

Protocol

Optimized CRISPR–Cas9 System for Genome Editing in Zebrafish

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This protocol describes how to generate and genotype mutants using an optimized CRISPR–Cas9 genome-editing system in zebrafish (CRISPRscan). Because single guide RNAs (sgRNAs) have variable efficiency when targeting specific loci, our protocol starts by explaining how to use the web tool CRISPRscan to design highly efficient sgRNAs. The CRISPRscan algorithm is based on the results of an integrated analysis of more than 1000 sgRNAs in zebrafish, which uncovered highly predictive factors that influence Cas9 activity. Next, we describe how to easily generate sgRNAs in vitro, which can then be injected in vivo to target specific loci. The use of highly efficient sgRNAs can lead to biallelic mutations in the injected embryos, causing lethality. We explain how targeting Cas9 to the germline increases viability by reducing somatic mutations. Finally, we combine two methods to identify F₁ heterozygous fish carrying the desired mutations: (i) Mut-Seq, a method based on high-throughput sequencing to detect F₀ founder fish; and (ii) a polymerase chain reaction–based fragment analysis method that identifies F₁ heterozygous fish characterized by Mut-Seq. In summary, this protocol includes the steps to generate and characterize mutant zebrafish lines using the CRISPR–Cas9 genome engineering system.



MATERIALS

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

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

Reagents

Agarose gel (1%, 2%, and 4% in gel running buffer of choice) and corresponding gel running buffer
We use 0.4 µg/µL ethidium bromide in all agarose gels.

AmpliScribe-T7 Flash Transcription kit (Epicentre), including all buffers

AmpliTaq DNA Polymerase with all the components (Applied Biosystems)

Annealing buffer for barcoding (2×) <R>

ATP, 10 mM (New England Biolabs)

Blue water <R>

CutSmart Buffer (New England Biolabs)

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dNTP mix (10 mM per nt) (New England Biolabs)
Ethanol (70% [prepared with RNase/DNase-free water], 95%–100% [RNA grade])
Formamide DI deionized (ultra pure) (American Bioanalytical)
mMessage mMachine SP6 Transcription Kit (Ambion) including all buffers
mMessage mMachine T3 Transcription Kit (Ambion) including all buffers
MS-222 (ethyl 3-aminobenzoate methanesulfonate salt [Sigma-Aldrich]), 500 mg/L in water
NaOH (100 mM)
NEBuffer 3.1 (New England Biolabs)
NotI (New England Biolabs)
Plasmids:
pT3TSnCas9n (Addgene 46757) (Jao et al. 2013)
pCS2-nCas9n-nanos 3'UTR (Addgene 62542) (Moreno-Mateos et al. 2015)
PCR primers (fragment analysis PCR [Step 41], Mut-Seq [Step 52], 10 μM)

Primers:

sgRNA primers:

Specific primer:

5'-*TAATACGACTCACTATA*[GGN₍₁₈₎]GTTTTAGAGCTAGAA-3'

Universal primer:

5'-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTT
AACTTGCTATTTCTAGCTCTAAAAC-3'

Mut-Seq primers:

Adapter_A: 5'(PO₄)-GATCGGAAGAGCACACGTCT-3'

Adapter_B: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

Adapter_C: 5'-AGGATGATACCGACCGAGATCTACACTCTTTCCCTACACGA-3'

In the single guide RNA (sgRNA) primers, italics indicate the T7 promoter and underlines indicate the complementary regions of the specific and universal primers. The “N(18)” sequence of the specific primer is determined as described in Steps 1 and 2.

QIAquick Gel Extraction Kit (QIAGEN)
QIAquick PCR Purification Kit (QIAGEN)
RNase/DNase-free water (Ambion)
RNeasy Mini kit (QIAGEN) including all buffers
Sodium acetate (3 M, pH 5.2)
T4 DNA ligase (New England Biolabs)
T4 DNA ligase buffer, 10× (New England Biolabs)
T4 polynucleotide kinase (PNK) (New England Biolabs)
Tris-Cl (1 M, pH 7.4)
TruSeq Barcodes: ScriptMiner Index PCR Primers (1–12) (Epicentre)
TURBO DNase (Ambion)
XbaI (New England Biolabs)
Zebrafish of desired genotypes

Equipment

Access to DNA analysis and sequencing facilities

See Steps 49, 51, and 75.

Benchtop cooler (–20°C)

Dry bath incubator

Fragment analysis software (e.g., GeneMarker)

Freezers (–20°C, –80°C)

Gel electrophoresis apparatus

Gel imager

GMAP read aligner (download from <http://research-pub.gene.com/gmap/>)

Incubators (28°C, 37°C)

Microcentrifuge

Microcentrifuge tubes (1.5 mL)

Mut-Seq pipeline (download from <http://protocol.crisprscan.org>)

Download on a machine with Python 3.2 (or more recent). A convenient Python installer such as Anaconda (<http://continuum.io/downloads>) can be used.

