Supplementary methods: A streamlined CRISPR pipeline to reliably generate zebrafish frameshifting alleles

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II) Identify CRISPR targets

Get to know the target gene:
Before beginning CRISPR design, it's important to have a good understanding of the gene you intend to mutate (the "target gene"). To begin, check the literature for existing phenotypic, expression, and domain information about the target gene:

- Check ZFIN (www.zfin.org) and the Mouse Genome Informatics (MGI) website (http://www.informatics.jax.org/) for expression patterns, existing mutants, and publications.
- Look for overlapping expression of orthologous genes in ZFIN and MGI. If there is a related protein with overlapping expression, consider targeting the ortholog as well.
- Check Pubmed for published functional analysis on your gene of interest.
- In particular, search for information that reveals functionally important portions of the target gene and/or protein.

Next, assemble a set of annotated files for the target gene's genomic, protein, and mRNA sequences:

- Obtain genomic sequence, with exon structure annotated.
    - o Check both Ensembl and NCBI, because sequences sometimes differ in the two databases. If major differences are found, compare genomic structure of the genes with homologues from other species to identify the best zebrafish sequence.
    - o To export annotated genomic sequence from NCBI, search for the target and species of interest in the "Gene" category. Open the desired gene page, and under the "genomic regions, transcripts, and products" section click the "genbank" link. To export this sequence, copy the text from "LOCUS" to "//", and paste into a text file. This exported file can be imported into viewers capable of reading GenBank files, such as Geneious (proprietary) or SnapGene viewer (freeware).
    - o To export annotated genomic sequence from Ensembl, search for the target gene and click the link to the chromosomal location. Click "export data", and change the format to "GenBank". Various features can be selected or deselected according to taste, but make sure that at minimum "Vega Gene Information" and "Variation Features" tracks are selected. Click "Next", and then click "text". Exported sequence can be pasted into a text file, and imported into viewers.
    - o The genomic sequences exported from Ensembl contain annotations of alternative splice forms. Make note of exons that are sometimes skipped, particularly if exon skipping is shown in Vega annotations.
    - o Look for alternative splicing in other species, even if those exons are not shown to have alternative splicing in zebrafish. Multi-species comparisons of exon structure can also be done using the UCSC genome browser (https://genome.ucsc.edu/) by searching for the human version of the target gene, and using the "configure" button to show the "vertebrate chain/net". Sequences can be exported from this browser using the "View" then "DNA" buttons. If alternative exon usage is found in some species, note this on the zebrafish sequences.

- Obtain protein sequences from a variety of species, and use these to identify conserved features of the target gene.

- Protein sequence files can be exported from Ensembl or NCBI, similar to genomic sequence files. In NCBI, these sequences can be found under the "mRNA and Protein" section of the gene page.
- In addition, annotated protein files can be directly imported to Geneious software using the "NCBI" search function. Simply search for your gene name and *Danio*, identify the correct sequence, and copy-paste the file into your desired folder.
- Blast the human and zebrafish protein sequences against the zebrafish genome to search for hidden duplicates and orthologs. You can BLAST sequences using several databases, including www.ensembl.org and www.phylogeny.fr.
- Check both Ensemble and NCBI for protein domain annotations.
- In the absence of published data, protein domains can also be identified using bioinformatics tools such as Pfam (http://pfam.xfam.org/).
- Align protein sequence using freeware (e.g. http://www.ebi.ac.uk/Tools/msa/clustalo/) or proprietary software (e.g. Geneious). For these alignments, we typically use sequences from zebrafish (including duplicates if present), at least one other fish, a *Xenopus* species, chicken, mouse, and human.
- Examine alignments of orthologous proteins to identify more putative domains, which may be annotated on proteins from other species.
- Annotate strongly conserved regions of the zebrafish protein, even if they do not contain known domains.
- Obtain mRNA sequence, and annotate with information about genomic and protein structure.
  - mRNA sequence with 5' and 3' untranslated regions annotated can be obtained in a manner similar to protein sequences.
  - Annotate the mRNA file with exon boundaries identified above, and highlight exons that are sometimes skipped.
  - Annotate the mRNA file with sequences encoding conserved protein features and domains.
  - This annotated mRNA file should now contain information about genomic sequences (exon structure), mature splice form of the mRNA, and the protein that is encoded by the mRNA. It will be used to identify the ideal region for CRISPR mutagenesis.

Identify a target region

Once familiar with the target gene, it is important to hone in on the correct target region for mutagenesis. The target region is an exon, or portion of a large exon, found towards the 5' end of the target gene that optimally satisfies the criteria set forth below. Use the mRNA file annotated in the previous section to learn which region of the target gene will most likely cause a strong loss of function. We use the following guidelines, written in order of importance, to help identify the best region.

- The target region should be 5' to domains that you suspect may impart functionality.
- The target region should be 3' to potential alternative start sites (in frame ATGs or even CTGs[1] at the beginning of the gene). Alternative start sites can also be avoided by selecting a target region 3' to short deeply conserved sequences at the 5' end of a gene (e.g. signal peptides).
- Use RepeatMasker (http://www.repeatmasker.org/) to identify repeat sequences. These are usually rare in exons, but should be excluded from CRISPR target regions if present.
- The target region should be in a constitutive exon. Avoid exons that are sometimes excluded from transcripts unless your project focuses on function of a particular splice form.
- Preferred target regions are located on the 5' end of exons containing at least part of a domain that appears important to protein function.
- Preferred exons have few or no SNPs shown in Ensembl's "Variation Features" track.
- Avoid choosing the first exon in poly-exonic genes, because of the prevalence[2] of alternative first exon usage.
- Exons longer than 150 bp are likely to contain ideal CRISPR sites. If a shorter exon is chosen, be aware that short exons can be hot spots for alternative splicing.
- If you intend to use HRMA for CRISPR tests and mutant screening, it is important that the target region has a smooth melt curve. We find that uMelt software (https://www.dna.utah.edu/umelt/um.php [3]) is an excellent predictor. Smooth melt curves are sigmoidally shaped, have a single peak on the derivative curve, and have a plateau shaped melting profile. If the predicted melt curve is not smooth, trim the target region to one that does predict a smooth melt curve (preferably >150 bp).

Identify a target site

There are several websites that design targets for CRISPR mutagenesis. We have mainly used ZiFiT software [4, 5] for design, so this section follows the ZiFiT workflow. Other CRISPR design pages "CRISPR Design Tool" [6] (http://crispr.mit.edu/) and CHOPCHOP [7] (https://chopchop.rc.fas.harvard.edu/index.php) can also be used. It is especially helpful to check multiple sites if few or no target sites are found in the target region you have chosen based on the preceding criteria.

