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A High-Throughput functional genomics workflow based on CRISPR/Cas9-mediated targeted mutagenesis in zebrafish

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Abstract

Zebrafish is a popular model organism for studying development and disease and genetically modified zebrafish provide an essential tool for functional genomic studies. Numerous publications have demonstrated the efficacy of gene targeting in zebrafish using CRISPR/Cas9, and have included descriptions of a variety of tools and methods for guide RNA synthesis and mutant identification. However, most published techniques are not readily scalable to increase throughput. We recently described a CRISPR/Cas9-based high-throughput mutagenesis and phenotyping pipeline in zebrafish. Here, we present a complete workflow for this pipeline, including: target selection; cloning-free single-guide RNA (sgRNA) synthesis; microinjection; validation of the target-specific activity of the sgRNAs; founder screening to identify germline transmitting mutations by fluorescent PCR; determination of the exact lesion by Sanger or next-generation sequencing (including software for analysis); and genotyping in the F₁ or subsequent generations. Using these methods, sgRNA's can be evaluated in 3 days, zebrafish transmitting germline mutations can be identified within 3 months and stable lines can be established within 6

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months. Realistically, two researchers can target tens to hundreds of genes per year using this protocol.

Introduction

Advances in sequencing technologies have enabled the rapid identification of candidate human disease genes by genome-wide analysis studies (GWAS)¹ or whole-exome sequencing techniques². However, there is a large gap between identification of these candidate disease genes and their functional validation. Because of its many unique characteristics-- such as rapid development, transparent embryos, and high fecundity, and a fully annotated genome³--zebrafish has become a popular vertebrate model organism for the functional validation of disease genes. 70% of human genes have at least one conserved ortholog in zebrafish, and most of these genes perform similar functions in the fish³. Until recently, functional genomics mostly relied on mutants generated using random mutagenesis approaches⁴⁻⁷. However, the adaptation of targeted genome-editing tools, such as zinc-finger nuclease (ZFNs)^{8,9}, transcription activator-like effector nucleases (TALENs)^{10,11} or CRISPR/Cas9¹²⁻¹⁷ nucleases, has proven to be very effective in zebrafish. While ZFNs and TALENs clearly work, low throughput and laborious assembly has limited their adoption in zebrafish. In contrast, the RNA-guided CRISPR/Cas9 system is technically simpler-- guide RNAs are easy to design for either single or multiple target loci^{12,16}--and has made high-throughput, targeted mutagenesis in zebrafish feasible.

All these targeted mutagenesis methods generate double stranded breaks at the target site that can either be repaired by error-prone Non-Homologous End Joining (NHEJ) or, if a donor DNA is supplied, Homology Directed Repair (HDR). While HDR tends to be accurate and is generally used for generating knock-in alleles, the error prone NHEJ repair method often leaves insertions or deletions upon repair, which is exploited to generate gene knockouts.

Development of the Protocol

The use of CRISPR/Cas9 for genome editing has been demonstrated in zebrafish by many researchers¹⁸. We generated a genetically defined zebrafish strain to simplify target site selection¹⁹, a rapid assay (CRISPR-STAT) for determining the somatic activity of sgRNA²⁰, and a public database to curate published sgRNA targets and activities²¹ in the belief that the transfer of sgRNA information is faster and cheaper than transferring actual mutant zebrafish. We used our high-throughput mutagenesis pipeline to generate the largest published germline transmission dataset in zebrafish¹⁷. Here, we provide a detailed workflow for generating gene knockout zebrafish lines using CRISPR/Cas9 that is readily amenable to scaling from a few to hundreds of mutants.

The CRISPR/Cas9 system uses a single-guide RNA (sgRNA) that contains an 18 to 20-nt target sequence and directs Cas9 to the target site. The target sites can be selected using any available CRISPR/Cas9 target design web tool. To facilitate targeting in our lab, we generated UCSC Genome browser tracks that display all possible *Streptococcus pyogenes* Cas9 (*SpCas9*) target sites throughout the zebrafish genome including the number of

predicted off-target sites for each guide¹⁷. These tracks are available as a public data hub titled “Zebrafishgenomics.” Another issue that affects targeting efficiency is that inbred strains of zebrafish are generally unhealthy, thus most of the “standard” strains used by researchers possess polymorphism rates of approximately 1 in every 100 bases²². These variations could impact the target binding specificity as even a single mismatch in the target site could negatively impact sgRNA activity²³.

To improve our target efficiency, it would be advantageous to use a strain where all variations were known. We therefore created NHGRI-1, a highly fecund strain in which nearly all variations in the founding parents were documented by high-throughput sequencing. We now use the variation data (and the fish themselves) to select the target sites that avoid polymorphisms, helping to ensure success on the first attempt¹⁹. The variation data is also included in the UCSC genome browser public hub together with the predicted CRISPR/Cas9 target site tracks allowing for integration of the data.

We did not have to alter the well-established techniques for mRNA injections in zebrafish embryos, but we did develop a Microsoft Excel based calculator, CRISPR-CALC to simplify the calculations for the amount of sgRNA and Cas9 mRNA to be injected²⁰.

Because target mutagenesis efficiency can vary so widely, it was essential for us to develop a rapid method for evaluating sgRNA targeting efficiency. There are several methods to evaluate the sgRNA activity, the most common being the PCR amplification of the target site followed by subcloning and sequencing. We developed a scalable, high-throughput, and high-resolution CRISPR Somatic Tissue Activity Test (CRISPR-STAT) to evaluate the target activity in injected embryos using fluorescent PCR²⁰. We collect 8 uninjected and 8 injected embryos 24-48 hrs after injection and run a “three-primer” fluorescent PCR using gene specific primer pairs and a fluorescently tagged primer. The resulting fluorescently tagged amplicons are then separated using capillary electrophoresis on a Genetic Analyzer 3130xl or 3730xl. We then compare the fragment size peak(s) of injected and uninjected embryos to detect the fold reduction of WT-sized alleles. We found evaluating the somatic activity of the injected target maximized the likelihood of obtaining a genetic mutant. All targets where somatic activity was detected transmitted mutations through the germline.

Once sgRNA efficiency has been determined, the final step we needed to develop was rapid and simple genotyping of the F₁ progeny. We developed a PCR-based protocol using the same techniques as CRISPR-STAT, that robustly and rapidly identified frame-shifting mutations, and could, in parallel, be sequenced using indexed samples and “next-generation” sequencing¹⁷.

Overview of the Procedure

The experimental workflow for zebrafish target design, sgRNA synthesis, microinjection of sgRNA/Cas9, target somatic activity validation, and screening of mutants in the germline is summarized in Figure 1 and discussed in following sections.

Target selection and sgRNA Synthesis—We recommend selecting a minimum of two target sites because our data show that approximately 85% of the targets are sufficiently

active to generate germline mutations¹⁷, two targets ensures success in almost all cases. We also prefer to target a known functional domain of the protein whenever possible, or early exons common to all known splice variants. To select sgRNA sequences, we use the UCSC browser and our predicted sgRNA targets for T7 or Sp6 synthesis, opting for guides that avoid polymorphisms and have as few predicted off-targets as possible (<500 preferred).

For sgRNA synthesis, we use a cloning free, oligo-based method (Figure 2a). This requires a target specific DNA oligo (Top stand oligo, 55-57nt) and a “generic” DNA oligo for the guide RNA (Bottom strand ultramer, 80-nt). The target-specific oligo contains a 17-nt long T7 or SP6 promoter, the 18-20-nt target sequence and finally 20-nt of complementary sequences to the guide RNA. The two oligos are annealed and extended with DNA polymerase and the resulting product serves as a template for in vitro transcription. This method can synthesize 96 sgRNAs in 3-4 hours.

Microinjection—The sgRNA and Cas9 mRNA (or protein) are co-injected in 1-cell stage embryos. We routinely co-inject 25-50pg sgRNA per target and 150-300pg Cas9 mRNA. Injection of 50pg sgRNA/150pg Cas9 or 25pg sgRNA/300pg Cas9 resulted in similar germline transmission efficiencies. To make this pipeline high-throughput, we injected sgRNA/Cas9 mRNA in the yolk, however, in order to observe phenotypes in injected embryos we recommend injecting directly into the cell, which typically results in an increase in mutagenic efficiency and thus increased chance of simultaneous mutation in both alleles. However, cell injections are not recommended if the goal is germline transmission, as targeting essential genes with high mutagenesis efficiencies might result in poor survival of the founder fish.

