Chapter 6

Protein Tyrosine Phosphatase Studies in Zebrafish

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Abstract

The zebrafish is an ideal model for functional analysis of genes at the molecular, protein, cell, organ, and organism levels. We have used zebrafish to analyze the function of members of the protein tyrosine phosphatase (PTP) superfamily for more than two decades. The molecular genetic toolbox has significantly improved over the years. Currently, generating mutant lines that lack the function of a PTP gene is relatively straightforward by CRISPR/Cas9 technology-mediated generation of insertions or deletions in the target gene. In addition, generating point mutations using CRISPR/Cas9 technology and homology-directed repair (HDR) is feasible, albeit the success rate could be higher. Here, we describe the methods, including the tips and tricks, that we have used to generate knock-out and knock-in zebrafish lines in PTP genes successfully.

Key words Protein tyrosine phosphatases, PTP, Zebrafish, PTEN, SHP2, CRISPR/Cas9, Genome editing

1 Introduction

Intracellular processes are controlled by post-translational modifications (PTMs) of signaling proteins, enabling cells to act and respond to their environment. Phosphorylation and dephosphorylation of tyrosine residues in signaling proteins are orchestrated by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), which catalyzes the addition or removal of phosphate groups to or from tyrosine residues in proteins, respectively [1, 2]. Maintaining a balance between these phosphotyrosine regulators controls important processes, including proliferation, differentiation, and cell–cell adhesion. Aberrant intracellular signaling by altered PTP function has been linked to many human diseases, including developmental disorders, cardiovascular disease, and cancer [3, 4].

Over the years, the zebrafish (*Danio rerio*) has become an increasingly important vertebrate model for studying developmental biology and modeling human disease because of its many

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favorable characteristics [5]. For example, the zebrafish has emerged as an essential model for studying cardiovascular diseases. Although the human heart is morphologically distinct from the zebrafish heart, with a single circulatory system and only one atrium and ventricle, similarities are evident with respect to cardiac development, heart looping structure, and physiology [6]. The high degree of sequence conservation between human and zebrafish genes offers great potential for modeling human cardiovascular diseases [7]. Additionally, the external fertilization, rapid development, and embryonic translucency of zebrafish allow for a unique opportunity to visualize a broad range of biological processes by in vivo microscopy analysis of transgenic markers [8]. Since the development of the first transgenic zebrafish line containing a green fluorescent protein (GFP) sequence fused to the GATA-1 promotor sequence [9], numerous reporter lines have been generated that specifically stain a particular tissue or cell type (*see* www.zfin.org). An example of how fluorescent imaging techniques were instrumental for PTP studies in zebrafish was given by Stumpf et al. [10] by investigating the function of the tumor suppressor gene, phosphatase, and tensin homolog (PTEN). Confocal live imaging of homozygous *ptena/ptenb* double knock-out embryos in the transgenic *Tg(kdrl:eGFP)* background revealed hyperbranching of the intersegmental vessels in the absence of functional PTEN. Micro-injection of exogenous PTEN, but not PTEN phosphatase mutants, suppressed the hyperbranching phenotype emphasizing the requirement of PTEN phosphatase activity for normal embryonic angiogenesis [10, 11].

The *ptena/ptenb* double mutant described above was derived by target-selected gene inactivation (i.e., random mutagenesis), followed by resequencing of the gene of interest [12, 13]. Target-selected gene inactivation was effective but laborious, and since then, more directed technology has been developed to inactivate specific genes. Current genome editing strategies are based on nuclease systems, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9. All these technologies provoke a permanent mutagenic outcome. The CRISPR/Cas9-based method depends on an RNA–DNA interaction which provides multiple advantages over ZFNs and TALENs, including higher efficiency, more straightforward design for any genomic target, easier prediction of off-target sites, and the possibility to modify several genomic sites simultaneously [14, 15]. For these reasons, CRISPR/Cas9 technology is emerging as the superior technology to generate knock-out animal models. The advancements in CRISPR/Cas9 gene editing techniques in zebrafish include the generation of knock-in models, which enables functional analysis of proteins at the single amino acid level in embryonic development and disease. Generation of the first

CRISPR/Cas9-based zebrafish knock-in model was reported by Irion and colleagues, who applied homology-directed repair (HDR) of the alb^{b4} mutation by co-injection of Cas9 mRNA, in vitro synthesized single-guide RNA (sgRNA), and a circular template vector containing 875-bp homology arms [16]. More recently, we and others generated knock-in zebrafish models using single-stranded oligonucleotide HDR templates [17–20]. HDR templates diffuse faster into the nucleus, speeding up integration into the DNA, which is particularly important for zebrafish models because the blastomeres divide extremely fast, causing declined germline transmission [21, 22]. Although introducing specific mutations in the zebrafish genome has great potential for studying the functional effects of human disease-associated genetic variants, only a few studies have employed the CRISPR-Cas9 system to model human disease [23]. The first and foremost requirement when modeling human diseases is the availability of the gene in the model system. Homologs of all human PTPs have been identified in zebrafish except for *PTPN7* and *PTPN14* [24]. A search of the zebrafish genome (http://www.ENSEMBL.org) led to the identification of a small part of the *ptpn12* gene, which may suggest either that the rest of this gene is present but has not been annotated properly yet, or that the rest of the homolog of *PTPN12* is missing from the zebrafish genome altogether.

