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Pronuclear Injection-based Targeted Transgenesis

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Abstract

Microinjection of DNA expression cassettes into fertilized zygotes has been a standard method for generating transgenic animal models. While efficient, the injected DNA integrates *randomly* into the genome, leading to potential problems such as disruption of endogenous genes or regulatory elements, variation in copy number, and integration into heterochromatic regions that inhibit transgene expression. A recently developed method addresses such pitfalls of traditional transgenesis by *targeting* the transgene to predetermined sites in the genome that can safely harbor exogenous DNA. This method, called Pronuclear Injection-based Targeted Transgenesis (PITT), employs an enzymatic transfer of exogenous DNA from a donor vector to a previously created landing pad site in the animal genome. The DNA transfer is achieved through the use of molecular tools such as Cre-*LoxP* recombinase and PhiC31-*attB*/*P* integrase systems. Here, we provide protocols for performing PITT and an overview of the current PITT tools available to the research community.

Keywords

Pronuclear Injection; Targeted Transgenesis; PITT; Cre-*LoxP* recombination; PhiC31-*attB/P* integration

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INTRODUCTION

Transgenic animals are invaluable for studying gene function and modeling human disease. The canonical method to create transgenic animals involves injecting exogenous DNA of interest (DOI) into fertilized zygotes (also called pronuclei; PN). These injected zygotes are then transferred to recipient females for gestation, leading to the birth of transgenic founder offspring.

While efficient, this approach results in random integration of the DOI into the genome. The inability to control copy number and integration site can lead to multiple problems including disruption of endogenous genes, multiple integration sites, and repressed expression of the transgene by epigenetic silencing or proximity to heterochromatic regions and local regulatory elements.

To overcome the challenges of random DOI integration, the transgene can be targeted to a specific locus by using gene targeting to embryonic stem (ES) cells. Transgenic ES cells are then injected into blastocysts, which are transferred to surrogate mothers to create chimeric founder animals. However, this method still has pitfalls, as it is time consuming, labor intensive, and more expensive than PN injection-based transgenesis.

Using the best features from both of these standard techniques, we have developed Pronuclear Injection-based Targeted Transgenesis (PITT), a method in which the transgene can be inserted at a predetermined locus through PN injection (Ohtsuka et al., 2010). PITT includes two major steps. The first step involves generation of a “seed mouse” strain by inserting heterotypic recombination (*LoxP*) or integration (*attP*) sites at specific loci in the genome, which will ultimately serve as landing pads for DOI sequences. These seed mouse strains are currently available from us or RIKEN BRC mouse repository, as described in Table 3. In the second step, a donor DNA cassette that contains compatible *LoxP* or *attB* sites is injected into PN that have been isolated from the seed mouse strain. The donor cassette gets inserted at the landing pad sites through Cre-recombination or PhiC31-integration, respectively. An overall schematic of the PITT process is shown in Figure 1. Over the years, our group and other laboratories have improved this method and developed additional tools (Ohtsuka et al., 2012b, 2013, 2015; Tasic et al., 2011). This unit provides a detailed protocol for performing PITT.

The PITT method involves four major steps: (1) designing and building of PITT donor DNA constructs (**Basic Protocol 1**); (2) synthesis and purification of DNA and RNA components for microinjection (**Basic Protocol 2**); (3) isolation of embryos from seed mice, microinjection of PITT components, and embryo transfer (**Basic Protocol 3**); and (4) genotyping of offspring to identify transgenic founders (**Basic Protocol 4**). A general overview of these steps is presented in Figure 2.

BASIC PROTOCOL 1

Designing and building of PITT donor DNA constructs

This protocol involves two major steps: (a) Designing the PITT donor construct and (b) Building the PITT donor vector.

Designing the PITT donor construct—The PITT donor DNA construct contains the DOI flanked by elements needed for integration into the endogenous landing pad. Table 1 describes these required sequence elements. There are currently two PITT platforms available, based on the enzyme used for the efficient targeted integration: Cre-PITT and PhiC31-PITT. There is also a third platform that uses Flp-*FRT* recombination, but due to poor efficiency of this system, it is not used as a major PITT platform. We instead currently use Flp-*FRT* recombination as an additional tool to remove excess sequences (that come from the vector backbone) after generation of founder transgenic mice.

Platform selection depends on the DOI. For example, Cre-PITT would not be suitable if the DOI consists of *LoxP* sites, because it would result in aberrant recombination in the PITT donor DNA construct. Such a donor construct can be inserted using PhiC31-PITT. Similarly, presence of *attB*/*P* sites within the DOI would interfere with PhiC31-PITT, in which case Cre-PITT should be used.

A PITT donor vector consists of two major classes of sequence elements: DOI elements and PITT elements. The DOI is the primary transgenic DNA cargo that needs to be inserted into the genome, whereas PITT elements help achieve the targeted insertion of the DOI into the genomic landing pad. The DOI and PITT elements are typically assembled in a standard bacterial plasmid backbone that contains essential plasmid features, such as an origin of replication and an antibiotic selection marker. A few previously developed PITT donor vectors are listed in Table 2.

The composition of DOI elements depends on the transgenic project. A simple DOI may have an expression cassette with a promoter driving a cDNA and/or microRNA followed by polyA signal sequence. A more complex DOI may have an inducible reporter cassette followed by an internal ribosome entry site (or a viral 2A peptidase) with another expression cassette encoding a second reporter and a polyA terminator sequence.

In a donor vector, PITT elements (such as *LoxP* or *attB* sites) flank the DOI. The choice and architecture of PITT elements in a donor vector depends on the chosen recombinase/integrase system and the seed mouse strain. For example, heterotypic *LoxP* sites or *attB* sites are included if Cre-PITT or PhiC31-PITT are used, respectively. Our recommendations for compatible combinations of PITT elements needed in a typical PITT donor vector and the corresponding seed mouse landing pads that are available to the scientific community are listed in Tables 2 and 3. Schematics of some donor vectors and landing pads in PITT seed mouse strains are shown in Figures 3 and 4, respectively.

Once the DOI and PITT elements have been selected and the theoretical sequences of the donor vector have been designed, the vector can be built by custom synthesis from

commercial vendors (such as Bio Basic, Integrated DNA Technologies, GENEWIZ, GeneArt, GenScript, or other companies). Alternatively, DOI elements can be cloned into preexisting plasmid donor vectors. We have made available donor vectors with certain PITT elements, such as recombination/integration sites, as well as some commonly used DOI elements, such as promoters and polyA signal sequences (Table 2). These plasmids contain multiple restriction enzyme sites that enable cloning of the desired cDNA or expression cassette.

Building the PITT donor vector—This step involves standard molecular biology and recombinant DNA techniques for donor vector cloning. The protocol steps described below provide a choice between two types of cloning methods: a conventional restriction endonuclease (RE)-based method and a more modern technique called Gibson assembly.

Materials

Reagents and solutions

Donor vector backbone (selected from Table 2)

Restriction enzymes for cloning and confirming positive clones (refer to Table 2 to identify specific enzymes needed for cloning into the selected donor vector)

QIAquick PCR Purification Kit (Qiagen, catalog #28104)

Standard gel electrophoresis reagents including low melting agarose (such as SeaPlaque GTG Agarose from Lonza [catalog #50111] or comparable products from other vendors), ethidium bromide (such as from Sigma Aldrich, catalog # E1510), 50× Tris-Acetate-EDTA (such as from Thermo Fisher Scientific, catalog #BP1332-20), DNA gel loading dye, and DNA ladder

Modified TE: 10 mM Tris/0.1 mM EDTA (pH 8.0) (such as TE Buffer, 1× Solution pH 8.0, Low EDTA, from Affymetrix, catalog #75793)

Alkaline Phosphatase, Calf Intestinal (CIP; NEB catalog #M0290)

Phenol, TE-saturated (such as from Sigma-Aldrich [catalog #77607], Nacalai Tesque [catalog #26829-54], or comparable products from other vendors)

3M Sodium acetate (NaOAc) buffer solution (pH 5.2; such as from Sigma Aldrich [catalog #S7899], Nacalai Tesque [catalog #31150-64], or comparable products from other vendors)

Ethanol (200 proof ethyl alcohol, such as from Decon Laboratories, catalog #07-678-005)

High fidelity DNA polymerase (such as Phusion from NEB [catalog #M0530], KOD-plus from Toyobo [catalog #F0934K] or comparable products from other vendors)

Primers with appropriate overhangs to amplify the desired cDNA or expression cassette while adding flanking sequences for cloning

Quick Ligation Kit (NEB, catalog #M2200)

Gibson Assembly Master Mix (NEB, catalog #E2611)

Competent cells (such as 5-alpha Competent *E. coli*, High Efficiency, from NEB, catalog #C2987)

LB Agar containing appropriate antibiotics (e.g. +100 μg/ml ampicillin or +25 μg/ml kanamycin) (such as from Sigma-Aldrich, catalog #L3147)

Luria Broth (LB) medium (prepared as directed from MP Biomedicals, catalog #113002022) with appropriate drug selection

Plasmid Mini Kit (Qiagen, catalog #12125)

Primers for sequencing donor vector

Equipment

Standard equipment for agarose gel electrophoresis such as microwave for melting agarose, gel electrophoresis units, electrophoresis power supplies and gel imaging system (including LED gel illuminator)

Microcentrifuge

Vortex mixer

Thermocycler (BioRad T100 or equivalent)

Autoclave (if you are making your own LB media or plates)

Standard equipment for growing and harvesting small volumes of bacteria including culture tubes/flasks and 37°C shaking incubator

1. Decide on the optimal recombinase/integrase platform for your project. There are two major platforms for PITT: Cre and PhiC31. The Cre platform uses two heterotypic *loxP* sites (e.g. *JTZ17* and *Lox2272*) flanking a DOI. The PhiC31 platform has an *attB* site on one end of the DOI. If your DOI contains *LoxP* or *attB*, do not choose the Cre or PhiC31 platforms, respectively. You may also choose to use both platforms in tandem (Ohtsuka et al., 2015).
2. Select the appropriate plasmid backbone for your project based upon your platform of choice and the seed mouse you plan to use (refer to Table 2).
3. Digest the selected plasmid using the specified restriction enzyme (Table 2) according to the manufacturer's instructions.
4. Purify the digested plasmid fragments using the QIAquick PCR Purification Kit or gel purification as follows:
    a. Electrophorese digested DNA in a 1% low-melting agarose gel.
    b. Excise the desired DNA fragment from the gel under LED light (~100 μl). Add 2 volumes (~200 μl) of modified TE

to the sample and freeze at −80 °C for more than 20 minutes.

c. Thaw the sample at room temperature, and then spin it down using a microcentrifuge at 4,000–6,000 rpm for 10 seconds. Transfer the supernatant to a new tube.

5. Dephosphorylate the plasmid fragment with CIP at 37°C for 40 min.

This step is important to prevent self-ligation of the vector during cloning.

6. Clean the dephosphorylated DNA using the QIAquick PCR Purification Kit or phenol extraction and subsequent ethanol precipitation as follows:

a. Add an equal amount of TE-saturated phenol, vortex, and centrifuge at 12,000 rpm for 5 minutes.

b. Transfer the aqueous phase to a new tube and add 0.1 volumes 3M NaOAc (pH 5.2) and 2.5 volumes ethanol.

c. Precipitate by centrifuging at 12,000 rpm for 10 minutes at room temperature. Remove the supernatant and wash the pellet with 70% ethanol.

d. Centrifuge at 12,000 rpm for one minute at room temperature and remove the supernatant. Let the pellet air dry for 10 minutes.

e. Resuspend the dried pellet in modified TE (e.g. 2 μl).