Screening and verification of germline mutations—After evaluating the sgRNA activity in somatic tissues, the remaining injected embryos are raised to adulthood. We typically screen 6-8 founder fish for germline mutations using our 3-primer fluorescent PCR method (Figure 2b). Each founder (F₀) is either outcrossed to a wild-type fish or inbred with another founder, then 7-8 embryos from each outcrossed pair or 16-24 embryos from each inbred pair are screened for mutations in the F₁ generation. We have previously demonstrated the use of fluorescent PCR in screening germline mutations in a high-throughput manner^{17,24}. NHEJ generates small indels that the fluorescent PCR can readily detect, allowing us to quickly decide which founders to keep and raise their mutant F₁ progeny. We typically raise F₁ embryos for 2-3 independent alleles that carry frameshift mutations. While fluorescent PCR is a robust method of screening mutants quickly, it does not give the exact nature of the lesions, the screened alleles must be sequenced to get the precise mutation data. In addition, sequencing also avoids the risk of wrongly assuming any naturally occurring indel variation in the target area to be an introduced mutation. Therefore, we developed a multiplexed barcoded next-gen sequencing pipeline that allows pooling amplicons from up to 96 different embryos from a single target¹⁷. This sequencing pipeline uses the same gene-specific primer set used in fluorescent PCR (Figure 2b) saving time and expense.

Use of fluorescent PCR for genotyping—The F₁ adults (and subsequent generations) are genotyped after fin amputation and performing the same “3-primer” fluorescent PCR method as described above.

Phenotyping is done in the F₂ generation by inbreeding two heterozygous F₁ adults and the phenotype-genotype correlation is established by genotyping again using fluorescent PCR and observing the presence or absence of the WT and mutant alleles. We also demonstrated that phenotyping can be done in the F₁ generation and the mutants (compound heterozygotes) can be phenotyped in a non-Mendelian, but robust manner¹⁷. Based on our data, the average rate of obtaining compound heterozygotes by inbreeding two injected founder fish is 11%, but when the founders are prescreened and only positive founders are inbred the average rate is $\approx 36\%$ ¹⁷.

Advantages and Limitations

The mutagenesis pipeline described here is inexpensive, high-throughput and 6-7 $\times$ more efficient than TALEN's or ZFN's in generating germline mutations. This pipeline has been used in generating mutants for more than 500 targets. The primary goal of this pipeline is to generate knockout mutations, however, this method could also be used to select efficient sgRNAs using CRISPR-STAT for targeted knock-in experiments.

As with all methods, our approach also has some limitations. The frequency of variants (~ 1 in 100) in the zebrafish genome means that fluorescent PCR used to rapidly identify mutations can generate false positive data. We therefore recommend sequence confirmation of all identified mutations to verify the precise nature of detected indels. Since not every lab has direct access to a capillary electrophoresis instrument, DNA samples can be submitted to a sequence analysis facility (commercial or in-house core) for fluorescent PCR analysis and Sanger sequencing. We also recommend using variant data from the zebrafish genome when designing primers to avoid known variants in the target amplicons.

Another limitation is the number of useful targets in the zebrafish genome. When using T7 or SP6 to synthesize the sgRNA, target sequences must start with GG (T7) or GA (SP6). Further, CRISPR/Cas9 requires that the target site precedes an NGG Protospacer Adjacent Motif (PAM) sequence (not included in the actual target sequence). The PAM sequence requirement can be relaxed by using alternative Cas9s from different bacteria with different PAM requirements, and the 5' GG or GA requirement can be addressed using different lengths of targets (19nt, 18nt)^{25,26}, chemically synthesizing the sgRNA, or using Csy4 to trim off leader sequences²⁷.

A generally-recognized limitation of CRISPR/Cas9-based genome editing is off-target cleavage. We previously detected a germline mutation in one off-target site out of 25 predicted off-target sites tested¹⁷. Off-target cleavage is generally not a significant concern in zebrafish as outcrossing animals typically segregate unlinked mutations. However, off-target cleavage can be reduced either by using a “nickase” version of Cas9 that requires two adjacent sgRNAs²⁸ (increasing specificity) or using a truncated guide RNA²⁹. We have generated a zebrafish codon optimized version of the Cas9 nickase (pT3TS-nls-zCas9-

nlsD10A) (addgene # 78634) plasmid, however, we have not systematically compared its on-target and off-target mutagenic activities to wild-type Cas9. Recently, two different versions of spCas9 were developed with reported higher specificity than the wild-type Cas9^{30,31}, we have generated zebrafish codon optimized versions of spCas9 VQR (addgene #78662) and spCas9 EQR (addgene#78663), but we have not yet tested their efficacy in zebrafish.

Alternative methods

In zebrafish two other targeted mutagenesis tools, ZFNs and TALENs, have been used extensively¹⁸. We compared the germline transmission efficiencies of ZFNs, TALENs and CRISPR/Cas9 in zebrafish. The average germline transmission efficiency for CRISPR/Cas9 was 28% using this protocol compared to ZFNs and TALENs with 5% and 4% respectively¹⁷. CRISPR/Cas9 is not only 6-7 times more effective but can also be used effectively in a high-throughput manner. The higher germline transmission efficiencies of CRISPR/Cas9 reduce the animal husbandry work, which not only increases the throughput but also saves time and makes it more affordable. Multiplexed CRISPR/Cas9 mutagenesis (i.e. targeting multiple loci simultaneously) can be achieved by adding additional sgRNAs to the injection mix. This is much more difficult with ZFNs or TALENs due to costs and the time associated with making them. In terms of mutagenic diversity, ZFNs generate deletions, insertions and complex mutations (i.e. a mixture of deletion and insertion), TALENs mostly generate deletions or complex mutations with fewer simple insertions. CRISPR/Cas9 generates deletions, insertions, and complex mutations similar to ZFNs^{17,32}.

Experimental Design

Choice of Cas9

There are multiple plasmids encoding Cas9 available, we have not tested all of them, but we recommend using the zebrafish codon optimized Cas9 by Jao et al¹⁶. The Cas9 protein available from the multiple commercial sources can also be used. We find the mutagenesis rates to be comparable with Cas9 mRNA or Cas9 protein. Moreno-Mateos et al. used a Cas9-nanos version targeting preferentially to the germ cells²⁶. While its activity is lower than WT Cas9, it is enough to generate germline mutations with a reasonable efficiency without harming the somatic tissue. We have not directly tested this option so we only present it as a possible approach.

Controls

We use sgRNA targeting the *tyrosinase* (*tyr*) gene as a positive control (Supplementary Table 1). The phenotype associated with the *tyr* knock-down is reduced pigmentation which can easily be observed at 48hpf. The *tyr* sgRNA can also be used to check the quality of Cas9 mRNA, we recommend testing every new batch of Cas9 mRNA with this target.

