Protocol

CRISPR–Cas9 Genome Editing in *Nothobranchius furzeri* for Gene Knockout and Knock-In

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The African turquoise killifish *Nothobranchius furzeri* has recently gained interest as an emerging vertebrate model system for the study of aging, owing to its naturally short life span and generation time. Here, we provide a step-by-step guide for effective genome engineering using the CRISPR–Cas9 system to generate loss-of-function (i.e., knockout) alleles and for precise editing (i.e., knock-in) of short sequences into the genome. Using this approach, a new stable line can be created within several months. The killifish's tough chorion, rapid growth, and short life span are considered in this protocol and account for the key deviations from similar protocols in other fish models.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Agarose (Life Technologies 16500-500)
Buffer for agarose gel electrophoresis
DNA ladder, 100-bp or 1-kb (Promega G2101 or G5711)
DNase I
DNase/RNase-free distilled H_2O (Invitrogen 10977-015)
dNTP solution set, 25-mM (NEB N0446S)
EndoFree Maxiprep kit (QIAGEN 12362)
Ethanol, 100% (Rossville Gold Shield Chemical)
Ethidium bromide (Thermo Scientific 15585011)
Gene-specific genotyping primers (100 μM stock concentration, standard desalted, Sigma-Aldrich; see Step 5)
GlycoBlue Coprecipitant, 15 mg/mL (Invitrogen AM9516)

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From the African Turquoise Killifish collection, edited by Anne Brunet.

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HDR DNA repair template (optional; see Steps 7 and 38)

For detailed instructions, please see Harel et al. (2016) and Astre et al. (2022a).

Methylene blue embryo solution <R>
Miniprep kit (QIAGEN 27104)
mMessage mMachine SP6 Ultra kit (Life Technologies AM1340)
NaOH, 50 mM (Sigma-Aldrich 221465)
NEB buffer 2.1 (NEB B7202S)
NotI restriction enzyme (NEB R0189S)
PCR purification kit (QIAGEN 28104)
pCS2-nCas9n plasmid (Addgene Plasmid 47929) (Jao et al. 2013)
Phenol red solution, 0.5% (Sigma-Aldrich P0290)
QIAquick nucleotide removal kit (QIAGEN 28304)
Sanitizing solution <R> (optional; see Step 41)
Sexually mature *Nothobranchius furzeri,* GRZ strain

For detailed protocols on how to raise killifish and how to determine if they are sexually mature, please see Dodzian et al. (2018) and Astre et al. (2022a).

Because killifish are a vertebrate organism, their experimental use and care must be approved and monitored by relevant institutional ethics guidelines.

Sodium acetate, 3 M, pH 5.5 (Ambion AM9740)
T4 DNA polymerase (NEB M0203S)
TE buffer (Thermo Fisher AM9849) (optional; see Steps 21 and 31)
TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific K0441)
Tris HCl, 1 M, pH 8 (Sigma-Aldrich RES3098T-B701X)
Universal reverse oligonucleotide (100 μM stock, standard desalted, Sigma-Aldrich): AAAAGCACC GACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCT AGCTCTAAAAC

This oligonucleotide is used to generate all gRNA templates.

Variable forward oligonucleotide (100 μM stock, standard desalted, Sigma-Aldrich; see Step 4): TAATACGACTCACTATA[**GG-(N)18**]GTTTTAGAGCTAGAAATAGCAAG

The interchangeable bold target sequence is unique for each gRNA.

Equipment

Agarose gel apparatus
Borosilicate microcapillaries with filament (Sutter BF100-58-10)
Breeding sand (Agat Minerals Ltd. FL S250)
DNAase/RNAase-free disposable plastic pestles (Alex Red CSP001002)
Dumont tweezers #5 (WPI 500342)
Fine mesh strainer, 8-in (OXO Good Grips 38991)
Fish nets
Gilson PIPETMAN (Fisher Scientific F123600G, F144801G, F123601G, and F123602G)
Injection apparatus (Applied Scientific Instrumentation), which includes an MHC model magnetic stand, an MMPI model pressure injector, a foot switch to pulse the injected solution into the embryos, an MM 33 micromanipulator (Marzhauser Wetxlar 00-42-101-0000), and an M-PIP model micropipette holder (Applied Scientific Instrumentation), assisted by a back-pressure unit (Warner Instrument)
Injection mold (well depth: 1.1 mm; well width: 0.95 mm)

See (Harel et al. 2016) for a 3D printing design, in STL or DWG file formats.

