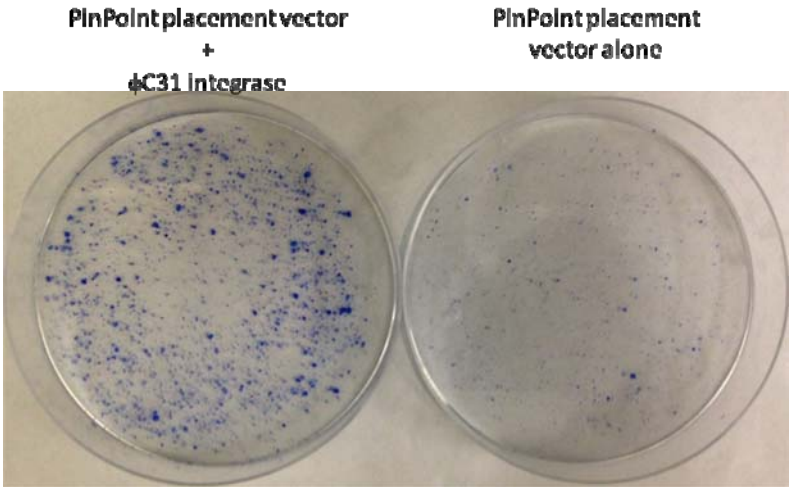

Fig. 4. Methylene Blue Stained G418-Resistant HEK293T Colonies Obtained via Co-Transfection of ϕC31 Integrase expression and PinPoint placement vectors

C. Vector Map Details for PinPoint-FC System

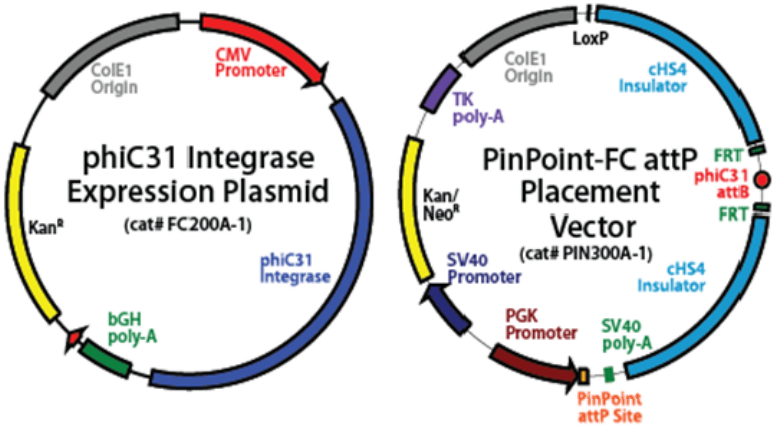

Fig. 5. Vector Maps for ϕC31 Integrase Expression and PinPoint *attP* Placement Plasmids

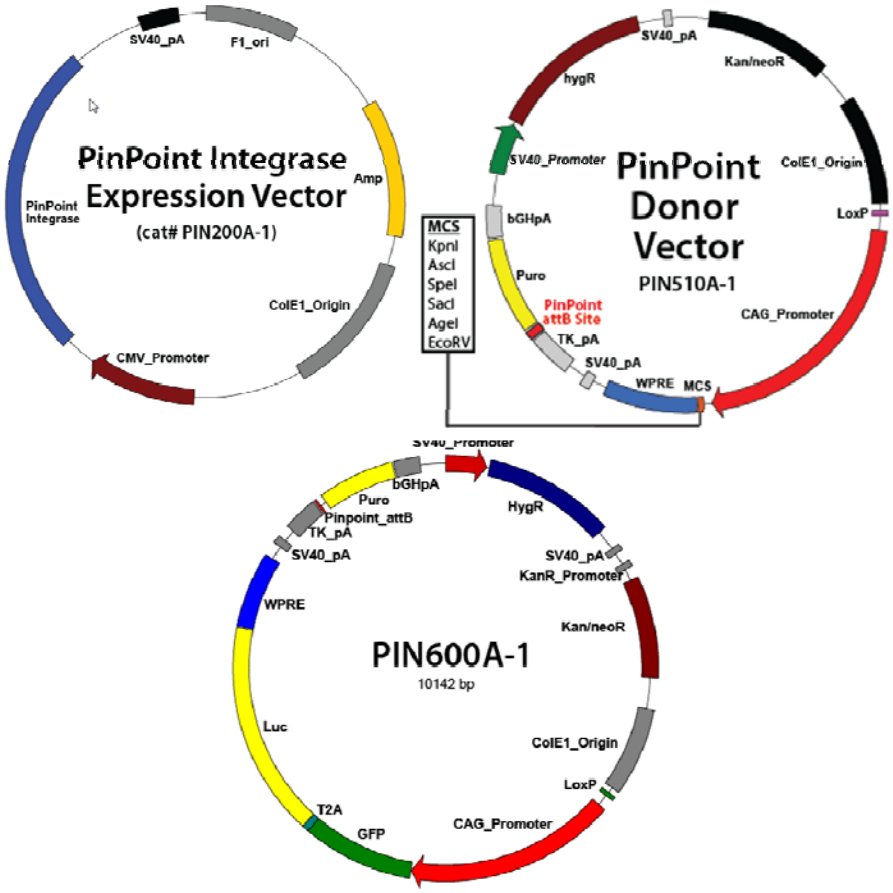

Fig. 6. Vector Maps for PinPoint Integrase and Donor Vectors (PIN200A-1, PIN510A-1, PIN600A-1)