Materials

Reagents

- Zebrafish (*Danio rerio*): Wildtype (WT) or transgenic zebrafish can be obtained from the Zebrafish International Resource Center (ZIRC). CAUTION All zebrafish work was approved by National Human Genome Research Institute Animal Care and Use Committee at the National Institutes of Health (OLAW Assurance # A-4149-01)
- Custom-designed top strand Oligonucleotides (Integrated DNA Technologies), designed as described in Procedure step 6.
- Universal Bottom Strand Ultramer (5' – AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAC – 3') (Integrated DNA Technologies)
- Custom PCR Primers (Integrated DNA Technologies, Examples listed in Supplementary Table 1), designed as described in Procedure steps 7-8.
- pT3TS-nls-zCas9-nls plasmid¹⁶ (Addgene, cat no. 46757, <https://www.addgene.org/46757>)
- Qiagen Plasmid Maxi Kit (Qiagen, cat no. 12162)
- XbaI and associated buffers (NEB, cat no. R0145S)
- Nuclease free water (Quality Biological, cat no. 351-029-131)
- QIAquick PCR purification kit (Qiagen, cat no. 28104)
- MinElute Reaction Cleanup Kit (Qiagen, cat no. 28204)
- mMessage mMachine T3 Transcription Kit (Ambion, cat no. AM1348)
- RNA Clean and Concentrator-5 kit (Zymo, cat no. R1016) CRITICAL Other kits may not work as well due to the small size of the RNA being purified.
- Ethyl alcohol, Pure (EtOH) (Sigma, cat no. 459844)
- 10× TAE Buffer (Quality Biological, cat no. 351-009-131)
- UltraPure Agarose (Invitrogen, cat no. 16500)
- UltraPure Ethidium Bromide (Invitrogen, cat no. 15585-011) CAUTION Carcinogenic. Wear gloves when handling.
- 5× DNA Gel Loading Solution (Quality Biological, cat no. 351-028-661)
- GeneRuler 1kb DNA ladder (Thermo, cat no. SM0311)
- TE pH 8.0 (Quality Biological, cat no. 351-011-131)
- 10mM dNTP mix (Clontech, cat no. S1477)

- Phusion High Fidelity DNA Polymerase and associated 5×Buffer (NEB, cat no. M0530S)
- HiScribe T7 Quick High yield RNA synthesis Kit (NEB, cat no. E2050S)
- MAXIScript SP6 Transcription Kit (Thermo, cat no. AM1308)
- 3M Sodium Acetate pH5.2 (Quality Biological, cat no. 351-035-721)
- GeneRuler 50bp DNA ladder (Thermo, cat no. SM0371)
- Mineral oil (Sigma, cat no. M5904)
- Egg water (see REAGENT SETUP)
- Instant Ocean Aquarium Salt (Petco, cat no. 77763)
- Extraction Solution (Sigma, cat no. E7526)
- Tissue Preparation Solution (Sigma, cat no. T3073)
- Neutralization Solution B (Sigma, cat no. N3910)
- Sodium Hydroxide (NaOH) (Sigma, cat no. S5881)
- TRIS-HCl 1M pH 8.0 (Quality Biological, cat no. 351-007-101)
- AmpliTaq Gold DNA Polymerase (Life Technologies, cat no. 4311820)
- GeneAmp 10×PCR Buffer II &MgCl₂ (Life Technologies, cat no. 4379878)
- 100mM dNTP mix (25mM each) (Life Technologies, cat no. N8080261)
- GeneScan 400HD ROX dye Size Standard (Life Technologies, cat no. 402985)
- Hi-Di Formamide (Life Technologies, cat no. 4440753) CAUTION Carcinogenic and target organ and reproductive toxicity. Wear gloves when handling.
- M13-FAM Primer-/56-FAM/TGTAAAACGACGGCCAGT,100mM (Integrated DNA Technologies)
- POP-7 Polymer for 3130×l/3170×l Genetic Analyzers (Life Technologies, cat no. 4352759/4335615)
- Sequencing Buffer 10×(VWR, cat no. 97063-502) This is a cheaper alternative to the Life technology sequencer buffer.
- ExoSAP-IT PCR Product Cleanup (Affymetrix, cat no. 78201)
- BigDye Terminator v3.1 and associated buffers (Life Technologies, cat no. 4337455)
- DyeEx 96 kit (Qiagen, cat no. 63183)
- Ovation Ultraflow Library System (NuGEN, part 0329)

Equipment

- Microcentrifuge (Cole Palmer, cat no. EW-17415-15)

- Block heater at 37°C (Fisher Scientific, cat no. 05-400-200)
- NanoDrop Spectrophotometer 1000 (Thermo)
- Owl D2 Wide-Gel Electrophoresis System (Thermo cat no. D2)
- 96-well PCR plates (Thermo cat no. AB0600)
- PCR plate spinner (VWR, cat no. 89184-608)
- Veriti 96 well Thermal Cycler 0.2mL (Life technologies, cat no. 4479071)
- Microinjection apparatus (World Precision Instruments)
- Peak Glass Scale, calibrated lines on glass, 50mm (SPI Supplies, cat no. 9761-AB)
- Glass capillaries (World Precision Instruments cat no. TW100F-4)
- Needle puller (Koph Instruments, model 710)
- Incubator at 28.5°C (Torrey Pines Scientific cat no. IN30)
- ABI 3130xl or 3730xl Genetic Analyzer with a 36cm or 50cm array (Life Technologies) Set up a run module for CRISPR-STAT and Fragment Analysis based on the instrument and capillary size using the parameters in Supplementary Table 2.
- GeneMapper Software (Life Technologies, cat no. 4475073) Follow instructions in Box 1 to set up the Panel Manager and the Analysis Method Editor.
- MiSeq System (Illumina)
- AmpliconDIVider bioinformatics pipeline (see Equipment Setup).

Reagent Setup

Oligonucleotides (Top Strand and PCR Primers)—Resuspend the oligonucleotides to a final concentration of 100μM using TE pH 8.0. After resuspension they should be stored at -20°C indefinitely.

Bottom Strand Ultramer—Resuspend the oligo to a final concentration of 100μM using TE pH 8.0. After resuspension, store as 20μl aliquots at -80°C indefinitely. **CRITICAL** Do not freeze thaw the aliquots. Use freshly thawed aliquot each time and discard the unused part. Resuspension in water should be avoided due to a high degree of secondary structure and the potential for the 3' hairpin structure to be cleaved off, severely reducing efficiency of Cas9 activity.

pT3TS-nls-zCas9-nls Plasmid—The plasmid DNA is purified using the Qiagen Plasmid Maxi Kit. Store plasmid DNA at -20°C indefinitely.

NaOH solution—Dissolve 10g NaOH in 50 mL water to make a 5M stock solution. To make a 50mM working solution, mix 500μl 5M NaOH to 49.5mL water. Store at room temperature (25°C) for up to 6 months.

Egg water—Add 1.92g Instant Ocean to 32L RO water. Store at room temperature (25°C) for up to 6 months.

Fluorescent PCR Master mix—To prepare 50mL, combine the following: 7.94mL 10× PCR Buffer II, 7.94mL 25mM MgCl₂ Solution, 795μl 100mM dNTP blend (25mM each), 635μl AmpliTaq Gold, 32.69mL Nuclease free water. Aliquot the Fluorescent PCR Master mix into ~40 aliquots of 100μl (for PCR of up-to 16 samples) and ~45 aliquots of 1mL (for 96-well plate) aliquots and store at -20°C indefinitely.

Equipment Setup

Amplicon DIVider bioinformatic pipeline requirements—The following programs must be in a directory in the \$PATH, with the version at least as recent as those tabulated below. ampliconDIVider is developed to be run from the Linux command line, it may also be run on OS X. ampliconDIVider can run within a Sun Grid Engine environment, but it is not required that it do so.

Program	Version	Source
Perl	v.5.8.8	https://www.perl.org/get.html
Cutadapt	v.1.3	https://github.com/marcelm/cutadapt/
Novoalign	v.3.02.07	http://www.novocraft.com/support/download/
Bam2mpg and mpg2vcf.pl		http://research.nhgri.nih.gov/software/bam2mpg/ Follow the steps in file 'INSTALL' to install it.
Sam2pairwise	v.1.0.0	http://www.github.com/mlafave/sam2pairwise
Samtools	v.0.1.19-44428cd	http://www.htslib.org/
Bamtools	v.2.3.0	https://github.com/pezmaster31/bamtools
Bgzip		http://www.htslib.org/download/

Procedure

Design and selection of CRISPR/Cas9 targets and fluorescent PCR primers

Timing 1 hour – 1 day

1. Access the UCSC genome browser tracks we created by going to <http://genome.ucsc.edu/cgi-bin/hgHubConnect>. CRISPR/Cas9 targets can be designed using any of the target design tools such as CRISPRSCAN²⁶, a comprehensive list of sgRNA design tools is listed in Varshney et al¹⁸.
2. Select “ZebrafishGenomics” track and click “connect” and then select a genome assembly (e.g. danRer10 or danRer7, danRer10 recommended).
3. The ZebrafishGenomics track will be added, Select one of the following three options: All targets (all targets), GA targets (N18-20) (CRISPR/Cas9 targets that start with GA for use with an SP6 promoter), and GG targets (N18-20) (CRISPR/Cas9 targets that start with GG for use with a T7 promoter) (Supplementary

Figure 1). We recommend selecting the GG targets if possible, since T7 polymerase is more efficient for RNA synthesis than Sp6.

