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

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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. The cell then repairs the resulting DSBs using either homology-directed repair (HDR) or the error-prone non-homologous end joining (NHEJ) pathway. Both DNA repair pathways can be leveraged in different ways to introduce desired modifications at the target 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 Cas9-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, U2OS, 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.

Keywords

CRISPR-Cas9, Gene Editing, Genome Engineering, Cas9, Transfection, Transfection Optimization, TransIT®-X2, TransIT®-mRNA, Mismatch Assay, T7 Endonuclease

Introduction

Genome editing technologies such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) system have proven to be invaluable research tools for generating precise genome alterations. All three systems enable DNA modifications by creating a double-stranded break (DSB) at the target locus. ZFNs and TALENs both utilize endonuclease domains fused to modular DNA binding proteins which can be programmed to target specific genomic locations. In contrast to ZFNs and TALENs which rely on protein-DNA interactions to afford target recognition, Cas9 is targeted to DNA via a short guide RNA which forms Watson-Crick base pairs with the target DNA[1-3]. Targeting of Cas9 to different loci only requires delivery of Cas9 and the appropriate guide RNA sequence, making the CRISPR/Cas9 system a highly facile technology for genome engineering[6,7].

The development of CRISPR/Cas9 technology into a genome editing platform stems from basic research into the mechanisms of adaptive immunity in prokaryotes[4]. In bacteria and archaea, CRISPR arrays and Cas genes normally function to provide protection against viral and plasmid invasion by targeting and silencing foreign nucleic acids in a sequence-specific manner[4,5]. CRISPR arrays are genetic segments characterized by identical repeats interspersed with non-repetitive sequences called spacers. The sequence of each spacer corresponds to fragments of foreign DNA (protospacers) acquired through exposure to previous viral or plasmid DNA. Upon subsequent viral and plasmid invasion, the CRISPR locus is transcribed and processed into short CRISPR RNAs (crRNAs) that guide the Cas endonuclease to digest the complementary genomic target sequence of the invading pathogens. CRISPR–Cas systems are highly diverse and can be classified into three major types (I–III). In type I and II systems, a short nucleotide sequence adjacent to the protospacer called the protospacer adjacent motif (PAM) is required for target recognition. The PAM is crucial for the CRISPR–Cas system to discriminate between the invading pathogen genome and the CRISPR locus in the host genome, which does not contain the PAM[8].

The CRISPR type II system from *Streptococcus pyogenes* was the first system to be adapted for genome engineering in mammalian cells by inducing sequence-specific DSBs at desired genomic locations[3]. In this system, the guide RNA consists of two parts: the CRISPR RNA (crRNA) which forms base pairs with the target DNA and a trans-activating crRNA (tracrRNA) which forms base pairs with the crRNA and triggers Cas9 cleavage of target DNA. In order to perform genome editing, the Cas9 endonuclease and the two-part gRNA, consisting of a constant tracrRNA and a target specific crRNA, must be introduced to cells. The guide RNA can be supplied separately as crRNA and tracrRNA or as a single chimeric oligonucleotide referred to as a single guide RNA (sgRNA). Once inside the cell, the protospacer sequence at the 5' end of the crRNA will direct Cas9 to a specific target DNA site immediately 5' of a PAM and guide double-strand DNA cleavage (Figure 1). Cas9-induced DSBs are repaired by either homology-directed repair (HDR) or nonhomologous end joining (NHEJ). In HDR, DNA lesions are repaired using homologous sequence as a template. Therefore, researchers can take advantage of this repair pathway to make specific alterations in the host genome by supplying a donor DNA

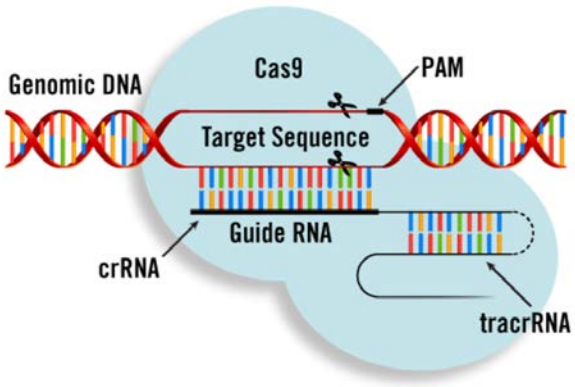

Figure 1. CRISPR/Cas9 Genome Editing. The Cas9 endonuclease (blue) is targeted to DNA by a guide RNA which can be supplied as a two-part system consisting of crRNA and tracrRNA or as a single guide RNA, where the crRNA and tracrRNA are connected by a linker (dotted line). Target recognition is facilitated by the protospacer-adjacent motif (PAM). Cleavage occurs on both strands (scissors) 3 bp upstream of the PAM.

