

Step-by-Step Protocol for CRISPR-Cas9 mediated genomic deletions in Zebrafish

Index

I.	Target Sites Selection	2
II.	Design sgRNA Oligos & Primers.....	2
III.	Anneal Oligos	3
IV.	RNA Transcription	4
V.	Microinjection of Zebrafish Embryos	5
VI.	DNA Extraction.....	6
VII.	PCR Amplification	7
VIII.	F0 Screening & Sequencing.....	7
IX.	F1 Generation, Genotyping and Confirmation	8

I. Target Sites Selection

1. Determine your region of interest within the zebrafish genome.
2. Source DNA sequence and design working template in SnapGene or preferred software. This should include a minimum of 1000bp before and after the genomic region to delete.

II. Design sgRNA Oligos & Primers

For each deletion, we recommend designing 4 sgRNAs, 2x in 5' and 2x in 3' of the genomic region of interest.

First define two sgRNAs in 5':

1. We recommend using the CRISPRScan website to identify suitable sgRNA oligos. (<https://www.crisprscan.org/>)
2. Select 'Submit a Sequence' and input the DNA sequence upstream of the 5' target region (~500-1000bp), including the first 50bp of the 5' sequence of the genomic region of interest.
3. Ensure species selected is 'Zebrafish – Danio rerio', effector protein selected is 'Cas9-NGG' and promoter used for synthesis is 'in vitro T7 promoter'.
4. Select 'Get sgRNAs' and CRISPRscan will list all possible CRISPR targets for your given sequence.

The screenshot shows the CRISPRscan website interface. At the top, there are navigation tabs: 'By Gene', 'Submit sequence', 'Browser tracks', 'Protocol', 'Citing', 'Help', and 'CRISPRscan'. The 'Submit sequence' tab is active. A text area contains a DNA sequence. Below it, there are dropdown menus for 'Species' (Zebrafish - Danio rerio), 'Effector protein' (Cas9 - NGG), and 'Promoter' (In vitro T7 promoter). A 'Get sgRNAs' button is highlighted. To the right of the button are 'OK' and 'Example' links. Below the input fields, a genomic map shows a scale from 28,234,600 to 28,236,000. Below the map, there are dropdowns for 'Genbank (SnapGene)' and 'All gRNAs', and an 'Export' button. The main content area displays a table of CRISPRscan results. The table has columns: 'CRISPRscan score', 'Locus', 'Target sequence', 'Off-targets' (CFD, All, Seed), and 'Site type'. The first row is highlighted in green. The 'Site type' section on the right shows the 'gRNA' sequence highlighted in red.

CRISPRscan score	Locus	Target sequence	Off-targets			Site type
			CFD	All	Seed	
57	21:28234703-28234725 (-)	GGAGGACGCTGCTCCATTACAGG	1.87	0	0	Site type GG18NGG Genome GGAGGACGCTGCTCCATTACAGG gRNA GGAGGACGCTGCTCCATTCA Oligo taatagcactactataGGAGGACGCTGCTCCATTAgtttagagctagaa Off-targets (top 30 shown out of 102)
60	21:28234704-28234726 (-)	TGGAGGACGCTGCTCCATTACAGG	1.59	0	0	
56	21:28234826-28234848 (+)	GCGATCAAACTCGGAAAGGTGG	3.04	0	0	
56	21:28234874-28234896 (+)	AGAGCTGAGAGACGACATCCGG	5.39	0	0	
73	21:28234893-28234915 (-)	GTATGTTGGCGGATCCCACCG	1.34	0	0	
58	21:28234904-28234926 (-)	GCTAGTGACTGTATGTTGCCGG	5.51	0	0	
69	21:28234938-28234960 (-)	GGAGGTCAAGTCTAAGAGAAGGG	6.89	0	0	
68	21:28235076-28235098 (-)	GCGCTTCGCCATCCACTGTGGG	4.35	0	0	
57	21:28235088-28235110 (+)	TGGCGAAGCGCAACTGAAGACGG	3.25	0	0	
65	21:28235232-28235254 (+)	AGGGTCCATAGCGAACCATTGG	0.81	0	0	

Note: The sgRNA candidates are chosen based on the sequence and the potential off-target binding. To help determine which sgRNA to pick, two types of scores are provided. The first, CRISPRscan score, predicts the *in vivo* activity of the sgRNA with a high score indicating a greater cutting efficiency. The second, CFD (Cutting Frequency Determination) score, predicts the potential off-target binding for each sgRNA, with the lowest score indicating least off target binding. For more information visit the help page on CRISPRscan website.

