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Efficient CRISPR/Cas9 Delivery Using Nucleofector® Technology

Comparison of Plasmid- and RNP-based Editing

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Introduction

CRISPR (clustered, regularly inter-spaced, short palindromic repeats)/Cas9 technology facilitates genome editing at unprecedented efficiencies. This system uses a guide RNA (gRNA) to direct a Cas9 nuclease to the designated genomic target. Cas9 then creates a double-strand break (DSB) that facilitates the introduction of particular modifications using endogenous cellular repair systems. The non-homologous end joining (NHEJ) repair pathway can be harnessed to introduce targeted indels for out-of-frame, loss-of-function variants. Alternatively, if an exogenous donor template is introduced along with the CRISPR reagents, repair of the DSB by homology directed repair (HDR) can introduce precise sequence modifications as specified by the user-defined donor template.

The ultimate goal when using the CRISPR/Cas9 platform is targeted genomic modification limited only to the site of interest. Transient expression of CRISPR/Cas9 is traditionally supplied to cells through plasmid DNA (pDNA). An alternative approach is to supply CRISPR/Cas9 ribonucleoprotein (RNP), which is generated using purified Cas9 protein pre-complexed with *in vitro* transcribed gRNA. Although the stability of pDNA over RNP is technically convenient, there are applications when an RNP-based approach may be more suitable.

For instance, when investigating therapeutic applications (e.g. *ex vivo* correction of a disease genotype), strategies that mitigate off-target effects, however rare they may be, are typically applied. The relative stability of pDNA in the cell is a drawback in this scenario, because CRISPR/Cas9 nuclease activity delivered via pDNA can persist for as long as 72 hours – increasing the potential off-target window.[1] Off-target events can occur in the presence of tolerated mismatches sufficient for CRISPR/Cas9 activity at unwanted sites or due to random integration of pDNA. In contrast, nuclease activity via RNP delivery plateaus after 24 hours, limiting the exposure to potential off-target events.[1] Indeed, in a pool of cells, known off-target sites are unedited when RNPs are used in place of traditional pDNA.[1,2] Moreover, the use of RNPs compared to pDNA results in an overall higher survival of transfected human embryonic stem cells.[1] Thus, the use of RNPs can diminish spurious editing and, in hard-to-transfect cell lines, toxicity.

However, it is important to highlight that a well-designed gRNA, with at least 3 bp of mismatch between the target site and any other site in the genome, is unlikely to cause off-target cutting in a targeted clone.[3,4] Off-target nuclease activity at a clonal level most often occurs when optimal design principles cannot be implemented due to low sequence complexity or restricted coordinate requirements (e.g. knock-in of a substitution). Thus, although off-target events are rare, there are, on occasion, sequence contexts and experimental needs that benefit from an alternative approach. Moreover, the need to mitigate and/or abolish the potential for any off-target editing is even more of a concern in the therapeutic area, in which millions of cells (rather than clonal populations) will likely be introduced into a patient.

This led us to develop a high-efficiency method to deliver CRISPR/Cas9 RNPs for gene modification. While lipofection-based delivery of reagents works for a variety of cell lines, some cell types, such as suspension cells, are not efficiently transduced by this method. Thus, we developed a Nucleofection-based protocol that is suitable across most cell types for efficient and targeted gene editing.

Materials and Methods

Cell Culture

Mouse Neuro-2A and human K-562 cell lines were cultured according to ATCC guidelines with the addition of Gibco™ GlutaMAX™ (Thermo Fisher Scientific) and 50 µg/mL Gibco™ Penicillin-Streptomycin (Thermo Fisher Scientific).

RNP Complexing

A one-step synthesis was used to assemble a single chain gRNA (20 nt + tracrRNA).[5] RNA was synthesized overnight at 37°C using T7 RNA polymerase (NEB) and purified using the MEGAclear™ Kit (Invitrogen). 100 pmol (16 µg) of Cas9 protein (Berkley Microlab) and 200 pmol of *in vitro* synthesized single chain gRNA were complexed at room temperature for 10 minutes.

