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

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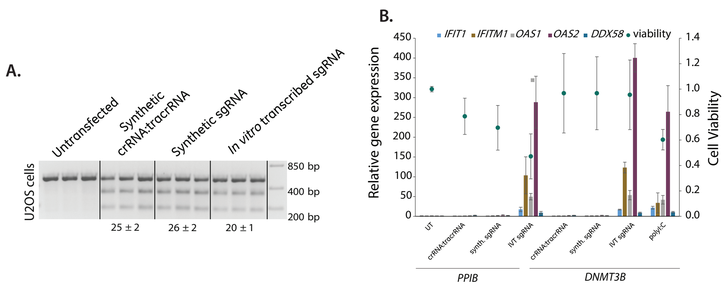
Abstract

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 to cause a double-strand break at the target site. In the native system, a DNA-targeting CRISPR RNA (crRNA) is duplexed with the trans-activating crRNA (tracrRNA) to guide the Cas9 protein; these small RNAs can be chemically synthesized as transfection-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 phosphorothioates linkages (MS) on the 5′ and/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 RNAs 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, or into 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

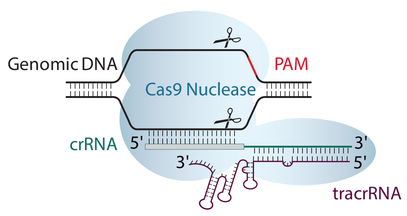


A. Synthetic crRNA:tracrRNA performs similarly to synthetic sgRNA and in vitro transcribed (IVT) sgRNA in U2OS cells when co-transfected with Dharmacon™ Edit-R™ Cas9 nuclease protein NLS (Kelley, M.L., *et al.* 2016. Versatility of chemically synthesized guide RNAs for CRISPR-Cas9 genome editing. *J. 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.

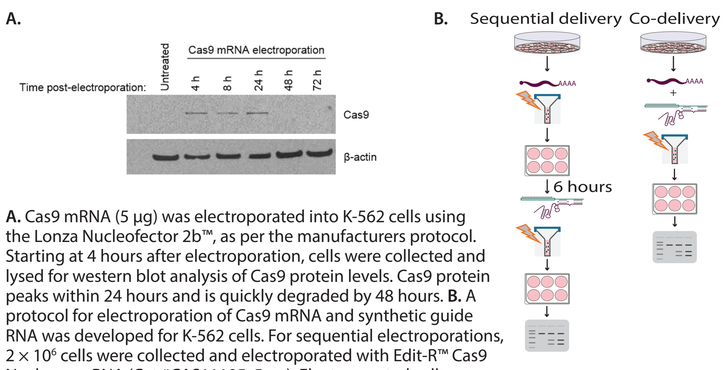
Synthetic crRNA:tracrRNA for DNA-free CRISPR-Cas9 gene editing

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

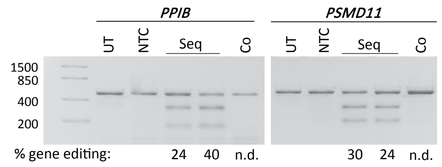


Development of an electroporation protocol for synthetic guide RNA and Cas9 mRNA



A. Cas9 mRNA (5 µg) was electroporated into K-562 cells using the Lonza Nucleofector 2b™, as per the manufacturers protocol. Starting at 4 hours after electroporation, cells were collected and lysed for western blot analysis of Cas9 protein levels. Cas9 protein peaks within 24 hours and is quickly degraded by 48 hours. **B.** A protocol for electroporation of Cas9 mRNA and synthetic guide RNA was developed for K-562 cells. For sequential electroporations, 2×10^6 cells were collected and electroporated with Edit-R™ Cas9 Nuclease mRNA (Cat #CAS11195; 5 µg). Electroporated cells were plated for 6 hours, collected and electroporated with synthetic guide RNA (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 guide RNA, as described above, in a single electroporation. Electroporated cells were plated and incubated for 72 hours then analyzed for gene editing.

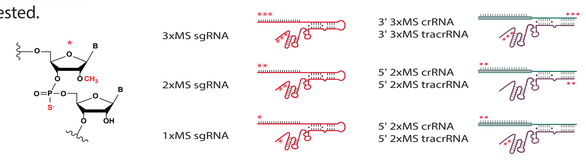
Sequential electroporation is required for delivery of unmodified crRNA:tracrRNA and Cas9 mRNA



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 *PSMD*11 (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.