5. Select the most appropriate sgRNA based on the scores and genomic location. It is advised to design at least two pairs per target. The sgRNA sequence to insert into our template (see below) will be visible in the right-side bar (outlined in red).

Note: The sgRNA sequence may present a 1bp mismatch in the first two base when compared to its genomic target sequence. Be careful to select the sgRNA sequence with the mismatch and not the genomic region during the next step of the protocol.

6. Insert the selected 20bases sgRNA into the following sequence (or in **supplemental file S3**) to design the “top-oligo” to order (Use same orientation as presented in the website). Save the file with an appropriate oligos name and complete laboratory primer database.

TAATACGACTCACTATA_20bp_sgRNA_from_CRISPRscan_GTTTATAGAGCTAGAAATAGCAAG
TTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT

Final length = 117bp

T7 Promoter = TAATACGACTCACTATA

Constant Region = GTTTATAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT

Important Note: An annotated SnapGene template file containing the full length sgRNA oligo sequence to order is included as **Supplemental Figure 3**. Please simply insert the 20bp sgRNA sequence in the defined region for generating the final oligos to record and order.

7. Design the reverse complement named “bottom-oligo” and complete laboratory oligo database.
8. Order “top-” and “bottom-oligos”.
9. Once genomic regions and corresponding sgRNA have been selected, update the working DNA template by annotating the genomic regions targeted by the selected sgRNAs.
10. Repeat step 2-9 to further select 2x target/sgRNAs in the flanking 3’ region of your sequence of interest.
11. Once the 4x sgRNAs have been designed and mapped on your DNA sequence, design “screening” forward and reverse primers surrounding the desired deletion and located no less than 100-150bp from the nearest CRISPR site (For schematics see **figure 1**).

Note: Ideally, design primers that will lead to amplicons of 250-500bp when the desired deletion would occur.

III. Anneal Oligos

1. Reconstitute oligos to a concentration of 200uM with ddH₂O.
2. Anneal forward and reverse oligos by combining the following reagents:

Reagent	Final Concentration	Amount
Forward (5') Oligo (200 μM)	50 μM	5μL
Reverse (3') Oligo (200 μM)	50 μM	5μL
10 × NEBuffer2.1	1 x	2μL
ddH ₂ O	n/a	8μL
Total	n/a	20μL

3. In a thermocycler, heat for 5 min at 95°C without additional steps and let cool down for 1hour with the lid on.
4. Purify DNA template using Invitrogen PureLink Quick Gel Extraction and PCR Purification Kit (Cat #: K220001) following manufacturers’ procedures outlined below:
 - i. Add 4 volumes of Binding Buffer to 1 volume of PCR reaction (50–100μL) and mix well.
 - ii. Add sample in Binding Buffer, above, to the PureLink Spin Column in a Wash Tube.
 - iii. Centrifuge the PureLink Spin Column at room temperature at 10,000 × g for 1 minute. Discard the flow through and replace the PureLink Spin Column into the tube.

- iv. Add 650µL of Wash Buffer containing ethanol to the PureLink Spin Column.
- v. Centrifuge the PureLink Spin Column at room temperature at $10,000 \times g$ for 1 minute. Discard the flow-through from the Wash Tube and replace the PureLink Spin Column into the tube.
- vi. Centrifuge the PureLink Spin Column at maximum speed at room temperature for 2–3 minutes to remove any residual Wash Buffer.
- vii. Discard the Wash Tube and place the PureLink Spin Column in a clean 1.7-mL PureLink Elution Tube (supplied with the kit).
- viii. Add 15µL of ddH₂O to the centre of the PureLink Spin Column and incubate at room temperature for 1 minute.
- ix. Centrifuge the PureLink Spin Column at maximum speed for 1 minute. Remove and discard the PureLink Spin Column.

