Video Article

Efficient Production and Identification of CRISPR/Cas9-generated Gene Knockouts in the Model System *Danio rerio*

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Abstract

Characterization of the clustered, regularly interspaced, short, palindromic repeat (CRISPR) system of *Streptococcus pyogenes* has enabled the development of a customizable platform to rapidly generate gene modifications in a wide variety of organisms, including zebrafish. CRISPR-based genome editing uses a single guide RNA (sgRNA) to target a CRISPR-associated (Cas) endonuclease to a genomic DNA (gDNA) target of interest, where the Cas endonuclease generates a double-strand break (DSB). Repair of DSBs by error-prone mechanisms lead to insertions and/or deletions (indels). This can cause frameshift mutations that often introduce a premature stop codon within the coding sequence, thus creating a protein-null allele. CRISPR-based genome engineering requires only a few molecular components and is easily introduced into zebrafish embryos by microinjection. This protocol describes the methods used to generate CRISPR reagents for zebrafish microinjection and to identify fish exhibiting germline transmission of CRISPR-modified genes. These methods include *in vitro* transcription of sgRNAs, microinjection of CRISPR reagents, identification of indels induced at the target site using a PCR-based method called a heteroduplex mobility assay (HMA), and characterization of the indels using both a low throughput and a powerful next-generation sequencing (NGS)-based approach that can analyze multiple PCR products collected from heterozygous fish. This protocol is streamlined to minimize both the number of fish required and the types of equipment needed to perform the analyses. Furthermore, this protocol is designed to be amenable for use by laboratory personal of all levels of experience including undergraduates, enabling this powerful tool to be economically employed by any research group interested in performing CRISPR-based genomic modification in zebrafish.

Video Link

The video component of this article can be found at https://www.jove.com/video/56969/

Introduction

The conservation of molecular machinery across eukaryotes underlies the power of using model organisms for research. Many of these model systems facilitate the use of reverse-genetic approaches such as targeted gene knockouts to characterize the contribution of a gene product to a biological or disease process of interest. Gene disruption techniques in organisms such as zebrafish have historically relied on targeted introduction of frameshift mutations that result from imprecise repair of DSBs[1,2]. When a DSB is introduced into the genome, the DNA lesion is repaired through one of two pathways that are universally present in nearly all cell types and organisms: non-homologous end joining (NHEJ) and homology-directed repair (HDR)[3,4]. The imprecise nature of the NHEJ machinery frequently produces indels of various lengths[5,6,7,8,9]. Introduction of frameshift mutations in the coding sequence of a gene can produce a premature stop codon, which often renders the gene nonfunctional.

Early genome engineering strategies in zebrafish to promote indels included meganucleases, zinc-finger nucleases, and transcription activator-like effector nucleases, all of which utilized DNA-protein interactions to target a nuclease to a specific genomic target where it introduced a DSB[10,11,12,13,14,15]. However, these technologies are often difficult to apply due to the laborious and complex engineering needed to generate a nuclease that targets the DNA sequence of interest. Unlike previous strategies, CRISPR-based gene editing does not rely on protein-DNA interactions for targeting. Instead, the CRISPR-associated (Cas) endonuclease is directed via an RNA guide that uses nucleotide base pairing interactions to target a genomic site of interest[16,17,18,19,20,21]. Due to the simplicity of designing a RNA guide with the desired base pairing interactions for targeting it is relatively easy to target the Cas endonuclease to the desired locus. The type II CRISPR system in particular has been widely developed for genome editing applications due to several advantageous features including use of a single multidomain Cas nuclease (Cas9) that requires interaction with DNA to stimulate endonuclease activity and use of a single guide RNA (sgRNA) to target it to the cognate DNA sequence[18]. The sequence requirements necessary for targeting of the cognate sgRNA are well understood[19], and the desired sgRNA is easily generated by *in vitro* transcription. The simplicity and robustness of the CRISPR/Cas9 approach greatly facilitates targeted genetic modification in zebrafish and a wide variety of other organisms.

The enhanced ability to undertake targeted genome editing in zebrafish as a result of developing CRISPR-based reagents has significantly increased the opportunity to study processes emblematic of vertebrate organisms such as development of the central nervous system. The

zebrafish genome contains orthologs of 70% of the protein-coding genes found in the human genome as well as 84% of genes associated with diseases in humans[22]. Zebrafish development exhibits several key qualities that enhance its use in reverse genetic studies: the embryos are laid in large clutches, develop externally from the mother making them amenable to genetic manipulation by microinjection, and adult zebrafish sexually mature by 3 months of age, allowing for rapid propagation of desired lines[23].