4. Search for your gene, and select a target in the region of interest.
5. Click on the target to obtain the target sequence. The targets are color-coded, the darker the color, the lower the score (i.e. fewer predicted off targets). A target in the region of interest with the lowest score is preferred. **CRITICAL STEP:** It is best to select a minimum of 2 targets since only 85% of sgRNAs are active enough for significant germline transmission¹⁷.
6. Obtain the selected target sequence; add the T7 promoter (for GG targets) or SP6 promoter (for GA targets) sequence 5' to target sequence, and a 20-nt overlapping sequence at the 3' end. **CRITICAL STEP:** Do not include the PAM sequence in the target sequence of the oligo.

T7 Promoter (17nt):	TAATACGACTCACTATA
SP6 Promoter (17nt):	ATTAGGTGACACTATA
Overlapping gRNA sequence:	GTTTGTAGAGCTAGAAATAGC

The final assembled top strand oligo sequence will be as follows:

(T7)
 TAATACGACTCACTATAggnnnnnnnnnnnnnnnnnnnnnGTTTGTAGAGCTA
 GAAATAGC

(Sp6)
 ATTTAGGTGACACTATAgannnnnnnnnnnnnnnnnnnnnnGTTTGTAGAGCTA
 GAAATAGC

Order these oligos either as 10 mM scale (desalted) or 500-picomole scale (available in 96-well plate format).

7. Design primers to amplify a 200-325bp product surrounding the targeted region of the gene with the CRISPR/Cas9 target roughly in the middle of the amplicon. (Figure 2b) **CRITICAL STEP:** If possible design primers in an exon sequence. If intronic sequence must be used avoid repetitive sequences and stretches of the same nucleotide in order to avoid off-target priming or multiple target artifacts. It is very important that the WT PCR products give a single peak when performing fluorescent PCR to avoid false positives.
8. Add the M13F sequence (5'-TGTAACACGACGGCCAGT-3') to the 5' side of the forward primer and a PIG-tail sequence (5'-GTGTCTT-3') to the 5' side of the reverse primer. (Figure 2b)

Primers will be as follows:

Forward: 5' – TGTAACACGACGGCCAGT (n)₁₈₋₂₂ – 3'

Reverse: 5' – GTGTCTT (n)₁₈₋₂₂ – 3'

The PIG-tail sequence was first described by Brownstein et al.³³ for accurate genotyping of human DNA samples using short tandem repeat mapping and adopted by Sood et al.²⁴ to the genotyping of indels generated by targeting nucleases in zebrafish.

9 Order the top strand oligos, fluorescent PCR primers, M13FAM primer and universal bottom strand Ultramer.

In vitro transcription and purification of Cas9 mRNA

Timing 1 day

Hint: Steps 10-19 and 20-27 can be carried out in parallel.

10 Digest 5µg of circular pT3TS-nls-zCas9-nls plasmid DNA with XbaI in 50 µl of total volume at 37°C for 2 hours.

11 Purify the digested DNA with the QIAquick PCR purification kit following the manufacturer's protocol and verify digestion on a 1% (wt/vol) agarose gel in TAE buffer using standard protocols. A successful digest should produce a single 7.3kb band (Figure 3a, lane 2).

12 Quantify the digested DNA using a Nanodrop spectrophotometer. The final concentration should be > 150ng/µl with an A_{260/280} ratio range of 1.7-1.9.

TROUBLESHOOTING

13 Set up the in vitro transcription reaction as follows using the mMessage mMachine T3 Transcription Kit:

Component	Amount (µl)	Final concentration
2× NTP/CAP	10	1×
10× Reaction Buffer	2	1×
Linearized DNA template (from Step 11) 1µg		
Enzyme Mix	2	
Nuclease free water	variable	
Final	20	

14 Incubate reaction at 37°C for 2 hours.

15 Add 1µl TURBO DNase (included in the mMessage mMachine T3 Transcription kit) and mix well.

16 Incubate reaction at 37°C for 15 minutes.

17 Purify the RNA transcription reaction using the RNA Clean and Concentrator-5 kit following the manufacturer's instructions with an elution volume of 20µl nuclease free water.

18 Quantitate RNA using a Nanodrop. The concentration should be above 500ng/µl with a A_{260/280} ratio range of 2.0-2.3.

TROUBLESHOOTING

19 Run ~500ng purified RNA on a 1% (wt/vol) agarose gel in TAE buffer using standard protocols. A successful transcription should produce a band running at approximately 1.6kb (Figure 3a, lane 3). Aliquot *Cas9* mRNA into aliquots of 5µl with a concentration >500ng/µl.

TROUBLESHOOTING

PAUSE POINT *Cas9* mRNA aliquots can be stored at -80°C for months. Freezing and thawing is not recommended, therefore, thaw required number of aliquots for each use.

Synthesis of guide RNA using cloning-free methods

Timing 4-5 hours

20 Setup the oligo annealing reactions using option A for single sgRNA synthesis or option B for multiplex sgRNA synthesis. Top strand refers to the oligo that contains the target site. Bottom strand refers to the universal Ultramer that will add the crRNA-tracrRNA sequences that Cas9 will recognize.

A. Single guide synthesis

- i. Dilute 100µM stock solutions of top and bottom-strand oligonucleotides to a final concentration of 10µM using nuclease free water.
- ii. Setup the oligo assembly reaction as follows:

Component	Amount (µl)	Final concentration
5× Phusion Buffer	10	1×
10mM dNTP (2.5mM each)	1	0.2µM
Top Strand Oligo Mix (10µM)	1	0.2µM
Bottom Strand Ultramer (10µM)	1	0.2µM
Phusion DNA Polymerase	0.5	
Nuclease free water	36.5	
Final	50	

B. Multiplex synthesis

- i. Mix equimolar concentration (10µM each) of different top oligos (up to 12).
- ii. Set up the oligo assembly reaction as follows:

Component	Amount (µl)	Final concentration
5× Phusion Buffer	10	1×
10mM dNTP (2.5mM each)	1	0.2µM
Top Strand Oligo (10µM)	1	0.2µM
Bottom Strand Ultramer (10µM)	1	0.2µM
Phusion DNA Polymerase	0.5	
Nuclease free water	36.5	
Final	50	

21 Anneal and extend the oligos in a thermocycler using the following conditions:

Cycle Number	Condition
1	98°C, 2 min
2	50°C, 10 min
3	72°C, 10 min
4	4°C, hold

22 Run 3µl of the oligo assembly reaction along with 1Kb Plus DNA Ladder on a 2.5% (wt/vol) agarose gel in TBE buffer using standard protocols. The expected size of a correctly assembled oligo is 117-120bp (Figure 3b, lanes 1 and 2).

TROUBLESHOOTING

23 Following gel verification of the correct size product set up the sgRNA synthesis reaction using the NEB T7 High yield RNA synthesis kit (option A) or MAXIScript SP6 Transcription Kit (option B) depending on the promoter sequence in the assembled oligo.

A. NEB T7 High yield RNA synthesis kit

i. Set up the T7 reaction mix as tabulated below.

Component	Amount (µl)	Final concentration
NTP Buffer Mix	5	1×
Assembled oligo (Step 20)	4	unknown
T7 RNA polymerase	1	
Final	10	

B. MAXIScript SP6 Transcription Kit

i. Set up the SP6 reaction mix as tabulated below.

Component	Amount (µl)	Final concentration
Nuclease Free Water	4	
10× Transcription Buffer	1	1×
Assembled oligo (Step 20)	2	unknown
10mM ATP	0.5	
10mM CTP	0.5	
10mM GTP	0.5	
10mM UTP	0.5	
SP6 Enzyme Mix	1	
Final	10	

24 Incubate the reaction in a thermocycler as follows:

Cycle Number	Condition
1	37°C, 4 hours
2	4°C, hold

25 Add 1µl DNase (included in both the T7 High yield RNA synthesis kit and the MAXIScript SP6 Transcription Kit) to the reaction and incubate at 37°C for 20 minutes.