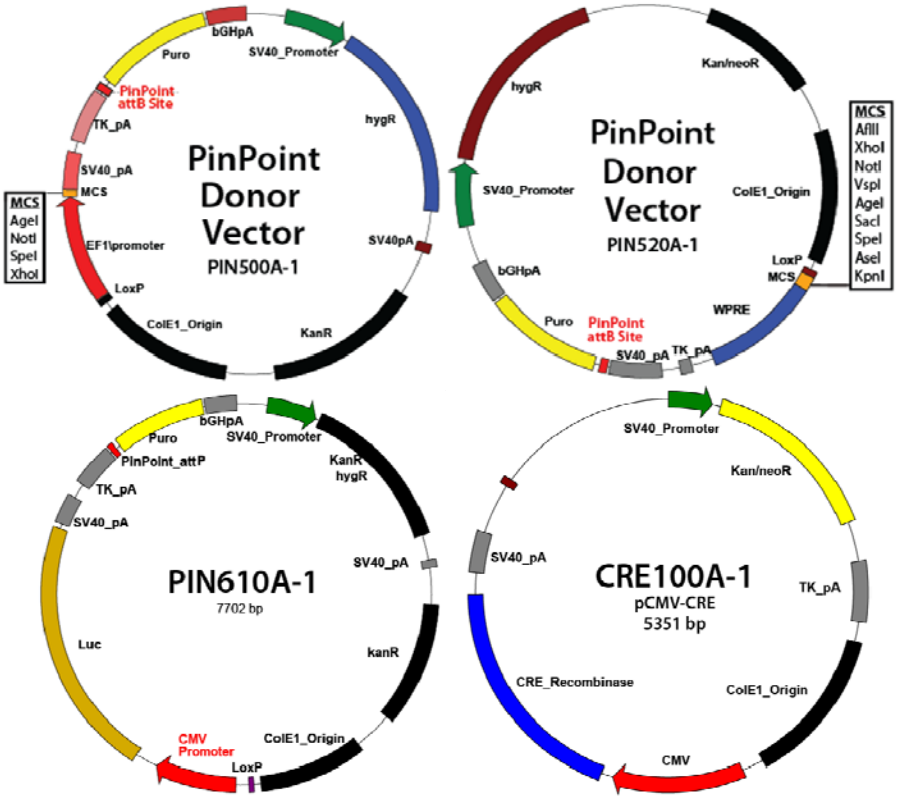

Fig. 7. Vector Maps for Additional Plasmids for use with PinPoint-FC System (PIN500A-1, PIN520A-1, PIN610A-1, CRE100A-1)

D. Overview and Protocol for PinPoint-FC System

The initial step is to create PinPoint *attP* containing platform cell lines. This is done through the co-transfection of ϕC31 integrase plasmid (Cat# FC200PA-1) with the PinPoint-FC *attP* placement plasmid (Cat# PIN300A-1) in the target cell line. Transfected cell lines will be selected as G418 (Neomycin) resistant colonies and picked for expansion as platform cell lines for subsequent retargeting using the PinPoint integrase and donor vectors (**see Section IV**).

The retargeting step is the final step of the process, where a donor vector bearing an *attB* site and transgene of interest will target the placed PinPoint *attP* site. Successful retargeting results in the puromycin resistance gene fragment in the donor vector to be introduced under the control of the PGK promoter in the PinPoint *attP* plasmid, resulting in puromycin-resistant colonies which can be clonally expanded and characterized for relevant phenotype(s) of interest.

Notes:

1) Plasmids should be transformed and propagated in high-quality competent cells (>1x10^9 CFUs/µg) prior to use according to the manufacturer's recommended protocols. Plasmids can be grown in LB+Kanamycin (50ug/ml). For extraction of plasmid DNA from maxipreps, we recommend the use of endotoxin-free columns and buffers for optimal results.

2) 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.

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

4) For selection of target cells, we strongly recommend testing different concentrations of 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.

5) In order to limit the number of colonies resulting from random integration of the PinPoint *attP* placement plasmid, it is recommend to use the ϕC31 integrase plasmid at a 50:1 ratio (w/w) over the PinPoint *attP* plasmid. For example, for transfection of HEK293T cells using Lipofectamine 2000, a 50:1 ratio of ϕC31 integrase to PinPoint plasmid (2 µg to 40ng) successfully integrated the PinPoint plasmid in a single-copy fashion with a very low incidence of random integration.

Day 0

1. 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 positive and negative control vectors, if desired.

Day 1

2. When cells are ~60-80% confluent, co-transfect target cells with ϕC31 integrase and PinPoint *attP* placement plasmid at a 50:1 molar ratio (e.g. 2 µg of ϕC31 integrase and 40ng of PinPoint plasmid) using the transfection reagent of your choice.

Day 2

3. Split co-transfected cells at 1/10 and 1/20 ratios in 100mm plates, and replace transfection medium with complete growth medium including antibiotics.

Day 3

4. Add recommended amount of neomycin (G418) suitable for optimal selection of the transfected cells (400 – 1000 µg/ml is a suggested range)

Days 4-14

5. Untransfected cells will begin to die and colonies will begin to emerge from cells that were successfully transfected and showing G418 resistance. 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.

6. When the clones become confluent, split cells for seeding into a single well of a 6-well plate for the optional Plasmid Rescue assay (see below) and 100mm plate/T-75 flask for continued propagation. Remaining cells can be frozen down for archival purposes.

E. Details of 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 kanamycin (50 µg/ml) antibiotic selection plates

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.

III. Generation of PinPoint Platform Cell Lines using the PinPoint-HR™ System

A. Background for the PinPoint-HR System

Recent advances in genome engineering tools, especially Transcriptional Activator-Like Nuclease (TALE-Nuclease) (Miller *et al.* 2011, Cermak *et al.* 2011) and Cas9/CRISPR systems (Jinek *et al.* 2012, Mali *et al.* 2013), have made notable impact on approaches for controlled, precise modification of eukaryotic genomes. While there are notable differences between the two major systems with respect to how they work, the end results are very similar in that both TALE-Nuclease and Cas9/CRISPR systems generate targeted dsDNA breaks which can be utilized for homology-directed recombination using a suitable donor template. In this application, the donor vector must contain homologous sequences at the 5' and 3' end of the DNA sequence being targeted by TALE-Nuclease or Cas9, with the internal sequence being an expression cassette containing the PinPoint site, which can be subsequently retargeted using PinPoint donor vectors (**see Section IV**). This approach, while less efficient than PinPoint-FC based placement and more time-consuming (e.g. screening for HR-positive cells, etc.), provides the ultimate in placement control to introduce the PinPoint site into any defined locus of a target genome.

