piggyBac® transposase DNA modification system

User Manual for Mammalian Cell Applications

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1. Introduction and Background

A. The *piggyBac* Tranposase DNA Modification System

piggyBac (PB) is a highly efficient, non-viral means of DNA integration into target genomes. To date, the *piggyBac* transposon has been utilized in cell line engineering & bioprocessing, yeast metabolic engineering, plant modification, cell reprogramming and differentiation among others. The *piggyBac* DNA modification system enables you to:

- Use simple transfection to manipulate a variety of genomes
- Engineer cell lines for high level protein production
- Complete non-viral gene delivery efficiently and economically
- Revert modifications to the genome in a scarless, Footprint-Free® manner

The *piggyBac* transposon is a mobile genetic element that efficiently transposes between vectors and chromosomes via a "cut-and-paste" mechanism (Figure 1). During transposition, the *piggyBac* transposase recognizes transposon-specific inverted terminal repeat sequences (ITRs) located on each end of the transposon. Subsequent to ITR recognition, the *piggyBac* transposase excises ("Cut") the transposon from the transposon vector which is then efficiently integrated ("Paste") into random, highly expressed TTAA genetic locations. The *piggyBac* transposons have an enormous cargo limit with over 200 kb demonstrated.

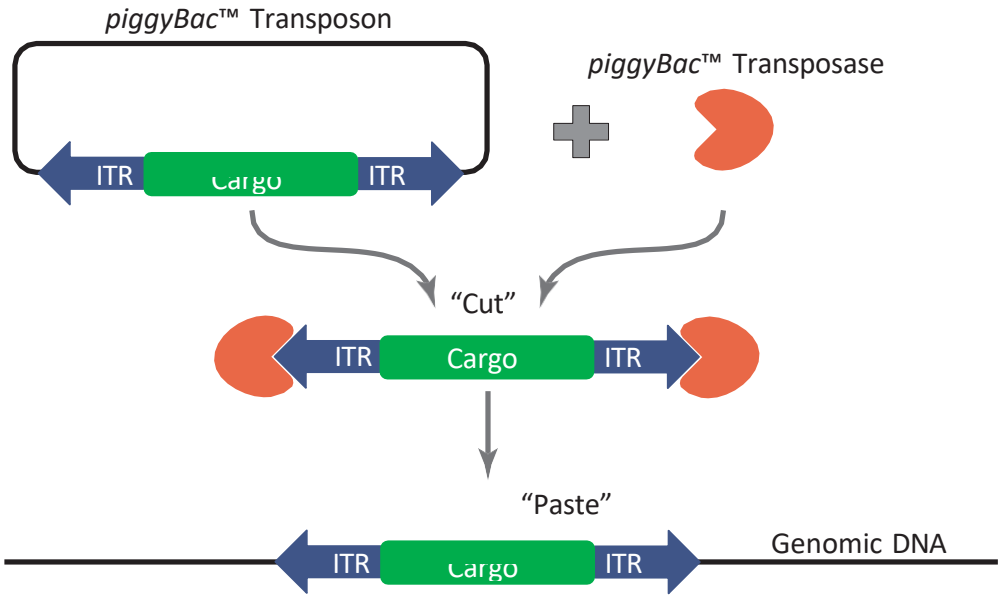

Figure 1: *Mechanism of the piggyBac transposase/transposon gene modification system.*

If desired, genomes containing an inserted *piggyBac* cargo can be transiently re-transfected with the excision-only *piggyBac* (PBx) vector to remove the cargo in a scarless, or Footprint-Free manner.

