Chapter 21

Targeted Mutagenesis in Zebrafish Using CRISPR RNA-Guided Nucleases

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Abstract

In recent years, the zebrafish has become a critical contributor to various areas of biomedical research, advancing our fundamental understanding of biomedicine and helping discover candidate therapeutics for human diseases. Nevertheless, to further extend the power of this important model organism requires a robust and simple-to-use genome editing platform that will enable targeted gene knockouts and introduction of specific mutations identified in human diseases into the zebrafish genome. We describe here protocols for creating insertion or deletion (indel) mutations or precise sequence modifications in zebrafish genes using customizable CRISPR-Cas9 RNA-guided nucleases (RGNs). These methods can be easily implemented in any lab and may also potentially be extended for use in other organisms.

Key words CRISPR, Cas9, Gene-editing, Genome engineering, Zebrafish, T7E1

1 Introduction

Over the last few years, the field of genome engineering has generated several breakthrough technologies for constructing site-specific DNA nucleases [1–4]. Customizable site-specific DNA nucleases can be used to create double-stranded DNA breaks (DSBs) at investigator-specified genomic loci. These DSBs can be repaired by an error-prone non-homologous end joining (NHEJ) mechanism, resulting in random-length insertions or deletions (indels) at the site of the break [5–13]. Alternatively, DSBs can also be repaired via homology-directed repair (HDR) using a homologous "donor" template, resulting in a precise modification of the sequence [12–14]. The donor template for HDR can be in the form of either plasmid DNA [15–18] or synthetic single-stranded oligodeoxynucleotides (ssODNs) [19–23]. These plasmid and ssODN template forms respectively contain several hundred base pair (bp) or less than 100 bp homology arms flanking the genomic site of modification. Nuclease-based approaches enable editing of endogenous genes in a

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wide range of model organisms, including many where targeted genome manipulations were inefficient (e.g., zebrafish, rats, fruit flies, and *C. elegans*) [5–7, 9, 24–33]. Among the various platforms described to date, the CRISPR-Cas9 RNA-guided nucleases are one of the simplest to implement [16, 25, 34–37].

The Type II CRISPR system of *Streptococcus pyogenes* is used by various bacteria as a mechanism to inactivate foreign DNAs [38, 39]. It has been previously shown that the Cas9 endonuclease can function together with a programmable synthetic single guide RNA (sgRNA) to induce targeted site-specific DSBs [38]. The nuclease activity of the Cas9 protein is directed by the first 20 nt of the ~100 nt sgRNA, which are complementary in sequence to one strand of the genomic target DNA site. One additional requirement for the genomic target DNA site is that strand of DNA opposite to the one that interacts with the sgRNA must also contain a proximal protospacer adjacent motif (PAM), which for the *S. pyogenes* Cas9 must be of the form NGG or NAG [38, 40].

We have previously shown that microinjection of sgRNA and Cas9-encoding mRNA into one-cell stage zebrafish embryos results in high rates of somatic indel mutations at 9 of 11 tested endogenous gene target sites [25]. In addition, RGN-mediated indel mutations can be transmitted through the zebrafish germ line efficiently [21]. Furthermore, the RGN system may also be used with single-stranded oligodeoxynucleotides (ssODNs) to introduce precise sequence modifications [21]. Another benefit of this system is the ease with which multiple genes can be targeted at once by introducing several sgRNAs simultaneously ([41] and Hwang, unpublished results).

There are several considerations when designing a gene-targeting experiment in zebrafish using a CRISPR-Cas9 system. Originally, sgRNAs expressed from our vectors begin with a 5′-GG dinucleotide due to the sequence requirements of the T7 promoter we use for in vitro transcription. We have shown that sgRNAs with a 5′-GG overhang that bear either 18 or 20 nucleotides of complementary sequence can still confer efficient target site cleavage in most cases [21]. This simple strategy expands the targeting range of our CRISPR-Cas9 system from 1 in every 128 bp of random DNA sequence to 1 in every 8 bp. Secondly, it is still unclear why some synthesized sgRNAs failed to efficiently induce indels at their genomic targets [21, 25]. One possibility is that certain sgRNA sequences might form secondary structures that either cannot bind to Cas9 or base pair with DNA targets [21]. Thus, we recommend making and testing more than one sgRNA vector per target gene. Finally, several studies have suggested that CRISPR-Cas9 targeted to different sequences may show different ranges of off-target activities [40, 42–44]. Thus, as with any genome editing experiment (regardless of the particular nuclease platform used), one should always perform control experiments to confirm that any phenotype observed is indeed linked to a specific mutation.