The elution tube contains the purified DNA product which can be stored at 4°C for immediate use or -20°C for long term.

IV. RNA Transcription

Critical: For RNA Synthesis, use molecular grade and nuclease-free reagents. We highly recommend to clean workbench and pipettes with RNase neutralising solution before commencing work.

1. Transcribe sgRNA using the Ambion MEGAscript T7 Transcription Kit (Cat #: AM1354) as follows:
 - i. Combine reagents below in RNase-free 0.2mL PCR tube at room temperature in the order shown. This amount is for a single reaction. Reactions may be scaled as required.

Reagent	Amount
10 x Reaction Buffer	0.5µL
ATP (75mM)	0.5µL
CTP (75mM)	0.5µL
GTP (75mM)	0.5µL
UTP (75mM)	0.5µL
Template DNA (~800ng)	<2µL
T7 Enzyme Mix	0.5µL
Water (Nuclease-free) to 5µL final volume.	
Total	5µL

- ii. Mix reagents briefly and incubate reaction at 37°C overnight.
 - iii. To remove the DNA template, add 1µL of TURBO DNase and 14µL of RNase/DNase-free H₂O. Incubate for a further 20 minutes at 37°C.
2. Purify sgRNA using Zymo Research RNA Clean & Concentrator-5 Kit (R1015) or similar. Manufacturers' procedures were followed with minor variations as outlined below:
 - i. Bring volume of sgRNA to a minimum of 50µL by adding RNase/DNase-free H₂O.
 - ii. Add 2 volumes of RNA Binding Buffer to sample and mix.
 - iii. Add an equal volume of ethanol (95-100%) and mix.
 - iv. Transfer the sample to the Zymo-Spin Column in a collection tube and centrifuge at 4000-5000 x g for 1 minute. Discard the flow-through.
 - v. Add 400µL of RNA Prep Buffer to the column and centrifuge at 4000-5000 x g for 1 minute. Discard the flow-through.

- vi. Add 700 μ L of RNA Wash Buffer to the column and centrifuge at 8000-10,000 x g for 1 minute. Discard the flow-through. Repeat wash step.
 - vii. Carefully transfer column into 1.7mL RNase-free tube and add 15 μ L of RNase/DNase-free H₂O. Let column sit for 2 minutes.
 - viii. Centrifuge at 4000-5000 x g for 1 minute and collect flow-through.
The eluted RNA can be used immediately or stored at -80°C.
3. Quantify concentration of sgRNAs using Nanodrop ND-1000 Spectrophotometer or similar. All samples should be measured in duplicate. Good quality RNA should generate a 260/280 ratio of ~2.0 and 260/230 ratio in the range of 2.0-2.3.
 4. Run a 2% Sodium Borate (SB) agarose gel at 200V for ~20 minutes to confirm integrity of sgRNAs.

V. Microinjection of Zebrafish Embryos

Injections are made using Cas9 protein from New England Biolabs (Cat#: M0646).

1. Separate female and male zebrafish in 1L breeding tanks, in a ratio of 2:3 respectively. Zebrafish should be separated in the afternoon the day before the injection with a minimum of 2 breeding tanks.
2. Prepare the mixture of sgRNAs and Cas9 protein (400pg) using the calculations provided in the table below or use the template provided in **Supplemental Table 3** to modify/do your own mix. Phenol red (0.5%) is used to visualise the injections and assess if the mixture ends in the yolk or in the cell. It is critical to always keep tubes on ice.