7. Create your DOI.

a. If you are amplifying cDNA or an expression cassette for your DOI, use a high fidelity polymerase and primers with suitable overhangs.

For RE-based cloning, the overhangs should include RE sites that are compatible with the donor plasmid cloning site. For Gibson assembly, 15–80 bp overhangs that overlap the ends of the digested plasmid backbone should be used (refer to The NEBuilder Assembly Tool to design optimal primers (http://nebuilder.neb.com)).

b. If you are custom synthesizing your DOI, include flanking sequences at both ends of your sequence that reflect the same overhangs in **step 7a**.

Make sure to take the intended DOI orientation into account when designing primers.

8. **If you amplify your DOI by PCR:** Electrophorese PCR products to confirm the correct size amplicon.

9. **RE-based cloning method only:** Digest ~50–300 ng of the DOI fragment using the specified restriction enzyme(s) according to manufacturer's instructions.

10. **For all DOIs amplified by PCR and/or digested by restriction enzymes:** Clean the DNA sample using the QIAquick PCR Purification Kit or by gel purification, phenol extraction, and ethanol precipitation as follows:

    a. Electrophorese digested DNA in a 1% low-melting agarose gel.

    b. Excise the desired DNA fragment from the gel under LED light (~100 μl). Add 2 volumes (~200 μl) of modified TE to the sample and freeze at −80 °C for at least 20 minutes.

    c. Thaw the sample at room temperature, then spin it down using a microcentrifuge at 4,000–6,000 rpm for 10 seconds. Transfer the supernatant to a new tube.

    d. Add an equal amount of TE-saturated phenol, vortex, and centrifuge at 12,000 rpm for 5 minutes.

    e. Transfer the aqueous phase to a new tube and add 0.1 volumes 3M NaOAc (pH 5.2) and 2.5 volumes ethanol.

    f. Precipitate by centrifuging at 12,000 rpm for 10 minutes at room temperature. Remove the supernatant and wash the pellet with 70% ethanol.

    g. Centrifuge at 12,000 rpm for one minute at room temperature and remove the supernatant. Let the pellet air dry for 10 minutes.

    h. Resuspend the dried pellet in modified TE (e.g. 2 μl).

11. Assemble your digested plasmid backbone (**step 6**) and DOI insert (**steps 7b or 10**).

    a. **For RE-based cloning**: Mix the vector and DOI fragments together and ligate using the Quick Ligation Kit (follow manufacturer's instructions and incubate at room temperature for 5 minutes).

    b. **For Gibson Assembly**: Incubate samples with Gibson Assembly Master Mix according to manufacturer's instructions. In brief, place mix in a thermocycler at 50°C for 60 minutes. Store samples between 4°C and -20°C until transformation.

12. Transform sample into competent *E. coli* cells and spread mixture on an LB plate containing the appropriate antibiotic. Incubate at 37°C overnight.

For projects where the DOI may render the plasmid unstable, transformation into a different bacterial strain, such as SURE (stop unwanted rearrangement events) E. coli, or growth at 30°C instead of 37°C, may be warranted.

13. Pick colonies, grow in 5 ml LB media with the appropriate antibiotic while shaking at 37°C overnight, and extract plasmids using the Qiagen Plasmid Mini Kit.

    To avoid another transformation for positive clones, you can save the remaining bacterial cultures at 4°C until confirming transformants with positive clones.

14. Confirm correct clones using restriction digestion analysis and sequencing.

BASIC PROTOCOL 2

Synthesis and purification of DNA and RNA components for microinjection

Microinjection components for PITT include the donor vector and an mRNA encoding Cre or PhiC31. While PITT donor vectors are unique for supporting a specific transgenic project, there are well-established plasmids available for generating mRNA encoding the enzymes (such as pBBI and pBBK, see Table 2 and Figure 5). You can also directly inject plasmids with DNA encoding the recombinase or integrase enzymes, but we have found empirically that mRNA is ~5-fold more efficient for PITT (Ohtsuka et al., 2013). We have observed that the plasmid is inferior to mRNA because the delay in translation of the recombinase/integrase results in a higher likelihood of mosaicism in founder mice and therefore less efficient germline transmission (Ohtsuka et al., 2013). Furthermore, injection of the Cre and PhiC31-encoding plasmids may even result in their own undesired insertion into the genome. For these reasons, the protocol below describes the mRNA technique.

Materials

Reagents and solutions

Donor vector, transformed into *E.coli* (from **Basic Protocol 1**)

Vector for mRNA synthesis of recombinase/integrase, transformed into *E.coli*

- Cre platform: pBBI, vector for iCre mRNA synthesis (addgene plasmid #65795; https://www.addgene.org/65795/; (Ohtsuka et al., 2013))
- PhiC31 platform: pBBK, vector for PhiC31o mRNA synthesis (addgene plasmid #62670; https://www.addgene.org/62670/; (Ohtsuka et al., 2015))

Luria Broth (LB) medium (prepared as directed from MP Biomedicals, catalog #113002022) with appropriate drug selection

HiSpeed Plasmid Midi Kit (Qiagen, catalog #12643)

Phenol, TE-saturated (such as from Sigma-Aldrich [catalog #77607], Nacalai Tesque [catalog #26829-54], or comparable products from other vendors)

3M Sodium acetate (NaOAc) buffer solution (pH 5.2; such as from Sigma Aldrich [catalog #S7899], Nacalai Tesque [catalog #31150-64], or comparable products from other vendors)

Ethanol (200 proof ethyl alcohol, such as from Decon Laboratories, catalog #07-678-005)

Modified TE: 10 mM Tris/0.1 mM EDTA (pH 8.0) (such as TE Buffer, 1× Solution pH 8.0, Low EDTA, from Affymetrix, catalog #75793)

Ultrafree-MC Filter (HV, 0.45 μM pore size; Millipore, catalog #UFC30HV00)

Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, catalog #P11496)

1.5 ml RNase-free microfuge tubes

*Xba*I to linearize mRNA synthesis vector (NEB, catalog #R0145)

Nuclease-free water (not DEPC treated; Ambion, catalog #AM9937)

Standard gel electrophoresis reagents including low melting agarose (such as SeaPlaque GTG Agarose from Lonza [catalog #50111] or comparable products from other vendors), ethidium bromide (such as from Sigma Aldrich, catalog # E1510), 50× Tris-Acetate-EDTA (such as from Thermo Fisher Scientific, catalog #BP1332-20), DNA gel loading dye, and DNA ladder

Phenol/chloroform/isoamyl alcohol (25:24:1, v/v; pH 7.9, such as from Ambion [catalog #AM9730] or Nacalai Tesque [catalog #25970-14])

Chloroform (such as from Nacalai tesque [catalog #08402-55] or comparable products from other vendors)

mMESSAGE mMACHINE T7 Ultra Kit (Ambion, catalog #AM1345)

MEGAclear Transcription Clean-up Kit (Ambion, catalog #AM1908)

Microinjection buffer (5 mM Tris pH 7.4, 0.1 mM EDTA)

> Dilute to final concentration from 1M Tris-HCl (Sigma Aldrich, catalog #T2663) and 0.5M EDTA (Sigma Aldrich, catalog #E7889) into sterile-filtered water (Sigma Aldrich, catalog #W1503). Sterilize using a 0.22 μM filter.

Equipment

Autoclave (if you are making your own LB media or plates)

Standard equipment for growing and harvesting large volumes of bacteria including culture tubes/flasks, 37°C shaking incubator, and centrifuge with rotor and bottles for harvesting cells

Vortex mixer

Microcentrifuge

NanoDrop spectrophotometer and/or fluorescence microplate reader

Standard equipment for agarose gel electrophoresis such as microwave for melting agarose, gel electrophoresis units, electrophoresis power supplies and gel imaging system

Preparation of plasmid DNA

1. Prepare donor and mRNA-encoding vectors using the Qiagen HiSpeed Plasmid Midi Kit according to the manufacturer's protocol.

   We recommend growing these transformed bacterial strains in 200 ml LB medium with appropriate drug selection prior to harvesting (to achieve ~50–100 μg yield).

2. After concluding the Qiagen HiSpeed Plasmid Midi Kit protocol with TE elution, precipitate the DNA.

   a. Add an equal amount of TE-saturated phenol, vortex, and centrifuge at 12,000 rpm for 5 minutes.

   b. Transfer the aqueous phase to a new tube and add 0.1 volumes 3M NaOAc (pH 5.2) and 2.5 volumes ethanol.

   c. Precipitate by centrifuging at 12,000 rpm for 10 minutes at room temperature. Remove the supernatant and wash the pellet with 70% ethanol.

   d. Centrifuge at 12,000 rpm for one minute at room temperature and remove the supernatant. Let the pellet air dry for 10 minutes.

   e. Resuspend the dried pellet in 50–100 μl modified TE. Place the DNA sample at 4°C overnight.

3. Filter the donor vector using a pre-equilibrated Ultrafree-MC filter at 12,000 × g for 2 min at 4 °C.

   Filtering is critical to avoid clogging the injection needle during microinjection of one-cell stage embryos.

4. Determine plasmid concentration using the Quant-iT PicoGreen dsDNA Assay Kit.

   The final concentration may range from 200–2000 ng/μl.

5. Store plasmids at -20°C until ready to use.

Preparation of mRNA

6. Transfer 30 μg of the mRNA synthesis vector (e.g. 30 μl × 1 μg/μl) to an RNase-free 1.5 ml microfuge tube. Add 120 units *Xba*I and incubate at 37°C

for 3–3.5 hours in a total volume of 120 μl. Check 0.5 μl by agarose gel electrophoresis for complete digestion.

7 Wash and precipitate the digested sample.

- **a.** Add 280 μl nuclease-free water and 40 μl 3M NaOAc (pH 5.2) to the digested sample.
- **b.** Add 450 μl phenol/chloroform/isoamyl alcohol and vortex.
- **c.** Centrifuge at 12,000 rpm for 5 minutes at room temperature.
- **d.** Collect ~440 μl aqueous phase and add 450 μl chloroform.
- **e.** Vortex and centrifuge at 12,000 rpm for 5 minutes at room temperature.
- **f.** Collect ~435 μl aqueous phase and add approximately 2.0 volumes (~870 μl) ethanol.

  At this step, the DNA is visible in the solution.
- **g.** Centrifuge at 12,000 rpm for 10 minutes at room temperature. Remove the supernatant and wash the pellet with 70% ethanol.
- **h.** Centrifuge at 12,000 rpm for 1 minute at room temperature and remove the supernatant. Let the pellet air dry for 10 minutes.
- **i.** Resuspend the dried pellet in 23 μl nuclease-free water.

8 Use 1 μl DNA (diluted in 9 μl nuclease-free water) to determine the linearized plasmid concentration by NanoDrop or the Quant-iT PicoGreen dsDNA Assay Kit.

9 Dilute the sample to ~0.5 μg/μl with nuclease-free water.

10 Synthesize mRNA transcripts using the mMESSAGE mMACHINE T7 Ultra Kit according to the manufacturer's protocol with the following adjustments to scale up the reaction for a large amount of stock solution:

- **a.** When combining *in vitro* transcription reagents included in the kit, make 100 μl total transcription reaction, using 5 μg of the linearized plasmid.
- **b.** Incubate the mixed sample at 37°C for 3 hours, add 5 μl TURBO DNase I, and incubate again for 15 minutes at 37°C.
- **c.** Divide the sample into two 52.5 μl aliquots.

We have omitted the polyA tailing procedure following in vitro transcription because our plasmids already include a polyA sequence.