In early vertebrate evolution, zebrafish and other teleost fish species have undergone a whole genome duplication [25, 26]. Since then, some, but not all, of the duplicated chromosomes have been lost from the zebrafish genome, yielding two paralogs of many genes, including genes encoding PTPs [24, 27]. Duplicated genes were identified for 14 PTPs providing an opportunity to study the sub-functionalization of the gene. Bonetti et al. demonstrated that duplication of the *PTPN11* gene leads to zebrafish *ptpn11a* and *ptpn11b* encoding Shp2a and Shp2b. Whereas knock-out of the *ptpn11a* gene causes severe developmental defects and is embryonically lethal, the *ptpn11b* gene is dispensable throughout development. Yet, rescue experiments indicate functional redundancy for the two paralogs because injection of *ptpn11a* or *ptpn11b* rescues defects in *ptpn11a* mutants. *Ptpn11a* is expressed constitutively during development, whereas *ptpn11b* is expressed at very low levels during early development. Endogenous Shp2b levels are insufficient to compensate for the loss of Shp2a, but the expression of exogenous Shp2b does rescue the phenotype [28].

Loss of SHP2 function is lethal in mice and zebrafish. Elevated activity of SHP2 also induces developmental defects. Therefore, broadening our understanding of the SHP2 function is essential. SHP2 is dynamically involved in multiple human diseases, including Noonan syndrome (NS), Noonan Syndrome with multiple lentigines (NSML), juvenile myelomonocytic leukemia (JMML),

and metachondromatosis (MC) [17, 29]. NS is an autosomal dominant disorder characterized by over 100 gain-of-function (GOF) variants in *PTPN11* and other RAS/MAPK pathway genes. Initially, we modeled NS transiently by micro-injection at the one-cell stage of synthetic mRNA encoding Shp2 variants with mutations identified in human patients [28, 30, 31]. Subsequently, our laboratory developed and characterized a novel genetic zebrafish model with the NS Shp2-D61G mutation. Micro-injection of the CRISPR/Cas9 complex and single-stranded oligonucleotide HDR templates at the one-cell stage led to the identification of founder fish and the establishment of a stable line. These Shp2 mutant zebrafish recapitulate multiple typical NS features, including short stature, craniofacial anomalies, and JMML-like myeloproliferative neoplasia [17]. This zebrafish line provides a valuable preclinical model for the development of future therapies. Considering the elegance by which the Shp2-D61G genetic model recapitulates human disease features, our laboratory is eager to explore CRISPR/Cas9 technology further to establish additional zebrafish lines, which will facilitate the analysis of genotype–phenotype correlations associated with NS. Accordingly, we have successfully generated multiple knock-out and knock-in models currently being characterized. In this chapter, we provide details of the method that we have used to generate stable genetic knock-out and knock-in zebrafish models.

2 Materials

2.1 Fish Husbandry

1. Tanks (e.g., Aqua Schwarz GmbH).
2. Mesh nets (e.g., Aqua Schwarz GmbH).
3. Butterfly net.
4. Single boxes (e.g., Aqua Schwarz GmbH SD-881).
5. Single boxes nets (e.g., Aqua Schwarz GmbH 006-0620).
6. Scalpel (e.g., Swann-Morton No. 23).
7. Tweezers.
8. Ice bucket.
9. 10-cm Petri dishes
10. Transfer pipet (e.g., Sarstedt—Ref. 86.1171).
11. Tricaine methanesulfonate (MS-222) (0.1%).
12. E3 medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$.
13. Methylene blue (0.01%).

2.2 Genotyping by polymerase chain reaction (PCR)

1. PCR strip.
2. 96-well plates
3. Taq DNA polymerase.
4. dNTPs (25 mM).
5. Proteinase K (20 mg/mL).
6. Sequencing stickers.
7. SZL lysis buffer: 10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.005% NP-40, 0.005% Tween-20, and 0.01% Gelatine.
8. 5× Green GoTaq Flexi Reaction buffer (e.g., Promega—M8911)
9. Q5® Hot Start High-Fidelity DNA Polymerase, and 10× reaction buffer (e.g., Bioke—M0493S).
10. Gene-specific primers.
11. T7 forward primer: 5′-TAATACGACTCACTATAGGG-3′.
12. M13 reverse primer: 5′-CAGGAAACAGCTATGAC-3′.
13. LB agar plates + 50 μg/mL ampicillin.
14. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (20 mg/mL).
15. 5-Bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal) (20 mg/mL).