Pasteur pipettes

PCR tubes (0.2 mL), strip or plate

sgRNA prediction tool (at <http://www.crisprscan.org/>)

Spectrophotometer (e.g., NanoDrop)

Thermocycler

Zebrafish embryo microinjection system

METHOD

Design of sgRNA (Fig. 1)

1. Predict sgRNA(s).

Three methods to design sgRNAs are described below; each is adapted to a specific situation. Sequences including the sgRNA primer that contains T7 promoter, sgRNA, and tail are provided (Fig. 2A).

Although the CRISPRscan model (Moreno-Mateos et al. 2015) has been designed in zebrafish and tested on both zebrafish and Xenopus, predictions for multiple species are provided on the CRISPRscan website. These predictions directly apply the zebrafish model without further validation.

Case 1: Targeting the coding sequence of a gene in a species included on CRISPRscan.org (Fig. 1A)

i. In CRISPRscan.org, go to the (1) “By gene” tab.

ii. Choose species of interest (2) and “Gene” in (3).

Alternatively, CRISPRscan.org can be used to target a specific transcript/isoform by selecting “Transcript” instead of “Gene” in (3).

iii. Enter gene name or ID in (4). In case this name is incomplete, choose a gene from the list of choices offered. Get sgRNAs by clicking on (5).

Results in panel (6) for the selected gene will include sgRNAs for all transcript isoforms (coordinates are reported in relation to the cDNA coding sequence). Sorting by column in panel (6) is available by clicking on column header. Presence of off-targets in the genome and canonical (GGN(18)) sites are indicated with a tick (check mark).

Case 2: Targeting an intergenic or noncoding (e.g., 3'-UTR) region in a species included on CRISPRscan.org

i. In CRISPRscan.org, go to the “UCSC tracks” tab (Fig. 1A (1)).

ii. Click on the species of interest to load tracks into the UCSC genome browser.

iii. Within the UCSC genome browser, navigate to the region of interest.

iv. Retrieve an sgRNA sequence by clicking on its name.

Case 3: Targeting a species not included on CRISPRscan.org (Fig. 1B)

i. On CRISPRscan.org, go to the (1) “Submit sequence” tab.

ii. Copy and paste your sequence of interest in (2).

iii. Click on (3) “Get sgRNAs” to sgRNAs in panel (4).



FIGURE 1. sgRNA design with CRISPRscan.org (A) for a gene and (B) for any sequence. (A) Precomputed sgRNAs for multiple species (1) and genes or transcripts (2) are displayed on panel (6). (B) User can enter a sequence (or multiple sequences in FASTA format) in (2) to identify and score potential sgRNAs.

2. Select sgRNA(s).

Most appropriate sgRNAs should have (i) a maximal CRISPRscan score of at least 55, ideally >70 (Moreno-Mateos et al. 2015), (ii) close proximity to the targeted site, and (iii) absence of off-target sites. If there are no sgRNAs that satisfy all of these criteria (e.g., when targeting short elements such as miRNAs or transcription factor binding sites), first use a lower CRISPRscan score threshold to select an sgRNA. If no such sgRNAs exist, choose two sgRNAs with the highest possible score flanking the short element to induce a deletion that spans the two sgRNAs.

sgRNA Generation (Fig. 2A)

sgRNA DNA Template Synthesis: Fill-in PCR and DNA Purification

3. Assemble the following polymerase chain reaction (PCR) master mix on ice in a PCR tube (volumes shown are for one reaction):
- 2.5 μL PCR Buffer II (10×) (AmpliTaq DNA Polymerase kit)
 - 0.5 μL dNTP mix (10 mM per nt)
 - 1.5 μL MgCl₂ (25 mM) (AmpliTaq DNA Polymerase kit)
 - 1 μL Universal primer (10 μM)

