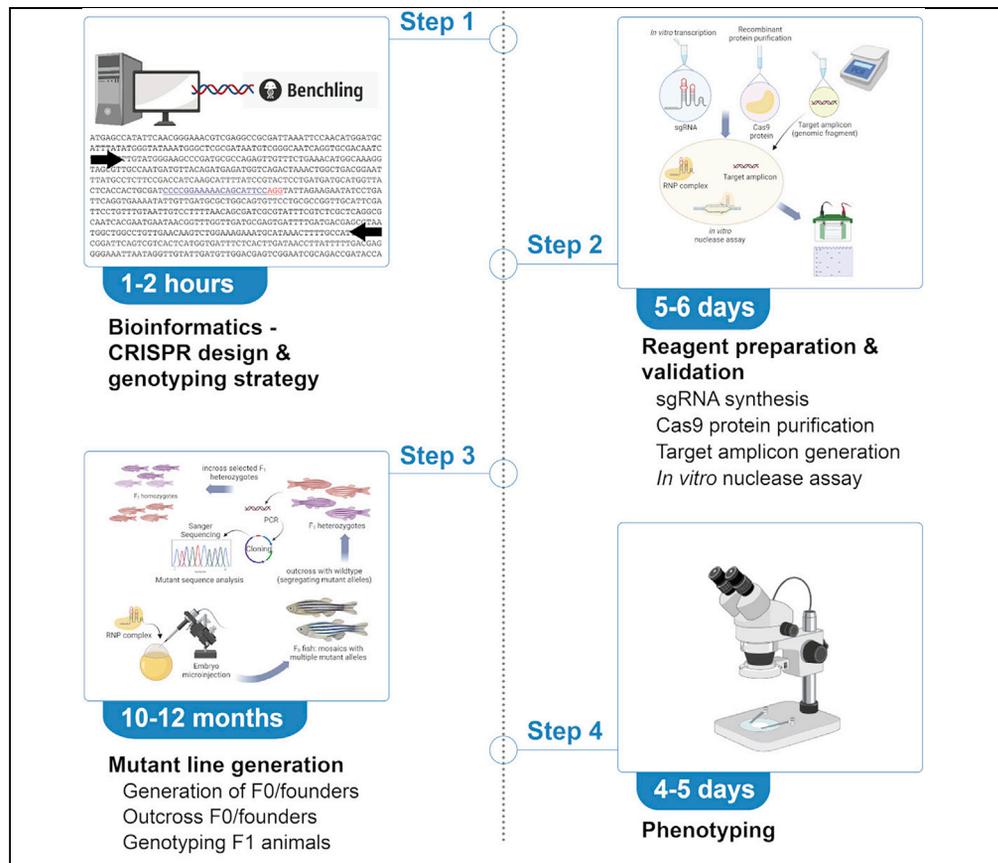


## Protocol

# CRISPR-Cas9-induced gene knockout in zebrafish



The application of CRISPR has greatly facilitated genotype-phenotype studies of human disease models. In this protocol, we describe CRISPR-Cas9-induced gene knockout in zebrafish, utilizing purified Cas9 protein and *in vitro*-transcribed sgRNA. This protocol targets the PHLPP1 gene in an Indian wild-caught strain, but is broadly applicable. Major factors influencing protocol success include zebrafish health and fecundity, sgRNA efficiency and specificity, germline transmission, and mutant viability.

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

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### Highlights

Detailed protocol for CRISPR-Cas9-induced targeted gene knockout in zebrafish

Use of in-house purified Cas9 protein and *in vitro*-synthesized single-guide RNA

Heteroduplex mobility assay for quick genotyping, with sequencing for confirmation

Broadly applicable to a range of target genes and zebrafish strains

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## Protocol

## CRISPR-Cas9-induced gene knockout in zebrafish

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<https://doi.org/10.1016/j.xpro.2022.101779>

## SUMMARY

The application of CRISPR has greatly facilitated genotype-phenotype studies of human disease models. In this protocol, we describe CRISPR-Cas9-induced gene knockout in zebrafish, utilizing purified Cas9 protein and *in vitro*-transcribed sgRNA. This protocol targets the PHLPP1 gene in an Indian wild-caught strain, but is broadly applicable. Major factors influencing protocol success include zebrafish health and fecundity, sgRNA efficiency and specificity, germline transmission, and mutant viability.

For complete details on the use and execution of this protocol, please refer to Balamurugan et al. (2022).

## BEFORE YOU BEGIN

1. Review the procedures for working with zebrafish. While substantial literature is available, the Zebrafish Book (Westerfield, 2000) is a widely used source of information.
2. Review the background and application of targeted genetic engineering strategies in zebrafish (Li et al., 2016).
3. Ensure availability of healthy zebrafish breeding pairs with a reliable setup for housing and breeding, comprising the following.
  - a. A housing facility in which a daily light/dark cycle can be maintained.
  - b. An adult housing system with physical and biological water filtration and UV disinfection, preferably with some level of automation and daily partial water replacement.
  - c. Breeding tanks and a temperature-controlled incubator (to maintain a temperature of 28°C) for housing embryos and larvae.
  - d. Water tanks or a system for housing juveniles prior to moving them into the adult housing system. Temperature control, aeration, filtration, and daily partial water replacement are needed for such a setup.
  - e. A reverse osmosis water purification system is preferable for ensuring consistent water quality.
4. Ensure the quality of feed, water, and any additives.
  - a. Live feed (brine shrimp) is preferable over dry feed.
  - b. Water temperature, pH, and conductivity need to be maintained within the desired range.
  - c. Ensure daily removal of organic waste and other debris, and prevent nitrite and nitrate accumulation.
5. Ensure that embryos can be collected immediately after fertilization, at least within 30 min of starting the breeding.



- a. Because any heritable genetic modification needs to be transmitted via germline cells (sperm and egg precursors), such modifications should be made in embryos at the single-celled stage (zygote).
6. A stereo microscope with the ability to visualize and allow manual sorting of non-viable and viable embryos and larvae.
7. A microinjection setup for introducing the CRISPR reagents into zebrafish embryos; a manual microinjection rig at a zoom stereo microscope is sufficient for this.
8. Compliance with all relevant animal ethics regulations and approval of the protocol(s) by the relevant institution(s) prior to study initiation. All protocols described here were approved by the Institutional Animal Ethics Committee of Dr. Reddy's Institute of Life Sciences, Hyderabad, India, and are consistent with national and international guidelines for zebrafish care and use.
9. The protocol described here targets the zebrafish PHLPP1 gene, in an Indian wild-caught zebrafish strain bred over multiple generations in-house. However, it is potentially applicable to any zebrafish gene, and any wild type strain.