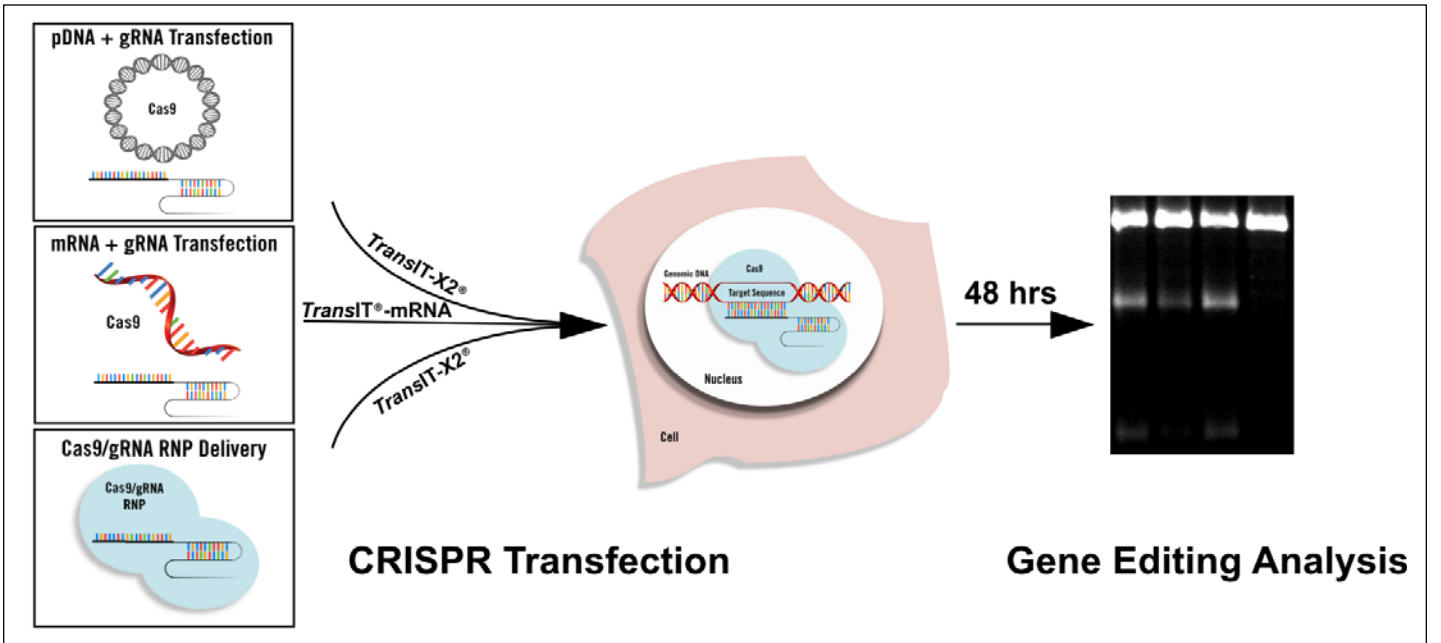

Figure 2. **Cas9 encoding plasmid, mRNA, or protein and synthetic crRNA:tracrRNA co-transfection optimization experimental workflow.** Guide RNA targeting *PPIB* gene was formed by incubating synthetic tracrRNA (Dharmacon) and synthetic crRNAs targeting *PPIB* gene in exon 2 (Dharmacon) in Opti-MEM® 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; MilliporeSigma) or Cas9 encoding mRNA (Cas9 mRNA with 5-methylcytidine and pseudouridine modification; TriLink BioTechnologies) using *Trans*IT-X2® Dynamic Delivery System (Mirus Bio) or *Trans*IT®-mRNA Transfection Kit (Mirus Bio) 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 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. Transfection optimization was performed in 24-well plate format varying 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 (New England Biolabs).

template that has homology with the sequence flanking the DSB. The NHEJ repair mechanism is more error prone and often results in the introduction of small insertions and deletions (indels) causing frameshift mutations or premature stop codons to produce gene knockouts[6].