Numerous protocols are available that describe a variety of approaches to generate and identify CRISPR-derived indels in zebrafish[24,25,26,27,28,29,30,31]. However, many of these procedures are time intensive, require access to expensive equipment, and can be challenging for labs with limited expertise. The steps described herein provide a simple, robust, and economical CRISPR/Cas9-strategy to engineer zebrafish knockout lines. This protocol describes the use of a highly efficient kit to synthesize sgRNAs using DNA oligonucleotides (oligos), similar to other approaches that have been previously described[32]. The described protocol includes two steps in particular that greatly simplify analysis of CRISPR-mutated lines: step-by-step use of the PCR-based HMA[33] to easily determine the presence of genome modifications, and sequencing analysis of heterozygous zebrafish to rapidly and easily determine the nature of multiple indels in an economical fashion. In addition, step-by-step instructions are included for robust selection, reliable production, and injection of guide RNAs. The steps provided here exemplify a robust, relatively inexpensive protocol that enables laboratory personnel with a range of expertise to contribute to the identification of gene knockouts in zebrafish.

Protocol

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Purdue Animal Care and Use Committee (PACUC number 08-031-11).

1. Design of Template-specific Oligos for Guide RNA Production

1. Select the target region of interest to be modified in the coding region of the gene. This should be close to the 5' end of the gene to generate a truncated protein, but not so close such that a subsequent in-frame start codon enables production of a protein with a modest N-terminal truncation.
   NOTE: One way to preclude this possibility is to scan the downstream coding region of the gene for an in frame start codon. In addition, using a guide RNA that targets the middle of a large exon, can help to identify PCR primers for subsequent scoring of the resulting indel.
2. Identify potential guide sites in the genomic region of interest using a guide RNA selection program (see **Table of Materials**)[34,35,36]. Set the browser data to *Danio rerio* and the protospacer adjacent motif (PAM) sequence to 5'- NGG -3'.
   NOTE: Ideal gRNA sequences contain a 5′-G in the first position of the gRNA for efficient T7 *in vitro* transcription. If no acceptable guide is found with a G at the 5′ position, the 5′ base of another guide can be altered to a G or a G can be added onto the 5′ end of the guide RNA, but this may reduce cutting efficiency[37]. To maximize the cutting efficiency, an optimal guide sequence has 40-80% GC content (higher is better), and contains a G at the 20th position, adjacent to the PAM, but is not required[38]. An example of an ideal targeting sequence: 5′- G $(N)_{18}$ G -3′ -NGG (NGG is the PAM). In addition to examining the results from the guide RNA selection program to identify optimal guide RNAs as described above, care should be taken to avoid guide RNAs with predicted strong off-target effects which greatly complicate downstream analysis. In particular, guide RNAs with predicted off-target effects that fall within coding regions should be excluded, and the total off-target sites predicted should be minimized.
3. From the output of the guide RNA design tool, exclude the PAM sequence (5′- NGG -3′); it is not used for targeting but comprises the recognition sequence for Cas9 cleavage.
4. To the remaining 20 nucleotides (nts), add the T7 promoter sequence and the overlap sequence (region complementary to a scaffold oligo used to synthesize full length sgRNAs supplied in the recommended *in vitro* transcription kit) in the order indicated below to obtain a 54 nt oligo: T7 promoter sequence: 5′- TTCTAATACGACTCACTATA -3′; Guide RNA sequence 5′- G $(N)_{18}$ G -3′; Overlap sequence: 5′- GTTTTAGAGCTAGA -3′
5. Identify PCR primers that flank the predicted cut site (Cas9 cleaves 3 nt upstream of the PAM sequence) at a distance of 50–150 base pairs (bp) each from the cut using web-based software (see **Table of Materials**).
   NOTE: These will be used in a later step for measurement of cutting efficiency. If no suitable primers are identified using these constraints, another guide RNA site may need to be considered.
6. Order 54 nt oligonucleotides to produce the guide RNA and PCR primers for analysis of the target sites (see **Table of Materials**).
   NOTE: As an optional positive control, it may be helpful to produce a sgRNA targeting a gene necessary for production of pigment to verify the performance of this protocol using an easily scored visual phenotype (see **Figure 1** for representative results). A common target is the gene *tyrosinase*, using the oligo (guide RNA sequence is underlined)[39]: 5′- TTCTAATACGACTCACTATAGGACTGGAGGACTTCTGGGGGTTTTAGAGCTAGA -3′

2. Preparation of CRISPR-reagents for Embryo Microinjection

1. **Order commercially available Cas9 protein (see Table of Materials).**
   NOTE: Injection of Cas9 mRNA can also be used to generate indels in zebrafish[40,41], however zebrafish embryo microinjection with Cas9 protein has been shown to be more efficient[32,42].
   1. Suspend the Cas9 protein in the supplied buffer to generate a 1 mg/mL solution. Store the solution in injection-ready aliquots in PCR tubes at -80 °C to minimize the number of freeze-thaw cycles. To generate a 5 µL injection solution, 2 µL of 1 mg/mL Cas9 solution is used, therefore the Cas9 can be aliquoted in 2 µL aliquots using PCR strip-tubes.

2. Synthesize the sgRNA using the sgRNA *in vitro* transcription kit (see **Table of Materials**). Perform the *in vitro* transcription as per the manufacturer's instructions.
   NOTE: Maintain RNase-free technique during all synthesis, clean-up, and injection solution preparation steps. For example, use disposable gloves and change them frequently, use tubes and tips that are certified RNase-free, and clean surfaces and pipettes with commercially available solutions to decontaminate labware (see **Table of Materials**).