2.3 Injection

1. Magnetic stirrer.
2. Low protein binding tubes 0.5 mL (e.g., Eppendorf—022431064).
3. Recombinant SpCas9 (5 μg/μL).
4. sgRNA (200 ng/μL),
5. HDR template (200 ng/μL).
6. PCR purification kit (e.g., Thermo Scientific GeneJET PCR Purification Kit—K0702).
7. Protein dilution buffer: 25 mM phosphate buffer, pH 8.0, 500 mM NaCl, 1.25 mM $MgCl_2$, RNAse free H_2O, and 2 mM beta-mercapto-ethanol.
8. KCl (1 M).
9. Phenol red (50%).
10. Ethanol (70%).

2.4 Gel Electrophoresis

1. Ultrapure agarose (e.g., Invitrogen—16500-500).
2. 10× Tris-borate-EDTA (TBE) buffer: 0.89 M Tris, pH 8.3, 0.89 M H_3BO_3, and 20 mM EDTA

3. Ethidium bromide (1%).
4. 50-bp DNA ladder (5 μg/mL) (e.g., Fisher Scientific—SM0372).

2.5 Equipment

1. Brightfield stereomicroscope with an ocular micrometer (e.g., Leica M165 FC).
2. Thermomixer.
3. Transilluminator.
4. Vortex.
5. pH meter.
6. Nanodrop (e.g., Thermo Scientific Nanodrop One).
7. Thermal cycler.

3 Methods

3.1 Preparation—Design of sgRNA

For CRISPR/Cas9-mediated knock-out and knock-in approaches, we use protocols based on the publication by Gagnon et al. about CRISPR/Cas9-mediated mutagenesis [32], as outlined in Fig. 1. Selecting a suitable sgRNA near the target site depends on the location of specific recognition motifs necessary for the binding of the Cas9 protein. Since we use the Cas9 nuclease, we have designed sgRNAs containing a protospacer adjacent motif (PAM) site.

1. Go to the online tool https://chopchop.cbu.uib.no/ to select a target site for CRISPR/Cas9-directed mutagenesis.
2. Fill in the requested boxes, and hit the button "Find Target Sites!" (*see* **Note 1**).
3. Zoom into your exon of interest by scrolling the mouse (*see* **Note 2**).
4. Select a guide sequence near your desired cut site with the highest possible ranking (*see* **Notes 3**–**6**).
5. Select the guide sequence by clicking the colored bar (*see* **Note 7**).
6. Order the target sequence in the 5′–3′ direction (*see* **Notes 8** and **9**).
7. Dissolve the sgRNA in MilliQ water to 100 ng/μL and store in aliquots at −80 °C.

3.2 Preparation—Preparation of HDR Template

A homology-directed repair (HDR) template is injected together with the sgRNA and Cas9 protein to induce specific mutations rather than knock out the gene of interest.