### Institutional permissions

All protocols described here were approved by the Institutional Animal Ethics Committee of Dr. Reddy's Institute of Life Sciences, Hyderabad, India and are consistent with national and international guidelines. The facility is registered with and approved by the national-level apex committee CPCSEA, and has current NIH-OLAW animal welfare assurance for foreign institutions.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Bacterial and virus strains</b>		
E. coli Rosetta (DE3)	Novagen	Catalog # 70954
<b>Chemicals, peptides, and recombinant proteins</b>		
100 bp DNA Ladder	Takara	Catalog # 3422A
1 M Tris Hydrochloride Buffer (1 M Tris HCl) pH 8.0	SRL (Sisco Research Laboratories)	Catalog # 55904
2-Mercaptoethanol	SRL	Catalog # 83759
30% Acrylamide / Bis-acrylamide Mix Solution (Ratio 29:1)	SRL	Catalog # 67394
Ammonium persulfate	VWR Life Sciences	Catalog # JT0762-01
Ampicillin	SRL	Catalog # 61314
Boric acid	HiMedia	Catalog # MB007
Brilliant Blue R 250	SRL	Catalog # 93473
Bromophenol blue	SRL	Catalog # 93676
Calcium chloride dihydrate	HiMedia	Catalog # MB034
Chloramphenicol	HiMedia	Catalog # CMS218
Chloroform	SRL	Catalog # 96764
DNase I, RNase-free	Thermo Scientific	Catalog # EN0521
dNTPs	SRL	Catalog # 94903
EDTA Free Acid extrapure AR	SRL	Catalog # 73816
Ethanol	Spectrum Chemical Manufacturers	Catalog # 64-17-5
Ethidium Bromide	Spectrum Chemical Manufacturers	Catalog # 1239-45-8
Ethyl 3-aminobenzoate methanesulfonate	Chempure	Catalog # CRL6202
Glacial acetic acid	SRL	Catalog # 59788
Glycerol	SRL	Catalog # 77453
Glycine	SRL	Catalog # 64072
HEPES	SRL	Catalog # 63732
HiScribe™ T7 High Yield RNA Synthesis Kit	New England Biolabs	Catalog # E20405
Imidazole	HiMedia	Catalog # GRM1864
Isopropyl-β-D-Thiogalactopyranoside (IPTG)	SRL	Catalog # 54110
Luria Broth Miller	SRL	Catalog # 29817

(Continued on next page)

<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Lysozyme	Merck	Catalog # 105281
Macro-Prep High S Resin	Bio-Rad	Catalog # 1560030
Magnesium chloride	SRL	Catalog # 1349117
Magnesium sulfate heptahydrate	HiMedia	Catalog # GRM683
Methanol	SRL	Catalog # 96446
N,N,N,N-Tetramethyl Ethylenediamine (TEMED)	SRL	Catalog # 52145
NEB 3.1	New England Biolabs	Catalog # B6003S
Ni-NTA His60 Ni Superflow resin	Takara	Catalog # 635676
Phenol: Chloroform: Isoamyl Alcohol	SRL	Catalog # 69031
Phenol Red	SRL	Catalog # 79311
Phenylmethanesulfonyl Fluoride	Merck	Catalog # 93482
Potassium chloride	Merck	Catalog # P9541
Proteinase K	Takara	Catalog # 740506
Q5® High-Fidelity 2x Master Mix	New England Biolabs	Catalog # M0492L
RNase A	Thermo Scientific	Catalog # AM2270
RNase-Free Water	Takara	Catalog # 9012
Sea Salt	Instant Ocean	Catalog # SS15-10
Sodium Bicarbonate	SRL	Catalog # 36328
Sodium chloride	HiMedia	Catalog # MB023
Sodium dodecyl sulfate	HiMedia	Catalog # GRM6218
Sodium acetate	HiMedia	Catalog # MB048
Taq DNA Polymerase	GeNei	Catalog # MME23L
Tris Base	Merck	Catalog # 10708976001
Tris Buffer	SRL	Catalog # 71033
Triton X-100	SRL	Catalog # 64518
Tryptone	SRL	Catalog # 32007
Yeast extract	SRL	Catalog # 34266
<b>Experimental models: Organisms/strains</b>		
Zebrafish ( <i>Danio rerio</i> ) Indian wild caught strain; Males and Females; Newly fertilized embryos to adults (~3–12 months)	Wild Caught and Bred In-House	ZFIN
<b>Oligonucleotides</b>		
PHLPP1-targeting sgRNA	Commercial Provider	N/A
sgRNA Template Forward Oligo	Commercial Provider	N/A
sgRNA Template Tail Oligo	Commercial Provider	N/A
<b>Recombinant DNA</b>		
pSHS207	Addgene (Jennifer Doudna Lab)	RRID:Addgene_101199
<b>Software and algorithms</b>		
Benchling Web-Based Application	Benchling	RRID:SCR_013955
Interference of CRISPR Edits (ICE) Web-Based Tool	Synthego	Synthego ICE
<b>Other</b>		
1.7 L Slope Breeding Tank	Tecniplast	Catalog # ZB17BTISLOP
Analytical Balance	Sartorius	Catalog # CPA225D
Artemia Cysts	INVE Aquaculture	Inve
Barnstead lab line L-c Incubator	Barnstead International	Catalog # 403-1
BioSpectrometer	Eppendorf	Catalog # 6135000009
FemtoJet Programmable Microinjector	Eppendorf	Catalog # 5253000025
Frippak PL+150 Ultra Feed	INVE Aquaculture	Inve
Frippak PL+300 Ultra Feed	INVE Aquaculture	Inve
Gel Imaging System	Azure Biosystems	Catalog # Azure 600
Mastercycler® Nexus X2 Thermal Cycler	Eppendorf	Catalog # No. 6337000027
Microloader	Eppendorf	Catalog # 5242956003
Microcentrifuges	Eppendorf	Catalog # 5424R

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mini Centrifuge	Genaxy	Catalog # 6KS
Piezo Drill Tip	Eppendorf	Catalog # EP5195000095
PowerPac	GE Biosciences	Catalog # GE EPS301
SPINIX Vortex Shaker	Tarsons	Catalog # 3020
SteREO Discovery V8 stereomicroscope	ZEISS	Catalog # 495015-0012-000
ZebTEC rack with Active Blue technology	Tecniplast	Catalog # ZB52550SAABXS2

**MATERIALS AND EQUIPMENT**

**Oligonucleotide Sequences**

PHLPP1-targeting sgRNA:

5' GGGACGTATAACGTGCGGAA 3'

PHLPP1 sgRNA-target site PCR forward primer:

5' TGATTGAGCCTCGACATTATC 3'

PHLPP1 sgRNA-target site PCR reverse primer:

5' CTCATACTTCAGCCTCCACATC 3'

sgRNA Template sgRNA (forward) Oligo:

5' TAATACGACTCACTATAGGGACGTATAACGTGCGGAAGTTTTAGAGCTAGAA 3'

sgRNA Template Tail (reverse) Oligo:

5'AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTATTTTAACTTGCTATTCTAGCTCTAAAAC 3'

**E3 medium (Zebrafish Embryo Culture)**

Reagent	Final concentration	Amount
NaCl	5 mM	0.29 g
KCl	0.17 mM	0.013 g
CaCl <sub>2</sub>	0.33 mM	0.044 g
MgSO <sub>4</sub>	0.33 mM	0.018 g
ddH <sub>2</sub> O	N/A	1 L
<b>Total</b>	<b>N/A</b>	<b>1 L</b>

Room temperature storage, up to one month.