3. **Purify the synthesized sgRNA using an ammonium/acetate precipitation using RNase-free technique.**
   NOTE: Alternatively, sgRNAs can be purified using a variety of commercially available column-based RNA clean up kits for a relatively modest cost.
   1. Add 25 µL of 5 M ammonium acetate and vortex to mix thoroughly.
      NOTE: Ammonium acetate solution is commercially available (see **Table of Materials**), or a 5 M solution can be made in house by adding 385.4 mg of molecular grade ammonium acetate to 1 mL of RNase-free water and stored at -20 °C.
   2. Add 150 µL of 200-proof nuclease-free ethanol to each sample. Place the reaction in a -80 °C freezer for a minimum of 20 min.
      NOTE: The samples can be stored overnight at -80 °C, but will not significantly increase the total RNA yield.
   3. Centrifuge the samples at maximum speed (>16,000 x g) in a 4 °C microcentrifuge for 20 min.
   4. Remove the supernatant carefully by slowly pipetting off the liquid, ensuring the RNA pellet is not disturbed.
   5. Add 1 mL of 70% ethanol (created by diluting nuclease-free ethanol in RNase-free water) and gently mix the tube by inverting it several times to wash residual salt from the tube.
   6. Repeat the centrifugation step for 7 min.
   7. Remove the supernatant by first pipetting off most of the solution using a P1000 pipette, then use a P200 pipette to remove as much solution as possible without perturbing the pellet. Dry the RNA pellet in a clean space, such as a laminar flow hood or a bench top, being careful to avoid RNase contamination, for 15 min or until no more liquid drops are visible in the tube.
   8. Resuspend the pellet in 30 µL of RNase-free water, quantify the product (for example using a spectrophotometer), and aliquot the solution for long-term storage in a -80 °C freezer.
      NOTE: Typical concentrations range from 800–2,500 ng/µL.

4. **(OPTIONAL) Verify that full-length RNA has been generated using urea/PAGE. Alternatively, use an agarose gel to verify that the RNA is intact.**
   NOTE: However, if using an agarose gel a larger amount of gRNA must be run to visualize the RNA, and the length cannot be accurately determined. When analyzing the efficiency of target cutting after injection of the reagents into the fish, if there is no or little cutting present then the sgRNA should be checked for degradation.
   1. Cast an 8% polyacrylamide gel in TBE with 40% polyacrylamide (19:1) and 8 M urea using RNAse-free technique for the solutions and equipment[43].
      NOTE: Commercially available materials can be used to clean equipment (see **Table of Materials**).
   2. After the gel has completely solidified (approximately 30 min), equilibrate the gel by placing it in TBE running buffer and performing electrophoresis for 30 min at 5 V/cm.
   3. Mix 300–500 ng of sgRNA with an equal volume of 2x RNA gel loading dye (see **Table of Materials**). Using a P1000, clear the wells of any debris by pipetting running buffer in each well several times. Load the solution(s) and run the gel at 10 V/cm for 2.5 h.
      NOTE: A marker lane here is useful to visualize the length of the RNA but is not required; generally it is readily apparent if full length RNA has been synthesized (**Figure 2**).
   4. Visualize the bands using a nucleic acid stain (see **Table of Materials**).
      NOTE: sgRNA bands should appear as a single band, whereas smearing indicates RNA degradation (**Figure 2**).

3. Microinjection of CRISPR-components into Zebrafish Embryos

1. Set up breeding tanks the night prior to injecting by placing the number of desired males and females (typically 2 females and 1 or 2 males) in a breeding tank with a divider in place[44].
2. Prepare a microinjection plate with 1.5% agarose in 1x E3 media (see **Table of Materials**) with 0.01% methylene blue (a fungicide) by pouring 35 mL of the melted agarose into a 10 cm Petri dish and gently lay a plastic mold to create wedge-shaped troughs into the solution, tapping the mold to eliminate air bubbles.
3. Allow the agarose to set, and store the dish with a small amount of media and wrapped in paraffin film to prevent the plate from drying out at 4 °C.
   NOTE: Injection plates are reusable for several weeks, until the wells become deformed or dry, or the plate begins to grow mold.
4. On the morning of injecting, thaw purified sgRNA and Cas9 protein on ice. Remember to handle all materials with gloves to prevent RNase contamination and to use RNase-free tips and tubes.
5. Generate a 5 µL injection solution by combining Cas9 protein and the sgRNA in a 2:1 ratio of Cas9:sgRNA to obtain final concentrations of 400 pg/nL Cas9 protein and 200 pg/nL sgRNA. Incubate the Cas9/sgRNA solution at room temperature for 5 min to allow the Cas9 and sgRNA to form a ribonucleoprotein complex. Add 0.5 µL of 2.5% wt/vol phenol red solution (see **Table of Materials**), and RNase-free water to a final volume of 5 µL.
   NOTE: The ionic strength of the solution has been shown to affect the solubility of the Cas9/sgRNA complex, therefore the addition of KCl may increase the cutting efficiency of sgRNAs that exhibit low indel formation[28].
6. Make an injection needle by pulling a 1.0 mm glass capillary using a micropipette puller. Cut the tip of the freshly-made needle using a new razor blade or forceps to obtain an angled opening that will easily pierce the chorion and yolk sac.
7. Place the needle in a micromanipulator attached to a microinjector with the air source turned on. Under a light microscope using the magnification suitable for the calibration determined for the particular apparatus, adjust the injection pressure until the needle consistently ejects a 1 nL solution into a Petri dish filled with mineral oil.
   NOTE: The quality of the needle is critical. Practice producing a needle and injecting into the yolk sac of embryos until this is skill is mastered before attempting further experiments[45].
8. Remove the divider and allow the fish to breed for approximately 15 min.
   NOTE: Longer breeding times will produce more embryos, however the injection should be completed while the embryos are at the 1-cell stage to maximize the chance that Cas9 cutting will occur early and therefore decrease genetic mosaicism. Embryos can be injected at later stages (2–4 cell stages), but this may possibly decrease the germline transmission rate of the modified allele.
9. Collect the eggs using a strainer and rinse them into a 10 cm Petri dish using 1x E3 media with 0.0001% methylene blue. Examine the health of the eggs under the light microscope, removing any unfertilized eggs and debris.