The appropriate delivery method of Cas9 and gRNA is critical for performing effective genome editing in the targeted cells. Current systems for delivering Cas9 include plasmid DNA, mRNA, and ribonucleoprotein (RNP) complex. Each method has its own set of advantages and limitations. The delivery of Cas9 as plasmid DNA offers a simple and low-cost approach; however, many studies have shown a higher degree of off-target cleavage events using this method[10,11]. Transfection of Cas9 mRNA enables rapid gene expression, and eliminates the risk of insertional mutagenesis[12]. Delivery of Cas9 protein/guide RNA ribonucleoprotein (RNP) complex results in the most rapid pulse of genome editing activity and reduces the possibility of off-target cleavage events[13]. Cas9 mRNA and RNP formats provide an efficient delivery method to cell types that are resistant to transfection with plasmid DNA[7]. To date, the delivery of the Cas9 and gRNA remains challenging in many cell types, particularly primary and suspension cells[7]. To overcome these limitations, we optimized conditions for transfection of CRISPR/Cas9 components using combinations of plasmid DNA, mRNA, Cas9 protein and gRNA in a variety of mammalian cells (Figure 2). Our results identify the most effective methods for intracellular delivery of Cas9 and gRNA resulting in highly efficient genome editing in hard-to-transfect cells.

Results

Transfection optimization for Cas9 mediated genome editing by plasmid and gRNA delivery.

Transfection optimization was performed in HEK293T/17 and U2OS in a 24-well plate format using *Trans*IT-X2® Dynamic Delivery System. Cells were co-transfected with 0.5 µg of Cas9 encoding plasmid and varying concentrations of gRNA targeting the human *PPIB* gene using 2 µL of *Trans*IT-X2® Dynamic Delivery System per well of a 24-well plate. Genomic DNA was harvested from the transfected cells at 48 hours post-transfection and the region spanning the gRNA target site was PCR amplified and analyzed for gene editing efficiency by a mismatch detection assay using T7 Endonuclease I (T7EI). Under these conditions, the concentration of gRNA complex that yielded the highest gene editing efficiency in HEK293T/17 and U2OS cells is between 25-50nM yielding cleavage efficiencies of 43 and 32%, respectively (Figure 3).

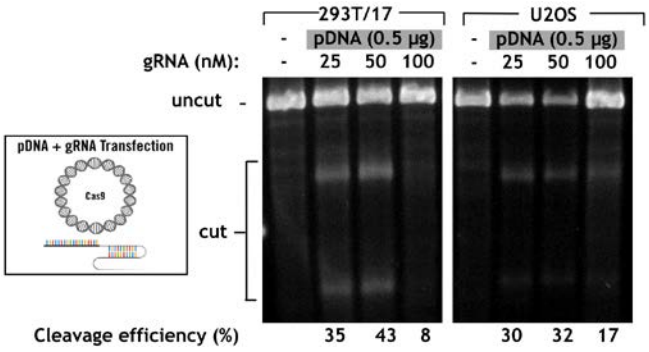

Figure 3. Efficient Genome Editing with Cas9 Plasmid DNA and gRNA Oligonucleotides. HEK293T/17 and U2OS cells were co-transfected with 0.5 µg of Cas9 encoding pDNA and 50nM PPIB targeting 2-part gRNA using *Trans*IT-X2® Dynamic Delivery System (2 µl/well of a 24-well plate). A T7E1 mismatch detection assay was used to measure cleavage efficiency at 48 hours post-transfection.

Transfection optimization for Cas9 mediated genome editing by mRNA and gRNA delivery.

Cas9 mRNA and two-part gRNA transfection optimization was performed in HEK293T/17 and U2OS in a 24-well plate format using *Trans*IT®-mRNA Transfection Kit. Cells were co-transfected with 0.5 µg of Cas9 encoding mRNA (5methyl cytidine, pseudo uridine) and varying concentrations of gRNA targeting the human *PPIB* gene using 0.5 µL of *Trans*IT®-mRNA Transfection Reagent and 0.5 µL of mRNA Boost Reagent per well of a 24-well plate. The use of modified base mRNA decreases the innate immune response to long RNA and is also believed to enhance translation thereby increasing overall expression levels[17]. 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 T7EI. The concentration of gRNA complex that yields the highest gene editing efficiency in HEK293T/17 and U2OS cells is 50nM, yielding cleavage efficiencies of 67 and 41%, respectively (Figure 4).