11 Recover mRNA using the MEGAclear Transcription Clean-up Kit according to the manufacturer's protocol with the following adjustments:

a. Treat each aliquot as its own sample throughout the purification.

b. When eluting RNA, select RNA elution option 2, and perform the second optional elution procedure for a final volume of 100 µl for each eluate.

c. Combine eluates from both aliquots (for a total of 200 µl) prior to doing the optional ethanol precipitation step with ammonium acetate (NH_4Ac).

We recommend performing a 15-minute centrifugation step at 4°C.

d. Resuspend the pellet in 50–100 µl nuclease-free water (included in the kit).

12 Filter the mRNA using a pre-equilibrated Ultrafree-MC filter at 12,000 × g for 2 min at 4 °C.

13 Quantify mRNA concentration by NanoDrop and confirm good RNA quality by 1% agarose gel electrophoresis. A representative gel image of iCre and PhiC31 mRNA preps is shown in Figure 5.

14 Dilute the purified mRNA to approximately ~400–500 ng/µl and dispense 5 µl aliquots into RNase-free tubes. Store at -80°C until use.

Preparation of microinjection solution

15 Thaw frozen stocks of the donor vector (200–2000 ng/µl) and mRNA (400–500 ng/µl) on ice. Gently mix the solutions by tapping the tube and centrifuge at 14,000 rpm for 3 minutes at 4°C.

16 Take 2 µl of donor vector from the top of the stock and dilute it with microinjection buffer to a concentration of about 20 ng/µl (at least twice as concentrated as desired for the final solution concentration of 10 ng/µl).

17 Take 2–4 µl of mRNA from the top of the stock and dilute it with microinjection buffer to a concentration of about 1–2 ng/µl for iCre and/or 15–30 ng/µl for PhiC31 (at least twice as concentrated as desired for the final solution concentration of 0.5–1 ng/µl for iCre and 7.5–15 ng/µl for PhiC31).

Optional: At this stage, the mRNA can be filtered again using the Ultrafree-MC filter.

18 Centrifuge the diluted donor vector and mRNA solutions at 14,000 rpm for 3 minutes at 4°C.

19 Carefully transfer the appropriate volume (typically about 5–40 µl) of both the donor vector and mRNA solutions from the top of the tubes to a single RNase-free 1.5 ml tube. Mix gently by pipetting, and adjust final concentration with microinjection buffer if needed to make the final microinjection solution (typically ~100 µl).

20 Check the quality and concentration of nucleic acids using agarose gel electrophoresis on 3 µl of injection solution.

21 Either use the injection solution immediately or store at -80°C until use.

BASIC PROTOCOL 3

Isolation of embryos from seed mice, microinjection of PITT components, and embryo transfer

Targeted transgenesis follows similar technical steps to traditional transgenesis, but is unique in that it utilizes zygotes isolated from seed mice as opposed to from wild type animals. These seed mouse strains have been previously developed by us (Ohtsuka et al., 2010, 2012a, 2015) and other groups (Tasic et al., 2011), so the generation of PITT seed mice is not described in this unit. If a given project requires development of a new seed mouse, it can be made using standard gene targeting in mouse embryonic stem cells (Behringer et al., 2014; International Society for Transgenic Technologies, 2011) or more rapidly with CRISPR-based approaches (Quadros et al., 2015). The basic steps for this protocol include isolation of one-cell stage embryos from seed mice, microinjection of PITT components into mouse embryos, and transfer of injected embryos into pseudo-pregnant mice. These steps follow standard mouse transgenesis protocols that have been described previously (Pease and Saunders, 2011; Behringer et al., 2014). Notably, they are very similar to those described in a previous unit in *Current Protocols in Human Genetics* (see **Basic Protocol 3** in Harms et al., 2014), and are given below with slight modifications relevant to PITT. We also extend techniques from the previous unit by providing an alternative protocol for producing fertilized zygotes in place of steps 1–11 (see **Support Protocol 1**).

Materials

Animals

Egg donors (wild type C57BL/6 female mice; procured at 3 weeks old from Charles River Laboratories in Wilmington, MA).

Homozygous seed female mice (bred in-house), instead of wild type females, can also be used. However, we have found that hemizygous embryos survive better than homozygous embryos after microinjection.

Stud males (homozygous seed mice; 3–6 months old)

A list of seed mouse strains can be found in Table 3.

Pseudo-pregnant recipients (Crl:CD1(ICR) female mice; purchased at 5–6 weeks of age from Charles River Laboratories, Wilmington, MA; typically used for experiments when they are about 8 to 12 weeks of age)

Vasectomized males (CD1 male mice; purchased at 5–6 weeks old from Charles River Laboratories, Wilmington, MA; vasectomized as previously described (Behringer et al., 2014))

Hormones

Pregnant Mare's Serum Gonadotropin (PMSG; National Hormone and Peptide Program, Harbor–UCLA Medical Center, Torrance, CA)

Human Chorionic Gonadotropin (hCG; National Hormone and Peptide Program, Harbor–UCLA Medical Center, Torrance, CA)

Hormones are supplied as lyophilized vials of 2000 IUs. Upon first use, reconstitute in 2 ml PBS (Millipore, catalog #BSS-1006-B). Aliquot this 20× stock solution (100 IU/100μl) into 100 μl vials and store at -80°C. On the day of the hormone injection, one vial is thawed and diluted to 2 ml with PBS to get a final concentration of 5 IU/0.1 ml. Each animal is administered 0.1 ml of this solution. The leftover solution is discarded.

Reagents and solutions

35 × 10 mm Falcon Tissue Culture Dish (Corning, catalog #353001)

EmbryoMax M2 Medium (1×), liquid, with phenol red (M2; for embryo handling and microinjection; Millipore, catalog #MR-015-D)

EmbryoMax M2 Medium (1×), liquid, with phenol red and hyaluronidase (hyaluronidase; for dissociation of the cumulus oophorus complex; Millipore, catalog #MR-051-F)

EmbryoMax KSOM Medium (1×) with ½ amino acids (KSOM; for embryo incubation; Millipore, catalog # MR-106-D)

Falcon IVF/Organ Culture Dish (Corning, catalog #353653)

Flexipet Pipette (130 μm, for collecting embryos; Cook Medical, catalog #K-FPIP-1130-10BS-5)

Glass capillaries (4 in/1 mm) (World Precision Instruments, catalog #TW100F-4)

MicroFil 28 gauge/97mm long (World Precision Instruments, catalog #MF28G)

Microinjection buffer (5 mM Tris pH 7.4, 0.1 mM EDTA)

Dilute to final concentration from 1M Tris-HCl (Sigma Aldrich, catalog #T2663) and 0.5M EDTA (Sigma Aldrich, catalog #E7889) into sterile-filtered water (Sigma Aldrich, catalog #W1503). Filter sterilize using a 0.22 μM filter.

1-cc tuberculin syringe

Nunc Lab-Tek Chamber Slide System (Lab-Tek, catalog #177372)

EmbryoMax Filtered Light Mineral Oil (Millipore, catalog #ES-005-C)

Holding micropipets (Origio, catalog #MPH-SM-20)

Equipment

Individually Ventilated Cages (IVCs; Allentown, Lab Products, or Tecniplast)

Heraeus HERAcell 150i Tri–gas incubator, equipped with Coda Inline filters

Standard surgical equipment such as scissors, fine forceps, suturing material, anesthesia chambers, etc. (refer to Behringer et al., 2014)

Large slide warmer (Spectrum Scientifics, catalog #3875)

Dissecting scope (two examples are included below):

- Leica MZ 9.5
  - Condenser lens: PLAN 0.5×, model #10 446 157
  - Base: Model #10 445 367
  - Tilt head
  - Heating glass (Live Cell Instrument, catalog #HG-T-Z002) with temperature controller (Live Cell Instrument, CU-301)
- Nikon SMZ1000
  - Condenser lens: PLAN APO 1× WD70
  - Base: C-DSDF, model #1002364
  - Mid-Piece: C-FMC, model #1009459
  - LV-TV Camera port
  - Eyepiece: P-BERG w/c-w15/16 eyepiece, model #1007501
  - Heating glass (Live Cell Instrument, catalog #HG-T-Z002) with temperature controller (Live Cell Instrument, CU-301)

Mouth pipetting apparatus (assembled as previously described: Gurumurthy et al., 2016).

Glass pipette puller (Sutter Instrument Co. model #P97), outfitted with a 2.5mm × 2.5mm Box filament (catalog #FB255B)

Microinjection scope (two examples are included below):

- Leica DM IRB, equipped with Narishige IM 300 microinjector, and Leica manual manipulators
  - Eyepiece: HC PLAN 10×/22 w/tilt, model #11 507 804
  - Condenser lens: .30 S70
  - Objectives:

C PLAN 4×/.10, model #11 506 074

N PLAN L20×/0.40 CORR, model #11 506 057

N PLAN L40×/0.55 CORR, model #11 506 059

Heating glass (Live Cell Instrument, catalog #HG-T-Z002) with temperature controller (Live Cell Instrument, CU-301)

Nikon Eclipse TE 2000-E with DIC, equipped with Narishige IM 300 microinjector and NT-88-V3 micromanipulators

Condenser lens: LWD 0.52

Objectives:

PLAN 4×/0.10 WD30

PLAN APO 10×/0.45 WD4.0

PLAN FLUOR ELWD 20×/0.45 DIC L/NI

PLAN FLUOR ELWD 40×/0.60 DIC M/NI

Heating glass (Live Cell Instrument, catalog #HG-T-Z002) with temperature controller (Live Cell Instrument, CU-301)

Isolation of one-cell stage embryos from seed mice

1. House mice in Individually Ventilated Cages (IVCs) on a 14-10 light cycle (on at 06:00, off at 20:00).
2. Intraperitoneally inject 10–20 donor female mice, each with 5.0 IU PMSG (in 0.1 ml volume) around 12:00 on Day 1.
3. On Day 3, approximately 48 hours after the PMSG injection, intraperitoneally inject each female mouse with 5.0 IU hCG (in 0.1 ml volume). Breed with stud males overnight.
4. On the morning of Day 4 (around 8:00), prepare the following dishes:
   a. Oviduct collection dish: 35 mm tissue culture dish with 2 ml M2 media (one dish per up to 10 females).
   b. Hyaluronidase dish: 35 mm tissue culture dish with 1.5 ml hyaluronidase media (one dish per up to 10 females).
   c. Wash dish: 35 mm tissue culture dish with 1.5 ml M2 media (at least two per injection batch).
   d. KSOM rinse dish: 35 mm tissue culture dish with 1.5 ml KSOM media (pre-equilibrated).

e. Incubation dish: IVF/organ culture dish with 1 ml KSOM (two per injection batch, both pre-equilibrated).

f. Embryo Transfer dish: 35 mm tissue culture dish with 1.5 ml M2 media.

5 Euthanize plugged donor females by your institutional animal care and use committee's approved method. Euthanasia is performed approximately 20 hours after hCG injection (about 8:00 on Day 4).

6 Surgically dissect out the oviducts and place in the oviduct collection dish. Maintain tissue samples at 37°C on a heated slide warmer. Make sure all oviduct dissections are completed in less than 10 minutes after euthanasia.

7 Once all oviducts have been collected, clear the working space and begin dissociating the cumulus-oocyte complexes (COC), one at a time.

The following steps are performed under a dissecting scope maintained at 37°C.

a. Place oviducts in the hyaluronidase dish.

b. Dissect the COCs by disrupting the ampulla with a pair of fine forceps.

c. Continue to process the remaining oviducts while working efficiently.

If all oviducts cannot be processed in under 10 minutes after they are dissected, it is better to plan a dissection of fewer animals in the future.

