GE Healthcare

CRISPR-Cas9 genome editing utilizing chemically synthesized RNA

Michael Delaney, Kaizhang He, Eldon Chou, Amanda Haas, Žaklina Strezoska, Melissa L. Kelley, and Anja van Brabant Smith
Dharmacon, part of GE Healthcare, 2650 Crescent Drive, Lafayette, CO 80026, USA

Introduction

The CRISPR-Cas9 system allows researchers to quickly edit genes for functional gene knockout in mammalian, fish, and plant genomes, among others. Consequently, this has dramatically transformed biological research. Cas9 nuclease and a guide RNA are required for CRISPR-Cas9 genome engineering; however, these components can be utilized in different reagent formats, depending upon the application. Vector-based guide RNA reagents utilize an expressed chimeric single guide RNA (sgRNA), while synthetic reagents can be either sgRNA or a two-RNA system of CRISPR RNA (crRNA) and tracrRNA as the guide RNA component. Chemical RNA synthesis has been applied for the rapid generation of either crRNA and tracrRNA or sgRNA. This allows for direct delivery into cells for unique gene editing applications such as high throughput arrayed screening or experiments that benefit from DNA-free components.

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

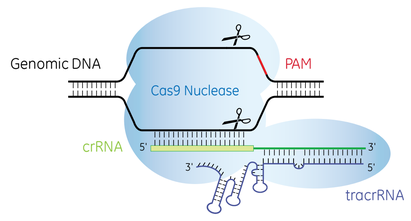


Figure 1. Illustration of Cas9 nuclease (light blue), programmed by the crRNA (green):tracrRNA (blue) complex cutting both strands of genomic DNA 5' of the protospacer-adjacent motif (PAM) (red).

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

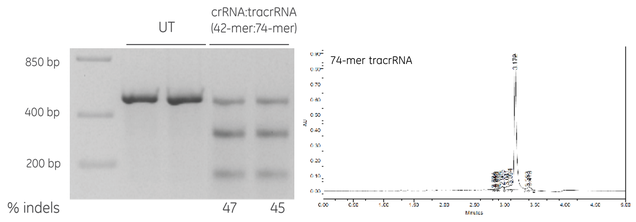


Figure 2. Demonstration of gene editing observed with the synthetic dual RNA system (crRNA:tracrRNA) and HPLC trace of a high-quality tracrRNA made with 2'-ACE chemistry after purification.

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.

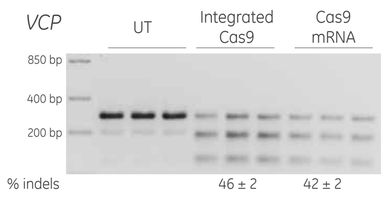
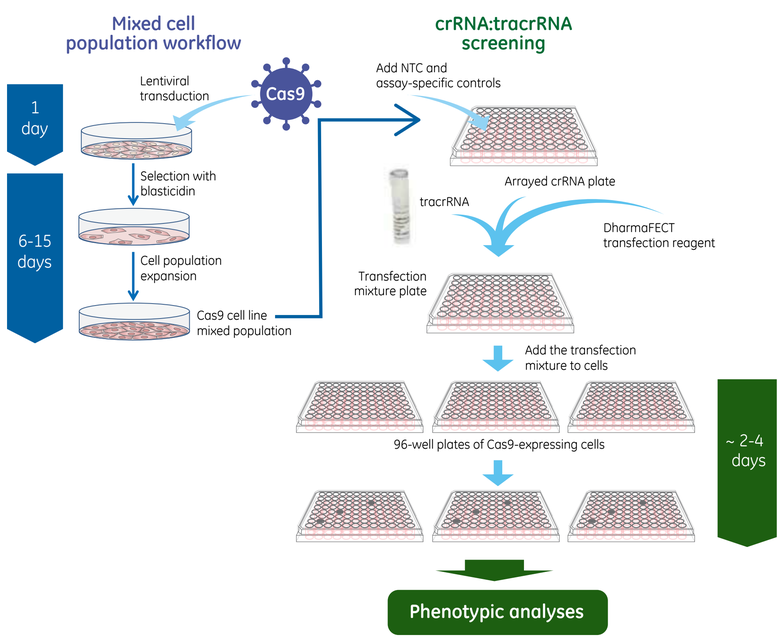


Figure 3. Synthetic crRNAs targeting *VCP* gene were transfected into U2OS-CAG-Cas9 stable cells and into U2OS cells with Dharmacon™ DharmaFECT™ Duo transfection reagent (Cat #T-2010-xx) and Edit-R™ Cas9 Nuclease mRNA (Cat #CAS11195). Cells were harvested 72 hours after transfection and a mismatch detection 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

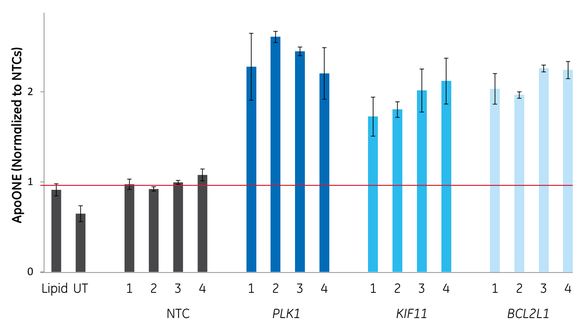


Figure 4. U2OS-(Ubi)EGFP-Cas9 stable cells were seeded at 10,000 cells/well in 96-well format one day prior to transfection. Cells were transfected with crRNA:tracrRNA (25 nM) using DharmaFECT 4 transfection reagent (0.2 µL/well; Cat #T-2004-xx). The Casp3/9 homogeneous assay (ApoONE, Promega) was preformed 48 hours post-transfection.

