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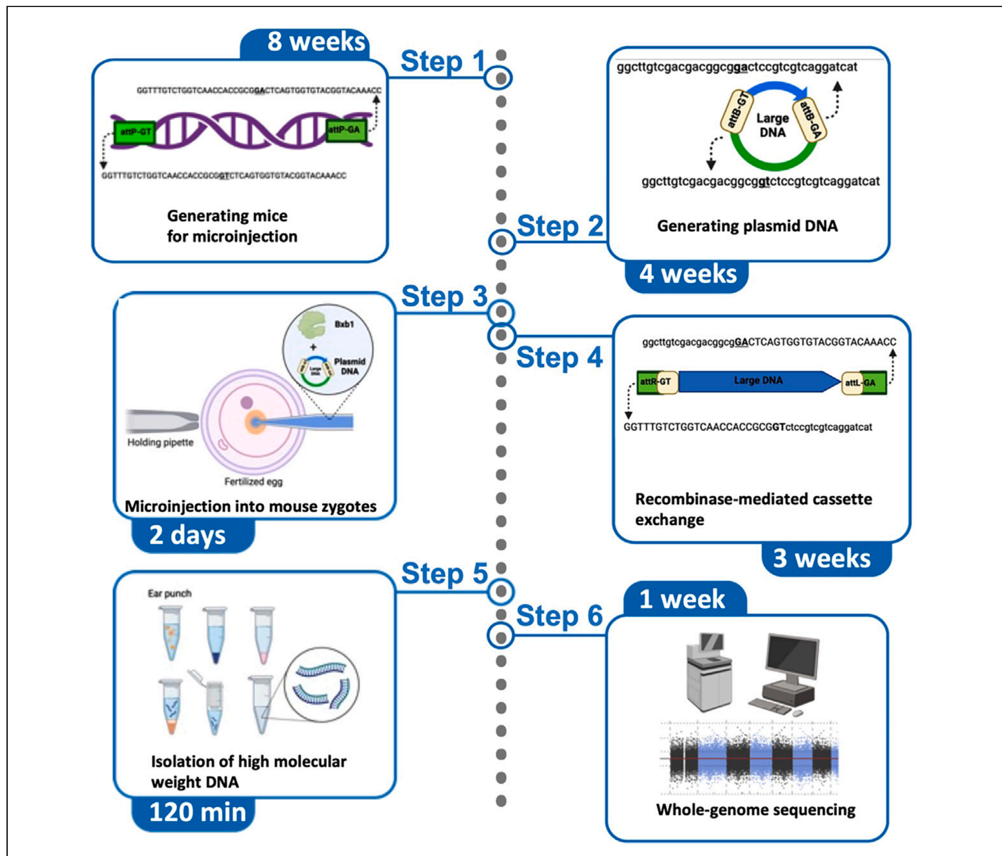
Protocol

# Protocol for large DNA transgenesis in mice using the Cas9+Bxb1 toolbox

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Highlights

Steps to improve efficiency with optimal Bxb1 mRNA and plasmid donor DNA concentrations

Instructions on using PCR for fast and effective screening of transgenic candidates

Guide to obtaining founder mice from a single microinjection with 10%–20% efficiency

Precise integration of DNA constructs greater than 3 kb into mouse zygotes is difficult. Here, we present a protocol for large DNA transgenesis in mice using the Cas9+Bxb1 toolbox. We describe steps for choosing mouse strains with preplaced attachment sites. We then detail procedures for microinjecting mouse zygotes with the plasmid donor DNA construct to generate transgenic mice by recombination-mediated cassette exchange. This protocol has the potential for application in exploring the functional implications of large structural variations in cancer.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

# Protocol for large DNA transgenesis in mice using the Cas9+Bxb1 toolbox

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## SUMMARY

**Precise integration of DNA constructs greater than 3 kb into mouse zygotes is difficult. Here, we present a protocol for large DNA transgenesis in mice using the Cas9+Bxb1 toolbox. We describe steps for choosing mouse strains with pre-placed attachment sites. We then detail procedures for microinjecting mouse zygotes with the plasmid donor DNA construct to generate transgenic mice by recombination-mediated cassette exchange. This protocol has the potential for application in exploring the functional implications of large structural variations in cancer.**

**For complete details on the use and execution of this protocol, please refer to Low et al.[1] and Hosur et al.[2]**

## BEFORE YOU BEGIN

**Timing: 4–8 weeks**

The protocol below describes the specific steps for large DNA transgenesis in the following strains of mice: C57BL/6J, NOD, and NSG. However, this protocol can be applied to any mouse strain with the dual heterologous attachment sites (*attP-GT* and *attP-GA*) in the genome.

1. Based on the research question, choose an appropriate inbred mouse strain (Bxb1.v2 mice) with dual heterologous attachment sites (*attP-GT* and *attP-GA*). For instance, *B6.RosaBxb-GT/GA or NSG.RosaBxb-GT/GA.*

*attP-GT* sequence: GGTTTGTCTGGTCAACCACCGCG**GT**CTCAGTGGTGTACGGTACAAACC

*attP-GA* sequence: GGTTTGTCTGGTCAACCACCGCG**GA**CTCAGTGGTGTACGGTACAAACC.

2. 30 female and 30 male homozygous Bxb1.v2 mice are required for a single microinjection experiment.

*Note:* It takes approximately 4–8 weeks to generate ∼60 Bxb1.v2 mice from four to five homozygous breeding pairs.

3. Make sure the donor DNA plasmid has dual heterologous attachment sites (*attB-GT* and *attB-GA*). Perform maxi-prep and store DNA at −20°C.

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*Note:* The *attP-GT*/*attP-GA* and *attB-GT*/*attB-GA* are complementary or cognate attachment sites.

4. Three days before microinjection, administer 5 IU of PMSG intraperitoneally to 24–28-day-old donor females.
   a. Inject 5 IU of hCG intraperitoneally 47 h after PMSG delivery.
   b. Pair donor females with validated C57BL/6J stud males at a 1:1 ratio immediately after hCG injection.
   c. Check for copulation plugs around 7:00 AM the next morning and separate females with plugs to collect zygotes.
   d. Euthanize via cervical dislocation. Excise oviducts and place them into M2 medium for a brief wash.
   e. Transfer oviducts into M2 medium with 300 μg/mL hyaluronidase.
   f. Release the oocyte clutch by puncturing the ampulla and incubate in hyaluronidase containing M2 medium until cumulus cells detach (usually < 5 min).
   g. Pick up zygotes and transfer them to a dish with fresh M2 medium.

   ⚠ CRITICAL: Examine embryos for viability and fertilization; select healthy and fertilized zygotes.

   h. Wash selected zygotes through two fresh M2 medium washes.
   i. Place the zygotes in microdrops of K-RVCL-50 medium under mineral oil within a COOK MINC incubator and incubate for 24 h until they are ready for microinjection.
5. On the day of microinjection, pull a glass capillary tube using a micropipette puller to create a fine needle.
   a. Set up a microinjection system comprising a microscope, micromanipulators, and a microinjector.
   b. Clean microscope slides to serve as the injection platform.
   c. Transfer collected zygotes to 150–200 μL of M2 media on a microscope slide.
   d. Use a holding pipette to stabilize the zygote while positioning it for injection.