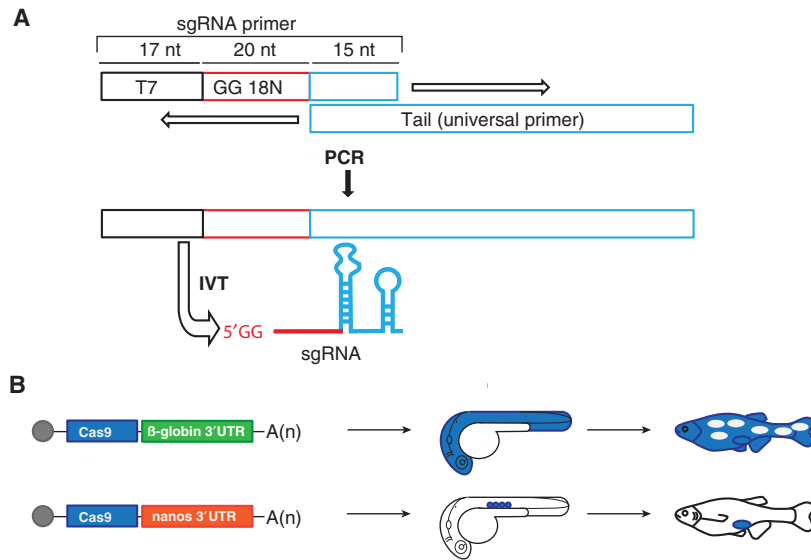


FIGURE 2. sgRNA generation and *Cas9-nanos* mutagenesis strategy. (A) PCR method to obtain a 117-bp template for sgRNA in vitro transcription (IVT). Each sgRNA primer contains the T7 promoter (green box), the 20 nt of the specific sgRNA–DNA binding sequence (red box), and a constant 15-nt tail which is used to anneal with an 80-nt reverse oligonucleotide (universal primer) to add the sgRNA constant 3' end (light blue box). (B) Schema illustrating the *Cas9-nanos* 3'-UTR approach. Injection of *Cas9-nanos* will concentrate the expression in the germ cells (blue circles) and injection of *Cas9-β-globin* will induce mutations in the soma and generate mosaic fish. (Adapted by permission from Macmillan Publishers Ltd: *Nature Methods* [Moreno-Mateos et al. 2015], © 2015.)

1 μL sgRNA primer from Steps 1 to 2 (10 μM)
 0.2 μL AmpliTaq polymerase (5 U/μL)
 18.3 μL RNase/DNase-free water
 25 μL total

If two or three sgRNAs are used to target one particular gene in the same injection, the sgRNA primers can be mixed in equimolar concentrations and 1 μL of this mix (10 μM) should be used in the reaction.

4. Enter the following program in the thermocycler:

1 cycle	95°C	3 min
30 cycles	95°C	30 sec
	45°C	30 sec
	72°C	30 sec
1 cycle	72°C	5 min

5. Place the samples into the thermocycler and run the program.

6. After the program has completed, run an aliquot (5 μL of the sample) on an ethidium-bromide-stained 2% agarose gel. Visualize the PCR product (117 bp) on a standard gel imager to ensure proper amplification.

7. Clean up the rest of the PCR product (20 μL) with the QIAquick PCR Purification Kit.

The following steps are based on the protocol for this kit, and the referenced buffers are contained in the kit.

Bind DNA to column

i. Add 5 volumes buffer PB to the PCR reaction (1 volume) and mix.

If the color of the mixture is orange or violet, add 10 μL 3 M sodium acetate, pH 5.2, and mix. The color of the mixture will turn yellow.

ii. Place the QIAquick column in the provided 2-mL collection tube.

iii. To bind DNA, apply the sample/PB buffer mixture to the column.

- iv. Centrifuge at 18,000g for 1 min.
- v. Discard the flowthrough and place the column back into the same tube.

Wash DNA bound to column

- vi. To wash, add 750 μ L buffer PE to the column.
- vii. Centrifuge at 18,000g for 1 min.
- viii. Discard the flowthrough and place the column back into the same tube.
- ix. To remove residual ethanol, centrifuge at 18,000g for 1 min.

Elute DNA from the column

- x. Place the QIAquick column in a clean 1.5-mL microcentrifuge tube.
- xi. To elute the DNA add 30 μ L of RNase/DNase-free water to the center of the membrane.
- xii. Let the column stand for 1 min and then centrifuge at 18,000g for 1 min at room temperature.

DNA can be stored at -20°C .

sgRNA In Vitro Transcription (IVT) Reaction

This protocol is based on the AmpliScribe-T7 Flash Transcription kit (Epicentre).

8. Vortex and thoroughly mix all the reagents.
Keeping the reaction buffer at room temperature is very important because the spermidine in chilled 10 \times reaction buffer can precipitate the template DNA and causes the reaction to fail.
9. Setup IVT reaction at room temperature while keeping the enzyme solutions in a -20°C bench-top cooler (volumes shown are for one reaction). Incubate for 5–6 h at 37°C .

6.3 μ L sgRNA DNA template (from Step 7)
1.8 μ L ATP (100 mM; from AmpliScribe kit)
1.8 μ L CTP (100 mM; from AmpliScribe kit)
1.8 μ L GTP (100 mM; from AmpliScribe kit)
1.8 μ L UTP (100 mM; from AmpliScribe kit)
2 μ L DTT (100 mM) (AmpliScribe kit)
2 μ L AmpliScribe-T7 Flash 10 \times Reaction buffer
0.5 μ L RiboGuard RNase Inhibitor (AmpliScribe kit)
2 μ L AmpliScribe T7-Flash Enzyme Solution
20 μ L total

10. Add 1 μ L TURBO DNase (2 U/ μ L).
11. Incubate for 20 min at 37°C .

6.3 μ L of sgRNA DNA template is ~ 120 – 150 ng.

The reaction is also efficient if it is performed with half the volume of each component.

sgRNA Precipitation

12. Add 80 μ L RNase/DNase-free water.
13. Add 10 μ L of 3 M sodium acetate pH 5.2 and mix by vortexing.
14. Add 300 μ L RNA-grade 95%–100% ethanol and mix by vortexing.
15. Incubate for 1 h at -80°C .
16. Centrifuge at 16,100g for 30 min at 4°C .

