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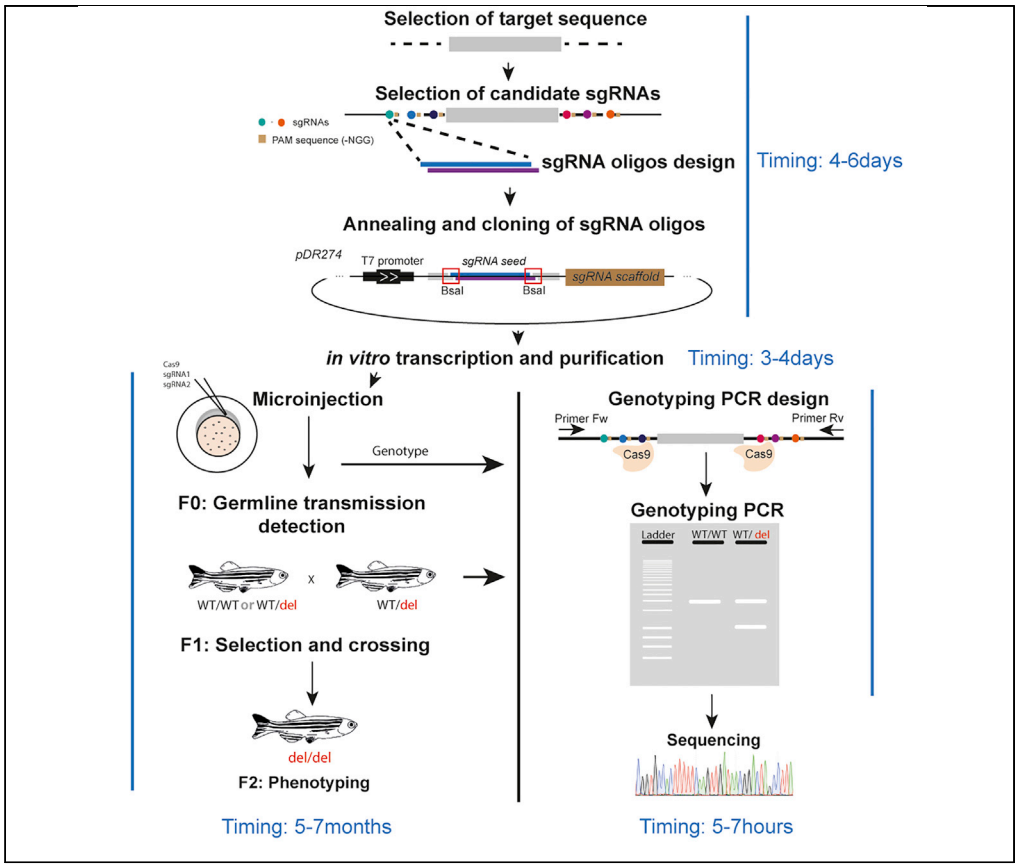
CRISPR-Cas9-Mediated Genomic Deletions Protocol in Zebrafish



Since its first application for site-directed mutagenesis, the CRISPR-Cas9 system has revolutionized genome engineering. Here, we present a validated workflow for the generation of targeted genomic deletions in zebrafish, including the design, cloning, and synthesis of single-guide RNAs and Cas9 mRNA, followed by microinjection in zebrafish embryos and subsequent genotype screening for the establishment of a mutant line. The versatility and efficiency of this pipeline makes the generation of zebrafish models a widely used approach in functional genetics.

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HIGHLIGHTS

Step-by-step details from design to production of target-specific sgRNAs

Microinjection of sgRNAs/Cas9 complexes into one-cell-stage zebrafish embryos

PCR-based genotyping of *in vivo* deletions

Cost- and time-effective pipeline for establishment of a zebrafish stable mutant line

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CRISPR-Cas9-Mediated Genomic Deletions Protocol in Zebrafish

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SUMMARY

Since its first application for site-directed mutagenesis, the CRISPR-Cas9 system has revolutionized genome engineering. Here, we present a validated workflow for the generation of targeted genomic deletions in zebrafish, including the design, cloning, and synthesis of single-guide RNAs and Cas9 mRNA, followed by microinjection in zebrafish embryos and subsequent genotype screening for the establishment of a mutant line. The versatility and efficiency of this pipeline makes the generation of zebrafish models a widely used approach in functional genetics.

For complete details on the use and execution of this protocol, please refer to Amorim et al. (2020).

BEFORE YOU BEGIN

The following protocol can target any sequence of the form: 5′- **GG-N18-NGG**-3′. **GG-N18** will be included in the transcribed sgRNA and confers the sequence specificity of the target region. The **–NGG** (Photospacer Adjacent Motif, PAM) will only be present in the genomic DNA, working as a constraint for the binding and nuclease activity of *Streptococcus pyogenes* Cas9 (SpCas9; hereafter referred as Cas9) (Cong et al., 2013). **GG-** is required for the optimal transcription by T7 RNA polymerase, which will later be used for the *in vitro* synthesis of the sgRNAs. It is possible to use sgRNAs that have a mismatch in the first two -**GG**- without affecting the targeting of Cas9 to the genomic target site.

Note: sgRNAs can be commercially synthesized; in that case, no "GG-" is required.

Selection of Target Sequence

Timing: 1 h

1. Choose your target sequence in the zebrafish genome. You can search for a DNA sequence in a genome browser such as UCSC Genome Browser (https://genome.ucsc.edu/) (Kent et al., 2002) (Figure 1). For this, in the website:
   a. Select: Genomes - zebrafish
   b. Search for a specific gene or genomic coordinates (e.g., Nog2E3 genomic coordinates – chr24:40,310,715-40,311,254; danRer7)
   c. Zoom on your sequence of interest

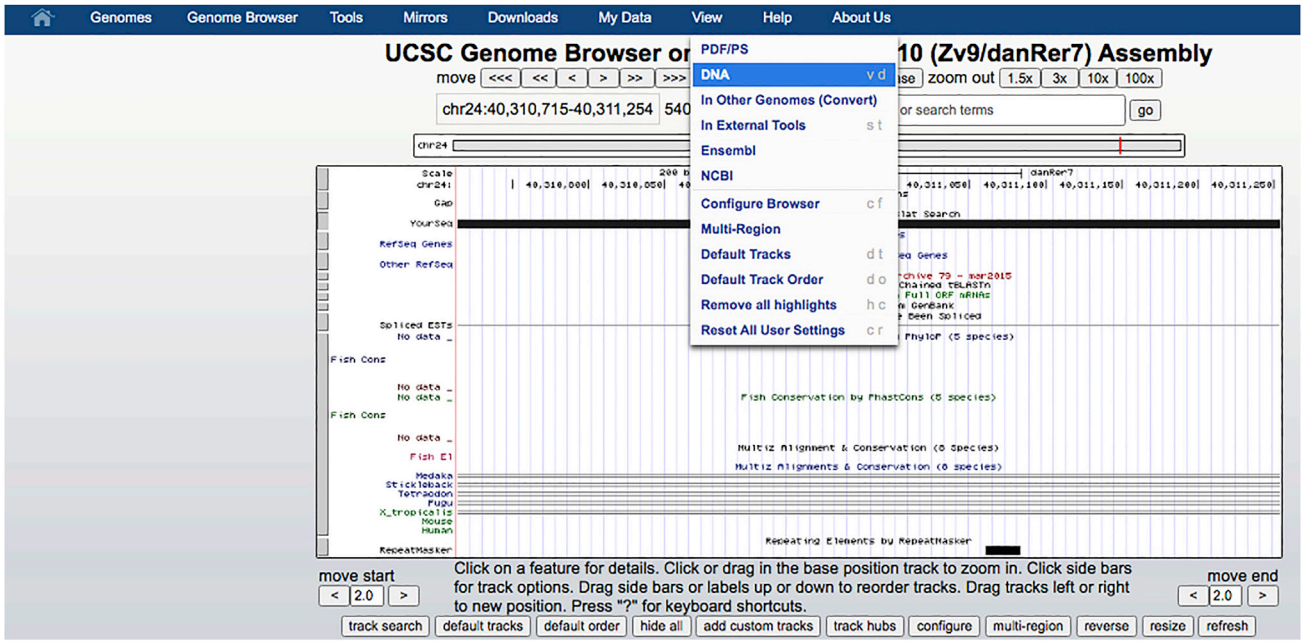

Figure 1. Snapshot of the UCSC Genome Browser Website Showing How to Get a DNA Sequence of Interest, Using the Example Shown (Nog2E3)

d. Select: View → DNA
e. Select: get DNA
f. Copy and save the obtained sequence

Note: This protocol has been tested for the generation of deletions up to 2 kilobases (kb). If your target sequence is bigger than 2 kb, we recommend following this protocol as guideline, taking into account that lower efficiency of the genomic deletion is possible (see Troubleshooting).

sgRNA Oligos Design

Timing: 1–2 h

⚠ CRITICAL: To generate a genomic deletion in a given sequence (for example, a gene or a cis-regulatory element), you need to design a pair of sgRNAs located upstream and downstream of your target region. We advise to design at least 3 pairs per target sequence.

