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DNA-free CRISPR-Cas9 genome engineering in zebrafish

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Introduction

The CRISPR-Cas9 system permits researchers to quickly edit genes for functional protein knockout in mammalian, fish and plant genomes, among others, and consequently has dramatically transformed biological research. The CRISPR-Cas9 system requires exogenous Cas9 nuclease to be delivered into the cell, which can be accomplished through transfection of an expression plasmid, mRNA or protein, or through transduction with lentiviral particles. DNA-based Cas9 constructs, while appropriate for many applications, may result in unwanted integration events. Lentiviral delivery results in integration of the Cas9 expression cassette into the cell's genome, and transfection of a Cas9 plasmid may result in the insertion of vector sequence at the site of the double-stranded break when the genomic DNA is repaired through the NHEJ pathway. The use of Cas9 mRNA or protein avoids any unwanted integration, and in combination with using synthetic crRNA and tracrRNA, results in a completely DNA-free gene editing system. Here we demonstrate successful gene editing using DNA-free CRISPR-Cas9 reagents for gene knockout in zebrafish. Zebrafish embryos from a stable transgenic line were injected with Cas9 nuclease mRNA, synthetic tracrRNA, and crRNA designed to target GFP. A mismatch detection assay confirmed efficient gene editing, and successful functional protein knockout was confirmed by loss of GFP fluorescence.

Synthetic crRNA:tracrRNA for CRISPR-Cas9 gene editing

Why dual RNAs?

Most like the natural bacterial system

crRNA – synthetic RNA comprising 20 nt target-specific sequence and fixed *S. pyogenes* repeat sequence

tracrRNA – Long synthetic RNA which hybridizes with crRNA, a universal component (not target-specific)

Why synthetic?

Easier for researcher (no cloning, sequencing, etc.)

DNA-free guide RNA: transient, fewer off-target effects, less toxic

Enables high-throughput applications like arrayed screening

Provides possibility of chemical modifications to enhance functionality

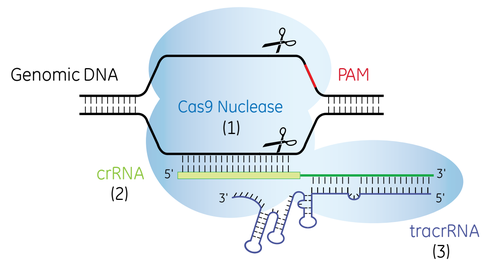


Figure 1. Illustration of Cas9 nuclease (light blue), programmed by the tracrRNA (blue) : crRNA (green) complex cutting both strands of genomic DNA 5' of the protospacer-adjacent motif (PAM) (red).

High-quality synthetic RNA for gene editing

RNA synthesis using Dharmacon's 2'-ACE chemistry shows improvements when compared to traditional 2'-silyl (TBDMS or TOM) protection strategies:

- Faster coupling rates, higher yields, greater purity
- Superior ease of handling
- CRISPR-Cas9 requires synthesis of long RNAs
  crRNA – synthetic 42 mer RNA, customized to target sequence
  tracrRNA – synthetic 74 mer RNA, synthesized in large batches for higher yields and purity

With traditional chemistries, it can be challenging to accurately and efficiently synthesize RNA greater than ~70 bases. Using Dharmacon's 2'-ACE chemistry, long RNA can be routinely synthesized and is ideal for generating synthetic sgRNAs.

- **Single guide RNA** – synthetic 99 mer

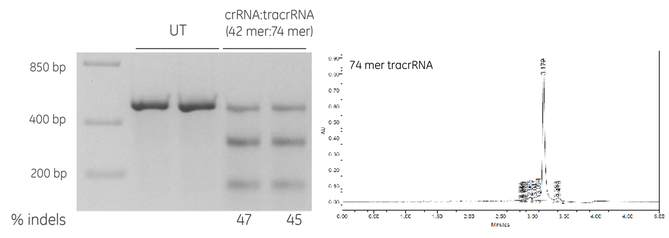


Figure 2. Demonstration of gene editing observed with the two RNA synthetic system (crRNA:tracrRNA) and mass spectrometry trace of a high-quality tracrRNA made with 2'-ACE chemistry after purification.

