Improve genome editing outcomes in biologically relevant cell models

Introduction

With increasing expansion into research areas of more biological relevance, existing molecular and cellular techniques need to be improved. Invitrogen™ Lipofectamine™ 3000 Transfection Reagent, a new reagent developed to improve delivery and enable use of new technologies, can be used in more relevant systems enabling faster and more reliable outcomes. The area of genome editing is rapidly growing and requires more advanced techniques to maximize its potential applications. Transcriptional activator-like effector nucleases (TALENs) and technology derived from clustered regularly interspaced short palindromic repeats (CRISPRs) allow precise cleavage of DNA at specific loci. However, the effectiveness of these tools is contingent upon the intrinsic properties of the locus of interest, efficient delivery, and the painstaking downstream processes of generating stable cell lines and knockout models to study the phenotypic effects of such genetic modifications. During the development of Lipofectamine 3000 reagent, we assessed transfection of HepG2 and U2OS cell lines with TALEN and CRISPR vectors designed using Invitrogen™ GeneArt™ Gene Synthesis services to target a specific locus. We observed improvements in transfection efficiency, mean fluorescence intensity, and genomic cleavage. These advancements in delivery help minimize painstaking downstream workflows, enable easier stem cell manipulation, and enhance site-specific insertion of transgenes into the cellular genome.

Materials and methods

Plasmid design and preparation

Invitrogen™ GeneArt™ Precision TALs and GeneArt™ CRISPR Nuclease Vectors were designed using the GeneArt™ web design tool (**thermofisher.com/tals**). The forward and reverse TALENs contain the FokI nuclease and target the *AAVS1* safe harbor locus. The all-in-one CRISPR vector system contains a Cas9 nuclease expression cassette and a guide RNA cloning cassette that target the *AAVS1* safe harbor locus, combined with a downstream orange fluorescent protein (OFP) reporter. Invitrogen™ pcDNA™3.3 vector was also used as a negative control throughout the assay. The plasmids were transformed into competent *E. coli* cells. Clones were analyzed and sequenced for specificity and then purified using an Invitrogen™ PureLink™ HiPure Plasmid Filter Maxiprep Kit to ensure low endotoxin activity and high-quality DNA.

Cell culture

Cells were cultured using Gibco™ DMEM, high-glucose, with Gibco™ GlutaMAX™ Supplement and 10% fetal bovine serum for 4–5 passages after thawing; cells were dissociated using Gibco™ TrypLE™ Express Enzyme and seeded in a 12-well plate at 2 x 10^5 cells per well in 1 mL complete medium to ensure 70–90% confluence on the day of transfection.

Transfection

Transfection with Lipofectamine 3000 reagent and Invitrogen™ Lipofectamine™ 2000 Transfection Reagent was compared. For transfection with Lipofectamine 3000 reagent (Figure 1), in separate tubes, 1.5 µL of Lipofectamine 3000 reagent and 1 µg of DNA were each diluted in 50 µL Gibco™ Opti-MEM™ Reduced-Serum Medium; then 2 µL P3000™ reagent was added to the diluted DNA. The diluted DNA with P3000 reagent was added to the diluted Lipofectamine 3000 reagent and incubated at room temperature for 5 minutes. Then 100 µL of the resulting complex was added to cells in complete medium. The procedure was the same for Lipofectamine 2000 reagent, except that the amount of transfection reagent was increased to 3 µL and no additional reagent was added to the diluted DNA before adding it to the diluted Lipofectamine 2000 reagent. All downstream analysis was performed 72 hours posttransfection.

Microscopy and flow cytometry

OFP expression from the CRISPR vector was determined by flow cytometry and microscopy. An Invitrogen™ EVOS™ FL Imaging System was used to acquire images with the RFP filter. Cells were then dissociated 72 hours posttransfection with TrypLE Express Enzyme and analyzed using a BD Accuri™ C6 Flow Cytometer with an FL-2 filter and blue laser.

