Applications of chemically modified synthetic guide RNA for CRISPR-Cas9 genome editing

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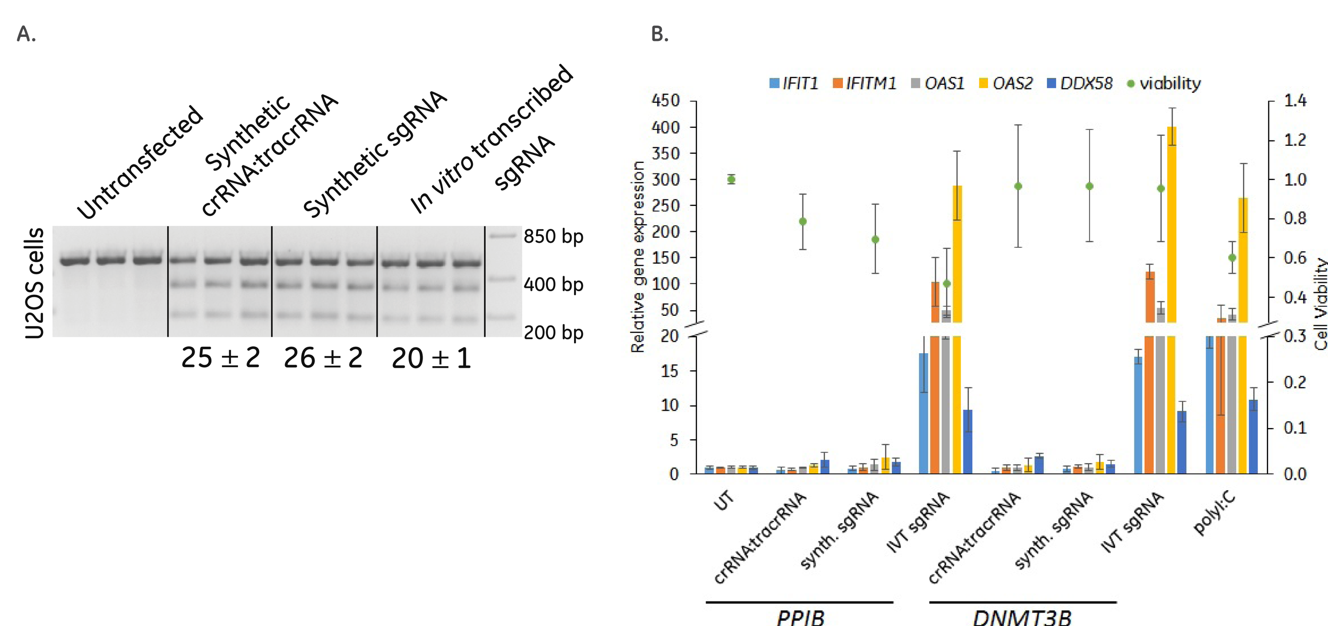
Abstract

The bacterial CRISPR-Cas9 system has been applied in mammalian cells to efficiently disrupt genes through the formation of targeted DNA double-strand breaks. The Cas9 nuclease is directed to DNA using a guide RNA (gRNA), either as the native dual-RNA system 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 gRNA, including *in vitro* transcribed (IVT) gRNA, synthetic sgRNA or synthetic crRNA:tracrRNA. While IVT sgRNAs can elicit an immune response, synthetic sgRNA or synthetic crRNA:tracrRNA have little to no effect on the immune response and permit chemical modifications to be incorporated to the RNA for increased stability. Here we present chemical modification of synthetic crRNA:tracrRNA with one to three 2'-O-methyl and phosphorothioates (MS) on the 5' and/or 3' ends. These modified RNAs were co-delivered into cells with Cas9 mRNA or Cas9 protein using electroporation. Some modification patterns were found to significantly improve CRISPR-Cas9 gene editing when used with Cas9 mRNA compared to the unmodified versions, yet most modifications did not significantly increase gene editing when used with Cas9 protein. Transfection reagent-mediated delivery of these modified gRNAs into a stably expressing Cas9 cell line resulted in similar editing efficiencies as the unmodified synthetic gRNAs, and cellular toxicity was observed with certain modification patterns. 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 MS modifications are required for experiments with co-electroporation of Cas9 mRNA and synthetic gRNA, yet have no impact on editing efficiency when delivered with lipid-based transfection reagents.