8 Once the last COC has been expelled from the ampulla, collect individual oocytes from the dish using the mouth pipetting apparatus.

The first set of oocytes should begin to dissociate in the time it takes to harvest the rest of the COCs.

9 Using a 130 μm Flexipet pipette, transfer the oocytes to the wash dish (avoiding as much of the hyaluronidase media and as many of the cumulus cells as possible). This will inactivate the residual hyaluronidase. This step may be repeated a second time in order to remove residual cumulus cells and hyaluronidase.

10 Pool all harvested zygotes into a fresh wash dish. Collect the zygotes one by one using a flexipet pipette. Count the number of zygotes and unfertilized oocytes. Record this information to determine fertilization efficiency.

Zygotes can be distinguished from unfertilized oocytes by the presence of two pronuclei.

11 Transfer only fertilized eggs (zygotes) to the KSOM rinse dish to wash the residual M2 media from the embryos. Next, transfer them to the incubation

dish until needed (typically 30 minutes to 1 hour). Culture the incubation dish at 5% CO_2 to maintain a pH range of 7.23–7.42.

The time until microinjection should not exceed more than two hours, as zygotes that advance past the one-cell stage are no longer suitable for injection.

Microinjection of PITT components into mouse embryos

Microinjection needles: Injection capillaries are made fresh on the morning of the microinjection using the pipette puller. It is essential to use sterile technique to keep the capillaries nuclease-free. The following program is used:

Glass	Heat	Pull	Velocity	Pressure	Time
#TW100F-4	Ramp +5	70	120	200	100 delay

Embryo microinjection: PITT requires both a cytoplasmic and nuclear injection into embryos, because the mRNA must be translated in the cytoplasm and the donor DNA needs to be delivered to the nucleus for targeted integration. This injection approach is very similar to the protocols followed for mouse genome editing using CRISPR/Cas (Harms et al., 2014).

12 Prewash a 28 gauge MicroFil three times with sterile microinjection buffer.

13 Backfill 5–6 injection needles with 1–2 μl of microinjection solution each (from **Basic Protocol 2**, ***Preparation of Microinjection Solution***) using the prewashed MicroFil connected to a 1-cc tuberculin syringe.

14 Affix the injection needle to the microinjector. The remaining prefilled needles should be stored on ice in a needle holder as an additional precaution to prevent RNA degradation during this step.

The needle holder is made from a 150 mm tissue culture dish (Falcon, catalog #351058), outfitted with a 0.25 cm-diameter rod-shaped model of clay. The injection needles filled with the solution are pushed into the clay and the entire storage unit is placed directly in contact with the ice bath.

15 The following parameters are programmed into the Narishige IM 300 microinjector:

Injection pressure	Balance	Hold	Clear	Clear Hold	Inj. time
20 psi	2.2 psi	14 psi	0.20 sec	0.30 sec	0.08 sec

16 Prepare an injection slide using a Lab-Tek chamber and by making two side-by-side 150 μl drops of M2 media. Flatten these drops into discs with a pipette tip to minimize their height. Overlay the flattened drops with ~1 ml of mineral oil. Maintain the temperature at 37°C with the heating glass.

17 We usually inject about 25–30 zygotes per batch. Transfer zygotes to the injection slide. All zygotes must be injected within 10 minutes so the number of zygotes taken per batch depends on efficiency of the injector. A beginner

may start with as few as 4 to 6 per batch and the most experienced technician can inject as many as 50 zygotes in 10 minutes.

18 Check the general morphology of the zygotes under the microscope for presence of zona pellucida, pronuclei, and both polar bodies. Discard embryos that contain more than two pronuclei.

19 Prior to injection, make sure that the needle is open by placing the injection needle next to an embryo.

- **a.** Press the “clear” button on the injector.
- **b.** If the embryo rotates freely, the needle is free of any obstruction.
- **c.** If the embryo does not move, gently remove the tip of the injection needle using a scraping motion against the holding pipette. Check the needle again for flow rate. The needle should be discarded if the embryo moves too much, but can be used if the embryo rotates freely. If the needle is still obstructed, try breaking off more of the tip.

20 Using the holding micropipet, place the first zygote in position and fix by applying negative pressure.

21 Align the embryo and the microinjection needle so that both the opening of the needle and the pronucleus of the embryo are in focus.

22 Perform the microinjection.

- **a.** Maintain positive pressure on the injection needle at all times.
- **b.** Penetrate the zona pellucida and oolemma with the injection needle. Move forward into the closest pronucleus.
- **c.** A slight swelling of the pronucleus may be seen once the plasma membrane is penetrated. Otherwise, press the injection foot pedal to observe a slight swelling of the pronucleus.
- **d.** Retract the tip of the needle to the cytoplasm and inject another volume of microinjection solution. Carefully withdraw the capillary from the zygote if it hasn’t already been removed from the force of the cytoplasm injection.

23 Proceed with all remaining zygotes.

24 After all zygotes have been injected, use the mouth pipetting apparatus to collect and transfer them to the Embryo Transfer dish.

25 Remove lysed zygotes.

26 Incubate surviving zygotes at 37°C in KSOM until embryo transfer (usually within the next 1–2 hours).

Every new batch of microinjection solution should be assessed for toxicity by culturing about 30 injected zygotes overnight. In a successful injection session, 90–95% of zygotes should progress to the two-cell stage. If the solution batch is toxic, extensive lysis may be visible within an hour post injection.

Transfer of injected embryos into pseudo-pregnant mice

27 Transfer injected embryos into pseudo-pregnant female mice.

a. Obtain pseudo-pregnant mice by mating 8–12 week old CD1 females to vasectomized CD1 males on the day before microinjection between 12:00–16:00.

b. On the morning of the injection day, use plug-positive females for oviduct transfers.

Typically, 10–20 CD1 females are bred in each session to obtain an average of 4–8 plugged females.

c. Transfer viable manipulated embryos into the oviducts of pseudo-pregnant foster mothers following established surgical procedures as previously described (Behringer et al., 2014).

About 15–25 injected embryos are transferred per female. The optimal number of embryos transferred is 18 total per female (9 per side).

SUPPORT PROTOCOL 1

Production of fertilized eggs through *in vitro* fertilization (IVF)

As an alternative to steps 1–11 in **Basic Protocol 3**, zygotes may be produced by *in vitro* fertilization using sperm from homozygous PITT seed mice as described here. IVF is advantageous because it reduces the number of stud mice needed and provides more scheduling flexibility for the researcher. We have also found that IVF produces a large number of synchronized and high quality embryos for microinjection.

Please note that the hormone treatment in this IVF protocol is slightly different from the natural mating protocol provided above (e.g., hormone concentrations, doses and timings of hormone administration). This is because these techniques are performed in different laboratories (natural mating: from CB Gurumurthy's laboratory in the United States; IVF: from Masato Ohtsuka's laboratory in Japan). Although slightly different, these protocols seem to work most optimally based on the hormones, animal sources, and housing conditions in these labs.

Materials

Animals

Stud males (homozygous seed mice; 3–6 months old)

A list of seed mouse strains can be found in Table 3.

Egg donors (wild type C57BL/6 female mice; procured at 7 weeks old from CLEA Japan, Inc., Tokyo, Japan).

Homozygous seed female mice (bred in-house), instead of wild type females, can also be used. However, we have found that hemizygous embryos survive better than homozygous embryos after microinjection.

Hormones

Pregnant Mare's Serum Gonadotropin (PMSG; ASKA Animal Health Co., Ltd, Tokyo, Japan)

Human Chorionic Gonadotropin (hCG; ASKA Animal Health Co., Ltd, Tokyo, Japan)

Hormones are supplied as lyophilized vials. These are reconstituted in saline (Otsuka Normal Saline, Otsuka Pharmaceutical Factory, Inc.) to a final concentration of 7.5 IU/0.2 ml and stored at −30°C until use. Each animal is administered 0.2 ml of this solution. The leftover solution is discarded.

Reagents and Solutions

35 × 10 mm Falcon Tissue Culture Dish (Corning, catalog #353001)

HTF (Human Tubal Fluid) (ARK Resource, Kumamoto, Japan)

EmbryoMax M2 Medium (1×), liquid, with phenol red (M2; for embryo handling and microinjection; Millipore, catalog #MR-015-D)

1 ml Tuberculin Syringe with needle (26G × ½″; TERUMO, catalog #SS-01T2613S)

Liquid paraffin (Nacalai Tesque, catalog #26137-85)

Equipment

Standard surgical equipment such as scissors, fine forceps, suturing material, anesthesia chambers, etc. (refer to Behringer et al., 2014).

Dissecting microscope (e.g., Olympus SZ11) with a hot plate (e.g., KM-1, Kitazato)

Heraeus HERAcell 150i Tri–gas incubator, equipped with Coda Inline filters

1. Intraperitoneally inject 7.5 IU of PMSG (in a 0.2 ml volume) into about 15 female mice at 18:00 on Day 1.

   The later injection time for IVF (compared to the earlier hormone treatment for natural mating) allows for the microinjection and surgery procedures to fall at a reasonable time (16:30 – 19:30) on the day of IVF.

2. After 48 hours, on Day 3 at 18:00 pm, intraperitoneally inject 7.5 IU of hCG (in a 0.2 ml volume) into female mice.

3. On the morning of Day 4, (8:00–8:30), about 30 min before egg collection, dissect the cauda epididymides from a stud homozygous male seed mouse as previously described (Takahashi and Liu, 2010)

   Instead of using the freshly-isolated epididymides as a source of sperm, cryopreserved sperm samples may also be used. However, we have observed that using cryopreserved sperm leads to poor fertilization rates and poor quality embryos.

4. Cut the epididymides and use forceps to transfer the sperm into a 35 mm dish containing 37°C HTF medium (300 μl). Incubate the sperm in a 5% CO_2 incubator for 1–1.5 hours to allow for capacitation.

5. During sperm incubation, sacrifice the super-ovulated females following your institutional animal care and use committee's approved method of euthanasia. Surgically remove the oviducts, and place them into 35 mm dishes containing M2 medium.

6. Introduce the egg–cumulus cell complex into the medium by teasing the ampulla of the oviduct with a 26-gauge needle. Transfer the egg–cumulus cell complex to HTF drops (250 μl) and cover with liquid paraffin in a 35 mm dish.

7. At 9:30, add 10 μl cultured sperm to the HTF drop containing oocytes. Using a dissecting microscope, confirm sperm motility. Incubate in a 5% CO_2 incubator to allow IVF to occur.

8. Determine success of IVF 5–6 hours later by observing fertilized pronuclei.

   With freshly isolated sperm, the fertilization rate should be greater than 80%.

9. Proceed to microinjection starting at 16:30 (**Basic Protocol 3, starting at step 12**).

BASIC PROTOCOL 4

Genotyping of offspring to identify transgenic founders

Although performing a Southern blot is traditionally considered the gold standard for confirming successful targeted cassette insertions, PCR is reliable for identifying PITT founder mice. While the exact genotyping strategy depends on the PITT project, primer sets must be designed to amplify both landing pad-DOI junctions in order to identify the precise insertion site. Additional primer sets should be used to probe the DOI. A schematic of this standard PCR-based strategy is shown in Figure 6.

With the exception of the chosen primer set, all genotyping PCRs follow the same standard protocol steps. There are two major steps in this protocol: (a) mouse tail DNA extraction and (b) PCR amplification followed by agarose gel electrophoresis.