26 Purify the RNA synthesis reaction using the RNA Clean and Concentrator-5 kit following the manufacturer's instructions, and elute with 20µl of nuclease free water. The concentration should be > 100ng/µl with a A260/280 ratio range of 2.0-2.3. Alternatively, use 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol to precipitate the sgRNA. Both methods, i.e. ethanol precipitation or purification using the kit work well, the ethanol precipitation being the cost-effective approach.

TROUBLESHOOTING

27 Run ~500ng on a 2% (wt/vol) agarose gel in TAE buffer using standard protocols. Two bands of approximately 100bp and 200bp size indicate successful sgRNA synthesis. The 200bp band is due to the secondary structure of the sgRNA (Figure 3c, lanes 1 and 2)

TROUBLESHOOTING

PAUSE POINT sgRNA can be stored at -80°C for several months.

Microinjection workflow

Timing 2 hours

28 Setup matings of Zebrafish the night before scheduled injections.

29 On the morning of injections set up the injection apparatus. Prepare injection solutions on ice as follows or use the CRISPR-Calculator. http://research.nhgri.nih.gov/CRISPRz/downloads/CRISPR_CALC.xlsx

Component	Amount (µl)	Final concentration
SgRNA	variable	17.7ng/µl (for 25pg injections) or 35.4 ng/µl (for 50pg injections)
<i>Cas9</i> Mrna	variable	212.2 ng/µl
Nuclease free water	variable	
Final	10	

CRITICAL STEP Each embryo should be injected with 50pg sgRNA and 300pg *Cas9*mRNA for single targets and 25pg sgRNA and 300pg *Cas9*mRNA for multiplex targeting. To avoid toxicity, the total amount of mRNA injected/embryo should not exceed 500pg.

30 Load the mixed RNA solution into a capillary injection needle and inject 1.4nL per embryo (Figure 4) into ~100 embryos. Injections into the yolk are faster and usually sufficient for generating a mutant, however, we have observed that injecting into cell results in higher mutagenic activity (Figure 5a-f). Cell injections are also recommended if it is desirable to observe the phenotype in the injected embryos.

31 Save uninjected embryos from the same clutch as controls and place embryos into a 28.5°C incubator.

32 At 3-6 hpf, remove the unfertilized and injection-damaged embryos. Place embryos back into the 28.5°C incubator.

33 At 24hpf count normal looking, deformed, and dead embryos in all injection groups as well as control group to assess toxicity of the injections. For optimal results, >50% of the injected embryos should show normal development similar to uninjected embryos. Remove dead and deformed embryos and replace water with fresh egg water. Either proceed to step 34 or place embryos back into the 28.5°C incubator overnight. CRISPR-STAT can be performed on 24 hpf embryos, but we prefer to wait until 48 hpf since extra tissue results in more consistent DNA extraction.

TROUBLESHOOTING

Determining the somatic activity of injected targets (CRISPR-STAT)

Timing 1-2 days

34 At 24-48hpf, euthanize 8 embryos from each injection group and 8 uninjected wildtype embryos. Remaining embryos should be kept in 28.5°C incubator.

35 Using a 96 well plate place 1 embryo per well, using 1 column for each injection group.

36 Extract genomic DNA from the embryos as described in Box 2. **CRITICAL STEP** if embryos are still within chorions place the plate into a -20°C freezer until samples are completely frozen (~30 min). Allow them to thaw at room temperature (RT) and proceed with genomic DNA extraction. This freeze thaw cycle will burst the chorions, allowing the solutions access to the embryo.

37 Prepare primer mix as follows:

Component	Amount (μl)	Final concentration
TE pH 8.0	485	
Forward M13F tailed primer (100μM)	5	1μM
Reverse PIG-tailed primer (100μM)	5	1μM
M13-FAM primer (100μM)	5	1μM
Final	500	

38 Add 6μl of primer mix to a 100μl aliquot of PCR master mix. Note: The volumes given here are for 16 samples/primer set and can be adjusted depending upon the number of samples being processed. Typically we analyze 16 samples per target (8 control and 8 injected) allowing us to analyze 6 targets on a 96 well plate. **CRITICAL STEP:** Before detecting CRISPR/Cas9 activity we recommend that each primer pair be tested with 8 WT control samples to ensure no endogenous in/del polymorphisms are present in the amplicon.

39 Using a fresh 96 well plate add 5μl of the PCR Master mix/primer mix to each well.

40 Add 1.5µl of diluted DNA (Box 2) to the 5µl of PCR Master mix/primer mix. Vortex plate and briefly spin down the plate. **CRITICAL STEP:** We use 5 µl mastermix to keep the cost low, but the reaction volume may be increased to 10 or 20 µl.

41 Run the fluorescent PCR using the following conditions:

Cycle Number	Denature	Anneal	Extend
1	94°C, 12 min		
2-41	94°C, 30 sec	57°C, 30 sec	72°C, 30 sec
42			72°C, 10 min
43			4°C, hold

PAUSE POINT PCR products can be frozen at -20°C for up to a week.

42 Make 1mL of a 1:50 dilution of GeneScan 400HD ROX™ using Hi-Di™ Formamide.

43 Add 10ul of the ROX/Hi-Di mixture to a 96 well plate that will fit onto an ABI 3130xl or 3730xl Genetic Analyzer.

44 Add 2.5µl of fluorescent PCR product (Step 41) to the plate containing the ROX/Hi-Di mixture.

45 Heat the plate at 95°C for 5 minutes using a thermocycler.

46 Load plate onto an ABI 3130xl or 3730xl Genetic Analyzer and run using the CRISPR-STAT module parameters.

47 After the run is complete analyze data using the GeneMapper Software Package to determine the CRISPR/Cas9 mutagenesis activity. (Figure 5g-i)

TROUBLESHOOTING

48 Grow injection groups where embryos display high or low activity (from here on referred to as F₀) for founder screening. Growing the embryos with high activity will make recovering germline mutations fairly easy by screening <10 F₀ fish. Recovering germline mutations from low activity targets may require screening of additional F₀ fish.

Screening for germline transmitting founders by fluorescent PCR

Timing 3-4 days

49 Perform pairwise crossings with F₀ fish and WT fish. Collect embryos and incubate at 28.5°C for 2 days.

50 At 2dpf collect 7-8 embryos from each founder, placing 1 embryo per well in a 96 well plate. Use 1 empty well each for a positive control (WT DNA) and a negative control (No DNA). This allows 12 F₀ fish to be screened per plate.

51 Extract genomic DNA as described in Box 2.

52 Add 60µl of primer mix (Step 37) to a 1ml aliquot of PCR Master mix. Note:

The volumes given here are for 96 samples and can be adjusted depending upon the number of samples being processed.

53 Setup the reaction and run fluorescent PCR and capillary electrophoresis as described in Steps 39-45.

54 Load plate onto an ABI 3130xl or 3730xl Genetic Analyzer and run using the Fragment Analysis module parameters.

55 After the run is complete analyze data using GeneMapper Software Package to determine CRISPR/Cas9 induced mutations in the progeny of each F₀ fish. Each sample should give a peak of the expected size (WT peak). Some or all samples may give a second peak, indicating presence of a mutant allele. The difference in size between the WT peak and the mutant peak indicates the size of the insertion (larger than the WT peak) or deletion (smaller than the WT peak) generated by the CRISPR/Cas9. (Figure 6) TROUBLESHOOTING

56 Choose F1 embryos with frameshift mutations (not multiples of 3) to establish knockout mutant lines. Ideally establish two lines by growing F1 embryos from 2 independent founders.

CRITICAL STEP Perform sequence validation of the chosen mutant alleles, as it is possible to have the same size mutation by fluorescent PCR but a different mutation at the nucleotide level. This can be done by Sanger sequencing or multiplex sequencing as described in step 65.

Identification of heterozygote adults from the F₁ generation and genotyping of subsequent generations by fluorescent PCR

Timing 1-2 days

57 Perform Fin Amputations (Figure 7) using the Animal Care and Use Committee approved protocol and put each sample into a well of a 96 well plate. We recommend up to 94 fin amputations/plate leaving wells for a positive (WT DNA) and negative (no DNA) control.