N/A: not applicable.

**Lysis Buffer (Cas9 purification)**

Reagent	Final concentration	Amount
TRIS pH 8	20 mM	0.24 g
NaCl	500 mM	2.92 g
Glycerol	10%	10 mL
Triton X-100	0.25%	0.25 mL
Lysozyme	1 mg/mL	100 mg
ddH <sub>2</sub> O	N/A	95 mL
<b>Total</b>	<b>N/A</b>	<b>100 mL</b>

Storage at 4°C.

N/A: not applicable.

**Ni-NTA Column Wash Buffer (Cas9 purification)**

Reagent	Final concentration	Amount
TRIS pH 8	20 mM	0.24 g
NaCl	500 mM	2.92 g
Glycerol	10%	10 mL
ddH <sub>2</sub> O	N/A	90 mL
<b>Total</b>	<b>N/A</b>	<b>100 mL</b>

Storage at 4°C.

N/A: not applicable.

### Ni-NTA Column Elution Buffer (Cas9 purification)

Reagent	Final concentration	Amount
TRIS pH 8	20 mM	0.24 g
NaCl	500 mM	2.92 g
Glycerol	10%	10 mL
Imidazole	250 mM	2.6 g
ddH <sub>2</sub> O	N/A	90 mL
<b>Total</b>	<b>N/A</b>	<b>100 mL</b>

Storage at 4°C.

N/A: not applicable.

### Cation Exchange Column Equilibration Buffer (Cas9 purification)

Reagent	Final concentration	Amount
HEPES	20 mM	2.38 g
KCl	150 mM	5.60 g
ddH <sub>2</sub> O	N/A	500 mL
<b>Total</b>	<b>N/A</b>	<b>500 mL</b>

Storage at 4°C.

N/A: not applicable.

### Cation Exchange Column Wash Buffer (Cas9 purification)

Reagent	Final concentration	Amount
HEPES	20 mM	2.38 g
KCl	250 mM	9.31 g
ddH <sub>2</sub> O	N/A	500 mL
<b>Total</b>	<b>N/A</b>	<b>500 mL</b>

Storage at 4°C.

N/A: not applicable.

### Cation Exchange Column Elution Buffer (Cas9 purification)

Reagent	Final concentration	Amount
HEPES	20 mM	2.38 g
KCl	1,000 mM	37.26 g
ddH <sub>2</sub> O	N/A	500 mL
<b>Total</b>	<b>N/A</b>	<b>500 mL</b>

Storage at 4°C.

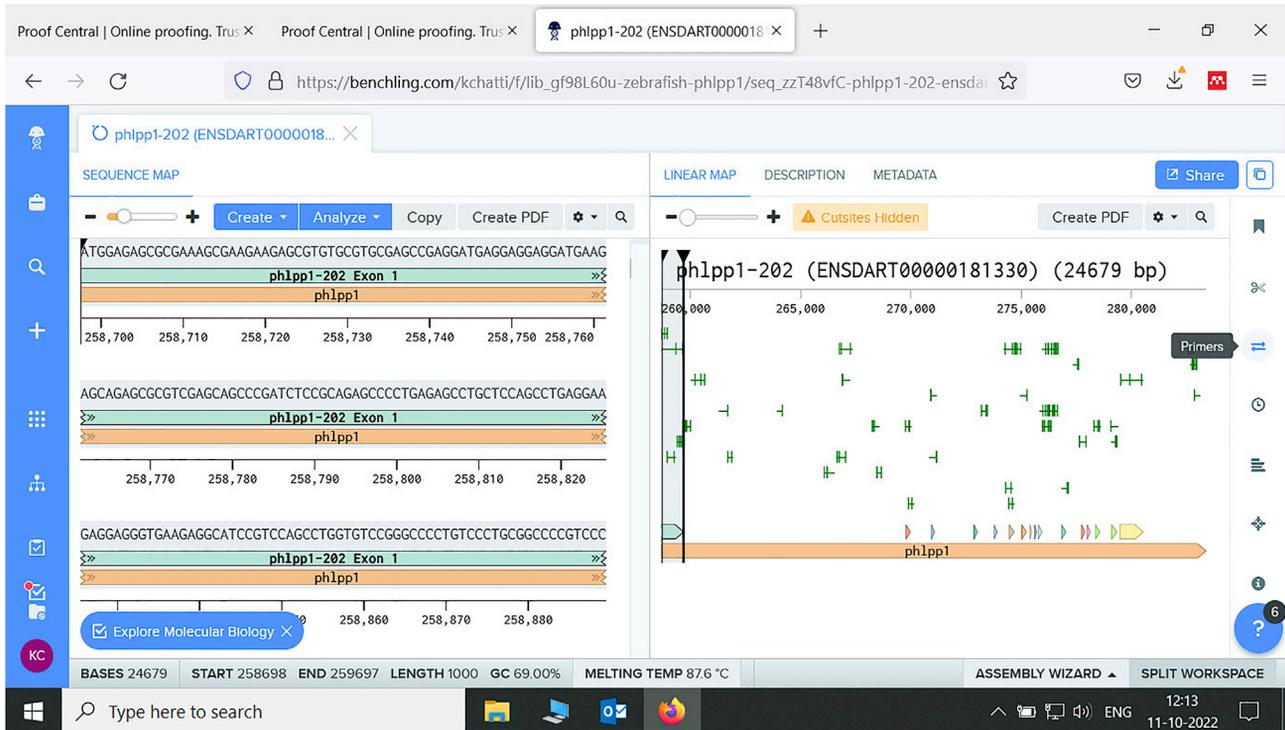
N/A: not applicable.