- Navigate to the ZiFiT website (http://zifit.partners.org/ [4, 5]), and follow links to the "CRISPR/Cas nucleases" site.
- Copy/paste the target region from the target gene's genomic sequence file into the text box.
- Select "T7 Promoter" which will ensure that the CRISPR target begins with "GG", the preferred starting bases for T7 RNA polymerase. This criterion can be relaxed if no hits are returned that satisfy the guidelines below.
- If a suitable target beginning with GG is not found, then de-select "T7 promoter", and find a target preferably beginning with NG or GN; alter the sequence to read "GG" [8] (see example on page 7). Alternatively, the Sp6 promoter can be used, which lacks the GG requirement. A recent report indicates that the Sp6 promoter generates highly active gRNA [9].
- Specify the length of target site, typically 20 bp. Recent reports have shown that 17 bp or 18 bp target lengths work well, and may even improve mutagenesis specificity [10].
- Select "Zebrafish" for species.
- Click "Identify target sites".
- Click "Identify potential off target sites". Eliminate candidate targets that have off-target sites containing only 1 bp or 2 bp mismatches compared to the target. Ensure that the correct target (0 bp mismatch at the correct genomic location) is returned in this list of "off target" locations.
- Annotate a few non-overlapping targets with low predicted off-targeting on the mRNA sequence file.
- Eliminate candidate CRISPR target sites with an obvious stretches of self-complementary sequence that might cause hairpin formation.
- Prioritize candidate CRISPR targets that overlap a restriction enzyme site, since restriction analysis provides an alternative method to assay mutagenesis and genotype. Restriction sites can be identified using NEBcutter (http://tools.neb.com/NEBcutter2/) or other platforms. Alternatives to HRMA are particularly important when genotyping incrosses of heterozygotes (see Notes on page 23).
- Select the most 5' preferred CRISPR target(s), and annotate onto genomic sequences.
- Annotate the Protospacer Adjacent Motif (PAM), which is the short sequence (NGG) following the CRISPR site where cleavage occurs.
- Generate HRMA primers to genomic sequences surrounding the most 5' CRISPR target site (see page 6), and check that the amplicon sequence predicts a smooth melt curve using uMelt software.
- If the 5' furthest candidate CRISPR target within the target region passes the above selection, then use it to make CRISPR gRNA for mutagenesis. If not, then assess other possible candidate targets in a 5' to 3' direction.
- In our experience, the majority (>60%) of genes we have targeted are effectively mutagenized using the first gRNA we construct. A second CRISPR gRNA targeting another site can provide a backup for mutagenesis, as well as an independent allele.

Design primers for HRMA and sequencing
Before designing a gRNA, ensure that you can design good primers flanking the CRISPR target site for HRMA and sequencing. Sequencing primers are rarely problematic, but we have needed to re-design several CRISPR targets that could not be paired with good primers for HRMA.

Primer design for HRMA is similar to regular primer design, and we use standard primer design software (e.g. Primer3 http://bioinfo.ut.ee/primer3/). In addition, we suggest the following guidelines for reliable analysis.

- Check for predicted SNPs in the target region using the "variation features" track on Ensembl. If there are SNPs predicted in the PCR product, we recommend sequencing the amplicon from the fish you intend to inject. If SNPs are already segregating in the background, HRMA of CRISPR-injected fish will be complicated.
- The product size should be 100-160 bp; with these short PCR products, small sequence changes can produce large changes in melting temperature.
- Ensure that at least 15 bp are present between each primer and the CRISPR target site, so longer insertions or deletions (indels) can be detected.
- Test HRMA primers for predicted melt curves using "uMelt" software (https://www.dna.utah.edu/umelt/umelt.html [11]). Using this software, ensure that the predicted PCR product gives a smooth melt curve, with only one melt peak. If multiple melt peaks are predicted on wild-type DNA, then analysis of mutant DNA will be difficult.
- We order HRMA primers from Operon at 10 nMole scale, with salt-free cleanup.

Primers for sequencing reactions also can be made using standard primer design software. Use the following guidelines to design successful primers for sequencing reactions on heterozygotes.
- Ensure that at least 50 bp are present between each sequencing primer and the CRISPR target site.
- Ensure that at least one of the primers for sequencing is found in the same exon as the CRISPR target site. Intronic sequence and untranslated regions (UTRs) sometimes contain silent indels, which can complicate lesion identification.
- We order sequencing primers from Operon at 10 nMole scale, with salt-free cleanup.

Example of CRISPR target design: *six1a*
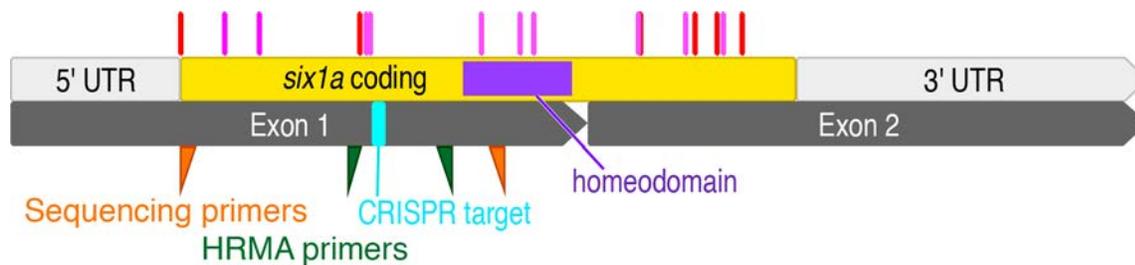
We uploaded and analyzed *six1a* genomic sequences from Vega (OTTDARG00000022888), and NCBI (Gene ID: 494168); the two databases showed perfect agreement so we only uploaded NCBI sequences for mRNA and protein files. *six1a* is a two exon (gray) gene. The protein encoded by *six1a* is strongly conserved across chordates, with >91.6% identity in pairwise comparisons between species (lamprey *six1, Danio six1a, Danio six1b, X. laevis six1*, chicken *Six1*, mouse *Six1*, human *SIX1*). Although we typically avoid targeting the first exon, we chose to target the first exon of *six1a* because this exon contains a deeply conserved homeodomain (purple). Ensembl prediced no SNPs in the coding sequence. To target downstream of any potential alternative start sites, we marked the transcript with in-frame AUG's (red) and CUGs (pink), and selected a target region between these sites and the homeodomain:

GAAGGCGCACTATGTTGAGGCGGAAAAGTTGCGGGGGGCGCCCGCTAGGCGCTGTGGGCAAATA
TCGTGTGCGCAGGAAATTCCCTTTGCCCCGTACTATCTGG

Using ZiFit software, we identified target sites towards the 5' end of this region. One target site (GGGGCGCCCGCTAGGCGCTG) worked well with a T7 promoter, but was likely to make hairpins, and caused low to no mutagenesis. A second, non-overlapping, target site (aqua) was selected at the 5' end of the target region:

Target site [PAM]: GAAGGCGCACTATGTTGAGG[CGG]

This target is incompatible with T7 promoter, so we modified it to accommodate the promoter (GgAGGCGCACTATGTTGAGG). Alternatively, we could have left the target unchanged, and used an Sp6 promoter instead of T7.

We designed HRMA primers (six1a_HRM_F: GCACAACCACCCGAAGATG and six1a_HRM_R: CTCGCCGTCCCAGATAGTAC) (green triangles) to amplify sequences around this *six1a* target. These primers predict a 145 bp product and a smooth melt curve using uMelt software which we confirmed using HRMA. We also designed sequencing primers (six1a_Seq_F: ATGTCAATCTTGCCCTCGTTCG and six1a_Seq_R: TGGATAAGGGTTGTGCGTGTAC) (orange triangles) that amplify within the first exon of *six1a* and produce a 447 bp product centered near the CRISPR target site. We ordered short oligos to clone this CRISPR target into pDR274 (see page 10), and used the resulting plasmid to generate gRNA for six1a mutagenesis. We also describe an alternative, cloning-free, method to generate gRNAs (see page 8) that we now use routinely.

IIIa) Cloning-free method to generate gRNA
Based closely on protocols described in Hruscha et al [12].

Design Guide-oligos

"Guide-oligos" are synthetic DNA strands, which are used to generate templates for guide RNA synthesis. Guide-oligos are made of three main parts: a T7 promoter, a variable ***targeting sequence***, and a Cas9 binding scaffold. A short clamp is provided to stabilize the 5' end of double-stranded sequence. The full sequence of a generic guide-oligo is shown below:

GCGTAATACGACTCACTATA***GGNNNNNNNNNNNNNNNNNN***GTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTT

To design a guide-oligo, simply replace the underlined region in the above sequence with your target sequence. T7 transcribes the last base (G) of its promoter, and also prefers a G in the next position. These two Gs are included in the target sequences identified by ZiFiT software, when the "T7 promoter" option is selected. The underlined example above is shown with 18 N's, plus the invariant 5' GG for a 20 bp target, but target lengths may vary. To design gRNA with an Sp6 promoter, replace the green sequence with "ATTTAGGTGACACTATA", and then add targeting sequence without the GG restriction [9].