10. Set aside 10–15 embryos as an uninjected control in a separate, labeled Petri dish.
11. Using a transfer pipette, gently line up the eggs on the injection plate warmed to room temperature.
12. Under a dissection microscope at 2.5X magnification, inject 1 nL of the solution into the yolk sac of each embryo to inject a total of 400 pg of Cas9 protein and 200 pg of sgRNA.
    NOTE: To increase cutting if desired or necessary, increase the final concentration of Cas9 protein to 800 pg/nL and of sgRNA to 400 pg/nL in the injection solution; however, this may also increase off-target cutting and/or decrease embryo health. Cutting efficiency may also be increased by injecting directly into the cell[46]. However, injection into the yolk sack is technically less demanding and gives sufficient cutting to produce fish with high germline transmission (>70% of offspring containing a modified allele).
13. Return the injected embryos to a properly labeled Petri dish, cover them with 1x E3 media with methylene blue, and put them in an embryo incubator set to 28 °C.
14. At 24 h post fertilization (hpf), inspect the health of the injected embryos, removing dead or abnormally developing individuals and change the media (See **Figure 3**). Check the rate of survival against the uninjected control.
    NOTE: When targeting a nonessential gene, less than 10% lethality is expected relative to the uninjected control. If elevated levels of lethality are observed in the guide-injected populations compared to the uninjected control, it may indicate that the targeted gene is essential for development, or off-target effects are leading to failed development. Reducing the amount of injected CRISPR-reagents may be necessary or generation of a new sgRNA with reduced off-target effects may be required.
15. Return the embryos to the incubator and continue growing the embryos to 72 hpf, changing the media daily to maintain embryo health.