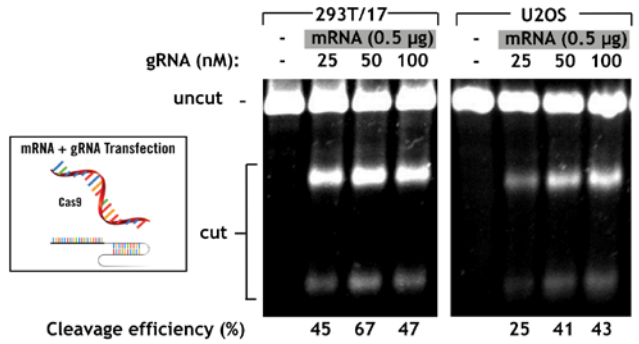

Figure 4. Efficient Genome Editing with Cas9 mRNA and gRNA Oligonucleotides. HEK293T/17 and U2OS cells were co-transfected with 0.5 µg of Cas9 encoding mRNA, 5meC, ψ and 25nM of PPIB targeting 2-part gRNA using *Trans*IT®-mRNA Transfection Kit (0.5 µl/well of 24-well plate of both mRNA Reagent and Boost Reagent). A T7E1 mismatch detection assay was used to measure cleavage efficiency at 48 hours post-transfection.

Transfection optimization for Cas9 mediated genome editing by Cas9 RNP complex delivery.

RNP complex transfection optimization was performed in HEK293T/17 and U2OS in a 24-well plate format using *Trans*IT-X2® Dynamic Delivery System. To form an effective RNP complexes, First, two part gRNA (crRNA and tracrRNA) were incubated in Opti-MEM® serum free medium for 10 minutes at room temperature; second, Cas9 protein were added and incubated for 10 minutes; third, *Trans*IT-X2® Dynamic Delivery System were added and incubated for 15 minutes; Finally, the transfection complexes were added to the cells growing in normal growth medium. Genomic DNA was 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 T7EI. The optimal level of gene editing is achieved in HEK293T/17 and U2OS by delivery of a Cas9 RNP complex comprised of 25nM Cas9 protein and 50nM gRNA, resulting in cleavage efficiency of 57 and 54%, respectively (Figure 5).

Cas9 mediated genome editing in hard-to-transfect cells.

Empirical evidence has revealed that some cell types are resistant to chemical transfection methods. There is a lack of understanding as to why these cells are hard-to transfect and it may be dependent on the type of macromolecule that is being delivered. Cell types that frequently fall into this category include: primary cells, hematopoietic and stem cells. To address the need for genome editing in these areas, we used three cell types, K562, primary Normal Human Dermal Fibroblasts (NHDF), and induced human pluripotent stem cells (HiPSC) as model for hard-to-transfect cell types. Transfection optimization was performed in K562, primary NHDF, and Human iPSC cells. 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 (plasmid DNA and RNP) or *Trans*IT®-mRNA Transfection Kit (mRNA). Human iPSC (ATCC-DYS100) were grown in a 6-well plate and co-transfected with varying amounts of Cas9 encoding plasmid, 2 µg of Cas9 encoding mRNA, varying concentrations of Cas9 protein and PPIB targeting gRNA with varying amounts of *Trans*IT-X2® Dynamic Delivery System or *Trans*IT®-mRNA Transfection Kit. 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 K562 and iPSC cells are achieved by delivery of a Cas9 RNP complex composed of 25nM Cas9 protein and 50nM gRNA, yielding cleavage efficiencies up to 20%. In contrast, the optimum level of gene editing is attained in primary NHDF cells co-transfected with 0.5 mg of Cas9 encoding mRNA and 25-50 nM gRNA to yield 30% cleavage efficiency (Figure 6).

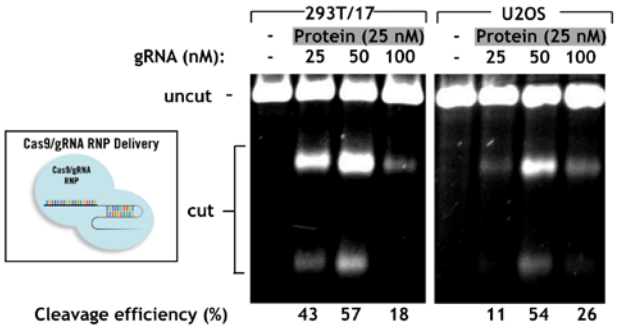

Figure 5. Efficient Genome Editing with Cas9 + gRNA Ribonucleoprotein Complexes. The RNP complex of PPIB targeting 2-part gRNA and Cas9 protein was delivered into HEK293T/17 and U2OS cells using *Trans*IT-X2® Dynamic Delivery System (1 µl/well of a 24-well plate). A T7E1 mismatch detection assay was used to measure cleavage efficiency at 48 hours post-transfection. High levels of gene editing are achieved in cells that were transfected with an RNP complex comprised of 50nM of gRNA and 25nM of Cas9 protein.