Note: We recommend to design high GC content (40%–60%) sgRNA sequences, as these are shown to be more effective (Liu et al., 2016).

2. To find hypothetical CRISPR-Cas9 sgRNA targets in your sequence, use "http://www.crisprscan.org" (Moreno-Mateos et al., 2015) (Figure 2). In the website:
   a. Select: Submit Sequence
   b. Paste your sequence of interest
   c. Select: Species - zebrafish (*Danio rerio*), effector protein – Cas9 (NGG) and promoter used for synthesis – *in vitro* T7 promoter
   d. Select: get sgRNAs

Note: In the results list, capital letters will show the sequence to be included in the sgRNA design (Figure 2).

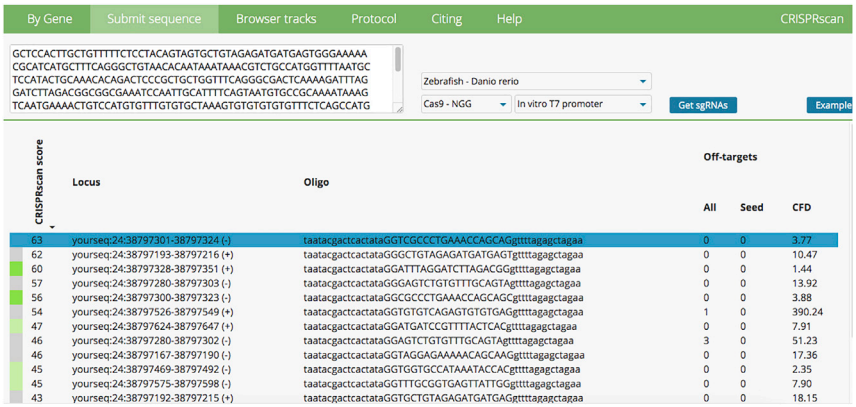

Figure 2. Results List for sgRNA Oligos in CRISPRscan

Highlighted in orange is an example of a sgRNA target site (Nog2E3sg1). The upstream "GG" base pairs are required for *in vitro* transcription. The combination of CRISPRscan and CFD (Cutting Frequency Determination) scores are designed to help select the best candidate sgRNAs, with high affinity for the targeted sequence and low off-targets prediction. The capital letters in the table represent the sgRNAs sequence; the seed region refers to the 12 bp upstream of the PAM sequence and is crucial for genomic binding and target specificity (Cong et al., 2013). For a detailed explanation and bibliography about sgRNA scores, off-targets, and seed definition, we encourage you to visit the CRISPRscan help page (https://www.crisprscan.org/?page=help).

⚠ CRITICAL: The software calculates the best sgRNA candidates based on the sequence and potential off-target binding, presenting the results as a list. Two types of scores are provided: CRISPRscan score (Moreno-Mateos et al., 2015) and Cutting Frequency Determination (CFD) (Doench et al., 2014). The CRISPRscan score has been experimentally validated to predict sgRNA activity *in vivo*: the higher the score, the higher the cutting efficiency prediction (Moreno-Mateos et al., 2015). The CFD score predicts potential off-target binding of each sgRNA candidate. The best candidates should have low CFD scores (Figure 2). For more detailed information, we recommend to explore the http://www.crisprscan.org website.

Note: CRISPRscan sgRNA prediction tracks are also directly available in the UCSC Genome Browser -https://www.crisprscan.org/?page=track.

Note: Alternative websites such as CHOPCHOP (https://chopchop.cbu.uib.no/) or Synthego (https://www.synthego.com/) can also be used in the design of sgRNA oligos.

3. **Example :** In a recent publication, we have targeted a cis-regulatory element downstream of the *nog2* gene in zebrafish (Amorim et al., 2020) (see also Expected Outcomes). By analyzing this sequence in http://www.crisprscan.org, we selected several upstream and downstream sgRNAs pairs. As an example, we will show the design of one of the pair of selected sgRNAs to target the Nog2E3 sequence: **Nog2E3sg1** (Figure 2, highlighted), GG**TCGCCCTGAAACCAGCAG.** For the design of complementary oligos to clone in pDR274:
   a. **Oligo 1** will be the sequence obtained in "crisprscan.org" in capital letters with the addition of TA 5′ of this sequence: 5′ TAGGTCGCCCTGAAACCAGCAG 3′
   b. **Oligo 2** will be the reverse-complementary sequence starting after the first 2 GG, then adding AAAC 5′ of this sequence.
      i. Sequence after the GG: 5′ TCGCCCTGAAACCAGCAG 3′
      ii. Reverse-complement: 5′ CTGCTGGTTTCAGGGCGA 3′
      iii. Add AAAC 5′: 5′ AAACCTGCTGGTTTCAGGGCGA 3′
   c. sgRNA oligos for Nog2E3sg1:

oligo 1: 5′ TAGGTCGCCCTGAAACCAGCAG 3′

oligo 2: 5′ AAACCTGCTGGTTTCAGGGCGA 3′

Note: sgRNA oligos can be purchased through companies such as Sigma-Aldrich (https://www.sigmaaldrich.com/) or IDT (https://eu.idtdna.com/pages). Oligos can be ordered as desalted and minimal amount.

4. To transcribe the sgRNAs, use the pDR274 vector (Addgene #42250) (Hwang et al., 2013). This strategy implies the annealing of two complementary oligos that contain the sequence of the target site and overhanging sequences compatible with two restriction sites of the BsaI endonuclease (Butkus et al., 1985). In the pDR274, the two BsaI-sites generate non-compatible ends, which allows directional cloning of the annealed oligos.

Note: There is an alternative way to transcribe sgRNAs, in a vector-free manner, based only in PCR amplification (Fernandez et al., 2018).

Annealing and Cloning of sgRNA Oligos

Timing: 4–6 days

5. **Day 1**: Anneal oligos 1 and 2.
   a. Mix the following reagents:

Reagents	Amount
Oligo 1 (from 100 μM stock)	1.5 μL
Oligo 2 (from 100 μM stock)	1.5 μL
ddH_2O (double-distilled water)	23 μL
Annealing buffer	24 μL

   b. Heat for 5 min at 95°C and let cool down slowly to 22°C–25°C.

6. Linearize pDR274 plasmid by BsaI digestion (Thermo Fisher Scientific #ER0291).
   a. Mix the following reagents:

Reagents	Amount
Vector	5 μg
Restriction buffer (10×)	3 μL
BsaI (10 units/μL)	1 μL
RNase-free water (NzyTech #MB11101)	up to 30 μL

   b. Incubate at 37°C for 1–2 h. Alternatively, you can perform digestion for 12–18 h, overnight.

7. **Day 2**: Confirm linearization of the plasmid by loading 10% of the reaction (3 μL with 0.6 μL of 6× NZYDNA Loading Dye, NzyTech #MB13101) on a 1% agarose gel and running in TAE buffer 1× (see Materials and Equipment).