Materials

Reagents and solutions

Cell Lysis Soution (Qiagen, catalog #158908)

Proteinase K (20 mg/ml, such as from 5 PRIME, catalog #2900150)

Protein Precipitate solution (Qiagen, catalog #158912)

Ethanol (200 proof ethyl alcohol, such as from Decon Laboratories, catalog #07-678-005)

DNA Hydration Solution (Qiagen, catalog #158914)

PCR 2× master mix (such as GoTaq Hot Start Green Master Mix from Promega [catalog #M5122] or other comparable vendors)

Nuclease-free water (such as from Thermo Fisher Scientific, catalog #BP561-1)

Primer mix

Mix equal volumes of forward and reverse primers from 100 pmol/μl stocks, resulting in a final mix that is 50 pmol/μl with respect to each primer. (To be used at a ratio of 1 μl/100 μl of PCR master mix)

Standard gel electrophoresis reagents including 1% TAE agarose gel with ethidium bromide, 50× Tris-Acetate-EDTA (such as from Thermo Fisher Scientific, catalog #BP1332-20), DNA gel loading dye, and DNA ladder.

Equipment

Heat block

Vortex mixer

Microcentrifuge

Thermocycler (BioRad T100 or equivalent)

Standard equipment for agarose gel electrophoresis such as microwave for melting agarose, gel electrophoresis units, electrophoresis power supplies and gel imaging systems

Mouse tail DNA extraction

1 Collect ~2–3 mm tail pieces from potential founder mice and wild type controls in 1.5 ml microcentrifuge tubes. Add 300 μl Cell Lysis Solution containing 3 μl Proteinase K and incubate at 65°C overnight.

To save time and reagent loss, make a master mix for the lysis solution and Proteinase K. This can then be distributed to 300 µl aliquots in separate microcentrifuge tubes.

2. Cool to room temperature. Add 100 µl of the Protein Precipitation Solution and vortex thoroughly for ~20 seconds.
3. Place the tubes on ice for 2–3 minutes, and then centrifuge at 13,000 rpm for 2–4 minutes.
4. Transfer supernatants to new tubes containing 800 µl of ethanol and mix by inverting the tubes 8–10 times.
5. Centrifuge at 13,000 rpm for 2 to 4 minutes.
6. Discard the supernatant, add 800 µl of 70 % ethanol, invert 8–10 times.
7. Centrifuge at 13,000 rpm for 2–4 minutes, and then discard supernatant.
8. Centrifuge at 13,000 rpm for 1 minute.
9. Manually aspirate the remaining 70% ethanol using a 200 µl pipette tip and air dry the DNA pellet for ~5 minutes (do not exceed 8 minutes).

   It is important to change tips between samples to avoid cross contamination.
10. Add 50 µl DNA Hydration Solution to the pellet and mix by flicking the side of the tube. Incubate the tubes at 65°C for 15–30 minutes to solubilize the DNA.

PCR amplification and agarose gel electrophoresis

11. Perform PCR for all primer sets on potential transgenic founder mice and wild type control DNA samples.
12. Use the PCR 2× master mix manufacturer's protocol to determine the exact parameters for your PCRs. The basic steps for GoTaq Hot Start Green Master Mix are outlined below:
    a. Combine PCR master mix, nuclease-free water, mouse tail DNA (~1 µl, from **step 10**), and primer mix (both forward and reverse) for a total volume of 15 µl/sample.
    b. Run the PCR reactions in a thermocycler. While the annealing temperature may slightly vary depending on the primer set, the following are standard PCR conditions:
       i. 95°C for 2 minutes
       ii. 35 cycles: 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute
       iii. Hold at 4°C.
13. Run all PCR products on a 1% TAE agarose gel.

14 Analyze gels to identify mice genotypes.

c Ensure that no amplicons are visible for any reactions using wild type control DNA samples.

d Identify transgenic founders as mice that demonstrate the correct size amplicons for all assayed primer sets.

Example genotyping results are provided in Figure 6.

COMMENTARY

Background Information

Historical perspectives on random and targeted transgenic technologies—

Transgenesis has revolutionized many fields of biology including genetics, gene regulation, medicine, and bioengineering since its inception in the early 1980s (Jones, 2011). The initial successes in developing this technology were reported by Drs. Jon Gordon and Frank Ruddle, who performed the first genetic transformation by injecting DNA into mouse pronuclei, resulting in the integration and stable germ line transmission of the exogenous DNA (Gordon et al., 1980; Gordon and Ruddle, 1981). However, this technology suffered from the random nature of exogenous DNA integration, including variation in copy number and chromosome position effects at the site of integration, which led to inconsistent gene expression (Hogan, 1983). To overcome this challenge, the next generation of transgenesis techniques involved insertion of transgenes into embryonic stem cells at a specific site in the genome and subsequent injection of targeted ES cells into blastocyst-stage embryos (Gossler et al., 1986). However, this technique required generation of transgenic embryonic stem cells including electroporation/transfection, selection, and screening, in addition to an additional generation of breeding to ensure germline transmission in the chimeric founders. These additional steps made the ES cell-based approach laborious and expensive. Taking the best features from these techniques, we decided to inject transgenic DNA into pronuclei, like traditional transgenesis, but direct integration to a specific site, like ES cell-based approaches. To facilitate targeted integration, our laboratory first developed a seed mouse containing a special landing pad in its genome that would enable efficient and targeted insertion of an injected DOI sequence in the presence of a recombination enzyme (Ohtsuka et al., 2010). We systematically tested and characterized a set of heterotypic *LoxP* sites to direct a DOI to a specific genomic locus using a non-reversible Cre recombination-mediated cassette exchange mechanism, and first described this technique, including the ideal landing pad and compatible DNA elements that would be suitable for such targeted transgenesis, in 2010 (Ohtsuka et al., 2010). Soon after, another group adapted pronuclear injection-based targeted transgenesis to be used with *attB/P* sites and PhiC31 integrase (Tasic et al., 2011). Our lab subsequently improved the PITT tools including developing a multiplexed PITT system that constitutes Cre-*LoxP*, PhiC31-*attB/P*, and Flp-*FRT* systems (Ohtsuka et al., 2015).

Critical Parameters

Here we address critical parameters of the microinjection solution and the seed mouse to maximize PITT efficiency.

Microinjection solution—PITT insertion efficiency and the viability of injected embryos are highly sensitive to the quality and concentration of nucleic acids in the microinjection solution.

Nucleic acid quality: We recommend filtering the nucleic acid solution with the Ultrafree-MC filter prior to preparing the injection mixture to prevent potential clogging of the injection needle. The concentration should be quantified after this step to account for decreased yield post filtration. It is also important to ensure high integrity of the synthesized mRNA. We recommend evaluating mRNA quality by gel electrophoresis both after mRNA synthesis and after injection mixture preparation. It is important to check mRNA quality after mRNA synthesis, because the synthesized mRNA solution contains variable amounts of incompletely synthesized RNAs. As a result, the concentration of the mRNA solution does not fully reflect the number of intact mRNA molecules within a given sample. Incomplete mRNA synthesis can be detected as a smear in the gel. Given that there may be trace RNase contamination in the donor vector solution, the microinjection solution should also be evaluated for mRNA integrity after it has been stored at room temperature or 4°C for 24 hours.

Nucleic acid concentration: Success of PITT is highly dependent on the concentration of nucleic acids. Although we have described the optimal concentrations of mRNA and donor vector for our own system, the ideal concentration should be optimized for each unique system. This is especially important because there is only a narrow range of recombinase/integrase concentrations that will maximize insertion efficiency while preserving embryo survival. Concentrations may vary because the methods of calculating nucleic acid concentration differ depending on the lab preference (NanoDrop, PicoGreen, UV absorbance, etc). In addition, regardless of the concentration of mRNA, the amount of recombinase/integrase translated in injected zygotes depends on the vector used for mRNA synthesis (e.g., Cre vs. iCre, PhiC31 vs. PhiC31o, or vector containing a polyA sequence vs. no polyA sequence and subsequent polyadenylation by the mMESSAGE mMACHINE T7 Ultra Kit).

To determine the optimal concentration of mRNA in your system, inject pronuclei with microinjection solutions that test a range of mRNA concentrations. Develop these pronuclei *in vitro* and check the survival rate at the blastocyst stage (see Supplementary Tables S3 and S4 in Ohtsuka et al., 2010, Table S1 in Ohtsuka et al., 2015). Select the maximum concentration before blastocyst viability is compromised by more than 40–60%. This should be performed for each new batch of mRNA as well as for each unique microinjectionist to account for person-to-person technical variability. Given the investment in time and reagents to perform this optimization, we recommend preparing large batches of mRNA so one optimization can be performed for many experiments.

While we have observed that increasing sizes of the DOI decreases insertion efficiency, we have not empirically defined the acceptable range for donor vector size. To date, we have performed PITT with donor vectors up to 14.4 kb in size (Ohtsuka et al., 2013).

Seed mouse—While we have generated many seed mice that have been used successfully for PITT, we have observed that some of these strains are less efficient at integrating exogenous DOI than others. Seed mouse parameters that may influence efficiency include 1) landing pad locus, 2) recombinase/integrase system, and 3) genetic background.

Landing pad locus: *Rosa26* is the frequently used genomic locus for targeted donor vector insertion because it has been well established as an open chromatin region that is conducive to both efficient DOI integration and expression of inserted genes. However, several other loci can serve as landing pads including *H11*, *H2-Tw3*, *Hprt*, *TIGRE*, *Actb* and *AAVS1*.

Recombinase/integrase system: Although there are three available PITT platforms (Cre, PhiC31, and Flp), only Cre and PhiC31 systems have been proven to work *in vivo*. The Flp recombination system has been successfully used *in vitro*, but not yet in mouse embryos. In our experience, Cre and PhiC31 are equally effective at mediating targeted transgenesis. These two systems demonstrate comparable insertion efficiencies from embryonic stem cell studies (Ohtsuka et al., 2015). Furthermore, combining the Cre and PhiC31 platforms together, as has been done in our TOKMO-3 seed mouse, increases the targeted insertion efficiency (Ohtsuka et al., 2015). To use this *i*mproved PITT (*i*-PITT) strategy, mRNAs from both platforms should be added to the microinjection solution for simultaneous translation of Cre and PhiC31 in injected fertilized eggs.

Seed mouse genetic background: It has been reported that mouse genetic background influences traditional transgenic rates. For example, FVB/N mice are known to be highly susceptible to transgenesis whereas C57BL/6 strains have lower integration efficiencies (Auerbach et al., 2003). While we have not empirically tested the influence of genetic background on PITT, we predict that strain differences may influence insertion efficiency, rate of successful transplantation, and embryo survival. The genetic backgrounds of our seed mice are reported in Table 3.

Troubleshooting

If PITT mice are not obtained for a specific project, the following factors should be checked.

1. Confirm that the donor vector, recombinase/integrase mRNA, and seed mouse are compatible. Ensure that the PITT elements in the DOI, recombinase/integrase of choice, and docking sites in the landing pad are all a part of the same Cre-PITT or PhiC31-PITT platform. To determine reagent compatibility for a given platform, please refer to our suggestions in Tables 2 and 3.
2. Ensure that you have evaluated the quality and concentration of nucleic acids in your microinjection solution. Our suggestions for assessment and optimization may be found in the critical parameters section of this protocol.
3. Consider the possibility that expression of the transgene of interest may affect embryo viability. If embryo health is influenced by expression of a

specific transgene, engineer an inducible construct that will spatially and/or temporally control expression.

If the above factors are satisfactory, then we recommend repeating the microinjection with more (150–200) zygotes. Alternatively, instead of transferring injected fertilized eggs to pseudo-pregnant surrogate females at the two-cell stage, eggs may be cultured *in vitro* until they develop into blastocysts, which can then be genotyped to screen for targeted insertion prior to transplantation. It is also possible to increase the concentration of nucleic acids in the injection solution or inject a greater volume of the solution if embryo viability has not been compromised at the current concentration (determined by if more than 60% of injected zygotes develop into blastocysts and/or more than 15% of transplanted eggs survive to birth).

