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Protocol for in vivo CRISPR screening targeting murine testicular cells.

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Authors

Noguchi, Yuki
Maruoka, Masahiro
Suzuki, Jun

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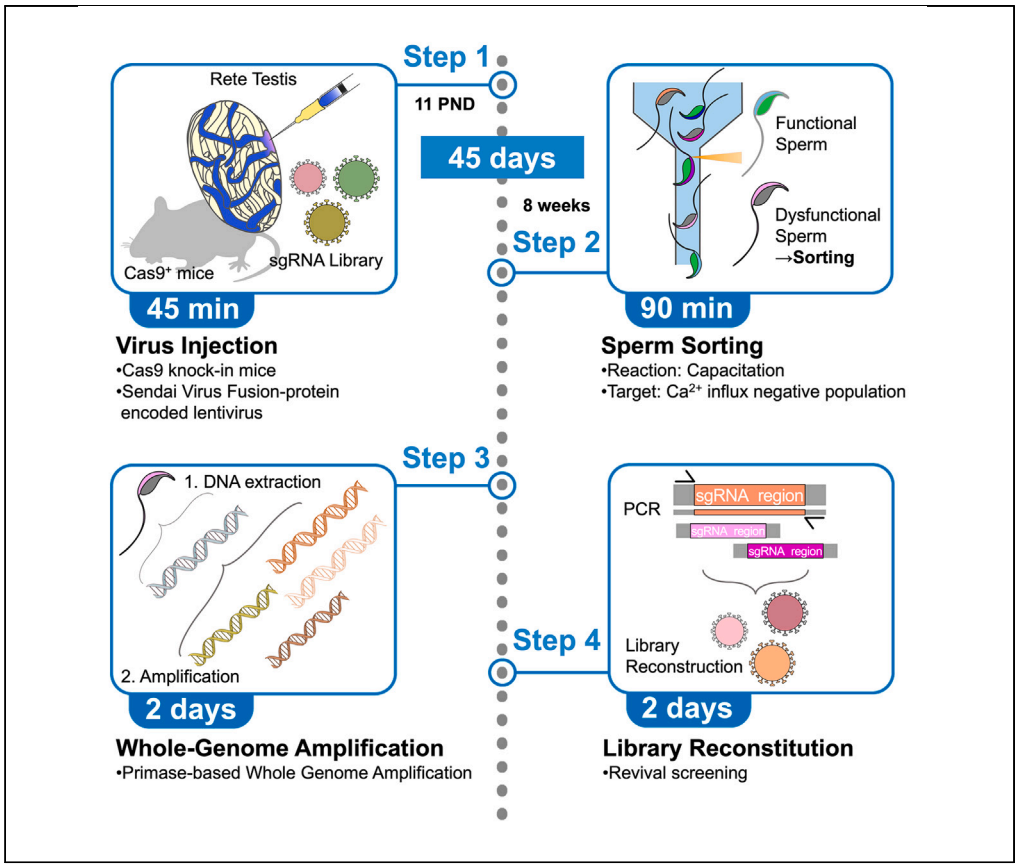

In vivo genome-wide screening elucidates tissue-specific molecular events. Here, we present a protocol for an *in vivo* genome-wide CRISPR-Cas9 single-guide RNA (sgRNA) library screening technique optimized for mouse testicular cells to investigate spermatogenesis. We describe steps for virus injection, sperm sorting, and primase-based whole-genome amplification. We then detail procedures for library reconstruction using a "revival screening" technique. Our approach reveals intricate spermatogenesis processes and is adaptable for diverse tissue-specific studies.

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

Yuki Noguchi, Masahiro Maruoka, Jun Suzuki

jsuzuki@icems.kyoto-u.ac.jp

Highlights

Protocol for *in vivo* genome-wide screening targeting murine testicular cells

Method for introducing lentivirus-based sgRNA library into male germ cells

Instructions for applying the revival screening method to testis

Pipeline for analyzing sgRNA library results

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Protocol for *in vivo* CRISPR screening targeting murine testicular cells

Yuki Noguchi,[1,2,5] Masahiro Maruoka,[1] and Jun Suzuki[1,2,3,4,5,6,*]

[1]Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Yoshida-Honmachi, Sakyoku, Kyoto 606-8501, Japan

[2]Graduate School of Biostudies, Kyoto University, Konoe-cho, Yoshida, Sakyoku, Kyoto 606-8501, Japan

[3]Center for Integrated Biosystems, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan

[4]CREST, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

[5]Technical contact

[6]Lead contact

*Correspondence: jsuzuki@icems.kyoto-u.ac.jp
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SUMMARY

***In vivo* genome-wide screening elucidates tissue-specific molecular events. Here, we present a protocol for an *in vivo* genome-wide CRISPR-Cas9 single-guide RNA (sgRNA) library screening technique optimized for mouse testicular cells to investigate spermatogenesis. We describe steps for virus injection, sperm sorting, and primase-based whole-genome amplification. We then detail procedures for library reconstruction using a "revival screening" technique. Our approach reveals intricate spermatogenesis processes and is adaptable for diverse tissue-specific studies.**

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

BEFORE YOU BEGIN

For succeeding at *in vivo* genome-wide screening, it is critical to establish the following conditions: (1) Prepare for the well-qualified initial library pool. (2) Purify the highly-titered lentivirus for introducing the sgRNA into spermatogenic cells with high efficiency. (3) Familiarize the method to inject a virus into the testis via seminiferous tubules. (4) Establish the condition enabling the highly-covered sgRNA library screening. Here, we explain the way to optimize each step.

Prepare for sgRNA library and evaluate the clone number

Timing: 3 days

Note: Reference: Maruoka et al.[2]

1. Prepare 40 LB agar plates (150 mm diameter) supplemented with ampicillin (100 μg/mL, Wako, A634315).
2. Thaw electrocompetent cells of *E.coli* (MegaX DH10B T1^R (Invitrogen, C640003)) on ice.
3. Prepare the electroporation mix by mixing 20 μL electrocompetent cells, 1 μL GeCKO v2 Mouse CRISPR Knockout Pool A or Pool B Library[3] (50 ng/μL), 49 μL of 10% glycerol on ice, respectively.

Note: The mixture is separately prepared in Pool A and Pool B.

Note: Prepare 10% glycerol at the time of use to prevent the DNA (plasmid) contamination.

4. Transfer 70 μL whole mixture into cuvette (1 mm Gap NEPA cuvette (Nepagene, EC-001S)) chilled on ice for electroporation.

⚠ CRITICAL: Confirm that there are no bubbles on the surface.

5. Conduct the electroporation reaction by the NEPA Porator Electroporator (Nepagene, K03191).

⚠ CRITICAL: Carefully wipe off water drops around the cuvette and set it on the electrode.

Note: 2,600–2,700 V, Resistance should be around 2.5–3.0 Ω.

6. Transfer the electroporated cell mixture into 25 mL pre-warmed (32°C) recovery medium.

Note: Recovery medium is supplied with MegaX DH10B $T1^R$ (Invitrogen, C640003).

7. Preculture at 32°C for 2–3 h.
8. Inoculate 1 mL precultured cell mixture onto 150 mm diameter LB agar plates by conlarge stick.

Note: Twenty dishes are used per Pool.

9. Incubate at 32°C for 16 h.
10. Add 5 mL DPBS (Nakalai, 14249–24) onto each plate.
11. Scrape and collect all colonies by conlarge stick.
12. Purify plasmid by QIAGEN Plasmid Maxi Kit (QIAGEN, 12162).
13. Evaluate the clone number.
    a. Prepare serial dilutions (1,000- and 10,000-fold dilutions) of the precultured cell solution.

Note: These concentrations were optimized to accurately count the number of *E. coli* colonies. The resulting 1×10^7 clones represent a 100-fold increase over the original sgRNA library clone numbers.

    b. Inoculate 20 μL of each diluted solution onto 100 mm diameter LB agar plates.

Note: Final dilutions: 50,000 and 500,000 fold.

    c. Incubate at 32°C for 16 h.
    d. Count colony numbers.

Note: Appropriate clone number should be over 1×10^7 clones, which represent a 100-fold increase over the original sgRNA library clone numbers.

Generate lentivirus and determine lentivirus titer

Generate lentivirus for testicular injection

Timing: 3 days

Note: Reference: Li et al.[4]

Note: Sendai-Virus Fusion (SVF) protein is used for enhancing the lentiviral infectivity into male germ cells.[5,6]

Note: Based on our experiments to determine the transfection methods, calcium phosphate precipitation is the best transfection method that can produce high-titer lentivirus.

14. Seed HEK293T cells at 4×10^6 cells / 7 mL DMEM (Wako, 043–30085) with 10% fetal bovine serum (FBS) and penicillin/streptomycin solution (Nacalai, 26253–84) onto sixteen 100 mm diameter dishes.
15. Incubate cells in 37°C, 5% CO_2 incubator for 24 h.
16. One hour before transfection, the old media is exchanged for 5 mL fresh medium without FBS and penicillin/streptomycin.
17. Prepare for the plasmid mix according to the indicated table below:

Note: To perform transfection for 16 dishes, prepare for 2 tubes.

Plasmid mixture		
Reagent	Amount/sample	Amount (8 samples)
Targeted plasmid	17 μg	136 μg
pCMV-SVF-P2A-VSVG-pRSV-REV	10 μg	80 μg
pCAG-HIVgp	10 μg	80 μg
DDW	Up to 450 μL	Up to 3,600 μL (in 50 mL tube)

18. Load 400 μL of 2.5 M $CaCl_2$ solution onto the bottom of plasmid mix.
19. Vortex for 10 s.
20. Drop 4 mL of 2×BBS (BES buffered saline) onto mixture with a mild vortex (Scientific Industries, SI-0286, power:3).