4. Analysis of Efficiency of Indel Formation Using an HMA

1. Collect two sets of five embryos from the injected plates grown to 72 hpf into microcentrifuge tubes and collect one set of five embryos from an uninjected control.
2. Anesthetize the embryos by adding 0.004% MS-222 (tricaine) and wait 2 min.
3. To extract the gDNA, gently pipette the media off each embryo set and add 45 µL of 50 mM NaOH. Incubate the embryos at 95 °C for 10 min.
4. Remove embryos from the heat source and cool to room temperature. Add 5 µL of 1 M Tris-HCl pH = 8, and vortex the samples vigorously (5–10 s). Centrifuge the solution at max speed (>16,000 x g) in a room temperature microcentrifuge for 3 min. Transfer the supernatant to a clean, labeled tube and store the gDNA at -20 °C DNA for up to 6 months.
   NOTE: DNA fragments of approximately 900 bp and smaller will be generated through use of this protocol.
5. Set up a 50 µL PCR reaction using 2 µL of the prepared gDNA from each sample (including an uninjected control) per the instructions included with the polymerase, using the previously designed primers flanking the predicted cut site.
6. Purify the PCR products using a PCR clean up kit (see **Table of Materials**), elute the samples in 30 µL of water or elution buffer and quantify the DNA using a spectrophotometer.
7. Reanneal all 30 µL of each purified PCR product by placing the tubes in a floatable rack in a boiling water bath (approximately 150 mL in a 500 mL beaker). After 3 min, turn off the heat source and allow the solutions to cool to room temperature, about 1 h.
   NOTE: This step first denatures the DNA and then allows the strands to reanneal randomly to generate possible heteroduplex bands, or mismatched double-strand DNA that contains polymorphisms created by CRISPR-mutagenesis and therefore have an altered electrophoretic mobility compared to homoduplexes. The last cycle of PCR or ramp-down program in the thermocycler can also be used to reanneal products[47,48], but use of the boiling bath may yield improved resolution of heteroduplex products.
8. Add 5 µL of 6x loading dye (see **Table of Materials**) to the reannealed PCR solutions.
9. Cast a 15% polyacrylamide/TBE gel using 30% polyacrylamide (29:1). After the gel has set, place it into an electrophoresis apparatus with TBE running buffer. Using a P1000, clear the wells of any debris such as residual salts or gel fragments that can obstruct the wells by gently pipetting buffer up and down into the wells several times.
10. Load 500 ng of the reannealed PCR products, and load a control (sample from uninjected fish) next to each set of sgRNA samples. Run the gel at 150 V for 2.5 h or until the dye front is at the bottom of the gel.
11. Visualize the bands using a nucleic acid stain (*e.g.*, ethidium bromide or SYBR green (see **Table of Materials**)).
12. Examine the band pattern for each control and CRISPR-injected pool of embryos.
    NOTE: The appearance of multiple bands that run slower in the injected versus uninjected lanes indicates formation of novel heteroduplex products (**Figure 4**). The presence of novel heteroduplex DNA indicates that indels were generated by the CRISPR-injection. Reduction in the homoduplex band intensity in the injected solutions of approximately 50% or greater is generally sufficient to result in sufficient germline transmission. Additionally, extra bands are sometimes identified in the uninjected fish, and should not be considered heteroduplex bands when observed in the injected fish.
13. Choose the injections with highest cutting efficiencies relative to the uninjected control for each target; embryos from these injections can be used to grow up fish to look for indels causing premature stop codons.
    NOTE: For highly efficient cutting (reduction in the homoduplex band by approximately > 50% intensity), screening 20–30 adult fish should be sufficient to obtain germline transmission.
    For sgRNAs that generate less cutting, more adult fish may be needed to increase the likelihood of identifying fish that exhibit germline transmission of modified alleles containing a premature stop codon. If indel formation is not observed it may be necessary to redesign the sgRNA to a different region of the gene. If no heteroduplex band formation is observed, the sgRNA may have degraded, and the sgRNA quality should be verified using a urea/PAGE gel.

5. Identification and Propagation of Knock-out Lines

1. To identify a potential founder parent fish, perform tail clips of the adult F0 fish (grown to approximately 2.5–3 months) to identify presence of indels. Anesthetize the fish in 0.62 mM tricaine (see **Table of Materials**), then use a clean, sharp razor blade to remove approximately 1/2-3/4 of the tail fin.
2. Place the tail in 45 µL of 50 mM NaOH, and return the fish to a recovery tank. Perform the gDNA extraction as described (steps 4.2–4.4). Once the fish resumes normal swimming behavior, place the fish back on flowing system water until the nature of the indel is identified.
   NOTE: It is critical to ensure that individual fish are identifiable, and can be appropriately matched to the results of the tail clips.

3. As described (steps 4.5–4.9), perform a HMA on the tail clip gDNA to determine if the fish was modified by the CRISPR injection (**Figure 5**). To identify a founder fish, breed the adults that exhibit heteroduplex bands from tail gDNA to wild-type fish. Collect the embryos and grow them to 72 hpf.
4. At 72 hpf, collect 10 embryos and place each embryo in an individual tube. Perform a gDNA extraction as described above (steps 4.2–4.4) using 11.25 µL of 50 mM NaOH and 1.25 µL of 1 M Tris pH = 8.
5. Repeat the PCR and electrophoresis to identify heteroduplex bands as described above (steps 4.7–4.13) to determine if indel alleles have been passed on to this generation (**Figure 6**).
6. Based on the percent transmission observed in single embryos using the HMA, grow an average of 20–30 embryos obtained by the cross for each CRISPR-lines of interest to adulthood.
   NOTE: This number may be increased or decreased depending on the frequency of germline transmission.
7. Perform tail clips of the adult F1 fish to identify presence of indels as described (steps 5.1–5.2). As described (steps 4.5–4.11), perform an HMA on the tail clip gDNA to determine if the fish carries an indel (**Figure 7**).
8. **For fish that contain a heteroduplex band, prepare the DNA for sequencing analysis.**
   1. Perform PCR using new primers to amplify a 300–600 bp PCR product centered around the cut site.
   2. Use a PCR purification kit to clean up the DNA, and elute in 30 µL. Examine the DNA on an agarose gel to ensure a single band is present.
      NOTE: Some heteroduplex banding may be present on the agarose gel and appears as a small smear or double band just above the PCR product at the expected size.
9. If one to three indels are analyzed, sequence the PCR products using Sanger sequencing and determine the sequence of the indel using a bioinformatics tool[49]. Otherwise, the determination of the sequence of multiple PCR products using NGS analysis (see **Table of Materials**) is more economical.
10. Determine if a premature stop codon has been obtained by analyzing the sequence using a bioinformatics tool (see **Table of Materials**).
11. Place the fish containing desired mutant allele into a new tank with appropriate labels.
12. Design PCR primers for specific indel alleles for future genotyping needs. These primers should span the mutated sequence and not amplify the wild-type sequence.
13. In-cross heterozygous zebrafish to generate a segregating population that will contain 25% knock-out lines.