   ⚠ CRITICAL: Ensure proper alignment of the needle tip with the pronucleus's focal plane. Visual confirmation of entry into the pronucleus is achieved by observing swelling.

   e. Once the injection process is complete, collect the zygotes and gently wash them in three separate 30 μL drops of equilibrated K-RCVL.
   f. Subsequently, place them in culture within a COOK MINC benchtop incubator set at 37°C, 5% $CO_2$ in a humidified atmosphere.
   g. Following the microinjection, move the altered zygotes to the oviducts of the pseudopregnant mice. For further details, refer to Qin et al.[3] and Low et al.[4]

   *Note:* Do not place more zygotes on a slide than can be injected within a 20- to 30-min timeframe.

   ⚠ CRITICAL: Ensure high-quality pseudopregnant recipients for accommodating injected zygotes.

   ⚠ CRITICAL: Conduct transfers on the same day of injection, preferably at the one-cell stage, to minimize the zygotes' time in an artificial milieu. Throughout the process, follow sterile techniques diligently to avoid contamination.

6. All the experiments must be approved and conform to the relevant institutional guidelines and regulations.

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### Institutional permissions

The Institutional Animal Care and Use Committee (# 22022) at The Jackson Laboratory approved all experimental procedures.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| Bxb1 mRNA at 1,000 ng/µL | TriLink (Custom order) | For Bxb1 mRNA sequence, refer to materials and equipment table. |
| RNasin Plus ribonuclease inhibitor | Promega | Cat# N261A |
| SDS, 20% solution, RNase-free | Ambion | Cat# AM9820 |
| Proteinase K molecular biology grade | New England Biolabs | Cat# P8107S |
| Cresol Red | Sigma-Aldrich | Cat# 114472 |
| Nuclease-free water | Integrated DNA Technologies | Cat# 11-05-01-04 |
| IDTE pH 8 (10 mM Tris, 0.1 mM EDTA) | Integrated DNA Technologies | Cat# 11-05-01-09 |
| IDTE pH 7.5 1× TE (10 mM Tris, 0.1 mM EDTA) solution | Integrated DNA Technologies | Cat# 11-05-01-05 |
| RNaseZap RNase decontamination solution | | Cat# AM9780 |
| SeaKem LE agarose | Lonza | Cat# 50004 |
| 10× TAE buffer | Thermo Fisher Scientific | Cat# 15558042 |
| Quick-Load Purple 1 kb DNA ladder (size range: 500 bp to 10 kb) | New England Biolabs | Cat# N0552L |
| SYBR Safe DNA gel stain | Thermo Fisher Scientific | Cat# S33102 |
| PMSG—Pregnant mare serum gonadotrophin | ProSpec | HOR-272 |
| Human chorionic gonadotropin (hCG) | ProSpec | HOR-250 |
| Hyaluronidase | Sigma | H3506-1G |
| M2 Mouse embryo handling medium | Zenith Biotech | ZFM2-100 |
| Bovine serum albumin (used at 1 mg/mL with M2 medium) | Sigma-Aldrich | A2153 |
| Embryo culture medium | Cook Medical | K-RVCL-50 |
| Sterile-filtered Tyrode's solution, acidic | Sigma-Aldrich | T1788 |
| Silicone fluid | Clearco | PSF-20cSt |
| Opti-MEM reduced serum medium | Gibco | Cat# 31985 |
| KSOM medium KSOMaa, Evolve | Zenith Biotech | ZEKS-050 |
| 10-beta competent *E. coli* | New England Biolabs | Cat# C3019H |
| SOC outgrowth medium | New England Biolabs | Cat# B9020S |
| Terrific broth | RPI | Cat# T15050-1200 |
| Carbenicillin disodium salt | MP Biomedicals | MP21950922 |
| Kanamycin sulfate | Thermo Fisher Scientific | Cat# 15160054 |
| Critical commercial assays | | |
| Phusion high-fidelity DNA polymerase | New England Biolabs | M0530L |
| Q5 Site-Directed Mutagenesis Kit | New England Biolabs | Cat# E0554S |
| HighPrep PCR; 50 mL | MagBio | AC-60050 |
| Plasmid Maxi Kit | QIAGEN | Cat# 12163 |
| Monarch Plasmid Miniprep Kit | New England Biolabs | Cat# T1010L |
| dsDNA Broad Range Qubit kit | Thermo Fisher Scientific | Q32853 |
| Genomic DNA TapeStation kit | Agilent | 5067-5366 |
| Monarch HMW DNA extraction kit for tissues | New England Biolabs | Cat# T3060L |
| Experimental models: Organisms/strains | | |
| Mouse: *B6.RosaBxb-GT/GA* | The Jackson Laboratory | Strain# 36152 |
| Mouse: *B6A.RosaBxb-GT/GA* | The Jackson Laboratory | Strain# 36153 |
| Mouse: *NOD.RosaBxb-GT/GA* | The Jackson Laboratory | Strain# 36181 |
| Mouse: *NSG.RosaBxb-GT/GA* | The Jackson Laboratory | Strain# 36151 |
| Software and algorithms | | |
| SnapGene | SnapGene | https://www.snapgene.com/ |

(*Continued on next page*)

