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A simple validation and screening method for CRISPR/Cas9-mediated gene editing in mouse embryos to facilitate genetically modified mice production

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Abstract

Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) is a genome engineering method for generating site-specific editing in target genes in a variety of species. It is a common tool for generating mouse models of different diseases. However, detecting target modifications in mouse embryos can be time-consuming and expensive. Accordingly, developing a screening method to confirm gene modification may be useful. We propose herein an evaluation method (cleavage assay – CA) for CRISPR/Cas9-mediated gene editing in preimplantation mouse embryos that allows us to detect mutants efficiently and later on initiate in vivo production without the extensive number of samples needing to be sent for Sanger sequencing and animal usage. Our method is based on the inability of the RNP complex to recognize the target sequence after CRISPR-mediated genome editing due to modification of the target locus. It allows us to establish gene edited mice in a user-friendly fashion with a limited number of mice usage by confirming each step of CRISPR-mediated gene editing of mouse embryos and, therefore, can be considered as a supportive tool to existing procedures for verification of successful CRISPR/Cas9-mediated gene alterations in mouse embryos and further mutant production.

Troubleshooting

Mouse hormone stimulation and zygote preparation:

1 Inject intraperitoneally (IP) 10 females C57BL6/CBA/H (F1) (or strain of choice), 10–12-weeks old mice with 0.1 ml PMSG (7.5 IU) at 11:00–13:00 (day 1).

2 After 48 h, IP female mice with 0.1 ml hCG (7.5 IU). After the hCG injection, mate the females with stud males (C57BL6/CBA/H) overnight (day 3).

3 The next morning, check the female mice for the presence of a vaginal plug (day 4).

4 On the morning of day 4, prepare the followings:

a)
Oviduct collection dish: 35 mm dish with 2 ml M2 medium.
b)
Hyaluronidase dish: 35 mm dish with 400 µl (150 IU/ml, Millipore hyaluronidase).
c)
Wash dish: 35 mm dish with 30 µl drops of M2 medium under mineral oil.
d)
KSOM culture dish: 35 mm dish with 20 µl drops of KSOM medium under mineral oil, and put it into the incubator. KSOM dishes are prepared approximately 30 minutes prior to use. KSOM medium pH changes rapidly outside the incubator.
e)
Wash dish with drops of Opti-MEM under the mineral oil.
f)
Tools for mouse dissection: forceps, scissors and preparation needle.

5 Euthanize donor females using institution-approved methods. This process is performed approximately 20 h post-hCG (about 8:00 on day 4).

6 Dissect out the oviducts and place them in a culture dish containing pre-warmed M2. Maintain the tissue samples at 37.5 °C on a heated stage. Ensure all females are dissected in less than 10 minutes post-euthanasia.

7 Move each oviduct into 400 µl of pre-warmed M2 medium with hyaluronidase and, using a stereomicroscope, disrupt the oviduct by using a pair of forceps at the swollen ampulla region to release cumulus-oocyte complexes. Pin down the oviduct with one pair of forceps and gently tear the ampulla with the preparation needle. Remove the disrupted oviduct and repeat the process for the remaining oviducts. Wait for the cumulus cells to dissociate from the zygotes. This will take from 30 seconds to a couple of minutes.

8 Using a mouth pipette, transfer the zygotes to a warm M2 wash dish. Pass the embryos through 3 drops of M2 medium. This will inactivate the residual hyaluronidase. Transfer as little hyaluronidase/cumulus cells as possible during this process.

9 (Optional) Repeat the washing step to remove residual cumulus cells and hyaluronidase.

10 Remove any unfertilized or fragmented oocytes and place the remaining fertilized zygotes into pre-equilibrated KSOM medium at 37.5 °C in a 5% CO2 incubator until ready for electroporation.

Electroporation:

11 Guide RNA (gRNA) is prepared in a 0.5 ml tube by mixing 0.6 µl (100 µM) crRNA and 0.6 µl (100 µM) ATTO550 labeled tracrRNA each with 1.8 µl Nuclease-Free Duplex Buffer (Cat. #11-01-03-01; IDT, Coralville, USA).

12 The reaction is incubated at 95°C for 3 minutes and cooled down for 30 minutes after turning off the heat block to generate annealed gRNA on the heat block.

13 Add 0.96 µl of NLS-Cas9 (IDT; 61 µM) to generate the RNP complex and 6.04 µl of Opti-MEM I (Total 10 µl).

14 Set up the electroporator (Genome Editor, BEX, Tokyo, Japan) according to the manufacturer's instructions. A pair of platinum block electrodes (BEX, Tokyo, Japan; length: 10 mm, width: 3 mm, height: 0.5 mm, gap: 1 mm) was used and placed under a stereoscopic microscope.

15 Set up the electroporation conditions to 30 V (3 msec ON + 97 msec OFF) × 10 times.

16 Fill the gap of the electrode with 5 µl of Opti-MEM containing the RNP complex.

17 Wash the collected zygotes cultured in KSOM medium with Opti-MEM three times to remove the culture medium. Then wash three times with Opti-MEM containing the RNP complex.

18 Place the zygotes (up to 40) in a line in the electrode gap with a mouth pipette and perform the electroporation.

19 After electroporation, immediately collect the zygotes from the electrode chamber and wash them four times with M2 medium, followed by three washes in KSOM medium. Thereafter, culture the zygotes in KSOM medium at 37 °C and 5% CO2 for 24 h, until

two-cell stage embryo formation (or, if testing new target loci, until the blastocyst stage to genotype them).

