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Simple electroporation for efficient CRISPR/Cas9 genome editing in murine zygotes V.4

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Electroporation of zygotes represents a rapid alternative to the elaborate pronuclear injection procedure for CRISPR/Cas9-mediated genome editing in mice. However, current protocols for electroporation either require the investment in specialized electroporators or corrosive pre-treatment of zygotes which compromises embryo viability. Here, we describe an easily adaptable approach for highly efficient introduction of specific mutations in mice by electroporation of intact zygotes using a common electroporator with synthetic CRISPR/Cas9 components and minimal technical requirement. This protocol works efficiently with zygotes from a variety of genetic backgrounds and is compatible with other CRISPR nucleases like Cas12a.

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protocol

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Zygote, Electroporation, CRISPR, Cas9, Cas12a, Mouse model

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Use only embryo-grade reagents.

Embro culture media (M2, M16, KSOM/AA) may be purchased or prepared in-house as published (Behringer et al., 2014). Mouse pre-implantation embryos are incubated in pre-equilibrated M16 or KSOM/AA in a CO_2 incubator (5% CO_2, 37°C, 95% humidity).

Embryo-grade standard $T_{10}E_{0.1}$ (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) injection buffer is prepared as described (e.g., Chu et al., 2016).

Mouse zygotes are collected from the oviducts of superovulated females as described in published protocols (Behringer et al., 2014). To avoid developmental impairment is recommended to use only zygotes of high quality (i.e., presence of polar bodies and pronuclei).

This protocol works efficiently with zygotes from any source (generated by natural mating or IVF as well as freshly harvested or frozen-thawed) and from various genetic backgrounds like C57BL/6N, C57BL/6J, FVB/N and SWISS mice. Frozen zygotes may be purchased from commercial vendors (e.g., Kit Eazygote from Janvier Labs).

Instead of Cas9 other CRISPR nucleases like Cas12a can be used as well. For Cas12a use the same concentration of reagents in the electroporation mix as described for Cas9. Cas12a does not require gRNA annealing as it works without a tracRNA. It is advisable to include the IDT electroporation enhancer at 4 µM (i.e., 0.8µl of 100 µM in 20 µl of total mix) and to use the mix immediately after preparation for the electroporation as Cas12a can have indiscriminate ssDNA nuclease activity. For the same reason the ssODN should be stabilized with with PS (Phosphorothioate) bonds at each end (the last 3 nucleotide bonds are sufficient).

References:
Behringer, R., et al., (2014) Manipulating the mouse embryo : a laboratory manual, Fourth edition. ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
Chu, V.T., et al., (2016) Efficient generation of Rosa26 knock-in mice using CRISPR/Cas9 in C57BL/6 zygotes. BMC Biotechnol 16, 4.

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MATERIALS

Alt-R® S.p. Cas9 Nuclease V3 **Integrated DNA Technologies Catalog #1081058**

Alt-R® A.s. Cas12a Ultra Nuclease **Integrated DNA Technologies Catalog #10001272**

Alt-R® CRISPR-Cas9 tracrRNA **Integrated DNA Technologies Catalog #1072532**

Alt-R® CRISPR-Cas9 crRNA **Integrated DNA Technologies**

ssODN (Ultramer DNA Oligonucleotides) **Integrated DNA Technologies**

Alt-R® Cas12a Electroporation Enhancer **Integrated DNA Technologies Catalog #1076300**

Opti-MEM™ Reduced Serum Medium **Thermo Fisher Scientific Catalog #31985062**

M2 medium **Merck Millipore Sigma Catalog #M7167**

KSOM **Merck Millipore Sigma Catalog #MR-106-D**

M16 Medium **Contributed by users**

T10E0.1 Buffer **Contributed by users**

Electroporation Cuvette 1mm **BioRad Sciences Catalog #1652089**

60 mm Center Well Organ Culture Dish **Corning Catalog #353037**

guide RNA annealing

1 Resuspend lyophilized crRNA, tracrRNA and ssODN in $T_{10}E_{0.1}$ buffer to 100 µM (e.g., 5 nmol in 50 µl)
(Store at -80 °C until use)

2 Combine 5 µl crRNA (100 µM) and 5 µl tracrRNA (100 µM) in a nuclease-free PCR tube to yield an equimolar crRNA:tracrRNA duplex solution of 50 µM

3 Heat to 95 °C for 5 min and cool down at 5 °C/ min in a thermocycler
(crRNA:tracrRNA duplex can be stored for months at -80 °C)

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Preparation of the electroporation mix

4 Add 1.6 µl crRNA:tracrRNA duplex and 1.3 µl Cas9 nuclease to 15.1 µl Opti-MEM in a nuclease-free tube and vortex
(Instead of Cas9 other CRISPR nucleases like Cas12a can be used as well. See Guidelines & Warnings)

5 Incubate mix at room temperature for 10 min
00:10:00

6 Place tube on ice, add 2 µl ssODN and vortex
(The ssODN may be left out if desired but must subsequently be compensated by 2 µl Opti-MEM to reach a total electroporation mix of 20 µl)

7 Quick-spin at 4 °C and keep tube on ice until use

8 Summary of the 20 µl electroporation mix:

A	B	C	D
Reagent	Stock concentration	Final concentration	Volume
crRNA:tracrRNA duplex	50 µM	4 µM	1.6 µl
Cas9 nuclease	61 µM (10 µg/µl)	4 µM	1.3 µl
ssODN (optional)	100 µM	10 µM	2.0 µl
Opti-MEM	-	-	15.1 µl

Electroporation of zygotes

9 Collect zygotes from the oviducts of superovulated females as described in published protocols

10 Wash the zygotes in five drops of M2 medium

11 Wash up to 50 zygotes in one drop of Opti-MEM
(work quickly as zygotes may suffer from extended incubation in Opti-MEM)

12 Transfer zygotes with as little media as possible to the 20 µl electroporation mix
(e.g., by first transfering the 20 µl electroporation mix onto a culture dish and subsequently

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adding the zygotes)

13 Using a 100 µl pipette set to 21 µl volume transfer the entire drop of electroporation mix including the zygotes into a pre-warmed (37 °C) 1 mm electroporation cuvette
(Ensure retrieving all zygotes by quickly aspirating the entire drop. Slow aspiration will leave zygotes behind)

14 Insert the cuvette into a standard electroporator (e.g., BioRad Gene Pulser Xcell electroporator)

15 Apply two square wave pulses at 30 V and 3 ms duration with a 100 ms interval

16 Retrieve the zygotes by flushing the cuvette with 100 µl M2 medium using a 100 µl pipette into a culture dish (e.g. 60 mm Center Well Organ Culture Dish)

17 Wash the cuvette with 100 µl M2 medium

18 Transfer all zygotes to a new culture dish containing pre-incubated microdrops of culture medium covered by oil. Wash the zygotes in three drops prior to culture in a fourth drop of culture medium.
(Alternatively, a dish with 500 µl pre-incubated culture medium without oil may be used)

19 Incubate zygotes in an incubator until the two-cell stage and transfer the developed embryos into pseudopregnant foster mice
(Embryos may also be transferred at the one-cell stage)

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