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, some 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

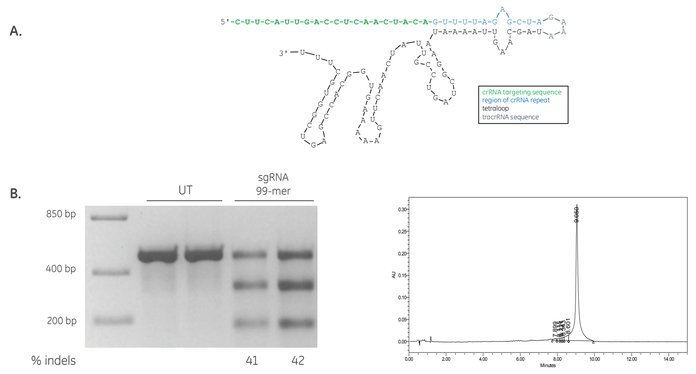


Figure 5. 2'-ACE chemistry was used to synthesize a 99-mer sgRNA targeting *PPIB* (**A**), which was then purified by HPLC (trace shown in **B**). A U2OS cell line stably expressing Cas9 nuclease from the CAG promoter was plated at 10,000 cells per well in 96-well format one day prior to transfection. sgRNA (25 nM) was transfected into duplicate wells using DharmaFECT 3 transfection reagent (0.25 µL/well; Cat #T-2003-xx). 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**).

Conjugated single guide RNA for CRISPR-Cas9 gene editing

Access to some long synthetic RNA sequences can be difficult due to the nature of solid-phase oligonucleotide synthesis. The challenging aspects of yield, purity, and cost help to complicate the synthesis of high quality long RNAs (~ 100 nt) on a high throughput scale.

- Ligation approaches are a viable option to gain access to longer sequences.
- Click Chemistry is bio-orthogonal and does not interfere with any other functional groups found in RNA
- Active groups can be introduced chemically on the ends of the RNA during solid-phase synthesis allowing for the ability to perform conjugations on much larger scales than other conventional enzymatic approaches.
- Can be carried out with 2'-ACE protected RNA allowing for improved solubility and handling of the RNA.
- Conjugation process produces an unnatural triazol linkage that may interfere with the recognition and activity of the Cas9 protein.

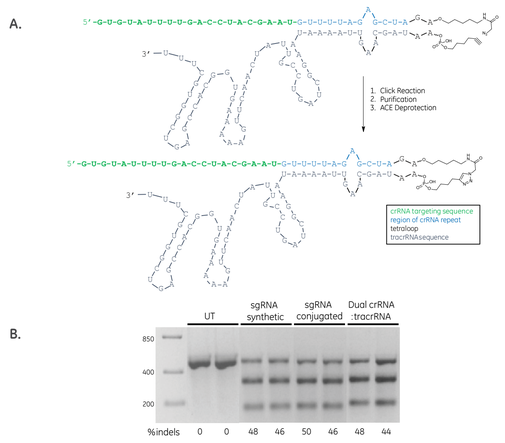


Figure 6. 2'-ACE chemistry was used to synthesize each component for the conjugated 99-mer. The 65-mer portion is based upon the tracrRNA sequence and is modified with a 5'-hexynyl group. The 34-mer portion is based upon the crRNA sequence and contains a 3'-aminohexyl group that is modified with azidoacetic acid NHS ester prior to the Click conjugation step illustrated in **A. B.** Comparison of the gene editing ability of fully synthetic sgRNA 99-mer, conjugated sgRNA 99-mer, and a synthetic dual RNA system (crRNA:tracrRNA). Cell line, transfection conditions and detection methods are identical to those listed in Figure 5. The level of gene disruption of the conjugated sgRNA 99-mer is comparable to the dual RNA system and the fully synthetic sgRNA 99-mer resulting in high efficiency indel formation.

K. He, E.T. Chou, S. Begay, E.M. Anderson and A. van Brabant Smith. Conjugation and Evaluation of Triazole-Linked Single Guide RNA for CRISPR-Cas9 Gene Editing. *ChemBioChem.* **17**, 1-5 (2016).

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
- The use of conjugative methods to produce guide RNAs provides an alternative approach to access difficult to synthesize guide RNAs but does not exceed 2'-ACE chemical synthesis in yield and purity

gelifesciences.com/dharmacon