2'-O-methyl phosphoroxythioate linkage-modified synthetic guide RNAs for efficient CRISPR-Cas9 genome editing and reduced cellular toxicity

Tiana Stansty, Megan Basila, Hidevaldo B. Machado, Emily Anderson, Eldon T. Chou, Melissa L. Kelley, Anja van Brabant Smith

Dharmacon, A Horizon Discovery Group Company, Lafayette, CO, USA

Abstract

The discovery of the bacterial CRISPR-Cas9 system has drastically changed the way we perform genome engineering and significantly shortened the time to results. With this system, a Cas9 endonuclease is targeted to DNA through a complementary guide RNA (gRNA) to cause a double-strand break at the target site. In the native system, a DNA-targeting CRISPR gRNA (crRNA) is delivered into a stably expressing Cas9 cell line. The placement of modifications on these regions can be chemically synthesized as transcription-ready gene-editing reagents. Alternatively, the crRNA and tracrRNA can be linked together to create a chimeric single guide RNA (sgRNA). For DNA-free editing, sgRNA can be delivered as in vitro transcribed (IVT) RNA or synthetic RNA in combination with Cas9 mRNA or Cas9 protein. An issue arises with IVT sgRNA due to it causing an immune response, which contrasts with synthetic guide RNAs which elicit no immune response. Here, we present chemical modification of both synthetic crRNA:tracrRNA and sgRNA with one to three 2'-O-methyl and phosphorothioate linkages (MS) on the 5' or 3' ends of the RNAs. We examined the stability of these RNAs by measuring their gene editing efficiency using an electroporation protocol in which guide RNA are co-delivered with Cas9 mRNA. Additionally, we assessed modification patterns for their ability to improve gene editing when delivered to cells with electroporation or lipid-mediated transfection along with Cas9 protein. Cas9 mRNA is delivered to a stably expressing Cas9 cell line. The placement of modifications on guide RNAs must be considered for the respective application. While co-electroporation experiments with Cas9 mRNA require modifications in certain positions on the guide RNA for stability, other applications do not, but in some cases may still show modest increases in gene editing efficiency. Importantly, some modification patterns caused increased cell death. Therefore, we have established a minimal guide RNA modification pattern for efficient gene editing and reduced toxicity in all applications.

Synthetic guide RNAs show comparable editing to in vitro transcribed sgRNA, but elicit no immune response

Stabilizing modifications of synthetic guide RNAs for resistance to degradation by nucleases

For sequential (Seq) electroporation, Cas9 mRNA was electroporated and followed 6 hours later with either Edit-R® PF8 Synthetic crRNA Control Kit (Cat# UK-007508) or Edit-R® predesigned crRNA targeting PSMD11 (Cat# CR-011367-04) and Edit-R® tracrRNA (Cat# 002000). When unmodified crRNA:tracrRNA reagents are co-delivered (Co) with Cas9 mRNA, gene editing was undetectable for both gene targets. NTC = Edit-R® crRNA Non-targeting Control #1 (Cat# U-007501), UT = untreated, n.d. = not detected.

Modification of the single-stranded regions of crRNA:tracrRNA is sufficient for stabilization in co-electroporation with Cas9 mRNA

Chemical modification of guide RNAs did not consistently improve gene editing in co-electroporation with Cas9 protein

Guide RNA modifications do not improve Cas9 activity in a stably expressing cell line, but some patterns negatively affect cell viability

Development of an electroporation protocol for synthetic guide RNA and Cas9 mRNA

C-terminal delivery

A. Synthetic crRNA:tracrRNA performs similarly to synthetic sgRNA and in vitro transcribed (IVT) sgRNA in U2OS cells when co-transfected with Dharmacon® Edit-R® Cas9 nuclear protein. NLS (Kelley, M. L., et al. 2016. Versatility of chemically synthesized guide RNAs for CRISPR-Cas9 genome editing. J. Biochem. 159, 73-80). B. Synthetic guide RNAs do not elicit an immune response, as IVT sgRNA does. When IVT sgRNAs are delivered into a stably expressing Cas9 cell line, major genes involved with an immune response are up-regulated.

Marker-free stable cell lines

% gene editing 20 40 60 80 100

Relative gene expression 0 0.2 0.4 0.6 0.8 1.0

% gene editing 20 40 60 80 100

Relative gene expression 0 0.2 0.4 0.6 0.8 1.0

Conclusions

- Stabilizing modifications on synthetic guide RNAs (crRNA:tracrRNA or sgRNA) to resist nuclease degradation are required for co-electroporation with Cas9 mRNA
- Modifications can improve gene editing when electroporated as RNPs, but this may be target sequence specific and not a universal benefit
- Modified and unmodified synthetic guide RNAs show no difference in gene activity when delivered by lipid transfection reagents into a Cas9 stable cell line
- Some modification patterns cause increased cellular toxicity
- Modification of the single-stranded regions of the guide RNAs modestly increases gene editing in lipid co-transfection with Cas9 mRNA or Cas9 protein while also stabilizing the RNAs for co-electroporation with Cas9 mRNA

References


Synthetic crRNA:tracrRNA performs similarly to synthetic sgRNA and in vitro transcribed (IVT) sgRNA in U2OS cells when co-transfected with Dharmacon® Edit-R® Cas9 nuclear protein NLS (Kelley, M. L., et al. 2016. Versatility of chemically synthesized guide RNAs for CRISPR-Cas9 genome editing. J. Biochem. 159, 73-80). B. Synthetic guide RNAs do not elicit an immune response, as IVT sgRNA does. When IVT sgRNAs are delivered into a stably expressing Cas9 cell line, major genes involved with an immune response are up-regulated.

Marker-free stable cell lines

% gene editing 20 40 60 80 100

Relative gene expression 0 0.2 0.4 0.6 0.8 1.0

Conclusions

- Stabilizing modifications on synthetic guide RNAs (crRNA:tracrRNA or sgRNA) to resist nuclease degradation are required for co-electroporation with Cas9 mRNA
- Modifications can improve gene editing when electroporated as RNPs, but this may be target sequence specific and not a universal benefit
- Modified and unmodified synthetic guide RNAs show no difference in gene activity when delivered by lipid transfection reagents into a Cas9 stable cell line
- Some modification patterns cause increased cellular toxicity
- Modification of the single-stranded regions of the guide RNAs modestly increases gene editing in lipid co-transfection with Cas9 mRNA or Cas9 protein while also stabilizing the RNAs for co-electroporation with Cas9 mRNA

References