Note: 2×BBS is prepared according to the RIKEN's protocol (https://dnaconda.riken.jp/Form_PDF/IntPrepen.pdf)

2×BBS for calcium phosphate transfection	
Reagent	Amount
BES (Nacalai, 08889–92)	2.665 g (final: 50 mM)
NaCl (Wako, 191–01665)	4.091 g (final: 280 mM)
150 mM Na_2HPO_4 solution (Wako, 193–0286)	2.5 mL (final: 1.5 mM)
DDW	up to 250 mL
pH	6.95

⚠ CRITICAL: pH should be adjusted to 6.95.

21. Vortex for 3 s.
22. Pulverize the calcium-phosphate precipitates into smaller particles by applying physical stress through bubbles using an electronic pipette (Drummond, 4000100).
23. Incubate at RT for 30 min.
24. Apply 1 mL plasmid solution onto HEK293T cells gently.
25. Incubate cells in 37°C, 5% CO_2 incubator for 6 h.
26. Exchange media for 6 mL fresh media (with 10% FBS and without penicillin/streptomycin) with 9 mM sodium butyrate (Wako, 156-54-7).
27. Incubate cells in 37°C, 5% CO_2 incubator for 40–44 h.
28. Collect the virus supernatant into two 50 mL tubes.
29. Centrifuge at 900 × *g*, 4°C, 10 min.
30. Filtrate virus supernatant with 0.45 μm PES filter (Merck Millipore, SLHP033NK) into 50 mL tube (final: 2 × 50 mL tube) for removing cell debris.

31. Divide all of the virus supernatant into eight 15 mL tubes.

Note: The final volume is approximately 10.5 mL per tube.

32. Load 1 mL of 20% sucrose solution onto the bottom of each tube.[4]

Note: Upper layer contains viral supernatant and bottom layer contains sucrose solution.

33. Centrifuge at 20,000 × *g*, 4°C, 4 h.
34. Aspirate supernatant while leaving a small amount of sucrose solution (about 50 μL).
35. Detach viral pellet from one tube using 100 μL HBSS, using the same 100 μL HBSS, repeat this step for all viral pellets.

Note: In this step, there should be 8 viral pellets in the 100 μL HBSS.

36. Detach remained viruses from one tube using 100 μL HBSS, using the same 100 μL HBSS, repeat this step for all remained tubes.

Note: In this step, there should be 100 μL HBSS including remained viral particles.

37. Combine solutions from 35 and 36 (total: 200 μL) and resuspend the viral pellets well.

Note: The final volume of the virus solution is approximately 600 μL because sucrose solution is remained at step 34 (total: 400 μL).

38. Gather every virus solution onto 900 μL of 20% sucrose in one 1.5 mL tube.
39. Centrifuge at 20,000 × *g*, 4°C, 2 h.
40. Thoroughly resuspend the pellet with 98 μL HBSS (Nacalai, 17461–05) by pipetting around 500 times.

Note: This step aims to completely dissociate virus pellet into single particles.

Note: Take care not to generate bubbles.

41. Split each solution in 12 μL × 8 tubes (for injection) and 1 μL × 2 tubes (for titer evaluation), respectively.

Note: One aliquot is for two times injections (5 μL per one injection).

42. Store at −80°C.

Pause point: Virus can be stored at −80°C for at least 1 year.

Determine lentiviral titer

Timing: 4 days

There are two methods to determine the lentiviral titer based on marker genes (encoding fluorescent protein or puromycin-resistant protein).

Note: Appropriate viral titer unit for testicular cell infection is over 5×10^7 infectious titer units (IFU)/mL from both methods.

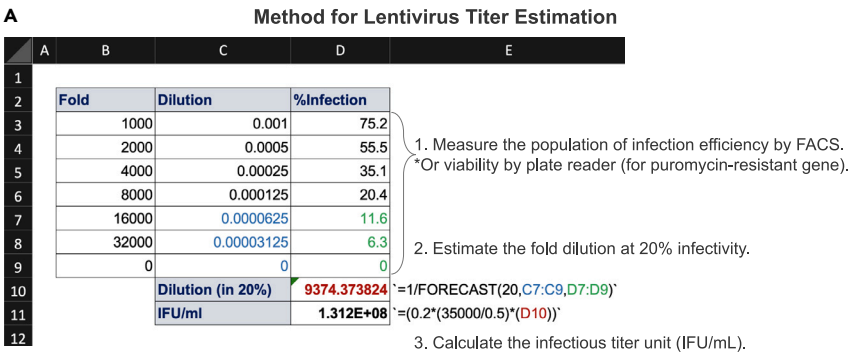

Figure 1. Workflow for determining viral titer

Numbering at right indicates the step for estimating viral titer in Microsoft Excel (A).

Note: Viral titer was determined using the following protocol with some modifications: LV-MAX Lentiviral Production System USER GUIDE (Gibco, Cat.A35684, Publication No.MAN0017000).

43. Evaluate fluorescent protein-encoded virus (Method A).

Note: In Method A, we designed an assay to analyze the infectivity using FACS, requiring a sufficient number of cells. Hence, we used 24 wells (3.5×10^4 cells/well). Conversely, in Method B, we designed an assay to measure viability using an MTT-based assay (Cell Count Reagent SF [Nacalai, 07553–15]), where a 96-well plate (7×10^3 cells/well) is the appropriate choice.

a. Seed HT-1080 cells onto 24 multi-well plates (Nunc, 140675) at 3.5×10^4 cells/well.
b. After 4 h incubation, aspirate medium.
c. Apply 500 μL virus solutions serially diluted with DMEM (0, 1,000, 2,000, 4,000, 8,000, 16,000, 32,000-fold dilution, Wako, 043–30085) to cells.
d. Incubate at 37°C, 5% CO_2 for 72 h.
e. Detach cells with 100 μL of 0.25% trypsin EDTA (Wako, 209–16941).
f. Collect cells by adding 400 μL DMEM with 10% FBS.
g. Analyze the percentage of fluorescent protein-positive population by FACS Lyric (BD).
h. Calculate the infectious titer unit (IFU/mL) by the following formula:

$$\mathrm{IFU}/\mathrm{mL} = \frac{(20(\%)/100) \times 35{,}000(\mathrm{cells}) \times \text{estimated fold dilution(at 20\%)}}{0.5(\mathrm{mL})}$$

Note: The estimated fold dilution of 20% fluorescent protein-positive is calculated by the linear approximation in Microsoft Excel using values for infection efficiency under 20% (Figures 1A and 1B).

Note: Appropriate viral titer unit for testicular cell infection is over 5×10^7 IFU/mL.

44. Evaluate puromycin-resistant protein-encoded virus (Method B).
a. Seed HT-1080 cells onto 96 multi-well plates (Nunc) at 7×10^3 cells/well in triplicate.
b. After 4 h incubation, aspirate the medium.
c. Apply 100 μL virus solution serially diluted with DMEM (0, 1,000, 2,000, 4,000, 8,000, 16,000, 32,000-fold dilution) to cells.
d. Incubate at 37°C, 5% CO_2 for 24 h.
e. Change to 100 μL new medium with 10 μg/mL puromycin (InvivoGen, ant-pr-1).
f. Incubate at 37°C, 5% CO_2 for 72 h.

g. Add 10 μL Cell Count Reagent SF (Nacalai, 07553–15) to each well.
h. Incubate at 37°C, 5% CO_2 for about 1 h.
i. Quantify the cell viability by SYNERGY H1 microplate reader (BioTeK) at 450 nm absorbance.
j. Calculate the infectious titer unit (IFU/mL) by the following formula:

$$IFU\,/\,mL = \frac{(20(\%)/100) \times 7{,}000(cells) \times \text{estimated fold dilution(at 20\%)}}{0.1(mL)}$$

Note: The fold dilution of a 20% viable population compared to a non-treated population under puromycin is estimated using the approximate linearization method in Microsoft Excel, based on infection efficiency values of 20% or lower.

Optimize the infection efficiency in testicular cells

Training of the seminiferous tubular injection

Timing: 45 min

Note: References: Ikawa et al.,[7] Shinohara et al.,[8] and Ogawa et al.[9]

45. Prepare a borosilicate glass injection pipette with 1 mm outer diameter, 0.6 mm inner diameter, and 90 mm length (Narishige, GDC-1) by Next Generation Micropipette Puller (SHUTTER INSTRUMENT).

Note: Glass tips should be sharpened by steel micro tweezers.

46. Add 1.0 μL trypan blue (Wako, 72-57-1) to 12 μL virus solution thawed on ice.

Note: Trypan blue indicates the accuracy of the injection.

Note: The virus solution is prepared according to the 'generate lentivirus and determine lentivirus titer/generate lentivirus for testicular injection' sections.

Note: Appropriate viral titer unit for testicular cell infection is over 5×10^7 IFU/mL.

Note: Here, we will describe TagBFP-encoded lentivirus.

47. Fill the pipette with 5 μL virus solution.
48. Inject the virus into the seminiferous tubules.
    a. Anesthetize 11 Post Natal Day (PND) mice by intraperitoneal injection of 1–2 μL anesthetization solution.

Note: Anesthetization solution contains 1 mg/mL Medetomidine (ZENOAQ, WAK0001, Domitor), 5 mg/mL Midazolam (Astellas, 4987-211-76210-0, Dormicum Injection 10 mg), and 5 mg/mL Butorphanol (Meiji Seika Pharma, WAK-52850, Vetorphale).

Note: We have not tried another anesthesia in this experiment, but we think that the advantages of this anesthesia are ease of use, as mice do not awaken from anesthesia for a long time, death is rare, and no special inhalation equipment is needed.

    b. Open the abdomen, and mildly pull out the testis (Figure 2A).
    c. Remove the fat pad around the seminiferous tubules (Figure 2B).

