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

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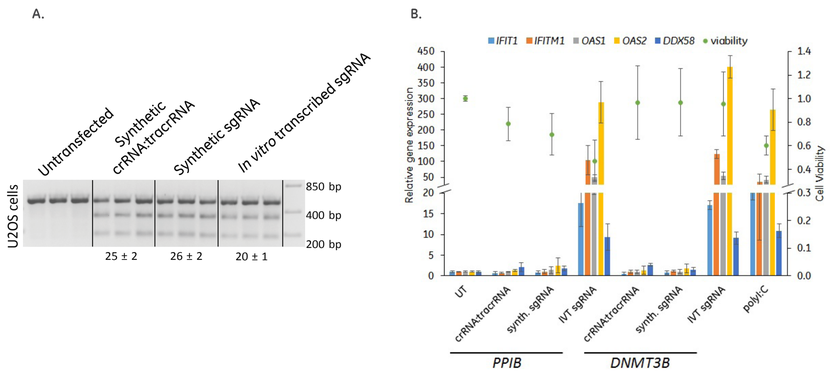
Abstract

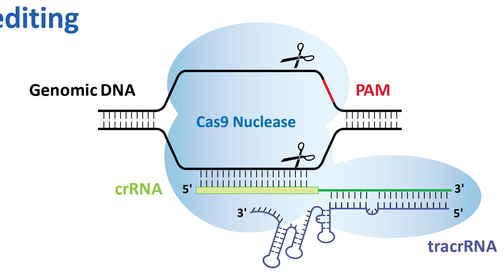
The ability to modify the genome of organisms and mammalian cells has been available for decades to scientists. However, the understanding of the mechanism of bacterial adaptive immunity by the CRISPR-Cas9 system, and its applicability to eukaryotic cells, has revolutionized the genome engineering field. Gene disruption and knock-ins can be easily obtained through the formation of precise DNA double-strand breaks with the CRISPR-Cas9 system. In this system, the Cas9 nuclease targets the genomic DNA using a guide RNA, provided as either the native dual-RNA consisting of a DNA-targeting CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), or a chimeric single guide RNA (sgRNA) created through the fusion of crRNA and tracrRNA. DNA-free genome engineering can be achieved by using Cas9 mRNA or Cas9 protein with a guide RNA, such as in vitro transcribed (IVT) sgRNA, synthetic sgRNA or synthetic crRNA:tracrRNA. Synthetic sgRNA or crRNA:tracrRNA offer an advantage over IVT sgRNA by providing little-to-no effect on the cellular immune response in addition to allowing the incorporation of chemical modifications to increase the RNA stability. We have chemically modified both synthetic sgRNA and crRNA:tracrRNA with one to three 2'-O-methyl nucleotides and backbone phosphorothioate linkages (MS) on the 5' and/or 3' ends. These modified guide RNAs were delivered into cells alone or with Cas9 mRNA or Cas9 protein using electroporation or lipid-mediated transfection. In electroporation experiments, some modification patterns were found to significantly improve CRISPR-Cas9 gene editing when co-delivered with Cas9 mRNA compared to the unmodified versions, yet most modifications did not significantly increase gene editing when used with Cas9 protein. Lipid-mediated transfection of modified guide RNAs into a Cas9-expressing cell line resulted in similar editing efficiencies as the unmodified synthetic guide RNAs; however, certain modification patterns resulted in increased cellular toxicity. Of the modifications that were nontoxic, some patterns showed modest improvement in editing efficiency when co-transfected with Cas9 mRNA or Cas9 protein. Overall, our results indicate that the placement and number of MS modifications on synthetic guide RNAs are important for increased gene editing by co-electroporation with Cas9 mRNA, but are not necessarily required for lipid-mediated transfection as some chemical modification patterns can increase cellular toxicity and may provide only modest improvement in gene editing efficiency.

Synthetic guide RNAs show comparable editing to *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-transfected with Dharmacon™ Edit-R™ Cas9 nuclease protein NLS (Kelley, M.L., *et al.* 2016. Versatility of chemically synthesized guide RNAs for CRISPR-Cas9 genome editing. *J. Biotech.*, **233**, 74–83). B. Synthetic guide RNAs do not elicit an immune response, while 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.

