Optimization of DNA, RNA and RNP Delivery Methods for Efficient CRISPR/Cas9 Mediated Mammalian Cell Engineering

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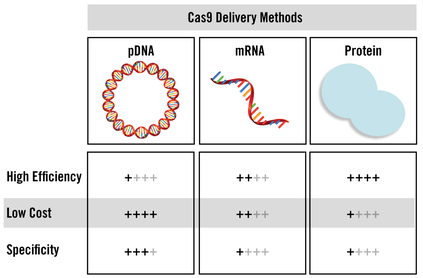
Abstract

The CRISPR/Cas9 genome-editing platform is a versatile and powerful technology to efficiently create genetically engineered living cells and organisms. This system requires a complex of Cas9 endonuclease protein with a gene-targeting guide RNA (gRNA) to introduce double-strand DNA breaks (DSBs) at specific locations in the genome. DSBs are then repaired by the error-prone Non-Homologous End Joining (NHEJ) pathway, resulting in insertions and/or deletions (indels) which disrupt the targeted locus.

The success of CRISPR genome editing experiments is limited by the intracellular delivery and expression of Cas9 protein and gRNA. Many methods for achieving CRISPR mediated cleavage have been identified and the choice of DNA, RNA or ribonucleoprotein (RNP) format is dictated by experimental goal and cell type. Transfection of each type of molecule requires specific considerations for efficient functional delivery. We performed transfections using different combinations of molecules including: plasmid DNA, messenger RNA, Cas9 protein and gRNA to maximize targeting of the Cyclophilin B (PPIB) gene in HEK 293T/17 and other mammalian cell types. Our results extend the utility of the CRISPR/Cas9 system by identifying optimal transfection conditions for intracellular delivery of Cas9 and gRNA in different formats.

Transfection optimization for maximal gene editing efficiency

CRISPR/Cas9 mediated genome engineering is a powerful tool that enables researchers to modify genomic DNA rapidly and efficiently. However, in many genome editing experiments, the greatest challenge is to achieve successful delivery of three CRISPR components including Cas9 endonuclease, CRISPR RNA (crRNA), and trans-activating crRNA (tracrRNA) required for gene editing in mammalian cells.



Cas9 endonuclease can be expressed in mammalian cells by delivering plasmid, mRNA and Cas9 protein. Each type of molecule has certain advantages and disadvantages as shown in the table on the left. Depending on experimental conditions, researchers may consider plasmid DNA, mRNA, or Cas9 protein as their preferred molecule for Cas9 delivery. Here we optimized the transfection conditions to maximize gene editing efficiency and successful delivery of plasmid DNA, messenger RNA, Cas9 protein and gRNA into a variety of mammalian cells including hard-to-transfect cells. The data demonstrates the optimal formats and transfection reagents for Cas9 delivery in certain cell types.

Experimental workflow

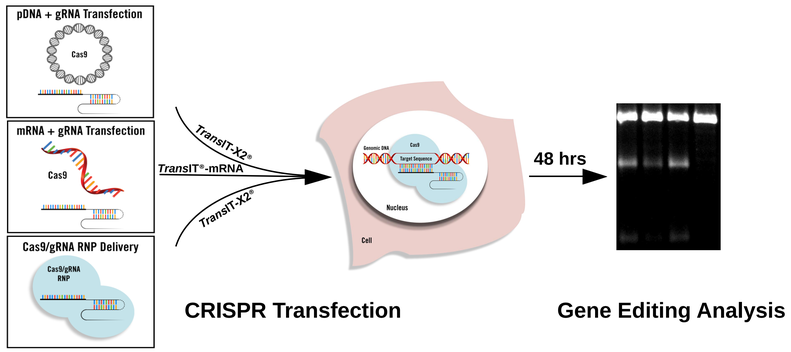


Figure 1. Cas9 encoding plasmid, mRNA, or protein and synthetic crRNA:tracrRNA co-transfection optimization experimental workflow. Guide RNA (gRNA) targeting PPIB (Cyclophilin B) gene was formed by incubating synthetic tracrRNA (Dharmacon) and synthetic crRNAs targeting PPIB gene in exon 2 (Dharmacon) in OPTI-MEM serum free medium (ThermoFisher Scientific) for 10 minutes at room temperature. The PPIB targeting gRNA was then co-transfected into several different mammalian cell lines with either Cas9 encoding plasmid (pCas9-GFP; Sigma) or Cas9 encoding mRNA (Cas9 mRNA with 5-methylcytidine and pseudouridine modification; TriLink BioTechnologies) using *Trans*IT-X2® Dynamic Delivery System (Mirus Bio LLC) or *Trans*IT®-mRNA Transfection Kit (Mirus Bio LLC) respectively. Alternatively, PPIB targeting gRNA was used to form an RNP complex with purified bacterial expressed recombinant Cas9 protein (PNA Bio) by incubating the two components in OPTI-MEM® serum free media (Fisher Scientific) for 10 minutes at room temperature. The PPIB targeting Cas9 RNP was then transfected into several different mammalian cell lines using *Trans*IT-X2® Dynamic Delivery System (Mirus Bio LLC). Transfection optimization was performed in 24-well plate format varying transfection duration, the transfection reagent amount, the concentration of gRNA, and Cas9 encoding molecule. Genomic DNA was then harvested from the transfected cells at 48 hours post-transfection and the region spanning the gRNA target site was PCR amplified and analyzed for level of gene editing efficiency by a mismatch detection assay using T7 Endonuclease I (T7EI).