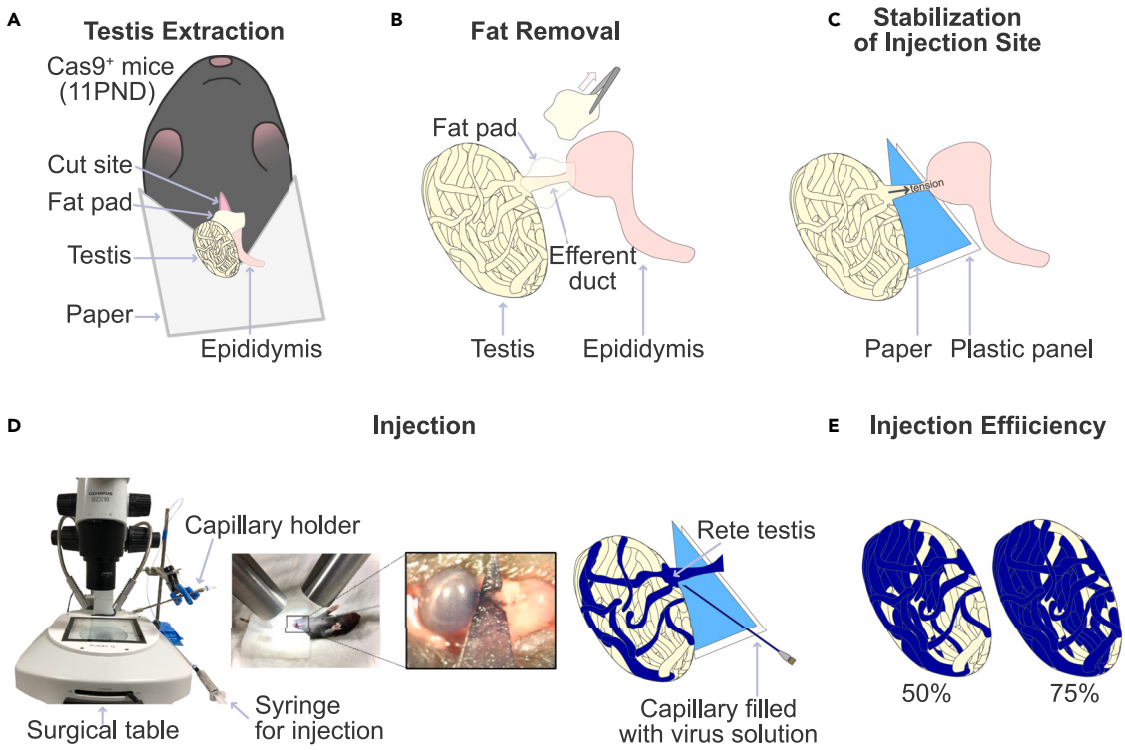

Figure 2. Procedure of seminiferous injection of lentivirus

(A) Condition just before testicular injection.

(B) Procedure of fat pad removal.

(C) Position of the testis, epididymis, and inserted paper and plastic panel.

(D) Position of capillary holder for injection (left), the image of seminiferous injection of lentivirus with trypan blue solution (middle) and detailed illustration (right). Image is from Noguchi et al.[1] Space size differs from others (such as A, B, and C).

(E) Evaluation of injection efficiency. 50% (left), 75% (right).

Note: It is recommended that the testis and epididymis are detached by tweezers at first, and subsequently fat pad is carefully removed.

⚠ CRITICAL: Be careful not to damage the efferent duct when removing the fat pad.

d. Insert the plastic panel and the colored paper beneath the efferent duct (Figure 2C).

Note: The plastic panel aims to stabilize the efferent duct while pulling the both sides of the testis and epididymis.

Note: The colored panel aims to visualize the efferent duct.

e. Inject virus solution via rete testis (Figure 2D).

Note: It is appropriate to keep another testis as an uninfected negative control.

⚠ CRITICAL: Check that around 75% area is filled with virus solution following the trypan blue distribution (Figure 2E).

49. Suture the abdomen and warm the mouse until awakening.
50. Keep mice until analysis for 1 week.

Evaluate the infection efficiency

Timing: 2 days

51. Isolate testicular cells.
    a. Open the abdomen of euthanized mice.
    b. Extract testes while removing the fat pad and epididymis.
    c. Remove the outer skin to expose the seminiferous tubules.
    d. Dissociate testes by incubating with 1.5 mL dissociation buffer at 37°C for 30 min while continuously shaking (at 200 rpm).

    Note: Dissociation buffer: DPBS (Nacalai, 14249–24) including 0.2 U/mL Liberase (Roche, 5401119001), 5 U/μL DNase I (Takara, 2270A) and 5 mM $MgSO_4$ (Wako, Cat. 131–00405)

    e. Add 150 μL FBS (final: 10%) and 6.0 μL of 500 mM EDTA (Wako, 349–01863) (final: 2 mM).
    f. Pipet twenty times with 1,000 μL tip to dissociate testicular cells.
    g. Filter cells with pluriStrainer 40 μm (pluriSelect Life Science UG & Co.KG, 43–50040-51).
    h. Wash twice with 500 μL DPBS (Nacalai, 14249–24).
52. Evaluate the infection efficiency in type A spermatogonia (SPG).
    a. Fix the testicular cells with 4% Paraformaldehyde (Wako, 30525-89-4) in DPBS (Nacalai, 14249–24) at RT for 10 min.
    b. Wash once with chilled 500 μL DPBS on ice.
    c. Resuspend cells with chilled 300 μL DPBS on ice.
    d. Pour 700 μL chilled complete EtOH (Wako, 057–00451).

    Note: Make sure that two layers exist. The upper layer contains EtOH, and the bottom layer contains the cell suspension. The border between each layer should be visible.

    e. Vortex vigorously.
    f. Incubate on ice for 30 min.
    g. Wash twice with 300 μL FACS incubation buffer.

    Note: FACS incubation buffer: DPBS including 1% donkey serum (Sigma, D9663), 5 mg/mL Probumin (Merck Millipore, 821001), and 0.01% Proclin 950 (Sigma, 46878-U).

    h. Resuspend cells with 100 μL primary antibody solution.

    Note: Primary antibody solution: FACS incubation buffer containing anti-Plzf antibody (Santa Cruz, sc-28319, 1:50) and anti-tRFP (Evrogen, AB223, 1:100) for TagBFP detection.

    i. Incubate at 4°C overnight (12–24 h) with gentle shaking.

    Note: We utilized the MicroMixer E−36 (TAITEC, 0027765–000) at approximately 30% power of the low configuration.

    j. Wash cells with 300 μL FACS incubation buffer.
    k. Resuspend with 100 μL secondary antibody solution.

    Note: Secondary antibody solution: FACS incubation buffer containing Alexa Fluor 488-conjugated anti-mouse IgG (for Plzf) and Alexa Fluor 594-conjugated anti-rabbit IgG (for TagBFP) antibodies (Invitrogen, 1:400) and DAPI (Dojindo, D523, 1:1,000).

    l. Incubate at RT for 1 h.

m. Wash cells with 300 μL FACS incubation buffer.
n. Evaluate the TagBFP-positive population in Plzf-positive type A SPG with FACS Aria IIIu (BD).

Note: Expected infection efficiency is 30%–50%.

Confirm the CRISPR activity by evaluating indels

Timing: 2 weeks

53. Inject the SVF-encapsulated lentivirus encoding sgRosa26 and TagRFP into Cas9 knock-in mice testes at 11 PND via the seminiferous tubule.

Note: The virus solution is prepared according to the 'generate lentivirus and determine lentivirus titer/generate Lentivirus for testicular injection' sections.

Note: sgRosa26 targeted sequence is 5′-GTTACATACACCACAAATC-3′.

54. Extract and dissociate testes at 21 PND according to the 'evaluate the infection efficiency' section.
55. Wash cells twice with 300 μL FACS incubation buffer.
56. Resuspend cells with 100 μL primary antibody solution.

Note: Primary antibody solution: FACS incubation buffer containing anti-Basigin (Bsg) antibody (BioLegend, 123701, 1:100).

Note: Anti-Bsg antibody is used for capturing the population of male germ cell.[10]

57. Incubate on ice for 30 min.
58. Wash cells twice with 300 μL FACS incubation buffer.
59. Resuspend cells with 100 μL secondary antibody solution.

Note: Secondary antibody solution: FACS incubation buffer containing Goat anti-rat IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Invitrogen, A-21247, 1:400) and DAPI (Dojindo, D523, 1:1,000).

60. Incubate on ice for 30 min.
61. Sort $TagRFP^+$ / Bsg^+ germ cells by FACS Aria IIIu.

Note: We believe that it is preferable to purify genomic DNA (gDNA) from at least 1×10^4 cells. Under this condition, if we successfully purify gDNA and perform PCR using one-hundredth of the obtained gDNA as a template, we will be able to analyze gDNA of 100 cells, which we consider sufficient to determine INDEL efficiency.

62. Extract genomic DNA (gDNA) by the QIAamp DNA mini kit (QIAGEN, 51306).
63. Amplify the Rosa26 region with PrimeSTAR GXL DNA Polymerase (Takara, R050A) using the following primers and the master mix.

Note: This polymerase is the most effective for amplifying targeted regions, based on comparisons with other polymerases

Fwd: GGCTTATCCAACCCCTAGACAG.

Rev: CTCCCTGGACTGAGAATAGGC.