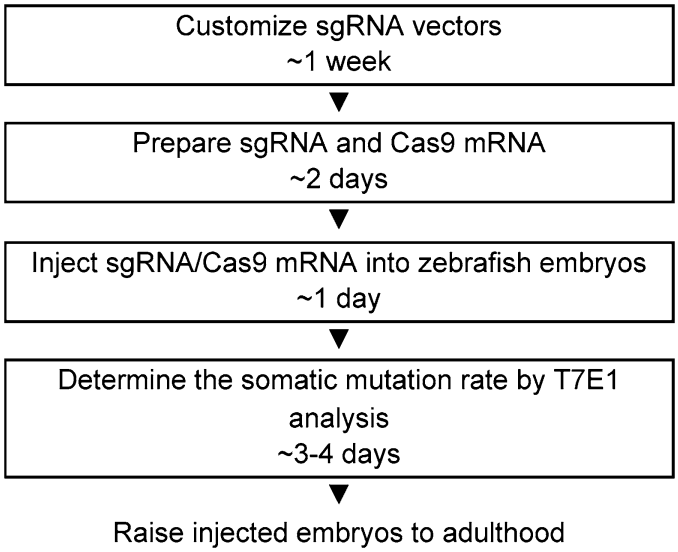


Fig. 1 The steps for conducting targeted mutagenesis in zebrafish embryos using the customizable CRISPR-Cas9 system are shown in the *boxes*. Protocols for each of these steps are provided in this chapter

In this chapter, we describe our protocols for constructing customized sgRNA vectors. Our system requires the synthesis of only two short complementary oligodeoxynucleotides that encode the 20 nt of complementarity with the target site. Ligation of these annealed oligos into our parental sgRNA vector yields the final plasmid that is ready for in vitro transcription. We also describe a simple method of detecting somatic indel mutations using T7 endonuclease I (T7E1), a mismatch-sensitive enzyme (Fig. 1). Alternatively, somatic indel mutations may be detected by subcloning and sequencing of the target loci, or by restriction digest analysis, both of which have been previously described [7]. In addition, ssODNs containing a predetermined mutation may be co-injected with sgRNA and Cas9-encoding mRNA (*see* Subheading 3.4) [21]. Following the successful demonstration of somatic mutations induced by the CRISPR-Cas9 system, injected embryos may be raised to adulthood and screened for germ line mutations at the target loci. In summary, the CRISPR-Cas9 system is simple to use and enables researchers to rapidly initiate experiments designed to create nearly any desired alteration in zebrafish.

2 Materials

2.1 Construction of Customized Single-Guide RNA (sgRNA) Vectors

1. The sgRNA vector pDR274 may be obtained through Addgene, a nonprofit plasmid repository (http://www.addgene.org/).
2. Two DNA oligos containing the sequences generated from the ZiFiT Targeter webserver (http://zifit.partners.org).

3. 10× NEBuffer 2 (B7002, New England Biolabs, Ipswich, MA): 50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9.
4. A dry bath with a removable heat block.
5. BsaI (R0535, New England Biolabs) supplied with 10× NEBuffer 4. There is also a high fidelity version BsaI-HF™ (R3535, New England Biolabs) available.
6. An agarose gel electrophoresis apparatus.
7. QIAquick PCR Purification kit (Qiagen, Valencia, CA) supplied with Buffer EB.
8. A spectrometer for measuring DNA concentration.
9. T4 DNA Ligase and 2× Rapid Ligation Buffer in the LigaFast™ System (Promega, Madison, WI).
10. Chemical or electric competent bacterial cells.
11. 6-well LB/kanamycin agar plates: Add 7.5 g agar into 0.5 L of LB. Autoclave. Add 0.5 mL of 30 mg/mL kanamycin stock solution after it cools down but before it solidifies (~60 °C). Mix well and pour 3 mL into each well of 6-well plates. Store at 4 °C for up to 2 months.
12. Sterile bacterial culture tube.
13. LB/kanamycin medium: Add 0.5 mL of 30 mg/mL kanamycin stock solution to 500 mL of autoclaved LB. Store at 4 °C for up to 2 months.
14. QIAprep Spin Miniprep kit (Qiagen).
15. M13F Primer, 5′-ACTGGCCGTCGTTTTAC-3′.
16. QIAGEN Plasmid Mini kit (Qiagen).

2.2 Production of sgRNA

1. DraI (R0129, New England Biolabs) supplied with 10× NEBuffer 4.
2. An agarose gel electrophoresis apparatus.
3. QIAquick PCR Purification kit (Qiagen) supplied with Buffer EB.
4. A spectrometer or NanoDrop for measuring DNA and RNA concentration.
5. MAXIscript® T7 kit (Life Technologies, Grand Island, NY).
6. 5 M ammonium acetate, nuclease-free.
7. Ambion® nuclease-free water (Life Technologies), filtered, not DEPC-treated.
8. 100 % ethanol.
9. Nuclease-free 70 % ethanol, prepared using 100 % ethanol and nuclease-free water.

2.3 Production of the Cas9 mRNA

1. The Cas9 expression vector JDS246 may be obtained through Addgene (http://www.addgene.org/).
2. QIAGEN Plasmid Purification kit (Qiagen).
3. PmeI (R0560, New England Biolabs) supplied with 10× NEBuffer 4 and 100× BSA.
4. An agarose gel electrophoresis apparatus.
5. QIAquick PCR Purification kit (Qiagen) supplied with Buffer EB.
6. A NanoDrop or spectrometer for measuring DNA and RNA concentration.
7. The mMESSAGE mMACHINE T7 Ultra kit (Life Technologies).
8. 100 % ethanol and 70 % ethanol prepared with nuclease-free water.