A white pellet should be observed.

17. Discard the supernatant carefully and add 750 μL 70% ethanol to rinse.
18. Centrifuge at 16,100g for 5 min at 4°C.
19. Repeat Steps 17–18.
20. Remove the supernatant carefully and dry the pellet 5 min to evaporate the ethanol.
21. Resuspend in 100 μL RNase/DNase-free water.
22. Dilute 5 μL in 20 μL RNase/DNase-free water (1/5 dilution). Make several aliquots.
23. Use 1 μL of the 1/5 dilution to quantify the sgRNA in a spectrophotometer.
This protocol yields ~100 μg of sgRNA. Therefore, the concentration in the dilution sample (Step 22) should be ~200 ng/ μL .
24. Store sgRNA aliquots at –80°C.
We do not recommend freezing and thawing the sgRNAs more than two to three times.

Cas9 mRNA Production

Although regular Cas9 (Cas9- β -globin) will induce mutations in the soma and germ cells, Cas9-nanos 3' UTR (Cas9-nanos) will concentrate the mutations in the germ cells (Fig. 2B; Kopranner et al. 2001; Mishima et al. 2006), reducing the possible toxicity or lethality caused by the mutations in the studied gene (Moreno-Mateos et al. 2015).

Linearized Plasmid Template Generation

25. To linearize Cas9 plasmids, set up the following reactions:

Cas9 (Cas9- β -globin)	Cas9-nanos
2 μg pT3TSnCas9n	2 μg pCS2-Cas9-nanos
3 μL CutSmart Buffer	3 μL NEBuffer 3.1
1 μL XbaI (10 U)	1 μL NotI (10 U)
\times μL RNase/DNase-free water	\times μL RNase/DNase-free water
30 μL total	30 μL total

26. Incubate for 2 h at 37°C.
27. Run the reaction in an ethidium-bromide-stained 1% agarose gel.
28. Visualize the linearized plasmid on a gel imager using long-wavelength UV and excise the fragment with a clean scalpel blade.
29. Place the gel slice in a labeled microcentrifuge tube.
30. Purify the linearized plasmid using a QIAquick Gel Extraction Kit.
The following steps are based on the protocol for this kit, and the referenced buffers are contained in the kit.

Bind DNA to column

- i. Excise the DNA fragment from the agarose gel.
- ii. Weigh the gel slice and add 3 volumes QG buffer to 1 volume gel (1 mg = 1 μL).
- iii. Incubate at 50°C, vortexing every 2 min until the gel slice has dissolved completely (~6–7 min).
If the color of the mixture is orange or violet, add 10 μL 3 M sodium acetate pH 5.2 and mix.
- iv. Add 1 volume isopropanol to the sample and mix.
- v. Place a QIAquick spin column in a provided 2-mL collection tube
- vi. Apply the sample to the QIAquick column and centrifuge 1 min at 18,000g at room temperature. Discard the flowthrough and reuse the collection tube.
Samples >750 μL should be loaded and centrifuged again.

Wash DNA bound to column

- vii. Add 500 μL Buffer QG to the column.
- viii. Centrifuge for 1 min at 18,000g. Discard the flowthrough and reuse the collection tube.
- ix. Add 750 μL PE buffer to the column. Let stand 2–5 min after addition of the PE buffer.
- x. Centrifuge for 1 min at 18,000g. Discard the flowthrough and reuse the collection tube.
- xi. To remove residual PE buffer, centrifuge for 1 min at 18,000g at room temperature and discard the collection tube.

Elute DNA from the column

- xii. Place the QIAquick column in a clean 1.5-mL microcentrifuge tube.
- xiii. To elute the DNA add 30 μL of RNase/DNase-free water to the center of the membrane.
- xiv. Let the column stand for 4 min and centrifuge 1 min at 18,000g.

IVT DNA template can be stored at -20°C .

Cas9 In Vitro Transcription and Purification

This protocol is based on the mMessage mMachine (T3 for Cas9 and SP6 for Cas9-nanos) Transcription Kit (Ambion).

31. Thoroughly vortex the 10 \times Reaction Buffer and the 2 \times NTP/CAP.
32. Store the 2 \times NTP/CAP (ribonucleotides) on ice, but keep the 10 \times Reaction Buffer at room temperature while assembling the reaction.
Keeping the buffer at room temperature is very important because the spermidine in chilled 10 \times reaction buffer can precipitate the template DNA and cause the reaction to fail.
33. Setup IVT reaction at room temperature while keeping the enzyme solution in a -20°C benchtop cooler. Add the reagents in the following order (volumes shown are for one reaction):

6 μL linearized plasmid (this is ~ 200 – 300 ng of DNA)
10 μL NTP/CAP (2 \times)
2 μL Reaction buffer (10 \times)
2 μL Enzyme Mix
20 μL total

34. Incubate for 2 h at 37°C .
35. Add 1 μL TURBO DNase (2 U/ μL).
36. Incubate for 20 min at 37°C .
37. Stop the reaction by freezing the tube in liquid nitrogen and keeping it at -80°C , or continue with the mRNA purification immediately.
38. Purify the RNA using an RNeasy Mini kit (QIAGEN).