PCR master mix for sgRosa26 indel investigation	
Reagent	Amount/sample
5 × PrimeSTAR GXL Buffer	5 μL
5 μM Forward Primer	0.5 μL (final: 0.1 μM)
5 μM Reverse Primer	0.5 μL (final: 0.1 μM)
10 mM dNTP Mixture (2.5 mM each)	2.0 μL
PrimeSTAR GXL DNA Polymerase	1.0 μL
gDNA	10 ng
DDW	up to 25.0 μL

PCR cycle conditions for library coverage evaluation			
Steps	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	35
Annealing	60°C	15 s	
Extension	68°C	20 s	
Hold	10°C	∞	1

64. Extract the amplified DNA using FastGene Gel / PCR Extraction Kit (Nippon genetics, FG-91302).
65. Sequence the amplified DNA by Sanger sequencing at Eurofins Genomics Japan, Inc.
66. Detect indels by CRISP-ID software (http://crispid.gbiomed.kuleuven.be/).[11]

Note: INDELs are analyzed as Figure 1C in Noguchi et al.[1]

Note: Expected INDEL efficiency is 30%–50% from our quantitative analysis of protein knockout efficiency (See Figure 1D in Noguchi et al.[1]).

Note: For quantitatively evaluating knockout effect, we suggest flow-cytometry based protein expression analysis (see Figure 1D in Noguchi et al.[1]).

Evaluate sgRNA library coverage

Timing: 2 months

Library introduction

67. Introduce the Pool B GeCKO v2 Mouse CRISPR Knockout Pooled Library[3] (containing 62,804 unique guide sequences) lentivirus into 11 PND Cas9 knock-in mice testes via seminiferous tubules.

Note: The pooled library is prepared according to the 'prepare for sgRNA Library and evaluate the clone number' section.

Note: The virus solution is prepared according to the 'generate lentivirus and determine lentivirus titer/generate Lentivirus for testicular injection' sections.

Note: The virus injection is conducted according to the 'optimize the infection efficiency in testicular cells/training of the seminiferous tubular injection' section.

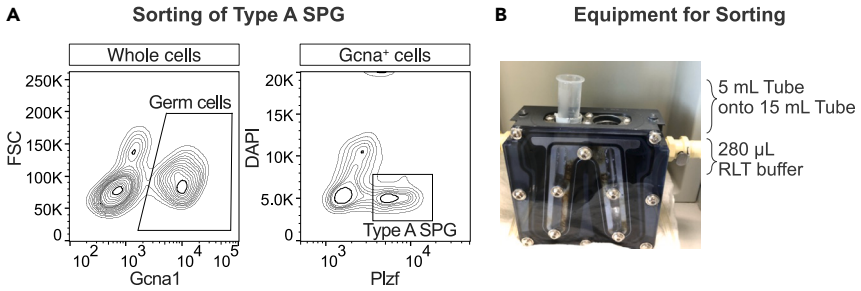

Figure 3. Condition of Type A SPG sorting

(A) Diagram for gating male germ cells (Gcna1$^+$; left) and type A SPG (Plzf$^+$/2 chromosome; right).

(B) Equipment for sorting.

68. Perform the testicular cell sorting in 3 or 7 days after injection.

Testicular cell sorting

69. Extract dissociated testicular cells in the following groups: 1. Three testes from three days treatment, 2. Nine testes from three days treatment, 3. Three testes from one week treatment, 4. Nine testes from one week treatment.

Note: Step 1 is conducted by the 'optimize the infection efficiency in testicular cells/evaluate the infection efficiency' section.

70. Permeabilize cells with 70% EtOH (Wako, 057–00451) on ice for 30 min.

Note: Step 2 is conducted by the 'optimize the infection efficiency in testicular cells/evaluate the infection efficiency' section.

71. Wash twice with 300 µL FACS incubation buffer.
72. Resuspend with 100 µL primary antibody solution.

Note: Primary antibody solution: FACS incubation buffer containing anti-Gcna1 (Abcam, ab82527, 1:200) and Plzf (Santa Cruz, sc-28319, 1:50) antibodies.

73. Incubate at 4°C overnight (12–24 h).
74. Wash twice with 300 µL FACS incubation buffer.
75. Resuspend with 100 µL secondary antibody solution.

Note: Secondary antibody solution: FACS incubation buffer containing Alexa Fluor 555 (for Gcna1)- and Alexa Fluor 488 (for Plzf)- conjugated secondary antibodies (Invitrogen, 1:400) and DAPI (Dojindo, D523, 1:1,000).

76. Incubate at RT for 1 h.
77. Wash twice with 300 µL FACS incubation buffer.
78. Sort the Gcna1$^+$ / Plzf$^+$ Type A SPG by FACS Aria IIIu into 5 mL tube containing 280 µL RLT buffer (QIAGEN, 79216) with a 100 µm nozzle (Figures 3A and 3B).

Note: The estimated number of sorted cells is 15,000–70,000 cells (median: 30,000 cells) per sample.

Note: The estimated total amount of lysate is 350 µL.

79. Invert 20 times.
80. Centrifuge at 1,000 × *g*, 4°C, 15 min.
81. Store lysate at −80°C.

Pause point: Lysate can be stored at −80°C until gDNA extraction.

gDNA extraction

82. Add 200 μg/mL Proteinase K (Sigma, P2308) and 200 μg/mL RNaseA (Sigma, R6513) to thawed lysate.
83. Incubate at 56°C for 2 h with vigorous shaking.
84. Add 350 μL buffer AL (QIAGEN, 19075).
85. Vortex for 5–10 s.
86. Add 350 μL absolute EtOH (final: 33%).
87. Shake the tube for 5–10 s vigorously.
88. Extract gDNA with QIAamp DNA mini kit (QIAGEN, 51306).

Note: The expected amount of gDNA is 500–1,000 ng from 3 testes and 3,000–6,000 ng from 9 testes.

Note: In the elution step, gDNA is eluted twice with 50 μL of 70°C pre-warmed double distilled water, and the first and second elutions were combined for the following analysis.

sgRNA amplification

89. The sgRNA regions were amplified by PCR using PrimeSTAR GXL (Takara, R050A) with the following primers and master mix:

Fwd: AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG

Rev: CTTTAGTTTGTATGTCTGTTGCTATTATGTCTACTATTCTTTCC

PCR master mix for library coverage evaluation

Reagent	Amount/sample
5 × PrimeSTAR GXL Buffer	5 μL
5 μM Forward Primer	0.5 μL (final: 0.1 μM)
5 μM Reverse Primer	0.5 μL (final: 0.1 μM)
10 mM dNTP Mixture (2.5 mM each)	2.0 μL
PrimeSTAR GXL DNA Polymerase	1.0 μL
gDNA	25 ng (3 testes) 250 ng (9 testes)
DDW	up to 25.0 μL

PCR cycle conditions for library coverage evaluation

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	98°C	10 s	28 (9 testes) 32 (3 testes)
Annealing	60°C	15 s	
Extension	68°C	15 s	
Hold	10°C	∞	1

90. Extract the amplicon using FastGene Gel / PCR Extraction Kit (Nippon genetics, FG-91302).

91. Add sample barcode with the following primers and master mix:

Fwd: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT.

(N_{4-7})TCTTGTGGAAAGGACGAAACACCG.

Rev: CAAGCAGAAGACGGCATACGAGATTCTACTATTCTTTCCCCTGCACTGT

Note: Fwd primer contains an adaptor sequence and four sample barcodes (N_4: CAAG, N_5: TAGCT, N_6: ATCGAC, N_7: GGTACAG)

PCR master mix for library coverage evaluation

Reagent	Amount/sample
5 × PrimeSTAR GXL Buffer	5 μL
5 μM Forward Primer	0.5 μL (final: 0.1 μM)
5 μM Reverse Primer	0.5 μL (final: 0.1 μM)
10 mM dNTP Mixture (2.5 mM each)	2.0 μL
PrimeSTAR GXL DNA Polymerase	1.0 μL
Purified sgRNA amplicon	20 ng
DDW	up to 25.0 μL

PCR cycle conditions for library coverage evaluation

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	98°C	10 s	15
Annealing	60°C	15 s	
Extension	68°C	15 s	
Hold	10°C	∞	1

92. Readout sgRNA by the next generation sequencing (NGS).

Note: To validate that your input library has nearly 100% sgRNA coverage, we recommend preparing and sequencing the original library plasmid (Pool A and Pool B) as an input control.

Note: we sequenced 51 bp using single-end sequencing. The total reads are around 10–20 million per sample.

sgRNA data processing

The sgRNA sequences were analyzed using our custom shell script, guide-caller version 1.0.0, accessible at our GitHub repository (https://github.com/SuzukiLab-icems). This process was part of a standard analysis workflow, including quality check with FastQC, sequence trimming with Cutadapt,[12] and sgRNA identification and quantification with MAGeCK.[13] Specifically, we utilized Cutadapt[12] to extract the 20 base pair (bp) sgRNA sequences from the initial 51 bp sequences through a two-step trimming process. The first trimming used parameters "-u {N_4:28 N_5:29 N_6:30 N_7:31}" to adjust the sequence lengths, and the second trimming employed "-u {N_4:-3 N_5:-2 N_6:-1 N_7:0}" for final length refinement. Following trimming, the sequences were aligned against a reference annotation list using MAGeCK.[13]

Note: We used MacBook M1 Pro with 16 GB memory as an analysis environment.

93. Clone the 'guide-caller v1.0.0' from GitHub repository.

```
(base) yourPC ~ % git clone https://github.com/SuzukiLab-icems/guide-caller.git

(base) yourPC ~ % cd guide-caller
```

94. Set up MAGeCK environment.

```
(base) guide-caller ~ % conda env create -f mageck.yaml
```

Note: For Mac users with an Apple silicon chip: use mageck.yaml. For Mac users with an Intel chip: use mageck_x86_64.yaml. For Linux users: use mageck_linux.yaml.

```
(base) guide-caller ~ % conda activate mageck
```

95. Download mageck-0.5.9.5.tar.gz from https://sourceforge.net/projects/mageck/ into 'guide-caller' directory.
96. Build mageck-0.5.9.5.