2.4 Injection of sgRNA and Cas9 mRNA into Zebrafish Embryos

1. Zebrafish: TU (Tübingen) or other wild-type strain may be obtained from the Zebrafish International Resource Center (http://zebrafish.org).
2. Breeding tanks with dividers.
3. A microinjection apparatus and an agarose plate for holding embryos during microinjection.
4. 0.5 % Phenol Red solution (P0290, Sigma, St. Louis, MO).
5. 1× Danieau solution: 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6; filter-sterilized. Store at 25 °C for 1 year.
6. Halocarbon oil (Sigma, St. Louis, MO).
7. A micrometer calibration slide.
8. E3 medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$. Store at 25 °C for 1 month.

2.5 Determining the Somatic Mutation Rate in Injected Zebrafish Embryos: T7 Endonuclease I (T7E1) Assay

1. PCR Lysis Buffer: Add 0.5 mL of 1 M Tris at pH 8, 0.2 mL of 0.5 M EDTA, 0.1 mL of Triton X-100, and water to 50 mL. Store at room temperature.
2. Proteinase K, Recombinant, PCR Grade (Roche Applied Science, Indianapolis, IN).
3. Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, Pittsburgh, PA).
4. 10 mM dNTPs (Life Technologies).
5. Gene-specific primers: Design primers for each sgRNA target site based on its flanking genomic DNA sequence. Each primer should anneal about 150–250 bp away from the expected double-stranded break location and extend toward it. Ideally, primers should have a melting temperature of approximately

60–65 °C and a length of 23–25 bp. Use free online software such as Primer3 (http://frodo.wi.mit.edu/primer3/) and OligoPerfect™ Designer (http://tools.invitrogen.com/content.cfm?pageid=9716) for designing the primers.

6. An agarose gel electrophoresis apparatus.
7. A thermocycler.
8. Agencourt AMPure XP (Beckman Coulter, Brea, CA) or QIAquick PCR Purification Kit (Qiagen).
9. A NanoDrop machine or a spectrometer for measuring DNA concentration.
10. T7 Endonuclease I (T7E1) (M0302, New England Biolabs).
11. A microfuge that can spin PCR strips or plates.
12. 0.25 M ethylenediaminetetraacetic acid (EDTA).
13. The QIAxcel system (Qiagen). Alternatively, a polyacrylamide gel electrophoresis apparatus, 15 % Tris–borate–EDTA (TBE) acrylamide gels (Bio-Rad, Hercules, CA), 100 bp DNA ladder (New England Biolabs), ethidium bromide, and a gel documentation system.

3 Methods

3.1 Construction of Customized Single-Guide RNA (sgRNA) Vectors

1. Find 23-basepair (bp) target sites in the form of 5′-GG-(N)18-NGG-3′ in your intended target region. You can use the web-based ZiFiT Targeter program (http://zifit.partners.org) to find sgRNA target sites and to generate the sequences of the DNA oligos required for constructing customized sgRNA vectors (Fig. 2) (*see* **Note 1**). If such a site does not exist in your target gene, it may be fine to allow up to two mismatches at the 5′ end.
2. Obtain complementary DNA oligos containing the sequences generated above from a DNA synthesis company such as Integrated DNA Technologies (Coralville, IA) or Life Technologies (Grand Island, NY). Anneal these two DNA oligos to make a double-stranded DNA fragment using the following protocol. Assemble 45 µL of 100 µM Oligo #1, 45 µL of 100 µM Oligo #2, and 10 µL of 10× NEBuffer 2 in an eppendorf tube. Place the tube in a dry bath with a removable heat block at 90–95 °C for 5 min. Remove the heat block with the eppendorf tube in it from the heating unit and allow it to cool gradually to room temperature (or below 37 °C). Continue to the next step or store the annealed oligonucleotides at −20 °C.
3. Linearize the sgRNA vector pDR274 with BsaI by adding 5 µg of the pDR274 DNA, 10 µL of 10× NEBuffer 4, 1 µL of BsaI