58 Extract genomic DNA from the fin tissue as described in Box 2.

59 Add 60µl of primer mix (Step 37) to a 1mL aliquot of PCR Master mix.

60 Using a fresh 96 well plate add 10µl of the PCR Master mix / primer mix to each well.

61 Add 3µl of diluted DNA to each well. Vortex plate and briefly spin down the plate.

62 Run fluorescent PCR and capillary electrophoresis as described in Steps 41-45. Store the remaining PCR reaction in -20°C for follow up sequencing.

63 Load plate onto an ABI 3130xl or 3730xl Genetic Analyzer and run using the Fragment Analysis module parameters.

64 After the run is complete analyze data using the GeneMapper Software Package to determine the mutation in each fish (Figure 6). The difference in alternate allele from the WT allele is the size of the insertion or deletion.

Sequencing of targeted loci

Timing 1-2 days

65 Sequence verify mutations using Sanger sequencing (option A) or barcoded multiplex sequencing (option B). Sanger sequencing (option A) is the preferred method for low throughput applications while barcoded multiplex sequencing (option B) is preferred for high throughput applications.

A. Sanger Sequencing of target loci

- i. Select the samples that need to be sequenced and transfer 4µl of the PCR reaction (Step 62) into a new 96-well plate. Include at least one sample with WT peak alone as a control.
- ii. Add 1µl ExoSAP-IT and 1µl nuclease free water to each reaction.
- iii. Using a thermocycler run the following:

Cycle Number	Condition
1	37°C, 15 minutes
2	80°C, 15 minutes
3	4°C, hold

- iv. Set up the Big Dye Terminator Reaction as follows:

Component	Amount (µl)	Final concentration
PCR/ ExoSAP-IT Reaction	4	
Sequencing primer (5µM)	1	0.5µM
BigDye Sequencing Buffer (5×)	2	1×
BigDye Terminator v3.1	1	
Final	10	

- v. Run the Big Dye Terminator reaction as follows:

Cycle Number	Denature	Anneal	Extend
1	96°C, 1 min		
2-27	96°C, 10 sec	50°C, 5 sec	60°C, 4 min
28		4°C, hold	

- vi. Clean up reactions using Qiagen DyeEx plates according to the manufacturer's instructions and run purified samples on an ABI 3130xl or 3730xl Genetic Analyzer using standard sequencing programs.
 - vii. Analyze the heterozygous sequence (Figure 8) by either reading the sequence manually or use a program such as PolyPeakParser³⁴.
- TROUBLESHOOTING**

B. Barcoded multiplex sequencing of targeted loci

- i. Amplify the target sites using the same gene-specific primer set in combination with a third M13-barcoded primer (Supplementary Table 3). Each sample should receive a unique barcode if the amplicons are from the same target but different embryos.

Component	Amount (μl)	Final concentration
TE pH 8.0	489	
Forward M13F tailed primer (100μM)	1	0.2μM
Reverse PIG-tailed primer (100μM)	5	1μM
M13-barcoded primer (100μM)	5	1μM
Final	500	

- ii. Setup PCR reaction as described in steps 38-40 using barcoded primer mix from step 65, option B, step i.

- iii. Run the PCR using the following conditions:

Cycle Number	Denature	Anneal	Extend
1	94°C, 12 min		
2-36	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec
37			72°C, 10 min
38			4°C, hold

- iv. Run 2-3 μl PCR amplicons on 2 % (wt/vol) agarose gel. Check the amplicon size with the predicted amplicon's size of each target.
- v. Take 3μl aliquots from each PCR reaction and pool them together. Purify using MinElute reaction cleanup kit following the manufacturer's instructions.
- vi. Use 100 ng purified pooled amplicons for Illumina MiSeq library preparation, with the Ovation Ultraflow Library System (or using the local core facility's preferred method). 300 base read pairs are generated on a MiSeq sequencer, shorter reads can also be used depending on the size of amplicons (see step 68-72 for input files and BOX 3 for output files).

Bioinformatics pipeline for barcoded multiplex sequencing data (ampliconDIVider)

Timing 1-2 days

66 Download the latest release of ampliconDIVider from <https://research.nhgri.nih.gov/software/amplicondivider>.

67 Ensure that all dependencies listed in the documentation are installed, and are within a directory in the \$PATH shell variable. Tip: The bam2mpg package also may require that the \$PERL5LIB variable be set to the appropriate lib/directory and exported.

68 Create input files: Create the barcode file (to be submitted with the ‘-b’ option). This is a six-column, tab-delimited file with the following format:

```
plate01 A01  cx35.4_T1_25_17051437-17051625  1M  1  AAAAAA
plate02 A01  cx35.4_T2_25_17051701-17051898  1M  1  AAAAAA
plate03 A01  cx35.4_T2_4_5106979-5107305      1M  1  AAAAAA
```

The first column indicates the name of the plate that held the sample, and the second column is the well ID within that plate. The third column serves as the ID of the target amplicon, and indicates both the amplicon name and its position. Underscores should be used instead of spaces, and coordinates should be represented as 1-based values (i.e., as if the first base of the chromosome were called base 1). Colons must not be used in this column. The fourth column is the ID of the founder individual (i.e., a means of identifying the sample/organism in which the nuclease in question initially cut). The fifth column is the ID of the progeny of the founder listed in the fourth column. If founder 1F had three progeny, different samples could have a 1, 2, or 3 in this field. It is not required that integers be used. The sixth field should contain the sequences of the barcode used for each sample, written 5′ to 3′.

69 Create an amplicon reference FASTA file based on the known genomic sequence of the target between your primer set (to be submitted with the ‘-f’ option). The name of each target sequence should match the corresponding entry in the third column of the barcode file, as described above (note that the FASTA name entry must begin with a > character, however). Avoid entering sequences that are nested within other entries in the same file, as this will cause certain valid alignments to be discarded. If your experiment requires you to look at nested entries, create and use the optional ‘-p’ file, as described in step 72

70 Create a novoalign index from the FASTA file (to be submitted with the ‘-x’ option). This can be done using the novoindex program that can be downloaded along with novoalign: novoindex desired_index_name.nix ~/path/to/amplicon_fasta.fa

Other gap-aware aligners like BWA or Bowtie2 are also options, but one need to modify the file ‘sh/novoalign.sh’, and create appropriate index from the FASTA file.

Timing: less than 1 min for small files (~1Mbp sequences), ~1 hour for a 2Gbp genome

71 (Optional) Create the ranges file (to be submitted with the ‘-r’ option). This is useful if one is only interested in deletion and insertion variants (DIV) that appear within a certain window of the amplicon sequence. The file should have three columns. The first column is the ID of the amplicon, and must match the third column in the barcode file. The second and third columns are the begin and end of the window of interest, respectively, and should both be 1-based coordinates. Here is some example lines:

```
cx35.4_T1_25_17051437-17051625 121 180
cx35.4_T2_25_17051701-17051898 67 126
cx35.4_T2_4_5106979-5107305 117 176
```

The file should have three columns. The first column is the ID of the amplicon, and must match the third column in the barcode file. The second and third columns are the beginning and end of the window of interest, respectively, and should both be 1-based coordinates.

72 (Optional) Create the file indicating the primers of long fragments (to be submitted with the ‘-p’ option). This is useful if one intends to look for large DIVs that occur between two of the amplicons described in the FASTA file from step 69

```
cx35.4_T1F/T2R_25:17051437-17051898 GTTTCAGTGGATTGGCTGGT
GCAGTGCTCTGATCTCCACA
```

```
man2a1_T2F/T1R_5:58517329-58543258
AGCTCCTACTGTGTTTGACTGC TGCATGCAGTTTCATGTTGA
```

```
man2b1_T2F/T1R_11:32098232-32100164
CTCAAGAAAATGCAGGTTTGC ATCCAGCATGCAGGTGTTTC
```

The first column is the amplicon ID, but it is not required to match the formatting used in the barcode file from step 68. Note the colons are permissible. The second and third column are the sequences of the primers used to amplify the long amplicons. Both are written 5’ to 3’.