1. Design an HDR template to incorporate the mutation of choice. The HDR template comprises the specific mutation

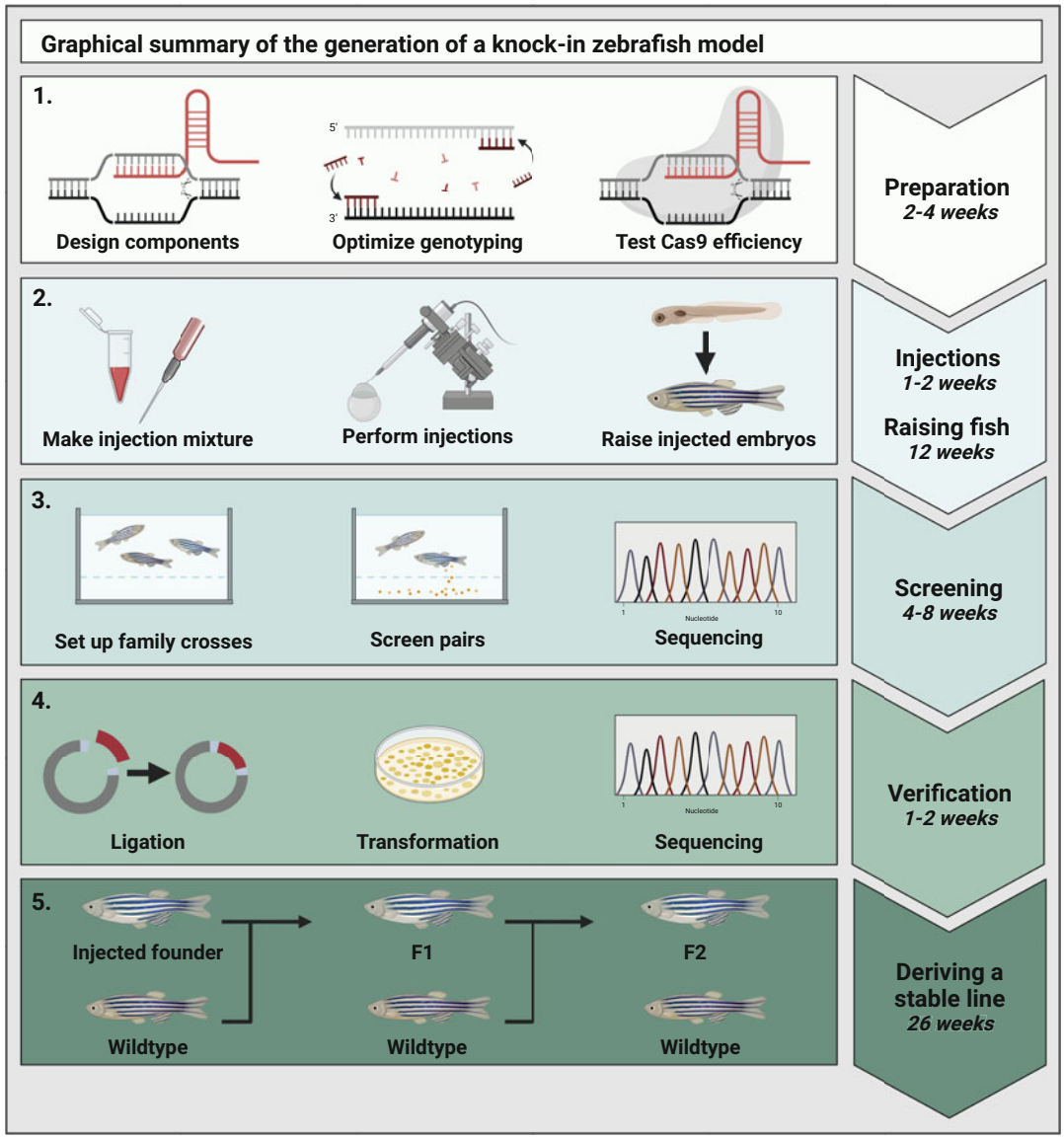

Fig. 1 Graphical overview of the steps involved in CRISPR/Cas9-based generation of zebrafish knock-in and knock-out models. Step 1. Preparation of injection mixture components and optimization of the genotyping strategy. Step 2. Composition and administration of the injection mixture. Step 3. Screening of the potential founder fish. Step 4. Verification of the mutation of interest by subcloning of the mutant transcript in pBlueScript. Step 5. Obtaining a stable line by outcrossing. (Created using BioRender (biorender.com))

you aim to introduce, two or three silent mutations (i.e., mutations that do not alter the amino acid sequence), and a left and right homology arm of >10 bases (Fig. 2). Altogether this amounts to approximately 60 bases (*see* **Note 10**).

2. Order as a standard oligo (*see* **Note 11**).

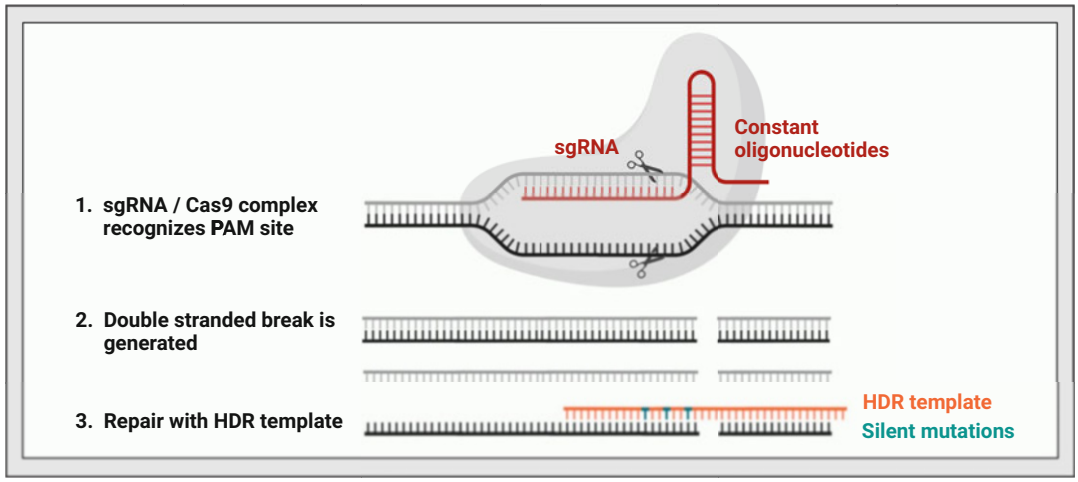

Fig. 2 Overview of the CRISPR/Cas9 genome editing approach for generating knock-in models. The one-cell stage eggs of the zebrafish are injected with a mixture including sgRNA, Cas9 protein, HDR template, and KCL. A complex of the sequence-specific sgRNA and the Cas9 endonuclease will recognize the target sequence and create a double-strand break. Repairing in the presence of the HDR template containing silent mutations will produce a new sequence that lacks a PAM site and hence is not *recognized by the sgRNA/Cas9 complex.* (Created using BioRender (biorender.com))

3. Dissolve the oligo and perform an additional PCR purification step (*see* **Note 12**).
4. Measure the concentration on a spectrophotometer and adjust it to 200 ng/μL.