Kimwipe (Kimtech Science)
Microcentrifuge

Microcentrifuge tubes
Micropipette Puller Model P-97 (Sutter Instrument Co.)
Nanodrop Spectrophotometer (Thermo Scientific)
Parafilm
Pasteur pipettes, plastic
PCR tubes
Petri dishes, 6-cm, 10-cm
Plastic tray for breeding sand, food grade (Peamit 22-100-0208)
Pneumatic PicoPump (WPI PV830)
Thermocycler

METHOD

Target Selection

1. Select a gene(s) of interest. If the gene of interest is poorly annotated and not present in CHOPCHOP, design gRNA targets by directly pasting a FASTA entry into the "Target" window in CHOPCHOP.
2. Go to CHOPCHOP (https://chopchop.cbu.uib.no/; (Labun et al. 2019)) and choose the following settings.
   i. Write the selected gene under the "**Target"** tab.
   ii. Choose *Nothobranchius furzeri* (Nfu_20140520/Jena, or NotFur1/Stanford) under the "**In**" tab.

   There are two available assemblies and it is helpful to compare both annotations for any given target.

   iii. Choose CRISPR/Cas9 under the "**using**" tab.
   iv. Choose knock-out under the "**For**" tab.
   v. Click on "**options**," choose the "**Cas9**" tab and under "**5′ required for sgRNA**" choose the option "GN or NG."
   vi. Move to the "**Primer**" tab and choose a product size of 400–600.
   vii. Send the query by clicking on the "**Find Target Site**."
3. Select two to three targets per gene from the list of possible target sites generated from the CHOPCHOP algorithm. These sites will be ranked by predicted efficiency and specificity.

   We recommend selecting gRNA targets which are within vertebrate-conserved regions using http://genome.ucsc.edu/. Specifically, this will assist in avoiding alternative translation start sites, which are conserved in eukaryotic genomes (Bazykin and Kochetov 2011).

   For a new gene of interest, we usually begin by injecting a mix of three different gRNAs together into approximately 10 eggs. We then identify the most efficient gRNA by sequencing and generate a stable line in a separate injection. At that stage we inject 30–50 eggs.
4. Ensure the target sequence starts with two G nucleotides (GG). If needed, replace either the first or the second nucleotide in the target sequence with a G. Remove the last 3 bp (the PAM sequence). This will result in a 20-bp-long sequence beginning with GG. Copy the new target sequence to the template below, in place of the square brackets:

   TAATACGACTCACTATA[—]GTTTTAGAGCTAGAAATAGCAAG.

   This is the gRNA template.
5. Consult the CHOPCHOP software, which will indicate several different pairs of primers for amplification and Sanger sequencing of the targeted area. Choose a set of primers that has ∼300–400 bp on each side of the predicted gRNA target.

Synthesis of gRNA

Conduct all steps involving RNA synthesis and handling in a clean environment, using RNase/DNase-free reagents.

6. Order all the oligonucleotides (100 µM stock concentration, standard desalted), including the variable and universal gRNA templates, and the genotyping primers (a total of three unique primers for each target, and a common universal gRNA template that is used for all targets).
7. Optional step: Prepare a homology-directed repair (HDR) DNA repair template as follows: Design an ssDNA template with homology arms of ~30 bases on each side, flanking the gRNA target site. Place introduced mutations as close as possible to the protospacer-adjacent motif (PAM) site. Purify the oligonucleotide using a QIAquick Nucleotide Removal kit. Elute the template in distilled H_2O at a final concentration of 100–200 µM and store at −20°C. For detailed instructions, please see Harel et al. (2016) and Astre et al. (2022a).
8. Add the following components to a PCR tube. Mix by pipetting.

Component	Amount	Final concentration
Universal reverse oligonucleotide (100 µM)	2 µL	20 µM
Variable forward oligonucleotide (100 µM)	2 µL	20 µM
NEB 2.1 buffer 10×	1 µL	1×
Nuclease-free H_2O	5 µL	
Total	10 µL	

9. Program a standard thermocycler as follows. Place the reaction from Step 8 in the thermocycler. Do not repeat any of the steps below.

Step number	Temperature	Time
1	95°C	30 sec
2	72°C	2 min
3	37°C	2 min
4	25°C	2 min
5	12°C	2 min
6	4°C	Forever

10. After the reaction has ended, add the following components to each reaction tube and mix by pipetting:

Component	Amount	Final concentration
dNTP mix (25 mM)	1.25 µL	1.25 mM
T4 DNA polymerase (3000 units/mL)	0.5 µL	0.15 units/µL
Nuclease-free H_2O	8.25 µL	
Total	10 µL	

11. Incubate the reaction for 20 min at 12°C in a thermocycler.
12. Purify the gRNA template using a standard PCR purification kit according to the manufacturer's protocol and elute in 30 µL of elution buffer (supplied in the kit).
13. Measure the concentration using a standard NanoDrop and expect a concentration of ~0.5 µg/mL. If desired, store the template for up to several months at −20°C.
14. Use 0.25 µL of the eluted template in a 5-µL TranscriptAid T7 High Yield Transcription Kit according to the manufacturer's instructions (one-quarter of the standard reaction size is sufficient). Carry out the reaction as described by the manufacturer.