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| --- | --- | --- |
| *Continued* | | |
| CodonCode Aligner | CodonCode Corporation | https://www.codoncode.com/aligner/ |
| NEBaseChanger version 1.3.3 | New England Biolabs | https://nebasechanger.neb.com/ |
| MFE Primer3 | Wang et al.[5] | https://mfeprimer3.igenetech.com/spec |
| Other | | |
| Autoclave | PHCbi, PHC Holdings | MLS-3751L-PA |
| Cook MINC Benchtop incubator | NA | NA |
| Shaker incubator | NA | NA |
| Microbiological incubator | Fisher Scientific | Fisherbrand Isotemp |
| Microwave oven | Fisher Scientific | Panasonic |
| Vortex mixer | Sigma-Aldrich | Z258423 |
| Sorvall RC 6 Plus superspeed centrifuge | Thermo Scientific | NA |
| Isotemp 205 digital water bath (65°C). | Fisher Scientific | FS-205 |
| Qubit fluorometer | Thermo Fisher Scientific | Q32857 |
| InGenius3 gel imaging and analysis system | Syngene | NA |
| C1000 Touch thermal cycler | Bio-Rad | 1851196 |
| Owl wide gel electrophoresis system | Thermo Fisher Scientific | D3-14 |
| Electrophoresis power supply | Fisher Scientific | FB300Q |
| Eppendorf benchtop centrifuge 5418R | USA Scientific | 4054-1137 |
| Petri dishes; 35 × 10 mm | Thermo Fisher Scientific | Cat# 53066 |
| Nunc cryogenic tubes, 1.8 mL | Thermo Fisher Scientific | Cat# 375418PK |
| ThermoMixer C | MilliporeSigma | EDPH99A536FE |
| ECM 830 square wave electroporation system | Harvard Apparatus | UX-02894-18 |
| 14 mL Falcon round-bottom tubes | Fisher Scientific | Cat# 14-959-11B |
| 96-well slotted ring magnet plate | Alpaqua | 001219, 96-well |
| Injectors: Narishige | Tritech Research, Inc. | IM-5A, IM-11-2 |
| Micropipette puller | Sutter Instrument Company | Model-P97 |
| Stereomicroscope Discovery.V8 | Zeiss | |
| Inverted microscope AxioObserver.D1 | Zeiss | |
| Micromanipulators | Eppendorf NK2 | |
| Thin-wall capillary tubes with filament | World Precision Instruments | TW100F-4 |
| Thin-wall capillary tubes without filament | World Precision Instruments | TW100-4 |
| Glass coverslips | Fisher Scientific | 12–545J |
| Aspirator tube assembly | Sigma-Aldrich | A5177 |
| Kimble pellet pestle motor | Fisher Scientific | K749540 |
| Agilent TapeStation | Agilent | G2991BA |
| Corning 70 µm sterile cell strainers | Fisher Scientific | 07-201-431 |
| Nalgene centrifuge bottles (wide mouth with sealing caps Style 3141, 250 mL capacity) | Sigma-Aldrich | Z353736 |
| Dissecting microforceps | Fisher Scientific | 08-953F |
| Electroporation cuvettes, sterile, 1 mm gap | Harvard Apparatus | 45-0124 |
| TempAssure PCR Flex-Free 8-tube strips | USA Scientific | *1402-4708* |
| RNase-, DNase-, and pyrogen-free Microcentrifuge tubes | VWR | NA |
| Microcentrifuge for 1.5/2.0/0.5 mL tubes and 0.2 mL tubes | USA Scientific | NA |
| Rainin LTS pipettes P1000, P200, P20, P2 | RAININ | NA |
| Rainin LTS compatible filtered pipette tips: 10 and 200 µL, 1,000 µL | Biotix/Fisher Scientific | NA |
| Nunc 15 mL conical sterile polypropylene centrifuge tubes | Thermo Fisher Scientific | Cat# 339651 |
| Nunc 50 mL conical sterile polypropylene centrifuge tubes | Thermo Scientific | Cat# 339653 |
| 5 mL, 10 mL, 25 mL, 50 mL sterile serological pipets. | Greiner Bio-One | NA |
| Microcentrifuge for 1.5/2.0/0.5 mL tubes and 0.2 mL tubes | USA Scientific | NA |

## MATERIALS AND EQUIPMENT

Timing: 2 h

| 1 L Pro-K lysis buffer pH 8.0 | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| 1 M Tris pH 8.0 | 10 mM | 10 mL |
| 5 M NaCl | 100 mM | 20 mL |
| 0.5 M EDTA | 10 mM | 20 mL |
| 10% SDS | 0.5% | 50 mL |
| Tween 20 | 0.05% | 0.5 mL |
| IDT Nuclease-Free Water | | 899.5 mL |
| Total | | 1000 mL |

Store at 24°C for up to 12 months.

*Note:* Add 1.5 μL of Proteinase K to 100 μL Pro-K lysis buffer.

| 5 mM Cresol Red solution | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Cresol Red | N/A | 1 g |
| Sucrose | N/A | 300 g |
| IDT Nuclease-Free Water | N/A | Up to 500 mL |

*Note:* Store in 1 mL aliquots at −20°C for up to 12 months.

| 20 mL Ready-to-use Phusion High-Fidelity DNA Polymerase PCR Mix | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| IDT Nuclease-Free Water | N/A | 13.4 mL |
| 5× High-Fidelity buffer **OR** | 1× | 4 mL |
| 5× High-Fidelity GC buffer | | |
| 10 mM dNTPs | 200 μM | 0.4 mL |
| Phusion High-Fidelity DNA Polymerase | 1 unit | 0.2 mL |
| Cresol Red | 5 mM | 2 mL |
| Total | | 20 mL |

20 mL PCR Mix is sufficient for ~1000 PCR reactions.
Dispense 19 μL of the Phusion PCR Mix into 0.2 mL PCR tubes and store them at −20°C for up to 12 months.

*Note:* Do not include primers in the PCR mix.

**Bxb1 sequence (Addgene # 51271):** ATGCCAAAAAAGAAAAGAAAAGTGTATCCCTATGATGT
CCCCGATTATGCCGGTTCAAGAGCCCTGGTCGTGATTAGACTGAGCCGAGTGACAGACGCCA
CCACAAGTCCCGAGAGACAGCTGGAATCATGCCAGCAGCTCTGTGCTCAGCGGGGTTGGGAT
GTGGTCGGCGTGGCAGAGGATCTGGACGTGAGCGGGGCCGTCGATCCATTCGACAGAAAGA
GGAGGCCCAACCTGGCAAGATGGCTCGCTTTCGAGGAACAGCCCTTTGATGTGATCGTCGCC
TACAGAGTGGACCGGCTGACCCGCTCAATTCGACATCTCCAGCAGCTGGTGCATTGGGCTGA
GGACCACAAGAAACTGGTGGTCAGCGCAACAGAAGCCCACTTCGATACTACCACACCTTTTG
CCGCTGTGGTCATCGCACTGATGGGCACTGTGGCCCAGATGGAGCTCGAAGCTATCAAGGAG
CGAAACAGGAGCGCAGCCCATTTCAATATTAGGGCCGGTAAATACAGAGGCTCCCTGCCCCC
TTGGGGATATCTCCCTACCAGGGTGGATGGGGAGTGGAGACTGGTGCCAGACCCCGTCCAGA
GAGAGCGGATTCTGGAAGTGTACCACAGAGTGGTCGATAACCACGAACCACTCCATCTGGTG
GCACACGACCTGAATAGACGCGGCGTGCTCTCTCCAAAGGATTATTTTGCTCAGCTGCAGGGA
AGAGAGCCACAGGGAAGAGAATGGAGTGCTACTGCACTGAAGAGATCTATGATCAGTGAGGC
TATGCTGGGTTACGCAACACTCAATGGCAAAACTGTCCGGGACGATGACGGAGCCCCTCTGG
TGAGGGCTGAGCCTATTCTCACCAGAGAGCAGCTCGAAGCTCTGCGGGCAGAACTGGTCAAG

ACTAGTCGCGCCAAACCTGCCGTGAGCACCCCAAGCCTGCTCCTGAGGGTGCTGTTCTGCGC
CGTCTGTGGAGAGCCAGCATACAAGTTTGCCGGCGGAGGGCGCAAACATCCCCGCTATCGAT
GCAGGAGCATGGGGTTCCCTAAGCACTGTGGAAACGGGACAGTGGCCATGGCTGAGTGGGA
CGCCTTTTGCGAGGAACAGGTGCTGGATCTCCTGGGTGACGCTGAGCGGCTGGAAAAAGTGT
GGGTGGCAGGATCTGACTCCGCTGTGGAGCTGGCAGAAGTCAATGCCGAGCTCGTGGATCTG
ACTTCCCTCATCGGATCTCCTGCATATAGAGCTGGGTCCCCACAGAGAGAAGCTCTGGACGCA
CGAATTGCTGCACTCGCTGCTAGACAGGAGGAACTGGAGGGCCTGGAGGCCAGGCCCTCTG
GATGGGAGTGGCGAGAAACCGGACAGAGGTTTGGGGATTGGTGGAGGGAGCAGGACACCG
CAGCCAAGAACACATGGCTGAGATCCATGAATGTCCGGCTCACATTCGACGTGCGCGGTGGC
CTGACTCGAACCATCGATTTTGGCGACCTGCAGGAGTATGAACAGCACCTGAGACTGGGGTC
CGTGGTCGAAAGACTGCACACTGGGATGTCCTAG.