Note: Run non-digested vector, side by side, as control. If the linearization is not complete, the linearized band and additional bands similar to the one in control will be visible in the gel. In this case, add 1 μL more of the BsaI enzyme and prolong the digestion reaction at 37°C for 1–2 h.

Figure 3. Stepwise sgRNA Design and Cloning into pDR274

8. Load all the remaining reaction on a 1% agarose gel, run in TAE buffer 1× and extract the linearized plasmid (2,125 bp) from the gel, using a gel extraction Kit (NZY GelPure; NzyTech #MB011). Quantify the DNA in a spectrophotometer (Nanodrop).

   Alternatives: other gel extraction kits can be used, such as QIAquick Gel Extraction Kit (QIAGEN #28704).

9. Ligate the linearized pDR274 to the annealed oligos (T4 Ligase; Thermo Fisher Scientific #EL0011) (see Figure 3).
   a. Mix the following reagents:

Reagents	Amount
Annealed oligos (from 100 µM stock)	1.5 µL
Plasmid	100 ng
T4 ligase buffer (10×)	1 µL
T4 ligase (5 units/µL)	0.5 µL
ddH_2O	complete to 10 µL

   b. Incubate at 22°C–25°C for 1 h or at 16°C for 12–18 h.

   Note: Do a "non-insert" ligation control reaction, by using water instead of annealed oligos. BsaI-linearized pDR274 vector has non-compatible ends, meaning that it cannot re-circularize without the presence of an insert.

10. **Day 3**: Transform competent bacteria (Thermo Fisher Scientific #C862003; alternatively, you can prepare home-made chemical competent cells with standard calcium chloride protocol) with 2 µL of the ligation reaction and respective control and plate the bacteria on kanamycin A

(Sigma-Aldrich #K1876) - supplemented LB agar plates (final concentration of kanamycin A – 50 μg/mL) (see Materials and Equipment). Incubate for 12–18 h at 37°C.

Note: pDR274 vector contains a kanamycin resistance gene.

Note: The non-insert transformation plate should be empty. If there are colonies in this plate, this means that the digestion of pDR274 occurred in only one of the two BsaI restriction sites, therefore allowing a circularization of the plasmid. In this case, repeat the cloning protocol from the linearization step (point 6).

11. **Day 4**: Perform a colony PCR to select bacterial colonies containing the plasmid with the cloned sgRNA oligos, using M13Fw and sgRNA Oligo1 as primers.

Note: M13Fw primer – 5′ TGTAAAACGACGGCCAGT 3′.

Note: When performing colony PCR, number the colonies analyzed by PCR. Colonies containing the insert will be the ones inoculated for DNA plasmid extraction preparation.

a. Mix the following reagents:

Reagents	Amount
Primer Fw (10 μM; final concentration 250 nM)	0.25 μL
Primer Rv (10 μM; final concentration 250 nM)	0.25 μL
NZT Taq II 2× Green Master Mix (NzyTech #MB358)	5 μL
ddH_2O	4.5 μL

b. Using a micropipette tip, touch a portion of a colony and introduce in the PCR reaction solution for each tube.

c. Run the colony PCR using the amplification protocol as follows:

Cycle step	Temperature	Time	Cycles
Initial denaturation	95	5 min	1
Denaturation	94	15 s	30–35
Annealing	*	20 s	
Extension	72	15 s	
Final Extension	72	5 min	1

* Annealing temperature should be optimized for each primer set based on the primer Tm; typically, it should be Tm-5°C.

12. Run PCR products plus 1–2 μL of Loading Dye in a 2% agarose gel, in TAE buffer 1×. A band of approximately 250 bp is expected if the cloning is successful.

Pause Point: Plates containing bacterial colonies can be kept at 4°C for several weeks.

13. Inoculate at least 2 positive colonies, separately, in 3–4 mL of liquid LB medium (see Materials and Equipment), supplemented with kanamycin A (Sigma-Aldrich #K1876) (final concentration 50 μg/mL) and incubate for 12–18 h at 37°C, with agitation (220 rpm).

Note: As the cloning strategy is highly efficient, it is usual to screen 5–6 colonies by colony PCR and proceed with 2 for plasmid extraction.

14. **Day 5**: Use grown cultures for plasmid extraction by using a DNA extraction kit (NZY Miniprep Kit; NzyTech #MB010).
15. Confirm correct cloned sequence (successful cloning without point mutations) by Sanger sequencing, using M13 Fw primer.

Pause Point: Plasmids can be kept at −20 °C for several months.

In Vitro RNA Transcription

Timing: 3–4 days

⚠ CRITICAL: The *in vitro* transcription protocols for sgRNA and Cas9 mRNA synthesis are slightly different. You can also opt to purchase or produce Cas9 protein.

⚠ CRITICAL: For purification of DNA and synthesis of RNA use molecular grade, nuclease-free reagents. For pipetting, use clean micropipettes and RNase-free micropipette filter tips. Cleaning of the benchtop with a RNase neutralizing solution (0.5% SDS) before RNA-related work is also recommended.

16. **Day 1**: For sgRNA synthesis, linearize the pDR274 containing the cloned sgRNA oligos using 1 μL HindIII restriction enzyme (10 units/μL; Thermo Fisher Scientific #ER0501). Set the digestion reaction as described before for BsaI enzyme. After this, perform phenol/chloroform purification.

Note: Use at least 3–4 μg of DNA (Miniprep) to perform linearization and RNA transcription.

17. Phenol/Chloroform purification
    a. Add RNase-free water to the DNA-miniprep (complete to 100 μL).
    b. Add 100 μL of phenol:chloroform:isoamyl alcohol (25:24:1; DNase, RNase-free) (Thermo Fisher Scientific #15593031).
    c. Vortex 30 s and centrifuge at 17,000 × *g* for 5 min.
    d. Collect the upper aqueous phase to a new clean 1.5 mL tube.
    e. Add 100 μL of chloroform (Fisher Scientific #C607SK-1).
    f. Vortex 30 s and centrifuge at 17,000 × *g* for 5 min.
    g. Collect the upper phase to a new clean 1.5 mL tube.
    h. Add 10 μL of NaOAc (Sigma-Aldrich #S2889) 3 M, pH 5.2, DEPC-treated (see Materials and Equipment), for each 100 μL of liquid.
    i. Add two volumes of ice-cold 100% EtOH.
    j. Precipitate at −20 °C for 12–18 h or at −80 °C for 2 h.
    k. Centrifuge at 4°C, at 17,000 × *g*, for 15 min.
    l. Remove all the liquid and let the pellet dry.
    m. Resuspend the pellet in 10–15 μL of RNase-free water.
18. **Day 2**: Quantify the DNA using a spectrophotometer and confirm linearization by loading digested vector into a 1% agarose gel. Use purified DNA to perform *in vitro* transcription (see below), with T7 RNA Polymerase (Thermo Fisher Scientific #EP0111).

Note: Run the non-digested vector in the same gel as a control for the linearization.

19. For Cas9 mRNA synthesis, linearize 3–4 μg of pCS2-nCas9n plasmid (Addgene #47929) (Jao et al., 2013) with 1 μL NotI restriction enzyme (10 units/μL; Thermo Fisher Scientific #IVGN0016), and use the same reaction conditions as described before for BsaI. After this, perform phenol/chloroform purification (see above).

20. Quantify DNA by spectrophotometer and confirm linearization by loading digested vector into a 1% agarose gel. Use purified DNA for *in vitro* transcription with SP6 RNA Polymerase (Thermo Fisher Scientific #EP0131).

Alternatives: you can use commercially available RNA transcription kits such as mMessage mMachine Transcription Kit (Thermo Fisher Scientific #AM1340).

21. *in vitro* RNA transcription reaction

Note: **T7 RNA Polymerase** (Thermo Fisher Scientific #EP0111) for **sgRNA**.

Note: **SP6 RNA Polymerase** (Thermo Fisher Scientific #EP0131) for **Cas9**.