ZiFiT outputs the targeting sequence in the correct orientation, but double-check that the sequence begins with GG. As another check, the CRISPR binding site ends just before the PAM sequence (NGG) following the target. This end should "point" towards the gRNA's Cas9 binding scaffold sequence, such that the PAM sequence will coincide with the terminal "GTT" of the scaffold sequence. If you plan to transcribe mRNA directly from this synthesized guide-oligo, be sure to reverse-complement your design. For consistency with previous publication [12], we purchase guide-oligos in the reverse-complemented orientation, but we transcribe from a PCR-amplified product instead of directly transcribing from the guide-oligo (see page 9).

Guide-oligos can be ordered as long primers, without special purification. We order guide-oligos, typically 119 bp long, from Operon at the 0.2 μM scale with salt-free cleanup. Oligos of this length can also be purchased from other vendors, such as Sigma or IDT.

Example: guide-oligo for *six1a*

Page 7 describes target site design.   Target[PAM]: ***Gg*AGGCGCACTATGTTGAGG[CGG]**

Insert target sequence into the guide-oligo template sequence:
GCGTAATACGACTCACTATA***Gg*AGGCGCACTATGTTGAGG**GTTTTAGAGCTAGAAATAGCAAGTT AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTT

Reverse complement to generate guide-oligo sequence (order this):
AAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTT CTAGCTCTAAAAC***CCTCAACATAGTGCGCCT*c*C**TATAGTGAGTCGTATTACGC

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PCR amplify guide-oligos
Because guide-oligos are lengthy, the oligo synthesis received from manufacturer isn't pure enough to use directly as templates for mRNA synthesis. A simple PCR converts the "dirty" guide-oligo into a clean template.

___Resuspend guide-oligo in low TE (10 mM Tris-HCl, 0.1 mM EDTA), to generate a 100 μM stock. Store this stock at -20˚C.
___Dilute this stock to 1 μM in sterile H_2O.
___Prepare PCR mix:

    ___10 μl 10 X Thermopol buffer   ___2 μl 10 mM dNTP
    ___2.5 μl 10 μM gRNA_Primer1/2   ___1.5 μl Taq (NEB M0267S)
    ___2μl 1 μM guide-oligo   ___79 μl Sterile H_2O.
    ___3 μl DMSO

      gRNA Primer 1: GCGTAATACGACTCACTATAG
      gRNA primer 2: AAAGCACCGACTCGGTGCCAC
___Split PCR into two 50 μl wells.
___Run PCR:
    95˚C 1 min (95˚C 15 sec, 60˚C 30 sec, 72˚C 20 sec) 40X, 72˚C 5 mins, 4˚C ∞
___ Run PCR product on a 3% agarose gel in 1x TBE at 180 volts for ~1 hour. Load 1μl on a narrow well for imaging, and the remaining 99 μl on a wide well for extraction. Expect a single bright band at 120 bp.

Gel extract PCR amplified guide-oligo
___Gel extract PCR product, using a Qiagen MinElute gel extraction kit (Qiagen 28604).
___Setup vacuum manifold (Qiagen 19413) with MinElute column and manifold adapters (Qiagen 19407).
___Cut out the 120 bp band from the 99 μl lane.
___Place in a 15 ml pre-weighed conical tube.
___Re-weigh the conical.
 ___Calculate the necessary volumes for isopropanol (same as gel volume), and Buffer QG (six times gel volume), as described in kit instructions.
___Add 6 gel volumes Buffer QG to the samples.
___Place at 50˚C for 15 minutes. Vortex occasionally.
___Add 1 gel volume isopropanol; mix by tilting a few times.
___Transfer to MinElute column, 700 μl at a time. Apply vacuum.
___Wash with 500 μl Buffer QG. Apply vacuum.
___Wash with 750 μl Buffer PE (apply Buffer PE to the column's lip to rinse away Buffer QG). Apply vacuum.
___Wash with 750 μl Buffer PE a second time. Apply vacuum.
___Place in collection tube, and spin 1 minute "empty" in a tabletop centrifuge
___Transfer columns to nuclease-free microfuge tubes.
___Add 12 μl H_2O (nuclease-free).
___Let sit 1 to 2 minutes.
___Spin 1 minute.
___Quantify sample via spectrophotometry (yield should be ~10-50 ng/μl). Don't be alarmed by low readings. The product is so short that 30 ng of template has higher molar concentration than 1 μg of a typical plasmid.

IIIb) Plasmid based method to generate gRNA

This method is based closely on protocols described in Hwang et al [13]. This plasmid-based method is more laborious than the cloning-free method, but is preferred by some labs. Both the plasmid-based and cloning free methods can be used to generate high quality gRNA.

Design and order insert-oligos
___Follow instructions above (page 5) to identify a target site.
___From the ZiFiT design page, copy/paste the "Oligo 1" and "Oligo 2" sequences for this target into an excel spreadsheet. These are the Insert-oligos.
___If using a different design program, the insert-oligos can be designed using the target site. To generate "Insert-Oligo 1", simply add "TA" 5' to the target sequence. To generate "Insert-Oligo 2", delete the GG at the beginning of the target sequence, then reverse complement the target sequence, and finally add "AAAC" 5' this sequence. When mixed together, the insert-oligos will create a double-stranded targeting sequence flanked by 4 bp single-stranded overhangs, compatible with the overhangs generated by BsaI digestion of pDR274 (see below).
___Order the insert-oligos as standard primers with salt-free cleanup.

Example: making insert-oligos to target *six1a*
Page 7 describes target site (***Gg*AGGCGCACTATGTTGAGG**) design.

Insert-Oligo 1 is: TA***Gg*AGGCGCACTATGTTGAGG**
Insert-Oligo 2 is: AAAC***CCTCAACATAGTGCGCCT***

Generate BsaI digested pDR274 fragment
___Obtain pDR274 from AddGene (plasmid #42250).
___Streak bacterial stock carrying pDR274 onto LB plates (10 g/L Tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar; pH 7.5) containing 50 μg/ml Kanamycin.
___Pick isolated colonies, and start an overnight culture in LB broth (10 g/L Tryptone, 5 g/L yeast extract, 10 g/L NaCl; pH 7.5) containing 50 μg/ml Kanamycin.
___Miniprep pDR274.
___Linearize pDR274 with BsaI-HF (New England Biolabs R3535S):
___10 μg plasmid   ___10 μl BsaI-HF   ___10 μl CutSmart Buffer   ___ Sterile H_2O to 100 μl
___Place at 37˚C for at least three hours.
___Purify linearized plasmid using QiaQuick PCR cleanup kit (Qiagen 28104). Use two columns to extract the sample, because each can purify up to 5 μg. Elute using 30 μl H_2O (nuclease-free) per column.
___Run 1 μl digested and undigested plasmid on a 1% agarose gel for diagnosis. Expect to see a single band at 2.1 kb.

Once prepared, a stock of linearized pDR274 can be used for many CRISPR cloning reactions.

Clone insert-oligos into pDR274

___Resuspend insert-oligos to create a 100 μM (1000x) stock solution of each oligo in Low TE (10 mM Tris-HCl, 0.1 mM EDTA).

___Mix 10 μl Insert-Oligo 1 and 10 μl Insert-Oligo 2 1000x oligo stocks in 80 μl Low TE to generate a 10 μM (100x) F/R stock.