## STEP-BY-STEP METHOD DETAILS

### Site-directed mutagenesis (SDM)

*NEB Q5 Site-Directed Mutagenesis Kit*

Timing: 7–9 days

Sequential insertion of heterologous attachment sites *attB-GT* and *attB-GA* into the plasmid donor DNA (Figure 1).

1. Use SDM to design and synthesize donor DNA with dual heterologous attachment sites (*attB-GT* and *attB-GA*) (Figure 1) using the Q5 Site-Directed Mutagenesis Kit.
2. The SDM Kit employs Q5 Hot Start High-Fidelity DNA Polymerase.

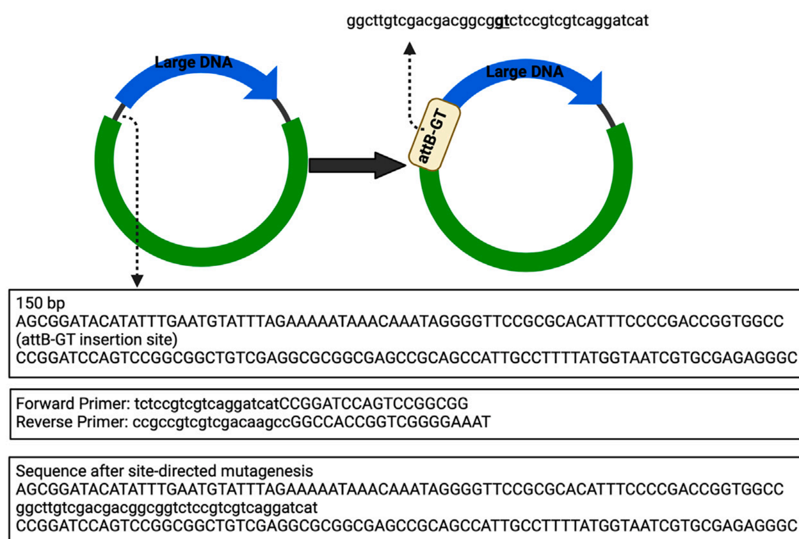
   ***Note:*** Primers are specially designed to enable the integration of attachment sites (*attB-GT* and *attB-GA*) into the given plasmid sequence (Figure 1).

*attB-GT* sequence: 5′ GGCTTGTCGACGACGGCGG**GT**CTCCGTCGTCAGGATCAT 3′.

*attB-GA* sequence: 5′ GGCTTGTCGACGACGGCGG**GA**CTCCGTCGTCAGGATCAT 3′.

3. For Primer Design, the NEBaseChanger version 1.3.3 tool (https://nebasechanger.neb.com/) is utilized.
4. Sequences before and after insertion of attachment sites are shown in Figure 1.
5. Post-PCR, the DNA product undergoes treatment with a unique Kinase-Ligase-DpnI (KLD) enzyme mix, which expedites circularization and template removal at 22°C–24°C.
6. Next, transform the plasmids (maximum size 20 kb) into NEB 5-alpha Competent E. coli cells.
7. Wait for a tube of NEB 10-beta Competent E. coli cells to thaw on ice for 10 min.
8. Add 5 μL (10–20 ng) of the plasmid to competent cells. Gently shake the tube 4–5 times to combine cells and DNA. Avoid vortexing. Keep the mixture on ice for half an hour. Do not stir.
9. Heat shock at 42°C for 30 s. Place on ice immediately for 2 min.
10. Add 950 μL of 22°C–24°C NEB 10-beta/Stable Outgrowth Medium into the mixture.
11. Place at 37°C in a shaker incubator for 60 min.
12. Retrieve selection plates with the appropriate antibiotic from the 4°C refrigerator and pre-warm them at 22°C–24°C for 30–60 min.
13. Spread 10 μL onto a selection plate and incubate overnight at 37°C.
14. Pick colonies using a sterile 200 μL pipette tip and add to 5 mL Terrific broth with appropriate antibiotic.

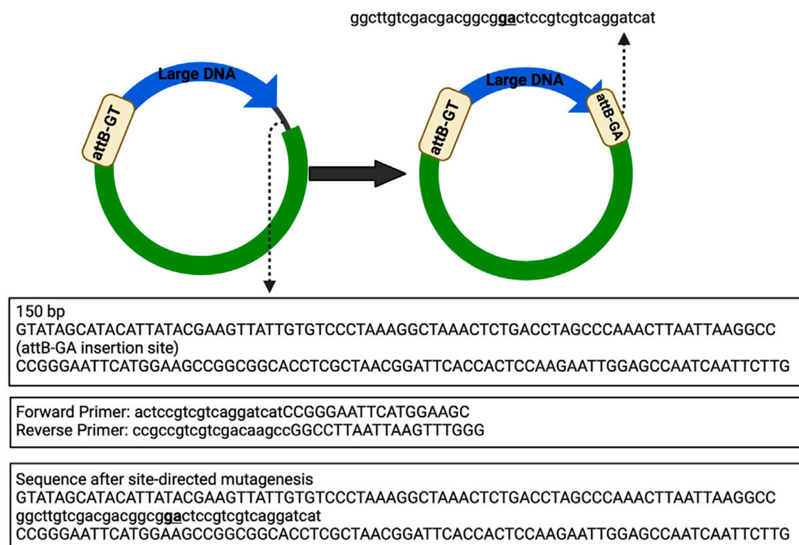
Incubate in a 37°C shaker for 6–8 h. Centrifuge at 3000 × *g* for 5 min, and proceed to DNA Mini-prep.




| STEP 1: PCR | 25 µL reaction |
|---|---|
| Q5 Master Mix | 12.5 µL |
| 10 µM Forward Primer | 1.25 µL |
| 10 µM Reverse Primer | 1.25 µL |
| Plasmid DNA (5 ng/µL) | 1 µL |
| Nuclease-free water | 9 µL |

| STEP | TEMP | TIME |
|---|---|---|
| Initial denaturation | 98°C | 30 sec |
| 30 cycles | 98°C | 10 sec |
| | 72°C | 20 sec |
| | 72°C | 15 sec/kb |
| Final Extension | 72°C | 5 min |
| Hold | 4°C | ∞ |

| KLD treatment* | 10 µL reaction |
|---|---|
| PCR product | 1 µL |
| 2X KLD reaction buffer | 5 µL |
| 10X KLD Enzyme mix | 1 µL |
| Nuclease-Free water | 3 µL |
| *Incubate for 5 min at 24°C | |




| STEP 1: PCR | 25 µL reaction |
|---|---|
| Q5 Master Mix | 12.5 µL |
| 10 µM Forward Primer | 1.25 µL |
| 10 µM Reverse Primer | 1.25 µL |
| Plasmid DNA (5 ng/µL) | 1 µL |
| Nuclease-free water | 9 µL |

| STEP | TEMP | TIME |
|---|---|---|
| Initial denaturation | 98°C | 30 sec |
| 30 cycles | 98°C | 10 sec |
| | 72°C | 20 sec |
| | 72°C | 15 sec/kb |
| Final Extension | 72°C | 5 min |
| Hold | 4°C | ∞ |

| KLD treatment* | 10 µL reaction |
|---|---|
| PCR product | 1 µL |
| 2X KLD reaction buffer | 5 µL |
| 10X KLD Enzyme mix | 1 µL |
| Nuclease-Free water | 3 µL |
| *Incubate for 5 min at 24°C | |