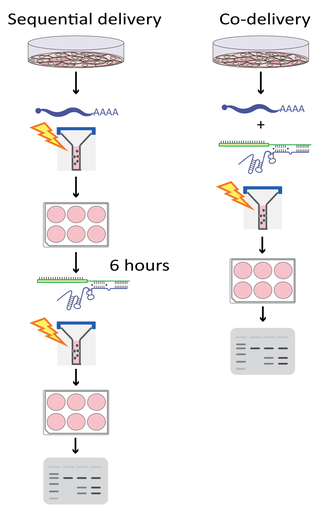
Synthetic crRNA:tracrRNA for DNA-free CRISPR-Cas9 gene editing



Benefits of Dharmacon™ Edit-R™ synthetic guide RNA

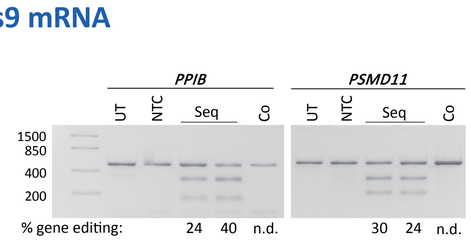
- Arrives ready to use (no cloning, sequencing, etc.)
- Transient, fewer off-target effects, less toxic
- Enables high-throughput applications (e.g., arrayed screening)
- Permits application of chemical modifications

Electroporation workflow for synthetic guide RNA and Cas9 mRNA



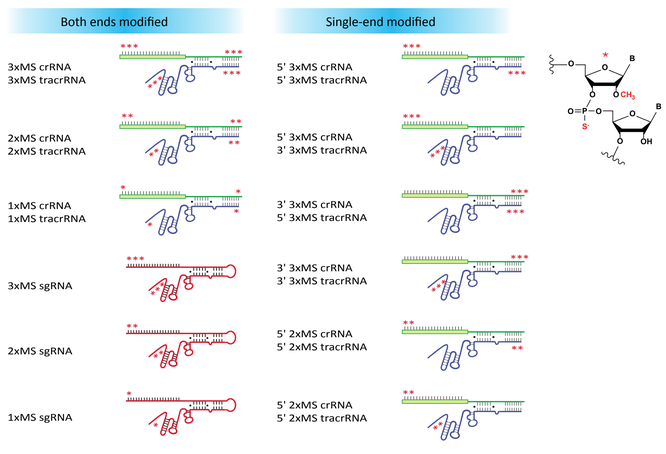
A protocol for electroporation of Cas9 mRNA and synthetic crRNA:tracrRNA was developed for K-562 cells. One day before electroporation, 6 × 10^6 cells were plated in a 150 mm dish. For sequential electroporations, 2 × 10^6 cells were collected and electroporated with Edit-R Cas9 Nuclease mRNA (Cat #CAS11195) using the Lonza Nucleofector 2b™, as per the manufacturers protocol. Electroporated cells were plated for 6 hours, collected and electroporated with crRNA:tracrRNA (5.4 µM). Cells were plated again and incubated for 72 hours and analyzed for gene editing. For co-delivery, K-562 cells were electroporated with Cas9 mRNA and crRNA:tracrRNA, as described above, in a single electroporation. Electroporated cells were plated and incubated for 72 hours then analyzed for gene editing.

Sequential electroporation is required for delivery of unmodified crRNA:tracrRNA and Cas9 mRNA



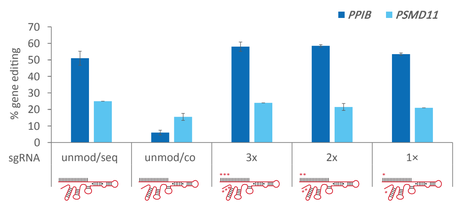
For sequential (Seq) electroporation, Cas9 mRNA was electroporated and followed 6 hours later with either Edit-R *PPIB* Synthetic crRNA Control Kit (Cat #UK-007050) or Edit-R predesigned crRNA targeting PSMD11 (Cat #CR-011367-04) and Edit-R tracrRNA (Cat #U-002000). When unmodified crRNA:tracrRNA are 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.

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



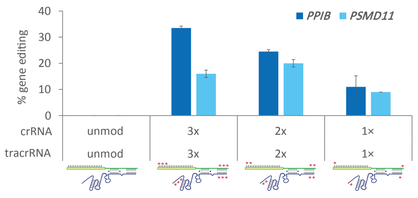
Synthetic guide RNAs were modified with one to three 2' O-methyl and backbone phosphorothioates (MS) on either the 5' or 3' or both ends of the RNA strands. All combinations of 5' and 3' end modifications on crRNA and tracrRNA were tested.

Modification of both ends of synthetic sgRNAs stabilize RNAs for co-electroporation with Cas9 mRNA



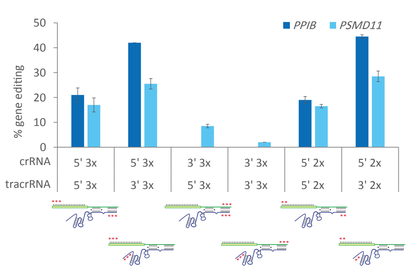
Cas9 mRNA and *PPIB* or *PSMD*11 sgRNA were co-electroporated in K-562 cells. Unmodified sgRNAs are most effective using a sequential electroporation protocol (unmod/seq) rather than co-electroporation (unmod/co). However, modification of both ends of the RNAs with 1-3xMS modifications (*) resulted in levels of gene editing similar to that obtained with sequential electroporation. Data are means ± SD.