Anticipated Results

PITT is used to generate targeted transgenic founder lines for a DOI of interest. While the outcome for every PITT project is unique depending on the selected DOI, seed mouse, and recombinase/integrase platform, here we provide the general range of anticipated results for targeted integration and gene expression of the donor cassette.

Targeted integration of donor cassette—Targeted integration efficiency depends on the PITT system used. We have observed efficiencies ranging from 1.9%–62.0% (Ohtsuka et al., 2010, 2012b, 2015). Efficiency will likely be greater than 10% in PITT projects that use mRNA injection instead of plasmid DNA encoding the recombinase/integrase and in projects that simultaneously employ both Cre and PhiC31 systems (Ohtsuka et al., 2013, 2015).

While random insertions could presumably occur using this technology, this only happens rarely compared to traditional transgenesis that uses a linear DNA fragment (~2.4% vs. 10–20%) (Ohtsuka et al., 2010; Fielder et al., 2010). Excluding a minor fraction of cases, almost all PITT mice will have the integrated allele at the expected locus as a result of the proper recombination/integration events. The insertion is clean, without any unintended insertions or deletions of genomic DNA. However, it is important to note that several insertion alleles can be anticipated when simultaneously using several platforms, such as Cre and PhiC31 in *i*-PITT (Ohtsuka et al., 2015).

Insertion of vector sequences at the landing pad—In some currently available PITT designs, insertion of the DOI leaves trace vector backbone sequences near PITT landing pads. These excess sequences include prokaryote-derived elements from the vector that can inhibit stable transgene expression. To ameliorate this problem, many PITT platforms include FRT elements in the donor vectors that allow for genetic removal of these excess sequences by crossing PITT Tg founder mice with transgenic mice carrying a ubiquitously expressed Flp recombinase transgene (Ohtsuka et al., 2010, 2015). An example of a Flp transgenic mouse from JAX Mice that can be used for such purposes is *B6.Cg-Tg(Pgk1-FLPo)10Sykr/J*.

Gene expression of donor cassette—Unlike traditional transgenesis, each transgenic founder generated from a given PITT project will express the inserted transgene to similar levels. This is because the transgene resides in a predetermined locus with a single copy configuration, which allows for consistent expression between transgenic siblings and from generation to generation.

Although a reliable transgene expression pattern can be obtained in PITT mice, the magnitude of cassette gene expression will vary between PITT projects based upon choice of locus, promoter, cDNA, and inclusion of additional sequence elements (such as multiple expression cassettes, microRNA sequences, internal ribosome entry sites, and polyadenylation signals). PITT can be used for ubiquitous expression, as well as tissue-specific expression when the DOI contains a tissue-specific promoter and is integrated into the *Rosa26* locus (Tsuchida *et al*, in press).

The one factor that may hinder reliable gene expression is the presence of proximal prokaryote-derived sequences from the donor vector backbone, which have been integrated as excess cargo in the landing pad. We have shown that the removal of this excess donor vector sequence ensures stable inheritance of cassette gene expression (Ohtsuka et al., 2010).

In the unforeseen case that a transgene does not exhibit the expected expression pattern, it is possible that unannotated regulatory sequences reside in the DOI, such as enhancers or silencers. Such unknown DNA interactions can be overcome by incorporating insulator sequences at the both sides of DOI (Madisen et al., 2015).

Time Considerations

Targeted transgenesis experiments performed through ES cell-based approaches require at least one year to generate chimeric mice, which must be bred to ensure germline transmission of the transgene. On the other hand, PITT takes about 3–4 months to generate a founder mouse line, which invariably transmits the transgene to offspring. A typical time frame for the PITT experimental procedures is outlined below and depicted in Figure 2:

Weeks 1 to 4: Designing and building of PITT donor DNA constructs

Weeks 3 to 6: Synthesis and purification of DNA and RNA components for microinjection

Weeks 4 to 6: Preparation of injection components and initiation of superovulation.

Weeks 7 to 9: Isolation of embryos from seed mice, microinjection of PITT components, and embryo transfer

Weeks 13 to 15: Genotyping of offspring to identify transgenic founders

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KEY REFERENCE

Some sections of this protocol were adapted from Chapter 1 of *Methods in Molecular Biology*: "Development of Pronuclear Injection-Based Targeted Transgenesis in Mice Through Cre-*LoxP* Site-Specific Recombination" (Ohtsuka, 2014).

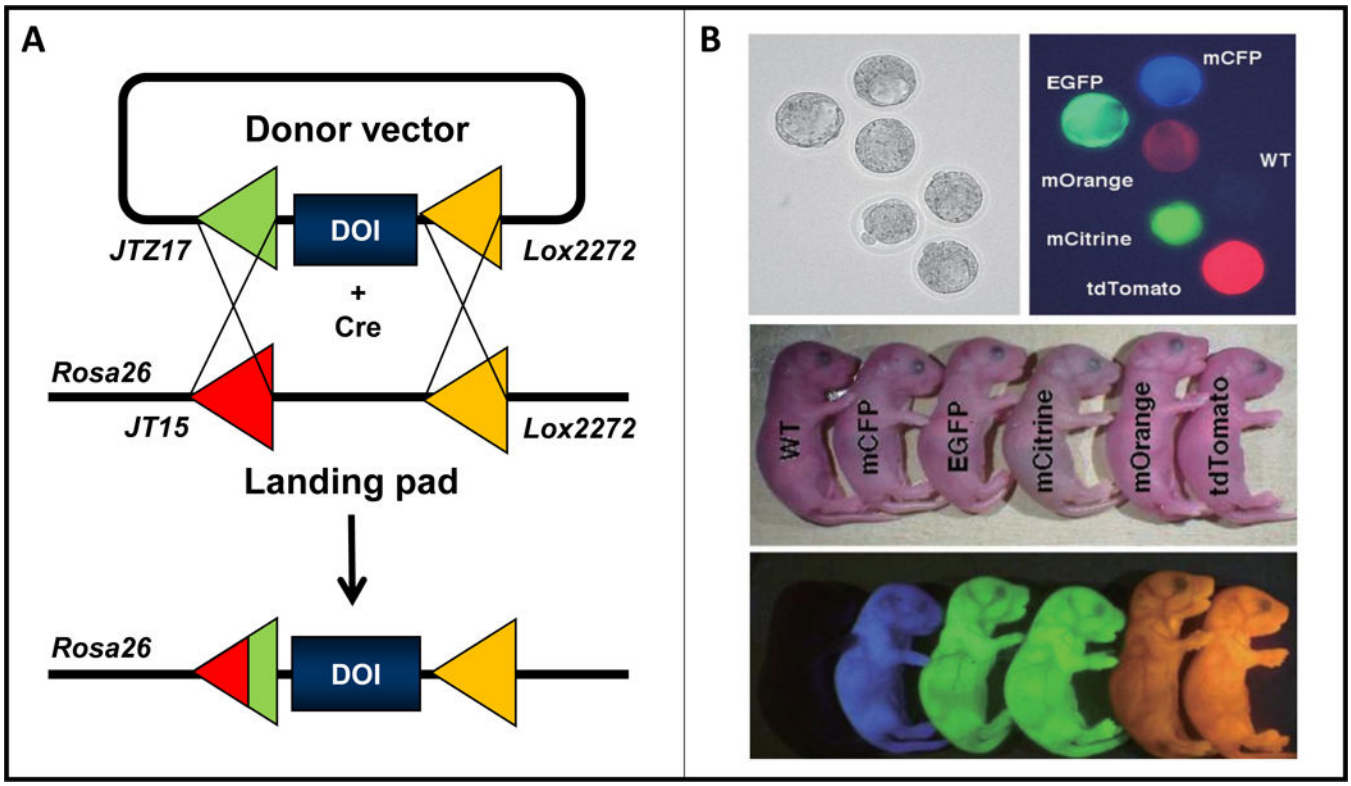


Figure 1. Schematic of the Cre-PITT system

(A) Donor vectors containing a project-specific DNA of interest (DOI) flanked by mutant *LoxP* sites are injected along with Cre (plasmid or Cre-mRNA) into fertilized eggs collected from seed mice. **(B)** Blastocysts (top) and neonates (bottom) resulting from PITT of CAG-driven fluorescent reporters at the *Rosa26* locus exhibit ubiquitous and high expression that is consistent between transgenic littermates and from generation to generation (adapted from Ohtsuka et al., 2010). The PhiC31-PITT platform follows a similar methodology except that it uses PhiC31 (instead of Cre) and *attB/P* (instead of *LoxP* elements).

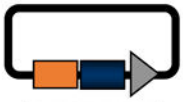
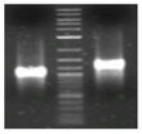
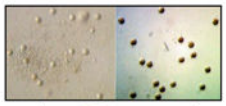
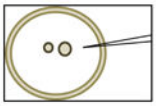
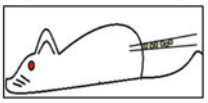
	Protocol Step		Basic Protocol	Time Consideration
1	Designing and building of PITT donor DNA constructs		1	Weeks 1-4
2	Synthesis and purification of DNA and RNA components for microinjection		2	Weeks 3-6
3a	Isolation of one-cell stage embryos from seed mice		3	Weeks 7-9
3b	Microinjection of PITT components into mouse embryos		3	
3c	Transfer of injected embryos into pseudo-pregnant mice		3	
4	Genotyping of offspring to identify transgenic founders		4	Weeks 13-15

Figure 2.
Overview of PITT steps.

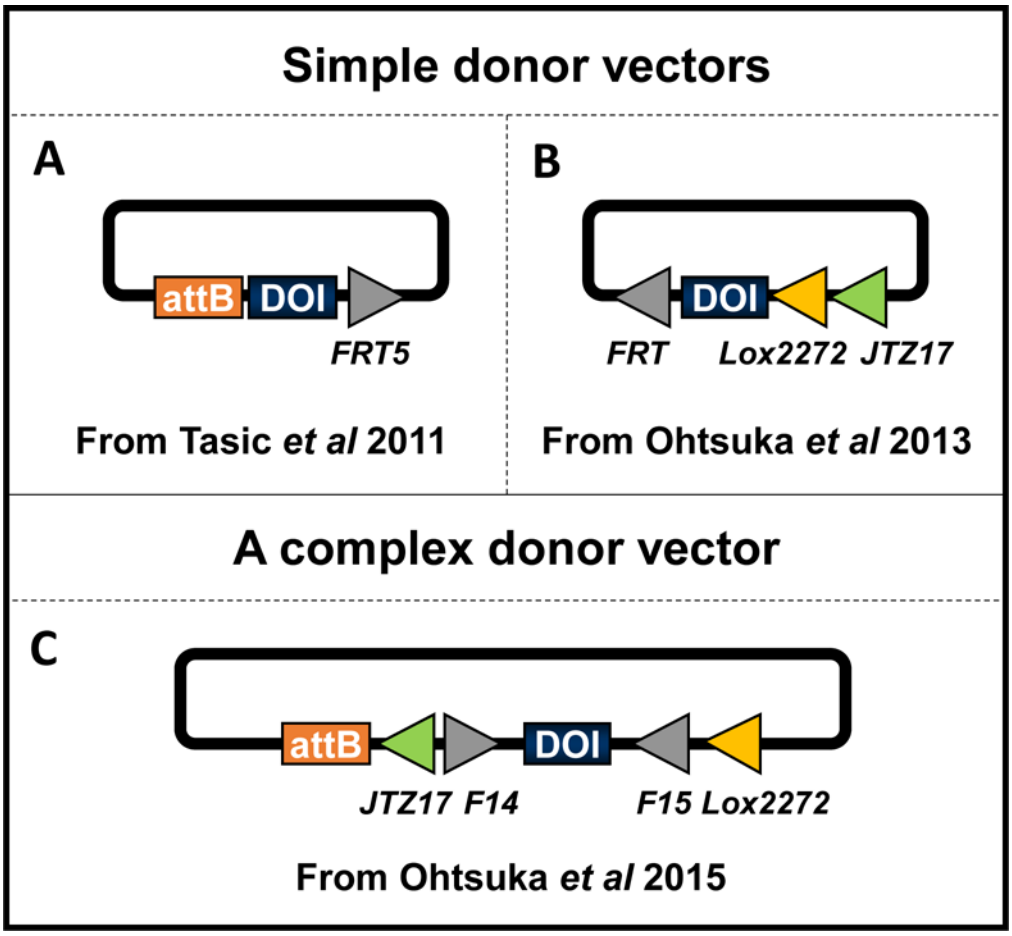


Figure 3. Examples of simple and complex PITT donor vectors

Simple PITT donor vectors contain either **(A)** *attB* elements for the PhiC31 platform or **(B)** *LoxP* elements for the Cre-PITT platform**. (C)** This complex PITT donor vector contains PITT elements for both platforms (Cre-*LoxP* and PhiC31-*attB*). While not a part of the PITT platform, FRT elements in donor vectors serve as a tool for removing extra sequences in the founder mice using Flp recombinase.