___Mix 10 μl of the 10 μM stock in 990 μl Sterile H_2O for 0.1 μM F/R working stock.

___In an 8-well strip, mix together:

    ___1 μl linearized pDR274

    ___1 μl T4 ligase (New England Biolabs M0202S, 400 U/μl)

    ___2 μl 10x T4 ligase buffer

    ___2 μl 0.1 μM insert-oligo F/R working stock

    ___14 μl H_2O (nuclease-free)

___Place in PCR machine, water bath, or incubator, and run at 16°C for 3 hours to overnight.

___Thaw competent bacteria, having a transformation efficiency of around 10^9 colony forming units per microgram, on ice.

___Dispense competent bacteria into three 25 μl aliquots in chilled microfuge tubes:

    ___Add 5 μl of ligation to one tube

    ___Add 5 μl of Sterile H_2O to a second tube

    ___Add 1 μl of Kanamycin-resistant positive control plasmid (10 pg/μl) to the third tube

___Leave on ice for 15-30 minutes.

___Heat shock at 42°C for 30 seconds.

___Add 125 μl SOC media (20g/L tryptone, 5g/L yeast extract, 10 mM NaCL, 10 mM gCl$_2$, 10 mM MgSO$_4$, 20 mM glucose).

___Shake the cells for one hour at 37°C.

___Plate 100 μl of ligation or H_2O control by spreading onto LB plates with 50 μg/ml Kanamycin. Only plate 1 μl of control plasmid.

___Let liquid absorb a few minutes, then place plates at 37°C overnight.

___Score transformations in the morning. Expect to find a few hundred colonies per plate per ligation, no colonies on the H_2O control plate, and about one thousand colonies on the positive control plate.

___Start two 3 ml overnight cultures per ligation, in LB containing 50 μg/ml Kanamycin. Grow overnight at 37°C while shaking or rotating.

___The next morning, create a frozen bacterial stock. Mix 750 μl of the overnight culture with 250 μl of 80% sterile glycerol in a cryo-safe screw cap vial, and store this stock at -80°C.

___Use the remaining culture to prepare plasmid DNA (miniprep).

___Sequence-confirm plasmids using M13F sequencing primer. Select a clone that carries the correct insert; this is the targeting plasmid.

Prepare targeting plasmid for gRNA synthesis

___Digest the targeting plasmid using DraI (New England Biolabs R0129S):

    ___5 μg plasmid   ___4 μl DraI   ___5 μl CutSmart Buffer   ___Sterile H_2O to 50 μl

___Place digestion mixture at 37°C for at least three hours.

___Run digests on a 1% agarose gel. Expect to see bands near 286 bp and 1.9 kb.

___Gel extract the 286 bp fragment as described on page 9.

IV) Generate guide RNA (gRNA) and *cas9* mRNA

Synthesize and cleanup gRNA
Prepare reaction mixture at room temperature, using templates generated on page 9 or page 11, and components provided by the MAXIscript T7 kit (Life Tech AM1312, or AM1310 for Sp6). Use RNAse-free technique throughout this protocol.

___Thaw dNTPs and templates on ice. Thaw TXN buffer at 40-50˚C. Keep T7 enzyme and RNAse inhibitor on ice.
___Mix together:
    ___ >30 ng template PCR product or linearized targeting plasmid
    ___1 μl ATP   ___1 μl UTP   ___1 μl CTP   ___1 μl GTP
    ___2 μl 10X TXN buffer
    ___2 μl T7 enzyme
    ___1 μl RNAse inhibitor
    ___ H_2O (nuclease-free) to 20 μl
___Place reaction at 37˚C for 3 hours to overnight.
___Add 1 μl Turbo DNAse.
___Incubate at 37˚C for 15 minutes.
___Add 2 μl 8M LiCl (nuclease-free, Sigma L7026) (see Note 1).
___Add 75 μl of pre-chilled 100% EtOH (nuclease-free. Fisher BP2818).
___Place at -80˚C for 1 hour to overnight.
___Spin 15 minutes at 4˚C.
___Wash with 200 μl pre-chilled 80% EtOH (nuclease-free).
___Spin 5 minutes at 4˚C.
___Remove all liquid.
___Let pellet dry 5-10 minutes.
___Resuspend in 30 μl H_2O (nuclease-free).
___ Quantify sample via spectrophotometry (concentration should be ~300-600 ng/μl)
___Run on a 3% agarose diagnostic gel (see Note 2):
    ___Pre-heat PCR machine to 72˚C.
    ___Mix 3 μl RNA, 2 μl H_2O (nuclease-free), 5 μl molecular biology grade formamide in an 8-well strip.
    ___Place strip in 72˚C PCR machine for 3-5 minutes.
    ___Load hot samples onto a 3% agarose gel, alongside 5 μl MassRuler DNA ladder (Fisher Fersm0403).
    ___Run gel 20-30 minutes at 160 V. Expect to a band below 80 bp, around 120 bp, and sometimes a faint "laddering" above 120 bp (see Note 3).
___Store immediately at -80˚C.

Notes
1. LiCl precipitation is less expensive than column-based methods, is simpler, is non-toxic, and in our experience gives better yields than column-based cleanup for small products.
2. Although less precise than an acrylamide gel, this diagnostic gel works well and is considerably simpler to prepare.
3. The extra bands may represent different folding structures, or annealed RNA.

Prepare linear pCS2-nCas9n plasmid

___Streak pCS2-nCas9n [14] (Addgene 47929) frozen stock onto LB plates containing 100 μg/μl Carbenicillin.

___Pick isolated colonies, and start 3 ml overnight culture in LB plus 100 μg/μl Carbenicillin.

___Perform a Qiagen midiprep of the pCS2-nCas9n plasmid.

___Linearize pCS2-nCas9n with NotI-HF (NEB R3189S):

___20 μg plasmid   ___10 μl NotI-HF ___10 μl CutSmart Buffer ___Sterile H_2O to 100 μl

___Place at 37˚C for at least three hours.

___Purify linearized plasmid using QiaQuick PCR cleanup kit (Qiagen 28104). Extract using two QuaQuick columns, which each have 10 μg capacity, and elute into 30 μl H_2O (nuclease-free) per column.

___Run 1 μl digested and undigested plasmid on a 1% agarose gel for diagnosis. Expect a single band at 8.2 kb in the digested sample.

Synthesize *nls-zCas9-nls* mRNA

___Thaw 2X NTP-CAP and linearized pCS2-nCas9n on ice. Thaw 10X Sp6 buffer at 37-50˚C. Keep Sp6 enzyme and RNAse inhibitor on ice (see Notes below).

___Assemble reaction mixture at room temperature:

     ___0.5-2 μg (up to 7 μl) linearized pCS2-nCas9n [14]

     ___2 μl Sp6

     ___2 μl 10X Sp6 buffer

     ___10 μl 2X NTP-CAP

     ___1 μl RNAse inhibitor

     ___H_2O (nuclease-free) to 20 μl

___Place reaction mixture at 37˚C for 4 hours.

___Add 1 μl Turbo DNAse.

___Place at 37˚C for 15 minutes.

___Add 80 μl H_2O (nuclease-free).

___Pre-mix 300 μl Buffer RA1 with 300 ml 100% EtOH (nuclease-free).

___Add 600μl RA1/EtOH mixture (from previous step) to sample. Pipette to mix.

___Transfer to Nucleospin RNA II columns.

___Spin 30 sec. Discard flow through.

___Add 350 μl Buffer MDB. Spin 1 min. Discard flow through.

___Add 200 μl Buffer RAW2. Spin 30 sec. Discard flow through.

___Add 600 μl Buffer RA3. Spin 30 sec. Discard flow through.

___Add 250 μl Buffer RA3. Spin 30 sec. Discard flow through.

___Spin column empty for 2 minutes.

___Transfer columns to new microfuge tubes.

___Add 40 μl H_2O (nuclease-free), and let sit 1-2 minutes.

___Centrifuge for 1 minute; flow-through is the *nls-zCas9-nls* product.

___Quantify product by spectroscopy. Concentration should be 300-800 ng/μl.

___Run 2 μl on a 1% agarose diagnostic gel. High quality *nls-zCas9-nls* mRNA shows prominent bands around 800, 1000, and 1500 nt, that may represent different folding structures.