Representative Results

The experimental approaches described in this protocol allow for efficient, cost-effective production of zebrafish knock-out lines using CRISPR/Cas9 technology. The following figures have been included in this article to facilitate interpretation and troubleshooting of the results obtained using this protocol. Following successful production and microinjection of CRISPR-reagents, the zebrafish embryos can be analyzed for overt phenotypes and for indel formation using HMA. A helpful control to visualize the success of the CRISPR-experiment is the use of the sgRNA described in step 1.5 to target the pigment-producing gene *tyrosinase.* Cas9-induced indel formation at *tyrosinase* results in loss of pigmentation and is easily scored by 48 hpf (**Figure 1**). Another helpful control to ensure that preparation of the CRISPR-reagents for injection has been successful, is to verify that full-length (120 nt) sgRNA has been synthesized using a denaturing polyacrylamide gel (**Figure 2**, Lane 1 and 2). If the RNA has been degraded it may appear as a smear, for example Lane 3 (**Figure 2**) shows degraded RNA that is not suitable for injection.

To analyze the indel formation frequency of genes targeted by CRISPR-Cas9 that do not result in overt phenotypes such as *tyrosinase,* HMA analysis is a simple and reliable method. sgRNA/Cas9 injected embryos analyzed using HMA results in the formation of heteroduplex bands, and reduction of the intensity of the homoduplex band (**Figure 4**). The presence of heteroduplex bands is further utilized in this protocol to identify potential founder fish from the microinjected embryos and as adults (**Figure 4** and **Figure 5**), to analyze the germline transmission efficiency of a founder (**Figure 6**), and to verify presence of an indel in a heterozygous F1 fish (**Figure 7**). The heterozygous fish that contain an indel are candidates for NGS to identify the nature of the indel and to determine if a premature stop codon is present in the coding region of the target gene.

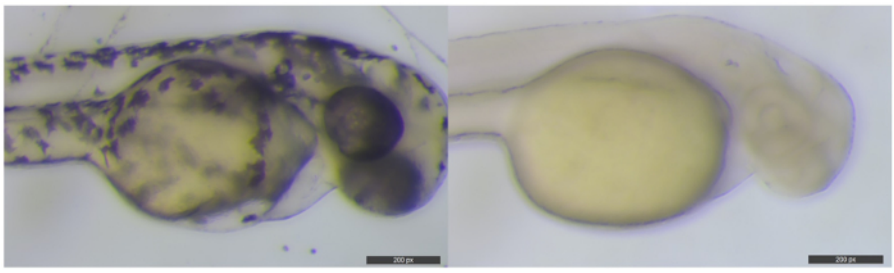

Figure 1: Zebrafish embryos exhibit a pigment defect when injected with a sgRNA targeting *tyrosinase* at the one-cell stage. (**A**) Wild-type, uninjected embryo at 48 hpf and (**B**) injected embryo at 48 hpf. Please click here to view a larger version of this figure.

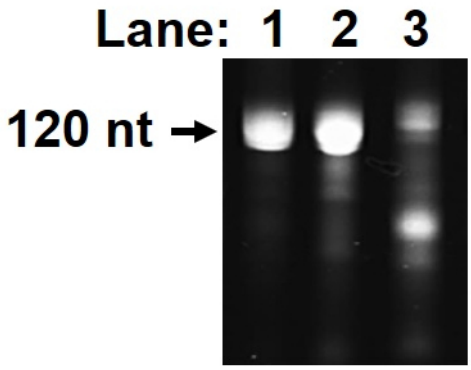

Figure 2: *In vitro* transcription of sgRNA using synthesis kit. Oligos were synthesized using *in vitro* transcription according to the sgRNA synthesis kit instructions. 500 ng of RNA was run on a urea/PAGE gel as described. sgRNA loaded in lanes 1 and 2 shows a band corresponding to the full length, intact 120 nt RNA. The sgRNA in lane 3 shows a degraded RNA sample that is not suitable for injection.

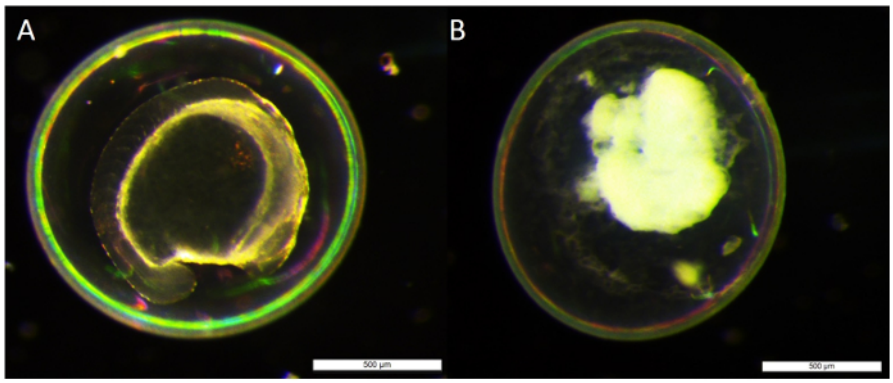

Figure 3: Comparison of the health of 24 hpf injected embryos. A living embryo (**A**) developed to 24 hpf, is easily distinguished from an embryo that has aborted development (**B**). Embryos that resemble (B) or have drastically altered features to (A), such as spinal curvature or altered head and eye development should be removed from dish. Please click here to view a larger version of this figure.