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

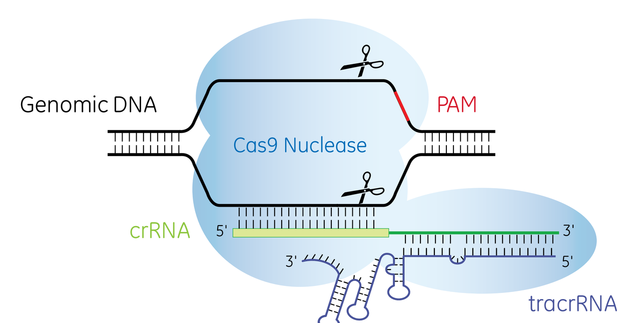


A. Synthetic crRNA:tracrRNA performs similarly to synthetic sgRNA and *in vitro* transcribed (IVT) sgRNA in U2OS cells when co-transfected with Dharmacon™ Edit-R™ Cas9 nuclease protein NLS (Kelley, M.L., *et al.* (2016) Versatility of chemically synthesized guide RNAs for CRISPR-Cas9 genome editing.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

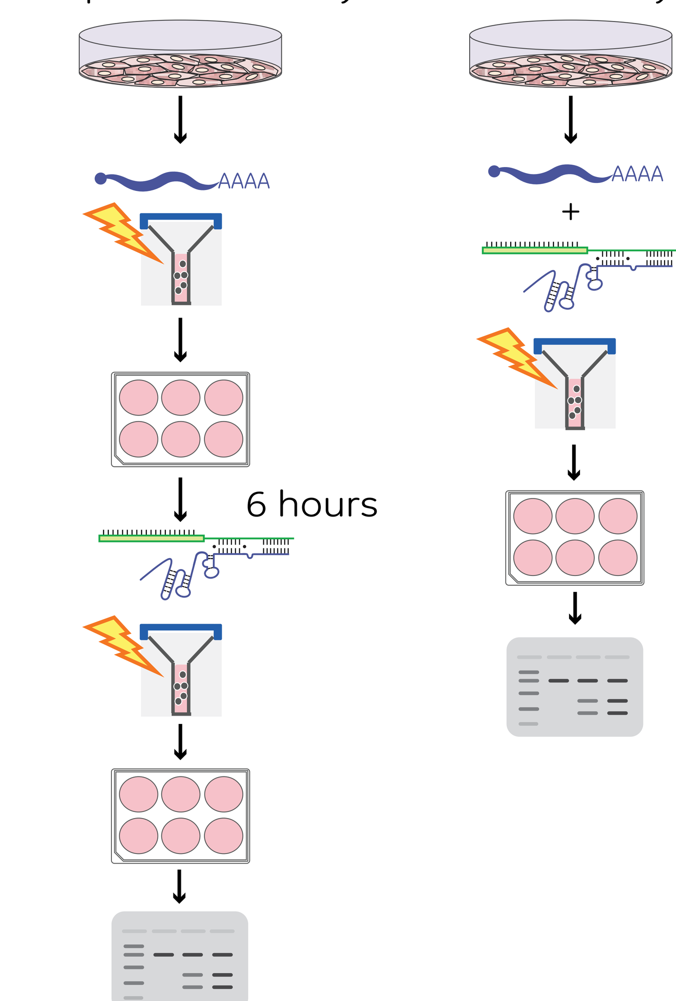
- Arrives ready to use (no cloning, sequencing, etc.)
- Transient, fewer off-target effects, less toxic
- Enables high-throughput applications (e.g., arrayed screening)
- Permits application of chemical modifications