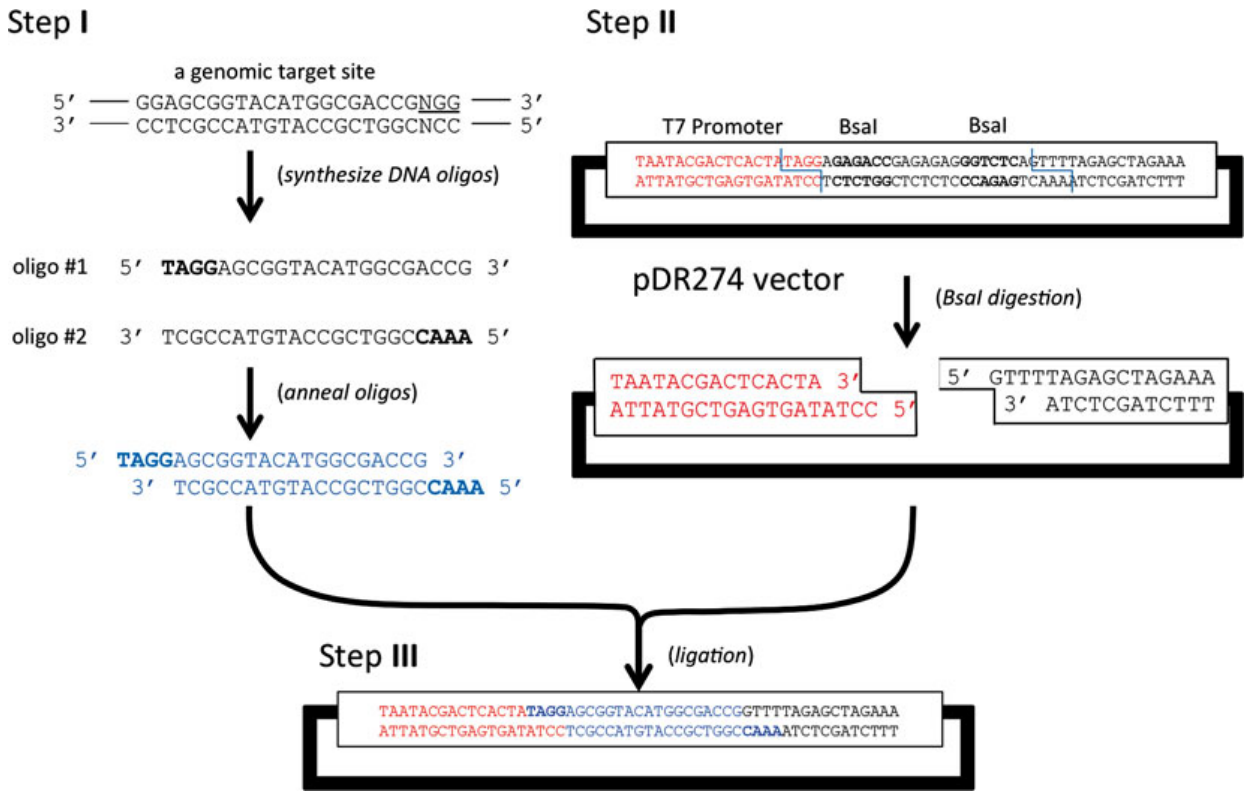


Fig. 2 Cloning customized target sequences into the sgRNA vector pDR274. Step I shows the DNA oligo sequences for an example target site. Notice that the 3′-NGG (called protospacer adjacent motif or PAM) is required at the target site but is not included in the oligo sequence. Step II shows the cohesive ends generated by BsaI digestion of pDR274. Step III shows the final sequences of the ligated vector

(10 U/μL) and sterile deionized water to a total volume of 100 μL in an eppendorf tube. BsaI should be added last. Incubate the reaction at 37 °C for 2–3 h.

4. Run 50 ng of the uncut vector DNA and 5 μL of the reaction mixture on a 1 % wt/vol agarose gel to verify that the digestion is complete. Purify the digested DNA using the QIAquick PCR Purification kit and elute the DNA with 50 μL of Buffer EB. Measure DNA concentration using a spectrometer and normalize to a final concentration of 5–10 ng/μL with Buffer EB.
5. Ligate the annealed oligos and the linearized vector by mixing 1 μL of the annealed oligos (from **Step 2**), 1 μL of the linearized vector DNA (from **Step 4**), 2.5 μL of 2× Rapid Ligation Buffer, and 0.5 μL of T4 DNA Ligase in an eppendorf tube. Incubate the reaction mixture at either room temperature for 1 h or 4 °C overnight. The ligated product can be used for transformation or stored at −20 °C.
6. Transform competent bacterial cells with 5 μL of the ligation product following the specific instructions for the cells that you are using. At the end of the transformation procedures,

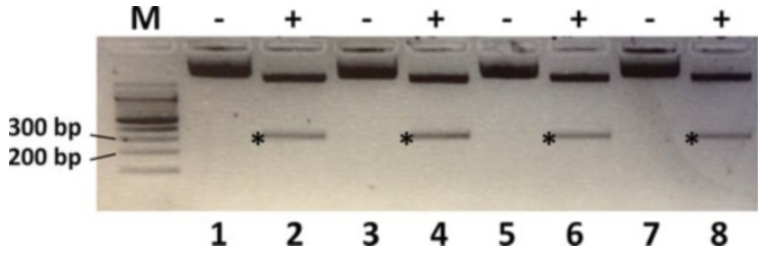


Fig. 3 A representative gel of DraI-linearized sgRNA vectors. Lane 1–2, 3–4, and 5–6 contain 3 sets of sgRNA vectors. *Minus*, undigested DNA; *plus*, DraI-digested DNA; *asterisk*, the fragments containing both the T7 promoter and the sgRNA sequences; M, 100 bp DNA ladder (New England Biolabs)

spread the cells on a LB/kanamycin plate and incubate the plate at 37 °C overnight.