The following steps are based on the protocol for this kit, and the referenced buffers are supplied in the kit:

Bind RNA to column

- i. Add to 80 μL RNase/DNase-free water to the IVT sample.
- ii. Add 350 μL Buffer RLT, mix well.
- iii. Add 250 μL 96%–100% ethanol and mix by pipetting. Do not centrifuge. Proceed immediately to the next step.
- iv. Transfer the sample to an RNeasy Mini spin column placed in a 2-mL collection tube. Centrifuge for 15 sec at 8000g. Discard the flowthrough carefully so that the column does not contact the flowthrough.

Wash RNA bound to column

- v. Add 500 μL Buffer RPE to the column. Centrifuge for 15 sec at 8000g. Discard carefully the flowthrough.
- vi. Add 500 μL Buffer RPE to the column. Centrifuge for 2 min at 8000g. Discard carefully the flowthrough.
- vii. Place the RNeasy spin column in a new 2-mL collection tube. Centrifuge for 1 min at max speed to eliminate any possible carryover of buffer RPE.

Elute RNA from the column

- viii. Place the RNeasy spin column in a new 1.5-mL collection tube. Add 30 μL RNase/DNase-free water to the spin column membrane.
- ix. Centrifuge for 1 min at 8000g to elute the RNA.
- x. Add the eluate from the previous step to the used column and centrifuge 1 min at 8000g to elute the RNA.
This step will elute more RNA and will concentrate it.
- xi. Make aliquots of 5 μL and use 1 μL to quantify the mRNA concentration in a spectrophotometer.
This protocol yields ~20–30 μg of sgRNA. Therefore, the concentration should be ~500–1000 ng/ μL .
- xii. Store sgRNA aliquots at -80°C .
It is not recommended to thaw and freeze mRNA more than two to three times.

Cas9–nanos will concentrate the mutations in the germ cells, whereas regular Cas9 will induce mutations in the somatic and germ cells (Fig. 2B).

39. Mix sgRNA plus Cas9 mRNA in 5 μL as final volume.

Working concentration should be 100 ng/ μL Cas9 mRNA and 20 ng/ μL per sgRNA (100 and 20 pg of Cas9 and sgRNA(s) will be injected, respectively).

40. Inject 1 nL directly into the cell during the early one-cell stage.

As a positive control, an sgRNA (5'-GGGGAAGGTTGATTATGCAC-3') targeting the albino (slc45a2) gene (involved in pigmentation) can be used (working concentration 10 ng/ μL). Using Cas9 results in 60%–70% embryos with albino-like phenotype (lack of pigmentation). Using Cas9–nanos, 70%–100% of the injected embryos show albino mutant clones in the retina.

Several sgRNAs targeting the same locus or different loci in the same injection can be used. When more than three to four sgRNAs are used together, 10–15 ng/ μL per sgRNA used is sufficient. In addition, the concentration of Cas9 can be scaled up to 200–300 ng/ μL .

Injections can also be performed using the purified protein (Gagnon et al. 2014). We used 300 pg of purified protein (PNA Bio) and 200 pg sgRNA. In our hands, there was no significant difference compared with mRNA/sgRNA injections, most likely because Cas9 mRNA is already polyadenylated and is therefore translated immediately upon injection.

Analysis of CRISPR–Cas9 Mutations: (A) Using Fragment Analysis

The low cost and high efficiency of the CRISPR–Cas9 system in zebrafish allows for the generation of multiple mutant lines in parallel. Below, we describe two methods to identify mutants: (A) Fragment analysis, a fast method recommended for identifying mutant alleles with nucleotide resolution for a few targeted genes/fish (Steps 41–51) and (B) Mut-Seq, a high-throughput method designed to identify the mutant allele sequences for multiple targeted genes (Steps 52–80).

Fragment analysis is a simplified version of Sanger DNA sequencing. In the sequencing method, hundreds of fluorescently labeled PCR fragments are separated with nucleotide resolution by capillary electrophoresis and the final result is a chromatogram with an equivalent number of peaks. Each peak represents one PCR fragment and the position of the peak along the axis is proportional to its nucleotide length. In the case of fragment analysis, after capillary electrophoresis only one peak (rather than hundreds of peaks) is observed in case of wild-type samples, and any extra peak indicates the presence of mutant alleles.

Fragment analysis can be performed in (i) F_0 injected embryos with CRISPR–Cas9 to check mutagenic activity, (ii) F_1 embryos from an F_0 fish injected with CRISPR–Cas9 to identify the adult F_0 founders transmitting the mutation, or (iii) fins clipped from adult F_1 to identify the mutant allele.