General electroporation workflow for synthetic crRNA:tracrRNA & Cas9 mRNA

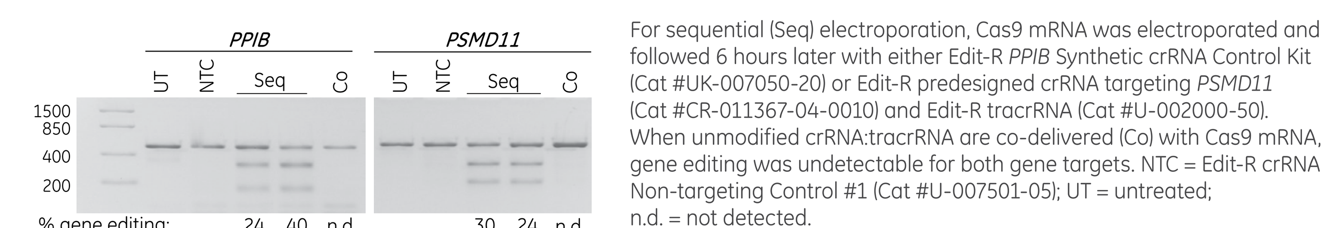
Sequential delivery

Co-delivery



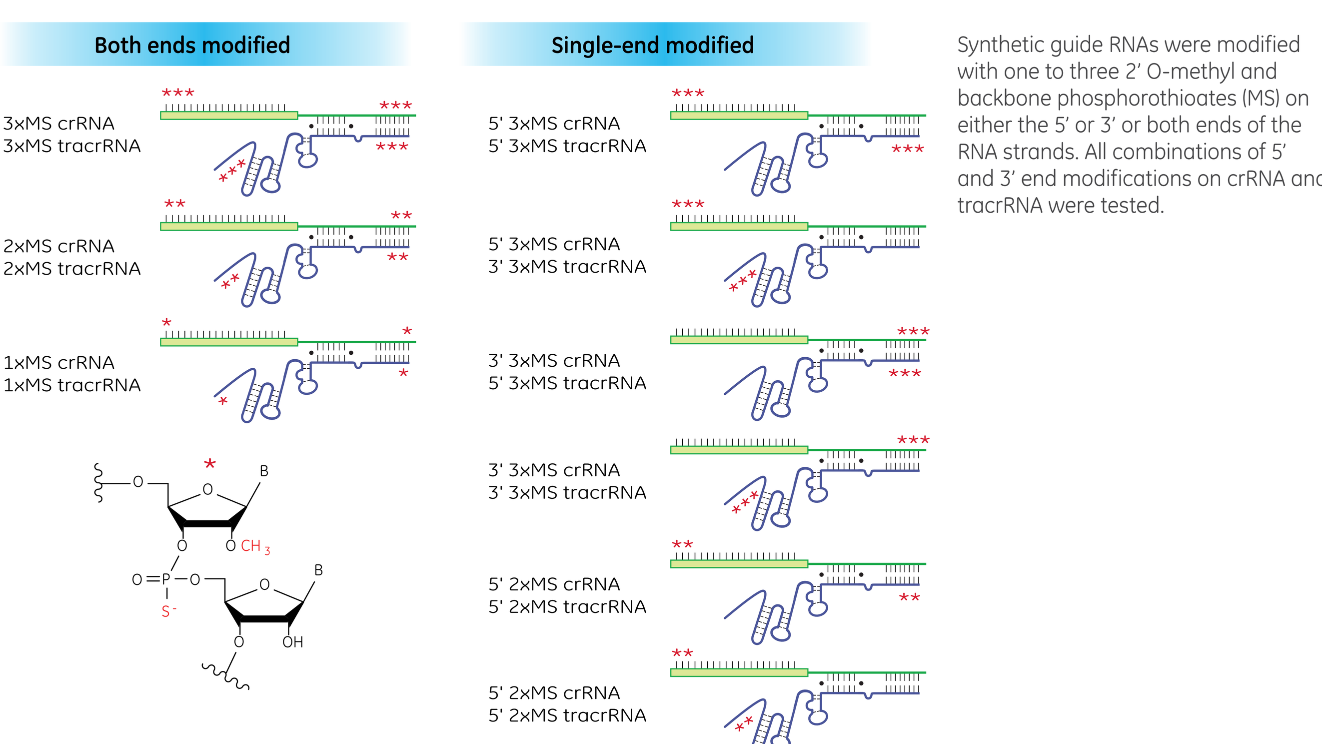
A protocol for electroporation of Cas9 mRNA and synthetic crRNA:tracrRNA was developed for K-562 cells. One day before electroporation, 6×10^6 cells were plated in a 150 mm dish. For sequential electroporations, 2×10^5 cells were collected and electroporated with Edit-R Cas9 Nuclease mRNA (Cat #CAS11195; 5 μg) 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 & Cas9 mRNA