7. Pick three colonies per transformation and inoculate each colony in a culture tube containing 1.5 mL of LB/kanamycin. Incubate the culture tubes at 37 °C overnight with agitation.
8. On the next day, transfer the bacterial culture to an eppendorf tube for plasmid DNA extraction using the QIAprep Spin Miniprep kit or any plasmid DNA miniprep kit. Verify the sequence of the extracted DNA using M13F primer (*see* **Note 2**). Save the culture tube at 4 °C for re-inoculation.
9. Re-inoculate the bacteria containing sequence-confirmed plasmid DNA by adding 3 mL of LB/Kanamycin to the previous culture tube (**Step 8**). Incubate the culture tube at 37 °C overnight with agitation. Extract plasmid DNA from the culture using the QIAGEN Plasmid Mini kit (*see* **Note 3**). Measure DNA concentration using a spectrometer. This is the customized sgRNA vector.

3.2 Production of sgRNA for Microinjection

1. Linearize your sgRNA vector with DraI by mixing 5 μg of the customized sgRNA vector DNA, 10 μL of 10× NEBuffer 4, 1 μL of DraI (10 U/μL) and sterile deionized water to a total volume of 100 μL in an eppendorf tube. DraI should be added last. Incubate the reaction at 37 °C for 2 h.
2. Run 2 μL of the reaction mixture on a 3 % wt/vol agarose gel to verify that the digestion is complete. The digested DNA should contain two fragments at 1.9 kb and 282 bp, respectively (Fig. 3) (*see* **Note 4**).
3. Purify the digested DNA using the QIAquick PCR Purification kit and elute the DNA with 50 μL of Buffer EB (*see* **Note 5**).
4. Measure DNA concentration using a spectrometer. Continue to the next step or store the purified DNA at −20 °C.
5. Produce sgRNA by in vitro transcription using the MAXIscript® T7 kit as follows (*see* **Note 6**). Assemble 1 μg of the linearized

sgRNA vector DNA, 2 μL of 10× transcription buffer, 4 μL of mixed NTPs (1 μL each of the ATP, GTP, CTP, and UTP solutions at 10 mM), 2 μL of T7 Enzyme mix, and nuclease-free water to a total volume of 20 μL in a nuclease-free eppendorf tube (*see* **Note 7**). Incubate the reaction at 37 °C for 2 h to overnight. At the end of incubation, add 2 μL of TURBO DNase and incubate at 37 °C for another 30 min. Add 1 μL of 0.5 M EDTA to stop the reaction.

6. Add 30 μL of nuclease-free water and 5 μL of 5 M ammonium acetate to the reaction mix and mix well (*see* **Note 8**). Subsequently, add 150 μL of 100 % ethanol and mix well. Incubate at −20 °C overnight or on dry ice for 0.5–1 h.
7. Spin the eppendorf tube at >10,000 rpm in a microcentrifuge at 4 °C for 30 min. Discard the supernatant and add 1 mL of nuclease-free 70 % ethanol. Spin the tube again using the same setting for 15 min. Remove the 70 % ethanol as much as you can. Put the tube in a chemical hood and leave the lid open for a few min until the pellet dries completely.
8. Add 11 μL of nuclease-free water to dissolve the pellet (*see* **Note 9**). Take 1 μL of the dissolved RNA solution for measuring RNA concentration using a spectrometer (*see* **Note 10**). Aliquot sgRNA into multiple nuclease-free eppendorf tubes (1 μL/tube) and store at −80 °C. Take 1 μL of the sgRNA, mix it with 5 μL of the Formaldehyde Loading Dye and run these samples on a 3 % wt/vol agarose gel to make sure that the sgRNA is not degraded.

3.3 Production of Cas9 mRNA for Microinjection

1. Extract Cas9 vector DNA (JDS246) using a Qiagen Plasmid Purification kit. Linearize Cas9 vector DNA with PmeI by mixing 5 μg of the vector DNA, 10 μL of 10× NEBuffer 4, 1 μL of 100× BSA, 1 μL of PmeI (10 U/μL), and sterile deionized water to a total volume of 100 μL. PmeI should be added last. Incubate the reaction at 37 °C for 2–3 h.
2. Run 50 ng of the uncut vector DNA and 2 μL of the cut DNA on a 1 % wt/vol agarose gel to verify that the vector DNA is completely digested.
3. Purify the digested DNA using the QIAquick PCR Purification kit and elute the DNA with 50 μL of Buffer EB. Measure DNA concentration using a spectrometer. Continue to the next step or store the purified DNA at −20 °C.
4. Synthesize Cas9 mRNA using the mMESSAGE mMACHINE® T7 Ultra kit following the steps below (*see* **Note 11**). Thaw 2× NTP/ARCA and 10× T7 reaction buffer at room temperature. Place 2× NTP/ARCA, but not 10× T7 reaction buffer, on ice immediately once thawed. Keep T7 Enzyme Mix on ice at all times. Vortex the 10× T7 reaction buffer to redissolve any

precipitate in the tube before using. Mix reagents in a nuclease-free eppendorf tube in the following order: 5 µL of T7 2× NTP/ARCA, 1 µL of 10× T7 reaction buffer, 1 µL of T7 enzyme mix, and 3 µL (approximately 1 µg) of linearized Cas9 vector (from **Step 3**). Gently flick the tube and microfuge briefly to collect all of the reaction mixture at the bottom of the tube. Incubate the tube at 37 °C for 2 h. At the end of the incubation, add 1 µL of TURBO DNase. Gently flick the tube to mix and microfuge briefly. Incubate the tube at 37 °C for additional 30 min.