Note: Define the version of mageck following your situation.

```
(mageck) guide-caller ~ % mkdir mageck-0.5.9.5 \

  && tar xzvf mageck-0.5.9.5.tar.gz \

  -C mageck-0.5.9.5 \

  --strip-components 1

(mageck) guide-caller ~ % cd mageck-0.5.9.5

(mageck) mageck-0.5.9.5 ~ % python setup.py install

(mageck) mageck-0.5.9.5 ~ % cd ..

(mageck) guide-caller ~ % rm -rf mageck-0.5.9.5 mageck-0.5.9.5.tar.gz
```

Note: Confirm that you can use 'mageck count' by the following command.

```
(mageck) guide-caller ~ % which mageck

~/miniforge3/envs/mageck/bin/mageck

(mageck) guide-caller ~ % mageck count -h

Usage: mageck count [-h] ...
```

97. Transfer fastq files in 'guide-caller' directory.
98. Confirm that fastq files are located in the appropriate directory.

```
(mageck) guide-caller ~ % ls

alignment_files

v1.0.0
```

```
your_directory

DEMO_v1.0.0

...
```

Note: We prepared for demo sample as stored in './DEMO_v1.0.0' directory. You can use this directory instead of 'your_directory'.

```
(mageck) guide-caller ~ % ls ./your_directory/*/*

./your_directory/your_sample1/your_sample1.fastq

./your_directory/your_sample2/your_sample2.fastq

...

./your_directory/your_sampleN/your_sampleN.fastq
```

99. Run the 'guide-caller v1.0.0.'

```
(mageck) guide-caller ~ % sh ./v1.0.0/guide_caller.sh \

  -i <your_directory> \

  -f <alignment_file> \

  -c <CPU>
```

Note: You can find the alignment files in the './guide-caller/alignment_file' directory as described below. You should specify each file name <*.csv> in the -f argument.

mmPoolA-B_fixed.csv.

hsPoolA-B_fixed.csv.

Within the GeCKO v2 sgRNA library, certain sgRNAs are linked to multiple genes simultaneously, including genes within the same family (for example, *Il11ra1*, *Il11ra2*, and *Gm13305*). In these instances, related genes were consolidated into a singular target, and the annotation list was updated accordingly to reflect these modifications for our analysis into '*_fixed.csv'.

Note: If you face the error due to an incompatible architecture (arm64 and x86_64 in Mac OX), please uninstall the specific package by 'pip uninstall <package>', and reinstall the code by 'pip install —no-binary <package> <package>.'

Note: Original alignment files can be downloaded into the 'alignment_file' directory by the following script

```
(mageck) guide-caller ~ % wget --directory-prefix=alignment_files {URL}
```

Note: URLs for alignment file are described below:

Human library A gRNA sequences): https://media.addgene.org/cms/filer_public/a4/b8/a4b8d181-c489-4dd7-823a-fe267fd7b277/human_geckov2_library_a_09mar2015.csv.

Human library B gRNA sequences): https://media.addgene.org/cms/filer_public/2d/8b/2d8baa42-f5c8-4b63-9c6c-bd98f333b29e/human_geckov2_library_b_09mar2015.csv.

Mouse library A gRNA sequences): https://media.addgene.org/cms/filer_public/d1/55/d1550427-2a8a-47fa-b120-fd33fa210663/mouse_geckov2_library_a_09mar2015.csv.

Mouse library B gRNA sequences): https://media.addgene.org/cms/filer_public/49/3d/493dd595-075c-4306-980c-3e17de298f39/mouse_geckov2_library_b_09mar2015.csv.

100. Check that the output files are generated.

```
(mageck) guide-caller ~ % ls ./your_directory/*/mageck_result/*.count.txt

./your_directory/your_sample1/mageck_result/your_sample1.count.txt

./your_directory/your_sample2/mageck_result/your_sample2.count.txt

...

./your_directory/your_sampleN/mageck_result/your_sampleN.count.txt
```

Coverage estimation and expected output

Here, we explain the way to count and visualize the number of detected sgRNA and targeted genes using our original custom script.

101. Make sure that script 'summarize_clone_coverage.py' is located in an appropriate directory.

```
(mageck) guide-caller ~ % ls ./utils/summarize_clone_coverage.py

summarize_clone_coverage.py
```

102. Prepare for 'meta_data_for_coverage.csv' in directory 'your_directory' for analysis as described below:

sample_id	treatment(day)	treatment(number)
Your_sample1	Day X	α Testes
Your_sample2	Day Y	β Testes
...	...	...
Your_sampleN	Input	Input

Note: Do not change the header columns ['sample_id' , 'treatment(day)' , 'treatment(number)'].

Note: You should prepare for "Input" sample.

Note: We prepared for demo sample as stored in './coverage_demo' directory. You can use this directory instead of 'your_directory.'

103. Check the contents of 'meta_data_for_coverage.csv.'

```
(mageck) guide-caller ~ % less ./<your_directory>/meta_data_for_coverage.csv

sample_id,treatment(day),treatment(number)
```

```
Your_sample1,Day X,α Testes
Your_sample2,Day Y,β Testes
...
Your_sampleN,Input,Input
./your_directory/meta_data_for_coverage.csv (END)
```

104. Run the script.

```
(mageck) guide-caller ~ % python ./utils/summarize_clone_coverage.py \
-m meta_data_for_coverage.csv \
-d <your_directory>
```

Note: You can see the summary information as described below if you use './coverage_demo' directory.

```
[SUMMARY: sgRNA clone coverage in Day X]
DayX:
id number
3 Testes 25906
9 Testes 44518
Input 60575
...
[Number of genes targeted by multiple sgRNAs in F2-1_1_D3N3]
Number of sgRNAs    Number of targeted genes
1                   5534
2                   8918
3                   5893
```

Note: If you want to perform coverage analysis using the demo sample generated in steps 27–34, you can do it using the following command:

```
(mageck) guide-caller ~ % python ./utils/summarize_clone_coverage.py \
  -m meta_data_for_coverage_demo.csv \
  -d DEMO_v1.0.0
```

105. Check that the output files are generated.

```
(mageck) guide-caller ~ % tree ./<your_directory>/
your_directory
```

```
├── your_sample1
│ ├── your_sample1_count_histgram.png
│ ├── your_sample1_sgRNA_multiplicity.png
│ └── mageck_result
│  └── your_sample1.count.txt
│
...
├── your_sampleN
│ ├── your_sampleN_count_histgram.png
│ ├── your_sampleN_ sgRNA_multiplicity.png
│ └── mageck_result
│ └── your_sampleN.count.txt
│
├── *_count_summary.csv
├── Gene_cumulative_plot_in_Day*.png
└── sgRNA_cumulative_plot_in_Day*.png
```

Note: '*_count_histgram.png' shows the normalized sgRNA counts (=Log_2(CPM+1)) in X-axis, and the total number of targeted genes in Y-axis (Please see Figures S2A and S2B in Noguchi et al.[1]).

Note: '*_ sgRNA_multiplicity.png' shows the sgRNA count per gene in X-axis, and the total number of sgRNA-targeted genes in Y-axis (Please see Figures S2C and S2D in Noguchi et al.[1]) .

Note: '*_summary.txt' summarizes the raw count of the detected sgRNA and corresponding gene.

Note: '*_count_summary.csv' shows the Log_{10}(CPM+1) count summary of the detected sgRNA and gene among each experimental condition.

Note: 'Gene_cumulative_plot_in_Day*.png' shows the targeted gene count (=Log_{10}(CPM+1)) in X-axis, and the cumulative gene count in Y-axis.

Note: 'sgRNA_cumulative_plot_in_Day*.png' shows the sgRNA clone count (=Log_{10}(CPM+1)) in X-axis, and the cumulative sgRNA clone count in Y-axis.

106. Evaluate whether your screening system satisfies the high coverage.

Note: In our screening, analysis of sgRNA clone coverage in three testes showed 42.3% coverage. When we expanded the study to include a total of nine testes, the coverage increased to 73.5%. Besides, analysis of gene coverage from the three testes showed that 78.2% of genes were covered, and that from the nine testes showed 96.1% (Please see Figures 2A and 2B in Noguchi et al.[1]). Additionally, one week after infection, the coverage remained stable at 67.8% for sgRNA clone coverage and 94.2% for sgRNA-targeted gene coverage (Please see Figures S2E and S2F in Noguchi et al.[1]) .