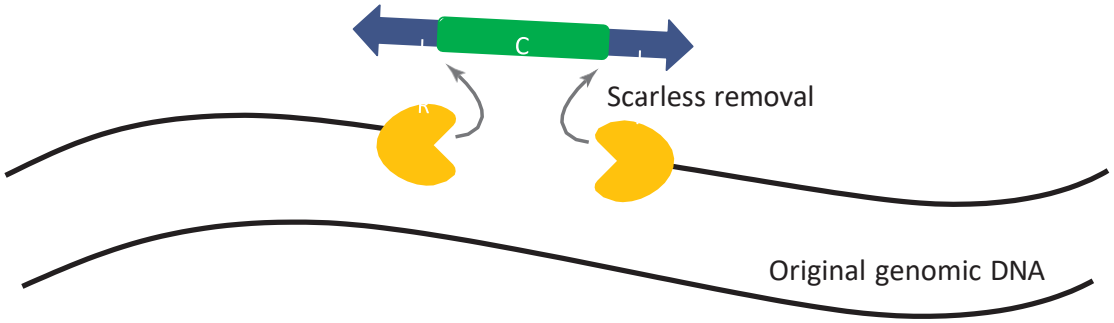

Figure 2: *Scarless, Footprint-Free removal of cargo with excision-only piggyBac (PBx)*

Using PBx with or without site-specific nucleases, including Cas-CLOVER, provides a clean and efficient method to select for desired gene modifications (Figure 3).