___Immediately store RNA at -80˚C in 800 ng aliquots.

Notes on *nls-zCas9-nls* mRNA synthesis

- We prefer to use a nuclear localized version of *cas9* that is optimized for codon usage in zebrafish (*nls-zCas9-nls*), which is easy to synthesize and shows high activity in zebrafish embryos [14].
- Use RNAse-free technique throughout this protocol.
- Use mMessage mMachine Sp6 kit (Life Tech AM1340) for synthesis, and "Nucleospin II RNA cleanup kit" (Machery Nagel 740955.50) to purify the RNA; the reagents listed above come from these two kits.
- When synthesizing 4.2 kb *nls-zCas9n-nls* mRNA, we do not use the MAXIscript kit recommended for gRNA synthesis, because the MAXIscript kit does not add a 5' cap and is not recommended for transcripts larger than 2 kb.

Ⅴ) Mutagenize F0 embryos, and test mutagenesis efficacy

Co-inject zebrafish embryos with gRNA and *nls-zCas9-nls* mRNA

___Prepare injection mixture on ice on the morning of injection:
    ___ gRNA to 40 ng/μl final concentration
    ___ *nls-zCas9-nls* mRNA to 80 ng/μl final concentration
    ___2 μl 1M KCl
    ___0.5 μl 0.5% Phenol Red
    ___H_2O (nuclease-free) to 10 μl
___Inject 1-2 nanoliters of injection mixture directly into the blastomere of early one-cell stage embryos. To ensure injection into this early stage, collect batches of embryos every 10-20 minutes and inject no more than 40 eggs per collection.
___Transfer embryos to 28.5°C incubator.
___Clean embryos a few hours after injection. Note numbers of healthy, deformed, and infertile embryos.

Lyse embryos

___Dechorionate 8 uninjected and 8 injected embryos at one to three days post fertilization.
___Transfer embryos to columns one and two of a 96 well plate. To ensure uniform volumes, embryos can be transferred using a p20 set to 2.5 μl and fitted with a p200 pipette tip. Using scissors or a razor, cut 2-3 mm off the end of this p200 tip, to accommodate the width of an embryo.
___Add 20 μl 1x Thermopol buffer (New England Biolabs, B9004S).
___Place in PCR machine. Run 95°C 10 minutes, 4°C ∞.
___Add 5 μl 10 mg/ml Proteinase K to each well.
___Run 55°C 1 hour, 95°C 10 minutes, 4°C ∞.
___Mix samples in each well.

Introduction to High Resolution Melt Analysis (HRMA)

The melt curve of a PCR product can be revealed using dyes which only bind to double-stranded DNA. Each DNA sequence produces a characteristic melt curve, which can be discerned from one another even when the sequences being compared differ by a single base-pair. This feature of DNA melt curves allows researchers to quickly identify heterozygous sequences in the progeny of CRISPR founders. Using HRMA, researchers can also quickly assess whether a target sequence has been mutagenized, which would result in a mixed WT/mutant population of PCR products with different melt characteristics than purely WT PCR products. Unlike most other means of mutant discovery, such as surveyor assays, HRMA has the ability to quickly distinguish two different mutants from one another without sequencing. HRMA requires researchers to use several curves which plot DNA fluorescence as a function of temperature or amplification cycle; below we offer explanations and guidelines for interpreting these curves.
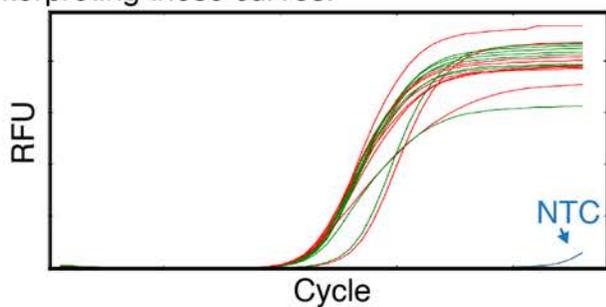
Amplification curve

The amplification curve shows the amount of DNA fluorescence in "Relative Fluorescence Units" (RFU) at each cycle of amplification. Any amplification of no template controls (NTCs) should be at least ten cycles later than wells with templates. Fluorescence increases as a function of double-stranded DNA quantity.
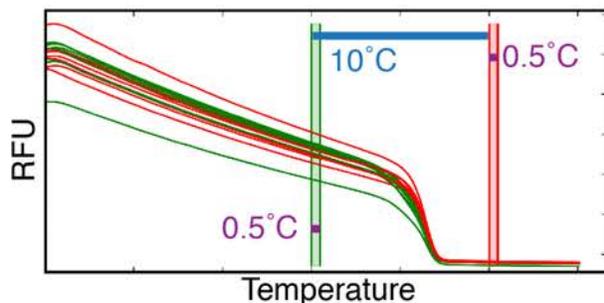
Melt curve

The melt curve is a plot of fluorescence intensity vs temperature. Sample amplification differences make it difficult to determine melt differences from this graph.
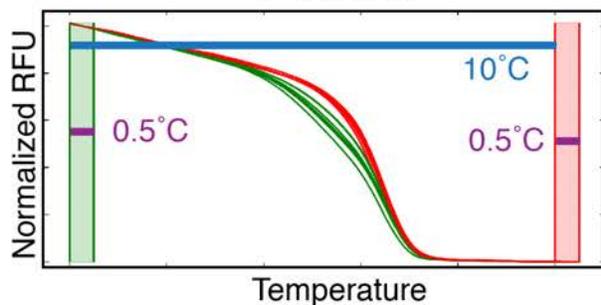
Normalized melt curve

The normalized melt curve allows melt profiles to be analyzed independent of amplification level. Normalized melt curves are generated by setting normalization borders before (green bar) and after (red bar) the peak melting temperature.

For consistency, we set the normalization borders 10°C apart, with widths of 0.5°C.
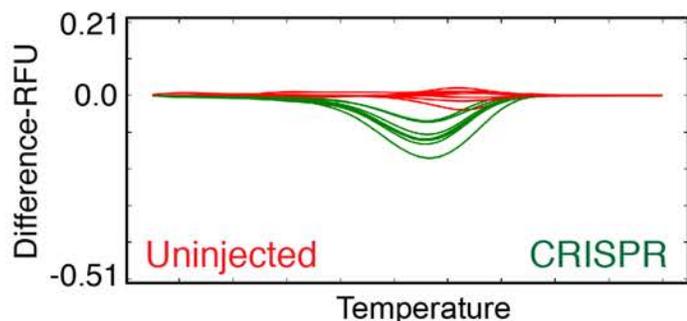
Difference curve

The difference curve sets a reference group as a constant RFU of "0" and allows easy visualization of how an experimental group differs from that reference (measured in "Difference-RFU").

Difference curves highlight small changes in melt temperatures, and also provide a metric for melt deflections to be quantitatively compared between experiments.

Expect most CRISPR injections to cause a downward deflection, because heteroduplex DNA typically melts at a lower temperature than WT (homoduplex) DNA.

All of our CRISPR injections so far have caused deflections between 0.21 and -0.51. We scale our difference curves to this range, so the relative mutagenesis of different CRISPRs can be compared. Injections that cause deflections of at least -0.1 difference-RFU result in a high founder rate.

Set up High Resolution Melt Reactions

Based on techniques described in Dahlem et al [11].