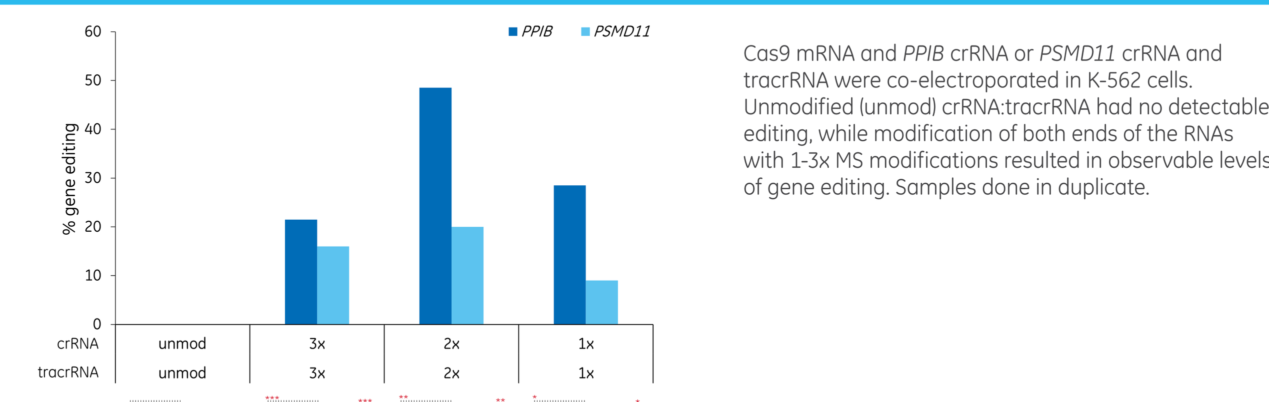
For sequential (Seq) electroporation, Cas9 mRNA was electroporated and followed 6 hours later with either Edit-R *PPIB* Synthetic crRNA Control Kit (Cat #UK-007050-20) or Edit-R predesigned crRNA targeting *PSMD11* (Cat #CR-011367-04-0010) and Edit-R tracrRNA (Cat #U-002000-50). When unmodified crRNA:tracrRNA 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-007501-05); UT = untreated; n.d. = not detected.

Modifications of synthetic guide RNAs for increased stability



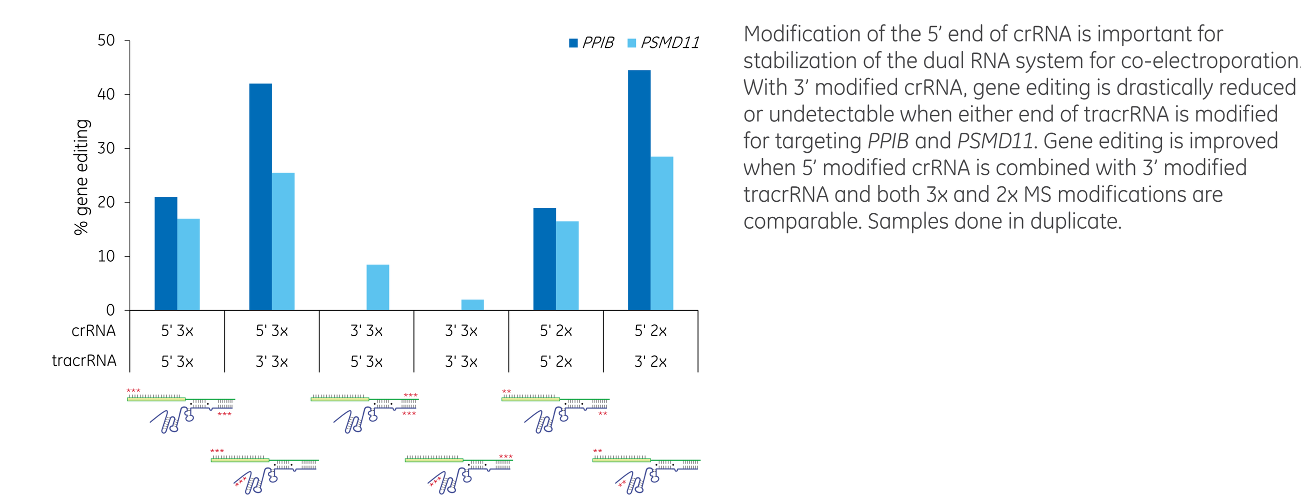
Synthetic guide RNAs were modified with one to three 2' O-methyl and backbone phosphorothioates (MS) on either the 5' or 3' or both ends of the RNA strands. All combinations of 5' and 3' end modifications on crRNA and tracrRNA were tested.

