CRISPR-Cas9 genome editing utilizing chemically synthesized RNA

Kaizhang He, Eldon Chou, Amanda Haas, Žaklina Strezoska, Melissa L. Kelley, and Anja van Brabant Smith 2650 Crescent Drive, Lafayette, CO 80026, USA

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. Besides Cas9 nuclease, the natural CRISPR-Cas9 system also requires two RNA components: CRISPR RNA (crRNA) comprised of spacer-derived sequence and of repeat-derived sequence and tracrRNA, which hybridizes to the crRNA through repeat-derived sequences. The crRNA:tracrRNA complex recruits the Cas9 nuclease and cleaves DNA upstream of a protospacer-adjacent motif (PAM). The crRNA and tracrRNA can be linked together with a loop sequence for generation of a chimeric single guide RNA (sgRNA). In a vector-based approach, cloning and sequence verification of each sgRNA vector can be laborious and time consuming, especially if the goal is to study tens or thousands of gene targets. Likewise, *in vitro* transcription of the sgRNA also requires additional time and quality control to ensure consistency in length and purity of the transcribed product. In contrast, chemical synthesis can easily be employed for rapidly generating the crRNA and tracrRNA molecules separately or a synthetic sgRNA for direct delivery into cells for gene editing.

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

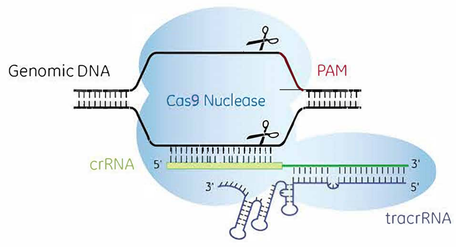

Figure 1. Illustration of Cas9 nuclease (light blue), programmed by the crRNA (green):tracrRNA (blue) complex cutting both strands of genomic DNA 5' of the protospacer-adjacent motif (PAM) (red).

High-quality synthetic RNA

RNA synthesis using GE Healthcare's Dharmacon 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 scale for higher yield and purity

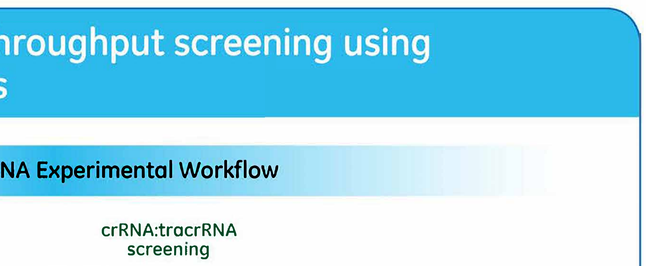

Figure 2. Demonstration of gene editing observed with the synthetic dual RNA system (crRNA:tracrRNA) and HPLC trace of a high-quality tracrRNA made with 2'-ACE chemistry after purification.

DNA-free gene editing efficiency using Cas9 mRNA and 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 3. Synthetic crRNAs targeting *VCP* gene were transfected into U2OS-CAG-Cas9 stable cells and into U2OS cells with Dharmacon™ 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.

General workflow for high throughput screening using arrayed synthetic dual RNAs

Arrayed crRNA Experimental Workflow

Arrayed synthetic crRNA:tracrRNA for phenotypic analysis

Knockout of *PLK1*, *KIF11* and *BCL2L1* by synthetic crRNAs induces apoptosis

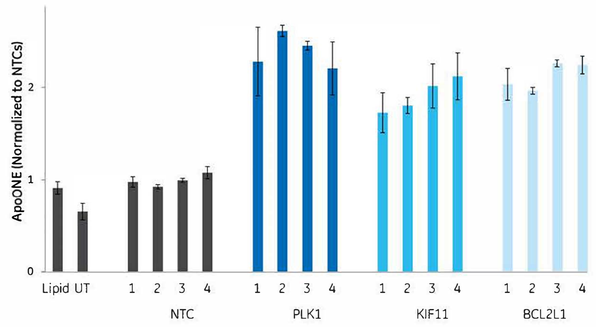

Figure 4. U2OS-(Ubi)EGFP-Cas9 stable cells were seeded at 10.000 cells/well in 96-well format one day prior to transfection. Cells were transfected with crRNA:tracrRNA (25 nM) using DharmaFECT 4 transfection reagent (0.2 µL/well). The Casp3/9 homogeneous assay (ApoONE, Promega) was preformed 48 hours post-transfection.