Nucleofection®

To estimate transfection efficiency in K-562 and Neuro-2a cells, 2 µg of pmaxGFP™ Vector (Lonza) was used. Nucleofection® efficiency was measured by FACS analysis on the MoFlo® MLS cell sorter (Cytomation) with Co-Lase 4 laser modification (Propel Labs). 200 ng of gRNA and 500 ng of Cas9 pDNA or pre-complexed RNP (1.6 or 8 µg) was added to 2.5×10^5 cells in a final volume of 20 µL of SF solution using 16-well strip cuvettes (Lonza 4D-Nucleofector® X Unit). RNP complexes have previously been reported to be effective at concentrations as low as 1.5 µg.[5] Programs DS-137 (Neuro-2A) and FF-120 (K-562) were used for Nucleofection®. For HDR, 100 pmol (4 µg) of ssODN was added to the pDNA or pre-complexed RNP solution prior to Nucleofection®. Cells were harvested 3–4 days post-Nucleofection for genomic DNA extraction.

Targeted Deep-sequencing Library

A two-step PCR was performed. First, the target genomic region was amplified using sequence-specific forward and reverse primers tailed with universal adapters. Second, the tailed amplicons were amplified using forward and reverse primers containing Illumina sequencing adapters tagged

with a unique index. PCR reactions were performed using EconoTaq® PLUS GREEN 2X Master Mix (Lucigen) in a final volume of 10 µL. Cycling conditions for the first PCR were 94°C for 2 minutes, followed by 35 cycles of 94°C for 15 seconds, 56°C for 15 seconds, and 72°C for 40 seconds, ending with a 2-minute final extension at 72°C. The second PCR was performed at 94°C for 2 minutes, followed by 5°- cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 40 seconds, with a 2-minute final extension at 72°C. All PCR products were pooled and gel purified using a 2% agarose gel. 2 × 250 reads were generated on an MiSeq Sequencing System (Illumina, Inc.).

Results

To generate a protocol for the delivery of RNPs into mammalian cells, mouse Neuro-2A and human K-562 cell lines were chosen. Both have high transfection efficiency by Nucleofection® for plasmid DNA and yield high frequencies of NHEJ and HDR events. A high activity gRNA (NHEJ >70%), previously validated using plasmid DNA, was chosen to target the mouse gene Dnajb6 in Neuro-2A cells and the human gene MFN2 in K-562 cells. To assay for transfection efficiency in each cell type, a GFP pDNA (pmaxGFP™ Vector) sample was used as a positive control. Indeed, K-562 and Neuro-2A GFP controls confirmed high transfection efficiencies of 96% and 64%, respectively (Figure 1).

Two concentrations of pre-complexed RNPs, 1.6 µg and 8 µg, were transfected using Nucleofection. Gene editing was evident for both concentrations of RNPs at all targets. The higher RNP concentration resulted in greater activity, with indel frequencies of 30% for MFN2 in K-562 and 14% for Dnajb6 in Neuro-2A, while the lower concentration (1.6 µg) yielded 2- to 4-fold fewer indels (Figure 2).

Interestingly, both targets had even higher NHEJ frequencies when ssODN donor template was present – 47% for MFN2 and 24% for Dnajb6 (with 8 µg RNP). The addition of non-homologous DNA with free ends has been reported to stimulate indel formation by RNPs, possibly by catalyzing a DNA damage response through excess amounts of molecules that appear as DNA breaks to the cell.[6] Targeted substitutions via HDR were made at MFN2 and Dnajb6 using ssODNs with 60 bp homology arms as donor templates. HDR was most effective using 8 µg of RNP at both sites, achieving 25% knock-in at the MFN2 locus and 2% at Dnajb6. There was a 3-fold increase in HDR for MFN2 when using RNPs compared to pDNA, suggesting this technique could improve knock-in efficiency at some sites. Although HDR was reduced for Dnajb6 using RNPs, it was comparable to pDNA at 8 µg of RNP when overall editing (HDR/HDR+NHEJ) is accounted – 7.6% for RNPs and 8.9% for Dnajb6 (Figure 2C). This is because overall NHEJ activity was lower using RNPs compared to pDNA, which is not unexpected given the shortened lifetime of RNPs in the cell. Reduced overall NHEJ is correlated with a reduction in off-target double strand breaks[7], consistent with the expectation of higher fidelity.