___Prepare HRMA mix.

Recipe for 16 samples:

___180 μl Precision Melt Mix (BioRad 172-5112)

___9 μl 10 μM HRMA primer pair (see Note 1)

___171 μl Sterile H_2O

___Vortex

Scalable recipe (total volume = V):

___1/2 V Precision Melt Mix

___1/40 V 10 μM HRMA primer pair

___19/40 V Sterile H_2O

___Vortex

___Transfer 18 μl HRMA mix to all 16 wells of Columns 1 and 2, plus wells 3A and 3B, of a 96 well plate (Bio-Rad; MLL9601) (see Note 2).

___Add 2 μl of embryo lysis (from Section VI) to wells of Columns 1 and 2 (see Notes 3 and 4).

___Add 2 μl sterile H_2O to wells 3A and 3B ("no template" controls).

___Cover plate tightly with a transparent seal (Bio-Rad; MSB1001), using the corner of a lab marker to press the seal into the gaps between wells.

___Vortex briefly to mix, and then briefly microfuge the plate.

___Place the plate in an HRMA-capable qPCR machine.

___Run the following program to generate amplification and melt data:

95˚C 3 mins, (95˚C 15 sec, 60˚C[†] 20 sec, 70˚C 20 sec*) 45x,

65˚C 30 sec, melt 65-95˚C [§], 95˚C 15 sec.

[†] Annealing temperature varies, but 60˚C works well for most primers.

* After 70˚C extension, image plate to generate amplification data.

[§] During melt steps, change temperature 0.2˚C per step, and hold each step for 5 seconds before imaging the plate, to generate the melt data.

___Analyze amplification and melt curves using instructions on next page.

___If HRMA confirms CRISPR activity, raise the remaining embryos.

Reaction assembly notes

1. HRMA primer design instructions are found on page 6.
2. When adding HRMA Mix to wells, use "positive pressure pipetting" to ensure uniform sample distribution. When pulling liquid from the master mix, compress the pipettor's plunger two stops, and suck up liquid through both the normal level and the blowout. When dispensing HRMA mix, expel only to the first plunger stop (no blow-out). Refill, while maintaining the blow-out reserve and expel to the first plunger stop again. Repeat, until all wells are filled.
3. Avoid bubbles when dispensing template; expel template only to the first plunger stop while pipettors are immersed in liquid. Then, drag the pipettor up the side of the plate while compressing the plunger to the second stop (blow-out).
4. Small differences in buffering can cause notable shifts in melt curves, and buffering can even be affected by the type of biological samples used. For instance, HRMA of wild-type fin clip DNA samples cannot be directly compared with HRMA of wild-type embryo DNA samples. Consistent results are obtained by keeping conditions constant for all samples within an experiment. For instance, the relative shape of wild-type versus heterozygous samples will appear the same when assayed using DNA from either fins or embryos.

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Analyze F0 melt curves

- The following steps are written for Precision Melt Analysis software on a CFX Connect machine from BioRad. Illumina's ECO machine and software also worked well for us, although the details of analysis differ slightly. In addition free software for HRMA is available online (https://www.dna.utah.edu/uv/uanalyze.html [15]); this software can produce normalized melt curves, but not difference curves.
- First, check the amplification curves using qPCR software. Exclude samples that did not amplify well. Samples with low amplification plateaus often show different melt curves from samples with high amplification plateaus, even if they are the same genotype. Be wary of samples with linear amplification, rather than sigmoidal amplification. "No template control" should not amplify, or should amplify at least ten cycles later than samples with DNA.
- Next, open a melt file in Precision Melt Analysis software.
- In this software, normalize the melt curves to a set scale. The degree of mutagenesis can be compared by plotting melt deflections on a "Difference curve", with a normalized scale (see page 16). To do this, set the borders of melt deflection (X-axis) to a 10°C range, centered on the mean melting point. Adjust the pre-melt and post-melt border widths to 0.5°C each. Ensure that the lower border is set before melting begins in CRISPR injected embryos (a downwards bubble in the melt curves). Adjust the Difference-RFU scale (Y-axis) to encompass 0.21 to -0.51 Difference-RFU. These borders ensure that CRISPRs with a wide range of activities can be compared with one another on the same scale.
- Mutagenesis creates heteroduplex DNA which lowers melting temperature, shifting melt curves of CRISPR-injected embryo away from the uninjected embryo's melt curves. Uninjected embryo melt curves should cluster tightly together, whereas successfully mutagenized CRISPR-injected embryos will produce a variety of melt deflections.
- In F0 embryos, mutagenic CRISPR activity should induce a melt deflection of -0.2 to -0.4 Difference-RFU. Melt deflections causing | 0.05 | Difference-RFU or less indicate poor CRISPR activity, and often are observed even among wild-type embryos.
- When exporting images of melt curves, generate a cleaner image by deselecting the Grid Line and Minor Tick checkboxes under curve chart options. Save the image as a jpeg file with a resolution of 300 dots per inch.

VI) Recover and sequence F1 founders

Screen founders
We use HRMA to screen CRISPR-injected founders for germline transmission of potential mutants (putants). This analysis reveals which CRISPR-injected fish transmit putants, how many putants are found per founder, and which putant embryos are the best candidates for sequencing.

___Outcross CRISPR-injected F0 fish to a wild-type line such as AB.
___Collect and clean embryos at a few hours post-fertilization, and raise at 28.5˚C.
___At 1 to 3 days post fertilization, dechorionate 16 embryos per founder and lyse these embryos as described on page 15.
___Similarly process a set of 8 wild-type embryos, which will provide a reference curve to distinguish new mutations from wild-type polymorphisms or variation within a single wild-type genotype; this is particularly important when screening strongly mutagenized CRISPR founders, which may produce few to no wild-type offspring.
___Set up HRMA reactions as described on page 17.
___Analyze the samples as shown on page 20.
___Put away identified carriers in individual containers. Segregate, or sacrifice, F0 fish that are unlikely to carry mutations.
___Raise the remaining embryos from identified founders.
___Once an ideal lesion is selected (page 22), use the rate of that lesion to calculate the total number of fish required to make a stock of at least 6 F1 carriers. For example, if 1/16 embryos shows the desired lesion, then raise at least 96 fish. Continue outcrossing the identified founder until you have raised this number of fish.
___At least once during the subsequent outcrosses, perform HRMA on 16 embryos to confirm the germline transmission rate for the desired lesion.

HRMA instructions for F1 screening

- Ensure that amplification occurs properly, as described on page 18.
- Open up the melt file in Precision Melt Analysis software.
- Once in melt analysis software, scale the graph based on all embryos from all founders (the "All Founders graph"). Identify a single embryo towards the center of the wild-type melt curves (the "Mean Wild-Type Sample").
- Next, divide the plate into "groups" by founder family. Scale graphs for each family to match scaling in the All Founders graph. Include the Mean Wild-Type Sample in each group.
- Most heterozygous mutants (both insertions and deletions) will melt at a lower temperature than wild-type and show negative deflections. Be wary of difference curves that show many embryos with positive deflections (indicating a higher melting temperature than wild-type). This usually means that the wrong sample has been selected as the wild-type reference.
- For each founder family, specify the Mean Wild-Type Sample as a new cluster and set that cluster as the reference. This way, each founder family can be directly compared to one another and to the total set of founders.
- The Precision Melt Analysis software automatically clusters melt curve types together, but these clusters often need manual polishing. Look closely at the curves, and adjust the melt-clusters as necessary.
- Wild-type and putant curves typically make smooth (if complex) lines on melt and difference curves. Jagged, irregular, curves are suspect and may represent problems in amplification; exclude these from further analysis.
- Be wary of melt clusters that show the same deflection shape in multiple founders, which may be background alleles. To check for melt clusters common to multiple founders, adjust the colors from the All-Founders graph to match those seen in single-family graphs; this manual color adjustment often separates distinct putants that would have otherwise appeared to cluster together.
- If HRMA clusters are ambiguous, we often run the HRMA PCR products (1μl of each sample) on a 3% agarose gel. Many indels are large enough to generate distinct band patterns that can be used to refine clustering.
- Each validated melt-cluster typically represents a shared lesion. Different melt clusters typically represent different lesions.
- Give each melt-cluster a name; these are your putants. We name putants by gene, then founder number, then a letter for each cluster. For instance, *six1a* CRISPR founder 7 gave rise to three putants: *six1a-7a*, *six1a-7b*, *six1a-7c*, the "putant A, putant B, and putant C" shown in Figure 1 of the accompanying article.
- Record the identity (putant name or wild-type) of each well.
- The best founders transmit at least two clear putants, at high rates. Raise offspring from these founders, and prioritize these putants for sequencing.