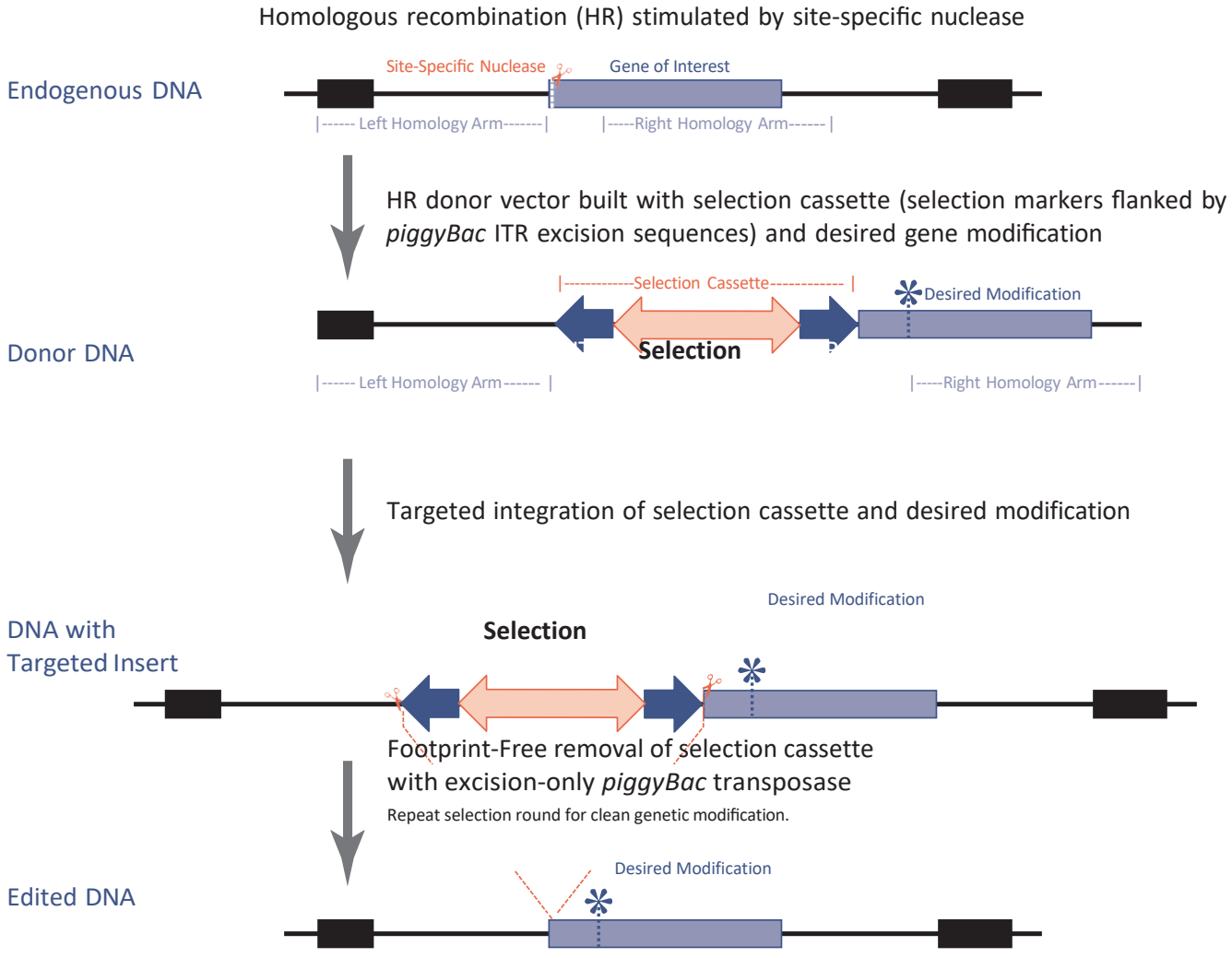

Figure 3: *Example of Editing DNA using piggyBac excision-only technology.*

2. Integration of the *piggyBac* Transposon

We suggest following manufacturers or user developed protocols for the introduction of DNA/RNA into your cell type of interest. The *piggyBac* system is titratable. More or fewer integrations can be achieved by varying the amounts of transposase and transposon, as appropriate.

A. Basic Protocol

Note: Below is an example integration protocol using *piggyBac* transposase mRNA and any transposon vector. Every cell line will respond differently to transfection and the introduction of foreign DNA. Be sure to determine the optimal transfection efficiency in your cell type (with a GFP vector for example) prior to beginning the integration protocol.

Co-transfect the Super *piggyBac* transposase with the *piggyBac* transposon vector:

1. Clone the desired cargo into the *piggyBac* transposon vector.
2. Sequence verify the clones.
3. Grow target cells to 60–80% confluency.
4. Prepare the transfection mixture. For one well of a 6-well dish combine:

Amount	Item	Recommendation
1.0 µg	PB Transposon vector	Start with a 1:1 molar ratio of transposase to transposon to determine baseline integration efficiency.
1.0 µg	PB Transposase mRNA	Calculate the molar ratio using your final transposon size.
x µL	Transfection reagent of choice	Follow manufacturer's protocol for RNA and/or consider electroporation
50 µL	serum-free or reduced-serum DMEM	

5. Mix by pipetting.
6. Allow complex formation by incubating the mixture for 15–30 minutes at room temperature.
7. Transfer the transfection mixture drop-wise to cells in culture wells and swirl to disperse.
8. Incubate the cells and the transfection mixture at 37°C in a CO_2 incubator. If lipofection was used, change media after 24 hours.
9. Check for positive integrations after 72 hours.
   - If you can, apply antibiotic selection or isolate cells by fluorescence-activated cell sorting (FACS), if applicable, to select for positive clones and measure expression of integrated cargo by standard RT-PCR methods.
   - If you did not use antibiotic or fluorescent markers, allow appropriate time for cells to remove trace amounts of remaining episomal transposon cargo before measuring expression of cargo by standard RT-PCR methods. Use non-transfected cells as negative control in RT-PCR.

3. Excision of *piggyBac* Transposon Cargo

Use excision-only *piggyBac* (PBx) mRNA to remove inserted cargo (see Figure 2). Increasing amounts of PBx will increase excision efficiencies. Negative selection, such as thymidine kinase/ ganciclovir or loss of a fluorescent marker, can also be utilized to enhance excision efficiencies.

a. Basic Protocol

Note: Below is an example excision protocol for mammalian cells with a Puromycin/Thymidine Kinase (Puromycin/TK) selection cassette. The protocol assumes that you have identified puromycin- resistant clones. Before beginning any selection protocol, you need to perform kill curves using your cell line and selection method of choice. Every cell line will respond differently to drug selection.

i. Mix approximately 2 x 10^6 cells with 10 µg PBx mRNA and

transfect according to the manufacturer's protocol using either a nucleofection device or lipofection reagents.