5. During the last incubation period, prepare the poly(A) tailing reaction master mix by combining 21.5 µL of nuclease-free water, 10 µL of 5× E-PAP buffer, 2.5 µL of 25 mM $MnCl_2$, and 5 µL of ATP solution in a nuclease-free eppendorf tube. Add the master mix to the in vitro transcription reaction mixture (**Step 4**). Gently flick and microfuge the tube. Aliquot 2 µL of the reaction mixture to a clean tube and label as "–polyA". Store this tube at –20 °C for a gel analysis later. Add 2 µL of E-PAP enzyme to the reaction mixture and incubate at 37 °C for 1 h. At the end of the reaction, aliquot 2 µL of the reaction mixture to another clean tube and label as "+polyA". Store this tube at –20 °C for a later gel analysis.
6. Add 25 µL of Lithium Chloride Precipitation Solution to the rest of the reaction mixture (*see* **Note 12**). Mix well and incubate the tube at –20 °C overnight or 0.5–1 h on dry ice. We recommend overnight incubation for a better yield.
7. During this incubation, conduct a gel analysis of the "–polyA" and "+polyA" labeled samples stored at –20 °C. Add 5 µL of the Formaldehyde Loading Dye into the 2 µL sample aliquots taken before and after the poly(A) tailing reaction (**Step 5**). Run the samples on a 1 % agarose gel (Fig. 4).
8. After the incubation (from **Step 6**), spin the tube at >10,000 rpm in a microcentrifuge at 4 °C for 30 min. Once the spin is done, check for the pellet at the bottom of the tube and remove the supernatant without disturbing the pellet. Add 1 mL of RNase-free 70 % ethanol and invert the tube several times. Centrifuge the tube at the same setting for 15 min. Check for the pellet once again and remove the solution completely without disturbing the pellet (*see* **Note 13**). Leave the tube with the lid open for 10–15 min until all of the solution evaporated.
9. Dissolve the RNA pellet with 20 µL of nuclease-free water. Once it is dissolved, keep the tube on ice at all time. Take 1 µL of the dissolved RNA solution for measuring RNA concentration using a spectrometer. Aliquot the Cas9 mRNA into multiple nuclease-free eppendorf tubes (1,500 ng/tube) and store at –80 °C (*see* **Note 14**).

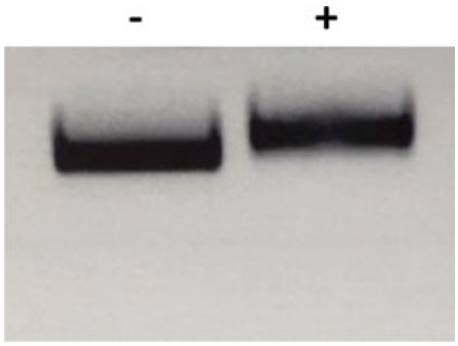


Fig. 4 A representative gel of in vitro transcribed Cas9 RNA. *Minus*, before the poly(A) tailing reaction; *plus*, after the poly(A) tailing reaction. Notice that the RNA bands are distinct and without smears. Meanwhile, the poly(A) tailing reaction causes an upshift of the RNA band due to an increase in its length

3.4 Injection of sgRNA and Cas9 mRNA into Zebrafish Embryos

1. Set up zebrafish for spawning the evening before injection. Place a divider in each breeding tank to separate males and females.
2. On the day of injection, prepare the injection solution by mixing a total of 62.5 ng of sgRNA, 1,500 ng of Cas9 mRNA, 0.5 μL of 0.5 % Phenol Red solution, and 1×Danieau solution to a total volume of 5 μL (*see* **Note 15**). Keep the injection solution on ice to prevent RNA degradation (*see* **Note 16**). In addition, for a predesigned sequence modification, add single-stranded DNA oligos to a final concentration of 1.66 μM (or 25 ng/μL) into the injection solution as the template for DNA repair (*see* **Note 17**). Do not change the final concentrations of sgRNA and Cas9 mRNA.
3. Prepare an agarose plate to hold embryos during microinjection. Calibrate the needle for injection as follows. Add a drop of halocarbon oil on the micrometer calibration slide. Magnify the stereoscope to see the scale. Adjust the injection pressure and/or injection time so that the diameter of each injection droplet is around 0.16 mm, which brings the volume of the droplet to be 2 nL. Once you finish the calibration, pull the divider out from a breeding tank and wait for the fish to spawn.
4. The RNA solution should be injected into the cytoplasm of zebrafish embryos at the one-cell stage. Once you collect the embryos from the breeding tank in E3 medium, orient the embryos in the furrows of the agarose plate so that the cytoplasm is facing toward the needle. Remove most of the E3 medium and leave only enough to cover the embryos (*see* **Note 18**). Carefully inject 2 nL of the RNA solution into the base of the cytoplasm where the nucleus is located. Save some non-injected embryos from each clutch as controls.