Prepare heterozygous DNA for sequencing
Set up PCR reactions using the sequencing primers designed on page 6.
___Prepare master mix, using Phusion polymerase (New England Biolabs M0530L).

For 8 samples: Scalable recipe (total volume = V):
___ 80 μl Phusion HF buffer   ___ 1/5 V
___ 12 μl DMSO   ___ 1/33 V
___ 10 μl sequencing primer pair (10 μM each)   ___ 1/40 V
___ 8 μl dNTP mix (10 mM each)   ___ 1/50 V
___ 6 μl Phusion Taq   ___ 1/67 V
___ Sterile H_2O to 400 μl   ___ 71/100 V

___Distribute 50 μl master mix per tube in an 8-well strip.
___Add 2 μl template to each well.
___Place in PCR machine. Run the following program:

98°C 1 min, (98°C 10 sec, 60°C[*] 20 sec, 72°C 30 sec[†]) 40x, 72°C 5 mins, 4°C ∞ .
[*] Annealing temperature varies, but 60°C works well for most primers.
[†] Vary elongation time as appropriate for product size.

___Run PCR products on a 2% agarose gel.
___Gel extract the PCR products, as described on page 9. Expect 10-100 ng/μl product.
___Prepare products for sequencing. Set up forward and reverse sequencing reactions for each sample.

Notes on sequencing setup:
- Begin by sequencing four putants per gene. With this many putants, there is a 99% chance of finding a frameshift, and an 80% chance of finding a frameshift into a preferred frame (see page 22), assuming an equal distribution of mutations in each frame.
- To confirm lesions, sequence two embryos carrying each of these four putations.

Analyze sequences from heterozygous DNA

___Closely examine wild-type and putant sequences. Wild-type sequence should be normal, with one prominent read per base location. Heterozygous sequence will initially be normal, but shifts to "doubled reads" at base locations following an indel (see example on page 24).

___Align sequences against an annotated genomic wild-type reference. The doubled reads should begin within the CRISPR target.

___On the aligned sequences, change 25-30 bp of the "doubled-reads" after frameshift to ambiguous DNA coding: G/T = K   A/C = M   A/G = R   C/T = Y   C/G = S   A/T = W

___In a text file, you can manually "subtract" wild-type sequences from the doubled reads, leaving behind mutant (see example on page 24). To do this, copy/paste wild-type sequence into one line of text, and ambiguous sequence into the next. On the third line of text, generate the mutant sequence by typing the nucleotide option *not* found in wild-type. Align the mutant sequence to wild-type sequence to assess whether the lesion introduces a frameshifting indel.

___Alternatively, sequence separation can be done automatically, using Insilicase's Heterozygous InDel software: (http://www.insilicase.co.uk/Web/HeterozygousInDels.aspx). See Notes below.

___Generate modified mRNA files carrying each lesion and pick the best alleles.

___Prioritize alleles that introduce early stop codons and minimal aberrant amino acids (see Notes and example below).

___Once an ideal lesion is identified, outcross the appropriate founder to generate a large F1 stock. In addition, raise a second frameshifting lesion to confirm mutant phenotypes.

___During these subsequent outcrosses, perform HRMA on 16 embryos at least once, to confirm that the correct founder was identified and to double-check allele frequency.

Notes on sequence analysis

- Ideal lesions shift into a frame with the shortest stretch of aberrant protein before a stop codon is reached. Most alleles at a particular CRISPR target will cause frameshifts beginning within a few amino acids of one another, so minimizing the number aberrant amino acids before the stop codon usually takes precedence over finding earlier lesions.
- This analysis correctly identifies lesions from a single sequencing read. However, to reduce the possibility of errors, we sequence in both forward and reverse directions from two individuals carrying the same putation.
- The Heterozygous InDel software accurately determines mutant sequence frame, but manual inspection can usually improve the borders of alignment.
- The Heterozygous InDel software doesn't always provide the correct result on the first try, but errors are usually obvious. If errors occur, first check that wild-type and heterozygous sequence line up correctly. If this does not fix the output, then enter a longer stretch of sequence (>30 bp).
- The Heterozygous InDel software works well with >20 bp of doubled-reads, but shorter sequences should be manually analyzed as described above.

Example of allele selection: choosing between three *six1a* alleles

Three alleles were recovered from one mutagenized *six1a* founder (see accompanying article). All three alleles have lesions in similar locations, causing frameshift at aa 93, 93, and 94. *six1a^{oz9}* introduces the fewest aberrant amino acids (12 aa) prior to termination, and is our preferred allele for further analysis. *six1a^{oz8}* introduces 37 aberrant amino acids, and will be maintained for complementation tests. The third allele also introduces 37 aberrant amino acids, but will not be maintained because of facility space considerations and the similarity of this allele to *six1a^{oz8}*.

VII) Confirm lesions in F2 homozygotes

Identify adult F1 carriers

___Clip the distal third of the caudal fin from adult F1 fish using established protocols (e.g., https://zfin.org/zf_info/zfbook/chapt7/7.8.html). Place each fin clip in separate wells of a 96 well plate.

___Add 50 μl 1x Thermopol buffer (New England Biolabs, B9004S) to each well.

___Place in PCR machine. Run 95°C 10 minutes, 4°C hold.

___Add 5 μl 10 mg/ml Proteinase K to each well.

___Run 55°C 1 hour, 95°C 10 minutes, 4°C hold.

___Mix samples in each well, then spin the plate to pellet the larger pieces of fin residue.

___Dilute the fin clip preparations 5-fold, by transferring 10 μl fin clip lysis to new wells containing 40 μl low TE. Mix each well containing diluted fin clip. This dilution reduces the buffer variation caused by differing sizes of fin clip, and improves HRMA consistency.

___Set up HRMA reactions as described on page 17. Be sure to include at least two wild-type fin clip controls. Expect to find the same putant curve shapes as were found during initial screening.

___Sequence confirm a few identified heterozygous fish with the ideal lesion, as described above. Use these sequence-confirmed individuals to start the next generation.

Identify homozygous mutants in F2

___Incross identified F1 adults carrying the desired lesion, after they have recovered from fin clip for at least one week.

___Lyse embryos after they have grown to at least 1 dpf, as described on page 15.

___Genotype embryos using HRMA or restriction enzyme-based genotyping. HRMA does an outstanding job of discerning heterozygotes from wild-type embryos, however homozygous wild-type DNA cannot always be distinguished from homozygous mutant DNA using HRMA (see Notes below). For this reason, a restriction enzyme-based genotyping protocol is preferred at this step.

Specific notes about using HRMA on the F2 generation

- DNA homozygous for deletion alleles should usually show a curve with a lower melting temperature than wild-type DNA. DNA homozygous for insertion alleles should melt at a higher temperature than wild-type DNA.
- Heterozygous DNA normalized melt curves will typically be nonparallel to both wild-type and mutant curves.
- The difference between homozygous wild-type curves and homozygous mutant curves is often much subtler than the difference between either homozygote and the heterozygote. The distinctiveness of heterozygous melt curves is likely caused by heteroduplex DNA complexes, found in heterozygous PCR products.
- HRMA on F2 progeny is sometimes complicated by competition in PCR reactions between wild-type and mutant products of different sizes which may dramatically alter the ratio of amplified products. This is a particular concern when the shortest HRMA PCR products (~100 bp) are used to genotype large insertions or deletions. For instance heterozygotes carrying a 14 bp deletion allele have sometimes amplified the short (WT: 99 bp, mut: 85 bp) PCR products used for HRMA as if they were mutants.