**Figure 1. Sequential site-directed mutagenesis to insert *attB-GT* and *attB-GA* into large donor DNA plasmids for Bxb1-mediated RMCE**

The plasmid undergoes a step-by-step modification process in which attP-GT (A) and attP-GA (B) sites are strategically inserted to flank the target DNA segment. Customized primers are specifically designed using the NEBaseChanger tool for the insertion of sequences. This tool facilitates the primer design process, ensuring precision in site-directed mutagenesis tasks and is specifically tailored for use with the Q5 Site-Directed Mutagenesis Kit. Sequences before and after the successful addition of the attachment sites are presented for comparison. It is important to note that the annealing temperature for the primers is uniquely determined and calculated by NEBaseChanger to complement the optimal conditions required by NEB's Q5 High Fidelity DNA Polymerase.

### Plasmid DNA Mini-prep

Timing: 2 h

Isolate high-quality plasmid DNA from 5 mL bacterial cultures using the Monarch Plasmid Mini-prep Kit.

15. Centrifuge 5 mL of bacterial culture at 1000 × *g* for 5 min, discard the supernatant, and remove any residual buffer.
16. Add 200 μL of Plasmid Resuspension Buffer to the pellet and mix well until no clumps are visible. Then, add 200 μL of Plasmid Lysis Buffer and gently invert the tube 5–6 times.
17. Add 400 μL of Plasmid Neutralization Buffer to neutralize the lysate. Do not vortex. Clarify the lysate by centrifuging for 2–3 min at 11,000 × *g*.
18. Transfer the supernatant to the spin column and centrifuge at low speed (3,000 × *g*) for 1 min.

*Note:* If the supernatant has not eluted completely, centrifuge for another 5 min at low speed. If necessary, increase the speed to 4,000 × *g*. Discard the flow-through.

19. Re-insert the column, add 200 μL of Plasmid Wash Buffer 1, and centrifuge for 1 min at 11,000 × *g*. Discard the flow-through.
20. Add 400 μL of Plasmid Wash Buffer 2 and centrifuge for 1 min at 11,000 × *g*. Discard the flow-through. Transfer the column to a clean 1.5 mL microfuge tube and centrifuge for 2 min at 11,000 × *g*.
21. Transfer the column to a clean 1.5 mL microfuge tube, add 20 μL of DNA elution buffer to the center of the matrix.
22. Wait for 5 min and centrifuge for 2 min at 11,000 × *g*.

*Note:* Heating the DNA Elution Buffer to 65°C improves the yield.

23. Determine the concentration using a Qubit fluorometer and proceed to validation by PCR and Sanger sequencing.

### Screening plasmids—Pcr, purification, and Sanger sequencing

Timing: 1.30 h

PCR strategy for screening plasmids after Site-directed mutagenesis. The strategy typically involves designing at least four sets of primers—a) flanking the *attB-GT* and *attB-GA* sites, b) the transgene, and c) the plasmid donor backbone.

#### *Phusion PCR*

24. Create a 1:10 dilution of Mini-prep DNA in Nuclease-Free Water. Use 2 μL of the diluted DNA for the PCR reaction.
25. Retrieve the 0.2 mL PCR tubes containing the Phusion PCR Mix (19 μL) from the −20°C freezer and place them on ice.
26. To each tube, add 1 μL of primer mix (comprising 0.5 μL of forward primer and 0.5 μL of reverse primer).
27. Incorporate 2 μL of diluted DNA into the mix. Gently mix the contents, centrifuge, and then load the samples into the thermal cycler.
28. Execute the Phusion PCR cycle as specified in Table 1.

**Table 1. Gradient PCR**

| Step | TEMP | Time |
|---|---|---|
| Initial denaturation | 98°C | 30 s |
| 30 cycles | 98°C | 10 s |
| | 48–72°C | 20 s |
| | 72°C | 15 s/kb |
| Final Extension | 72°C | 5 min |
| Hold | 4°C | infinite |

*Note:* Gradient PCR is employed to identify the optimal annealing temperature. Once the annealing temperature is determined, repeat the Phusion PCR at this optimal temperature.

### Magnetic beads purification protocol for PCR products

*MagBio HighPrep PCR*

**Timing: 1 h**

Efficient PCR product purification for downstream applications, including Sanger sequencing.

29. From a 22 μL PCR reaction, run 12 μL on a gel and reserve the remaining 10 μL for purification.
30. To the 10 μL PCR product, add 18 μL of thoroughly mixed beads (using a ratio of 1.8 μL beads per 1 μL PCR product).
31. Mix well, then perform a short spin on a tabletop centrifuge to collect the liquid at the bottom without pelleting the beads.
32. Allow the mixture to rest at 22°C–24°C for a minimum of 10 min.
33. Place the individual 0.2 mL PCR tube or 0.2 mL PCR strip tubes on the magnet and wait for at least 2 min. Note for PCR Strips: Secure the strips to the magnet using an empty 96-well pipette tip rack and a thin rubber band for stability.
34. Perform a wash by adding 100 μL of 70% Ethanol to each sample, then carefully aspirate using 200 μL pipet tips. Repeat this step two more times.
35. After the final wash, aspirate any residual ethanol while keeping the beads undisturbed.
36. Allow the ethanol to evaporate from the samples while they remain on the magnet, typically taking about 10-15 min.
37. Remove the individual 0.2 mL PCR tube or 0.2 mL PCR strip tubes from the magnet, ensure all ethanol has evaporated, then add 40 μL of Nuclease Free water. Mix thoroughly and perform a quick centrifugation.
38. Return the samples to the magnet and wait for at least 5 min.
39. Carefully transfer 5 μL the purified PCR product, without disturbing the beads, to a new PCR tube.
40. Add 1 μL of a 5 μM sequencing primer and submit samples for Sanger sequencing.

*Note:* Custom plasmids with the attachment sites can be synthesized, and sequence verified from reputable vendors.

### Plasmid DNA maxi-prep

*QIAGEN plasmid maxi kit*

**Timing: 2 days**

Fast and cost-effective method for extracting high-quality plasmid DNA from ~250 mL of bacterial culture.