Template for preparing multiple guides injection mix

XX - Not to modify
XX - Modify as required

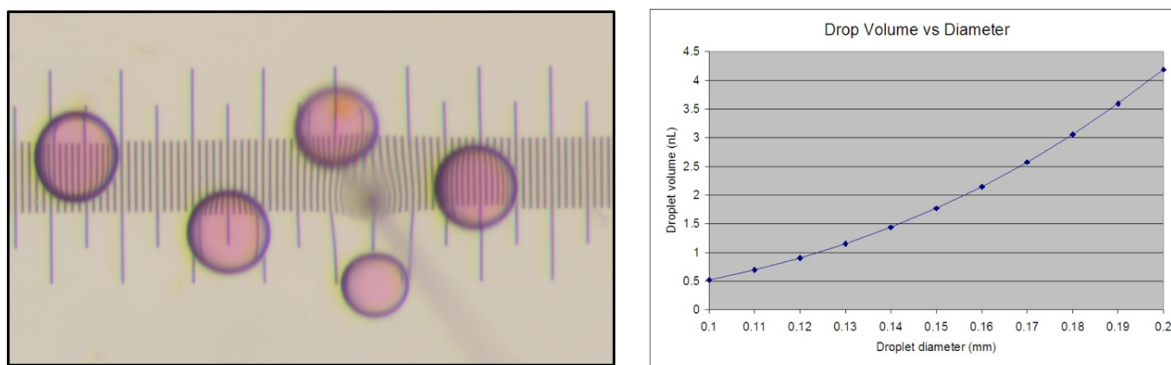
		μ M equivalent	
#number of guides per tube	5		
Total Volume required (ul)	10		
sgRNA length (bp)	117		
[conc] of sgRNA stocks (ng/ul)	1000	26.6	μ M
[conc] of NEB Cas9 stock (ng/ul=mg/ml)	3220	20	μ M
Cas9 protein conc wanted (ug/ml)	400	2.48	μ M
Ratio of Cas9	1		
Ratio of sgRNAs	4		
gRNA conc wanted - each guide (ng/ul)	74.62	1.984	μ M
Phenol red stock conc (%)	0.5		

ID	Mix	Vol each sgRNA	Vol Phenol Red	Vol Cas9	Buffer (x10)	vol H ₂ O
1	MIX_01 FOR 10 μ l	0.7	2	1.2	1	2.3

- Combine male and female fish (ideally within the first 2 hours after the lights turn on) and allow fish to begin mating. Monitor when fish start to lay eggs and collect fertilized eggs within 30 minutes.
- In the meantime, set-up microinjector with needle containing prepared sgRNAs/Cas9 mixture.

Note: To create needles for microinjection, glass capillaries (Harvard Apparatus Cat#: EC1-30-0038) are pulled using Sutter Instrument (Model P-97) Micropipette Puller.

- Calibrate injection volume to 1nL using a stereomicroscope and 0.01mm micrometre calibration ruler (ProSciTech Cat#: S81K). The diameter of the injected bolus must measure 0.12mm to ensure 1nL volume is injected (see example below).



- Inject mix into the yolk of embryos at one cell stage of development. We recommend injecting >200 embryos and keep some non-injected ones to evaluate the toxicity of the injection in case a repeat is required.
- Grow injected and non-injected embryos in E3 medium for 24 hours in incubator at 28°C.

VI. DNA Extraction

At 1 day post fertilisation (dpf), extract genomic DNA (gDNA) from individual and/or pooled injected embryos selected at random, with a minimum of 8 samples per injection condition. We also recommend extracting DNA from some un-injected embryos to use as DNA control.

Note: Depending on the size of the deletion, a wildtype amplicon may or may not be amplified in the PCR reaction. For large deletions (where no wt amplicon is present), we recommend to pool ~10 embryos together. On the other hand, for small deletions where a wt amplicon is present, we recommend assessing embryos individually as this may mask the signal from potential non-abundant deletion(s).