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



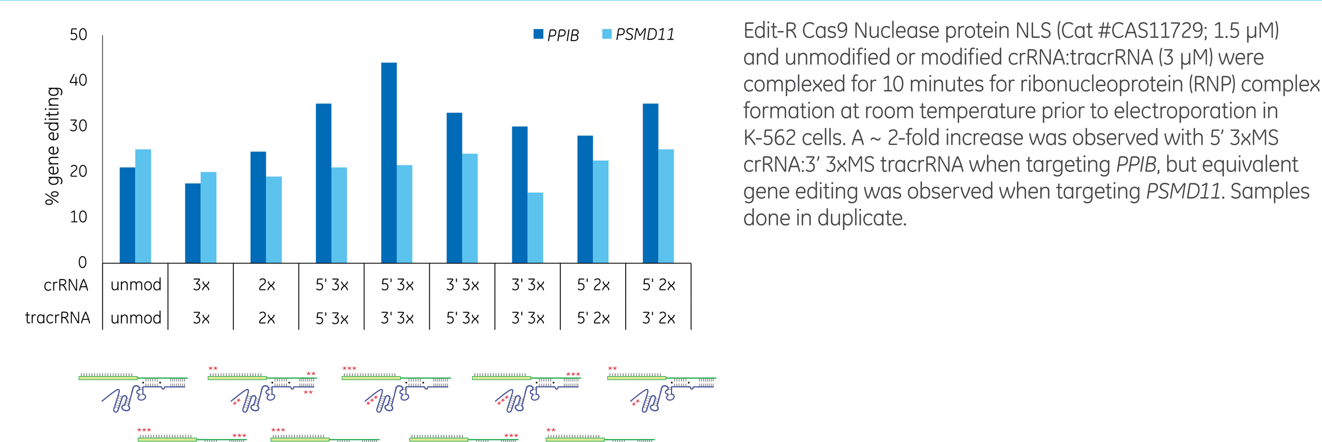
Cas9 mRNA and *PPIB* crRNA or *PSMD11* crRNA and tracrRNA were co-electroporated in K-562 cells. Unmodified (unmod) crRNA:tracrRNA had no detectable editing, while modification of both ends of the RNAs with 1-3x MS modifications resulted in observable levels of gene editing. Samples done in duplicate.