15. Add 1 µL of DNase I, mix gently, and incubate it for an additional 15 min at 37°C.
16. Add the following components to the tube in the order listed to precipitate the RNA. Mix well before and after adding the ethanol.

Component	Amount	Final concentration
Reaction mix	6 µL	
Nuclease-free H_2O	27.75 µL	
3M Sodium acetate	3.75 µL	0.1 M
GlycoBlue coprecipitant	0.5 µL	
Ethanol, 100%	75 µL	
Total	113 µL	

17. Incubate the mixture for at least 15 min at −20°C. The reaction can be placed overnight at −20°C with no decrease in yield.
18. Centrifuge the tube for 15 min at top speed in a prechilled 4°C microcentrifuge.
19. Remove the supernatant, centrifuge the tube for a few seconds at top speed in a microcentrifuge, and remove all the residual supernatant with a fine-tipped pipette (10-µL tip).
20. Let the pellet dry for 5 min. Do not overdry.
21. Resuspend the pellet in 30 µL distilled H_2O or TE.
22. Measure the concentration using the NanoDrop and adjust the stock concentration to 0.5–1 µg/µL with TE or distilled H_2O. It is possible to use RNA with a concentration as low as 0.2 µg/µL, as long as a final concentration of 30 ng/µL can be reached in the injection mix (Step 38), by adjusting the amount of water used.
23. Mix 2 µL of the purified gRNA with loading dye from the mMessage mMachine SP6 Ultra kit and resolve using standard agarose gel electrophoresis (1% agarose with ethidium bromide). Run the gel for 5–10 min total to limit RNA degradation. A discrete band should be seen, which indicates a nondegraded product. If the band is smeared, resynthesize the gRNA. The RNA is resolved for assessing integrity, and not for size estimation.
24. Prepare 2-µL aliquots of purified gRNA and store them for up to several months at −80°C.

Synthesis of Cas9 mRNA

25. Using a standard Maxiprep kit, purify the nCas9n expression DNA plasmid. Measure the concentration of the purified plasmid.

    See Troubleshooting.

26. Digest ~8–10 µg of nCas9n plasmid using a NotI restriction enzyme according to the manufacturer's instructions.
27. Following digestion, purify the nCas9n expression plasmid using a standard PCR purification kit. Elute in 50 µL of elution buffer.
28. Use the mMessage mMachine SP6 kit to transcribe capped and polyadenylated nCas9n mRNA from the purified plasmid template according to the manufacturer's instructions.
29. Add 1 µL of TURBO DNase (provided in the mMessage mMachine SP6 kit) to the reaction mixture, mix, and incubate for an additional 15 min at 37°C.
30. Precipitate the resulting mRNA using lithium chloride precipitation according to the mMessage mMachine SP6 kit protocol.
31. Resuspend the nCas9n mRNA pellet in 30 µL dH_2O or TE.
32. Place the tube for 10 min at 65°C to completely resuspend the nCas9n mRNA.

33. Run 2 µL of the purified nCas9n mRNA using the formaldehyde loading dye from the mMessage mMachine SP6 kit on a 1% agarose gel with ethidium bromide. A well-defined band should be seen, which indicates a nondegraded product. If the band is smeared, resynthesize the Cas9 mRNA. The RNA is resolved primarily for assessing integrity, and not for size estimation.

    See Troubleshooting.

34. Measure the mRNA concentration using the NanoDrop and adjust the concentration to ~1 µg/µL. Prepare 2-µL aliquots and store them for up to several months at −80°C.

Preparations before Injection

35. Prepare an injection plate by pouring 10 mL of 1.5% agarose into a 10-cm Petri dish. Wait for the hot agarose to cool for 5 min (as the heat might bend the injection mold), and then lay a clean plastic injection mold on the surface of the agarose, which will produce six troughs to hold eggs in position. For a 3D printing design in STL or DWG file formats, see Harel et al. (2016). Leave the mold in place until the agarose solidifies. Seal agarose plates with Parafilm and store for up to several weeks at 4°C. Discard plates if mold is observed.