Primer Design

41. Design primers to amplify an ~200–400-bp fragment covering the target(s) sgRNA sites.

Primers should prime at least 50 nucleotides away from the target(s) sgRNA sites. One of the primers needs to be ordered with a fluorescent label at the 5' end (usually FAM or HEX, which can be combined for multiplexing).

Embryo/Fin Collection

As a control, it is highly recommended to include at least one wild-type sample to identify the wild-type allele.

42. Collect the desired tissue using one of the following methods.

To collect embryos

- i. Collect embryos in a PCR tube strip or plate, one embryo per tube.
- ii. Remove all water with a glass Pasteur pipette.

To collect fins

- i. Anesthetize adult fish in diluted MS-222 (500 mg/L in water).
- ii. As soon as the fish fall unconscious, cut a small piece of the caudal fin and transfer the fin fragment with forceps to a PCR tube strip or plate.
- iii. Immediately put the fish back in a labeled, individual tank.

DNA Extraction

43. Add 100 μ L of 100 mM NaOH.
44. Incubate the embryos/fin fragments for 15 min at 95°C in a dry bath incubator.
45. Immediately place the embryos on ice, neutralize with 30 μ L of 1 M TrisCl, pH 7.4, and vortex to homogenize the sample.
46. In a new tube/strip, add 1 μ L of the extracted DNA solution and 19 μ L of water.

PCR

47. Assemble PCR reactions for each DNA sample:

2 μ L PCR Buffer II (10 \times) (AmpliTaq DNA Polymerase kit)
1 μ L dNTPs (10 mM)
1.2 μ L MgCl₂ (25 mM) (AmpliTaq DNA Polymerase kit)
0.5 μ L AmpliTaq DNA polymerase
1 μ L forward and reverse primers specific for each gene (one primer labeled with either FAM or HEX, 10 μ M mix)
1 μ L genomic DNA (the 1/20 dilution prepared in Step 46)
13.3 μ L RNase/DNase-free water
20 μ L total

48. Perform PCR using the following program:

1 cycle	95°C	2 min
34 cycles	95°C	10 sec
	50°C	30 sec
	72°C	30 sec
1 cycle	72°C	5 min

49. Assemble a PCR (0.2-mL) tube with 1 μ L PCR product plus 9 μ L formamide DI deionized and submit it to a DNA analysis facility for fragment analysis.

One FAM- and one HEX-labeled fragment can be combined in a single tube to reduce costs.

50. Analyze the data using GeneMarker (SoftGenetics) or other fragment analysis software. Compare the size of the wild-type copy to the targeted ones and identify the samples showing DNA deletion or insertion.

A DNA insertion will produce peaks of a larger size than wild type, while a DNA deletion will produce peaks of a smaller size compared with the wild-type peak (Fig. 3).

51. Sequence the PCR fragments from the desired samples to obtain the sequence of the allele.

Analysis of CRISPR–Cas9 Mutations: (B) Using Mut-Seq

Primer Design

52. Design primers to amplify a 130- to 150-bp fragment surrounding each sgRNA site.

Primers should prime at least 30–50 nucleotides away from the target(s) sgRNA sites. Multiple target sites can be analyzed in the same PCR fragment if they are <150 nucleotides apart.