Note: **Only for Cas9**: 5′ Cap (New England Biolabs #S1407S)

a. Mix the following and incubate at 37°C:
   i. 15 μL H_2O
   ii. 10 μL Transcription buffer 5×
   iii. 5 μL dithiothreitol (DTT) stock (50 mM) (NzyTech #MB03101)
   iv. 5 μL 10 mM NTP mix (Thermo Fisher Scientific #18109017), incubate at 37°C for 5 min.
   v. 5 μL 5′Cap (New England Biolabs #S1407S; **Only for Cas9 synthesis**), incubate at 37°C for 1 min.
   vi. Add 12 μL DNA (at least 1 μg), incubate at 37°C for 1 min
   vii. Add 1 μL RNase inhibitor (NzyTech #MB08401), incubate at 37°C for 1 min.
   viii. Add 2 μL RNA polymerase (**T7** or SP6) (20 units/μL), incubate at 37°C for 1 h.
   ix. Add 1 μL RNA polymerase, incubate at 37°C for 1 h.
   x. Add 1 μL DNase I (NzyTech #MB19901), incubate at 37°C for 30 min.

22. **Day 3**: Purify RNAs using a Sephadex column purification kit (illustra Probe Quant G-50) and subsequently purify samples by Phenol/Chloroform (as described before). Resuspend in 10 μL RNase-free water.
23. Quantify RNAs by spectrophotometry. Good-quality RNA should generate 260/280 ratio ~2 (>1.8 is acceptable) and 260/230 ratio in the range of 2.0–2.2. Run RNA in a 2% agarose gel at 80–100 V for ~30 min to confirm RNA quality.

Note: RNA is more susceptible to degradation than DNA. To run it in an agarose gel, clean the gel caster and use clean running buffer.

Note: A reasonable RNA yield resulting from the transcription protocol should be 150–1,000 ng/μL.

Note: Store RNA at −80 °C in small aliquots (e.g., 5 μL). Avoid multiple freeze-thaw cycles.

⚠ CRITICAL: The most important features of good-quality RNA are RNA integrity and RNA purity (absence of DNA contamination from the plasmid template). A smear in the gel means that RNA degradation is occurring, which will overestimate the RNA quantification and lower the efficiency of the injection. An extra band with higher molecular weight indicates plasmid contamination, which can cause toxicity in injected embryos.

Pause Point: RNA can be kept at −80 °C for several weeks until you proceed with the protocol.

Genotyping PCR Design

Timing: 30 min

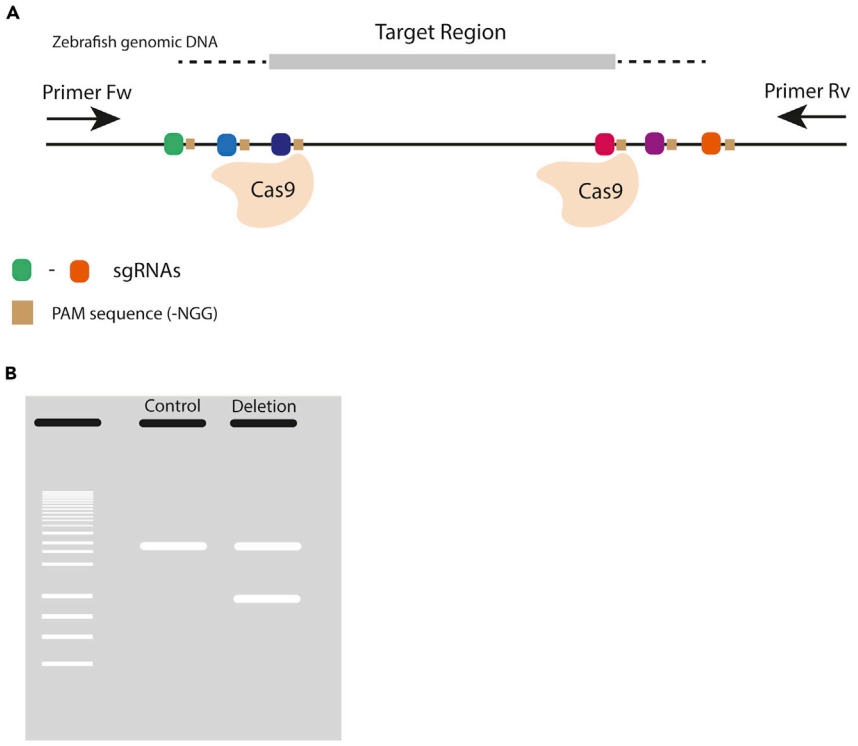

Figure 4. Genotyping PCR Design

(A) Target region showing candidate sgRNAs and genotyping primers.

(B) Representation of an agarose gel resulting from the genotyping PCR. The WT band present in both control and deletion lane serves as internal control for the PCR and represents the WT DNA sequence. The lower size band can be detected when the deletion occurs, resulting in a smaller DNA molecule. Injected animals, that can be mosaics for the deletion (F0), or fish resulting from crossing with WT animals (F1), heterozygous for the deletion, will generate two bands as genotyping PCR product.

24. Design Forward and Reverse primers flanking the region targeted by the sgRNAs for the genotyping PCR protocol. This PCR will be performed to confirm the occurrence of genomic deletions when using pairs of sgRNAs (see Figure 4).

⚠ CRITICAL: When designing genotyping primers, start by testing or optimizing their efficiency. If you are not able to produce robust and specific PCR products, you should design new ones.

Note: Online tools such as OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) (Kibbe, 2007) are helpful in primer design.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
One Shot Mach1 *E. coli* cells	Thermo	Cat #C862003
Chemicals, Peptides, and Recombinant Proteins		
5′Cap	New England Biolabs	Cat #S1407S
Acrylamide/bis-acrylamide	NzyTech	Cat #MB04501

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Agar	NzyTech	Cat #MB029
Agarose	NzyTech	Cat #MB027
Ammonium persulfate	NzyTech	Cat #MB03403
Boric acid	Sigma-Aldrich	Cat #B0394
BsaI restriction enzyme	Thermo	Cat #ER0291
$CaCl_2$	Sigma-Aldrich	Cat #449709
$CaCl_2 \cdot 2H_2O$	Sigma-Aldrich	Cat #223506
Chelex 100 resin	Sigma-Aldrich	Cat #95577
Chloroform	Fisher Scientific	Cat #C607SK-1
DNase I	NzyTech	Cat #MB19901
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich	Cat #D5758
Dithiothreitol (DTT)	NzyTech	Cat #MB03101
EDTA disodium salt	Sigma-Aldrich	Cat #03685
Glacial acetic acid	Sigma-Aldrich	Cat #A6283
Green Safe Dye	NzyTech	Cat #MB13201
HindIII restriction enzyme	Thermo	Cat #ER0501
Kanamycin A	Sigma-Aldrich	Cat #K1876
KCl	Sigma-Aldrich	Cat #P5941
Methylene blue	Sigma-Aldrich	Cat #50484
$MgCl_2$	Sigma-Aldrich	Cat #M8266
$MgCl_2 \cdot 6H_2O$	Sigma-Aldrich	Cat #M2670
NaCl	NzyTech	Cat # MB15901
NaOAc (acetic acid sodium salt)	Sigma-Aldrich	Cat #S2889
NaOH	Sigma-Aldrich	Cat #655104
NotI restriction enzyme	Thermo	Cat #IVGN0016
NTP mix	Thermo	Cat #18109017
NZY DNA 6× Loading Dye	NzyTech	Cat #MB13101
NZY Taq II	NzyTech	Cat #MB358
NZY Ribonuclease inhibitor	NzyTech	Cat #MB08401
Phenol red solution	Sigma-Aldrich	Cat #P0290
Phenol:chloroform:isoamyl alcohol (25:24:1)	Thermo	Cat #15593031
Proteinase K	NzyTech	Cat #MB019
RNase neutralizing solution (0.5% SDS)	n/a	n/a
SP6 RNA polymerase	Thermo	Cat #EP0131
T4 ligase	Thermo	Cat #EL0011
T7 RNA polymerase	Thermo	Cat #EP0111
Tetramethylethylenediamine (TEMED)	NzyTech	Cat #MB03501
Tricaine methanesulfonate	Sigma-Aldrich	Cat #E10521
Tris base	NzyTech	Cat #MB01601
Tryptone	Sigma-Aldrich	Cat #T9410
Water for molecular biology (RNase-free, DNase-free)	NzyTech	Cat #MB11101
Yeast extract	Sigma-Aldrich	Cat #Y1625