Sequence the homozygous mutation

___After genotyping F2 embryos, set up a PCR reaction for sequencing using homozygous wild-type and mutant embryo lysis for templates, as described on page 21.

___Submit PCR products for sequencing.

___Align wild-type and mutant sequences to predicted files. This time, there should be no "doubled reads", and sequence analysis uses standard methods (see example below).

___Once sequences are confirmed in F2 individuals, mutant construction is completed, and allele designation should be assigned. Each research group must obtain a unique identifier for their alleles from ZFIN (http://zfin.org/action/feature/line-designations); for instance, the Amacher lab uses "oz" alleles to designate the lines we generate at Ohio State University.

Example of sequence analysis on *six1a^oz9* heterozygotes and homozygotes

1) Assign "ambiguous" base names to doubled reads in heterozygous sequence:

WT
TGTTGAGGCGGAAAAGTTGCGGGGGCGCCCGCTAGG

heterozygote "Doubled reads"
TGTTGARRMGKWRMRGKKGCGGRRRMGYYSSSKRGG
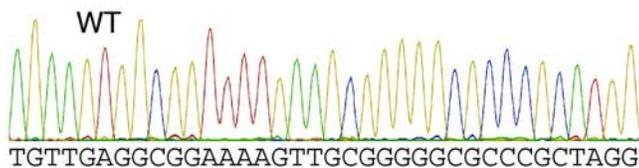
2) Subtract mutant from heterozygote:

WT: TGTTGAGGCGGAAAAGTTGCGGGGGCGCCCGCTAGG
het: TGTTGARRMGKWRMRGKKGCGGRRRMGYYSSSKRGG
mutant: TGTTGAAAAGTTGCGGGGGCGGAAAAGTTGCGGGGG
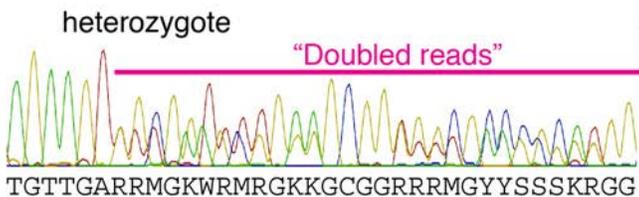
3) Align sequences:

WT: TGTTGA-----------GGCGGAAAAGTTGCGGGGG
mutant: TGTTGAAAAGTTGCGGGGGCGGAAAAGTTGCGGGGG

six1a^oz9 contains an 11 bp duplication

4) Heterozygous sequencing results are confirmed by homozygotes

WT
TGTTGA-----------GGCGGAAAAGTTGCGGGGGCGCCCGCTAGG

six1a^oz9
TGTTGAAAAGTTGCGGGGGCGGAAAAGTTGCGGGGGCGCCCGCTAGG
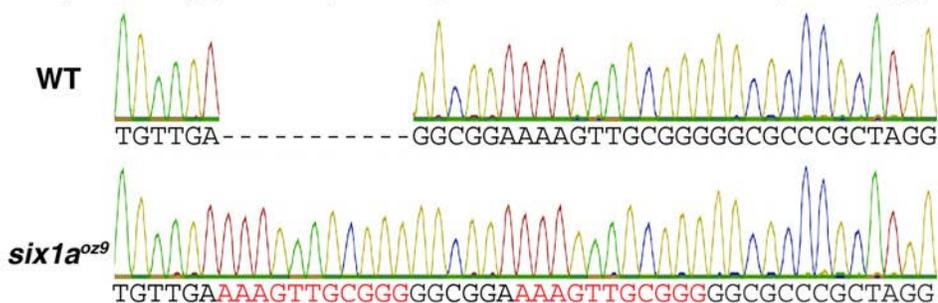
VIII) Validating the CRISPR pipeline

- CRISPR activity is sequence-specific. Previous deep sequencing analysis shows that off-target sites with 4-5 bp mismatches are mutagenized 25 to 50-fold less than the actual target site [12]. To extend this analysis of specificity, we tested two CRISPRs that differ from one another at only 2 bases. We found that both effectively mutagenized their intended target, but that mutagenesis at the alternative target site was undetectable by HRMA. In our experience, it is rare to find off-target sites with less than two basepair mismatch. However, if a CRISPR is identified with an off-target site having less than a 2 bp mismatch, we strongly recommend excluding that CRISPR during the design phase.

- Using CRISPR to a gene necessary for pigment formation (*tyrosinase*) [14], we find a good correlation between the degree of HRMA deflections and the degree of somatic mosaicism. For experimenters new to CRISPR mutagenesis, we recommend using a *tyrosinase* CRISPR as a side-by-side positive control.

- Somatic F0 CRISPR melt deflections also correlate well (R^2=0.43) with the number of putant HRMA curves transmitted through the germline. In our experience, the number of distinct putants recovered per founder can be calculated using the formula X=10.9Y, where

  > X= the average deflection (in | difference-CFU |) from the mean wild-type melt curve, assayed on F0 injected embryos

  > Y= the number of distinct putant classes found per founder in F1 outcross.

- Somatic F0 CRISPR melt deflections also predict (R^2=0.47) the total rate of putants per F1 embryo. In our experience, the number of putants found per embryo can be calculated using the formula X=2.0Y, where

  > X= the average deflection (in | difference-CFU |) from the mean wild-type melt curve, assayed on CRISPR injected F0 embryos

  > Y= the number of embryos in F1 outcross carrying lesions divided by the total number of embryos screened in F1 outcross.

- Putants analyzed on F1 embryos are good predictors of mutants recovered from F1 adults. For instance, all putant melt curve types identified to date from F1 embryos have also been seen when F1 adults are sequenced.

- For all lesions examined in both F1 and F2, the sequences found in F2 homozygotes have matched the mutant sequences predicted by F1 heterozygotes.

IX) Supplies and equipment

These are what we've tested in our lab; other versions may work too!

- CFX Connect system (Bio-Rad; 185-5201)
    - qPCR: "CFX manager Software" (Bio-Rad; 185-5201)
    - HRMA: "Precision Melt Analysis Software" (Bio-Rad; 184-5025)
- Ethanol (Fisher BP2818-100)
- Labnet MPS 1000 Mini Plate Spinner (Sigma; Z723533-1EA)
- LiCl, 8M solution (Sigma L7026)
- LB media and plates with 50 mg/ml Carbenicillin or 50 mg/ml Kanamycin
- MAXIscript T7 kit (Life Tech; AM1312)
- mMessage mMachine Sp6 kit (Life Tech; AM1340)
- Microseal 'B' Seals (Bio-Rad; MSB1001)
- Miniprep, QiaQuick PCR cleanup kit, MinElute gel extraction kits (Qiagen; 27104, 28104, 28604)
- Qiavac 24 plus vacuum manifold (Qiagen 19413) with VacConnectors (Qiagen 19407)
- Multiplate PCR plates Low 96-well, clear (Bio-Rad; MLL9601)
- Nucleospin RNA cleanup kit (Machery Nagel; 740955.10)
- pCS2-nCas9n (https://www.addgene.org/47929/)
- pDR274 (http://www.addgene.org/42250/)
- Proteinase K (Fisher; BP1700 100)
- Precision Melt HRM Supermix (Bio-Rad; 172-5112)
- T4 ligase with buffer (NEB; M0202S)
- Taq with Thermopol buffer (NEB; M0267S)
- Standard laboratory supplies for zebrafish research

X) Brief Bibliography

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