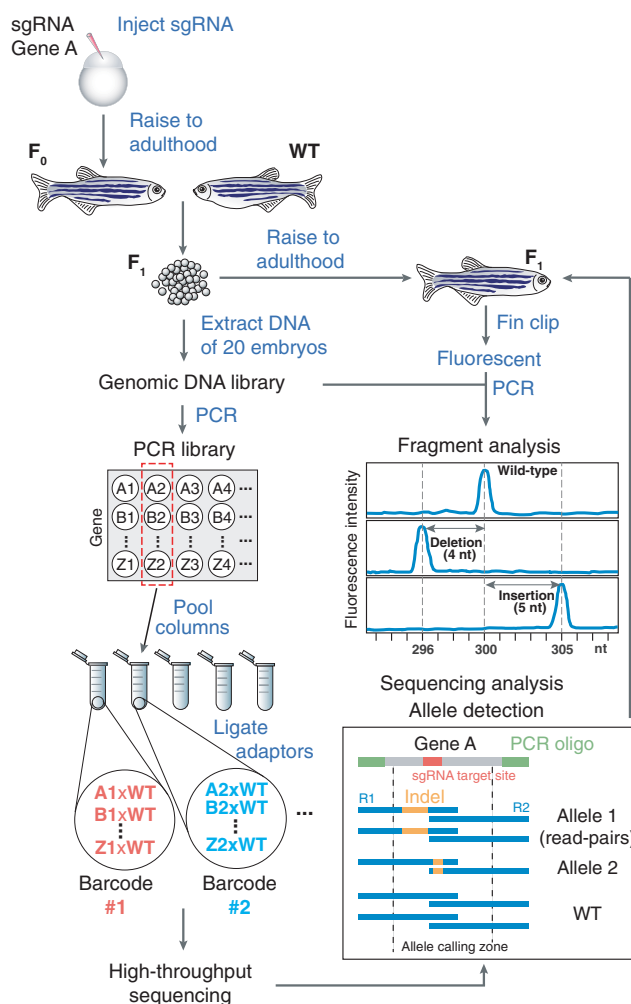


FIGURE 3. Identification of zebrafish mutants generated by the CRISPR–Cas9 system. One-cell-stage embryos are injected with the sgRNA targeting the desired gene (Gene A in the example). Those embryos are raised to adulthood (F₀) and outcrossed to wild-type (WT) fish. Twenty F₁ embryos are collected for DNA extraction and genotyping, while the remaining embryos are raised to adulthood. PCR amplicons covering the sgRNA target sites are generated for each gene target. Amplicons coming from different genes are pooled, barcoded, and sequenced, and then mutations are identified using Mut-Seq. Fragment analysis on fin clips is used to identify the F₁ heterozygous fish carrying alleles revealed by Mut-Seq.

Embryo Production and Collection

Day 0

53. In the afternoon, set crosses of F_0 CRISPR–Cas9-injected fish targeting (as example, gene A) to wild-type fish in crossing tank without using dividers (Fig. 3).

To reduce the number of crosses, F_0 CRISPR-injected fish targeting one locus (gene A) can be crossed to other F_0 CRISPR-injected fish targeting another locus (Gene B). At the end of the pipeline, it will uniquely identify the founder fish for each gene. However, once the F_0 is identified, it will be outcrossed to wild-type to clean the line of other mutations.

Day 1

54. Collect embryos ~ 2 h after the fish facility lights are turned on.
55. Place the embryos in a Petri dish with blue water and label it with the same identifier as the breeding tank holding the parents.
56. Place the plate with the embryos in an incubator at 28°C.
57. Set aside the breeding tank with the parents in a safe place in the fish facility.
58. Approximately 6 h after fertilization (at shield stage), collect 20 embryos from each clutch in a separate 1.5-mL tube (Fig. 3).

Grow the rest of the F_1 embryos to adulthood, because if the parent is carrying a germline mutation, these F_1 may carry the allele and can be genotyped as adults by fin clip and fragment analysis, to identify heterozygous fish and establish the mutant line.

DNA Extraction and PCR

59. Follow the steps explained above for DNA extraction and PCR (Steps 43–48).

A FAM/HEX-labeled primer is not required. It is important to use a DNA polymerase that introduces A-overhangs, because they will be used for ligating the adaptors. Alternatively, the PCR can be done with a proofreading polymerase and include an extra step of incubation with Taq polymerase to introduce the A-overhangs.

Sample Pooling

60. To reduce the number of samples to manipulate, pool the PCRs from clutches of different gene crosses to be sequenced using the same sequencing barcode.

As each PCR product will be specific for one specific genomic locus, it will be straightforward to identify the pair of origin even if they share the same sequencing barcode (Fig. 3).

Fish targeted in Gene A can be pooled with fish targeted in Gene B, C, ... Z, but other fish targeted in the same gene should never be pooled to avoid misidentification of the parent fish of origin (it will be impossible to assign the identified mutations in Gene A to the correct fish founder).

Phosphorylation

Perform Steps 61–65 on each PCR pool.

61. Purify the PCR using the QIAquick PCR Purification Kit as described above (Step 7.i–7.x).
62. Elute the PCR product as in Steps 7.xi–7.xii, but now use 45 μ L of water.
63. Add 5 μ L of 10 \times T4 DNA ligase buffer and 2 μ L of T4 PNK.
64. Incubate for 30 min at 37°C.
65. Heat-inactivate for 20 min at 60°C.

Barcoding

Each phosphorylated PCR pool (Steps 61–65) will be barcoded with Illumina barcodes.

Preparation of adapter

66. Mix:

- 12.5 μ L of Adapter_A (100 μ M)
- 12.5 μ L of Adapter_B (100 μ M)
- 25 μ L of 2 \times annealing buffer for barcoding

67. Heat for 1 min at 95°C and add to a floater in a beaker of boiling water. Let the water cool down to room temperature.

The final concentration of the mixture is 25 μ M.

Ligation of both adaptors to the PCR pool

68. After Step 67, add the following to the PCR pool from Steps 61–65:

- 0.5 μ L of 10 mM ATP
- 2 μ L adapter duplex (25 μ M stock from previous step)
- 2 μ L of T4 DNA ligase

69. Incubate 2 h at room temperature or overnight at 16°C.

70. Run a 2% agarose gel and cut out the ligated band (your PCR product +55 bp).

71. Slice the band from the gel and extract the DNA using the gel extraction kit (Step 30).

Final PCR to add barcodes

72. Perform PCR as described before (Steps 47–48) using Adapter_C and TruSeq barcodes. Use 2 μ L of the ligated PCR pools from the previous step (Step 71).