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Illustra ProbeQuant G-50	GE Healthcare	Cat #2890348
NZY GelPure	NzyTech	Cat #MB011
NZY Miniprep	NzyTech	Cat #MB010
Experimental Models: Organisms/Strains		
Zebrafish: WT (AB/TU)	n/a	n/a
Oligonucleotides		
Nog2E3sg1 Oligo1: TAGGTCGCCCTGAAACCAGCAG	Sigma-Aldrich	n/a
Nog2E3sg1 Oligo2: AAACCTGCTGGTTTCAGGGCGA	Sigma-Aldrich	n/a
M13 Forward primer: TGTAAAACGACGGCCAGT	Sigma-Aldrich	n/a
Recombinant DNA		
pDR274	Addgene	Cat #42250
pCS2-nCas9n	Addgene	Cat #47929
Software and Algorithms		
CRISPRscan	Moreno-Mateos et al. 2015	http://www.crisprscan.org
UCSC Genome Browser	Kent et al. 2002	https://genome.ucsc.edu/
Other		
Cooled incubator	Velp Scientifica	FOC2151
Microcentrifuge	Fisher Scientific	Accuspin Micro17R
Microinjector	Narishige	IM-300
Nanodrop 1000	Thermo	ND-1000
Stereomicroscope	Leica	KL300 LED
Thermo shaker	Grant Bio	PHMT
Thermocycler	Applied Biosystems	Veriti

MATERIALS AND EQUIPMENT

Note: Here we list the recipe for solutions required for this protocol. As alternative, solutions can be purchased, if commercially available.

- **Annealing Buffer** – 1 mL

Reagents	Final Concentration	Amount
Tris-HCl (pH 7.5) 1 M	100 mM	100 µL
NaCl 5 M	1 M	200 µL
0.5 M EDTA solution (pH 8.0)	10 mM	20 µL
ddH_2O	–	Adjust volume to 1 mL

Store at −20 °C for up to 2 years.

- **TAE Buffer** (agarose gel) – 1 L

TAE buffer is commonly prepared as a 50× stock solution for laboratory use. Dilute with ddH_2O to obtain a 1× working solution.

Reagents	Final Concentration	Amount
Tris base	2 M	242 g
Glacial acetic acid	5.71%	57.1 mL
0.5M EDTA solution (pH 8.0)	0.05 M	100 mL
ddH_2O	–	Adjust volume to 1 L

Store TAE 50× (stock solution) at 22°C–25°C for several months.
Store TAE 1× (working solution) at 22°C–25° for several weeks.

To prepare TAE 50×, mix:

- **TBE Running Buffer** (polyacrylamide gel) – 1 L

Reagents	Final Concentration	Amount
Tris base	0.9 M	108 g
Boric acid	0.9 M	55 g
0.5 M EDTA solution (pH 8.0)	20 mM	40 mL
ddH_2O	–	Adjust volume to 1 L

Store TBE 10× (stock solution) at 22°C–25°C for several months.
Store TBE 1× (working solution) at 22°C–25° for 1 week.

TBE buffer is commonly prepared as a 10× stock solution for laboratory use. Dilute with ddH_2O to obtain a 1× working solution.

To prepare TBE 10×, mix:

Reagents	Final Concentration	Amount
Tryptone	40 mM	10 g
Yeast extract	15.6 mM	5 g
NaCl	170 mM	10 g
Agar (for solid medium only)	60 mM	20 g
ddH_2O	–	Adjust volume to 1 L

Aliquot 300 mL in 500 mL bottles.
Autoclave (121°C for 20 min) and store at 22°C–25°C for several months.
For preparing LB agar plates follow standard procedures. Briefly, after autoclave (or melting in a microwave) allow the solution to cool down ~50°C. Add antibiotic(s) and swirl to mix. Pour ~20 mL per 10 cm Petri dish. Plates can be stored at 4°C for 1 month.

- **Lysogeny Broth (LB) medium** (liquid and solid) – 1 L

Mix:

- **E3 medium** (for zebrafish embryos) – 1 L

E3 medium is commonly prepared as a 60× stock solution for laboratory use. Dilute with ddH_2O water to obtain a 1× working solution. Methylene blue solution (Sigma-Aldrich #66725) should be

Reagents	Final Concentration	Amount
NaCl	0.3 M	17.4 g
KCl	10.7 mM	0.8 g
$CaCl_2 \cdot 2H_2O$	19.7 mM	2.9 g
$MgCl_2 \cdot 6H_2O$	24.1 mM	4.9 g
ddH_2O	–	Adjust volume to 1 L

Adjust the pH to 7.2 with NaOH.
Autoclave (121°C for 20 min) and store at 4°C for several months
To prepare 1× medium (working solution), dilute 16.5 mL of the 60× stock to 1 L. Add 100 µL of 1% methylene blue.
Store E3 and methylene blue at 22°C–25°C, protected from light, for several weeks.

added to the working solution to suppress fungal outbreaks in Petri dishes.

To prepare a 60× stock, dissolve the following ingredients in ddH_2O:

- **NaOAc solution, 3 M, pH 5.2 DEPC-treated** – 100 mL
  - Add 24.6 g of acetic acid sodium salt (NaOAc) to 70 mL of ddH_2O water.
  - Dissolve the acetic sodium acetate salt by adding a magnetic flea into the bottle and placing on a magnetic stirrer. It may take a few minutes to fully dissolve.
  - Adjust the pH to 5.2 by adding glacial acetic acid.
  - Top up the solution to 100 mL with ddH_2O water.
  - Treat with diethyl pyrocarbonate (DEPC): Briefly, add 0.2 mL of DEPC to 100 mL of solution. Shake vigorously and allow the solution to sit for 12–18 h in a fume hood. Autoclave the solution (121°C for 20 min) to inactivate DEPC.
  - Store at 22°C–25°C for several months.

⚠ CRITICAL: Wear gloves and use a fume hood when using DEPC.

STEP-BY-STEP METHOD DETAILS

Microinjection of Zebrafish Embryos

Timing: 2 days

1. **Day 1**: Separate female and male zebrafish in 1 L breeding tanks (Tecniplast), using a separating barrier, in a ratio of 3:2 (Figure 5). Use at least 2 breeding tanks for each injection condition. We recommend separating the fish in the afternoon.
2. **Day 2:** Prepare the mixture of Cas9 mRNA (150 ng/µL, final concentration) and sgRNAs (80 ng/µL, final concentration). For each µL of mixture add 0.2 µL of Phenol Red 0.5% (Sigma-Aldrich #P0290). Always keep tubes on ice.

   Note: You can fine-tune the final concentration for each sgRNA.

   Note: Phenol Red is used to color the mix solution, allowing to distinguish injected and non-injected eggs.

3. Remove the separating barriers, within the first 2 h after the lights of the fish facility are turned on, let the fish cross and monitor when they start to lay eggs. Then, collect fertilized eggs by using a strainer, within 20 min.

Figure 5. Example of a 1-L Breeding Tank Containing a Separating Barrier between Three Female and Two Male Adult Zebrafish

4. Inject 1–2 nL of the prepared mixtures of Cas9 and sgRNAs into one-cell stage embryos, using a microinjector (Narishige IM-300) and a stereomicroscope (Leica KL300). Ideally, Cas9+sgRNAs mix should be injected in the cell; however, we recommend to keep all injected embryos (injected in the cell, in the yolk, or in their interface).