Institutional permissions

The Animal Experiment Committee of Kyoto University approved all procedures employed in the present study (Permission Number: 49–4).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Plzf antibody	Santa Cruz	Catalog #: sc-28319 Clone: D-9 RRID: AB_2218941 Dilution: 1:50
Germ cell-specific antigen antibody [TRA98]	Abcam	Catalog #: ab82527 Clone: TRA98 RRID: AB_1659152 Dilution: 1:200
Anti-tRFP antibody	Evrogen	Catalog #: AB233 RRID: AB_2571743 Dilution: 1:100
PE anti-mouse CD147 antibody	BioLegend	Catalog #: 123707 Clone: OX-114 RRID: AB_2243692 Dilution: 1:100
Goat anti-rat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 555	Invitrogen	Catalog #: A-21434 RRID: AB_2535855 Dilution: 1:400
Goat anti-rat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 647	Invitrogen	Catalog #: A-21247 RRID: AB_141778 Dilution: 1:400
Donkey anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 488	Invitrogen	Catalog #: A-21202 RRID: AB_141607 Dilution: 1:400
Donkey anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 594	Invitrogen	Catalog #: A-21203 RRID: AB_2535789 Dilution: 1:400
Donkey anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 647	Invitrogen	Catalog #: A-31571 RRID: AB_162542 Dilution: 1:400
Donkey anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 488	Invitrogen	Catalog #: A21206 RRID: AB_2535792 Dilution: 1:400
Donkey anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 594	Invitrogen	Catalog #: A-21207 RRID: AB_141637 Dilution: 1:400
Chemicals, peptides, and recombinant proteins		
Fluo-4 AM	Dojindo	Catalog #: F311
Cell count reagent SF	Nacalai	Catalog #: 07553-15
Cellstain- PI solution	Dojindo	Catalog #: F378
Cellstain- DAPI solution	Dojindo	Catalog #: D523
NaCl	Wako	Catalog #: 7647-14-5
KCl	Wako	Catalog #: 7447-40-7
KH_2PO_4	Wako	Catalog #: 7778-77-0
$MgSO_4 \cdot 7H_2O$	Wako	Catalog #: 10034-99-8
Glucose	Wako	Catalog #: 50-99-7
60% Na-lactate solution	Wako	Catalog #: L1375
Na-pyruvate	Wako	Catalog #: 190-14881
Penicillin/streptomycin	Nacalai	Catalog #: 26253-84
HEPES-NaOH (pH7.4)	Wako	Catalog #: 345-06681
Fatty acid-free BSA	Sigma	Catalog #: A4612
$NaHCO_3$	Wako	Catalog #: 191-01305

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
$CaCl_2$	Wako	Catalog #: 038-24985
Critical commercial assays		
DPBS	Nacalai	Catalog #: 14249-24
HBSS	Nacalai	Catalog #: 17461-05
DMEM	Wako	Catalog #: 043-30085
Pre-washed sterilized glass capillary tubing with internal glass fiber	Narishige	Catalog #: GDC-1
QIAamp DNA mini kit	QIAGEN	Catalog #: 51304
QuantiFluor ONE dsDNA	Promega	Catalog #: E4871
MegaX DH10B $T1^R$ Electrocomp cells	Invitrogen	Catalog #: C640003
Next-generation micropipette puller	Sutter Instrument	Catalog #: P-1000
NEBuilder HiFi DNA assembly	NEB	Catalog #: E2621
4BB TruePrime whole genome amplification (WGA) kit	4basebio[14]	Catalog #: 380100
Deposited data		
Library coverage analysis	Noguchi et al.[1]	https://doi.org/10.5281/zenodo.10528508
sgRNA library pool A (input)	Noguchi et al.[1]	https://doi.org/10.5281/zenodo.10528508
sgRNA library pool A (1^{st} round)	Noguchi et al.[1]	https://doi.org/10.5281/zenodo.10528508
sgRNA library pool A (2^{nd} round)	Noguchi et al.[1]	https://doi.org/10.5281/zenodo.10528508
sgRNA library pool B (input)	Noguchi et al.[1]	https://doi.org/10.5281/zenodo.10528508
sgRNA library pool B (1^{st} round)	Noguchi et al.[1]	https://doi.org/10.5281/zenodo.10528508
sgRNA library pool B (2^{nd} round)	Noguchi et al.[1]	https://doi.org/10.5281/zenodo.10528508
Experimental models: Organisms/strains		
Gt(ROSA)26Sortm1.1(CAG-Cas9*,-EGFP)Fezh/J	The Jackson Laboratory (Platt et al.[15])	strain: # 026719 age: 11 PND sex: male
Recombinant DNA		
GeCKO v2 mouse CRISPR knockout pooled library	Sanjana et al.[3]	Addgene: #1000000052 and #1000000053
lentiGuide-Puro	Sanjana et al.[3]	Addgene: #52963
lentiGuide-TagBFP	Noguchi et al.[1]	N/A
lentiGuide-sgRosa26-TagRFP	Noguchi et al.[1]	N/A
pCAG-HIVgp	Miyoshi et al.[16]	RIKEN: RDB04394
pCMV-VSV-G-pRSV-REV	Miyoshi et al.[16]	RIKEN: RDB04393
pCMV-SVF-P2A-VSV-G-pRSV-REV	Noguchi et al.[1]	N/A
Software and algorithms		
CRISP-ID v1.1	Dehairs et al.[11]	http://crispid.gbiomed.kuleuven.be/
Cutadapt v4.1	Martin et al.[12]	https://github.com/marcelm/cutadapt
FastQC v0.12.1	Babraham Bioinformatics	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
FlowJo	BD Life Sciences	https://www.flowjo.com
guide-caller v1.0.0	Noguchi et al.[1]	https://github.com/SuzukiLab-icems/guide-caller
MAGeCK v0.5.9.4	Li et al.[13]	https://sourceforge.net/p/mageck/wiki/Home/

MATERIALS AND EQUIPMENT

Non Capacitation Medium (NCM) for sperm capacitation induction		
Reagent	Final concentration	Amount
1 M NaCl	101.6 mM	10.160 mL
1 M KCl	4.7 mM	0.470 mL
100 mM KH_2PO_4	0.4 mM	0.400 mL
1 M $MgSO_4 \cdot 7H_2O$	0.2 mM	0.020 mL
1 M Glucose	2.78 mM	0.278 mL
60% Na-lactate solution (mL)	23.3 mM	0.370 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
100 mM Na-Pyruvate	0.34 mM	0.340 mL
Penicillin/Streptomycin	1:1,000	1.000 mL
1 M HEPES-NaOH (pH7.4)	20 mM	2.000 mL
Fatty Acid-Free BSA	3 mg/mL	300 mg
DDW	N/A	74.162 mL
pH	7.4	N/A
Total	N/A	100 mL
Note: storage condition is 4°C for 6 months.		

2×Capacitation Medium (CM) for sperm capacitation induction		
Reagent	Final concentration	Amount
1 M $NaHCO_3$	50 mM	5.000 mL (Add before use)
1 M $CaCl_2$	4 mM	0.400 mL
1 M NaCl	101.6 mM	10.160 mL
1 M KCl	4.7 mM	0.470 mL
100 mM KH_2PO_4	0.4 mM	0.400 mL
1 M $MgSO_4 \cdot 7H_2O$	0.2 mM	0.020 mL
1 M Glucose	2.78 mM	0.278 mL
60% Na-lactate solution(mL)	23.3 mM	0.370 mL
100 mM Na-Pyruvate	0.34 mM	0.340 mL
Penicillin/Streptomycin	1:1,000	1.000 mL
Fatty Acid Free BSA	4 mg/mL	400 mg
DDW	N/A	79.562 mL
pH	7.4	N/A
Note: storage condition is 4°C for 6 months.		

Note: In screening, CM is diluted with the same amount of NCM.

Note: We refer to RIKEN's protocol (https://mus.brc.riken.jp/ja/wp-content/uploads/manual/IVF_with_frozen_sperm_ver4.pdf).

STEP-BY-STEP METHOD DETAILS

Virus packaging

Timing: 3 days

This section aims to generate lentivirus-based sgRNA library for introduction into mice testes.

1. Create highly titered SVF-LVs encapsulating GeCKO v2 Mouse CRISPR Knockout Pooled Library[3] (Pool A and B respectively).

   Note: Detailed method is described in the 'generate lentivirus and determine lentivirus titer/ generate lentivirus for testicular injection' section.

   Note: Appropriate IFU/mL is over 5×10^7 IFU/mL.

   Pause point: Virus stock can be stored at −80°C. Based on our experience, we can store the virus at −80°C for up to 1 year without freeze and thaw.

Virus injection

Timing: 45 min (per mouse)

This section aims to inject SVF-LVs encapsulated sgRNA library into testes via seminiferous tubules.

2. Inject the 5 μL virus solution into 12 testes of the Cas9 knock-in mice for each Pool.

Note: A detailed method is described in the 'optimize the infection efficiency in testicular cells/training of the seminiferous tubular injection' sections.

3. Wait for sperm maturation (8–9 weeks).

Sperm extraction and Ca^{2+} influx induction

Timing: 100 min (per two mice)

This section aims to extract and stimulate sperm after library induction and sort dysfunctional sperms that do not show Ca^{2+} influx.

Note: Reference: Xia et al.[17]

Note: Please see the detailed components of NCM and CM in 'materials and equipment.'

4. Pre-warm 500 μL NCM and 400 μL 2×CM in different 1.5 mL tubes on heat-block at 37°C per mouse.

⚠ CRITICAL: Add 20 μL of 1 M $NaHCO_3$ in 380 μL of 2×CM.

5. Extract sperms from the cauda epididymis into 500 μL pre-warmed NCM while keeping it on heat-block.
6. Gently invert 4–5 times.
7. Incubate on a heat block at 37°C for 5 min.

⚠ CRITICAL: During incubation, the tube cap should be opened to facilitate sperm reaction.

⚠ CRITICAL: During incubation, add 0.8 μL Propidium Iodide (Dojindo, P378) and 1.6 μL Fluo-4 AM (Dojindo, F311) into pre-warmed 2×CM.

8. Transfer 400 μL supernatant of sperm solution into pre-warmed 2×CM.

Note: This step aims to remove sperms without moving capability.

9. Gently invert 4–5 times.
10. Incubate at 37°C on a heat block for 60 min.

⚠ CRITICAL: During incubation, tube cap should be opened to facilitate sperm reaction.

11. Sorting by FACS Aria IIIu (BD).
    a. Pre-warm the chamber of the tube loading port at 42°C.
    b. Load sperm.
    c. Gate the targeted sperm population (PI^-/Fluo-4 AM^-) (Figures 4A, 4B, and 4C).
    d. Sort the targeted population into 280 μL RLT buffer (QIAGEN, 79216) directly.