B. Vector Map Details for PinPoint-HR System

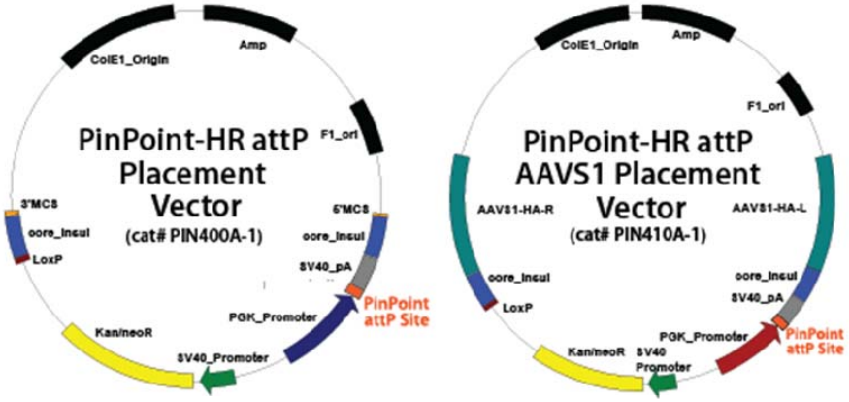

Fig. 8. Vector Maps for *attP* Placement Vectors – HR MCS and Human *AAVS1* Safe Harbor

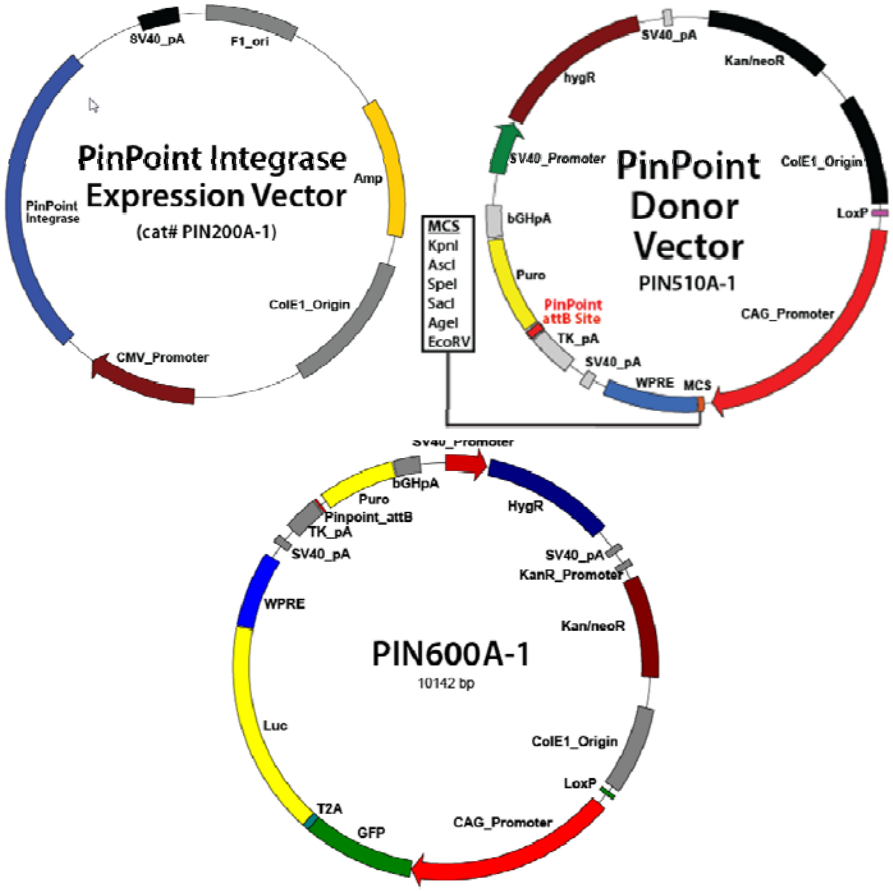

Fig. 9. Vector Maps for PinPoint Integrase and Donor Vectors (PIN200A-1, PIN510A-1, PIN600A-1)

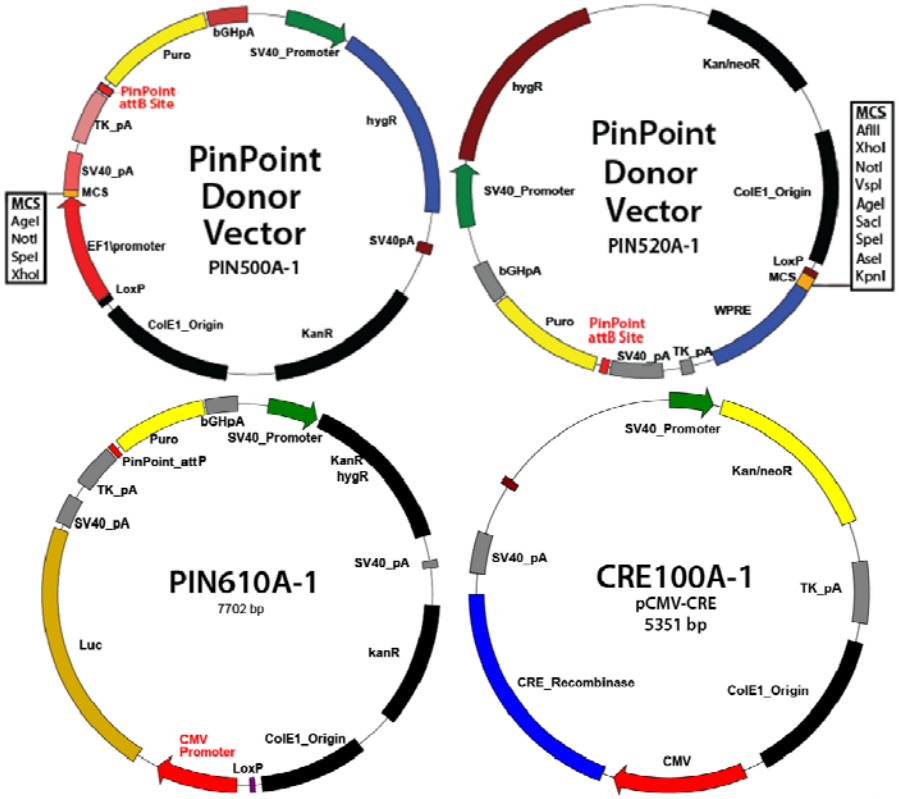

Fig. 10. Vector Maps for Additional Plasmids for use with PinPoint-HR System (PIN500A-1, PIN520A-1, PIN610A-1, CRE100A-1)