   Note: After crossing each fish tank and performing the injection, go back to the fish facility, collect the remaining embryos and carry on with the injection.

   Note: 1 μL of mixture should be enough to inject 200 embryos. We recommend to inject 200–400 embryos per condition. If in a single day is not possible to reach this range, we recommend to repeat the injections.

   Note: For each injected condition, grow non-injected embryos as control to determine the viability of embryo batches. You can also add a negative control for the experiment by injecting only Cas9, to test potential toxicity arising from the Cas9 microinjection.

5. Grow the embryos for 24 h in E3 medium (see Materials and Equipment) in an incubator at 28°C.

DNA Extraction and PCR Amplification

Timing: 5–7 h

6. Collect 3 batches of 8 embryos with 24 hpf using Pasteur pipette and transfer to 1.5 mL or 0.2 mL tubes.

   Note: Before proceeding with the protocol, inspect the health of the injected embryos and remove dead and abnormally developing embryos. Also, compare the lethality of injected embryos with the non-injected controls.

7. Perform DNA Extraction as described below (**DNA extraction from zebrafish embryos (24 hpf)**):

   Note: Alternative methods for DNA extraction can also be used (e.g., hot-shot NaOH-based method)

   a. Start by anesthetizing embryos using a tricaine methanesulfonate solution (Sigma-Aldrich #E10521; final concentration of 160 μg/mL).
   b. Add 70 μL of Chelex 100 resin (Sigma-Aldrich #95577).

⚠ CRITICAL: Homogenize the Chelex solution before use by swirling it. Cut the micropipette tip in order to pipette the chelex beads.

c. Add 5 µL of proteinase K (NzyTech #MB019) (from 10 mg/mL stock).
d. Incubate for 3 h at 56°C (in a thermomixer or thermocycler).
e. Incubate 10 min at 100°C to inactivate proteinase K.
f. Transfer the liquid upper phase containing the DNA to a new tube and store at −20°C. Do not take the chelex beads.

Note: There is no need to remove the embryos from the chorion to perform the DNA extraction.

Pause Point: You can keep extracted DNA at −20 °C for several months.

8. Use the resulting DNA as template for PCR amplification, with primers flanking the region targeted by the sgRNAs (see Genotyping PCR design; Figure 4).
   a. Mix the following reagents for genotyping PCR;

Reagents	Amount
Primer Fw (10 µM; final concentration 250 nM)	0.25 µL
Primer Rv (10 µM; final concentration 250 nM)	0.25 µL
NZT Taq II 2× Green Master Mix (NzyTech #MB358)	5 µL
DNA <	1 µL
ddH_2O	3.5 µL

   b. Run the genotyping PCR using the amplification protocol:

Cycle Step	Temperature	Time	Cycles
Initial denaturation	95	5 min	1
Denaturation	94	10–30 s	30–35
Annealing	*	15–30 s	
Extension	72	30–60 s/kb	
Final extension	72	5 min	1

* Annealing temperature should be optimized for each pair of primers, based on specific Tm; typically, it should be Tm-5°C.

9. Run 5 µL of the PCR product plus 1 µL of Loading Dye in a 1%–2% agarose gel. In the presence of a deletion, the PCR product will contain molecules of lower size than in controls (Figure 4).

⚠ CRITICAL: If lower size bands are not visible in the gel, this might be due to the lack of activity of one or more sgRNAs. In this case, you should test individual sgRNAs for functionality by performing the heteroduplex mobility assay (see Troubleshooting).

10. Grow the remaining injected fish to adulthood (F0 – parental strains) to be screened for germline transmission (Figure 6).

Note: The detection of the deletion in the PCR means that it is present in a mosaic fashion in at least 1 of 8 embryos.

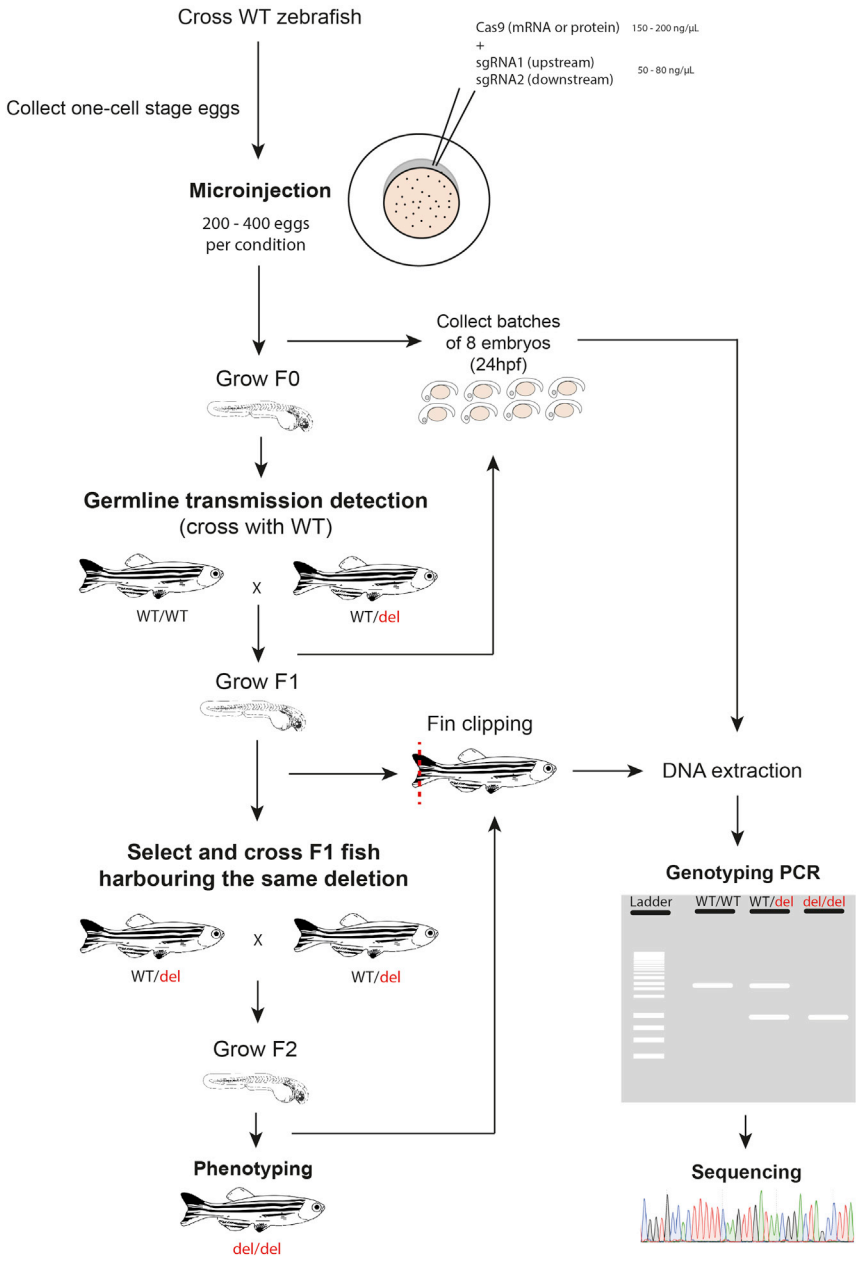

Figure 6. Pipeline from Microinjection to Establishing a Zebrafish Mutant Line

Establishing a Stable Mutant Line

Timing: 5–7 months

Mutagenesis is induced in zebrafish individuals, having a likelihood of affecting somatic and germline cells in a mosaic way. These initial individuals are termed Founders (F0). F0s might harbor different somatic and germline cells with different mutations, since different cells will go through independent mutagenesis events. To isolate mutations, F0s are crossed with wild type adults and the progeny is termed F1. F1 fish need to be genotyped, sequenced, and treated independently, since the recovered mutations might differ at the sequence level. In the case of CRISPR-Cas9, the system generates double-strand breaks (DSBs) at the designed targeted cleavage sites, being repaired by non-homologous end-joining pathway

(NHEJ) (Cong et al., 2013). NHEJ is an error-prone system that may result in deletion or insertion of few base pairs, a phenomenon known as "indels" formation, conferring a certain degree of randomness for each independent mutagenesis event. When using a pair of sgRNA for targeted deletion of a genomic sequence, as in the current protocol, indels can occur at both sgRNA cleavage sites. For this reason, when establishing a stable mutant line, it is essential to sequence the genotyping PCR bands to determine the exact sequence of the deletion of each F1 fish.