## STEP-BY-STEP METHOD DETAILS

### Design single-guide RNA and target region primers

⌚ Timing: Approximately 30 min (depending on the gene)

- Design sgRNA using the web-based application Benchling (screenshot in [Figure 1](#) below).
  - Access Benchling at [www.benchling.com](http://www.benchling.com) (create a free account).
  - Using the menu on the left, navigate to "Create CRISPR Guides".
  - Select the target gene by entering the gene name or ID, and the zebrafish genome from the drop-down menu. GRCZ11 ([Howe et al., 2013](#)) is the current latest genome release. Alternatively, it is possible to enter chromosomal co-ordinates, upload a sequence file, or paste the target sequence from a database.
  - Set the CRISPR guide design parameters. The defaults settings are single guide, with a length of 20 bases, and a 3' PAM site NGG.
  - The sequence is now displayed in the left window, with the guide design settings on the right.



**Figure 1. Screenshot of the Benchling web application used to design sgRNAs**

- f. The loaded sequence will be automatically annotated by the software to display the exons. In case the sequence was entered manually, the annotation will need to be done manually.
  - g. Select Exon 1, or the first exon common to all functional transcripts for the gene in the left window, and press the "+" icon in the right window to set the target genomic region and initiate gRNA design.
  - h. From the list of sgRNAs displayed, select 3 guides with top scores. Two kinds of scores are displayed – on-target and off-target scores, with high scores being better. First, order them by off-target scores and find those with sufficiently high on-target scores as well - a rule of thumb to follow is to have both scores >75. Clicking on the off-target score of an sgRNA displays the list of its off-targets with the mismatches shown in Red, and a corresponding score. A score <=1 with >=3 mismatches is suitable, and others should be excluded. In case the off-target and on-target scores are too low (<60), proceed to Exon 2 and repeat the process.
  - i. Once the desired sgRNAs are found, select and export them to a spreadsheet file. Benchling also has the option to design a strategy for cloning the sgRNA-coding sequence (using an Assemble option instead of Export) into an expression vector to be chosen from a drop-down menu.
2. Design PCR primers for the sgRNA target region(s).
    - a. Selecting the sgRNAs in the right window selects and shows the corresponding region in the genome sequence in the left.
    - b. In the menu on the right, click on Primers and proceed to create primers for the target regions. A Manual option or an automated Wizard option are available. From the primer sets listed, select a suitable one based on T<sub>m</sub>, GC%, and amplicon size (350–700 bp) among other parameters. Benchling also displays a Penalty score, with lower being better.

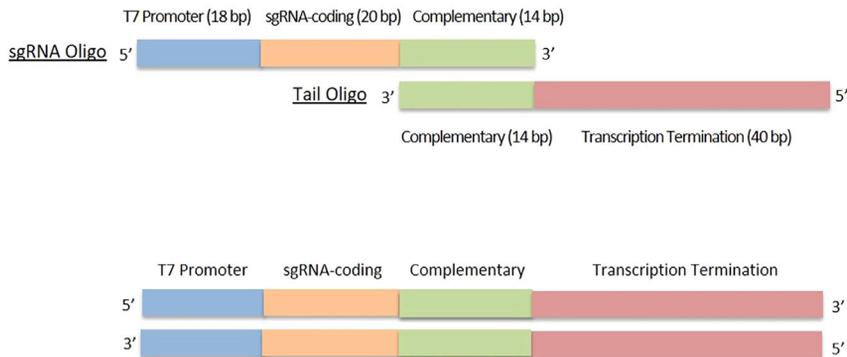
### Purification of Cas9

⌚ Timing: 4–5 days

3. Bacterial expression of Cas9.
  - a. Day 1: Transform *E. coli* Rosetta DE3 cells with a plasmid expression WT SpCas9 (pSHS207; Addgene Plasmid # 101199) (Jinek et al., 2012), plate on ampicillin-selective LB plates (100 µg/mL), and incubate overnight at 37°C.
  - b. Day 2: Inoculate a single colony into 5 mL of LB broth with ampicillin (100 µg/mL), and allow to grow at 37°C in a shaking incubator overnight.
  - c. Day 3: Sub-culture 2 mL of the above overnight culture into 100 mL LB broth with ampicillin (100 µg/mL) and grow at 37°C in a shaking incubator. Once the OD reaches 0.6, reduce the temperature to 18°C and incubate for 30 min for the culture to cool down. Induce SpCas9 expression by adding 0.5 mM IPTG and continue incubation at 18°C in the shaking incubator overnight.
  - d. Day 4: Assess Cas9 expression via SDS-PAGE of a 100 µL aliquot of the culture; centrifuge the aliquot at 5,000 g for 5 min, wash once with water, resuspend in 100 µL of 1× SDS-PAGE sample buffer and heat at 95°C for 5 min (it is useful to load different volumes, e.g., 10 µL, 20 µL, and 40 µL). Centrifuge the rest of the culture at 5,000 g for 10 min at 4°C to pellet the cells. Wash the cells three times with water or a suitable buffer. The cell pellet can be frozen at –20°C or –80°C at this stage.
4. Column Chromatography to purify Cas9 – step 3: Affinity Chromatography.
  - a. Day 4 or 5: Perform the steps from this point forward in a cold room or a refrigerated cabinet.
  - b. Lyse the pellet from above by resuspension in 5 mL of lysis buffer and sonication on ice, using sonication settings of 25-s pulses with 10-s pauses, for 15–25 cycles.
  - c. Centrifuge the lysate at 12,000 g for 15 min at 4°C. Transfer the supernatant to a fresh tube, and assess lysis via SDS-PAGE.
  - d. Equilibrate a 1-mL bed volume of Ni-NTA resin with loading buffer by pouring the resin into a 10-mL empty column suitable for gravity-based manual chromatography, uncapping the outlet and allowing the buffer to drain while maintaining a head of at least 0.25 mL on the top of the resin. Cap the column after equilibration with at least 5 column volumes of the buffer.
  - e. Gradually pour the lysate onto the settled equilibrated resin in the column, with minimal turbulence to the resin. Uncap the column and collect the flow-through in a single Falcon tube.
  - f. Wash the column with at least 5 column volumes of wash buffer, collecting the wash in a single Falcon tube.
  - g. Elute the bound Cas9 protein with 2.5 column volumes of elution buffer, and collect the elutions in 1-mL aliquots. Assess the purification via SDS-PAGE of all the collected fractions (Figure 5A).
5. Column Chromatography to purify Cas9 – step 4: Ion Exchange Chromatography.
  - a. Day 4 or 5: Pool the elutions from the above Affinity Chromatography step, and dilute 3-fold with equilibration buffer to reduce salt concentration before loading onto a cation exchange resin as below.
  - b. Pour cation exchange resin into an empty column for a 5-mL bed volume. Wash with 25 mL equilibration buffer.
  - c. Gently pour the pooled protein sample onto the column and collect the flow through in a single Falcon tube.
  - d. Wash the column with 25 mL wash buffer and collect the wash into separate single tubes.
  - e. Elute the bound protein with 10 mL elution buffer and collect 1 mL fractions. Assess the purification via SDS-PAGE of all the collected fractions (Figure 5B).
  - f. Dialyze the eluted protein into storage buffer, add glycerol to 30% and store at –20°C.