3.3 Preparation—Primer Testing

To screen for the desired mutation, it is a must to have a robust and reliable genotyping pipeline for each specific mutation. The primer pairs mentioned by the ChopChop online tool work very well in our hands. They are usually about 20 bases long and have a small region of interest to amplify. Before screening the primary injected zebrafish, we determine the most optimal PCR conditions for the primers on wild-type embryos and select a suitable sequencing primer.

1. Anesthetize wild-type embryos in MS-222 and transfer the embryos to a PCR strip.
2. Remove as much E3 medium as possible from the PCR strip.
3. Prepare the SZL lysis buffer by adding 5 μL proteinase K to 1 mL of buffer (final concentration of 100 μg/mL).
4. Add 50 μL SZL lysis buffer to the embryos in the PCR strip and ensure they are submerged within the liquid (*see* **Note 13**).
5. Incubate the embryos for 1 h at 60 °C followed by 15 min at 95 °C in a thermocycler.

Table 1
PCR program for gene-specific amplification

Step	Temperature	Time	
1. Denaturation	94 °C	1 min	
2. Denaturation 3. Annealing 4. Extension	94 °C Tm 72 °C	20 s 30 s 30 s	34 cycles
5. Final extension	72 °C	3 min	
6. Hold	12 °C	Infinite	

6. Primers arrive as a powder; dissolve them to a stock concentration of 100 μM by adding MilliQ.
7. Prepare working solutions by diluting an aliquot of the primers to 10 μM in MilliQ.
8. Vortex the lysate and prepare a fresh PCR strip including 1 μL lysate and 3.7 μL Green GoTaq Flexi Reaction buffer (5×), 0.2 μL dNTPs (25 mM), 0.2 μL Taq DNA polymerase, 4.7 μL MilliQ, and 0.1 μL of each primer (10 μM) (*see* **Note 14**).
9. Incubate the lysates according to Table 1 (*see* **Note 15**).
10. Prepare a 4% 1× TBE agarose gel and load 5 μL PCR product and 2 μL 50 bp ladder (5 μg/mL) to confirm the size of the product (*see* **Note 16**).
11. Send the PCR product, including forward or reverse primer, to your sequencing company of choice according to their requirements (*see* **Note 17**).

3.4 Preparation—Cas9 Concentration Test

Before the knock-out and knock-in injections, we determine the optimal concentration of the recombinant SpCas9 protein. In our experience, a low concentration is enough to induce correct cutting. However, it is essential to determine this for each new injection mixture.

1. Recombinant SpCas9 protein was provided to us by the lab of Prof. Geijsen, LUMC Leiden (http://divvly.com). We obtained the protein as a solution of 12.5 ng/μL and stored it at −80 °C (*see* **Notes 18** and **19**).
2. Prepare the protein dilution buffer according to the instructions of the manufacturer.
3. Freshly add beta mercapto-ethanol to a final concentration of 2 mM to the protein dilution buffer.
4. Thaw the SpCas9 protein stock at 22 °C in a thermoshaker.

Table 2
Concentrations and working solutions for preparing the injection mixture

	Stock concentration	Final concentration	Volume in μL
Cas9 protein	5 ng/μL	1.25 ng/μL	1.25
sgRNA	200 ng/μL	20 ng/μL	0.50
HDR template	200 ng/μL	40 ng/μL	1.00
KCl	1 M	200 mM	1.00
Phenol Red	50%	12.5%	1.25

sgRNA single-guide RNA, *HDR* homology-directed repair

5. Prepare a SpCas9 protein dilution range by supplementing the SpCas9 protein with the protein dilution buffer (*see* **Note 20**).
6. Aliquot the diluted SpCas9 protein in low protein binding tubes (*see* **Note 21**).
7. Freeze the aliquots at −80 °C.

3.5 Injections—Preparation of Injection Mixture and Injections

One should be skilled in injection handling to successfully microinject in one-cell stage embryos. The procedure, as well as the preparation of the injection plate, is performed according to Hale et al. [33].

1. Thaw and incubate a Cas9 aliquot in a thermoshaker at 22 °C.
2. Prepare the injection mixture according to Table 2 in low protein binding tubes. Add the Cas9 last and homogenize the mixture.
3. Incubate for 5 min at 37 °C.
4. Homogenize the injection mixture before insertion in the needle.
5. Inject injection mixture into one-cell stage zebrafish embryos as previously described by Hale et al. [33].