11. Test germline transmission by crossing F0 adults with WT animals and perform PCR amplification (as described before), in DNA extracted from 3 batches of 8 embryos.

Note: From a tank carrying 25 adult fish, we estimate that 3 are able to transmit the deletion to the progeny (approximately 10%).

12. Select F0 adults that are able to transmit the deletion to the progeny. Cross with WT animals and grow F1 (offspring of F0 generation).
13. Genotype F1 adult fish, by performing fin clipping, followed by DNA extraction and genotyping PCR (as described in the previous session). For **Fin Clipping:**
    a. Anesthetize fish using a tricaine methanesulfonate solution (Sigma-Aldrich #E10521). Dissolve in, at least, 100 mL of water from the zebrafish housing system, in a final concentration of 160 μg/mL.
    b. Move anesthetized fish to a 10 cm dish, without water.
    c. Cut a small portion of the fish tail (without reaching the tail bifurcation) with a sterile blade and put it in a clean 1.5 mL tube, on ice.
    d. Transfer the fish to water and let it recover from anesthesia.
    e. Keep fish separated from others and monitor its health over a period of 1 week.
    f. Use the collected fish tail to perform the genomic DNA extraction protocol.
14. Cut the deletion band of each positive sample from the agarose gel and extract them individually, using a gel extraction Kit (NZY GelPure; NzyTech #MB011). Sequence the PCR products by Sanger sequencing (Figure 7).
15. Select F1 fish harboring the same deletion and in-cross them.
16. Grow progeny (F2) to adulthood or directly evaluate phenotypes in homozygous embryos for the deletion.
17. Fin clip F2 and genotype by PCR (as described before).

Optional: Confirm genomic deletions of F2 by Sanger sequencing of extracted PCR bands.

DNA Heteroduplex Mobility Assay (HMA) (See Troubleshooting 4)

Timing: 3–4 days

The rational of this assay consists in identifying, from the same PCR reaction, amplicons that differ in few base pairs (Zhu et al., 2015). After a brief denaturation of DNA strands by heat, followed by slowly re-annealing, PCR products containing distinct amplicons will form mismatched heteroduplexes as well as perfectly matched homoduplexes of DNA. The WT genomic DNA will contain only WT alleles, thus forming homoduplex DNA. In the case of DNA from batches of embryos injected with a functional sgRNA, the PCR will amplify a mixture of "indel" (genomic insertions or deletions) mutations and WT alleles, generating both heteroduplexes and homoduplexes of DNA in the HMA assay. Usually, heteroduplexes of DNA migrate slower than homoduplex, allowing the visualization in a polyacrylamide (PAGE) gel (example shown in Figure 8).

18. **Day 1**: Separate WT fish in 1 L breeding tanks, as described before.
19. **Day 2**: Cross the fish and collect one-cell stage embryos.

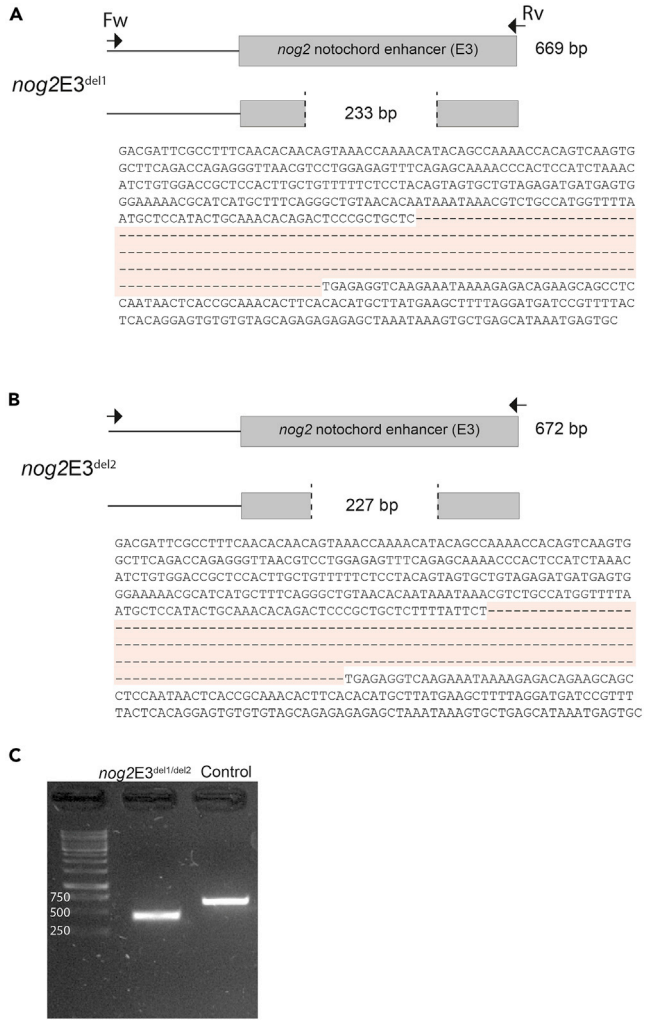

Figure 7. Two Similar Deletions in a *cis*-Regulatory Element, Downstream of the Zebrafish *nog2* Gene, Achieved through This Protocol

(A and B) Representation of two generated genomic deletions of the E3 enhancer (del1 and del2), with sizes of 233 and 227 bp, respectively.

(C) One heterozygous male and one female were crossed and their progeny was genotyped to select WT (control) and an heteroallelic combination of del1 and 2 (nog2E3del1/del2). Black arrows represent Fw and Rv primers, used in the genotyping PCR. Corresponding amplified bands are shown, in an agarose gel.

Adapted from Amorim et al. (2020).

20. Inject one-cell-stage embryos with a mixture of Cas9 (150–200 ng/μL final concentration) and single sgRNAs (50–150 ng/μL final concentration).
21. Collect 3 batches of 8 injected embryos and 1 batch of 8 non-injected embryos, as control for WT genomic DNA.
22. Perform DNA extraction using batches of embryos as samples (as described above).

Pause Point: Extracted DNA can be kept at −20 °C for several months.

23. Perform a PCR amplification using primers flanking the targeted regions (as described above for the genotyping PCR). You can use the primers designed in step 24 of Before You Begin or design new ones.

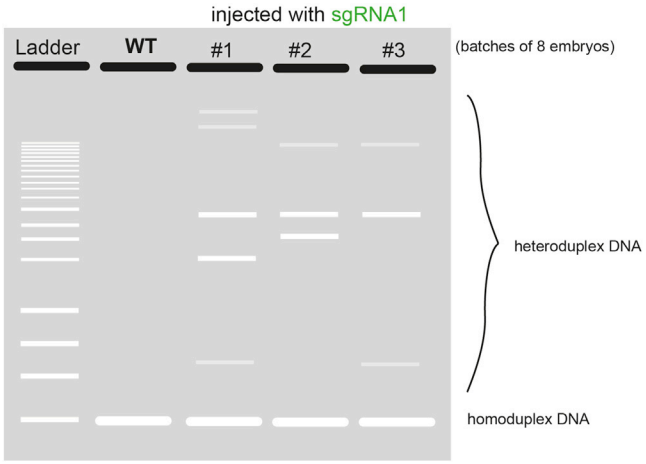

Figure 8. Representation of a PAGE Gel for Testing a Single sgRNA

Genomic DNA from a batch of 8 WT embryos will only contain WT alleles, forming homoduplex DNA (lane 2). DNA from 3 batches of 8 injected embryos, a mixture of "indel" mutations and WT alleles will be present, leading to the formation of heteroduplex DNA, indicating that the injected sgRNA is actively targeting the zebrafish genome. If more than one band is seen on the WT lane, this might be due to the presence of single nucleotide polymorphisms (SNPs) in the targeted genomic sequence. You should only extract and sequence heteroduplex bands that differ from those seen in the WT case.