C. Cloning of Homology Arms into PinPoint-HR *attP* Placement Vector

Since the PinPoint-HR *attP* placement vector relies on HR for guiding the vector to the desired target site in the genome to place the PinPoint site, the end-user must design suitable homology arms 5' and 3' of the genomic site being targeted by either TALE-Nuclease or Cas9 guide RNA. For "knock-in" of the PinPoint site, we suggest anywhere from 0.5kb-1kb of homologous DNA sequence 5' and 3' of the dsDNA break site induced by TALE-Nuclease or Cas9 guide RNA. An example diagram of the HR arm design for Cas9-mediated HR is shown below (Fig. 11).

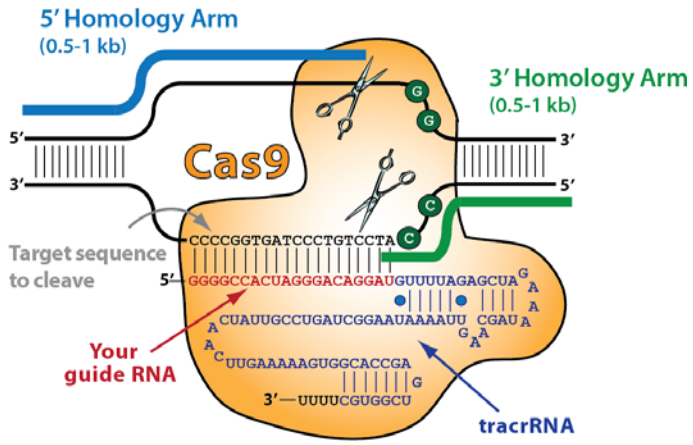

Figure 11. Schematic Diagram of Cas9-mediated Homologous Recombination (HR) Process Illustrating Homology Arm Design

For fast and efficient cloning of homology arms into the vector, we suggest the use of restriction enzyme, ligase-free methods such as SBI's Cold Fusion Kit. Cloning both of the arms into the placement vector will require sequential cloning reactions, one for each arm. Please refer to the user manual for the Cold Fusion Kit for details on the cloning process (http://www.systembio.com/molecular-tools/cold-fusion-cloning/literature)

For standard cloning strategies, please refer to the following protocol for more details. Since there are two homology arms that need to be cloned, the cloning will need to be repeated for each arm. The

1. PCR of homology arms

a) PCR amplify homology arms using proofreading PCR enzymes (e.g. Phusion) with primers containing compatible restriction sites to 5' MCS (for 5' homology arm) and 3' MCS (for 3' homology arm).
b) Run out PCR products on 1.5-2% agarose gel and extract correct size bands using a suitable gel extraction kit

2. Ligation of insert into vector

a) Digest the targeting vector using appropriate restriction enzymes for the homology arm being cloned and gel-purify the digestion product
b) Dilute gel-purified, digested vector to 10 ng/µl
c) Set up 10 µl ligation reactions for each control and test samples as below:

Volume	Item
1.0 µl	PinPoint *attP* Placement Vector

7.0 µl	5' or 3' homology arm PCR product (~70 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**

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

3. 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 kanamycin agar plates

4. Identify clones with the correct insert

a) Depending on the ratio of colony numbers for the test sample vs. the negative control sample, randomly pick 2 or 3 colonies and grow each clone in 100 µl of LB Broth with 100 µg/ml kanamycin at 37°C for 2 hours with shaking.

b) Use 1 µl of each bacterial culture for screening homology arm 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 1.5-2% 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-kanamycin broth, and purify the construct using an endotoxin-free plasmid purification kit. Sequence verification of the insert is optional.

i) Repeat the entire process with remaining homology arm to build the final placement construct

D. Overview and Protocol for PinPoint-HR System

The initial step is to create PinPoint *attP* containing cell line using a combination of TALE-Nuclease pairs or Cas9 along with a HR vector containing the PinPoint site to target a specific DNA locus of interest. We suggest building custom TALE-Nuclease Pairs using SBI's EZ-TAL™ kit (http://www.systembio.com/tale-effector-technology), or alternatively, use our Cas9 SmartNuclease Kit (http://www.systembio.com/genome-engineering-cas9-crispr-smartnuclease) for cloning in guide RNA sequences into the all-in-one Cas9 vector to target any site in the genome.

Once the method of inducing dsDNA breaks is determined, the user will co-transfect the TALE-Nuclease plasmid pair or Cas9-guide RNA vector with a suitable HR vector containing the PinPoint *attP* site and G418 (Neomycin) marker. After transfection, successfully transfected cells may be selected using G418, and resulting G418-resistant colonies can be picked for expansion to create platform cell lines. These cell lines can be retargeted using the PinPoint integrase and donor vectors (**see Section IV**).

The retargeting step is the final step of the process, where a donor vector bearing an *attB* site and transgene of interest will target the placed PinPoint *attP* site. Successful retargeting results in the puromycin resistance gene fragment in the donor vector to be introduced under the control of the PGK promoter in the PinPoint-HR *attP* plasmid, resulting in puromycin-resistant colonies which can be clonally expanded and characterized for relevant phenotype(s) of interest.