- Examine quality of embryos at 1dpf and discard any unfertilised eggs. Place 1-10 embryos in each tube of an 8-well PCR tube strip. Repeat for each injection condition.
- Prepare DNA Extraction Buffer by combining the following reagents:

Reagent	Amount
1M Potassium Chloride	500μL
0.5M Tris pH8.0	200μL
1M EDTA	10μL
20% IGEPAL	150μL
10% Tween-20	300μL
ddH2O to 10mL final volume.	
Total	10mL

- Thaw Proteinase K (10mg/mL) and add 1:200 dilution to DNA Extraction Buffer.

E.g., If using 2mL of Buffer, add 10μL of Proteinase K

4. Add 50μL of DNA Extraction Buffer containing Proteinase K to each sample tube.
5. Digest gDNA in Thermal Cycler at 55°C for 2 hours and 98°C for 10 mins.
6. The gDNA can be stored at 4°C for immediate use or -20°C for long term.

VII. PCR Amplification

Use the resulting DNA as a template for PCR amplification to confirm activity of injected sgRNAs.

1. Prepare PCR mix by combining the following reagents:
This amount is for a single reaction.

Reagent	Amount
H2O	18.375μL
Buffer 10x	2.5μL
MgCl ₂ (25mM)	1.5μL
dNTPs (10mM)	0.5μL
Forward Primer 10μM	0.5μL
Reverse Primer 10μM	0.5μL
Taq Polymerase (Amplitaq)	0.125μL
DNA	1μL
Total	25μL

2. Incubate samples in a Thermal Cycler using the amplification protocol:

Initial Denaturation	95°C	1:00
Denaturation	95°C	0:30
Annealing*	56°C	0:30
Extension	72°C	0:20
Repeat x 40 cycles		
Final Extension	72°C	1:00
	4°C	∞

*Annealing temperature should be optimised for each primer pair.

3. Run a 2% SB agarose gel at 200V for ~20 minutes.

Note: If a deletion is present, additional band(s) or smearing will be visible in comparison to the non-injected control (wt amplicon). If there is no deletion, only the wildtype amplicon should be present. **See schematics in Figure 1C.**

4. Once confirmed through PCR, grow remaining injected fish to adulthood (F0) to then be screened for germline transmission.

Note: If this validation fails to highlight/validate the cutting efficiency of the sgRNA, we recommend repeating with higher ratio Cas9/sgRNA (1:6) or ordering new guides.

VIII. F0 Screening & Sequencing

Out-cross individual adult F0 zebrafish to wild type adults and screen the resulting embryos to determine if the desired deletion has successfully been transmitted.

1. DNA extract embryos from each F0 out-cross as detailed above.
2. Amplify DNA using the same primers as the initial PCR screen and visualise on 2% SB agarose gel.
3. Identify F1 embryos carrying the desired deletion/mutation (presence of non-wild type band/s).
See schematics in Figure 1D.
4. Send positive amplicon for sequencing using the same forward and reverse primers. Gel extraction is recommended to avoid complex analysis of the sequencing results especially for samples with the presence of a wt band.
5. Analyze sequencing results either manually or with the aid of a web-based chromatograph reader such as Yost Tools or CRISP-ID to determine exact sequence of the deletion.
(<http://yosttools.genetics.utah.edu/PolyPeakParser/>; <http://crispid.gbiomed.kuleuven.be/>)
6. Once confirmed, out-cross these F0 adults to wt or line of interest and grow to generate heterozygotes.

IX. F1 Generation, Genotyping and Confirmation

Once F1 fish have reached adulthood, these must also be genotyped through fin clipping, DNA extraction and PCR.

For fin clipping:

1. Anaesthetise fish using 50% tricaine methanesulfonate solution in zebrafish housing system water with final concentration between 100-200 μ g/mL.
2. Once anaesthetised, move fish to petri dish without water.
3. Use a clean scalpel blade to cut a small portion (1-2mm²) of the tail fin and place in labelled 0.2mL PCR tube.
4. Place fish in separate tank with water from zebrafish housing system and monitor recovery.
5. Proceed with DNA Extraction and PCR as outlined previously. Once results are confirmed, fish with the desired mutation can be housed together and remaining fish culled.

Note: At this stage, it is recommended to reconfirm the deletion sequence via sequencing.