3.6 Screening

Following the injections, the embryos should be raised to adulthood. Survival of the embryos highly depends on the efficiency and/or toxicity of the sgRNA and HDR template. We noticed significantly higher lethality when the HDR template was co-injected compared to the injection of only Cas9 and sgRNA. Generation of a knock-in model by CRISPR/Cas9 and HDR results in mosaic adult zebrafish with genotypic variations from cell to cell. Not every cell harbors the desired mutation. We routinely select founders based on genotyping of F1 offspring embryos (Fig. 3). Once a founder has been identified, this mosaic founder will be outcrossed with a wild-type partner, and their offspring will be raised.

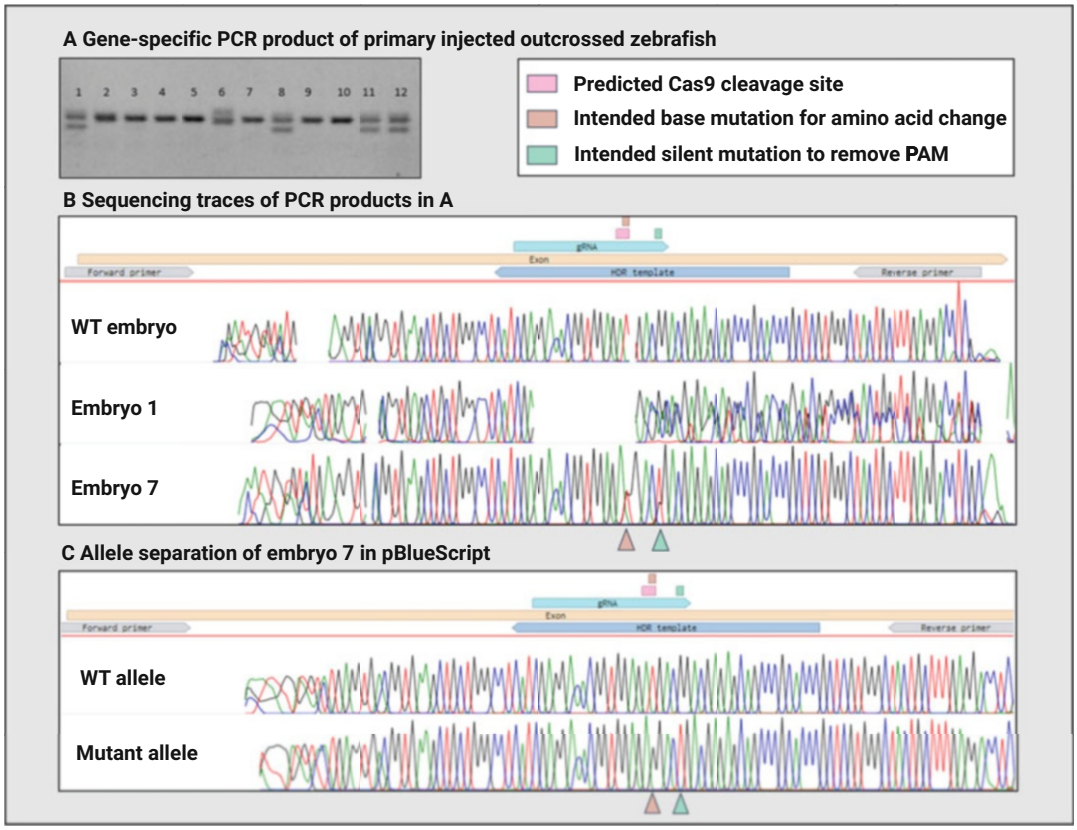

Fig. 3 Generation of knock-in zebrafish line. (**a**) Gene-specific PCR product of primary injected knock-in embryos and non-injected wild-type siblings. (**b**) Sequencing traces of the various gene-specific PCR products including a wild type, a deletion, and a correct incorporation. (**c**) Sequencing traces of wild-type and mutant alleles of embryo 7. (Created using BioRender (biorender.com))

1. Once the injection has been performed, distribute 60–80 injected eggs in a fresh dish with E3 and incubate at 28.5 °C until 5 dpf (*see* **Note 22**).
2. Remove unfertilized eggs at 24 hpf and check regularly for dead embryos (*see* **Note 23**).
3. Dechorionate the embryos at 48 hpf and clean the Petri dish from eggshells and other debris (*see* **Note 24**).
4. Move all healthy embryos containing a swim bladder to the nursery at 5 dpf and allow them to grow up to adulthood according to the standards of your fish facility (*see* **Notes 25**–**27**).
5. Determine the ratio of males and females in the injected colony at 12 weeks post-injection (*see* **Notes 28** and **29**).
6. Prepare a family cross once a week to ensure that sexual maturity is reached (*see* **Notes 30** and **31**).