41. Transform validated plasmids with the *attB-GT* and *attB-GA* attachment sites flanking the large Donor DNA.
42. Pick colonies using a sterile 200 μL pipette tip and add to 5 mL Terrific Broth with appropriate antibiotic.
43. Incubate in a 37°C shaker for 6 h and transfer 2 mL to a 2 L Erlenmeyer flask containing 250 mL Terrific broth. Incubate in a 37°C shaker for 16 h.
44. Transfer the bacterial culture into a 500 mL centrifuge and spin at 6000 × *g* for 15 min at 4°C.
45. Before you begin: Combine RNase A solution with Buffer P1, ensure a thorough mix, and store the mixture between 2°C–8°C.
    a. Add LyseBlue reagent into Buffer P1.
    b. Cool Buffer P3 to 4°C beforehand and inspect Buffer P2 for any SDS precipitation.
    c. Ready a QIAGEN-tip 10 mL with buffer QBT and let the column drain naturally.
    d. Warm the QF elution buffer to 65°C.
46. Thoroughly mix the bacterial pellet with 10 mL of Buffer P1 until it is completely dissolved.
47. Introduce 10 mL of Buffer P2, thoroughly mix by flipping the container 4–6 times, and let it sit at ambient temperature (15°C–25°C) for 5 min.
48. Add 10 mL of pre-cooled Buffer P3 and mix well by inverting 4–6 times (avoid using a vortex).

    *Note:* Stir until the mixture loses its blue color, then chill on ice for 20 min.

49. Centrifuge the sample at 6000 × *g* for 30 min at a temperature of 4°C. Activate the water bath and adjust it to 65°C.
50. Position a sterile 70 μm cell strainer atop a 50 mL centrifuge tube and filter the clear supernatant through it to segregate the cell pellet and avert filter blockage.
51. Administer the filtered liquid to the QIAGEN tip and let it permeate the resin by gravity.
52. Clean the QIAGEN-tip twice with 30 mL Buffer QC, allowing the buffer to pass through via gravity.
53. Warm the QF elution buffer to 65°C beforehand. Retrieve DNA with 15 mL of the heated elution buffer into a pristine 50-mL centrifuge tube.

    *Note:* Warming the elution buffer to 65°C considerably enhances the DNA yield.

54. Add 10.5 mL of isopropanol at 22°C–24°C to the eluted DNA and mix well.
55. Spin at a minimum of 15,000 × *g* for 30 min at 4°C. Gently pour off the supernatant.
56. Rinse the DNA pellet with 5 mL of 70% ethanol at 22°C–24°C and centrifuge at a minimum of 15,000 × *g* for 10 min. Pour off the supernatant with care.
57. Leave the pellet to air-dry for 20–30 min and then dissolve the DNA in 600–800 μL of IDT nuclease-free water.

    *Note:* There is no need for phenol-chloroform extraction of DNA. Furthermore, if the extraction is not performed correctly, remnants of phenol-chloroform can be toxic to mouse zygotes.

### Microinjection preparation

**Timing: 1 h**

Efficient method for the preparation of reagents for microinjection into mouse zygotes.

*Note:* It is essential to prepare the reagents on the day of the experiment.

58. Thaw all working stock reagents on ice, gently vortex, and then maintain on ice while working under RNase-free conditions.

Table 2. Renature program

| Temperature | Time |
|---|---|
| 90°C | 1 min |
| 80°C | 1 min |
| 70°C | 1 min |
| 60°C | 1 min |
| 50°C | 1 min |
| 40°C | 1 min |
| 30°C | 1 min |
| 20°C | 1 min |
| 10°C | 1 min |
| 4°C | 1 min |

59. Determine the concentration of Maxi-Prep plasmid DNA using the Qubit Fluorometer.
60. Centrifuge all reagents at 16,000 × *g* for 30 min at 4°C to sediment any microparticulates.
61. Rinse 0.2 mL PCR tubes with 200 mL of IDTE pH 7.5 and place the tubes on ice. Gently transfer the supernatant from the reagents to the pre-rinsed 0.2 mL PCR tubes.
62. Transfer the reagents in the following order: first, IDTE pH 7.5, and then Bxb1 mRNA. Gently mix, centrifuge, and perform 'Renature' on a thermocycler (see Table 2).

*Note:* Do not add plasmid DNA or RNasin Plus Ribonuclease Inhibitor prior to the Renature step.

63. Take the supernatant from the plasmid DNA and add it to the renatured sample.
64. Dilute RNasin Ribonuclease Inhibitor 1:10 in IDTE pH 7.5 and add 1.25 μL to the renatured sample. Gently mix and spin down.
65. Deliver 20 μL for microinjection prep (Table 3) on ice and reserve 5 μL for QC gel.
    a. After MIJ is completed, collect the unused portion of preps from MIJ and run it alongside working stocks in a QC gel (1% TBE). Bxb1 mRNA runs at ~1.5 kb.

## Steps for screening founder mice

**Timing: 1–2 weeks**

A simple and efficient method for screening the founder mice using multiple PCRs.

66. Expect the birth of mice approximately 20 days after implantation.
67. When the mice are weaned, around 3–4 weeks old, identify each animal and collect a tissue sample, either an ear notch or tail tip and place them in 0.2 mL PCR tubes.
68. Perform Proteinase-K lysis to extract DNA. Purification is not needed.
69. For Proteinase-K lysis, introduce 100 μL of lysis buffer to each tube to lyse the cells. Add 1.5 μL of Proteinase K to each sample to digest proteins.
70. Incubate the samples at 55°C overnight to ensure complete digestion. Heat the samples at 95°C for 3 min to denature the DNA, followed by cooling at 4°C for 5 min.
71. Keep the samples at −20°C until they are needed for PCR.
72. Create a 1:4 dilution of the tissue lysate in Nuclease-Free Water. Use 2 μL of the diluted lysate for the PCR reaction.
73. Conduct PCRs (Figure 2—PCRs 1–4) and use about one-third of the PCR product for agarose gel electrophoresis (e.g., for a 20 μL PCR reaction, use 12 μL for agarose gel, and 8 μL for sequencing).

*Note:* Conduct gradient PCRs to optimize PCR conditions (Table 2).

**Table 3. Microinjection preparation**

| | |
|---|---|
| IDTE pH 7.5 (10 mM Tris, 0.1 mM EDTA) | Up to 25 μL |
| Bxb1 Integrase mRNA | 120 ng/μL |
| Maxi-Prep Plasmid DNA | 25–35 ng/μL |
| RNasin Plus Ribonuclease Inhibitor (dilute 10×) | 0.2 U/μL |

74. Clean the PCR product (see magnetic beads purification protocol for PCR products) and utilize it for Sanger sequencing.
75. Examine the chromatograms against a reference.
76. For P0 animals, determine successful RMCE (PCR 2). The sequences for *attR-GT* and *attL-GA* are provided below.

*attR-GT*: 5′ GGTTTGTCTGGTCAACCACCGCG**GT**ctccgtcgtcaggatcat 3′.

*attL-GA*: 5′ ggcttgtcgacgacggcg**GA**CTCAGTGGTGTACGGTACAAACC. 3′.

*Note:* Probe-based assays can be designed to rapidly screen for founder mice, utilizing the specific *attR-GT* and *attL-GA* sequences in the mouse genome.

77. Verify the sequence of the transgene using at least three internal PCRs (PCR 3) and verify off-target integration using PCR 4. Rank P0 candidates based on data obtained from all the PCRs (Figure 2, PCRs 1–4).
78. Backcross potential candidates to the parental strain to produce N1 heterozygous offspring.
79. The N1 generation animals must be screened for clean germline transmission of the desired allele by repeating PCRs 2–4 and analyzing chromatograms. Be mindful of off-target integration.