We provide two HR-based plasmids containing the PinPoint *attP* site for use with TALE-Nuclease or Cas9 systems. The first vector is a complete HR vector (Cat# PIN410A-1) with 5' and 3' arms homologous to a 3' intronic sequence for the well-characterized human *AAVS1* (*PPP1R12C*) locus, which can be placed in the genome and subsequently retargeted by PinPoint integrase and donor vectors. Validated TALE-Nuclease expression plasmids compatible with Cat# PIN410A-1 for inducing a dsDNA break at the *AAVS1* site are also available (Cat #GE601A-1). The second vector (Cat # PIN400A-1) is a cloning vector that allows the user to clone in homologous sequences to any target sequence of interest, which allows for highly precise placement of the PinPoint site anywhere in the genome for subsequent retargeting. The user will need to provide their own TALE-Nuclease Pair or Cas9 guide RNA to target the specific DNA sequence of interest.

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 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

4) 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 positive and negative control vectors, if desired.

Day 1

5) When cells are ~60-80% confluent, co-transfect target cells with plasmid(s) encoding for TALE-Nuclease pairs (provided in the kit) or Cas9 gRNA for targeting human *AAVS1* or your gene of interest at a 1:1 ratio (e.g. 0.5 µg of Cas9/0.25 µg each of TALE-Nuclease and 0.5 µg of HDR plasmid) using the transfection reagent of your choice.

Day 2

6) Change out transfection media to complete growth media and incubate cells. Wait for at least 48-72 hours for HR recombination to occur

Day 4/5

7) Split co-transfected cells 1/10 into 100mm plates and allow one day for plating of the cells

Day 6

8) Add recommended amount of neomycin (G418) suitable for optimal selection of the transfected cells (400 – 1000 µg/ml)

Days 7-17

9) Untransfected cells will begin to die and colonies will begin to emerge from cells that were successfully transfected and showing G418 resistance. 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.
When the clones become confluent, split cells for seeding into 100mm plate/T-75 flask for continued propagation. Remaining cells can be frozen down for archival purposes.

IV. Targeting of Placed PinPoint Site with PinPoint Integrase and Donor Vectors

A. Overview of Targeting of the Placed PinPoint Site

Once platform cell lines containing the PinPoint site have been developed using either the PinPoint-FC or PinPoint-HR methods (covered in Sections II and III), one can target the PinPoint site using the PinPoint integrase and a donor vector of choice (see Sections II.C or III.C for donor vector details). This transfection-based reaction is very efficient and limited only by the transfection efficiency of the cell line being targeted. Co-transfection of the PinPoint integrase and a donor vector containing the insert sequence into a platform cell line will result in a specific recombination reaction between the placed PinPoint *attP* site and the *attB* site in the donor plasmid. This results in integration of the entire donor plasmid into (and only into) the PinPoint site. As a result of this reaction, correctly targeted donor vectors will have their promoterless puromycin-resistance gene now under control of the mouse PGK promoter present in the PinPoint placement vector, and will be resistant to puromycin when subjected to selection. Platform cell lines can be engineered to express different genes of interest at the exact same location for precise determination of underlying phenotypes, with very high specificity as the PinPoint integrase recognizes only its own *attP* site. This is a critical feature that distinguishes the PinPoint system from other targeted gene placement systems, which rely on other types of recombinases (e.g. R4) that often recognize pseudo-sites in the genome.

B. Validation Data for Targeting of Placed PinPoint site by Donor Vectors

After initial generation of the platform cell line with the placed PinPoint *attP* site, we have co-transfected the same HEK293T cells with a PinPoint positive control donor vector (Cat#PIN600A-1, CAG→GFP-T2A-Luciferase) bearing an *attB* site and the PinPoint integrase expression plasmid (Cat# PIN200A-1), resulting in GFP positive and puromycin-resistant colonies (Fig. 5). Verification of proper insertion of the donor vector into the placed PinPoint site was confirmed by performing junction PCR with primer pairs that span portions of both PGK promoter (in the PinPoint *attP* placement vector) and puromycin resistance gene fragment (in the donor vector) resulting in a defined PCR amplicon (Fig. 6).

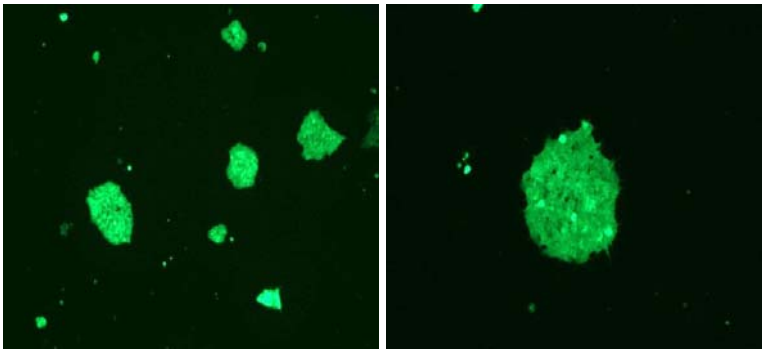

Fig. 12. GFP positive and Puromycin-resistant Colonies after Targeting with PIN600A-1 Donor Vector