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



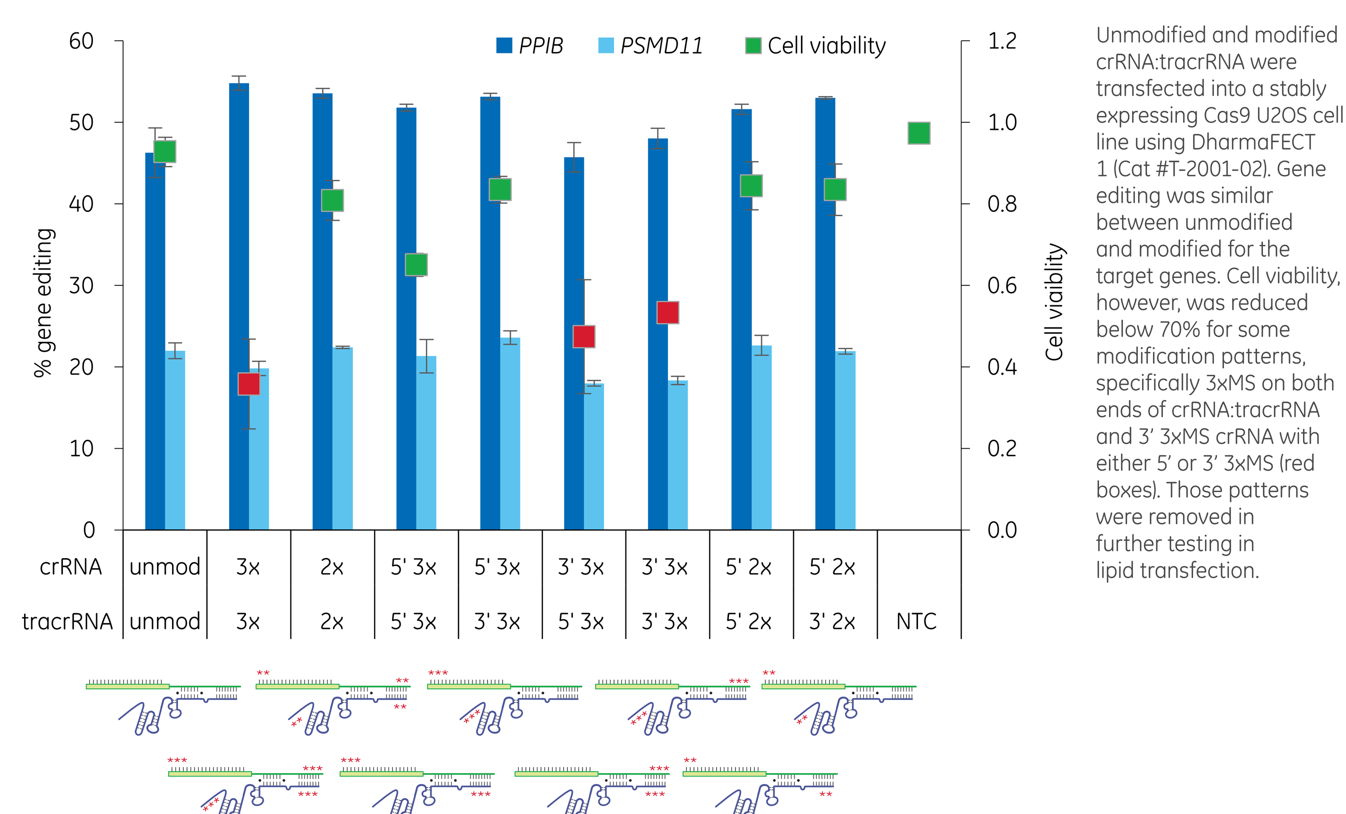
Modification of the 5' end of crRNA is important for stabilization of the dual RNA system for co-electroporation. With 3' modified crRNA, gene editing is drastically reduced or undetectable when either end of tracrRNA is modified for targeting *PPIB* and *PSMD11*. Gene editing is improved when 5' modified crRNA is combined with 3' modified tracrRNA and both 3x and 2x MS modifications are comparable. Samples done in duplicate.

Modification of crRNA:tracrRNA can increase gene editing in co-electroporation with Cas9 protein