Optimization of Cas9 Plasmid and gRNA delivery format

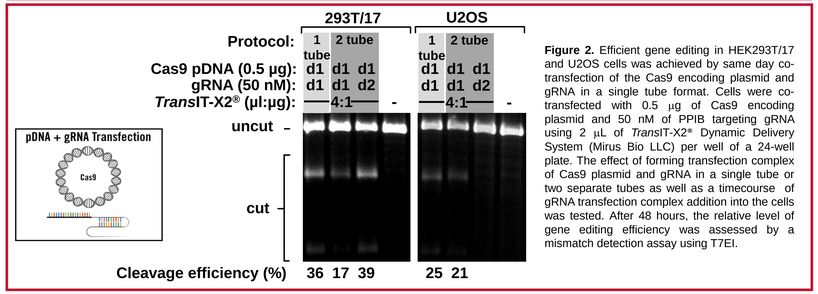


Figure 2. Efficient gene editing in HEK293T/17 and U2OS cells was achieved by same day co-transfection of the Cas9 encoding plasmid and gRNA in a single tube format. Cells were co-transfected with 0.5 µg of Cas9 encoding plasmid and 50 nM of PPIB targeting gRNA using 2 µL of *Trans*IT-X2® Dynamic Delivery System (Mirus Bio LLC) per well of a 24-well plate. The effect of forming transfection complex of Cas9 plasmid and gRNA in a single tube or two separate tubes as well as a timecourse of gRNA transfection complex addition into the cells was tested. After 48 hours, the relative level of gene editing efficiency was assessed by a mismatch detection assay using T7EI.

Cas9 mediated genome editing by plasmid and gRNA delivery

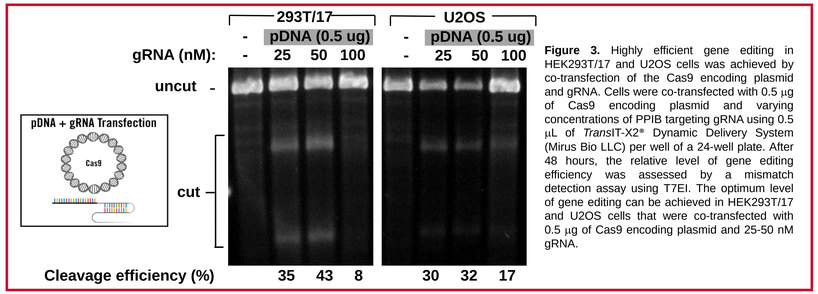


Figure 3. Highly efficient gene editing in HEK293T/17 and U2OS cells was achieved by co-transfection of the Cas9 encoding plasmid and gRNA. Cells were co-transfected with 0.5 µg of Cas9 encoding plasmid and varying concentrations of PPIB targeting gRNA using 0.5 µL of *Trans*IT-X2® Dynamic Delivery System (Mirus Bio LLC) per well of a 24-well plate. After 48 hours, the relative level of gene editing efficiency was assessed by a mismatch detection assay using T7EI. The optimum level of gene editing can be achieved in HEK293T/17 and U2OS cells that were co-transfected with 0.5 µg of Cas9 encoding plasmid and 25-50 nM gRNA.

Cas9 mediated genome editing by mRNA and gRNA delivery

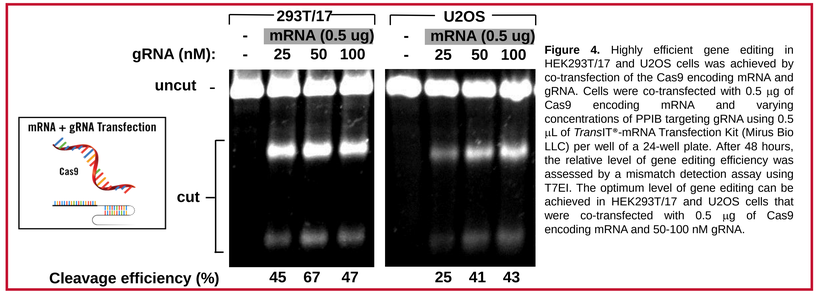


Figure 4. Highly efficient gene editing in HEK293T/17 and U2OS cells was achieved by co-transfection of the Cas9 encoding mRNA and gRNA. Cells were co-transfected with 0.5 µg of Cas9 encoding mRNA and varying concentrations of PPIB targeting gRNA using 0.5 µL of *Trans*IT®-mRNA Transfection Kit (Mirus Bio LLC) per well of a 24-well plate. After 48 hours, the relative level of gene editing efficiency was assessed by a mismatch detection assay using T7EI. The optimum level of gene editing can be achieved in HEK293T/17 and U2OS cells that were co-transfected with 0.5 µg of Cas9 encoding mRNA and 50-100 nM gRNA.