⚠ CRITICAL: Generate high molecular weight (HMW) gDNA from an N1 candidate mouse for whole-genome sequencing (see below).

80. Intercross N1 heterozygotes to generate homozygous mice.

## High-molecular weight DNA isolation

*NEB Monarch HMW DNA extraction kit for tissues*

Timing: 2 h

Isolation of high molecular weight DNA from mouse ear notch tissues for Nanopore whole-genome sequencing.

81. Collect two 2 mm ear punches from the founder mice in a 1.5 mL Eppendorf tube.
    a. Flash freeze the tubes in liquid nitrogen.
    b. Gently transfer the tubes into a −80°C freezer until ready for DNA isolation.
82. In a small tube, combine 20 μL of Proteinase K and 600 μL of HMW gDNA Tissue Lysis Buffer to create the lysis master mix.
83. Obtain two 2 mm ear punches and place it in a Monarch Pestle Tube. Keep the tube on ice. Centrifuge the tube to settle the tissue to the bottom.
84. Add 100 μL of lysis buffer to the tissue.
    a. Use a motorized pestle to grind the tissue for 30 s; it will not completely homogenize at this stage.
    b. Then, add 500 μL of lysis buffer and mix with a wide-bore pipette tip.
85. Incubate the tube at 56°C for 1 h with shaking at 2000 rpm in a Thermo mixer until the sample is completely lysed and clear. Increase the lysis time if necessary.

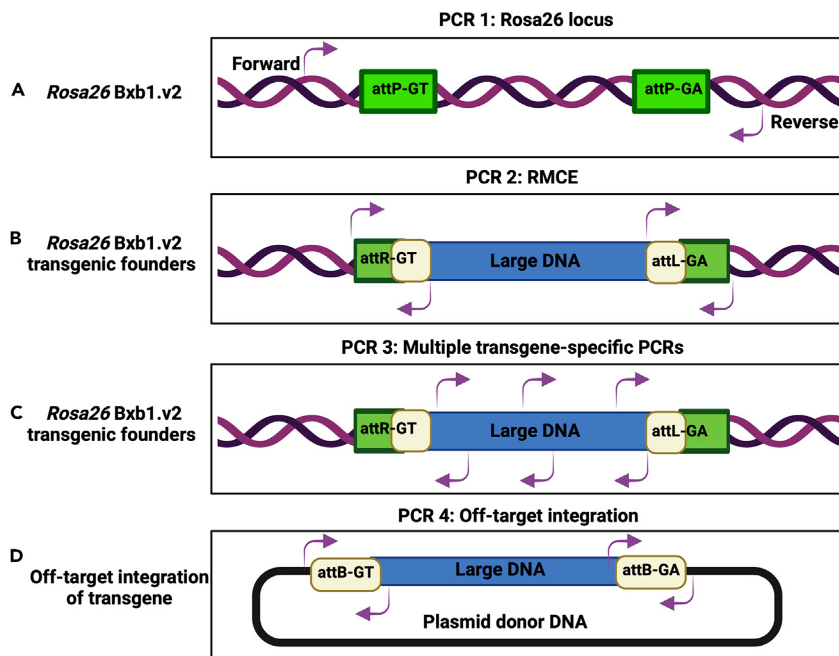



**Figure 2. PCR strategy for screening founder mice**

The strategy typically involves designing multiple primers that are specific to the *Rosa26* locus in the Bxb1.v2 mice, targeting the *attR* and *attL* sites, focusing on the inserted transgene, and addressing the plasmid donor backbone to determine off-target integration.

(A) **PCR 1:** Design primers flanking the *attP-GT* and *attP-GA* sites in the *ROSA26* locus of the Bxb1.v2 mice.

(B) **PCR 2:** Design primers spanning the *attR* and *attL* junctions. We recommend designing probe-based assays to screen the P0 animals to quickly identify potential candidates.

(C) **PCR 3:** Design multiple internal primers spanning the transgene.

(D) **PCR 4:** To detect the presence or absence of off-target integration, design primers for both inside/out left and inside/out right regions. For more detailed protocols and strategies please refer to Low et al.[4] and Hosur et al.[6]

86. Add 10 μL of RNase A to the tube and incubate for 10 min at 56°C with shaking at 2000 rpm in a Thermo mixer, then transfer 300 μL of Protein Separation Solution to the tube. Gently mix.
87. Centrifuge the tube for 10 min at 16,000g to separate the phases.
88. Prepare the plastics for Part II, including a Monarch Collection Tube II and a Monarch Bead Retainer for each sample.
89. Proceed with caution to transfer the upper aqueous phase containing the DNA to a Monarch 2 mL tube using a wide-bore pipette tip.

⚠ CRITICAL: Avoid transferring any of the interphase or lower organic phase.

90. Continue with DNA binding and elution.
91. Incorporate two DNA capture beads into each 2 mL Monarch vial that contains the DNA.
92. Add 550 μL of isopropanol into each tube and gently mix for 8 min at 10 rpm using a vertical rotating mixer. The DNA will bind to the beads during this step.

*Note:* The beads will fall to the bottom of the tube, so it is not necessary to centrifuge or remove every drop of supernatant. Remove what you can, add wash buffer, and invert gently to wash the beads and the walls of the tube.

93. Carefully remove and discard the supernatant without disturbing the beads.
94. Add 550 μL of gDNA Wash Buffer to each tube.
    a. Gently invert 2–3 times to wash beads, and then remove the supernatant.

b. Repeat the wash step once more with 500 μL of gDNA Wash Buffer.

95. Pour the beads into a Monarch Bead Retainer that has been appropriately labeled.

*Note:* Centrifuge the retainer in order to eliminate any remaining wash buffer.

96. Place the beads in a brand-new 2 mL Monarch tube.
    a. Add 50 μL of Elution Buffer II to the tube and incubate at 56°C for 5 min in a Thermo mixer with 300 rpm agitation to elute the DNA from the beads.
97. Place the bead retainer into a 1.5 mL microfuge tube designed with low DNA binding capabilities.
    a. Pour the glass beads and eluate into the bead retainer, ensuring a secure closure.
    b. As the pouring process advances, the eluate will flow through the bead retainer into the 1.5 mL tube.
98. After a short spin, transfer any remaining contents from the 2 mL tube into the bead retainer. Discard both the beads and the retainer afterward.

*Note:* To fully separate the eluate and the glass beads, centrifuge at 12,000g for 30 s.

99. Elute is now in the 1.5 mL tube.
    a. Pipette the eluate up and down with a wide-bore pipette tip a minimum of five to ten times, ensuring that any observable DNA aggregates are dispersed uniformly.
    b. Store the eluted DNA at −20°C until the sample is ready for whole-genome sequencing.