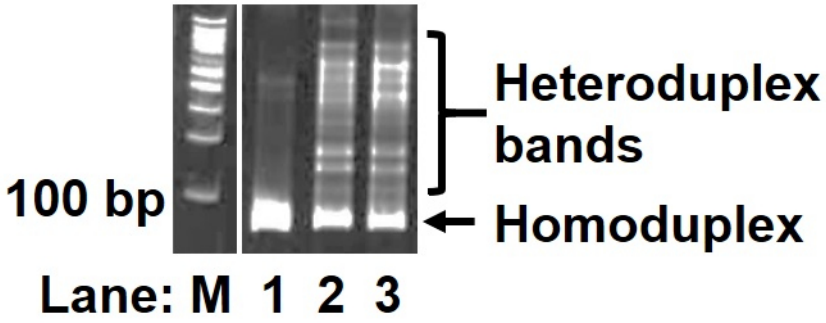

Figure 4: Heteroduplex mobility assay of sgRNA-Cas9 microinjected zebrafish embryos. Pools of 5 embryos per sample were collected at 72 hpf and gDNA was extracted. Heteroduplex analysis was performed as described, samples were loaded equally with 500 ng of DNA. Lanes: M = 100 bp marker; 1 = uninjected control; 2 = injection sample 1; 3 = injection sample 2. Expected band size = 98 bp.

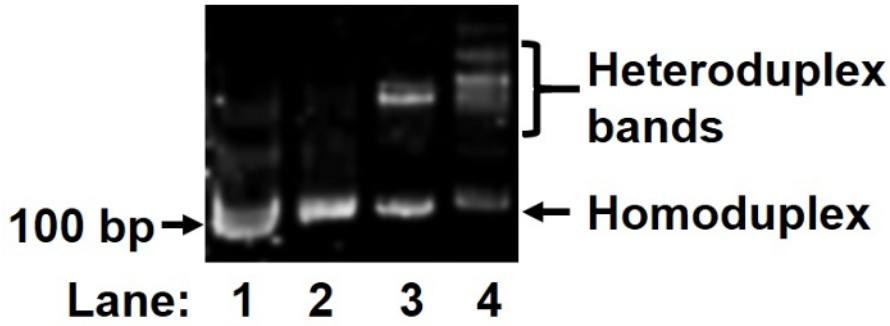

Figure 5: Heteroduplex mobility assay of gDNA extracted from the tail of an adult CRISPR-injected zebrafish. Embryos that were injected with an sgRNA and Cas9 protein were grown to adulthood (3 months). Fish B and C exhibit heteroduplex bands and were subsequently bred to identify germline transmitted indels; fish A was not used in subsequent analysis because it does not exhibit a positive heteroduplex band. Lanes: 1 = wild-type control; 2 = Fish A; 3 = Fish B; 4 = Fish C. Expected band size = 98 bp. Please click here to view a larger version of this figure.

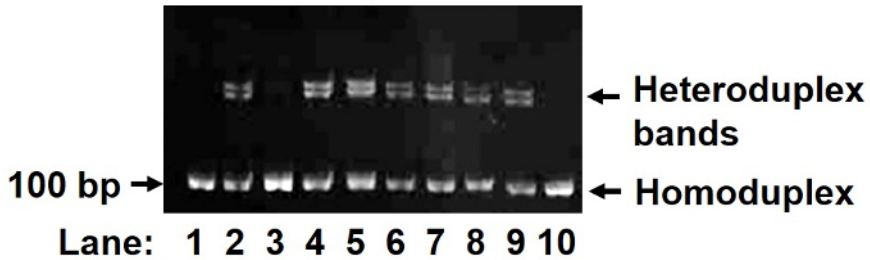

Figure 6: Heteroduplex mobility assay of single embryos generated by breeding a F0 CRISPR-injected zebrafish to a wild-type fish to identify germline transmitted indels. Zebrafish were mated, and the F1 embryos grown for 72 h. Single embryos were collected and heteroduplex analysis performed as described. Lanes: 1 = wild-type control; 2-10 = a single F1 embryo per lane. This gel shows that 7 out of 10 embryos show a positive heteroduplex band, indicating a germline transmission rate of 70% of the indel. Expected band size = 98 bp. Please click here to view a larger version of this figure.

