APPLICATION NOTE

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Microinjection of zebrafish embryos using Dharmacon™ Edit-R™ Cas9 Nuclease mRNA, synthetic crRNA, and tracrRNA for genome engineering

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Abstract
The rapid advances being made in gene editing utilizing the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) system have led to increasing interest in the application of this technology in zebrafish embryos for gene knockout. Using the Dharmacon™ Edit-R CRISPR-Cas9 DNA-free genome engineering platform, zebrafish embryos were injected with Cas9 Nuclease mRNA, synthetic tracrRNA and synthetic crRNA designed to target GFP in a zebrafish stable transgenic line. A mismatch detection assay was used to estimate gene editing efficiency, and successful functional protein knockout was confirmed by loss of GFP fluorescence.

Keywords
CRISPR-Cas9, gene editing, genome engineering, Cas9 mRNA, Edit-R, Cas9, direct injection, microinjection, zebrafish embryos, zebrafish

Introduction
The zebrafish (Danio rerio) is quickly becoming a preferred model system for biomedical research due to its high degree of sequence and functional homology with humans and relative ease of use in the laboratory. The zebrafish model is a genetically tractable system that undergoes a rapid external development, and therefore was initially utilized to study early developmental events. Currently, zebrafish research has expanded to a wide variety of basic science and clinical research settings, including modeling human genetic disease[1]. Zebrafish as a model for human disease is enhanced by the considerable genomic resources that exist, including a database of characterized mutant lines (zfin.org), and a complete and annotated genome.

Zebrafish are a relatively low cost research model, are highly amenable to genetic manipulation, and the molecular tools necessary to perform the targeted genomic editing are already available. As a result, zebrafish are an increasingly popular choice for target genomic modification using the CRISPR-Cas9 gene editing system.

Here we demonstrate DNA-free gene knockout of GFP in a stable transgenic zebrafish line by microinjection of the three components of the CRISPR-Cas9 system: Cas9 Nuclease mRNA, synthetic crRNA and synthetic tracrRNA.

Results
To determine the efficacy of the Edit-R CRISPR-Cas9 DNA-free genome engineering platform in zebrafish, approximately 100 single-cell stage embryos were microinjected with Cas9 mRNA and synthetic crRNA:tracrRNA, and approximately 50 control embryos were injected with Cas9 mRNA only. Survival was assessed at 1 and 2 days post-fertilization (dpf). Injected embryos had a high survival rate (WT = 97%, n=100; Control Injected = 96%, n=52; CRISPR Injected = 93%, n=94). At 2 dpf genomic DNA was extracted from eight of the embryos and analyzed for gene editing efficiency using a mismatch detection assay. Six of the eight embryos microinjected with all three components of the CRISPR-Cas9 system show estimated gene editing efficiency of 9-20% (Figure 1).

Detecting functional knockout of GFP was performed by imaging the zebrafish microinjected with Cas9 mRNA and crRNA:tracrRNA and comparing them to the embryos that were not injected. Figure 2 shows GFP and RFP fluorescence of neural crest cells. The zebrafish embryo that was not injected shows expression of both GFP and RFP in transgenic cells (Figure 2A). Following microinjection of CRISPR-Cas9 components targeting GFP, a loss or decrease in GFP fluorescence is observed in the zebrafish embryo, confirming successful gene knockout.