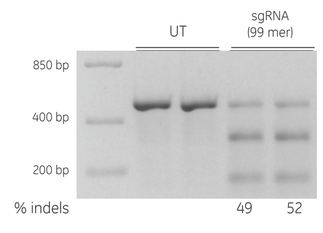


Figure 3. Demonstration of comparable editing observed with the synthetic sgRNA (99 mer) made with 2'-ACE chemistry.

Unwanted DNA integration events in plasmid-based gene editing experiments

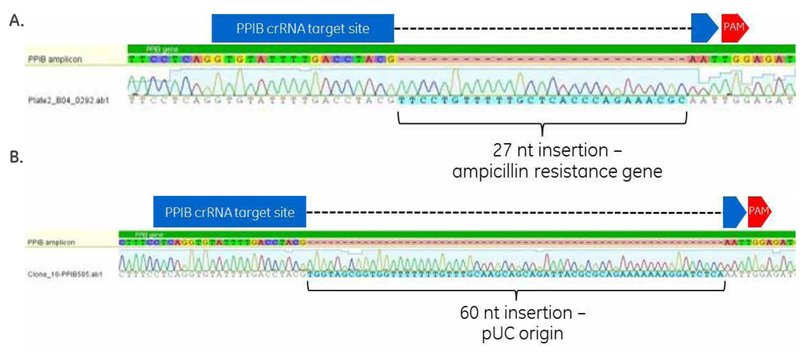


Figure 4. Sanger sequencing was performed on PCR products amplified from genomic DNA spanning the crRNA target site and analyzed for indels. Various indels were observed, but notably, several of the clonal lines contained insertions homologous to components of the Cas9 expression plasmid. Insertion of a 27 nt segment of the ampicillin resistance gene **(A)** and a 60 nt segment of the pUC origin **(B)** were detected at the crRNA cut site in the *PPIB* gene.

Gene editing efficiency using Cas9 mRNA & synthetic crRNA:tracrRNA

Using Cas9 mRNA with synthetic crRNA:tracrRNA allows for a completely DNA-free workflow and produces gene editing at levels comparable to Cas9-integrated lines. The DNA-free system reduces the concerns of unwanted integration as well as potential off-targets.

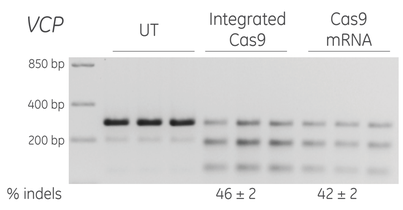
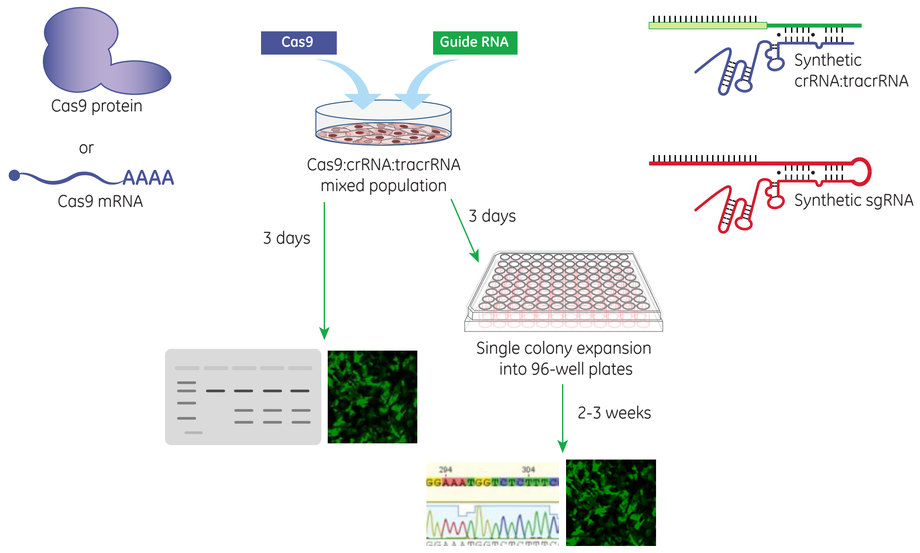


Figure 5. Synthetic crRNAs targeting *VCP* gene were transfected into U2OS-CAG-Cas9 stable cells and into U2OS cells with DharmaFECT™ Duo transfection reagent and Cas9 mRNA. Cells were harvested 72 hours after transfection and a mismatch detection assay (T7EI) was performed to estimate gene editing efficiency. The data indicate that Cas9 mRNA with synthetic crRNA:tracrRNA has comparable editing performance to the Cas9-integrated cell line.