**10% SDS-PAGE Gel Recipe**

Reagent	Amount for resolving gel	Amount for stacking gel
Water	4.1 mL	6.1 mL
0.5 M Tris pH 8.8	0 mL	2.5 mL
0.5 M Tris pH 6.8	2.5 mL	0 mL
30% Acrylamide/Bis-acrylamide mix	3.3 mL	1.3 mL
10% SDS	100 µL	100 µL
10% APS	32 µL	100 µL
TEMED	10 µL	10 µL



**Figure 2. sgRNA synthesis template and schematic**

Top Panel: Schematic of the oligonucleotides used to generate the template for sgRNA synthesis; Bottom Panel: Schematic of the annealed and extended sgRNA template, ready to be used for sgRNA synthesis via T7-driven *in vitro* transcription.

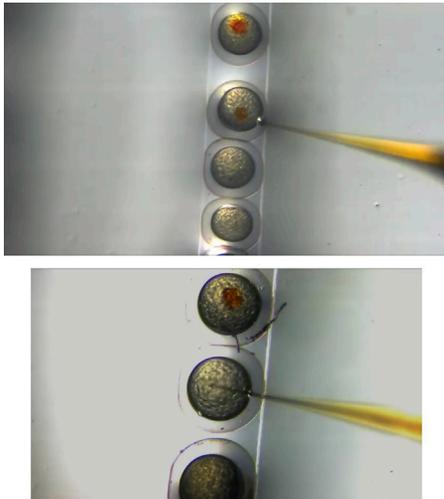
### ***In vitro* synthesis of single-guide RNA**

⌚ Timing: 3–4 days

6. Design oligonucleotides to generate a double-stranded DNA template encoding the sgRNA, and generate the template via overlap extension (Bowman et al., 2012).
  - a. Design a 52-nt oligonucleotide (sgRNA Oligo) comprising a T7 promoter sequence followed by a 20-nt guide RNA-coding sequence and a 15-nt sequence complementary to the Tail Oligo (depicted in Figure 2A below).
  - b. Design an 80-nt oligonucleotide (Tail Oligo) comprising a 15-nt sequence complementary to the sgRNA Oligo (depicted in Figure 2A below) followed by a sequence needed for transcription termination.
  - c. Obtain the above two oligonucleotides from a commercial provider.



**Figure 3. FemtoJet microinjection system (Eppendorf) with the microinjection settings shown**



**Figure 4. Zebrafish embryo microinjection**

Top Panel: View of zebrafish embryos during microinjection. The Phenol Red dye used as an additive for visualization is seen; Bottom Panel: Close-up view of zebrafish embryos during microinjection.

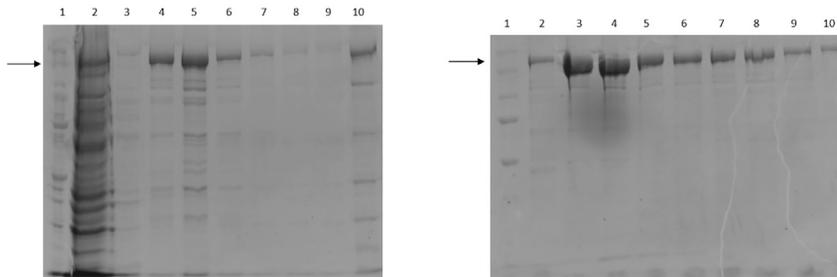
- d. Perform a PCR reaction as follows to generate the double-stranded DNA template (depicted in Figure 2B below).

PCR reaction mix	
Reagent	Amount
sgRNA Oligo (100 $\mu$ M)	1.25 $\mu$ L
Tail Oligo (100 $\mu$ M)	1.25 $\mu$ L
DNA Polymerase	2.0 $\mu$ L
10 $\times$ Buffer	10 $\mu$ L
dNTP (2.5 mM)	8 $\mu$ L
ddH <sub>2</sub> O	77.5 $\mu$ L

PCR cycling conditions

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	20 s	5
Annealing	52°C	20 s	
Extension	72°C	20 s	
Final extension	72°C	5 min	1
Hold	4°C	Until Removal	

- e. To the 100- $\mu$ L annealed and extended product (depicted above), add 10  $\mu$ L of 3 M sodium acetate pH 5.3 and 500  $\mu$ L of absolute ethanol and incubate for at least 2 h at  $-20^{\circ}$ C. Overnight is preferable.
- f. Centrifuge at 12,000 g for 10 min at 4°C, wash pellet with 70% ethanol, air dry, and dissolve in 10  $\mu$ L of water.
7. Use the template generated above in an *in vitro* RNA transcription reaction to generate the sgRNA.
- a. Perform a T7 RNA polymerase-driven *in vitro* transcription reaction as follows (the amount of DNA template used is typically at least 1  $\mu$ g).



**Figure 5. Cas9 protein purification**

Left Panel: Ni-Affinity chromatography of Cas9 protein. 1: Ladder. 2: Flow-through. 3: Wash. 4–9: Elutions. 10: Beads; Right Panel: Cation exchange chromatography of Cas9 protein following Ni-Affinity chromatography. 1: Ladder. 2: Flow-through. 3–10: Elutions.

#### Reaction mix

Reagent	Amount
DNA Template	3 $\mu$ L (1 $\mu$ g)
CTP	1 $\mu$ L
UTP	1 $\mu$ L
GTP	1 $\mu$ L
ATP	1 $\mu$ L
10 $\times$ Buffer	1 $\mu$ L
T7 Polymerase	1 $\mu$ L
DTT (0.1 M)	1 $\mu$ L