The indel spectrum at targeted sites in RNP pools is narrow compared to pDNA-edited pools. For MFN2, the same 1 bp insertion was found in both pools, while pDNA pools had high frequencies of two additional indels – a 1 and 2 bp deletion (Figure 3). This is possibly a consequence of the longer exposure to CRISPR/Cas9 delivered via pDNA. Similarly, the Dnajb6 target site had the same 1 bp insertion for both RNP- and pDNA-edited pools, while the pDNA-edited pool was found to have two additional high-frequency indels (2 bp deletions). Importantly, the predominant indel species is consistent between pools, indicating RNP-based delivery unlikely alters repair pathway selection. Instead, repair outcomes can differ, such as indel diversity and abundance, likely due to differences in CRISPR /Cas9 persistence between methods.

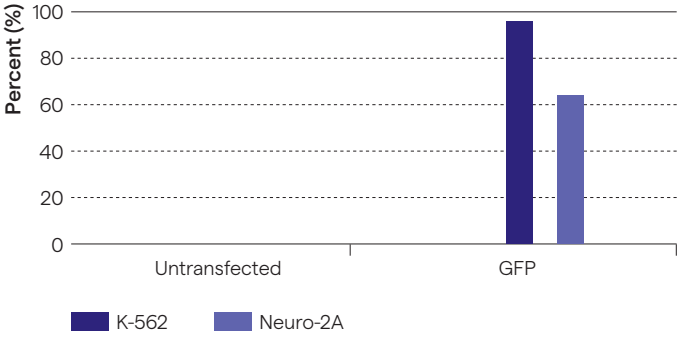
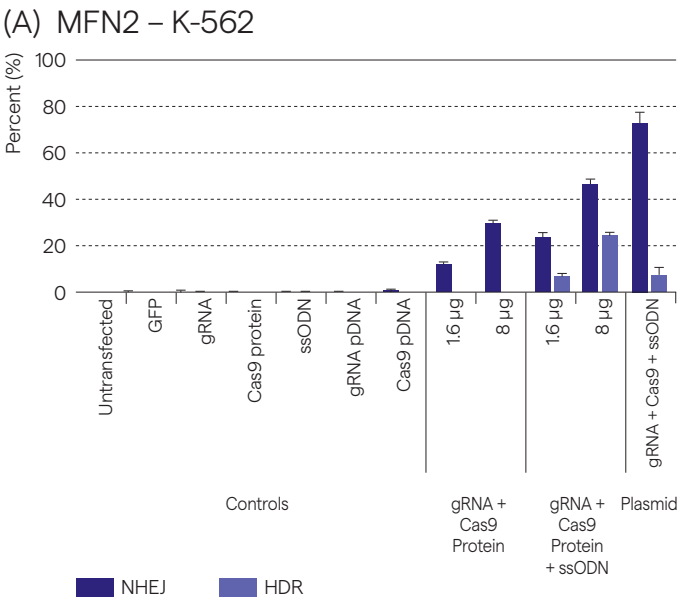
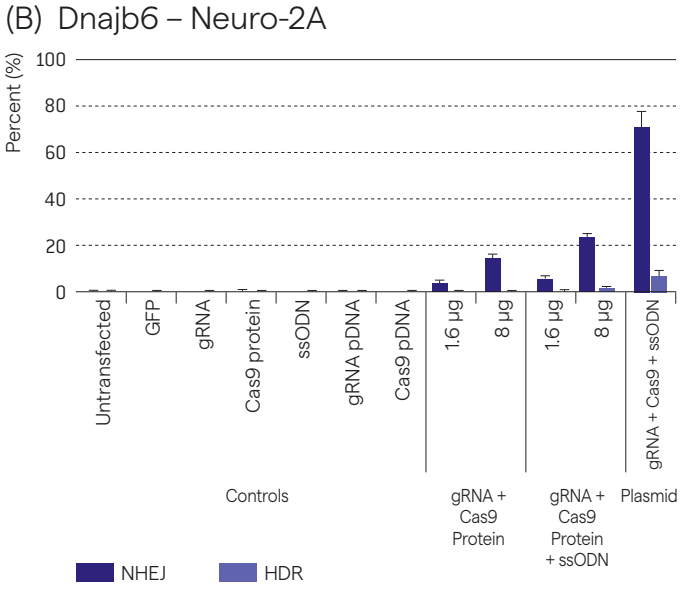


Figure 1.
GFP transfection efficiency. Percent transfection efficiency after Nucleofection® with pmaxGFP™ Vector for K-562 and Neuro-2A cells assayed by FACS analysis. Values represent one biological replicate.