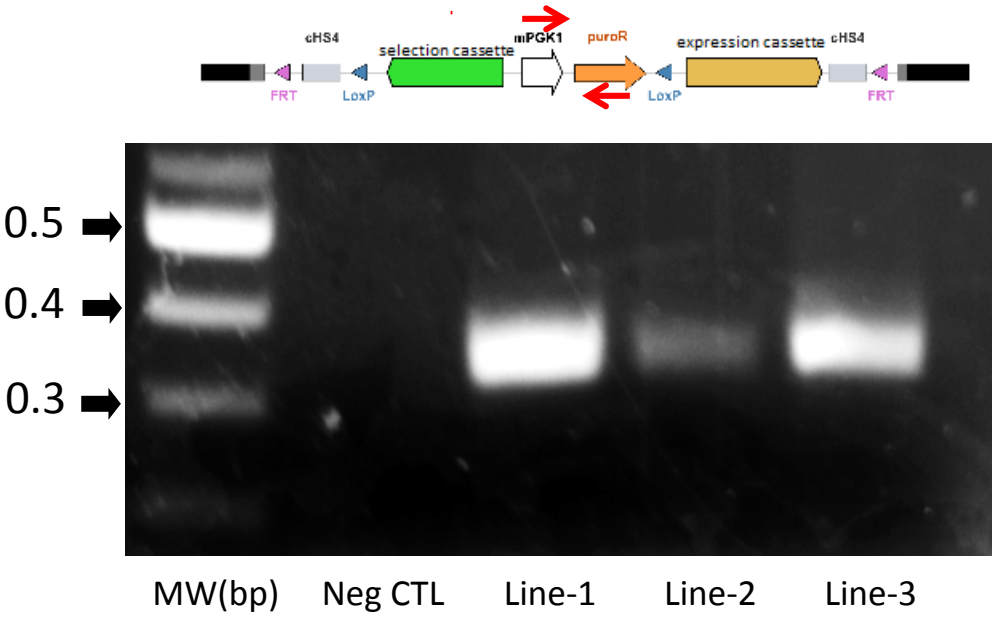

Fig. 13. Junction PCR analysis verified correct targeting of donor vector to the placed PinPoint *attP* site in HEK293T cells

C. Cloning of Inserts into PinPoint 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

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

Volume	Item
1.0 µl	PinPoint 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**

g) 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

a) 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 kanamycin agar plates.

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 of 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-kanamycin broth, and purify the construct using an endotoxin-free plasmid purification kit. Sequence verification of the insert is optional.

D. Protocol for Targeting the Placed PinPoint Site with Donor Vectors

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 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. For HEK293T cells, we have used a concentration of 0.5 µg/ml for selection.

4) It is recommend to use the PinPoint integrase plasmid (Cat# PIN200A-1) in a 1:1 ratio (w/w) with the donor. For example, for transfection of HEK293T cells containing a placed PinPoint *attP* site, using Lipofectamine 2000, a 1:1 ratio (0.1µg/0.1µg) of PinPoint integrase plasmid to donor plasmid successfully integrated the donor vector at the placed *attP* site.

Day 0

1) 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

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

Day 2

3) Split co-transfected cells at 1/2 and 1/5 ratios in 100mm plates, and replace transfection medium with complete growth medium including antibiotics.

Day 3

4) Next day, add recommended amount of puromycin suitable for optimal selection of the transfected cells.

Days 4-14

5) Untransfected cells will begin to die and colonies will begin to emerge 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.

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

7) Select the cell line(s) that give the desired level of transgene expression for further characterization and/or expansion. Please note that expression may vary from line to line depending on chromatin structure and/or epigenetic modifications surrounding the integration site.

8) The donor vector, including the backbone and selection marker, can be removed by the use of the *Cre* recombinase (Cat #CRE100A-1), leaving only a *loxP* site and the promoter/insert sequence. See Appendix A for further protocol details on Cre recombinase-mediated excision.

E. Verification of Insert Integration into PinPoint Site by Junction PCR

To verify that the retargeting of your cell line is correct, junction PCR can be employed to amplify the *attL* junction. This will require utilization of a nested PCR scheme:

a) Prepare a PCR Master Mix shown below:

Primary PCR Reaction	Amount (µL)
Genomic DNA (50 ng/µL)	1.0
5X iProof HF Buffer	5.0
10 mM dNTPs	0.5
10 uM Primer Mix 1	1.25
iProof Polymerase (2U/µl)	0.25
Water	17.0
Total	**25.0**

b) Proceed with PCR using the following “Touchdown” program:

PCR Settings		
1.	98C	30 sec
2.	98C	15 sec
3.	70-65C	30 sec (-0.5C/cycle)
4.	72C	15 sec
5.	Go to step 2, nine more times (10 total cycles)	
6.	98C	15 sec
7.	65C	30 sec
8.	72C	15 sec
9.	Go to step 6, 29 more times (30 total cycles)	
10.	72C	1 min
11.	4C	forever

c) After completion of the primary PCR program, take out 1 µl of the primary PCR reaction for use in the secondary PCR reaction and set up the reaction as following:

Secondary PCR Reaction	Amount (µL)
Primary PCR product	1.0

5X iProof HF Buffer	5.0
10 mM dNTPs	0.5
10 uM Primer Mix 2	1.25
iProof Polymerase (2U/µl)	0.25
Water	17.0
Total	**25.0**

d) Proceed with PCR using the following "Touchdown" program:

PCR Settings		
1.	98C	30 sec
2.	98C	15 sec
3.	66-61C	30 sec (-0.5C/cycle)
4.	72C	15 sec
5.	Go to step 2, nine more times (10 total cycles)	
6.	98C	15 sec
7.	61C	30 sec
8.	72C	15 sec
9.	Go to step 6, 29 more times (30 total cycles)	
10.	72C	1 min
11.	4C	forever

h) Take 5 µl of the PCR reaction and run it on a 1.2% agarose/EtBr gel in 1X TAE buffer. Expect ~300 bp PCR product, which confirms correct integration

attL Junction Primer Sequences

Primary PCR

Fwd
5'-CGTGCAGGACGTGACAAATG-3'

Rev
5'-GTGGGCTTGTACTCGGTAGC-3'

Secondary PCR

Fwd
5'-CTGAGCAATGGAAGCGGGTA-3'

Rev
5'- GGATGATCCTGACGACGGAG-3'

V. Pre-Placed PinPoint Cell Lines for Retargeting

A. Product Overview

In addition to the comprehensive vector collections for the PinPoint-FC and PinPoint-HR systems, SBI offers pre-made cell lines with the PinPoint site already integrated into the genome in a single locus, for use as platform cells ready for PinPoint-mediated retargeting. Currently available cell lines include the following:

1) Human Embryonic Kidney (HEK293T) (Cat # PIN320A-1)
2) Mouse iPSC derived from mouse embryonic fibroblasts (Cat # PIN340iPS-1).

We also offer kit formats for these platform cell lines, with everything needed to retarget the above cell lines with donor vectors and the PinPoint integrase. For the 293T platform cell line: Cat# PIN320A-KIT, and for the mouse iPSC platform line: Cat# PIN340iPS-KIT. For detailed product information on these cell lines and kit components, please refer to the product table in Section I.B.