## EXPECTED OUTCOMES

We optimized RMCE by employing a 20 kb plasmid donor DNA in accordance with the provided protocol, consistently achieving a success rate of approximately 10%. Plasmid DNA recovered from the Maxi-prep was approximately 1.5 μg/μL, and the concentration of high molecular weight genomic DNA obtained from two 2 mm ear punches was 66 ng/μL (total yield was ∼2 μg) (Figure 3). Notably, validation of successful RMCE can be conducted through whole-genome sequencing using ear notches, without the need to sacrifice the mice.

## LIMITATIONS

Currently, heterologous attachment sites in the *Rosa26* locus are available only in the following different strains: *B6.RosaBxb-GT/GA* (# 36152); *B6A.RosaBxb-GT/GA* (# 36153); *NOD.RosaBxb-GT/GA* (# 36181); *NSG.RosaBxb-GT/GA* (# 36151).

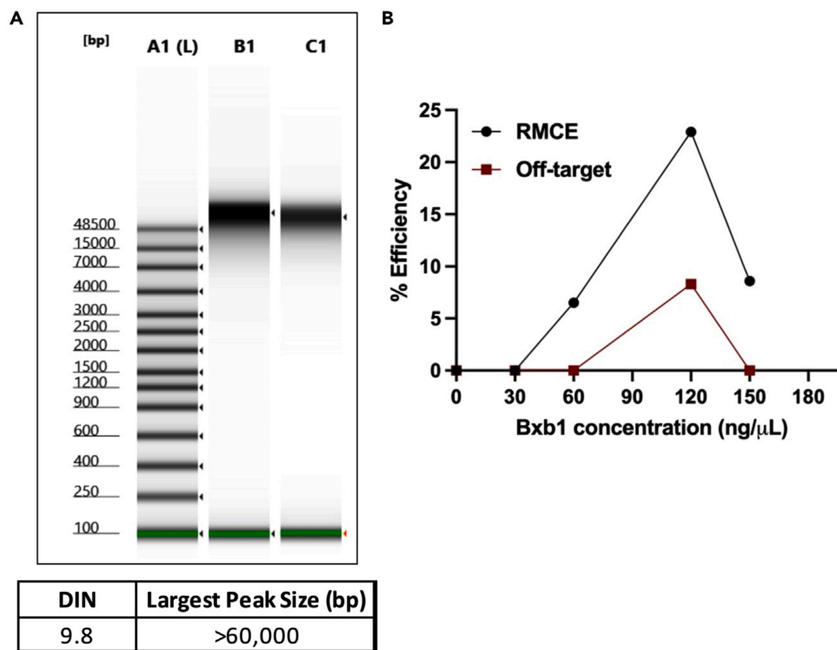
## TROUBLESHOOTING

### Problem 1

No potential founder mice carrying the large transgene.

### Potential solution

- To protect the integrity of genetic material, it is important to store RNA on ice when it is not kept at −80°C, thus maintaining its stability. Avoid subjecting reagents to multiple freeze/thaw cycles, as this can potentially reduce their effectiveness. Ensure the quality and integrity of DNA by keeping it at a temperature of −20°C before the MIJ process.
- Bxb1 mRNA degraded: RNase is present everywhere in a lab setting, so be cautious by cleaning the countertop, glassware, and plasticware used for MIJ prep. Use RNase-free tips and vials, and refrain from talking when handling RNA samples.
- Efficiently identify candidates by utilizing numerous PCR experiments, a fast and effective screening approach. Additionally, develop transgene-specific PCRs to validate sequences and identify the most appropriate candidates for subsequent investigation.




| DIN | Largest Peak Size (bp) |
|---|---|
| 9.8 | >60,000 |

**Figure 3. Expected results**

(A) A single high molecular weight peak, exceeding 60,000 base pairs, was observed, and the DNA integrity number (DIN) was approximately 9.8. Large peak sizes, exceeding 50,000 base pairs, and a DIN exceeding 8.5 are indicative of high-quality DNA. A1: Ladder, B1: HMW DNA from two 2 mm ear notches, C1: HMW DNA from three 2 mm ear notches.

(B) The efficiency of Bxb1-mediated RMCE in the *B6.RosaBxb-GT/GA* strain varied 6%–22%, depending on the concentration of Bxb1 used. When employing Bxb1 mRNA, we observed a significant improvement that was proportional to the injected concentration. As the concentration of Bxb1 increased, we noticed a continuous improvement in the efficiency of targeted gene integration. Our results indicate a critical level at values exceeding 60 ng/μL. Beyond this threshold, we observed an increase in off-target integration events, with up to 5% of genetic changes occurring at loci not intended to be altered. Additionally, we identified substantial toxicity at a dosage of 150 ng/μL. Concentrations between 60 ng/μL and 120 ng/μL proved to be optimal for maximizing on-target efficiency while minimizing the risk of off-target integration.

### Problem 2

Microinjection preps are difficult to inject/clogging of needle.

### Potential solution

- Microparticulates and dust in the microinjection preps can clog the needle. It is important to centrifuge the microinjection prep reagents at 16,000 × *g* for 10–20 min at 4°C and collect the supernatant to prepare the microinjection prep.
- If possible, move the unmodified or uninjected zygotes to the oviducts of the pseudopregnant mice, which could be a control group or a baseline to compare with manipulated embryos.
- High concentrations of DNA can clog the needles. In the DNA Maxi-prep procedure, dissolve the plasmid donor DNA in 600–800 μL of IDT nuclease-free water. Dilute the DNA 100-fold while determining the concentration using a spectrophotomer.

### Problem 3

Off-target integration.

### Potential solution

- Reduce the concentration of reagents, including plasmid donor DNA and Bxb1 mRNA.

- Identify off-target integrations by designing PCR assays that target the plasmid backbone. Furthermore, enhance the reliability of the outcomes by implementing two off-target integration PCRs on both ends of the plasmid backbone, thus providing an additional level of certainty (Figure 2).
- Isolate high-molecular-weight DNA from potential N1 candidates to investigate off-target integration of the transgene through whole-genome sequencing.

### Problem 4

Low survival rate/Toxicity.

### Potential solution

- Improve the MIJ preparation procedure by diluting the concentrated DNA, thus reducing the risk of toxicity to the zygote.
- If phenol-chloroform was used to extract DNA, remnants of phenol-chloroform can be toxic to the zygotes.
- High concentration of reagents, including Bxb1 mRNA and plasmid donor DNA can also be toxic.

### Problem 5

No PCR bands or non-specific bands are detected on the agarose gel

### Potential solution

- Too much DNA was added to the PCR reaction. Dilute tail DNA 1:4 in nuclease-free water and use 1 μL for PCR.
- Try to avoid GC regions. Use a GC-rich buffer when amplifying GC-rich (>60%) regions.
- For probe-based assays, dilute tail DNA 1:5 in nuclease-free water.
- Always run a gradient PCR to determine the optimal annealing temperature. Reduce the number of cycles to 30.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead contact Vishnu Hosur (email: Vishnu.hosur@jax.org).

### Technical contact

Valerie Erhardt (email: Valerie.erhardt@jax.org).

### Materials availability

All materials are available upon reasonable request.

### Data and code availability

This study did not generate any codes or analyze any data sets.

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## AUTHOR CONTRIBUTIONS

V.E., C.M.S., and V.H. wrote the manuscript. V.E. and C.M.S. performed the experiments. V.H. supervised the study.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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