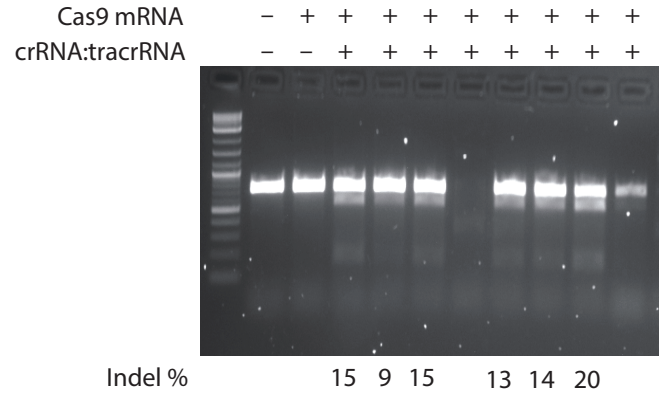

Figure 1. Zebrafish embryos microinjected with Edit-R Cas9 Nuclease mRNA and synthetic crRNA:tracrRNA have detectable editing events. Zebrafish embryos were microinjected with Edit-R Cas9 mRNA only (+/+ lane) or with Edit-R Cas9 mRNA plus crRNA:tracrRNA targeting GFP (+ lanes). Genomic DNA was prepared 2 days post-injection and PCR was performed with primers flanking the cleavage sites. A DNA mismatch assay with T7EI was performed and the samples were separated on a 2% agarose gel. Percent of insertions and deletions due to gene editing (Indel %) was estimated using ImageJ software and is shown at the bottom of the lanes. Targeted DNA cleavage using Cas9 mRNA programmed with crRNA:tracrRNA targeting GFP was achieved in 75% of the zebrafish embryos analyzed.

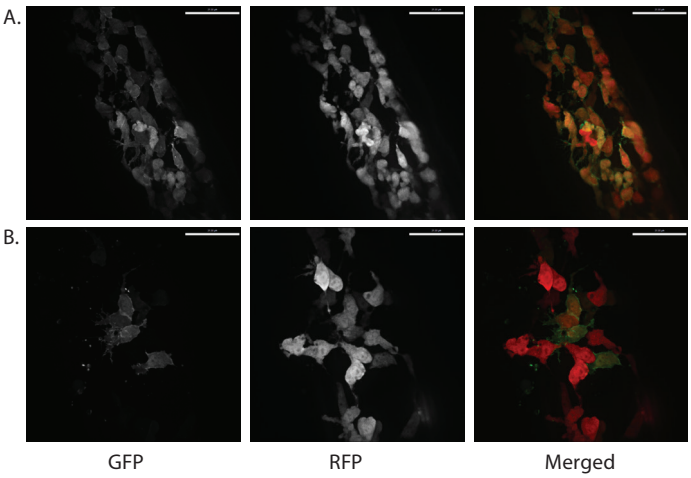

Figure 2. GFP knockout *in vivo* using Edit-R CRISPR-Cas9 DNA-free system. Dorsal view of Tg(Sox10:(PH)GFP; Sox10:tagRFP) zebrafish neural tube at 24 hpf. Images were collected on a Zeiss Axio Observer microscope equipped with a PerkinElmer UltraVIEW VoX spinning disk confocal system and Volocity imaging software (PerkinElmer) **A.** Neural crest cells expressing both GFP and RFP in zebrafish embryos injected with Cas9 mRNA only. **B.** Neural crest cells in embryos injected with Cas9 and crRNA:tracrRNA targeting transgenic gfp display mosaic GFP expression as a result of functional protein knockout. (37 µM bar for reference)

Discussion

Zebrafish are an excellent model system for studying genetic manipulations and relating these experimental results to human diseases. Here we demonstrated the utility of using a completely DNA-free gene editing system for *in vivo* microinjection of zebrafish embryos to knock out GFP in a stable transgenic line. Neural crest cells in the Tg(sox10:tagRFP; sox10:PH-GFP) transgenic zebrafish line express both RFP and GFP. Using the Edit-R CRISPR-Cas9 genome engineering platform with Cas9 mRNA and synthetic crRNA:tracrRNA, GFP was targeted for gene editing and functional protein knockout. A mismatch detection assay showed that 75% of the microinjected zebrafish embryos resulted in efficient gene editing (Figure 1). Importantly, since 75% of analyzed embryos that were injected showed gene editing, it is likely that the germ cell [one of thousands of cells at 24 hours post-fertilization (hpf)] has been mutated and can be used for generation of a stable transgenic line for further scientific interrogation. Additionally, we confirmed GFP knockout *in vivo* using fluorescent microscopy. Analysis of neural crest cells 24 hpf revealed highly mosaic expression of GFP in embryos microinjected with gene editing components (Figure 2). The successful gene editing demonstrated here with DNA-free CRISPR-Cas9 components shows one of the many new possibilities using CRISPR-Cas9 gene editing in model systems to find correlations to human diseases.