The PinPoint™ site was placed in these platform cell lines in a single locus using the PinPoint-FC system, and validated for single-copy insertion of the PinPoint *attP* site by Southern Blot analysis (Fig. 13).

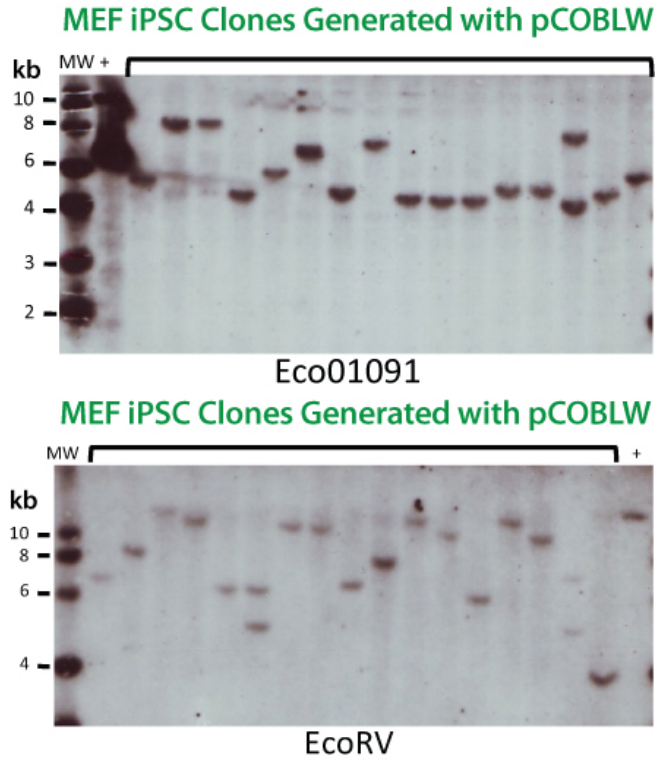

Fig. 14. Southern Blot Analysis of Genomic DNA isolated from Mouse Embryonic Fibroblast (MEF) clones integrated with PhiC31 Reprogramming Vector (Cat# FC305A-1) Reveals Single-Copy Insertion of Vector in Target Cells

B. Protocol for Culturing PinPoint HEK293T cells

The pre-made 293T cell line can be thawed, recovered, and frozen using standard conditions as outlined in the table below.

Complete Growth Medium	Freezing Medium
D-MEM, high Glucose	90% Complete Growth Medium
10% fetal bovine serum (FBS)*	10% DMSO
2 mM L-glutamine	
1% Penicillin/Streptomycin (10,000 I.U. Penicillin; 10,000 μg/ml Streptomycin)	

*FBS does not require heat inactivation

- **Thawing and Recovering Cells for Retargeting with PinPoint Donor Vector**

Use the following protocol to thaw cells to initiate the culture. The initial propagation of cells should be used to generate stocks to be frozen and stored for future use.

1) Remove the frozen vial of cells from liquid nitrogen and quickly thaw them by swirling in a 37ºC water bath. Try to keep the O-ring and cap of the vial out of the water, to prevent possible contamination. Wear eye protection.

2) Before cells are completely thawed, remove from 37ºC water bath and decontaminate outside of the vial with 70% ethanol.

3) Using sterile technique, transfer the cells to either a 100mm plate or T-75 cm^2 tissue culture flask containing 10 ml of complete medium at room temperature. Transfer entire contents of the vial to the tissue culture plate/flask and do not pipet cells up and down as this may kill the cells.

4) Swirl the plate/flask to evenly distribute cells. Incubate the flask at 37ºC, 5% CO_2 overnight to allow cells to attach to the bottom of the flask.

5) The following day, aspirate medium and replace with 10 ml fresh complete medium containing 400ug/ml of G418.

6) Incubate the cells at 37ºC, 5% CO_2 and check daily until they reach 80% confluency.

7) Once reach desired confluency, subculture the cells 1/5 to 1/10 into 100mm plates for transfection. It may be useful to have one plate devoted to freeze down for archival purposes.

8) When the cells reach target confluency of 60-80%, transfect cells with PinPoint integrase and appropriate donor vector using the protocol in Section IV.D of this user manual.

C. Protocol for Culturing PinPoint Mouse iPSC Cells

The pre-made mouse fibroblast iPSC cell line can be thawed and recovered, and frozen using the conditions as outlined below. 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-derived iPSC cells provided in the kit require separate feeder cells for proper culturing. The feeder cells need to be resistant to G418 and Puromycin for this application. Appropriate feeder cells for mouse iPS cell culture can be obtained from commercial sources (Amsbio, Cat# GSC-6004G). Please consult manufacturer's recommended protocols for plating and culturing MEF feeder cells. These feeder cells need to be prepared in advance and ready for use on the day of seeding mouse iPSC cells

- ### Growth Conditions for PinPoint 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

2. 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 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_2 until the cells reach 80% confluency. The mouse iPSC media must be changed every day.

3. 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. 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% CO2 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.

4. Freezing mouse iPS 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 $1x10^6$ 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.

5. Retargeting of mouse iPSC cells with Donor Vector

1) When the cells are ~60-80% confluent in 6-well plates, transfect cells using the PinPoint integrase and suitable donor vector using NucleoFection or similar transfection method per the protocol below (conditions adapted from Karow et al. 2011)
2) Use the MEF nucleofector kit I (Lonza) and the program T-20 according to the manufacturer's instructions.
3) Nucleofect with 3 µg **total DNA** (1.5 µg of PinPoint integrase and 1.5 µg of donor vector). The Neon transfection system from Life Technologies has also been demonstrated to work with similar efficiency.
4) After nucleofection, plate the cells in single wells of a six-well plate with MEF feeder cells already plated. Change media to mouse iPSC media.
5) Next day, switch to mouse iPSC media with 0.5 µg/ml of puromycin, and change the media every day.
6) Allow cells to form colonies (~10-14 days) in the plates, and pick individual colonies for characterization of relevant phenotype(s) of interest and/or expansion into 100mm plates if desired.