- Incubate the above mix at 37°C for 4.5 h (we have found this duration to work well, but it can vary between 0.5–5 h depending on the reaction efficiency).
- Add 0.5  $\mu$ L of DNase (1 unit/ $\mu$ L) and continue to incubate at 37°C for 40 min.
- Perform a phenol-chloroform extraction by making up the volume to 200  $\mu$ L with water, adding 100  $\mu$ L of acidic phenol and 100  $\mu$ L of chloroform, mixing by inverting and centrifuging at 10,000 g at 4°C for 10 min. Collect the supernatant and extract with an equal volume of chloroform. Collect the supernatant and proceed to RNA precipitation.
- Precipitate the RNA by adding 10  $\mu$ L of 3 M sodium acetate pH 5.3 and 500  $\mu$ L of absolute ethanol and incubating at least 2 h at –20°C (alternatively, 5–10 min at –80°C can be used).
- Centrifuge at 12,000 g for 10 min at 4°C, wash pellet with 70% ethanol, air dry, and dissolve in 10  $\mu$ L of RNase-free water.
- Quantify the RNA using a Nano drop UV spectrophotometer and verify integrity by resolving the RNA sample via 10% urea-PAGE (Summer et al., 2009) as follows. Dissolve 4.2 g of urea in 2 mL of water with agitation and boiling. After cooling to room temperature, add 2 mL of 5 $\times$  TBE, 3.3 mL of 30% Acrylamide / Bis-acrylamide Mix, 100  $\mu$ L of 10% APS, 10  $\mu$ L of TEMED and make up to 10 mL with water. Pour the gel and perform a pre-run for 20–30 min at 5 V/cm. Prepare the RNA sample as follows; take 0.5  $\mu$ L of RNA, add 4.5  $\mu$ L of water and 5  $\mu$ L of 2 $\times$  formamide dye. Heat the 10  $\mu$ L RNA sample at 95°C for 5 min and spin briefly. Flush the wells with 1 $\times$  TBE to remove urea, load the RNA samples, run until the dye front migrates 3/4<sup>th</sup> distance of the gel and stain with ethidium bromide (0.5  $\mu$ g/mL) for 10 min. Visualize RNA integrity using a gel imaging system.

#### Urea-PAGE Gel Recipe

Reagent	Amount
Urea	4.2 g
Water	2 mL
5 $\times$ TBE	2 mL

(Continued on next page)

**Continued**

Reagent	Amount
30% Acrylamide/Bis-acrylamide mix	3.3 mL
10% APS	100 $\mu$ L
TEMED	10 $\mu$ L
Water	To 10 mL

**5 $\times$  TBE Recipe**

Reagent	Amount
Tris Base	54 g
Boric Acid	27.5 g
0.5 M EDTA pH 8	20 mL
Water	To 1 L

**In vitro catalytic activity assay for assessing guide RNA efficiency**

⌚ Timing: 1 day

8. Perform an *in vitro* catalytic activity assay with purified Cas9, sgRNA, and the target region amplicon (Grainger et al., 2017).
  - a. Assemble a ribonucleoprotein complex by adding the following reagents and incubating the mix at 37°C for 10 min.

**Reaction mix**

Reagent	Amount
sgRNA (500 ng/ $\mu$ L)	1 $\mu$ L
NEB buffer 3.1	1 $\mu$ L
Cas9 (250 ng/ $\mu$ L)	1 $\mu$ L
Water	8 $\mu$ L

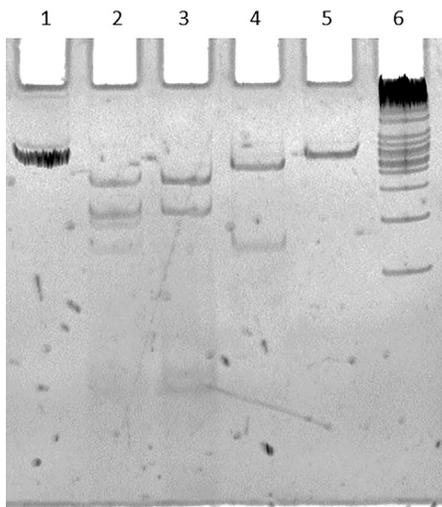
- b. Add 2  $\mu$ L of the target-region PCR amplicon to the above mix and incubate further at 37°C for 1 h. Add 1  $\mu$ L RNase A and incubate at 37°C for 30 min, and then add 1  $\mu$ L Proteinase K and further incubate at 37°C for 30 min.
- c. Resolve the sample on 10% Native PAGE and visualize by DNA staining. Cleavage of the target results in cut bands and thus a different band pattern compared to the control sample (target-region PCR amplicon alone) (Figure 6).
- d. Select one sgRNA (or a combination if multiple sgRNAs show desirable cleavage) based on a visual assessment of cleavage efficiency from the band patterns.

**10% Native PAGE Gel Recipe**

Reagent	Amount for resolving gel	Amount for stacking gel
Water	4.2 mL	6.2 mL
0.5 M Tris pH 8.8	0 mL	2.5 mL
0.5 M Tris pH 6.8	2.5 mL	0 mL
30% Acrylamide/Bis-acrylamide mix	3.3 mL	1.3 mL
10% APS	32 $\mu$ L	100 $\mu$ L
TEMED	10 $\mu$ L	10 $\mu$ L

**Generation of CRISPR-Cas9-induced PHLPP1-knockout zebrafish**

⌚ Timing: 10–12 months



**Figure 6. In vitro CRISPR catalytic activity assessment (Cas9-sgRNA RNP complex)**

1: Control. 2: sgRNA 1. 3: sgRNA 2. 4: sgRNA 1+2. 5: Control. 6: Ladder.

9. Zebrafish breeding and embryo collection.
  - a. On the evening before the day of microinjections, place 3–4 pairs of selected adult male and female zebrafish in a breeding tank, with males and females separated by a divider, and a partition separating the top and bottom half of the tank – this prevents the adults from accessing the laid eggs.
  - b. In the morning, remove the divider at first natural light or switch on a light source and remove the divider, to induce breeding. The laid eggs will be visible at the bottom of the tank. Because genetic modifications should be done at the single-celled stage, fertilized eggs should be collected as soon as possible, and the microinjections done within 30 min of fertilization.
  - c. Remove the partition and collect the eggs. Transfer fertilized eggs into a 95-mm Petri dish and wash several times in system water.
10. Microinjection of zebrafish embryos.
  - a. During the breeding step above, prepare for microinjection. Set the injection parameters (injection pressure 550 hPa, Time of injection 0.1 s and back pressure 10 hPa). We used an Eppendorf Femtojet microinjection system (shown in Figure 3 below) with a Zeiss Stereo Discovery V8 microscope in this protocol. Form a Cas9-sgRNA complex by adding reagents as below.

Reagent	Amount
sgRNA (500 ng/μL)	0.5 μL
TE buffer	1 μL
Cas9 (250 ng/μL)	2.5 μL
Water	6 μL

- b. Incubate the above mix at 37°C for 15 min. Add 0.05% phenol red to the mix to allow visualization during and after injection (as shown in the photograph below). Use of TE buffer in this step is optional, and we include it because it may help to stabilize the RNP complex. Load the mix into a microinjection needle from the back end using a micropipette fitted with a back-loader tip, and insert the back end of the needle into the injection tubing. Pre-pulled microinjection needles can either be bought or prepared from glass capillaries using a Needle Puller.
- c. Place the embryos in a Petri dish with slotted agarose and line the embryos along the slots (as shown in Figures 4A and 4B below). Position the above loaded needle appropriately and inject each embryo with approximately 10 nl of the loaded mix (if 10 nl results in toxicity and embryo death, decrease the volume to 5 nl or lower if possible). We typically inject a set of 80–100 embryos with the reagent mix, with buffer alone as control, and include

80–100 uninjected embryos as a control for embryo quality. Transfer the injected embryos to a dish with E3 medium and place the dish in a 28°C incubator.