Materials and methods

Materials required

- Edit-R Cas9 Nuclease mRNA (Cat #CAS11195)
- Edit-R tracrRNA (Cat #U-002000-xx)
- Edit-R synthetic crRNA targeting GFP (Dharmacon custom synthesis; previously published sequence[2])
- Microinjection setup (Microinjector, injection needles, injection trays)
- Single-cell zebrafish embryos, stable transgenic line Tg(sox10:tagRFP; sox10:PH-GFP)

Zebrafish microinjection

Prior to injections, a mixture containing the GFP crRNA (25 pg), tracrRNA (100 pg) and Cas9 mRNA (100 pg) was prepared on ice (see below). The injection mixture remained on ice until loading into the microinjection needle to improve mRNA stability. At the single-cell stage, cells were injected with 1-2 nL of the injection mix into the stable transgenic line Tg(sox10:tagRFP; sox10:PH-GFP), which express both red and green fluorescent proteins under the Sox10 promoter.

Reagent	Volume	Final concentration
KCl (2 M)	1.0 µL	0.2 M
Phenol Red (Sigma Cat #P0290)	1.0 µL	N/A
tracRNA (1.0 µg/µL)	1.0 µL	100 pg/nL
crRNA (GFP) (0.25 µg/µL)	1.0 µL	25 pg/nL
Cas9 mRNA (0.2 µg/µL)	5.0 µL	100 pg/nL
Water, nuclease free (Cat # B-003000-WB-100)	1.0 µL	N/A
Total Volume	10.0 µL	

Mismatch detection assay using T7 Endonuclease I

Genomic DNA was prepared from un-injected and injected embryos at 2 days post-fertilization (dpf) following a previously established method (https://zfin.org/zf_info/zfbook/chapt9/9.3.html). We PCR amplified a short genomic region (~ 700 bp) flanking the target site from the genomic DNA using Phusion™ High-Fidelity PCR Master Mix with HF Buffer (NEB Cat# M0531S) and GFP-specific primers (FWD: ATGGTGAGCAAGGGCGAGGAG and REV: CATGCCGAGAGTGATCCCGGC). Thermal cycling conditions were as follows:

Cycle steps	Temperature	Time	Cycle(s)
Initial denaturation	98 °C	1:00 min	1
Denaturation	98 °C	0:10 sec	34
Touchdown annealing	65°C–1 °C/cycle	0:10 sec	
Extension	72 °C	0:30 sec	
Final extension	72 °C	5:00 min	1

The PCR amplicons were purified using DNA Clean & Concentrator™–5 (Zymo Research Cat #D4003). To estimate the mutation rate for the GFP target site, a total of 200 ng of the purified PCR amplicon was denatured and slowly reannealed to facilitate heteroduplex formation. The reannealing procedure consisted of a 5 minute denaturing step at 95 °C, followed by cooling to 85 °C at –2 °C/second and further to 25 °C at –0.1 °C/second. The reannealed amplicon was then digested with 10 units of T7 Endonuclease I (T7EI; NEB Cat #M0302S) at 37 °C for 15 minute. The reaction was stopped by adding 1 µL of 0.5 M EDTA. The entire sample was resolved by electrophoresis through a 2% agarose gel and visualized by ethidium bromide staining. The band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD) for estimation of gene editing efficiency.

References

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If you have any questions, contact

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