7. Prepare single crosses of one potential founder and one easily recognizable wild-type and separate them overnight (*see* **Notes 32**–**35**).
8. Pool the male and female fish in the morning to start mating (*see* **Notes 36**–**38**).
9. Keep the pairs that produced eggs together in a single box and collect the eggs, one Petri dish per pair.
10. Collect the eggs in a fresh dish with E3 medium and count the number of eggs.
11. Remove the unfertilized eggs at 24 hpf.
12. Select a batch of healthy-looking embryos for lysis at 24 hpf or later (*see* **Notes 39** and **40**).
13. Collect the selected embryos in a Petri dish and sedate the embryos in 0.1% MS-222 before lysis.
14. Transfer the embryos to a PCR strip or 96-well plate and remove as much E3 medium as possible.
15. Lyse the embryos and perform PCR, agarose gel electrophoresis, and sequencing according to the method optimized as in Subheading 3.3 (primer testing).
16. Check the sequences (*see* **Note 41**).

3.7 Verification of the Mutation by Subcloning

Despite previous optimization steps, the sequencing results from the lysate of whole embryos may not be entirely clear. Additionally, it is crucial to confirm that, in the offspring, one allele is unaffected (derived from the wild-type fish) and the other allele (derived from the mutant founder fish) only has the desired mutation(s) and not unintended ones, such as partial deletions or multiple integrations of the HDR sequence. Therefore, we routinely analyze the sequence of each allele individually by subcloning a PCR fragment of the genomic DNA isolated from the embryo containing the mutation in pBluescript.

1. Digest pBluescript with a blunt-cutting enzyme (*see* **Note 42**).
2. Perform a Q5 PCR reaction on the lysate of embryos with suspected correct mutations using your gene-specific primers. Combine 1 μL lysate with 10 μL Q5 buffer (5×), 1 μL dNTPs (25 mM), 1 μL Q5 high-fidelity DNA polymerase (2000 units/mL), 32 μL MilliQ, and 2.5 μL of each primer (10 mM) (*see* **Note 43**).
3. Run a PCR reaction according to Table 3.
4. Perform agarose gel electrophoresis using 1 μL of the PCR product to confirm the presence and size of a single band.
5. Purify the product with the GeneJET PCR purification kit and measure the DNA concentration.

Table 3
PCR program for Q5 high-fidelity amplification

Step	Temperature	Time	
1. Denaturation	98 °C	30 s	
2. Denaturation 3. Annealing 4. Extension	98 °C Tm 72 °C	10 s 30 s 30 s	34 cycles
5. Final extension	72 °C	3 min	
6. Hold	12 °C	Infinite	

6. Ligate the product into the digested pBluescript II KS+ (*see* **Note 44**).
7. Transform bacteria with the ligated product and plate on LB Agar plates containing ampicillin, X-Gal, and IPTG. Incubate overnight at 37 °C.
8. Inoculate 12 white colonies (or as many as possible) into a 10 μL PCR reaction mix and perform a colony PCR using either gene- or plasmid-specific primers (*see* **Notes 45** and **46**).
9. Perform agarose gel electrophoresis to confirm the presence of the insert in the plasmid.
10. Send the PCR product with plasmid or gene-specific primer for sequencing.
11. Analyze the sequencing result: One bacterial colony should contain either the wild-type or mutant sequence.

To confirm the exclusive incorporation of the desired mutations in the target gene, we routinely analyze the sequence of the entire transcript of the target gene. To this end, mRNA is isolated from 5 dpf mutant embryos, and cDNA is generated. The entire Open Reading Frame of the target gene is amplified by RT-PCR and cloned into pBluescript, using the protocol described above. The complete sequence of at least four clones is established to verify that the desired mutations were inserted in the target gene and that no additional mutations were introduced in the process.

3.8 Deriving a Stable Line

We outcross our genetically modified founder fish at least twice before phenotypic characterization to reduce potential off-target effects caused by the CRISPR-Cas9 gene-editing procedure.

1. Once a founder fish is identified with the correct mutation, a suitable adult wild-type of similar size and age is selected (*see* **Note 47**).

2. Outcross the founder fish with a wild-type partner and raise the embryos (*see* **Note 48**).
3. Genotype the F1 adults at 12 weeks post-fertilization. A small piece of tissue is amputated from the caudal fin, lysed in 100 μL SZL lysis buffer with proteinase K, and further processed using the optimized genotyping protocol described in Subheading 3.3—primer testing.
4. Outcross the heterozygous adults with a wild-type partner to generate the F2 generation.
5. To ensure that the mutation is not lost over generations, every new generation is genotyped as described in Subheading 3.3—primer testing.