36. Pull needles from borosilicate capillaries. Our parameters have been optimized for the Micropipette Puller P-97 (with a trough filament), using filamented microcapillaries.

    Needles can be prepared while fish are mating; see Step 39.

37. Use the following parameters as a starting point:
    - Pressure: 450
    - Heat: 430
    - Pull: 40
    - Velocity: 40
    - Time: 250

    The needles should be able to penetrate the chorion fairly easily without bending. Successful injection will require some practice.

38. Prepare an injection mixture by mixing these components in a PCR tube.

Component	Amount	Final concentration
Purified gRNA	1 µL	30 ng/µL
nCas9n mRNA	1 µL	200–300 ng/µL
(Optional) HDR DNA repair template	1 µL	20 µM
Phenol red	2 µL	0.1% (wt/vol)
Nuclease free H_2O	to 10 µL	to 10 µL
Total	10 µL	

Keep the injection mixture on ice. Prepare up to an hour before injection. We recommend dividing the mixture into two tubes for better handling during the injection.

Mating and Egg Collection

In general, similarly to other laboratory fish, killifish are maintained in standard recirculating water systems, using 3- to 10-L tanks (brackish water is favored). Fish benefit from a rich diet and can reach sexual maturity as fast as 2–3 wk. For additional information about breeding, hatching, and genotyping of turquoise killifish for genome editing purposes, see Dodzian et al. (2018), Astre et al. (2022a), and Reichard et al. (2022).

39. On the morning embryos are to be injected, set up several breeding groups of sexually mature fish using nets (one male with two to three females in a single 3- to 10-L tank connected to the recirculating water system) as follows.

i. Prior to adding the fish, add a sand tray for each tank.

ii. Collect sand trays after 4.5 h. Separate the males from the females (in preparation for future matings).

iii. Separate the eggs from the sand using an 8-in. fine-mesh strainer, keeping the bottom of the mesh submerged in a tank with system water, and using circular movements. To collect eggs, use a plastic Pasteur pipette with a clipped tip (as eggs are slightly larger than the tip). Place eggs in a 6-cm Petri dish containing methylene blue embryo solution. Keep the dish at room temperature.

Ten breeding groups can yield around 100–200 eggs using the commonly used GRZ strain (Astre et al. 2022a). For optimal results, females and males should be housed apart, and mated frequently.

40. Inject eggs at the one- to two-cell stage (up to ~6 h after fertilization).

Injection

41. Discard any dead eggs, which will appear opaque (see Fig. 9 in Polačik et al. 2016). Remove dead eggs using a Pasteur pipette with a clipped tip. If desired, sanitize viable eggs by filling the plate with sanitizing solution for 5 min at room temperature. When changing solution, remove the existing solution using a Pasteur pipette, and pour in fresh solution. Wash eggs twice with methylene blue embryo solution.

We have observed that in some cases, sanitizing the eggs increases later survival by limiting bacterial infections.

42. Load ~5–7 µL of the injection mix into the injection needle (see Fig. 1) using capillary force by gently placing the backside of the pulled capillary into the injection mix.

43. Place ~5 mL of methylene blue embryo solution into the injection plate. Using a stereoscope and curved tweezers, place eggs into the injection troughs (about 10–15 per trough) and orient the fertilized eggs so that the cells are facing the needle (see Fig. 1 in Hartmann and Englert 2012). Remove embryos that have passed the two-cell stage.

44. Gently touch a Kimwipe with the tip of the needle to crack it.

45. Use the MM 33 micromanipulator (part of the injection apparatus, described in Astre et al. (2022a) to place the tip of the filled needle into the methylene blue embryo solution. Using the Pneumatic PicoPump, adjust the back pressure until a faint trail of injection mix is seen leaking into the water ("smoking gun"). If no mix is leaking into the methylene blue embryo solution, the pressure is not high enough or the needle is not broken (or is clogged).

46. Insert the needle into the cell and inject using the following parameters: 30 psi with 75-msec pulses (~2.5–10 pL of injection mixture). Ensure that a small red hue of phenol red is seen throughout the cell after injection. Injection parameters may need to be optimized for different needles and during injection. For more details, see Valenzano et al. (2011), Hartmann and Englert (2012), and Harel et al. (2016). If injecting a two-cell embryo, injection into one cell is sufficient.

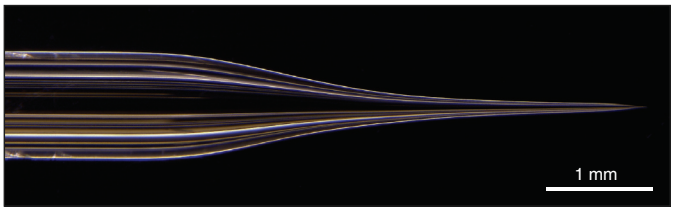

FIGURE 1. Overview of an injection needle. Note that the tip of the needle can be broken by gently rubbing it on a Kimwipe.