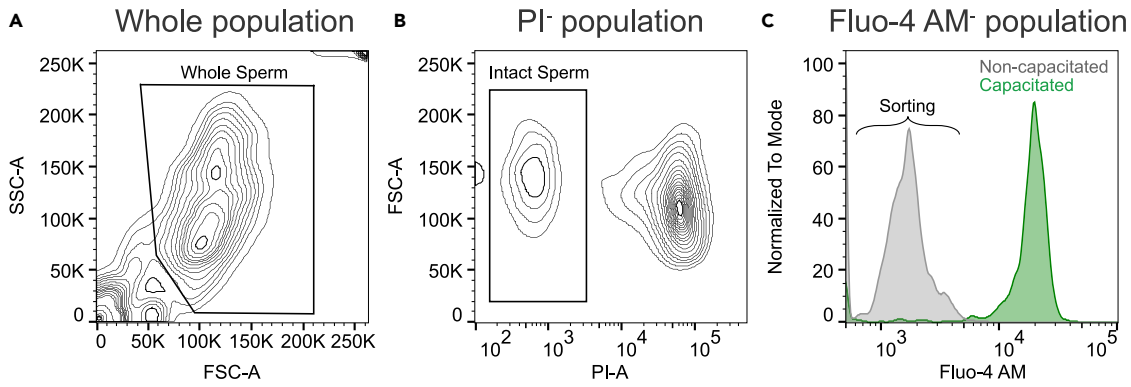

Figure 4. Diagram for Fluo-4 AM targeted sperm sorting

(A) Whole sperm population.

(B) Live sperms population distinguished by Propidium Iodide (PI).

(C) Fluo-4 AM negative and positive sperm population.

Note: 5 mL tube is used for sorting according to Figure 3B.

e. Pipet lysate until sorted solution and RLT buffer are completely mixed.
f. Spin-down the sample (1,000 × *g* , 4°C, 15 min).
g. Store at −80°C.

Note: The sorting procedure should be performed by keeping the flow rate at less than 5,000 events/sec.

Note: The expected number of sorted sperms is 20,000 sperms per testis.

Note: The final volume of lysate is expected to be 350 μL (280 μL RLT buffer + 70 μL sperms [20,000 sperms]).

Pause point: Lysate can be stored at −80°C.

Sperm gDNA extraction

Timing: 120 min (per 8 samples)

This section aims to extract sperm genomic DNA for primase-based whole genome amplification method.

12. Add 150 mM DTT (Wako, 048–29224), 200 μg/mL Proteinase K (Sigma, P2308) and 200 μg/mL RNaseA (Sigma, R6513) to sperm lysates thawed on ice.
13. Vortex for 5–10 s.
14. Incubate at 56°C for 2 h with vigorous shaking (170 rpm).
15. Add the same amount of the buffer AL (QIAGEN, 19075) to sperm lysates.
16. Vortex for 5–10 s.
17. Add the absolute EtOH (Wako, 057–00451) to sperm lysates (final: 33%).
18. Shake the tube for 5–10 s vigorously.
19. Extract gDNA with QIAamp DNA mini kit (QIAGEN, 51306).

Note: The elution process is conducted twice with 50 μL of 70°C pre-warmed DDW.

Note: Each elution process requires 5 min incubation at 70°C.

20. Combine all gDNA into two different tubes (Pool A and Pool B).
21. Measure the concentration of gDNA by QuantiFluor ONE dsDNA system (Promega, E4871).

Note: The expected concentration of the gDNA is 68.2 ± 12.4 pg/μL, with an average total amount of 67.90 ± 10.55 ng and viral copies of 78,492 ± 12,201 copies.

Note: The expected amount of gDNA solution is 800–900 μL.

Primase-based whole genome amplification

Timing: 2 days

This section aims to amplify sperm genomic DNA using a primase-based whole genome amplification method to proceed with PCR.

22. Expose the bench to ultraviolet for 30 min.

⚠ CRITICAL: UV exposure is essential to prevent DNA (Plasmid etc.) contamination.

Note: Steps 22–28 should be conducted on the clean bench.

Note: In this protocol, all gDNA is used (total amount of mixture: 1,372 μL).

23. Denature the gDNA by mixing 24.7 μL gDNA and 2.75 μL Denaturation buffer at RT for 3 min per sample.

Note: Denaturation buffer is 1 M KOH (Nacalai, 11679–55) solution. Manufacturer - supplied one is not used.

24. Quench the reaction by adding 6.85 μL Neutralization buffer per sample.

Note: Neutralization buffer contains 400 mM HCl (Nacalai, 11677–75) and 600 mM Tris-HCl pH 7.5 (Nippon Gene, 316–90221). Manufacturer - supplied one is not used.

25. Prepare for the mixture of primase-based whole genome amplification.

Master mix for whole-genome amplification

Reagent (every reagent from 4basebio[14])	Amount/sample
Reaction buffer	5.0 μL
dNTPs	5.0 μL
Enzyme 1	5.0 μL (final: 0.1 μM)
Enzyme 2	0.7 μL
Total	15.7 μL

Note: Prepare the required volume for your study (we prepared 40 samples).

26. Apply the master mix in PCR tube.
27. Add 34.3 μL denatured DNA solution.

Note: Final reaction volume: 50 μL.

28. Start the reaction by the following condition:

Steps	Temperature	Time
Multiple displacement	30°C	6 h
Enzyme deactivation	65°C	10 min
Hold	10°C	∞

29. Purify the amplified gDNA with QIAamp DNA mini kit.

Library reconstruction

Timing: 2 days

This section aims to reconstruct the sgRNA library using the revival screening method.

30. Prepare for the sgRNA amplicon and the linearized vector for library reconstruction.
    a. Amplify the sgRNA regions by PCR using PrimeSTAR GXL DNA Polymerase (Takara, R050A) with the following primers and master mix:
    Fwd: GTTTTAAAATGGACTATCATATGC.
    Rev: TATCCATCTTTGCACCCGGGC.

PCR master mix for library reconstruction	
Reagent	Amount/sample
5 × PrimeSTAR GXL Buffer	5 μL
5 μM Forward Primer	0.5 μL (final: 0.1 μM)
5 μM Reverse Primer	0.5 μL (final: 0.1 μM)
10 mM dNTP Mixture (2.5 mM each)	2.0 μL
PrimeSTAR GXL DNA Polymerase	1.0 μL
Whole genome amplified-gDNA	750∼1,000 ng
DDW	up to 25.0 μL

PCR cycle conditions for library reconstruction			
Steps	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	98°C	10 s	33
Annealing	60°C	15 s	
Extension	68°C	15 s	
Hold	10°C	∞	1

Note: Perform PCR using individual 24 tubes simultaneously to reduce the PCR bias.

Note: Each sample should be split into 24 tubes for PCR to minimize the biased amplification of the PCR product.

    b. Digest the plasmid at 37°C for 3 h using the following reaction mix.

PCR master mix for library reconstruction	
Reagent	Amount/sample
Targeted Plasmid (ex. lentiGuide-Puro)	3 μg
SmaI (NEB, R0141)	1.5 μL
NdeI (NEB, R0111)	1.5 μL
10 × Cutsmart (NEB, B6004S)	4.0 μL
DDW	up to 30.0 μL

Note: Fluorescent protein-encoded plasmid is useful for checking the infection efficiency during the screening by following the the 'optimize the infection efficiency in testicular cells/evaluate the infection efficiency' sections.

31. Extract the sgRNA region and linearized vector using FastGene Gel / PCR Extraction Kit (Nippon genetics, FG-91302).
32. Repeat step 31.

Note: The amount of elution buffer is described below.

Amount of elution buffer	1st round elution	2nd round elution
sgRNA region	100 μL DDW	100 μL DDW
Linearized vector	200 μL DDW	50 μL DDW

Note: The elution process requires the incubation at 70°C for 5 min.

33. Prepare the following master mix for Gibson assembly using NEBuilder HiFi DNA Assembly (NEB, E2621).

PCR master mix for library reconstruction	
Reagent	Amount/sample
Linearized vector (8031 bp)	50 fmol (=261 ng)
sgRNA region (425 bp)	100 fmol (=27.6 ng)
NEBuilder HiFi DNA Assembly	15 μL
DDW	up to 30 μL

34. Incubate at 52°C for 60 min.

⚠ CRITICAL: PCR tube and thermal cycler should be used.

35. Add 6 μL of 3 M Sodium Acetate (Nippon Gene, 316–90081) to the assembled plasmid.
36. Add 160 μL complete EtOH.
37. Incubate −80°C for 1 h.

Note: This process aims to purify gDNA by removing remaining materials (proteins and salt) to enhance the efficiency of electroporation.

38. Centrifuge at 20,000 × *g*, RT, 5 min.
39. Aspirate supernatant.
40. Add 100 μL of 70% EtOH.
41. Centrifuge at 20,000 × *g*, RT, 5 min.
42. Repeat steps 40–41.

43. Dry up the plasmid completely.
44. Resuspend with 100 μL of 10% glycerol.

Note: Prepare 10% glycerol at the time of use to prevent the DNA (plasmid) contamination.

45. Measure the concentration of DNA by QuantiFluor ONE dsDNA System (Promega, E4871).
46. Perform the electroporation and check the clone number.

Note: Detailed method is described in the 'prepare for sgRNA Library and evaluate the clone number' section.

Note: In this step, the electroporation mix is prepared by mixing 42 μL MegaX DH10B $T1^R$ (Invitrogen, C640003), 168 ng plasmid, and up to 147 μL with 10% glycerol.

Note: The estimated clone number is 1×10^7 clones.

47. Purify the plasmid by QIAGEN Plasmid Maxi Kit (QIAGEN, 12162).

Proceed with the next library screening

This section aims to enrich essential sgRNAs through repeated screening steps.

48. Repeat steps 1–47 using the reconstructed plasmid from step 47.

Data analysis

Timing: 30 min

This section aims to generate an sgRNA count summary, estimate sgRNA coverage, and detect enriched sgRNAs for identifying essential genes.