5. Pull the divider out from another breeding tank and repeat the process. Incubate both control and injected embryos at 28.5 °C overnight.
6. Examine the injected embryos at 24 h post-fertilization (hpf). Classify the injected embryos as normal, deformed, or dead by visual inspection under a stereoscope. Repeat with the control embryos. Record these numbers (*see* **Note 19**). Meanwhile, the injected embryos may exhibit specific phenotypes resulting from RGN-mediated knockdown of the target gene. In some cases it is necessary to reduce the concentration of sgRNA/Cas9 mRNA for injection if the goal is to obtain founder fish that carry transmittable germ line mutations. After injection and successful identification of somatic mutations (*see* Subheading 3.5), injected embryos that develop normally may be raised to adulthood and screened for founders.

3.5 Determining the Somatic Mutation Rate in the Injected Zebrafish Embryos: T7 Endonuclease I (T7E1) Assay

1. Manually dechorionate embryos with forceps. Array 10 injected embryos (for each combination of sgRNA/Cas9 mRNA) and 2 control embryos singly into each well of a 96-well PCR plate. Add 30 μL of PCR lysis buffer with 100 μg/mL of proteinase K. It is important to include 1–2 control embryos from the same clutch of embryos used for microinjection (*see* **Note 20**). Seal and incubate the plate at 50 °C overnight. The next day, gently agitate the plate to make sure that the embryos have been lysed completely. Incubate at 95 °C for 10 min to inactivate Proteinase K (*see* **Note 21**). Spin the plate at 3,000 rpm for 2 min at room temperature to bring down any debris. The supernatant contains genomic DNA and it is now ready to be used for PCR.
2. Set up 50 μL PCR reactions to amplify the genomic DNA encompassing the target site using gene-specific primers (*see* **Note 22**). Assemble 2 μL of the embryo lysate or 100 ng of the genomic DNA (from **Step 1**), 10 μL of 5× Phusion HF Buffer, 1 μL of 10 mM dNTPs, 2.5 μL of the forward primer (10 μM stock), 2.5 μL of the reverse primer (10 μM stock), 0.5 μL of Phusion Hot Start II High-Fidelity DNA polymerase, and sterile deionized water to a total volume of 50 μL. The enzyme should be added last. Use the following cycling condition or a cycling condition that has been optimized for your primer set: Step 1—98 °C, 2 min; Step 2—98 °C, 10 s; Step 3—65 °C, 15 s; Step 4—72 °C, 30 s; Step 5—go back to step 2 for 34 times; Step 6—72 °C, 5 min; Step 7—hold at 4 °C (*see* **Note 23**).
3. Run 2 μL of the PCR products on a 2–3 % agarose gel to verify correct amplification.
4. Purify the products using 90 μL of AMPure XP, elute in 30 μL of 0.1× Buffer EB and recover 25 μL. Alternatively, purify the

products using the QIAquick PCR Purification Kit and elute with 50 µL of Buffer EB. Measure DNA concentration using NanoDrop or a spectrometer. Continue to the next step or store the purified PCR products at −20 °C.

5. Set up the hybridization by mixing 200 ng of the purified PCR product, 2 µL of 10× NEBuffer 2 and sterile deionized water to a total volume of 19 µL in a new PCR plate (*see* **Note 24**). Put the plate in a thermocycler and run the following cycling condition: Step 1—95 °C, 5 min; Step 2—from 95 to 85 °C, −2 °C/s; Step 3—from 85 to 25 °C, −0.1 °C/s; Step 4—hold at 4 °C (*see* **Note 25**).
6. At the end of the hybridization cycle, microfuge briefly, add 1 µL of T7E1 to each well. Gently pipette up and down for ~10 times to mix thoroughly. Incubate the plate at 37 °C for exactly 15 min. Stop the reaction by adding 2 µl of 0.25 M EDTA.
7. Analyze the sizes of the DNA fragments using a QIAxcel system or on a 10 % polyacrylamide gel. If the former, the reaction products need to be purified using either AMPure XP or a QIAquick PCR Purification Kit before loading. For the former option, purify the reaction product using 36 µL of AMPure XP, elute in 20 µL of 0.1× Buffer EB and recover in 15 µL.
8. For analysis using QIAxcel, dilute 5 µL of purified T7E1 reaction product with 5 µL of QX Dilution Buffer. Run on QIAxcel using program OM500 and an injection time of 20 s.

   For analysis using a polyacrylamide gel, add 2 µL of 10× gel loading dye to the reaction product and run all of the samples along with the 100 bp DNA ladder on a 15 % TBE acrylamide gel. Stain the gel in water containing 0.5 µg/mL ethidium bromide at room temperature for 30 min. Inspect the gel using a UV light box. Compare the injected samples to the control samples and identify any additional restricted fragments of the expected sizes in the injected samples. The presence of such bands provides evidence of sgRNA/Cas9-induced mutations.
9. To calculate the somatic mutation rate, the sum of the area beneath the injection-specific cleavage peaks (expressed as a percentage of the parent amplicon peak, denoted fraction cleaved) is used to estimate somatic mutation rates using the following equation as previously described: % gene modification = 100× (1−(1−fraction cleaved)$^{1/2}$) [45]. The fraction cleaved may also be quantified based on the intensities of the parent amplicon band and the T7E1 cleavage bands as determined by a gel documentation system such as the Bio-Rad Gel Doc™ XR+ System with the Quantity One® 1-D Analysis Software.