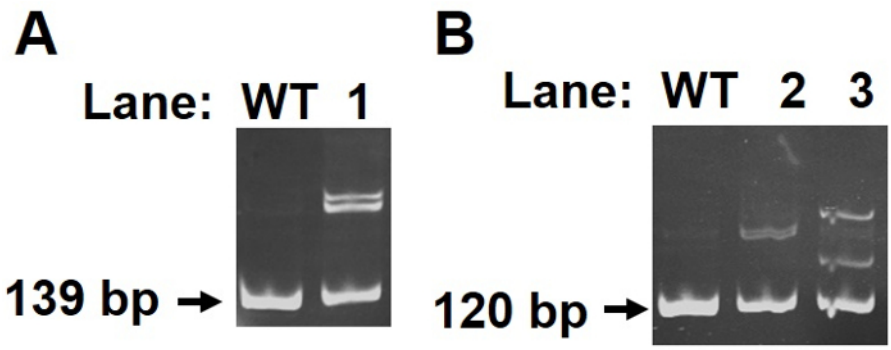

Figure 7: Heteroduplex mobility assay of adult F1 zebrafish tail clips. Adult F1 fish were scored by HMA to identify indels. Fish that exhibited a positive heteroduplex band were PCR amplified and submitted for wide sequencing analysis to determine the nature of the mutation. (**A**) Lanes: 1 = wild-type control; 2 = fish A (4 bp deletion, 1 bp mismatch). (**B**) These F1 fish were identified from the same founder, and yet show different heteroduplex patterning, indicating germline transmission of multiple modified alleles from a single founder. Lanes: 1 = wild-type control; 2 = fish B (4 bp insertion, 7 bp mismatch), 3 = fish C (4 bp deletion, 4 bp mismatch). Each of these indels created a premature stop codon in the coding sequence of the target gene, as determined by NGS. Please click here to view a larger version of this figure.

Discussion

This protocol describes the production of gene knockouts in the zebrafish vertebrate model system using CRISPR-Cas9 technology. A number of protocols have previously been described to undertake CRISPR-mediated genome engineering in zebrafish[15,25,26,50,51,52]. This protocol builds on previous efforts by combining a number of simple yet reproducibly consistent experimental techniques, in particular HMA and NGS of multiple heterozygous fish, to create a straightforward, economical, and experimentally robust protocol for CRISPR-mediated mutagenesis in zebrafish that is appropriate for labs staffed with personnel with a range of training and experience, as well as teaching labs.

Recommendations for design and synthesis of guide RNAs are included in this protocol. A major consideration in guide RNA design is the minimization of off-target effects. Several prediction algorithms have been developed to allow CRISPR-users to access computation tools with user-friendly graphical interfaces that predict both the activity of the on-target guide and the chance of off-target effects[34,35,36]. A specific advantage of the zebrafish system is lowered rates of off-target effects because the Cas9 is injected into the embryos and therefore expression is transient, which has been shown in mice to result in decreased off-target effects[53]. Nevertheless, off-target effects have been demonstrated

to occur in zebrafish[54]. One way to control for off-target effects is to phenotype founder zebrafish that have been generated by two independent guide RNAs that target the same gene, as these guides would be very likely to affect different off-target sites. An alternative method to minimize off-target effects that is not described in this protocol is the use of a mutated Cas9 that generates single strand breaks at the target DNA, which are repaired with high efficiency. Pairing DNA nicks within proximity of one another that are complementary to the opposite strands results in effective indel formation at the desired locus and minimizes off-target effects[55,56].

In addition to having different rates of off-target effects, different sgRNAs can have different rates of mutagenesis of the desired target[57,58,59]. This protocol uses HMA to analyze the efficiency of mutagenesis of a given sgRNA using heteroduplex band formation[33,40]. Heteroduplex bands are created by hybridization of PCR-generated DNA strands that contain mismatches, and can be easily resolved using gel electrophoresis. Unlike other methods commonly used to measure indel formation, such as the T7 endonuclease assay or high resolution melt analysis[25,26], HMA does not require an expensive enzyme to cut mismatched DNA, and does not require complicated analysis of PCR melt curves. Importantly, using HMA to verify high rates of indel formation in the injected population also enables the investigator to minimize the number of fish needed for subsequent production of knock-out lines, which reduces the cost of identifying a mutation with the desired characteristics.

The relative ease of generating CRISPR-based indels enables creation of multiple alleles of multiple genes at once. Web-based software is available for analysis of single mutations from heterozygous fish using Sanger sequencing of PCR products[49]. In the case where three or more CRISPR-mutated alleles are analyzed, NGS to characterize the nature of the indel is likely to be more cost effective to characterize the nature of the indel as this approach allows a pool of up to 50 different alleles to be characterized at once (see **Table of Materials**)[60,61,62]. Such economy of scale would likely be particularly useful in an undergraduate laboratory setting.

In summary, this protocol provides step-by-step directions for reproducibly generating high quality CRISPR-reagents (in particular, sgRNA) such that fewer adult fish need to be created and analyzed to successfully identify the mutant alleles of interest, which also reduces the time and cost of generating the desired lines. Importantly, this protocol has been designed such that it can be applied by laboratories with limited resources to produce mutant zebrafish in an affordable manner. Furthermore, we have found that this approach is suitable for undergraduates and thus expands the opportunities for education and training of undergraduate students interested in hands-on experience in CRISPR-based genome editing.

Disclosures

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