4 Notes

1. If your gene of interest is sequenced and well annotated, type the gene's name. It is also possible to manually enter the (genomic) sequence.
2. Above the exon of interest, several smaller bars are indicated, displaying the guide sequences.
3. Identified guide sequences are labeled with a number and color code indicating several parameters, including Cas9 efficiency score, number of off-target effects, self-complementary regions, GC content, and location within the gene (for further information, *see* https://chopchop.cbu.uib.no/instructions).
4. Some DNA regions provide many sgRNA options, while others contain a scarce amount of compatible sequence motifs. It is important to find a balance between the distance of the predicted Cas9 cleavage site to your mutation location and the ranking of the sgRNA.
5. The higher the number, the better the sgRNA works. However, proximity to the intended mutation site is, in this case, the most important.
6. To minimize off-target binding of the chosen sgRNA sequence, we run a search on the entire genomic DNA of the organism using the USCS genome browser BLAT option (https://genome.ucsc.edu/).
7. A new screen pops up, including the predicted cut site and multiple primer pairs to genotype the area of interest.
8. We use the Custom synthetic guide RNA option of Integrated DNA Technologies (IDT).
9. It is also an option to generate templates for sgRNA transcription [32].

10. Silent mutations should be included in the HDR template to prevent the binding of the sgRNA after the desired sequence is incorporated. If possible, we induce silent mutations in or near the PAM site to inhibit repetitive cleavage by Cas9.
11. We routinely use the Custom DNA oligo option of IDT.
12. We use the GeneJet purification kit.
13. For 4–5 dpf embryos, a short spin ensures that the embryos are not sticking to the plastic or floating above the liquid level.
14. When multiple reactions are performed, prepare a master mix.
15. We use the NEB Tm calculator (https://tmcalculator.neb.com/#!/main) to determine the annealing temperature of the PCR reaction.
16. Different Tm temperatures and a range of cycles should be used to optimize the PCR.
17. To optimize the sequencing, send both forward and reverse primers with variations of PCR product to determine the optimal primer and amount of PCR product.
18. The recombinant SpCas9 protein precipitates at 4 °C or when stored on ice, so make sure that the protein is kept at room temperature when in use or at −80 °C when stored.
19. A high-quality preparation of SpCas9 increases the success rate of DNA cleavage.
20. We prepared and tested protein dilutions of 1.25, 2.5, and 5.0 ng/μL.
21. According to the manufacturer, aliquoting in normal tubes results in significant protein loss.
22. To check the overall quality of the offspring, you should raise some uninjected embryos from the same batch.
23. Removing dead and unfertilized eggs is important for maintaining optimal growth conditions.
24. Some injection mixture might spill into the chorion egg during retrieval of the needle from the one-cell. This spilled injection mixture might result in “dirty eggs” with unwanted developmental defects.
25. Embryos lacking a swim bladder cannot swim to food and will not survive. To raise healthy, fertile adults, make sure only to select healthy embryos.
26. The survival ratio highly varies among the injected colonies. Some losses are inevitable and should be expected.
27. If the size of the embryos/ larvae within the colony is very different, it might be helpful to split the colony into two tanks, one with smaller larvae and one with larger larvae, to ensure that the smaller individuals will also get enough food to thrive.

28. Sometimes injected families are small, which may yield families with a biased gender ratio containing more females.
29. If the number of males in the colony is lower than that of females, add some similar age and size males to the colony to stimulate egg production.
30. We continue to perform family crosses until the family produces fertilized eggs for at least 2 consecutive weeks.
31. If the fish do not lay eggs in a mass crossing, they will likely not produce eggs in a single cross.
32. Start with the largest animals to give the smaller animals more time to grow.
33. Place the male below the net and the female above the net and put them together in the morning. Splitting the adults overnight will enhance the cue in the morning to spawn eggs.
34. When the potential founder has a dotted pigmentation pattern, one could use striped wild types and vice versa. Another option is using albino fish.
35. Ensure that the wild type is similar in age and size to the potential founder.
36. To induce egg spawning, we lift the net and create a slope or put shallow water in the net.
37. The female swims over the slope, and this might induce spawning.
38. If the female founder does not lay eggs, adding two males instead of one in a tank and one female might induce egg spawning.
39. We always select 12 embryos, but this number is arbitrary. More than 12 embryos may be analyzed if desired. However, we do not recommend analyzing fewer embryos.
40. Select embryos without a phenotype because they will likely survive until adulthood.
41. If all 12 embryos are wild type, the potential founder parent will be discarded.
42. We use pBluescript II KS+ and perform digestion with EcoRV.
43. Another blunt-ending polymerase may be used as well.
44. We generally perform the ligation using 20 ng pBluescript II KS+ with a vector:insert ratio of 1:9 and 1:18.
45. Use the same PCR mix and program as described in Subheading 3.3, replacing the 1 μL of lysate with MilliQ.
46. We generally use the T7 forward and M13 reverse primers since these are present in pBluescript.

47. In our experience, a weak phenotype is more easily visualized in the TL-wild-type background, whereas a very strong (lethal) phenotype may be less severe in the AB-wild-type background.
48. We usually find 1 or 2 embryos out of 12 with the correct mutation. Therefore, consider the number of F1 fish that you raise into adulthood. We typically grow up 120 embryos to adulthood.

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