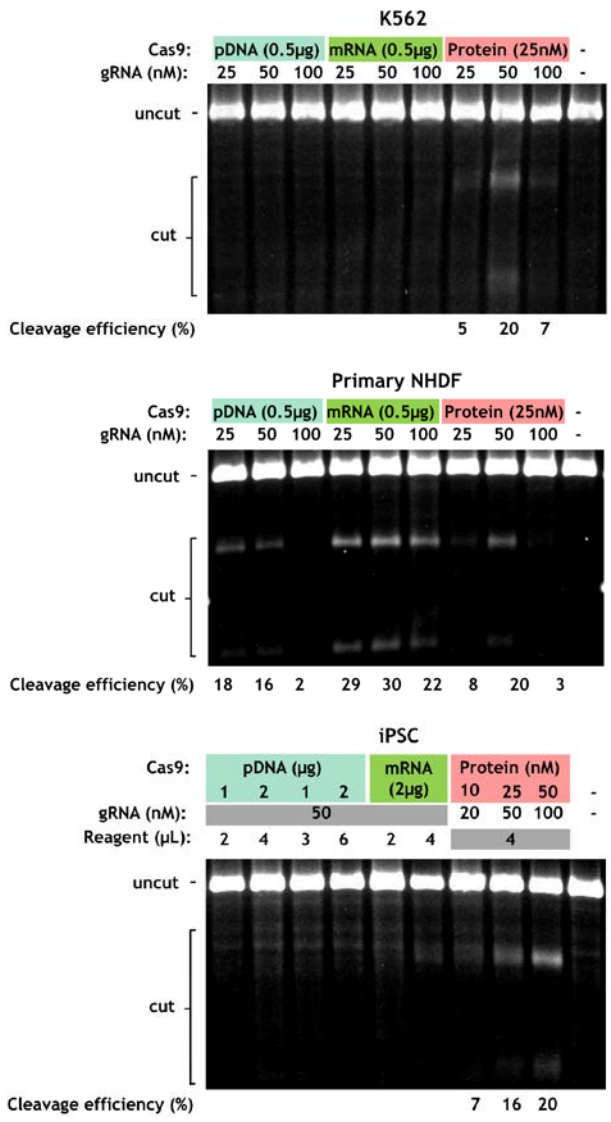

Figure 6. Efficient Genome Editing in Hard to Transfect Cells. *Trans*IT-X2® Dynamic Delivery System was used to deliver Cas9 pDNA/gRNA and Cas9 protein/gRNA (RNP complex). *Trans*IT®-mRNA Transfection Kit was used to deliver Cas9 mRNA/gRNA into K562, primary NHDF, and Human iPSC cells . A T7E1 mismatch assay was used to measure cleavage efficiency at 48 hours post-transfection.

Conclusions

The ability to utilize targeted nucleases to generate precise modifications in the genome holds great promise for basic research as well as gene and cellular therapies. Emerging therapeutic strategies using the CRISPR-Cas9 system to modify nucleic acids within disease-affected cells could enable the treatment of debilitating genetic diseases such as cystic fibrosis[14] and Duchenne muscular dystrophy[15]. Furthermore it could also be utilized to generate mutations that protect cells against infection. For example, HIV infection could be blocked through a loss of function mutation generated in the CCR5 receptor[16]. Efficient methods for delivery of Cas9 and guide RNA are essential to realizing the full potential of the CRISPR genome editing platform. Here we have optimized Cas9 and gRNA delivery methods using the *Trans*IT-X2® Dynamic Delivery System and the *Trans*IT®-mRNA Transfection Kit for generating desired gene alterations in a variety of mammalian cells including some difficult to transfect cells such as HiPSCs, K562, and primary NHDF. Several experimental parameters influencing the efficiency of genome editing were optimized to achieve the highest level of genome editing in each particular cell type. Numerous laboratories have demonstrated that the CRISPR-Cas9 system is robust and efficient. Researchers have flexibility in their experimental design which includes the delivery of Cas9 endonuclease in a variety of formats including: plasmid DNA, mRNA, and protein via a RNP complex. There are advantages and disadvantages to each system such as: cleavage efficiency, cost, and specificity. Herein, we have demonstrated that RNP formats for Cas9 delivery offers the highest gene editing efficiency, particularly in hard-to-transfect cells.

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