Decrease cell number and increase of mitotic index upon knockout of *PLK1* and *KIF11* by synthetic crRNAs in PC3-Cas9 stable cells

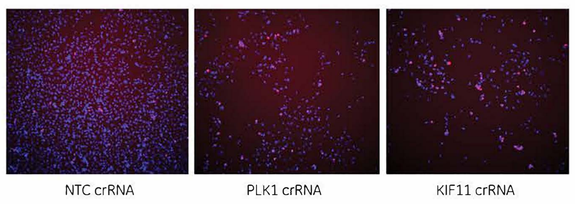

Figure 5. PC3-Cas9 stable cells seeded at 5,000 cells/well in 96-well format one day prior to transfection. Cells were transfected with crRNA:tracrRNA (25 nM) using DharmaFECT 4 transfection reagent (0.2 µL/well). Cells were fixed at 48 hours post-transfection, stained with anti-Phosho-Histone H3 (PH3) antibody (DY550-red) and Hoechst 33342 (blue), and analyzed on the IN Cell Analyzer 2200 imaging system (GE Healthcare).

Synthetic single-guide RNA for CRISPR-Cas9 gene editing

Although a synthetic dual RNA (crRNA:tracrRNA) system is very efficient and cost-effective for most applications, researchers working with *in vivo* and *ex vivo* models have indicated a preference for a sgRNA system. The advantages to using a synthetic sgRNA compared to plasmid-expressed or *in vitro* transcribed (IVT) sgRNA include:

- A single oligonucleotide arrives ready to use
- No cloning and sequencing steps or IVT reactions to perform
- Options for completely DNA-free gene editing when combined with Cas9 mRNA or Cas9 protein
- Potential for incorporation of chemical modifications

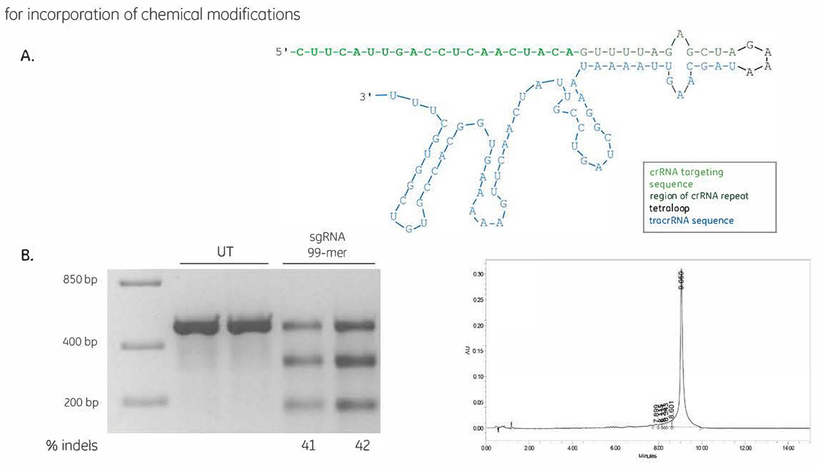

Figure 6. 2'-ACE chemistry was used to synthesize a 99-mer sgRNA targeting *PPIB* (**A**), which was then purified by HPLC (trace shown in **B**). A U2OS cell line stably expressing Cas9 nuclease from the CAG promoter was plated at 10,000 cells per well in 96-well format one day prior to transfection. sgRNA (25 nM) was transfected into duplicate wells using DharmaFECT 3 transfection reagent (0.25 µL/well). After 72 hours, direct cell lysis was amplified using primers surrounding the target site on the *PPIB* gene and gene editing efficiency was estimated using a mismatch detection assay (Dharmacon™ Edit-R™ Synthetic crRNA Positive Controls - Protocol). The 99-mer synthetic sgRNA for target gene editing resulted in high efficiency indel formation (**B**).

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

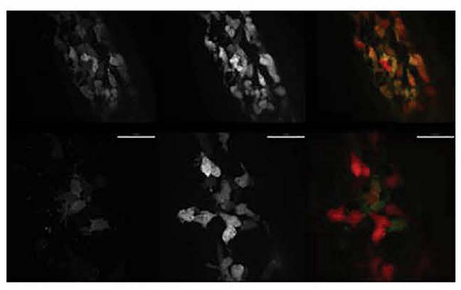

Figure 7. GFP knockout *in vivo* using Edit-R CRISPR-Cas9 DNA-free system. Dorsal view of Tg(Sox10:(PH) GFP; Sox10:tagRFP) zebrafish neural tube at 24 hours post fertilization. **A.** Neural crest cells expressing both GFP and RFP in zebrafish single-cell embryos were injected with only Cas9 mRNA. **B.** 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

- CRISPR-Cas9 gene editing using synthetic crRNA:tracrRNA or sgRNA is highly efficient and easy to use
- Synthetic crRNA:tracrRNA is suited for *in vitro* and *in vivo* applications, in particular, DNA-free approach with Cas9 mRNA or Cas9 protein
- Chemical synthesis of guide RNAs allows accurate and rapid production of arrayed crRNA libraries for high-confidence, loss-of-function screens

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If you have any questions directly related to this document, contact our experts:
UK +44 (0) 1223 976 000
USA +1 800 235 9880, +1 303 604 9499
Website horizondiscovery.com/contact--us