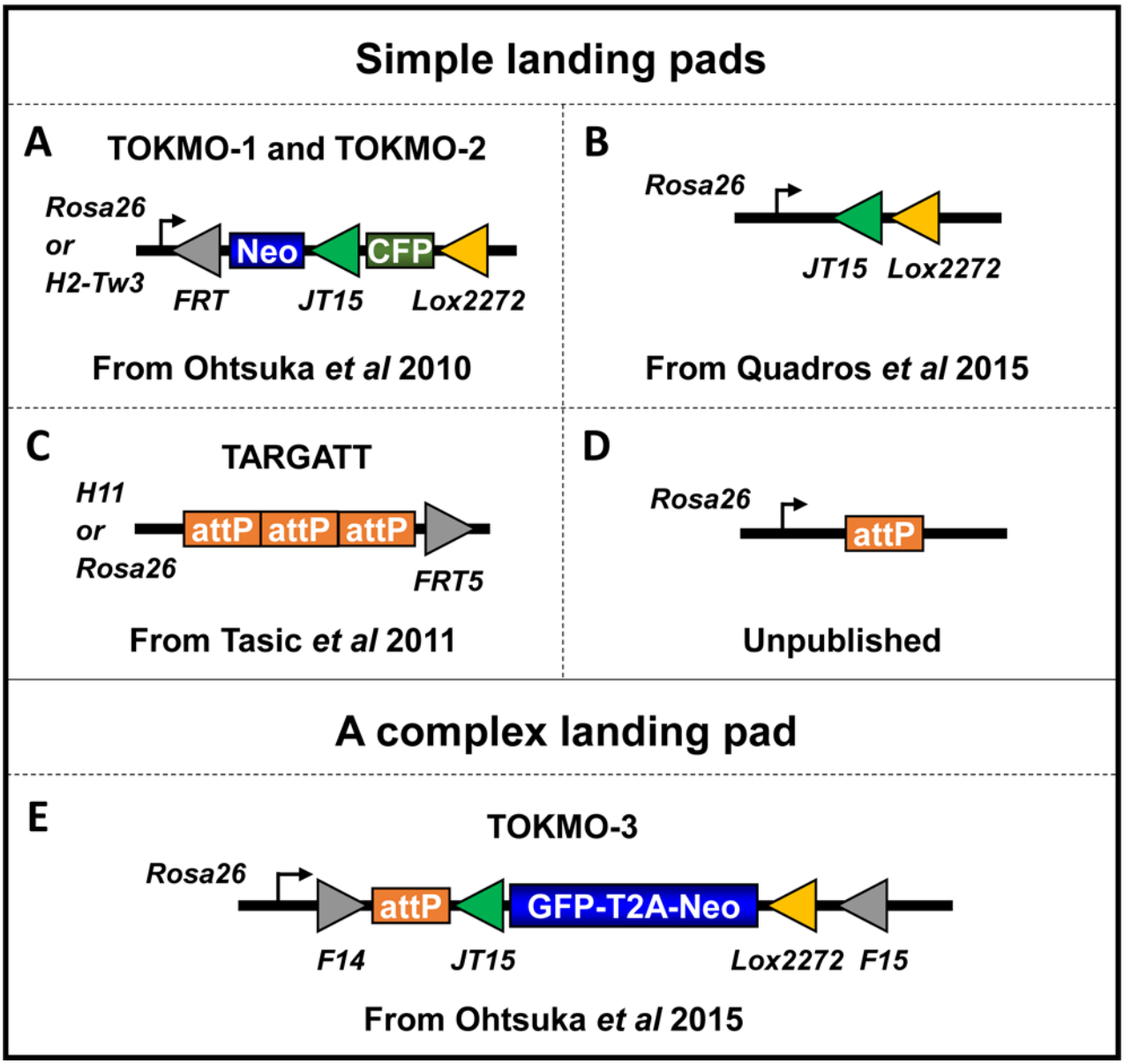


Figure 4. Examples of simple and complex landing pads in seed mouse strains

(A–D) Simple landing pads include the critical elements to facilitate targeted insertion for a single PITT-platform. Examples of landing pads include *LoxP* variant recombination sites (Cre-PITT; **A and B**) or *attP* integration sites (PhiC31-PITT; **C and D**). **(E)** Complex landing pads can include a combination of both *attP* and *LoxP* elements for using PhiC31-PITT and Cre-PITT platforms either independently or together. While not a part of the PITT platform, FRT elements in landing pads serve as a tool for removing extra sequences in the founder mice using Flp recombinase.

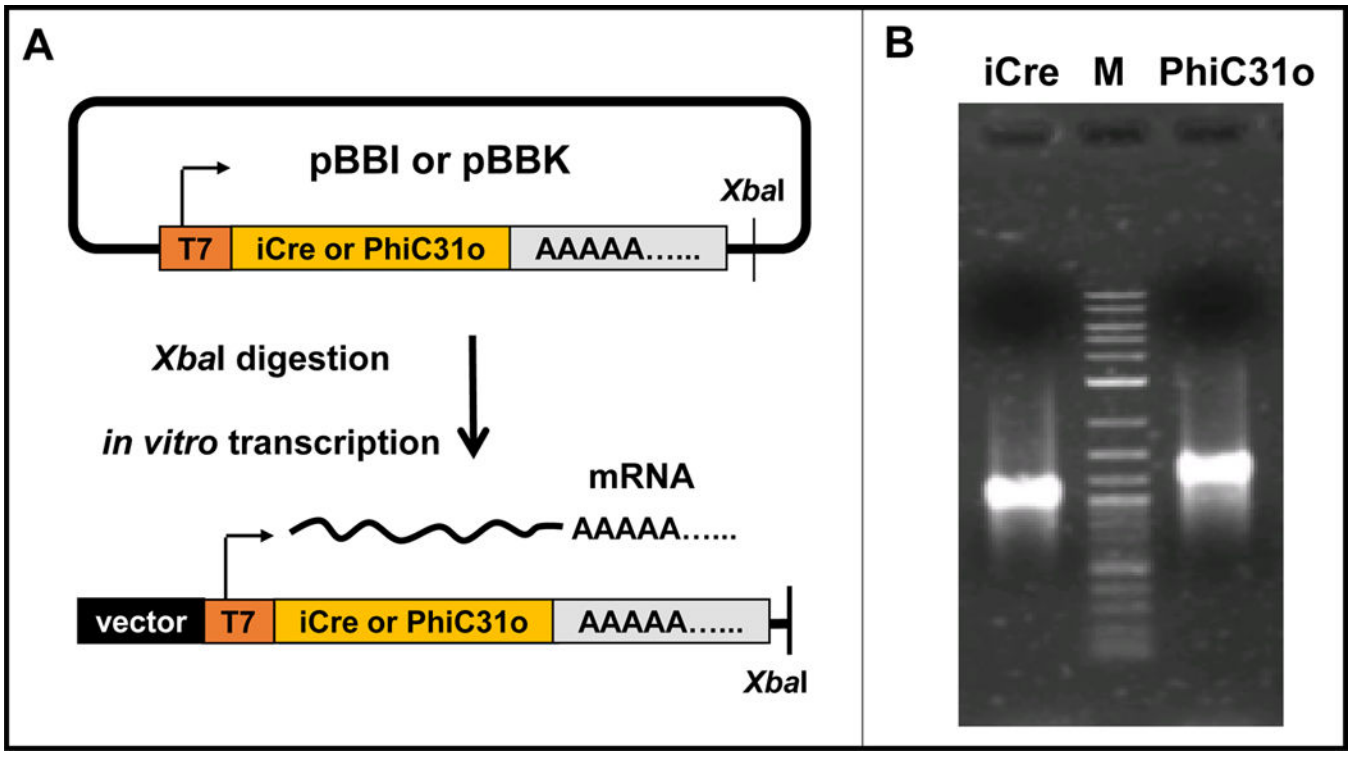


Figure 5. Generation of mRNA encoding Cre recombinase or PhiC31 integrase

(A) Plasmids (pBBI = iCre; pBBK = PhiC31o) are linearized with *Xba*I prior to T7 RNA polymerase-initiated *in vitro* transcription. **(B)** A non-denaturing agarose gel image shows 1 μg of synthesized mRNAs that are run alongside a 100bp DNA ladder (M). The approximate sizes for iCre and PhiC31o mRNAs are 1.3 kb and 2.1 kb, respectively. Note that the mRNAs do not migrate perfectly with the DNA molecular weight marker. For accurate size analysis, RNA markers may be included in the gel.