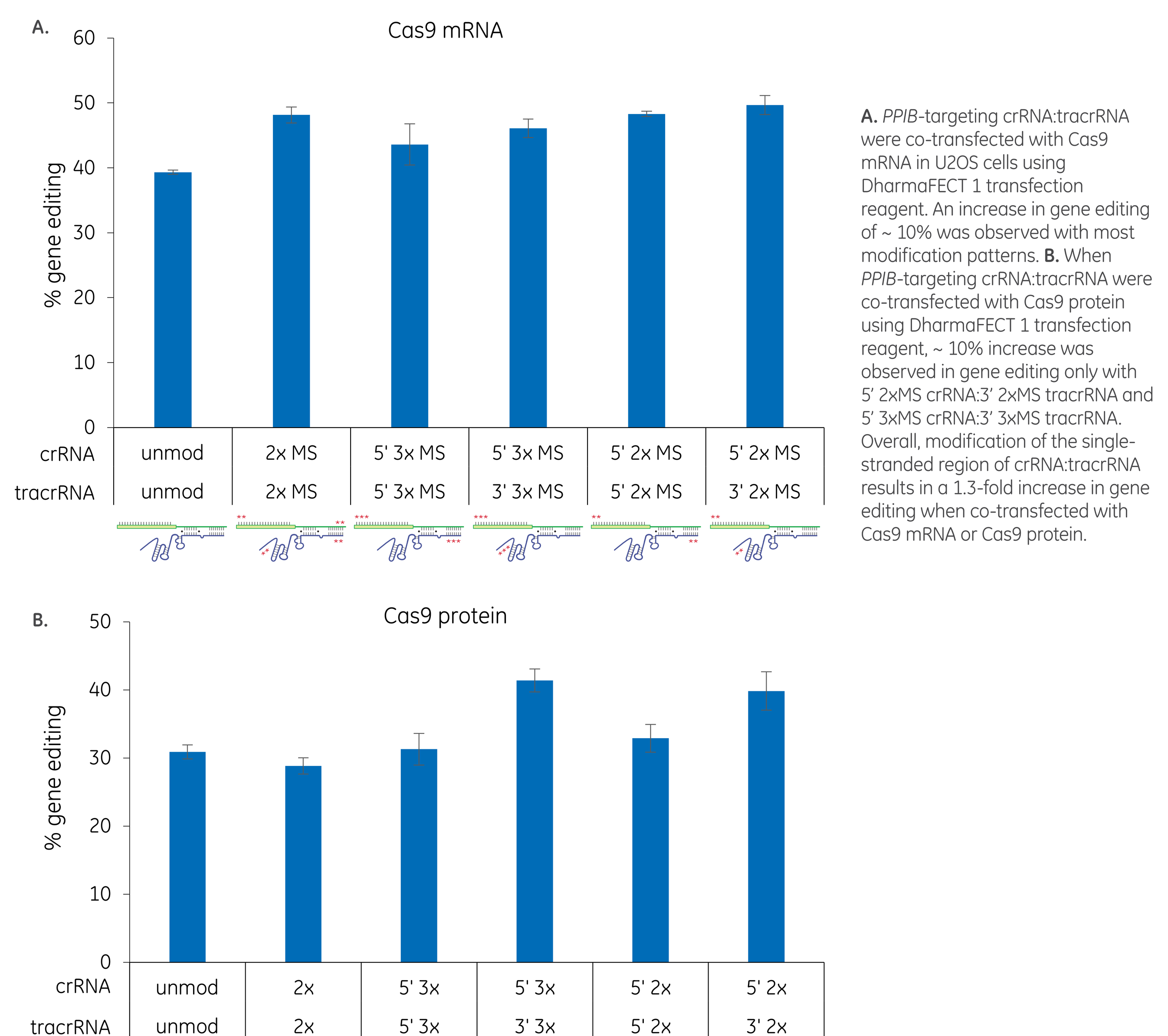
Edit-R Cas9 Nuclease protein NLS (Cat #CAS11729; 1.5 μM) and unmodified or modified crRNA:tracrRNA (3 μM) were complexed for 10 minutes for ribonucleoprotein (RNP) complex formation at room temperature prior to electroporation in K-562 cells. A ~ 2-fold increase was observed with 5' 3xMS crRNA:3' 3xMS tracrRNA when targeting *PPIB*, but equivalent gene editing was observed when targeting *PSMD11*. Samples done in duplicate.

RNA modifications do not affect gene editing activity with lipid transfection in 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 (Cat #T-2001-02). 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.

Some modification patterns show modest improvements in gene editing with co-transfection of modified crRNA:tracrRNA & Cas9 mRNA or Cas9 protein



A. *PPIB*-targeting crRNA:tracrRNA were co-transfected with Cas9 mRNA in U2OS 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 was observed in gene editing 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 a 1.3-fold increase in gene editing when co-transfected with Cas9 mRNA or Cas9 protein.

Conclusions

- Stabilizing modifications on crRNA:tracrRNA are required for co-electroporation with Cas9 mRNA
- Some modifications improve gene editing efficiency in co-electroporation with Cas9 protein for some gene targets
- Lipid transfection of crRNA:tracrRNA 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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