- d. Observe the embryos a few times over the next 3 h, and remove dead embryos.
11. Genotyping using Heteroduplex Mobility Analysis (HMA), selective breeding, and generating heterozygotes and homozygotes.
  - a. On the following day, collect 8 injected embryos and 1 uninjected (control) embryo in individual tubes and prepare lysate for HMA analysis as follows. Remove excess water from the tube, add 45 µL 50 mM NaOH and heat at 95°C for 25 min (a minimum of 10 min is needed) Vortex the tube to disintegrate the embryos, add 5 µL 1 M Tris-HCl pH 8.0, mix briefly and spin to pellet any debris. Use 0.5 µL of this lysate as input for a 5 µL PCR reaction as below.

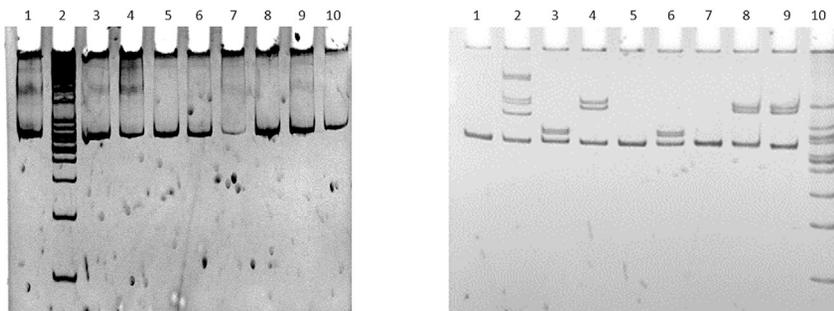
Reagent	Amount
2× Q5 mix	2.5 µL
FP (10 µM)	0.15 µL
RP (10 µM)	0.15 µL
Input	0.5 µL
Water	1.7 µL

#### PCR cycling conditions

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	10 s	35
Annealing*	62°C	10 s	
Extension	72°C	10 s	
Final extension	72°C	2 min	1
Hold	4°C	Until Removal	

\*Annealing temperature may be determined for each primer pair using the NEB Tm Calculator.

- b. Resolve the products using native PAGE, and visualize via DNA staining. Compared to control (un-injected embryos), additional bands will be visible if cleavage of the target region has occurred. Because of the presence of wild type DNA (target site cleavage may not have occurred in all embryos, and wild type alleles may be present in embryos with target site cleavage), heteroduplex formation will occur in the PCR products and these heteroduplexes will have different mobility patterns which can be visualized. The patterns above the main band may be of much lower intensity and smeared because the percentage of edited cells in the 24 hpf embryo may be low, and embryos with various types of edits (indels) are expected to be present (Figure 7A).
- c. Based on the above visual evidence of cleavage, if at least 30% of the embryos show heteroduplex mobility differences (are HMA-positive), it can be considered likely that the injected population of embryos have edits, and the embryos can be raised to adulthood. However, whether these fish carry the edits in the germline cells (are F0 founders) is crucial, and this cannot be determined for certain at this stage. Two strategies are possible for genotyping and selecting fish for further breeding, as described below.
- d. In one strategy, raise as many of the injected embryos as possible from step 11c to adulthood, breed them with wild type fish (backcross) using only F0 males or only F0 females in a given cross, and genotype a sample of the resultant embryos as described in step 11a above. These embryos represent the potential F1 or heterozygote generation. Because heterozygous embryos are expected to carry one wild type allele and one allele with target site disruption (and consequent NHEJ repair-mediated insertion or deletion), their HMA patterns

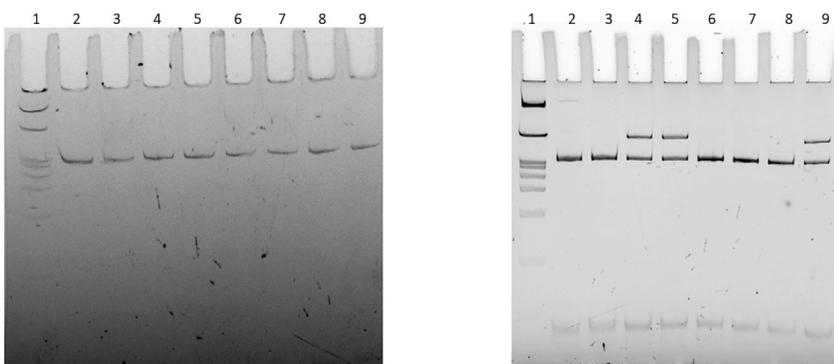


**Figure 7. HMA genotyping of F0 and F1 embryos**

Left Panel: HMA of F0 embryos (CRISPR-injected embryos). 1: Embryo 1 (HMA-Positive). 2: Ladder. 3: Embryo 2 (HMA-Positive). 4: Embryo 3 (HMA-Positive). 5: Embryo 4. 6: Embryo 5. 7: Embryo 6. 8: Embryo 7. 9: Embryo 8. 10: Control; Right Panel: HMA of F1 embryos (progeny of F0 adults and WT). 1: Control. 2: Embryo 1 (HMA-Positive). 3: Embryo 2 (HMA-Positive). 4: Embryo 3 (HMA-Positive). 5: Embryo 4. 6: Embryo 5 (HMA-Positive). 7: Embryo 6. 8: Embryo 7 (HMA-Positive). 9: Embryo 8 (HMA-Positive). 10: Ladder.

(heteroduplexes with mobility differences) will be prominent (Figure 7B). If the number of HMA-positive embryos and therefore the extent of germline transmission is at least 30%, raise these F1 embryos to adults (depending on available space), and genotype the adults as follows. Segregate a few of them by placing them in separate tanks, label the tanks to identify the fish, and genotype each one using tail clip DNA. This is done by clipping a small piece of the tail and performing the genotyping protocol as described in step 11a above. The HMA-positive adults are the F1 heterozygotes.