D. Validation of PinPoint Mouse iPSC Cell Lines

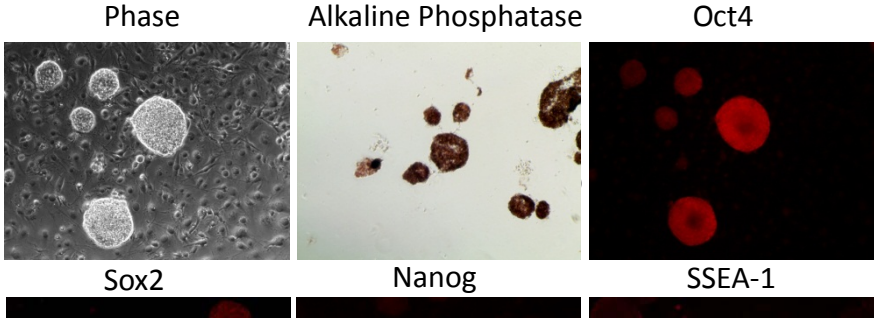

Fig. 15. Stem cell markers for Oct4, Sox2, SSEA-1 and Nanog were determined by immunocytochemistry using primary antibodies for SSEA1 (Millipore), Oct4 (Abcam), Sox2 (Abcam), and Nanog (Abcam) followed by Alexa Fluor fluorescent-labeled secondary antibodies (Invitrogen). Detection of Alkaline Phosphatase activity was performed using the AP Detection Kit (Millipore).

VI. Appendix

A. Protocol for use of Cre recombinase in excision of integrated donor vector backbone sequence

This protocol is designed to check for removal of the donor vector backbone and selection cassette upon excision using the Cre recombinase plasmid. The PCR primers have been verified for the Cat# PIN510A-1 and PIN600A-1 donor vectors. The use of other donor vectors will require designing of specific primer pairs to check for Cre excision.

Day 0

1) 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.

Day 1

2) Transfect cells with optimal amount of pCMV-Cre plasmid (Cat# CRE100A-1) using your transfection agent of choice. For example, for transfection of HEK293T cells containing a placed donor vector, using Lipofectamine 2000, 1 µg of the plasmid works very well to remove the donor vector. For other types of cells or using alternative transfection system, higher amounts of plasmid may be necessary.

Day 2

3) Next day, replace transfection media with complete growth media

Day 3/4

4) Split co-transfected cells at a very low density (~1:20) into 100mm plates

Day 5-14

5) Allow the cells to form single colonies on the plate. Note that colonies should not be puromycin-resistant if recombinase reaction has removed the donor vector backbone and selection cassette

6) Pick individual colonies and place them into 6-well plates for further expansion. When colonies have expanded, pick individual colonies for PCR-based analysis of Cre excision

7) Isolate genomic DNA from colonies using standard genomic DNA isolation kit of your choice.

8) Perform junction PCR to determine if the colony is Cre- excised using the conditions below.

The junction PCR has been validated with xxxx vectors.

Junction PCR Primers:

CreEx_F1(67/449) 5'- CCC TTT GAC TGA GAC TGA AAC CTG-3'

CreEx_R1(59/449) 5'-GGT AAT AGC GAT GAC TAA TAC GTA G-3'

a) Prepare a PCR Master Mix shown below:

Primary PCR Reaction	Amount (µL)
Genomic DNA (50 ng/µL)	1.0
5X iProof HF Buffer	5.0
10 mM dNTPs	0.5
10 uM Primer Mix 1	1.25
iProof Polymerase (2U/µl)	0.25
Water	17.0
Total	**25.0**

b) Proceed with PCR using the following "Touchdown" program:

PCR Settings		
1.	98C	30 sec
2.	98C	15 sec
3.	61-56C	30 sec (-0.5C/cycle)
4.	72C	15 sec
5.	Go to step 2, nine more times (10 total cycles)	
6.	98C	15 sec

7.	56C	30 sec
8.	72C	15 sec
9.	Go to step 6, 29 more times (30 total cycles)	
10.	72C	1 min
11.	4C	forever

c) After completion of the primary PCR program, take out 1 µl of the primary PCR reaction for use in the secondary PCR reaction and set up the reaction as following:

Secondary PCR Reaction

CreEx_F2(58/364) 5'- GTA TGC TAT ACG AAG TTA TCT TAA GC-3'

CreEx_R2(63/364) 5'- GGA AAG TCC CAT AAG GTC ATG T-3'

Secondary PCR Reaction	Amount (µL)
Primary PCR product	1.0
5X iProof HF Buffer	5.0
10 mM dNTPs	0.5
10 uM Primer Mix 2	1.25
iProof Polymerase (2U/µl)	0.25
Water	17.0
Total	**25.0**

d) Proceed with PCR using the following "Touchdown" program:

PCR Settings		
12.	98C	30 sec
13.	98C	15 sec
14.	60-55C	30 sec (-0.5C/cycle)
15.	72C	15 sec
16.	Go to step 2, nine more times (10 total cycles)	
17.	98C	15 sec

18.	55C	30 sec
19.	72C	15 sec
20.	Go to step 6, 29 more times (30 total cycles)	
21.	72C	1 min
22.	4C	forever

i) Take 5 μl of the PCR reaction and run it on a 1.2% agarose/EtBr gel in 1X TAE buffer. Expect ~365 bp PCR product, which confirms Cre excision

Optional: An alternative to junction PCR is to replica-plate individual colonies into wells +/- puromycin. Clones that grow in the absence of puromycin and do not grow in its presence will be Cre excised.

VII. References

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VIII. Technical Support

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IX. Licensing and Warranty Statement

Acknowledgement:
Design of the *AAVS1* TALE-Nuclease Expression vectors was performed by Dr. Jizhong Zou of the NIH Center for Regenerative Medicine, a Common Fund initiative of the U.S. National Institutes of Health.

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