Fluorescent signal detection and analyses:

20 The next morning (day 5), visualize the localization of the RNP complex (NLS-Cas9 and gRNA complex) with a Nikon A1R confocal (or other) microscope (Tokyo, Japan) at 24 h after electroporation. Analyze the confocal images using appropriate software.

Blastocyst DNA isolation and PCR:

21 Blastocyst-stage embryos are separately transferred using a mouth pipette to 0.2 ml PCR tubes.

22 Resuspend them in 20 µl of lysis buffer containing 100 mM Tris-HCl (pH 8.3), 100 mM KCl, 0.45% Tween 20, 0.02% gelatin, 125 µg/mL proteinase K.

23 Lysis is performed by incubation at 56 °C for 10 minutes, followed by 10 minutes of denaturation at 95 °C. Crude lysate is used for direct DNA amplification or stored at -20 °C.

24 The polymerase chain reaction (PCR) is performed using Terra™ PCR Direct Polymerase Mix (Cat. #639287; Takara Bio, Inc., Shiga, Japan) consisting of 12.5 µl of 2X Terra PCR Direct Buffer (with Mg2+, dNTPs), 0.5 µl (1.25 U) Terra PCR Direct Polymerase Mix, 1 µl (10 µM) of forward and reverse primers, 1.5 µl of DNA template, and sterile water up to25 µl. PCR conditions are: the initial denaturation step of 98°C for 2 minutes, followed by 36 cycles of 98°C for 10 seconds, 62°C for 15 seconds, 68°C for 1 minute, and a final extension of 68°C for 5 minutes.

25 After amplification is completed, load 2.5 µl of PCR product onto a 1.5% agarose gel containing 1X TAE (Tris-acetate-EDTA) buffer (Cat. #15558042; Invitrogen™, Waltham, USA).

26 Electrophoresis is performed at 100 V for 30 minutes with a ladder for precise sizing.

Preparation of gRNA for cleavage assay:

27 For the cleavage assay, guide RNA (gRNA) is prepared in a 0.5 ml tube by mixing 0.5 µl (100 µM) crRNA and 0.5 µl (100 µM) tracrRNA each with 49 µl Nuclease-Free Duplex Buffer.

28 The mixture is incubated at 95°C for 3 minutes and cooled down for 30 minutes after turning off the heat block to generate annealed gRNA on the heat-block. 61 µM NLS-

Cas9 (IDT) is diluted in Opti-MEM I to 1 µM concentration.

CLEAVAGE ASSAY of embryo-derived samples:

29 1 µl of gRNA, 1 µl of diluted NLS-Cas9 (1 µM in Opti-MEM I), 2.5 µl of PCR products, and 15.5 µl of Opti-MEM I are mixed and incubated overnight.

30 Electrophoresis is performed at 100 V for 30 minutes with a ladder for precise sizing, and the image is taken using the ChemiDoc XRS+ System (Bio-Rad) and Image Lab Software.

Embryo Transfer and Production of Mice:

31 After confirming the success of CRISPR-mediated editing, prepare pseudopregnant foster mothers (8–12 weeks old) by mating estrus-selected F1 female mice (or chosen strain) with vasectomized male mice the day before electroporation (day 3) and inspecting for a vaginal plug on the day of electroporation (day 4).

32 At day 5 anesthetize (in accordance with local regulations) a plug-positive foster female, put her on the heated stage (38 °C), and make a 5-mm skin incision parallel to the dorsal midline above the position of the left or right oviduct. The ovarian fat pad should become visible.

33 Pull out the ovary by fat pad and fix it with forceps.

34 Locate the infundibulum and tear the bursa around the ovary and oviduct using two fine forceps.

35 Transfer electroporated embryos to a drop of prewarmed M2 medium without mineral oil over it.

36 Load 8–10 embryos in the transfer mouth pipette in between 2 small air bubbles.

37 Insert the tip of the transfer capillary into the infundibulum and carefully release the embryos.

38 Perform an embryo transfer of 8–10 two-cell embryos that are electroporated with gRNA and Cas9 into each oviduct of 1.5 d.p.c. recipients.

39 Relocate the ovary and fat pad into the abdomen and suture the peritoneum and skin. After the procedure, place the recipient on a warm heat stage.

40 Repeat the procedure with the next plug-positive foster female until all embryos are transferred. Recipient mothers deliver pups at approximately 20.5 d.p.c.

41 When pups reach about 3 weeks of age, they can be weaned, sexed, and genotyped.

Confirmation CLEAVAGE ASSAY of Mice Samples and Genotyping:

42 Pieces of mouse tissue (e.g., tails or ears) for genotyping (up to 3 weeks old) are collected and separately transferred using forceps to 0.2 mL PCR tubes.

43 Resuspend it in 20 µl of lysis buffer of 100 mM Tris-HCl (pH = 8.3), 100 mM KCl, 0.45% Tween 20, 0.02% gelatin, and 125 µg/mL proteinase K.

44 Lysis is performed by incubation at 56 °C for 10 min followed by 10 min denaturation at 95 °C. Crude lysate is used for direct DNA amplification or stored at -20 °C.

PCR and Sequencing:

45 PCR and cleavage assays are performed following the same protocol as 24)-30), prior to sequencing. If successful editing is confirmed, the PCR products are sequenced using one of the primers via Sanger sequencing (Genomed S.A., Warsaw, Poland).

46 The result is analyzed by FinchTV 1.4.0 software to confirm modification by CRISPR.