- e. In the second strategy, raise as many of the injected embryos as possible from step 11c to adulthood, and genotype them after segregation and tail clipping as described in step 11d above. HMA-positive fish are considered F0 founders. Breed these founders with wild type fish (backcross as in step 11d above), raise the resultant embryos to adulthood, and identify F1 adult heterozygotes using tail clip genotyping as in step 11d above.
- f. To confirm the genotyping of the F1 heterozygotes identified using either of the strategies above, perform sequencing of the target region PCR products (in-house if available or using a commercial service provider), and analyze the sequence result using the ICE tool from Synthego. This tool predicts the likelihood of heterozygous status, and the presence of an indel



**Figure 8. HMA genotyping of F2 embryos**

Left Panel: HMA of F2 embryos (progeny of F1 adults) without denaturation and annealing. 1: Ladder. 2: Embryo 1 (WT Control). 3: Embryo 2. 4: Embryo 3. 5: Embryo 4. 6: Embryo 5. 7: Embryo 6. 8: Embryo 7. 9: Embryo 8; Right Panel: HMA of F2 embryos with denaturation and annealing. 1: Ladder. 2: Embryo 1 (WT Control). 3: Embryo 2. 4: Embryo 3 (HMA-Positive). 5: Embryo 4 (HMA-Positive). 6: Embryo 5. 7: Embryo 6. 8: Embryo 7. 9: Embryo 8 (HMA-Positive). The F2 HMA above shows that embryos 3, 4, and 8 are homozygous mutants. WT F2 samples (embryos 2, 5, 6, and 7) yield only a single band under both the above conditions. All the HMA data shown above is with embryos. Adult samples (tail or fin clips) is expected to yield similar results.

- (insertion or deletion) at the target site. Identify at least one pair of male and female F1 fish carrying an identical indel.
- g. Breed the above F1 fish pair (or pairs if multiple pairs can be identified) with each other, and genotype a set of embryos using HMA to verify Mendelian segregation as described but with one additional step (Foster et al., 2019). Visualize one half of the PCR product for each reaction via native PAGE. 50% of the samples are expected to be heterozygotes and should be HMA-positive. To the remaining 50% showing a single band (Figure 8A), add an equal volume of the control PCR product, and subject the mix to one round of denaturation and annealing as follows. In a thermal cycler, subject the samples to 85°C for 5 min and then to 25°C for 5 min. The samples can then be at 10°C until the next step.
  - h. Resolve and visualize the above products on native PAGE. Those that now show the HMA pattern are considered the F2 generation (homozygotes), and those that still show only one band are wild type (Figure 8B). Indels in both alleles will result in two bands in the presence of added wild type amplicon, but only one band otherwise. This is because no heteroduplex formation occurs when both alleles have the same indel (as is expected in homozygotes). To confirm, perform sequencing of the PCR products and compare with wild type sequence using ICE analysis as above or other sequence comparison methods. The disruption at the target site and the mutated sequence (insertion or deletion) should be evident from the analysis.
  - i. Raise the F2 embryos to adulthood (depending on available space), and genotype a set of them using tail clipping to identify the F2 homozygotes. The F0 founders, the F1 heterozygotes, and the F2 homozygotes can be housed and maintained as needed for experimental studies.

### EXPECTED OUTCOMES

Figures 5, 6, 7, and 8 detail the expected outcomes of this protocol.

### LIMITATIONS

Identification of efficient and specific guide RNA is dependent on the target sequence including the presence of a suitable PAM. Lack of a suitable PAM, or insufficient guide specificity or efficiency, may lead to unreliable targeting.

Presence of genomic variations in the target genomic region in wild-caught zebrafish in comparison to inbred strains. Because guide RNA design is based on database reference sequences (derived from inbred strains), such guides may not perform as expected and/or yield results differing from those in inbred strains.

In some cases, very low germline transmission or lack thereof may be observed, despite the positive HMA results in embryo or tail/fin clip genotyping. This can limit the efficiency of mutant generation or prevent it completely.

The use of an sgRNA-Cas9 protein complex may not be efficient and necessitate the use of an alternative (an sgRNA-Cas9 mRNA mix or an sgRNA-Cas9-encoding plasmid).

### TROUBLESHOOTING

#### Problem 1

Problems with microinjection due to fluctuation of the set pressure and time parameters on the device.

#### Potential solution

Cleaning the microinjection tubing per the manufacturer's instructions, or performing service and repair of the device may be needed.

### Problem 2

Toxicity and embryo death due to microinjection.

### Potential solution

Reducing the volume of microinjection (to 5 nl or less) is a potential solution. Assessing the toxicity of the reagents in a set of embryos is useful before initiating the actual protocol.

### Problem 3

Failure of PCR amplification of target region, or inconsistent PCR results while performing HMA genotyping.

### Potential solution

Use of polymerases such as Q5 or KOD, or redesign of PCR primers are useful options. The ultimate option is to change the target region, but this is rarely necessary.

### Problem 4

Presence of genomic variations in the wild-caught zebrafish strain used, relative to common inbred strains, which may confound the results.

### Potential solution

Sequencing of the CRISPR target genomic region in a set of the wild-caught zebrafish, and selecting only those that are consistent with the database reference sequence. While we have not encountered this problem, planning to sequence the target region is a potential solution if using wild-caught or other uncharacterized strains.

### Problem 5

Inefficient editing.

### Potential solution

The use of an sgRNA-Cas9 protein complex may result in inefficient editing. Alternatives include the use of a different sgRNA, use of different software for sgRNA design, use of an sgRNA-Cas9 mRNA mix or an sgRNA and Cas9-encoding plasmid(s).

### Problem 6

Inefficient germline transmission.

### Potential solution

Inefficient germline transmission despite evidence of editing in genotyping data (embryo or tail/fin clip DNA) may occur. While the reasons for this are unclear to us, speculative explanations are DNA repair during germ cell formation or apoptosis of edited germ cells. The only potential solutions, to the best of our knowledge, are use of a different sgRNA or a different target site.

### Problem 7

Lethality or loss of fecundity in the mutant zebrafish.

### Potential solution

Use of heterozygous mutants instead of the homozygous ones. If the heterozygotes also have such problems, one possible solution is the generation of a tissue-specific knockout.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kiranam Chatti ([kiranamc@drils.org](mailto:kiranamc@drils.org)).

## Materials availability

The zebrafish generated in this study are available from the corresponding author on request (subject to fulfillment of animal transfer regulations). No plasmids were generated, and oligonucleotide sequences are included in the article.

## Data and code availability

This study did not generate or analyze datasets or code.

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## AUTHOR CONTRIBUTIONS

R.M., K.B.: zebrafish experiments; K.Y.: Cas9 protein purification; R.R.: *In vitro* transcription and molecular biology experiments; K.C.: Overall experimentation supervision; K.C., K.P., A.S.: zebrafish experiments supervision; A.C.: zebrafish CRISPR-Cas9 design and troubleshooting; A.S.: *In vitro* transcription and molecular biology design and troubleshooting; K.C., A.S.: Writing and Editing; K.V.L.P., K.C., A.S.: Acquired funding.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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