49. Generate the count matrix by 'main.sh' in guide-caller v1.0.0.

Note: Detailed method is described in the 'evaluate sgRNA library coverage/sgRNA data processing' section (*_summary.txt in step 105).

50. Make sure that analysis script 'summarize_revival_screening.py' in an appropriate directory.

```
(mageck) guide-caller ~ % ls ./utils/summarize_revival_screening.py

summarize_revival_screening.py
```

51. Prepare for 'meta_data_for_revival_screening.csv' in directory 'your_directory' for analysis as described below:

sample_id	Pool*	Round**
Your_sample1	A	1
Your_sample2	A	2
...	...	...
Your_sampleN	AB	Input

Note: Do not change the header columns ['sample_id' , 'Pool' , 'Round'].

Note: Put the information of the 'Input' sample.

Note: *You should select A, B or AB. You can choose 'AB' if you want to perform screening with mixed Pool or want to integrate Pool A and B results.

Note: **This script is designed for pair-wise analysis. 1: before enrichment, 2: after enrichment, Input: Input library.

52. Run the script.

Note: We prepared for demo sample as stored in './revival_screening_demo' directory. You can use this directory instead of 'your_directory.'

```
(mageck) guide-caller ~ % python ./utils/summarize_revival_screening.py \
              -d <your_directory> \
              -m <meta_data> \
              -n <Number of top candidates which you want to show> \
              -f <Filter low count sgRNA>
```

Note: You can check each options definitions by 'python ./utils/summarize_revival_screening.py -h.'

Note: Here is the example command:

```
(mageck) guide-caller ~ % python ./utils/summarize_revival_screening.py \
              -d revival_screening_demo \
              -m meta_data_for_revival_screening.csv \
              -n 50 \
              -f TRUE
```

53. Check that the output files are generated.

```
(mageck) guide-caller ~ % ls ./your_directory
Summary of Enriched * in Pool*.csv
Enriched Genes in * vs. * from Pool*.png
Top_Candidates_In_Pool_*.png
```

Note: 'Summary of Enriched * in Pool*.csv' summarizes the result of pair-wise analysis between 1st (before enrichment) and 2nd (after enrichment) round of screen. If you perform the screening over three times, please run several times with the appropriate <meta_data>. Two files are output for showing enriched sgRNAs and Genes each.

Note: 'Enriched Genes in * vs. * from Pool*.png' showed the enrichment plot by indicated comparison. The data shows the enrichment rank on the X-axis and the enrichment score on the Y-axis (Please see Figures 2E and 2F in Noguchi et al.[1]). The enrichment score is designed to precisely show detected sgRNAs while maintaining significant Log_2FoldChange in the field over Log_2FoldChange = 1.5.[1]

Note: 'Top_Candidates_In_Pool_*.png' shows the names of detected genes, ordered as enrichment score (shown as magenta-colored dots), on the X-axis and the normalized sgRNA counts (=Log_2(CPM+1)) with input data according to the 1st and 2nd library screening results on the Y-axis (Please see Figure 2G in Noguchi et al.[1]).

54. Identify and extract your interest of genes.

EXPECTED OUTCOMES

In our initial screening phase, we isolated 209,591 and 191,023 Ca^{2+} uptake-negative spermatozoa from testes treated with Pool A and Pool B libraries, representing 8.3 ± 2.9% and 8.6 ± 2.2% of the initially introduced spermatozoa, respectively. In the subsequent second screening, we sorted 162,577 and 152,217 Ca^{2+} uptake-negative spermatozoa, accounting for 8.0 ± 2.3% and 8.8 ± 1.8% of the input, from the enriched Pool A and Pool B library-infected testes, respectively.

As a result of the NGS analysis, we enriched eighteen sgRNA-targeted genes in the second screening round from forty-two sgRNA-targeted genes in the first screening. A comparative analysis of the sgRNAs from both screening rounds identified nine sgRNA-targeted genes alongside a decrease in all non-targeting sgRNAs.

LIMITATIONS

This study has successfully demonstrated a proof of concept for the *in vivo* screening system aimed at the testis. However, it's crucial to highlight two main limitations of this system for future improvement.

Firstly, the effect of CRISPR-Cas9 editing on male germ cells presents a significant limitation. Our long-term monitoring of sgRNA-introduced male germ cells revealed a decline in the sgRNA-derived indel population over time despite ongoing lentivirus infectivity indicated by continuous fluorescent protein marker expression.[1] We suggest this reduction in the sgRNA population may be due to the activation of the DNA damage response,[18,19] particularly through the ATM-γH2AX axis,[20] a response typically confined to the nuclei during the leptotene and zygotene stages of germ cell development.[21] Cas9-induced DNA double-strand breaks are likely to initiate this response, resulting in a decrease in population where the sgRNA-mediated indel occurs. Future research should consider alternative strategies like CRISPR interference/activation (CRISPRi/a)[22,23] and CRISPR/Cas13[24,25] which may bypass the limitations of genome editing in specific cells, thereby improving screening effectiveness.

The second challenge is the technical difficulty in specifically targeting type A spermatogonia for sgRNA introduction. Although Sendai-Virus Fusion protein-pseudotyped lentivirus effectively facilitated sgRNA delivery to type A spermatogonia, there was unintended delivery to other germ cells or Sertoli Cells.[1] To address this, we treated Cas9 knock-in mice with Busulfan to deplete most germ cells, except for spermatogonia, before viral introduction. Nevertheless, we observed discrepancies in infection efficiency.[1] Future work should focus on the development of lentiviral vectors tailored for specific cell types to enhance the precision of investigations into cell type-specific biology and mechanisms.

TROUBLESHOOTING

Problem 1

Issue of low titer of lentivirus (related to the 'virus packaging' section).

Potential solution

- The pH of 2×BBS buffer is a critical factor. Please make sure that your 2×BBS buffer is pH 6.95.
- The condition of HEK293T cells is also an important factor. In our procedure, we checked cell growth in every passage (We seeded 4×10^6 cells onto a 150 mm diameter dish. After 48 h, cells were expanded to 2×10^7 cells). Basically, we renewed cells every 20 passages.
- Mycoplasmas contamination severely affects lentiviral titer.
- The condition of HT-1080 cells is also critical for accurately measuring the infectious titer unit. In our procedure, we checked the cell growth in every passage (We seeded 2×10^6 cells onto a 100 mm diameter dish. After 48 h, cells were expanded to 5×10^6 cells).
- Preincubation with serum-free DMEM at 37°C for 1 h is essential for pH equilibration (step 2 in 'generate lentivirus and determine lentivirus titer/generate lentivirus for testicular injection.').
- Prolonged vortex reduces the transfection efficiency. You should do less than 3 s (step 8 in 'generate lentivirus and determine lentivirus titer/generate lentivirus for testicular injection.').

Problem 2

Low lentivirus infection in testicular cells (related to the 'virus packaging' and 'virus injection' sections).

Potential solution

- The most critical factor is whether your injection has succeeded. We recommend that you ensure the trypan blue solution is filled in seminiferous tubules, not interstitial space.
- As an alternative way to check infection efficiency, the immunohistochemistry (IHC) method to detect fluorescent proteins is helpful. For further details, we described the protocol in Noguchi et al.[1]

Problem 3

Low sgRNA coverage in testicular cells (related to the 'evaluate sgRNA library coverage' sections).

Potential solution

- The most critical factor is infection efficiency. Please ensure your infection method toward testicular cells is robustly established according to 'problem 2' and the 'optimize the infection efficiency in testicular cells' section.
- The expected number of sorted cells in the testicular cell sorting step is limited. Therefore, it is important to sort cells directly into RLT buffer (QIAGEN) to prevent the loss using specially designed equipment, as indicated in Figure 3B.

Problem 4

Contamination of external DNA into a reconstructed library (related to from the 'sperm gDNA extraction' to the 'library reconstruction' sections).

Potential solution

- Contamination of external DNA, especially plasmid, is problematic in the library reconstruction step. To solve this issue, we strongly recommend using the newly prepared reagent from the 'sperm gDNA extraction' to the 'library reconstruction' steps in the step-by-step method details section.

- The primase-based whole genome amplification step is the most cautious point in a contamination issue. Mainly, plasmid contamination frequently occurs when performing on the open bench. Therefore, as we described in the 'primase-based whole genome amplification' step in the step-by-step method details section, we strongly recommend that this process should be done in a UV-treated clean bench with a newly prepared reagent and filter tips.
- A PCR hood is also an alternative equipment to avoid contamination.
- The other factor in the contamination issue is the contamination with the undigested linearized vector. Hence, we recommend that you digest the plasmid for at least 3 h–overnight (12–24 h) and separate it well using 0.5% agarose gel.

RESOURCE AVAILABILITY

Lead contact

For additional information, resource requests, and reagent inquiries, please contact the lead contact, Jun Suzuki, via email at jsuzuki@icems.kyoto-u.ac.jp. These requests will be addressed and fulfilled accordingly.

Technical contact

For additional information about our protocols, please contact Yuki Noguchi (nyuhki21@gmail.com), currently at University of California, Berkeley, and Jun Suzuki (jsuzuki@icems.kyoto-u.ac.jp).

Materials availability

The plasmids and cell lines developed during this research are accessible upon request by contacting the lead contact.

Data and code availability

All fastq files from this study have been deposited in the Zenodo repository and can be accessed via https://doi.org/10.5281/zenodo.10528508. Additionally, the analysis script used in this research is available in the Suzuki Lab GitHub repository at https://github.com/SuzukiLab-icems and also in the Zenodo repository, accessible through the same DOI link: https://doi.org/10.5281/zenodo.10528508.

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AUTHOR CONTRIBUTIONS

Y.N., M.M., and J.S. conceptualized the study, wrote drafts, and reviewed them.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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