Note: 5 µg is an appropriate starting point for difficult-to-transfect cells such as primary cells; for transformed cell lines use (i.e. HEK293) approximately 250 ng for 1 x 10^6 cells.

ii. Plate the cells onto a 6-well plate in 1:2, 1:4, and 1:6 dilutions in media without puromycin. If lipofection was used, change media after 24 hours.

iii. On day 4, count cells and plate 1 x 10^4 cells onto a 10-cm dish in media containing ganciclovir (Sigma Cat # G2536) at a concentration determined from your previously performed kill curve. Ganciclovir should not affect cells without the thymidine kinase (TK) gene.

iv. Change media every other day. Ganciclovir killing takes approximately 2–4 days for fast growing cells, but may take up to two weeks in some cell lines.

v. Check surviving clones for puromycin sensitivity and confirm appropriate cassette removal by standard molecular analysis methods.

Note: We recommend that you perform a literature search to find the appropriate conditions for your cell type prior to undertaking any gene editing project. Below are some published examples of the Footprint-Free Gene Editing Kit system:

- A selectable all-in-one CRISPR prime editing piggyBac transposon allows for highly efficient gene editing in human cell lines: https://pubmed.ncbi.nlm.nih.gov/34773059/
- piggyBac Transposon-Based Immortalization of Human Deciduous Tooth Dental Pulp Cells with Multipotency and Non-Tumorigenic Potential: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6801629/
- Efficient, footprint-free human iPSC genome editing by consolidation of Cas9/CRISPR and piggyBac technologies: https://pubmed.ncbi.nlm.nih.gov/27929521/
- Seamless gene correction of β-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and *piggyBac*: http://genome.cshlp.org/content/early/2014/07/30/gr.173427.114

4. Confirmation of Integrated *piggyBac* Transposon Cargo by Splinkerette PCR

The Splinkerette PCR assay locates and maps the exact location of each transposition event in the genome, and provides a copy number for the number of transposon integrations. Ask us about services or kits for the Splinkerette PCR assay.

The full reference for the Splinkerette PCR procedure is published:

- A high-throughput splinkerette-PCR method for the isolation and sequencing for retroviral insertion sites, *Nature Protocols*, Col.4 No5. 2009, Page 789–798. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3627465/

5. Frequently Asked Questions

Q. Is excision-only *piggyBac* (PBx) mutagenic?

A. No, the excision-only *piggyBac* is not mutagenic. It will only remove cargo flanked by the specific ITR sequences inserted previously. Removal of inserts by PBx is a scarless mechanism which restores the original wildtype sequence. This is a key advantage over the CRE recombinase system which is known to be highly mutagenic.

Q. How many copies of my integrated gene will be present?

A. The number of integration events will largely depend on the amounts and ratios of *piggyBac* transposase and transposon. Titrating these amounts can yield a majority of single integration events, or many integrations depending on your preference. Confirmation of integration events is also possible to verify the exact copy number present.

Q. How efficient are *piggyBac* expression vectors?

A. While integration using Super piggyBac transposase mRNA is highly efficient (80-100% of transfection efficiency), excision with PBx is somewhat less so. To monitor excision, we recommend inclusion of counterselection of unexcised cells.

6. Troubleshooting

If you see...	Then try this...
A lower-than-expected integration efficiency	Increase the amounts of transposase
A higher-than-expected integration efficiency	Decrease the amounts of transposase
Incomplete transposon excision across all cells	Multiple rounds of transfection may be required to completely remove all integrations.
Cell toxicity after transfection	Decrease RNA/DNA and transfection reagent amounts. Lower the amount of transposase used, as integration may have occurred in essential genomic locations.