47. Using a Pasteur pipette, collect the injected eggs into a 6-cm Petri dish filled with methylene blue embryo solution, cover with a lid, and keep them at 28°C.
48. After injection, monitor daily and remove dead embryos. Change the solution every few days, or even daily if it becomes clouded. Expect a survival rate of 50%.
    See Troubleshooting.

Evaluation of Editing Efficiency

49. Forty-eight hours after injection, extract genomic DNA from 10–15 injected embryos (individually) and a single naive age-matched embryo as a control (no manipulations are performed on control embryos).
    i. Using a Pasteur pipette, place individual fertilized eggs in separate microcentrifuge tubes.
    ii. Aspirate methylene blue embryo solution and add 20 µL of 50 mM NaOH.
    iii. Crush the embryos with a disposable pestle.
    iv. Incubate for 10 min at 95°C using a heat block.
    v. Neutralize with 2.2 µL of 1 M Tris HCl, pH 8. The solution is now referred to as extracted genomic DNA. Store extracted genomic DNA for up to a few months at 4°C.
50. To evaluate editing efficiency, perform direct sequencing of the amplified genomic region using the sequencing primers recommended by CHOPCHOP. Alternatively, estimate editing using any of the recently developed approaches, such as using restriction enzymes, high-resolution melting, and other approaches described in Astre et al. (2022a). Importantly, perform Steps 49–50 on a few embryos, and allow the rest to develop.
    See Troubleshooting.

Hatching and Breeding a Stable Line

51. After ~3 wk, hatch injected embryos, which are now termed F_0 founders. Additional information about hatching is provided in Harel et al. (2016), Dodzian et al. (2018), and Astre et al. (2022a).
52. Genotype F_0 founders using the approach described in Steps 49 and 50 using fin-clip or scales isolation as a source of DNA (Dodzian et al. 2018; Astre et al. 2022a).
53. Identify specific indels by direct sequencing of the amplified genomic region.
54. To generate stable lines, outcross mutant fish to dilute potential off-target mutations, and then breed fish carrying the desired mutation.

TROUBLESHOOTING

Problem (Step 25): The concentration of the DNA plasmid is too low.

Solution: If needed, carry out another maxiprep to obtain the amount of DNA plasmid required as a template.

Problem (Step 33): There is a problem with the RNA synthesis kit, resulting in degraded or absent mRNA.

Solution: The synthesis buffer in the mMessage mMachine SP6 kit is sensitive to repeated freeze–thaw cycles. Consider making aliquots of the buffer. Verify the expiration date of the kit.

Problem (Step 48): Low survival or abnormal development of injected embryos is seen.

Solution: Try any of the following.

- Inject less solution.
- Break the needle more gently or change the needle configuration.
- Lower the air pressure or decrease the strength of the injection pulse.
- Choose a different gRNA.

Problem (Step 50): Editing efficiency by a given gRNA efficiency is low.

Solution: Make sure to inject into the cells and not into the yolk (or perivitelline space). Alternatively, choose a different gRNA or remake the nCas9n mRNA.

DISCUSSION

This protocol provides an easy step-by-step guide for creating a stable line of genome engineered killifish. These approaches can be used for modeling age-related diseases (Harel et al. 2015, 2016, 2022), knocking-in disease variants (Harel et al. 2016), and exploring novel genetic pathways that extend vertebrate life span (Astre et al. 2022b). Using this method, a stable line may be generated within 2–3 mo with high editing efficiency (depending on injection skill and the specific efficiency of a given gRNA).

For precise editing of new sequences into the genome (i.e., "knock-in"), please see Harel et al. (2015, 2016) and Astre et al. (2022a).

RECIPES

Methylene Blue Embryo Solution

Component	Amount
Ringer's solution from tablets <R>	1000 mL
Methylene blue, 2.3% (Kordon 37344)	100 µL

PVPI Stock Solution

Component	Amount
Polyvinylpyrrolidone-iodine complex (Thermo Fisher Scientific 229471000)	10 g
Double-distilled H_2O	100 mL

Ringer's Solution from Tablets

Component	Amount
Double-distilled H_2O	1000 mL
Ringer tablets (Sigma-Aldrich 1155250001)	2 tablets

Sanitizing Solution

Component	Amount
Ringer's solution from tablets <R>	100 mL
PVPI stock solution <R>	500 µL

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