4 Notes

1. On the ZiFiT Targeter website, go to "Examples" → "CRISPR-Cas assembly using T7 promoter" for detailed instructions. In order to be cloned into the pDR274 vector, oligo#1 needs to have TAGG at the 5′ end, which is also a part of the T7 promoter. Oligo #2 needs to have AAAC at the 5′ end, enabling unidirectional cloning into the BsaI-digested pDR274 vector. All sgRNAs transcribed from the T7 promoter will have a GG dinucleotide at the 5′ end.
2. The sequencing results will show the T7 promoter and sgRNA sequences in reverse complement. The full DNA sequence of the pDR274 vector has been previously published [25]. Make sure that the sequenced DNA has a complete T7 promoter, the customized target sequence and the remaining of the guide RNA sequence. Based on our experience, 2 out of 3 colonies usually have correct sequences.
3. Plasmid DNA prep using gravity-flow columns often provides better quality DNA compared to spin columns. Based on our experience, better quality DNA leads to higher yields of sgRNA after in vitro transcription.
4. The smaller DNA fragment, which will be the template for in vitro transcription, contains both the T7 promoter and sgRNA sequences. There is no need to purify this fragment using gel purification.
5. Use a new bottle of Buffer EB to be sure that it is not contaminated with RNase.
6. Alternatively, sgRNA may be produced by using a MEGAshortscript™ T7 kit (Life Technologies, Grand Island, NY).
7. Mixed NTPs are made by mixing an equal volume of 10 mM ATP, CTP, GTP, and UTP from the kit.
8. Alternatively, add 30 μL of nuclease-free water and 25 μL of the lithium chloride precipitation solution (provided in the mMESSAGE mMACHINE® T7 Ultra kit) to precipitate sgRNA. You do not need to add 100 % ethanol subsequently. We recommend incubating the samples at −20 °C overnight for better yield.
9. We use Ambion® nuclease-free water that is not DEPC-treated. We found that injection of DEPC-treated water may induce embryo toxicity.
10. The yield of sgRNA is generally between 80 and 150 ng/μL. We always run 1 μL of sgRNA on a 3 % wt/vol agarose gel to check the integrity of the RNA. It should appear as a distinct band without smearing.

11. The synthesized Cas9 mRNA will contain a 5′ cap and a 3′ poly(A) tail in order to be translated in zebrafish embryos. The mMESSAGE mMACHINE® T7 Ultra Kit has all of the components that you would need. Alternatively, you can use a mMESSAGE mMACHINE® T7 Kit (Life Technologies, USA) and a Poly(A) Tailing Kit (Life Technologies, USA) for these reactions.
12. The volume of the Lithium Chloride Precipitation Solution is half of the volume of the reaction mixture.
13. It is critical to remove the ethanol completely so that the pellet will dry quickly. After removing the solution, one can microfuge the tube briefly and remove the remaining solution once more.
14. The yield of the RNA is generally between 1,000 and 2,000 ng/μL. We generally store RNA in multiple aliquots to prevent freeze-thaw cycles.
15. Multiple sgRNAs that target either the same gene or different genes can be co-injected for simultaneous generation of multiple gene mutations. In a multiplex experiment, the total amount of sgRNAs in the injection solution can be increased up to 100 ng/μL.
16. The injection solution should be freshly prepared. Do not freeze and reuse leftover injection solution.
17. The DNA oligo should contain the desired sequence modification and around 18–25 nt of flanking sequences that are homologous to the genomic target locus. It may be ideal to have the targeted sequence modification near the DSB, which is located at 3 base pairs upstream of the PAM. Using this method, we have successfully identified in injected embryos precise insertions of 3-4 nt, as well as, a precise single nucleotide substitution [21]. Although the efficiency of isolating a precisely modified allele may decrease when the size of the insertion increases, we have successfully inserted up to 40 nt using this method (Gonzales, unpublished data).
18. This is a very important step. Otherwise, embryos will roll around and it will be difficult to inject into the cell.
19. The toxicity of each sgRNA/Cas9 complex varies. Thus, the concentration of the Cas9 mRNA may be reduced to 100 ng/μL if the injection results in less than 50 % healthy embryos.
20. It is important to include non-injected embryos from the same clutches that were used for injections as controls. This will help identify if there is a polymorphism within the PCR amplicon that can result in a cleavage product in this assay.
21. You may use genomic DNA extracted from pooled embryos if you know there is no polymorphism in your target locus. In that case, add 30 μL of PCR lysis buffer with Proteinase K per embryo (e.g. 150 μL for 5 pooled embryos).

22. The primers should anneal to sequences around 150–200 bp upstream and downstream of the target site.
23. This is one PCR condition that generally works for us. It is very important to first optimize the PCR condition and obtain a very specific amplification product. The optimal conditions may differ for different gene targets.
24. It is important to start with the same amount (200 ng) of PCR products for all of the samples.
25. The hybridized DNA will be stable at 4 °C for a couple of hours.

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