73 Run the program within the ampliconDIVider/directory. Doing so will create a working directory in which the intermediate files will be kept; upon completion of the program, an output directory will be created to hold the final results. When running the program, it is recommended that the ‘-l’ and ‘-n’ options be used. ‘-l’ automatically calculates the read length and mean & standard deviation of the fragment length, and ‘-n’ allows you to specify a name for the working directory. The example BAM included in the repository can be used to test the script:

```
./ampliconDIVider_driver.sh -l -b Varshney_et_al_input/set1_barcodes -n
example -p
Varshney_et_al_input/set1_long_fragments -r Varshney_et_al_input/
set1_ranges -f
Varshney_et_al_input/set1_amplicons.fa -x ~/path/to/set1_amplicons.nix
example.bam
```

When running the program with your own data, replace these files with your own.

Timing: ~1 day for ~13M short read pairs.

74 Interpret the output using the information in Box 3.

Timing

Steps 1-9, Designing and selection of CRISPR/Cas9 targets and fluorescent PCR primers: 1 hour – 1 day

Steps 10-19, In vitro transcription and purification of *Cas9* mRNA: 1 day Steps 20-27, Synthesizing guide RNA using cloning-free methods: 4-5 hours Steps 20-27, Multiplexed sgRNA synthesis: 4-5 hours Steps 28-33, Microinjection workflow: 2 hours

Steps 34-48, Determining the somatic activity of injected targets (CRISPR-STAT): 1-2 days

Steps 49-56, Screening for germline transmitting founders by fluorescent PCR: 3-4 days

Steps 57-64, Identification of heterozygote adults in F1 generation and genotyping of subsequent generations by fluorescent PCR: 1-2 days Steps 65, Sequencing of targeted loci: 1-2 days

Steps 66-74 Bioinformatics pipeline for barcoded multiplex sequencing data (ampliconDIVider): 1-2 days

TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

Anticipated Results

The most critical part of the CRISPR/Cas9 mutagenesis pipeline is ensuring high-quality sgRNA and *Cas9* mRNA for injection into embryos. RNA quality should be assessed using a Nanodrop to determine concentration and purity (260/280 ratio) following by running an aliquot on a gel (Figure 3a, lane 3 and Figure 3c, lanes 1 and 2). High quality sgRNAs and *Cas9* mRNA is then injected into zebrafish embryos.

After injection and prior to large-scale screening, the activity at each sgRNA target site is measured by CRISPR-STAT, which should display a positive correlation with the observed phenotypes (Figure 5 d-f). Expect different mutagenic activities depending on whether *Cas9*/sgRNA were injected into the cell (Figure 5c) or into the yolk (Figure 5b). Different sgRNA targets will also show different levels of activity (Figure 5g-i), falling into one of three categories:

High activity

These targets give many peaks of strong intensity accompanied by a reduction or absence of the WT peak. Finding germline mutations from these targets is easy, since the majority of the founders transmit one or more mutations to their progeny (Figure 5g).

Low activity

These targets give several peaks of low intensity along with the dominant WT peak. Recovering desirable germline mutants from these targets may require screening of many founders (12 founders can be processed in a 96-well plate and usually that is sufficient to identify mutations in 1 or more founders) (Figure 5h).

No activity

These targets give a single WT peak similar to the uninjected controls. These targets rarely lead to germline mutations (Figure 5i).

Only embryos injected with high activity sgRNAs are raised and the mutations are screened in the F₁ generation. Based on our data, on average 1 of every 2 injected embryos will give rise to a germline transmitting founder and the average germline mutagenesis rate is 28% (number of embryos that transmit mutations divided by total number of embryos screened). To date, we have had only 2 genes for which a minimum of 2 targets were tested that were not successfully mutagenized using this protocol out of over 500 genes targeted. Thus, the pipeline described in this protocol is a robust approach for generating and identifying targeted mutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1**GeneMapper Software setup TIMING 30 minutes****Set up the Panel Manager as follows:**

1. Using Panel Manager create a new kit (Kit type Microsatellite). Name the kit “100-400bp FAM”.
2. Highlight the new “100-400bp FAM” folder and then click new bin icon to create a new bin. Name the new bin “100-400bp”.
3. Highlight the “100-400bp FAM” folder and then click the new panel icon to create new panel. Name the panel “Frag Analysis”.
4. Open the subfolder and highlight “Frag Analysis” and click new marker.
5. Fill in the new marker with the following settings. Any field not listed below should be left empty.

Marker Name: FAM

Dye Color: Blue

Min Size: 100

Max Size: 400

Marker repeat: 6

Marker Specific Stutter ratio 0.0

Comments: none

Ladder Alleles: false

Setup the Analysis Method Editor as follows:

1. Go to tools > GeneMapper manager > Analysis method tab
2. Click on New...
3. Select Microsatellite
4. Enter the following into the appropriate tabs. If a setting is not listed, use the default setting.

General tab

Name: 100-400bp

Peak Detector tab

Peak Detection Algorithm: Advanced

Peak Detection Threshold (these values can be changed based on the PCR product to eliminate background or increase sensitivity.)

B: 100

R: 20
Peak Quality Tab
Max Number of expected alleles: 6

Box 2**Extraction of genomic DNA from zebrafish embryos/fin tissues****TIMING** 45 minutes

Genomic DNA can be prepared using either the modified Sigma Extract-N-Amp Extraction procedure (method A) or the Sodium hydroxide extraction procedure (method B). The procedures below are written for extraction of genomic DNA from embryos; for fin tissues, simply double the volumes. We have used both methods and obtained similar results. However, we prefer the Sigma Extract-N-Amp Extraction method because it uses readymade solutions and is more consistent across samples, whereas a different number of sodium hydroxide extractions may be required to fully dissolve tissues in different samples. However, the Sodium Hydroxide Extraction procedure is a more cost effective approach.

A. Modified Sigma Extract-N-Amp Extraction procedure.

1. Mix 25µl of Extraction Solution and 7µl of Tissue Preparation Solution for an embryo. **CRITICAL** Use 2× volumes for all steps for fin tissues. (ie. 10 samples = 250µl Extraction and 70µl Tissue Preparation Solution)
2. Add 32µl of the Extraction/Tissue Preparation Solution mixture to each well. Make sure the embryo remains submerged in solution. **CRITICAL STEP** Do not vortex. Flick the plate gently if mixing is required.
3. Incubate the plate at RT for 10 minutes.
4. Incubate the plate at 95°C for 5 minutes. Tissue may not be completely dissolved at the end of the incubation. This is normal and will not affect performance.
5. Add 25µl of Neutralization Solution B to each well and mix by sealing and vortexing.
6. Dilute DNA 1:10 with nuclease free water for use in PCR reactions (main Procedure steps 40, 53, 61, or 65 option B).

PAUSE POINT Store stock and diluted DNA at -20°C for up to 4 weeks.

B. Sodium Hydroxide Extraction procedure.

1. Add 10µl of 50mM NaOH to each well of a 96-well PCR plate containing an embryo. **CRITICAL** Use 2× volumes for all steps for fin tissues.
2. Seal and heat at 95°C for 20 minutes.
3. Vortex samples until all tissue is dissolved.

CRITICAL STEP If tissue is still present after vortexing repeat Steps 2 -3 until all tissue is dissolved.

4. Add 10 μ l of 100mM Tris-HCl pH 8.0.
5. Dilute DNA 1:50 with nuclease free water for use in PCR reactions (main Procedure steps 40, 53, 61, or 65 option B).

PAUSE POINT Store DNA at -20°C for up to 4 weeks.