Cas9 mediated genome editing by Cas9 RNP complex delivery

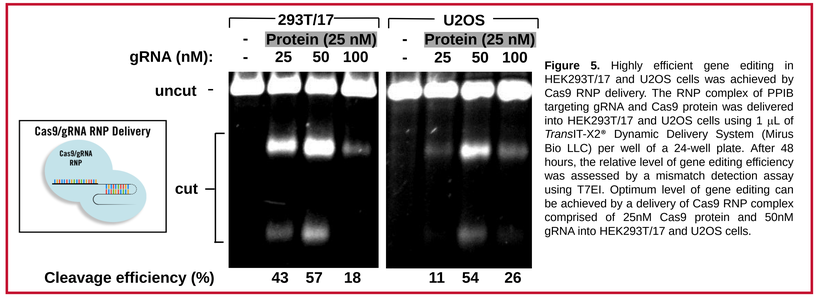


Figure 5. Highly efficient gene editing in HEK293T/17 and U2OS cells was achieved by Cas9 RNP delivery. The RNP complex of PPIB targeting gRNA and Cas9 protein was delivered into HEK293T/17 and U2OS cells using 1 µL of *Trans*IT-X2® Dynamic Delivery System (Mirus Bio LLC) per well of a 24-well plate. After 48 hours, the relative level of gene editing efficiency was assessed by a mismatch detection assay using T7EI. Optimum level of gene editing can be achieved by a delivery of Cas9 RNP complex comprised of 25nM Cas9 protein and 50nM gRNA into HEK293T/17 and U2OS cells.

Cas9 mediated genome editing in hard-to-transfect cells

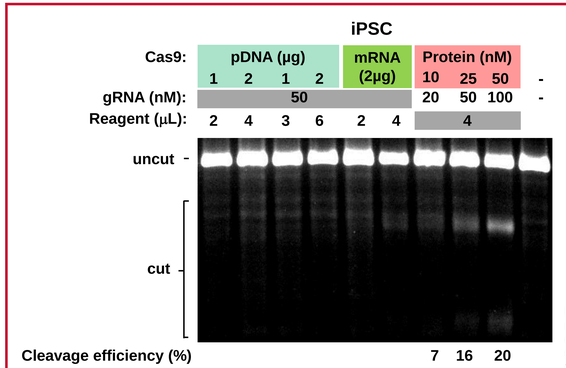


Figure 6. Highly efficient Cas9 RNP delivery mediated gene editing in hard-to-transfect cell lines. Human iPSC (ATCC-DYS100) growing in a 6-well plate was co-transfected with varying amount of Cas9 encoding plasmid, 2 µg of Cas9 encoding mRNA, varying concentration of Cas9 protein and PPIB targeting gRNA with varying amount of *Trans*IT-X2® Dynamic Delivery System or *Trans*IT®-mRNA Transfection Kit or (Mirus Bio LLC). K562 and primary NHDF cells growing in a 24-well plate were co-transfected with either 0.5 µg of Cas9 encoding plasmid, 0.5 µg of Cas9 encoding mRNA, 25 nM of Cas9 protein and varying concentrations of PPIB targeting gRNA delivered using the *Trans*IT-X2® Dynamic Delivery System or *Trans*IT®-mRNA Transfection Kit (Mirus Bio LLC). After 48 hours, the relative level of gene editing efficiency was assessed by a mismatch detection assay using T7EI. The highest level of gene editing in iPSC and K562 cells can be achieved by delivery of Cas9 RNP complex composed of 25nM Cas9 protein and 50nM gRNA. In contrast, optimum level of gene editing can be achieved in primary NHDF cells that were co-transfected with 0.5 µg of Cas9 encoding mRNA and 25-50 nM gRNA.

Conclusions

- **The delivery of Cas9 plasmid/gRNA or Cas9 RNP complex into mammalian cells can be achieved by using *Trans*IT-X2® Dynamic Delivery System**
- ***Trans*IT®-mRNA Transfection Kit is highly efficient at delivery of Cas9 mRNA/gRNA into mammalian cells**
- **Optimal formats and methods for Cas9 delivery differ among different cell types**
- **Delivery of Cas9 RNP complex using *Trans*IT-X2® Dynamic Delivery System achieved the highest gene editing efficiency in hard-to-transfect cells**