Note: The PCR product of this reaction will be sequenced. For this reason, you can opt to use a proofreading polymerase, such as iMaxII DNA Polymerase (Boca Scientific #25261). Be aware of the specific guidelines of different DNA polymerases, for performing the reaction.

Note: The size of the PCR product will influence the percentage of polyacrylamide to be used in the PAGE gel. We recommend short PCR products (150–500 bp). For a 200 bp PCR product, a 15% PAGE Gel is adequate.

24. Heat PCR products at 95°C for 5 min and perform re-annealing by slowly cooling down at 22°C–25°C, for another 5 min.
25. Run 3 μL the PCR product plus 0.6 μL of Loading Dye on a 2% agarose gel to confirm amplification.

Reagents	Amount
Acrylamide/bis-acrylamide (29:1; NzyTech #MB04501)	4.5 mL
TBE buffer 10×	1.2 mL
Ammonium persulfate (APS) solution (NzyTech #MB03403)	120 μL
Tetramethylethylenediamine (TEMED) (NzyTech # MB03501)	9.6 μL
ddH_2O	6.17 mL

Pause Point: PCR products can be kept at 4°C for several weeks.

26. **Day 3**: Load 2–10 μL of the PCR product plus 0.4–2 μL of Loading Dye (depending on the intensity of the band observed in the agarose gel) into a PAGE gel. **For PAGE gel preparation (12 mL, 15%),** mix the reagents as shown in the table below:

Note: The last reagents to be added should be TEMED and APS (as they catalyze polymerization). Then, quickly assemble the gel caster.

27. Run Gel in TBE running buffer (1×,) for 3 h, at 150 V.
28. Carefully disassemble the PAGE chamber and transfer the gel to a tray with 50 mL TBE 1×, supplemented with 2 μL Green Safe (NzyTech # MB13201). Incubate by swirling for 10 min to dye the gel for following DNA visualization.

Note: As Green Safe is light sensitive, protect the tray from light during incubations.

29. Remove the TBE and wash the gel in distilled water (2 times, 5 min each).
30. Visualize DNA under UV light, in a transilluminator. Cut the WT band (smaller – see Figure 8) and heteroduplex bands (bigger) and dissolve the extracted bands in Tris-EDTA or Tris 10 mM (use enough volume to cover the gel fragments). Incubate for 12–18 h at 37°C, shaking in a thermomixer (500 rpm).
31. Perform a PCR amplification with the extracted products as template (same protocol and primers as described in step 8 of Step-by-Step Method Details).
32. Sequence the amplicons by Sanger sequencing. If the tested sgRNA efficiently targets genomic DNA *in vivo*, overlapping signals will be observed in the sequencing chromatogram after the PAM sequence, due to a mixture of multiple PCR fragments.

Note: Sequencing the PCR products from all heteroduplex bands and comparing to the WT homoduplex band will confirm the targeting of the tested sgRNA.

EXPECTED OUTCOMES

In Amorim et al. (Cell Reports, 2020), we have identified a cis-regulatory element, downstream of the zebrafish *nog2* gene, able to drive GFP expression in the notochord, seen by enhancer reporter assays. Using this protocol, we were able to establish a mutant line for a partial deletion of this cis-regulatory element (E3 enhancer), by crossing F1 fish harboring very similar deletions (del1, del2; Figure 7).

LIMITATIONS

This protocol was designed to the specific use of *Sp*Cas9 (Cong et al., 2013), which binds exclusively to PAM sequences of the form –NGG, thus restricting the number of possible sequences to target in the zebrafish genome. However, Cas9 proteins from other organisms use PAMs with a different sequence as binding requirement. Thus, employing different Cas9 might overcome the limitation of the absence of the most common –NGG PAM from SpCas9. Two examples are the *Staphylococcus aureus* - *Sa*Cas9 (possible PAM sequences are - NGRRT or - NGRRN) (Ran et al., 2015) and *Neisseria Meningitidis* – *Nm*Cas9 (PAM sequence is - NNNNGATT) (Amrani et al., 2018).

TROUBLESHOOTING

Problem 1

Inefficient RNA synthesis (step 21 of Before You Begin)

Potential Solution

Inefficient RNA synthesis can be caused by too little or too much DNA input or low efficiency of RNA polymerase (caused by degradation, for example). This will result in a low RNA yield from the transcription protocol.

The RNA synthesis protocol can be optimized by:

- Titrating the initial amount of DNA (never below 1 μg);
- Adding a higher amount of RNA polymerase;

- Increase incubation times (RNA polymerase).

Problem 2

RNA degradation (step 23 of Before You Begin)

Potential Solution

When running RNA in a gel, the appearance of a smear indicates degradation. In this case, be sure all solutions and micropipettes are clean and nuclease-free. Also, use filter tips when dealing with RNA, in order to preserve its integrity.

> *Note:* You should also control RNA purity by spectrophotometry. A 260/230 ratio below 2 might indicate solvent contamination.

Problem 3

Low efficiency of genomic deletions (step 9 of Step-by-Step Method Details)

Potential Solution

If you are not able to detect deletions in injected embryos, there are several options to monitor the functionality of the assay:

- Perform control injections:
  - using Cas9 alone to monitor toxicity of the protein and the microinjection procedure overall.
  - using Cas9 with working sgRNAs as positive control. An option might be the use of sgRNAs targeting Green Fluorescent Protein (GFP) coding sequence, in a transgenic GFP line, expecting to see a reduction of GFP expression in mosaics. Two sgRNAs targeting GFP in the zebrafish model have been experimentally validated by (Auer et al., 2014).
- Adjust concentrations of sgRNAs and/or Cas9. Be aware that higher concentrations might lead to increased mortality due to toxicity.
- Take into account the genetic background of the fish: if it is not WT, this might affect viability. You can monitor toxicity by injecting Cas9 alone or with working sgRNA pairs (see above). Another approach is to reduce concentrations at minimum (usually 50 ng/μL of each sgRNA and 150 ng/μL of Cas9 mRNA) and increase number of injected eggs.

Problem 4

No genomic deletions observed (step 9 of Step-by-Step Method Details)

Potential Solution

If no genomic deletions are observed, this might be due to a lack of activity of one of the sgRNAs. To test individual sgRNA functionality, perform the DNA heteroduplex mobility assay (see above).

> *Note:* The heteroduplex mobility assay can also be used for the optimization of the protocol, by injecting different sgRNA concentrations and comparing efficiency.

Problem 5

No deletion band in the genotyping PCR, after ensuring both sgRNAs are functional. This might be caused by a suboptimal genotyping PCR (step 9 of Step-by-Step Method Details)

Potential Solution

- Optimize PCR amplification. For that:
  - Test several annealing temperatures
- Decrease elongation time to favor the amplification of smaller bands (the PCR amplification described above is optimized for WT band, which is of bigger size)

- Reduce number of embryos per batch to ensure that is a low frequency of deletion and not a PCR detection problem.

Problem 6

Lethality in homozygous embryos for the deletion (step 16 of Step-by-Step Method Details).

Potential Solution

Monitor the progeny from birth. By counting dead embryos, it is possible to identify when most of the deaths occur. If you are interested in embryos or larvae, perform phenotypic characterization at the desired time point and subsequently genotype, as described above. If death occurs before reaching adulthood, maintain the line in heterozygosity.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, José Bessa (jose.bessa@ibmc.up.pt)

Materials Availability

This study did not generate any unique reagents.

Data and Code Availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

J.B. conceived and supervised the project, with contributions from C.P. J.P.A., C.P., and J.B. wrote the paper. R.B.C. and A.G.M. gave significant contributions to the design of the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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