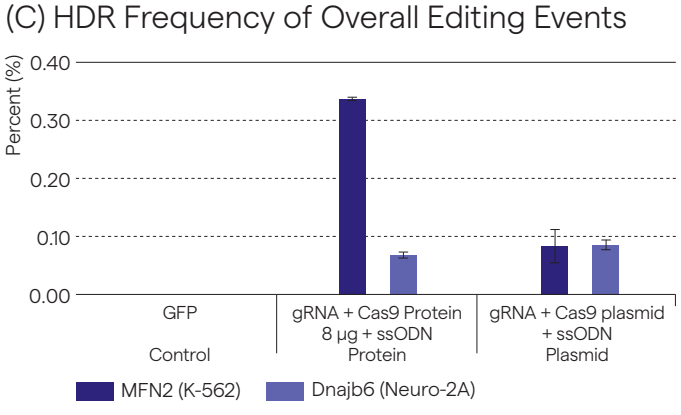


Figure 2.
Gene editing by RNPs compared to plasmid. Gene editing at MFN2 in K-562 cells (A, C) and Dnajb6 in Neuro-2A cells (B, C) by RNPs assayed by deep-sequencing. **A and B:** Percent of indels generated by non-homologous end joining (NHEJ) and targeted substitutions generated by homology directed repair (HDR) using a donor ssODN are shown. Data is normalized to untransfected control. **C:** HDR as a function of overall editing events (HDR/HDR+NHEJ) is also shown. Represented values are mean +/- SEM for three biological replicates.

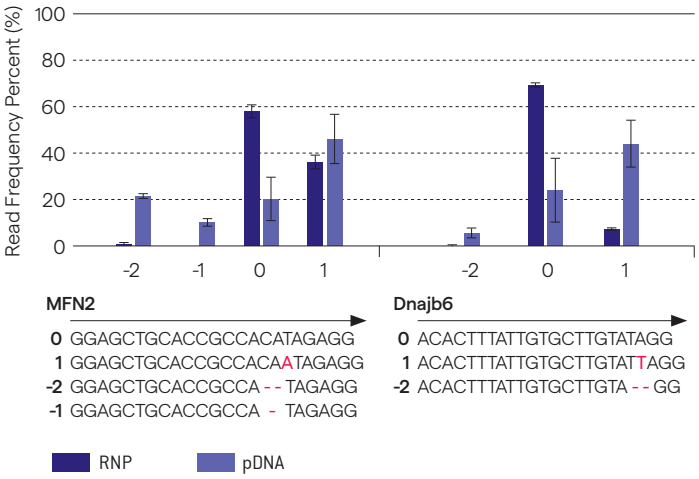


Figure 3.
Alignment and frequencies of indels generated at MFN2 and Dnajb6 assayed by deep- sequencing. The top most frequently occurring indels (>7%) are shown for MFN2 and Dnajb6. Note that comparison using RNP data is derived from 8 µg (+ssODN) pools. Represented values are mean +/- SEM for three biological replicates.

Conclusion

Nucleofection is a high-efficiency method of delivering reagents to a variety of cell types. Here, we show that Nucleofection can be used to efficiently deliver CRISPR/Cas9 RNPs for targeted gene editing. We find that 1.6 µg of pre-complexed RNPs are sufficient to generate indels via NHEJ, but 8 µg may be necessary to generate appreciable HDR using an ssODN (e.g. Dnajb6). Rates of NHEJ were highest for 8 µg of RNPs, suggesting this concentration is well suited for both knockout and ssODN-mediated knock-in projects. We demonstrate that HDR rates (relative to overall editing events) are comparable between pDNA and RNP, despite the shortened half-life of RNPs. For example, at the Dnajb6 site, 7.6% of RNP-edited alleles (frequency of HDR/frequency of HDR+NHEJ or 2%/26%) resulted in HDR compared to the 8.9% (7%/78%) of pDNA-edited alleles. In some cases, RNPs may even facilitate higher HDR rates as observed at the MFN2 locus. Additionally, the indel frequency and spectrum of RNP-edited pools have less diversity, adding yet another potentially useful criterion when selecting between methods.

The amounts of RNP and ssODN described here reflect starting concentrations that can be scaled up or down to achieve optimal results for other cell lines. Transfection of RNPs has less cellular toxicity than pDNA[1], thus, scaling up to determine at what concentration editing frequencies plateau for a given cell line is advisable – particularly for hard-to-edit cell lines (e.g. high ploidy lines such as HeLa).[8]

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