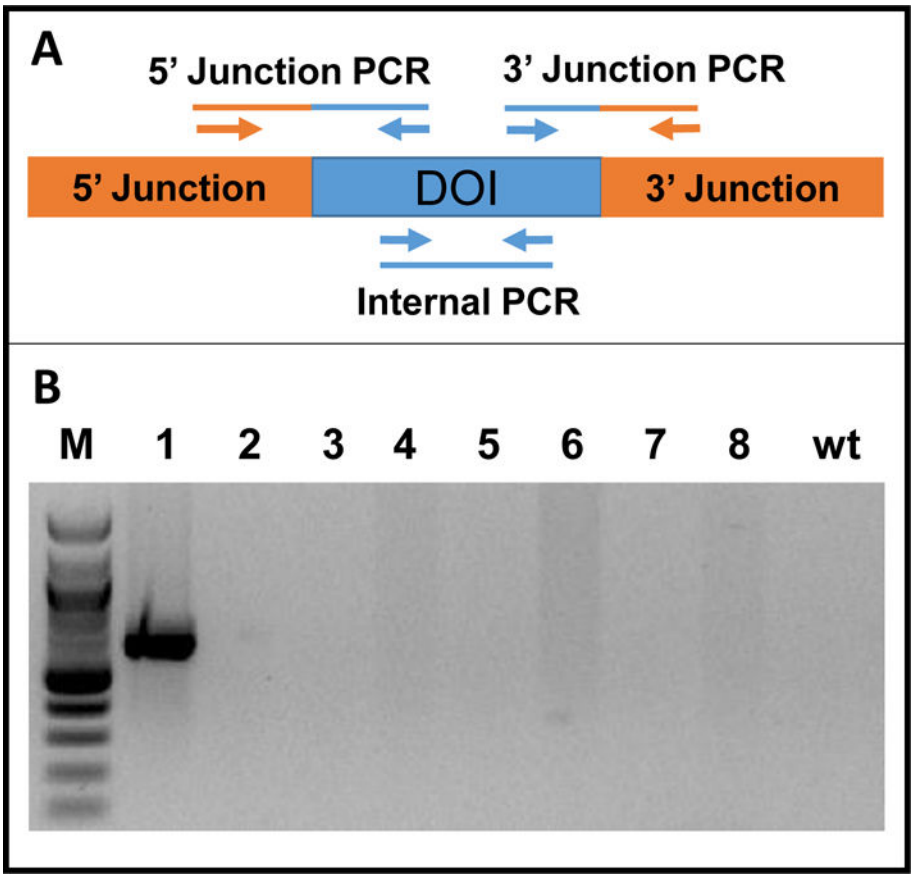


Figure 6. PCR-based genotyping of PITT transgenic founders

(A) Schematic of PCR sets typically used for genotyping: 5′ junction PCR, 3′ junction PCR, and internal PCR. Primer sets are designed to amplify 200–800 bp. **(B)** A sample agarose gel run with a 100 bp ladder (M) that demonstrates a transgenic founder identified by a 5′ junction PCR (lane 1) compared to other tested mice (2–8) and a wild type control (wt).

Table 1

Sequence elements used in the PITT donor vectors and seed mice

Sequence element in the PITT donor vector			Corresponding element in the landing pad of the seed mouse		Notes
PITT Platform	**Element**	**Sequence**	**Element**	**Sequence**	
Cre-PITT	*Lox2272*	ATAACTTCGTATAGGATACTTTATACGAAGTTAT	*Lox2272*	ATAACTTCGTATAGGATACTTTATACGAAGTTAT	Donor DNA between the *LoxP* variants gets inserted through Cre-recombination-mediated cassette exchange.
	JTZ17	ATAACTTCGTATAGCATACATTATAGCAATTTAT	*JT15*	AATTATTCGTATAGCATACATTATACGAAGTTAT	
PhiC31-PITT	*attB*	CCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCCAC	*attP*	GTAGTGCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAG	The entire donor plasmid gets inserted through PhiC31-integrase mediated integration.

Table 2

List of representative* PITT donor vectors and plasmids for mRNA synthesis

Vector name (Available from)	DNA of interest (DOI)	Landing pads (PITT Platform)	Vector features	Restriction site	Compatible seed mouse strains	References
pAOM (Request from Authors)	tdTomato expression cassette, "*CAG-tdTomato-pA*"	*JTZ17, Lox2272* (Cre-PITT)	Contains "*IRES-lacZ-pA*" and "*CAG-hyg-pA*" cassettes in a pUC119-based vector. Has amp^R for ampicillin resistance and *FRT* sites for Flp-mediated extra sequence excision.	tdTomato can be replaced with a different DOI using *Age*I and *Fse*I.	TOKMO-1 TOKMO-2	(Ohtsuka et al., 2010)
pAOT (Request from Authors)	eGFP expression cassette with a synthetic miRNA against the gene encoding tyrosinase, "*CAG-eGFP-miR(Tyr-1/2)-pA*"	*JTZ17, Lox2272* (Cre-PITT)	Contains "*IRES-lacZ-pA*" and "*CAG-hyg-pA*" cassettes in a pUC119-based vector. Also has amp^R for ampicillin resistance and *FRT* sites for Flp-mediated extra sequence excision.	eGFP can be replaced with a different reporter using *Age*I and *Bsr*GI. The miRNA can be replaced with a different miRNA using *Bam*HI and *Bgl*II	TOKMO-1 TOKMO-2	(Ohtsuka et al., 2010)
pA748 (Request from Authors)	Sucrose counterselection cassettes with GFPuv, "*sacB-GFPuv-sacB*" cassette	*JTZ17, Lox2272* (Cre-PITT)	Contains "*IRES-eGFP-pA*" and "*CAG-hyg-pA*" cassettes in a pUC119-based vector. Has amp^R for ampicillin resistance and *FRT* sites for Flp-mediated extra sequence excision.	The DOI cassette can be replaced with a different DOI using *Not*I.	TOKMO-1 TOKMO-2	(Ohtsuka et al., 2010)
pAWV (Addgene #62710)	tdTomato expression cassette with a synthetic miRNA against the GFP gene, "*CAG-tdTomato-miR(eGFP)-pA*"	*JTZ17, Lox2272* (Cre-PITT)	pBR322-based vector. Contains a "*CAG-FLPe-pA*" cassette to aid in self-removal of extra sequence by Flp-recombination. Has amp^R for ampicillin resistance.	The entire DOI cassette can be replaced with a different DOI using *Not*I. The miRNA region can be replaced with a different miRNA using *Sal*I and *Bgl*II.	TOKMO-1 TOKMO-2	(Ohtsuka et al., 2013; Miura et al., 2015)
pAWK (Addgene #62713)	Expression cassette for synthetic miRNA against the GFP gene, "*CAG-miR(eGFP)-pA*"	*JTZ17, Lox2272* (Cre-PITT)	pBR322-based vector. Contains a "*CAG-FLPe-pA*" cassette to aid in self-removal of extra sequence by Flp-recombination. Has amp^R for ampicillin resistance.	The entire DOI cassette can be replaced with a different DOI using *Not*I. The miRNA region can be replaced with a different miRNA using *Sal*I and *Bgl*II.	TOKMO-1 TOKMO-2	(Ohtsuka et al., 2013; Miura et al., 2015)
pBFD (Request from Authors)	Dre expression cassette from the Thy1 promoter, "*Thy1-Dre-pA*"	*JTZ17, Lox2272* (Cre-PITT)	pBR322-based vector. Has amp^R for ampicillin resistance and *FRT* sites for Flp-mediated extra sequence excision.	The entire DOI cassette can be replaced with a different DOI by *Eag*I.	TOKMO-1 TOKMO-2	(Ohtsuka et al., 2013)
pBDR (Addgene #62663)	Promoterless tdTomato cassette, "*tdTomato-pA*"	*JTZ17, Lox2272, attB* (Cre-PITT and/or PhiC31-PITT)	pIDTSMART-based vector. Has kan^R for kanamycin resistance and mutant *FRT* sites (*F14* and *F15*) for Flp-mediated extra sequence excision.	The entire DOI cassette can be replaced with a different DOI using *Age*I and *Eco*RI. The tdTomato can be replaced using *Age*I and *Fse*I.	TOKMO-3	(Ohtsuka et al., 2015)

Vector name (Available from)	DNA of interest (DOI)	Landing pads (PITT Platform)	Vector features	Restriction site	Compatible seed mouse strains	References
pBHL (Request from Authors)	Promoterless tdTomato cassette, "*tdTomato-pA*"	*JTZ17, Lox2272, attB* (Cre-PITT and/or PhiC31-PITT)	This plasmid is derived from pBDR but the order of *attB* and *JTZ17* is reversed.	The entire DOI cassette can be replaced with a different DOI using *Age*I and *Eco*RI. The tdTomato can be replaced using *Age*I and *Fse*I.	TOKMO-3	Unpublished
pBBI (Addgene #65795)	iCre expression cassette, *"T7-iCre-AAAA…"*	–	To be used for iCre mRNA synthesis. It is derived from pcDNA3.1 and has amp^R for ampicillin resistance.	Linearize with *Xba*I before mRNA synthesis.	–	(Ohtsuka et al., 2013)
pBBK (Addgene #62670)	PhiC31o expression cassette, *"T7-PhiC31o-AAAA…"*	–	To be used for PhiC31o mRNA synthesis. It is derived from pcDNA3.1 and has amp^R for ampicillin resistance.	Linearize with *Xba*I before mRNA synthesis.	–	(Ohtsuka et al., 2015)

Key: *AAAA…* = polyA stretch; amp^R = ampicillin resistance cassette; *CAG* = synthetic cytomegalovirus (CMV) early enhancer/ chicken ß-actin promoter/ rabbit ß-globin splice acceptor site for strong expression in mammalian cells; DOI = DNA of interest; *Dre* = encodes phage D6 recombinase; *eGFP* = encodes enhanced green fluorescent protein; *FLPe* = encodes enhanced FLP recombinase; *GFPuv* = encodes ultraviolet light-excitable green fluorescent protein; *hyg* = hygromycin-resistance cassette; *iCre* = encodes a codon-improved Cre recombinase; *IRES* = internal ribosome entry site; kan^R = kanamycin resistance cassette; *lacZ* = encodes ß-galactosidase; *pA* = polyA sequence; *PhiC31o* = encodes a codon-optimized PhiC31 integrase; *puro* = puromycin resistance cassette; *sacB* = encodes levansucrase and confers sensitivity to sucrose as counterselection; *T7* = T7 prokaryotic promoter; *tdTomato* = encodes a red fluorescent protein; *Thy1* = promoter frequently used for expression in neurons.

* Please note that this is not a comprehensive list of PITT donor vectors or plasmids for mRNA synthesis. Other versions of available plasmids may be found in our previous publications (Ohtsuka et al., 2010, 2012a, 2013, 2015). In addition, the construction of donor vectors for the R26 Cr4 mouse lines (described in Table 3) is in progress and these vectors can be requested from the authors. There are also other PhiC31-based vector systems reported in Tasic et al., 2011, which are available through Charles River Laboratories, USA, under the trade name TARGATT™ system.

Table 3

List of representative* PITT seed mouse strains

Common name	Strain name (Genetic background)	Landing pads used (PITT Platform)	Locus	Available from	References
TOKMO-1	*Gt(ROSA)26Sor<tm1Maoh>* (129/C57BL/6J mixed)	*JT15, Lox2272* (Cre-PITT)	*Rosa26*	Authors	(Ohtsuka et al., 2010)
TOKMO-2	*H2-T3<tm1Maoh>* (129/C57BL/6J mixed)	*JT15, Lox2272* (Cre-PITT)	*H2-Tw3*	Authors	(Ohtsuka et al., 2010)
R26 Cr4-Cre-PITT	*CRISPR(ROSA)(Cre-PITT)CBG>* (129/C57BL/6N mixed)	*JT15, Lox2272* (Cre-PITT)	*Rosa26*	Authors	(Quadros et al., 2015)
R26 Cr4-PhiC-PITT	C57BL/6N- *CRISPR(ROSA)(Cre-PITT)CBG>* (C57BL/6N)	*attP* (PhiC31-PITT)	*Rosa26*	Authors	Unpublished
TOKMO-3	C57BL/6N-*Gt(ROSA)26Sor<tm10(PITT)Maoh>* (C57BL/6N)	*JT15, Lox2272, attP* (Cre-PITT and/or PhiC31-PITT)	*Rosa26*	RIKEN BioResource Center, RBRC06517	(Ohtsuka et al., 2015)
R26 Cr4-CrePhiC-PITT	*CRISPR(ROSA)(Cre/PhiC31-PITT)CBG>* (129/C57BL/6N mixed)	*JT15, Lox2272, attP* (Cre-PITT and/or PhiC31-PITT)	*Rosa26*	Authors	Unpublished

* Please note that this is not a comprehensive list of PITT seed mice. Specifically, there are other PhiC31-based seed mice reported in Tasic et al., 2011, which are available through Charles River Laboratories, USA, under the trade name TARGATT™ system.