CRISPR-Cas9 DNA-free gene editing workflow for cell culture applications

The two-part RNA system combined with Cas9 mRNA or Cas9 protein allows for a completely DNA-free workflow that saves times and removes concern of unwanted genomic DNA integration.



Detect Cas9-induced double-strand DNA breaks: PCR-based detection assay, Sanger sequencing, phenotypic assay

Figure 6. Gene editing workflow with Dharmacon™ Edit-R™ Cas9 mRNA or Cas9 protein and Edit-R™ synthetic crRNA:tracrRNA or sgRNA (synthetic custom RNA). After three days, the transfected cell populations can be assessed for editing efficiency or phenotypic analysis or split for single colony expansion. Clonal cell lines can be harvested and characterized by Sanger sequencing and phenotypic analysis.

Zebrafish (*Danio rerio*) as *in vivo* model organism

- High degree of sequence and functional homology with humans
- Relative ease of use in the laboratory
- Genetically tractable vertebrate model system that undergo rapid external development
- Considerable genomic resources, database of characterized mutant lines (zfin.org)
- Complete and annotated genome
- Relatively low cost research model and highly amenable to genetic manipulation

CRISPR-Cas9 gene editing efficiency with mismatch detection assay

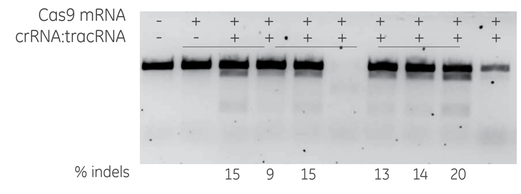


Figure 7. Zebrafish embryos were microinjected with Edit-R Cas9 mRNA only (+/- lane) or with Edit-R Cas9 mRNA plus crRNA:tracrRNA targeting GFP (+ lanes). Genomic DNA was prepared 2 days post-injection, PCR and DNA mismatch assay with T7EI was performed. Targeted DNA cleavage was achieved in 75% of the zebrafish embryos analyzed. Custom synthesis of Edit-R synthetic crRNA targeting GFP.

GFP knockout *in vivo* using Cas9 mRNA & synthetic crRNA:tracrRNA

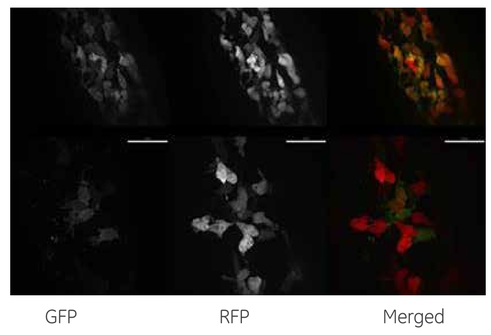


Figure 8. GFP knockout *in vivo* using Edit-R CRISPR-Cas9 DNA-free system. Dorsal view of Tg(Sox10:(PH) GFP; Sox10:tagRFP) zebrafish neural tube at 24 hours post fertilization. **A.** Neural crest cells expressing both GFP and RFP in zebrafish single-cell embryos were injected with only Cas9 mRNA. **B.** Neural crest cells in embryos injected with Cas9 mRNA and crRNA:tracrRNA targeting transgenic GFP display mosaic GFP expression as a result of functional protein knockout. (37 µM bar for reference)

Conclusions

- DNA-free gene editing using synthetic crRNA:tracrRNA is highly efficient and easy to use
- The removal of CRISPR-Cas9 DNA components increase experimental confidence while reducing potential off-targets
- Zebrafish are an amenable organism for genome engineering experiments

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