Modification of both ends of crRNA:tracrRNA stabilize the RNAs for co-electroporation with Cas9 mRNA



Cas9 mRNA and *PPIB* crRNA or *PSMD*11 crRNA and tracrRNA were co-electroporated in K-562 cells. Unmodified (unmod) crRNA:tracrRNA had no detectable editing, while modification of both ends of the RNAs with 1-3xMS modifications resulted in observable levels of gene editing. Data are means ± SD.

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

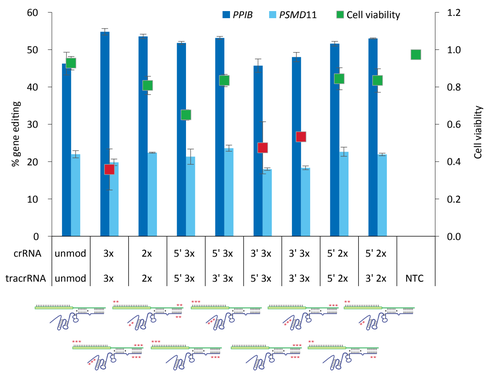


Modification of the 5' end of crRNA is important for stabilization of the dual RNA system for co-electroporation. With 3' modified crRNA, gene editing is drastically reduced or undetectable when either end of tracrRNA is modified for targeting *PPIB* and *PSMD*11. Gene editing is improved when 5' modified crRNA is combined with 3' modified tracrRNA and both 3x and 2xMS modifications are comparable. Samples done in duplicate.

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

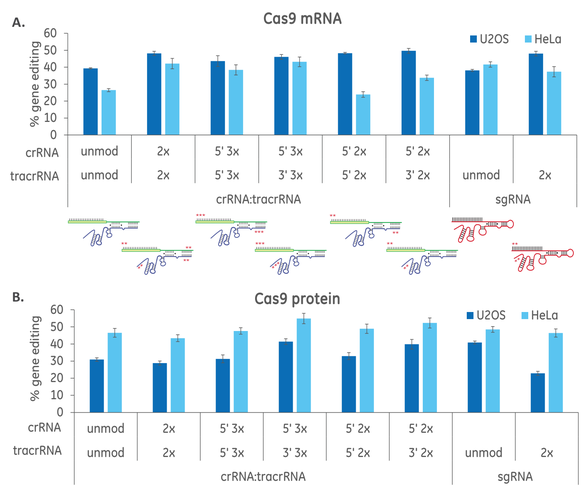
Edit-R Cas9 Nuclease protein NLS (Cat #CAS11729; 1.5 µM) and unmodified or modified guide RNAs (sgRNA or crRNA:tracrRNA; 3 µM) were complexed for 10 minutes for ribonucleoprotein (RNP) complex formation at room temperature prior to electroporation in K-562 cells. Despite a ~ 2-fold increase in PPIB gene editing observed with 5' 3xMS crRNA:3' 3xMS tracrRNA, unmodified or modified guide RNAs showed similar results for gene editing efficiency when used in co-electroporation with Cas9 protein. Data are means ± SD.

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



Unmodified and modified crRNA:tracrRNA were transfected into a stably expressing Cas9 U2OS cell line using DharmaFECT 1 transfection reagent (Cat #T-2001). Gene editing was similar between unmodified and modified for the target genes. Cell viability, however, was reduced below 70% for some modification patterns, specifically 3xMS on both ends of crRNA:tracrRNA and 3' 3xMS crRNA with either 5' or 3' 3xMS (red boxes). Those patterns were removed in further testing in lipid transfection. Higher cellular toxicity was also found with 3xMS sgRNAs (not shown).

Some modification patterns show modest improvements in gene editing with co-transfection of modified synthetic guide RNA with Cas9 mRNA or Cas9 protein



A. *PPIB*-targeting crRNA:tracrRNA or sgRNA were co-transfected with Cas9 mRNA in U2OS and HeLa cells using DharmaFECT 1 transfection reagent. An increase in gene editing of ~ 10% was observed with most modification patterns. **B.** When *PPIB*-targeting crRNA:tracrRNA were co-transfected with Cas9 protein using DharmaFECT 1 transfection reagent, ~ 10% increase in gene editing was observed only with 5' 2xMS crRNA:3' 2xMS tracrRNA and 5' 3xMS crRNA:3' 3xMS tracrRNA. Overall, modification of the single-stranded region of crRNA:tracrRNA results in ~ 1.2-fold increase in gene editing when co-transfected with Cas9 mRNA or Cas9 protein. Data are means ± SD.

Conclusions

- Stabilizing modifications on synthetic guide RNAs (crRNA:tracrRNA or sgRNA) to resist nuclease 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 difference in gene editing between unmodified and modified RNAs
- Some modification patterns are toxic to cells with lipid transfection
- Stabilization of the single-stranded regions of the dual RNAs modestly increases gene editing in lipid co-transfection with Cas9 mRNA or Cas9 protein

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