Box 3**Interpreting the output from ampliconDIVider (Timing: ~1 day for 4M read pairs)**

1. The main output file is `${BASE}.divs.gz`, in which `$BASE` is the basename of the BAM input. Each sample analyzed has a single entry, where a “sample” is defined as a unique combination of the amplicon ID, the founder ID, the progeny ID, and the barcode. If the `bam2mpg` variant caller detected any DIVs, the most frequently-occurring DIV is represented as a pairwise alignment, with the experimental structure on the top, and the reference on the bottom.
2. If the ‘-r’ option was used, there will also be a file called `${BASE}.range.div.table.gz` in the output directory. This file describes the variants that were detected in the window defined in the ‘-r’ file. Each line has nine columns: the first six are the same as those in the barcode file, and the seventh is a “group number” integer (the value used by the program to keep track of barcode/target combinations). The eighth column indicates if the DIV in the window defined for this sample was an insertion (I), deletion (D), or combination of both (C). Note that the insertion and deletion need not overlap in order to return a C designation. The ninth and final column provides a summary of the type, position, and size of the DIVs. Again, I and D stand for insertion and deletion, the middle value indicates the 1-based start site of the DIV, and the final value indicates how many bases were affected. Multiple DIVs are delimited by double colons. So, a result of “D_101_2::I_98_1” means there was a 1-base insertion at position 98, and a 2-base deletion at position 101.
3. If the ‘-p’ option was used, the output directory will have a file named `${BASE}.fulltrim.passbc.detectedlongfrag.${SHORTPRIMER}.top10.gz`. This file is intended to assist in determining the structure of large DIVs, and presents the 10 most frequent sequences for each target. The first column indicates how often that sequence appeared for a given amplicon, the second gives the amplicon ID, and the third is the 5’ to 3’ sequence matching the primers designated in the ‘-p’ file. Note that not all sequences will necessarily be presented as the “forward” strand.
4. Some of the intermediate files kept in the working directory may be of interest for checking the support for variants. The general naming scheme is provided below; the `${i}` value corresponds to the “group number” described in Box 3 step 2 that combines region and barcode information:
 - `region_barcode.${i}.bam`: The properly-paired, aligned reads
 - `region_barcode.${i}.mpg.gz`: The Most Probable Genotype scores, on which the variant calls were determined. Note that `bam2mpg` is

not deterministic, so multiple runs with the same sample may give slightly different results.

- `region_barcode.${i}.snv.vcf.bgz`: The single nucleotide variants, use: `gzip -c -d region_barcode.${i}.snv.vcf.bgz | awk '$5 != "." {print}'` to fetch SNV
- `region_barcode.${i}.div.vcf.bgz`: The deletion and insertion variants
- `region_barcode.${i}.freqdiv`: For samples in which a DIV was detected, this file contains the read information for the most frequently-occurring DIV-containing read in headerless SAM format.

Figure 1.
Overview of CRISPR/Cas9 mutagenesis pipeline.

Figure 2.

Schematic of sgRNA synthesis and 3-primer PCR strategies for fluorescent PCR or next-gen sequencing. a) sgRNAs are synthesized by annealing two oligos. The top strand is target-specific and the bottom strand is a generic oligo (Ultramer), both oligos are annealed and extended with a DNA polymerase and used as template for *in vitro* transcription. In this schematic, the T7 or SP6 promoter sequence is depicted in purple, 18-20 bp target sequence in orange (do not include NGG PAM site in this sequence), and remaining sgRNA sequence in green. b) Genotyping strategy by fluorescent PCR using an M13F-FAM primer or an M13F-barcoded primer and next-gen sequencing to determine lesions. Gene-specific primers are designed flanking the target site (200-325bp region). The M13F sequences are added to the forward primer, and the pigtail sequences are added to the reverse primer. Depending on

the application (fluorescent PCR vs Sequencing), the third primer is added to the PCR reactions to generate fluorescent or barcoded amplicons.

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Figure 3.

Agarose gel electrophoresis of Cas9 mRNA, target assembly and sgRNA. a) Restriction digest of *pT3SnCas9n* plasmid and *in vitro* transcription of Cas9 mRNA on a 1% (wt/vol) DNA agarose gel; lane M: Marker (1kb plus), lane 1: undigested plasmid, lane 2: digested plasmid, lane 3: Cas9 mRNA. A complete DNA digestion should give a single band at 7.3kb while a successful Cas9 mRNA synthesis should produce a band at ~1.6kb. b) Oligo assembly of sgRNA oligos. Target-specific oligos are annealed and extended with the generic gRNA oligo, and the assembled products are run on a 2.5% (wt/vol) agarose gel. Correctly assembled oligos should run at 117-120bp (lane 1, 2). Bottom strand oligo (oligo2) or top strand oligo will run at much smaller sizes (lane 3, 4). c) The assembled oligos were used as a template for *in vitro* transcription reaction. The sgRNAs are run on a 2.5% (wt/vol) DNA agarose gel. A successful sgRNA synthesis will produce 2 bands between 100bp and 200bp. The higher size band is due to secondary structure of the sgRNA (lane 2) and will disappear upon heating at 65°C (lane1), incorrect oligo assembly will not yield any RNA (lane 3 and 4).

Figure 4. Schematic using the diameter of a sphere to calculate the dose for injection into the embryos. When 10 pulses of the injector are equivalent to 0.3mm then the volume of 1 pulse is equal to 1.4nl.

Figure 5.

Evaluation of sgRNA activity by pigmentation phenotype and CRISPR-STAT plots. Comparison of uninjected (a, e) *tyr* sgRNA injected into the yolk (b, d) or cell (c, f) of embryos. Examples of sgRNA activity determined using CRISPR-STAT showing a highly active target (g), a target with low activity (h) and a target with no activity (i). CRISPR-STAT plots for an uninjected embryo for each sgRNA are shown in the left panels and for an injected embryo are shown in the right panels. The X-axis represents the size (in nucleotides) of the peaks and the red star denotes the position of the WT allele. The Y-axis shows the peak height (intensity) of the fluorescent PCR product. Data shown is for peaks within 20 nucleotides of the WT allele. All animal experiments were performed under an

approved animal study protocol by the National Human Genome Research Institute Animal Care and Use Committee at the National Institutes of Health.

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Figure 6. Fluorescent PCR plots of F1 embryos from an injected founder fish crossed with a WT fish. Wildtype samples show a single peak (top panel) whereas a heterozygous embryo shows 2 peaks (middle and bottom panels). The difference in the size of WT and mutant peaks indicates the size of the lesion. The X-axis represents the size of the peaks and the red star denotes the position of the WT allele. The Y-axis shows the peak height (intensity) of the fluorescent PCR product. All animal experiments were performed under an approved animal study protocol by the National Human Genome Research Institute Animal Care and Use Committee at the National Institutes of Health.

Figure 7. Fin amputation of an adult zebrafish caudal fin for genomic DNA extraction. Fin amputation before (a) and after (b) the cut to show roughly the amount typically removed for genomic DNA extraction. All animal experiments were performed under an approved animal study protocol by the National Human Genome Research Institute Animal Care and Use Committee at the National Institutes of Health.

Figure 8.

Chromatograms of a WT and heterozygous sample. After removal of the WT nucleotides from the heterozygous chromatogram the remaining sequence reveals the mutant allele sequence. In this example the mutation is a deletion of 2bp (GT).

Table 1
Troubleshooting table

Step	Problem	Possible reason	Solution
12	DNA concentration is low	Not enough DNA digested or poor clean up	Check plasmid DNA concentration and repeat digestion and cleanup.
18	Cas9 mRNA concentration is low	Poor synthesis	Repeat synthesis using RNase free techniques.
19	Cas9 RNA is not correct size	RNA is degraded	Repeat synthesis using RNase free techniques.
22	Assembled Oligo is not correct size	Oligos did not assemble correctly	Check oligo sequences and repeat reaction.
26	sgRNA concentration is low	Poor synthesis	Repeat synthesis using RNase free techniques.
27	sgRNA band is not detected	RNA is degraded or synthesis failed	Repeat reaction using RNase free technique.
33	All embryos are dead	Injection toxicity	Inject at a lower concentration.
47	No peaks	Primers did not work or FAM not incorporated	Run PCR product on an agarose gel to confirm that primers work. Design new primers checking M13 sequences.
	2 peaks in WT sample	Endogenous indel	Sequence amplicon and design new primers to avoid endogenous indel
	Multiple peaks in WT samples	Non-specific binding of primers	Optimize PCR conditions or design new primers
	No activity detected	Top strand oligo sequence was ordered incorrectly or sgRNA is not active	Check top strand oligo (PAM site should not be in the oligo sequence). Design another target
55	No mutations detected even though CRISPR-STAT was positive	Biallelic mutations caused injected embryos to die in case of the embryonic / larval lethal genes	Inject again at a lower dose (1/2 to 1/10 th of the sgRNA).
65 option A	Unable to determine exact mutation from the heterozygous samples	Mutation is either large indel or complex indel	Clone PCR product and sequence clones to identify exact mutation.