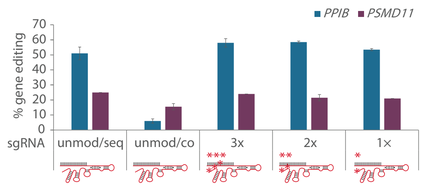
Stabilizing modifications of synthetic guide RNAs for resistance to degradation by nucleases

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 crRNA:tracrRNA stabilize the RNAs for co-electroporation with Cas9 mRNA

Cas9 mRNA and PPIB crRNA or PSMD11 crRNA and tracrRNA were co-electroporated in K-562 cells. Unmodified (unmod) crRNA:tracrRNA had no detectable gene editing, while modification of both ends of the RNAs with 1-3xMS modifications resulted in observable levels of gene editing.



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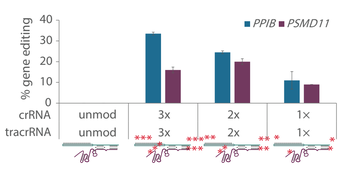
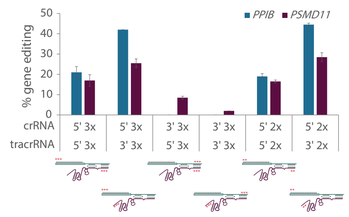
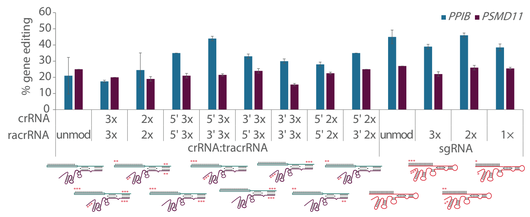


Modification of the single-stranded regions of crRNA:tracrRNA is sufficient for stabilization in co-electroporation with Cas9 mRNA

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 PSMD11. Gene editing is improved when 5′ modified crRNA is combined with 3′ modified tracrRNA and both 3x and 2xMS modifications are comparable.



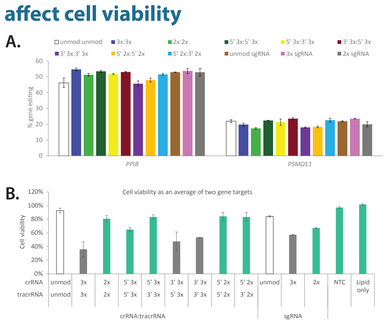
Chemical modification of guide RNAs did not consistently improve gene editing in co-electroporation with Cas9 protein



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. Unmodified or modified guide RNAs showed similar results for gene editing efficiency when used in co-electroporation with Cas9 protein. Data are means ± SD.

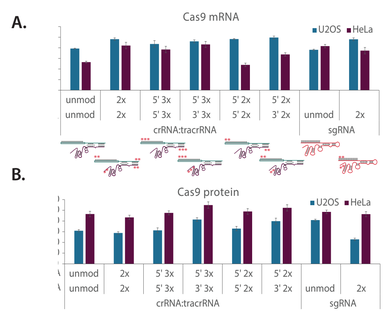
Guide RNA modifications do not improve Cas9 activity in a stably expressing cell line, but some patterns negatively affect cell viability

A. Unmodified and modified crRNA:tracrRNA or sgRNA (25 nM) 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 guide RNAs for the target genes. B. Cell viability was reduced to less then 70% for some modification patterns, specifically 3xMS on both ends of crRNA:tracrRNA and sgRNA and 3′ 3xMS crRNA with either 5′ or 3′ 3xMS (gray bars). Guide RNA with toxic modification patterns were removed in further testing in lipid transfection experiments.



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
- 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

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Reference M. Basila, M.L. Kelley, A. van Brabant Smith. Minimal 2′-O-methyl phosphorothioate linkage modification pattern of synthetic guide RNAs for increased stability and efficient CRISPR-Cas9 gene editing avoiding cellular toxicity. *PLoS ONE* **12**(11): e0188593. (2017). doi.org/10.1371/journal.pone.0188593