**Chemical modifications of synthetic guide RNA for enhanced RNA stability and reduced cellular toxicity in CRISPR-Cas9 genome editing**

Hidevaldo B. Machado, Megan Basila, Eldon T. Chou, Emily M. Anderson, Melissa L. Kelley, Anja van Brabant Smith Dharmacia, A Horizon Discovery Group Company, 2650 Crescent Drive, Lafayette, CO 80026, USA

**Abstract**

The ability to modify the genome of organisms and mammalian cells has been available for decades to scientists; however, the understanding of the molecular mechanisms of cellular response to the CRISPR-Cas9 system, and its applicability to mammalian cells, has revolutionized the genome engineering field. Gene disruption and knock-ins can be easily obtained following the formation of double-strand breaks with the CRISPR-Cas9 system. In this system, the Cas9 nuclease targets the genomic DNA using a guide RNA, provided in either the native dual RNA system consisting of a trans-activating crRNA (tracrRNA) and a crRNA targeting Cas9 (crRNA) or a single-stranded guide RNA elicits the Cas9 activity. A single-stranded guide RNA (crRNA) uses a Cas9 protein with a guide RNA, such as in vitro transcribed (IVT) sgRNA, synthetic sgRNA or synthetic crRNA:tracrRNA. Synthetic sgRNAs or synthetic crRNA:tracrRNA are also advantageous owing to the ability to introduce chemical modifications to the RNA strands. The chemical modifications are important for stabilization of the dual RNA complex, thus increasing the RNA stability. We have chemically modified both synthetic sgRNAs and CRISPR-Cas9 RNA with a variety of 2'-O-methyl modified bases and phosphorothioate linkages (MS) on the 5' or 3' ends. These modified guide RNAs were delivered into cells alone with Cas9 mRNA or with Cas9 protein using electroporation or lipofection. Electroporation experiments on unmodified and modified modification patterns were found to significantly improve CRISPR-Cas9 gene editing when co-delivered with Cas9 mRNA compared to the unmodified guide RNAs. Most modification patterns, even those that did not significantly increase gene editing when used with Cas9 protein, had no detectable editing, while modification patterns resulted in increased cellular toxicity. Some of these modifications showed most toxic patterns when co-delivered with Cas9 mRNA or Cas9 protein. Overall, our results indicate that the use of the various MS modifications on synthetic guide RNAs is important for increased gene editing by co-electroporation with Cas9 mRNA, but may not necessarily require for lipid mediated transfection as some chemical modification patterns can increase cellular toxicity and thus may limit only modest improvements in gene editing efficiency.

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

A. Synthetic crRNA:tracrRNA performs similarly to synthetic sgRNA and in vitro transcribed (IVT) sgRNA in U2OS cells when co-electroporated with Dharmacia® EdiR® Cas9 Nuclease protein (NIH-cells, NHL, et al., 2010). Unmodified chemically synthesized guide RNAs for CRISPR-Cas9 genome editing. A. Abstract, B. 86–90. A. Synthetic guide RNAs do not elicit an immune response, while NIH-CRISPR®_0174_0170_B synthesize MS crRNA:tracrRNA delivered into a stably expressing Cas9 cell line, major genes involved in an immune response are up-regulated.

**Synthetic crRNA:tracrRNA for DNA-free CRISPR-Cas9 gene editing**

A protocol for electroporation of Cas9 mRNA and synthetic crRNA:tracrRNA was developed for K562 cells. One day before electroporation, 6 x 10⁷ cells were plated in a 150 mm dish. For sequential electroporation, 2 x 10⁷ cells were collected and electroporated with EdiR Cas9 Nuclease mRNA (Cat# ACS1199P) using the Lonza Nucleofector™_0174_0170_B, as per the manufacturer’s instructions. Electroporated cells were plated 6 hours, collected and electroporated with EdiR crRNA (5 µM). Cells were plated again for 6 hours and were electroplated for simultaneous Cas9 mRNA and crRNA gene editing. For co-delivery, 5 x 10⁷ cells were electroporated with Cas9 mRNA and crRNA for 6 hours, collected and electroporated in a single-electroporation. Electroporated cells were plated then incubated for 72 hours then analysed for gene editing.

**Chemical modification of gene RNAs did not contribute to improve gene editing in co-electroporation with Cas9 protein**

For sequential (Seq) electroporation, Cas9 mRNA was electroporated and followed 6 hours later with either EdiR crRNA Synthetic crRNA Control Kit (Cat# ACS0075) or EdiR-predesigned crRNA targeting PPIB (Cat# ACS1287-04) and EdiR crRNA (Cat# ACS20023). When unmodified crRNA:tracrRNA are co-delivered (Co) with Cas9 mRNA, gene editing was undetectable for both gene targets. NTC = EdiR crRNA targeting Control Kit (Cat# ACS0075), UT = untransfected, n.d. = not detected.

**RNA modifications do not affect gene editing activity when transfected into Cas9-expressing cells, but some patterns negatively affect cell viability**

A. RNA targeting crRNA:tracrRNA or crRNA were co-electroporated with Cas9 mRNA in U2OS and Iken cells using Dharmacia® EdiR™ Cas9 Nuclease transfection reagent (Cat# AT-005). Gene editing was determined using a GeneArt U2OS-RED cells and a GeneArt U2OS DSB reporter cells. Gene editing was determined using a GeneArt U2OS-RED cells and a GeneArt U2OS DSB reporter cells. Data are means ± SD. Some modification patterns show modest improvements in gene editing with co-transfection of modified synthetic guide RNA with Cas9 mRNA or Cas9 protein.

**Conclusions**

- Stabilizing modifications on synthetic guide RNAs (crRNA:tracrRNA or sgRNA) to resist nucleic acid degradation are required for co-electroporation with Cas9 mRNA.
- Some modifications improve gene editing efficiency of some gene targets in co-electroporation with Cas9 protein.
- Lipid transfection of synthetic guide RNA in a Cas9 stable cell line shows no differences in gene editing between unmodified and modified RNA.
- Some modification patterns are toxic to cells with lipid transfection.
- Stabilization of the single-stranded regions of crRNA:tracrRNA is sufficient for stabilization in co-electroporation with Cas9 mRNA.

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