Setup three PCR reactions per sample at different cycles (start by testing 8, 10, and 12 cycles) to select the number of cycles that do not overamplify the sample.

73. Run a 2% agarose gel and cut out the desired band (your PCR product +128 bp)

74. Extract the DNA using the gel extraction protocol (Step 30).

75. Sequence in an Illumina Hi-Seq or Mi-Seq sequencer, paired end, with at least 75-nucleotide reads.

Sequence analysis

76. Install the Mut-Seq analysis pipeline:

- i. Download the mutseq program (<http://protocol.crisprscan.org>) on a machine with Python 3.2 (or more recent).

A convenient Python installer such as Anaconda (<http://continuum.io/downloads>) can be used.

- ii. Download the GMAP read aligner (<http://research-pub.gene.com/gmap/>) and install it.

77. Prepare Mut-Seq input:

- i. Organize sequencing FASTQ files by separating the FASTQ files for each sample (containing pooled PCRs) into folders (e.g., Pool1, Pool2).

- ii. Create a CSV (comma-separated value) file describing genes, oligo sequences to amplify them and in which sequencing pool they were added.

All sequences must be 5' to 3'. The first line of the final CSV must be a header line.

```
20.1% 350210 pair(s)
TTCAAGTCTAAATCTTTTTCTAGATTTAATTAGCTCAGAAAAATATATGACTTGTTTAATTTAGTATGATCATTACATAAATTATTTATTTGTAAGTGC
TTCAAGTCTAAATCTTTTTCTAGATTTAATTAGCTCAGAAAAATAT---GACTTGTTTAATTTAGTATGATCATTACATAAATTATTTATTTGTAAGTGC

6.2% 102102 pair(s)
TTCAAGTCTAAATCTTTTTCTAGATTTAATTAGCTCAGAAAAATATATGACTTGTTTAATTTAGTATGATCATTACATAAATTATTTATTTGTAAGTGC
TTCAAGTCTAAATCTTTTTCTAGATTTAATTAGCTCAGAAAAATATATA---CTTGTTTAATTTAGTATGATCATTACATAAATTATTTATTTGTAAGTGC
```

FIGURE 4. Mut-Seq program output for alleles of 4-nt deletion (top) and 3-nt deletion (bottom). In this example, the second allele is a 3-nt deletion, which results in an in-frame mutation inside a coding sequence. In this case, although highly frequent, fish bearing this allele have to be discarded. If targeting a noncoding region, fish bearing such an allele could be kept.

Example data:

Locus name	Pool (sequencing folder)	Forward oligo sequence	Reverse oligo sequence	Amplicon sequence
GeneA	Pool1,Pool2,Pool3	ACCA...	TGAC...	ACCA...GTCA
GeneB	Pool2,Pool3	TCCG...	ATGG...	TCCG...TACC

Corresponding CSV file:

```
"GeneA","Pool1,Pool2,Pool3","ACCA...","TGAC...","ACCA...GTCA"
"GeneB","Pool2,Pool3","TCCG...","ATGG...","TCCG...TACC"
```

78. Start the mutseq program. Specify the path to the CSV file created at the previous step using the `–path_loci` option and the path to the sequencing FASTQ files using the `–path_data` option.

The report is saved to the file specified using the `–report` option. For example:

Executing the mutseq program depends on installation. Please refer to <http://protocol.crisprscan.org> for details./mutseq.py-path_loci loci.csv-path_data /data/seq/flowcell1/Unaligned/Project_xx-report report.txt

All indels within the “Allele calling zone” (Fig. 3, lower right) are collapsed and counted with wild-type read-pairs. Allele frequency is estimated by the ratio of the number of collapsed read-pairs with same indels to the number of wild-type read-pairs.

79. Analyze the output of the mutseq program to select appropriate mutations.

For each locus, it will return the following:

- Number of mutant, wild-type, and aligned (i.e., aligned to the locus but neither mutant nor wild type, for example PCR dimers) read-pairs.
- Alleles sorted by frequency (see Fig. 4).

80. Proceed to fragment analysis to identify fish bearing the allele identified with Mut-Seq.

If the mutation is an insertion or deletion of over 10 nucleotides, the downstream analysis of the mutant line can be streamlined analyzing the genotyping PCRs by 4% agarose gel electrophoresis. This procedure will save the time and costs of fragment analysis. The only requirement is to design primers to generate PCR amplicons between 120 and 150 nucleotides long and perform the PCR reaction as described in Steps 47–48 (no need for FAM or HEX primer). In these conditions, a standard 4% agarose gel electrophoresis will have enough resolution to separate the mutant and wild-type bands.

RECIPES

Annealing Buffer for Barcoding (2×)

20 mM Tris-Cl, pH 7.8
100 mM NaCl
0.2 mM EDTA

Store for up to 1 yr at room temperature.

Blue Water

Methylene blue, 20 mL (0.1% [w/v] stock in H₂O)
Instant Ocean sea salt (Instant Ocean), 6 g
H₂O, 20 L



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