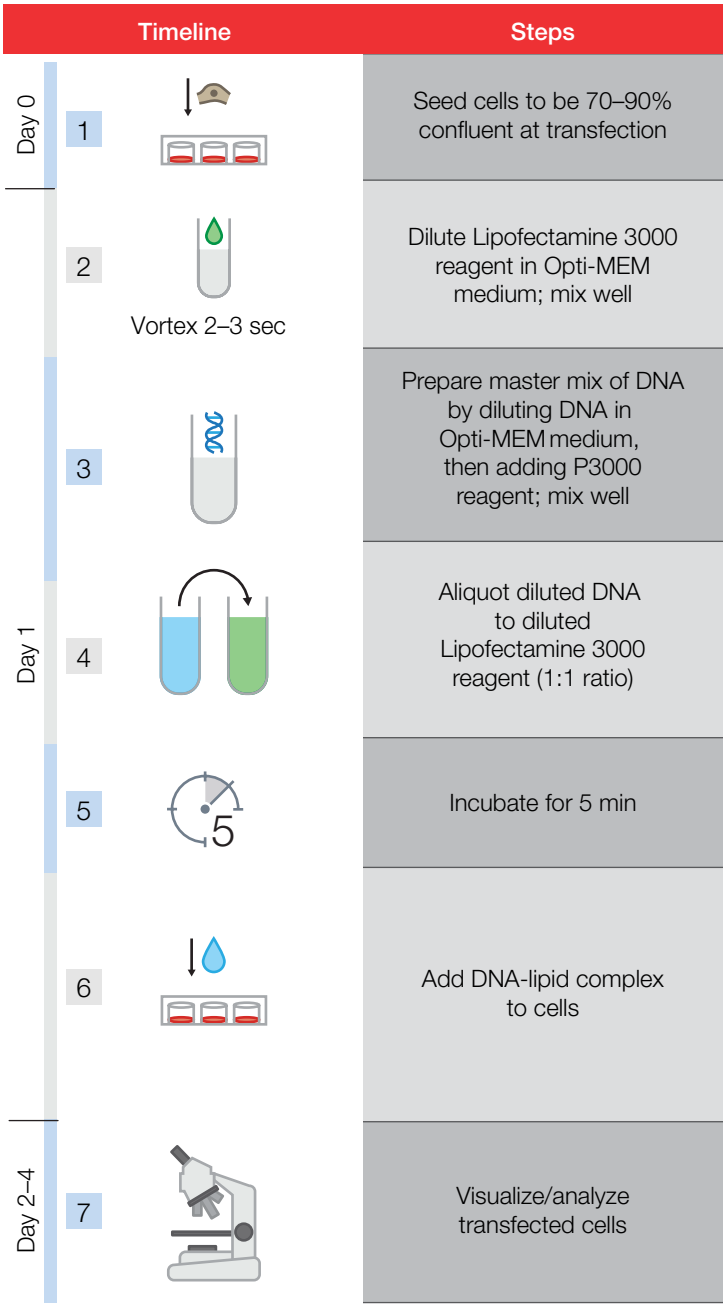

Figure 1. Lipofectamine 3000 reagent transfection workflow.

Genomic cleavage detection

The Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit provides a reliable and rapid method for the detection of locus-specific cleavage (Figure 2). Cells were dissociated with TrypLE Express Enzyme, washed with Dulbecco's phosphate-buffered saline, and pelleted. Cells were lysed with the Cell Lysis Buffer and Protein Degrader Mix from the Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit. The DNA was extracted and then PCR-amplified with forward and reverse primers. Denaturing and reannealing reactions were then performed to randomly anneal the mutated and unmutated PCR fragments. Detection enzyme (1 µL) was added, the mix was incubated for 1 hour at 37°C, and then the entire mix was electrophoresed in an Invitrogen™ E-Gel™ EX 2% agarose gel to determine the percent genome modification. AlphaView™ Software was used to determine cleavage efficiency using the formula: cleavage efficiency = 1 – [(1 – fraction cleaved)$^{1/2}$].

Results

U2OS cells, derived from human bone osteosarcoma, and HepG2 cells, derived from a human hepatocellular carcinoma, were transfected with Lipofectamine 3000 reagent. Both cell lines showed improved transfection efficiency and protein expression compared to transfection mediated with Lipofectamine 2000 reagent. Transfection efficiency and protein expression were assessed using a CRISPR construct that contains the OFP reporter gene. U2OS cells transfected with Lipofectamine 3000 reagent had 2-fold improved transfection efficiency (data not shown) and 4-fold improved fluorescence intensity (Figure 3A). HepG2 cells showed 20-fold improvement in transfection efficiency (data not shown) and 80-fold higher fluorescence intensity (Figure 3B). Most importantly, we observed increased TALEN- and CRISPR-mediated cleavage for the *AAVS1* target locus in both cell lines transfected with Lipofectamine 3000 reagent, demonstrating that increasing the transfection efficiency and, by implication, protein expression, will increase the cleavage rate of TALENs and CRISPR nucleases. U2OS cells transfected with Lipofectamine 3000 reagent showed 1.5-fold improved TALEN cleavage efficiency and slightly improved CRISPR cleavage (Figure 4A). HepG2 cells had 3-fold higher TALEN cleavage efficiency and 8-fold higher CRISPR cleavage (Figure 4B).

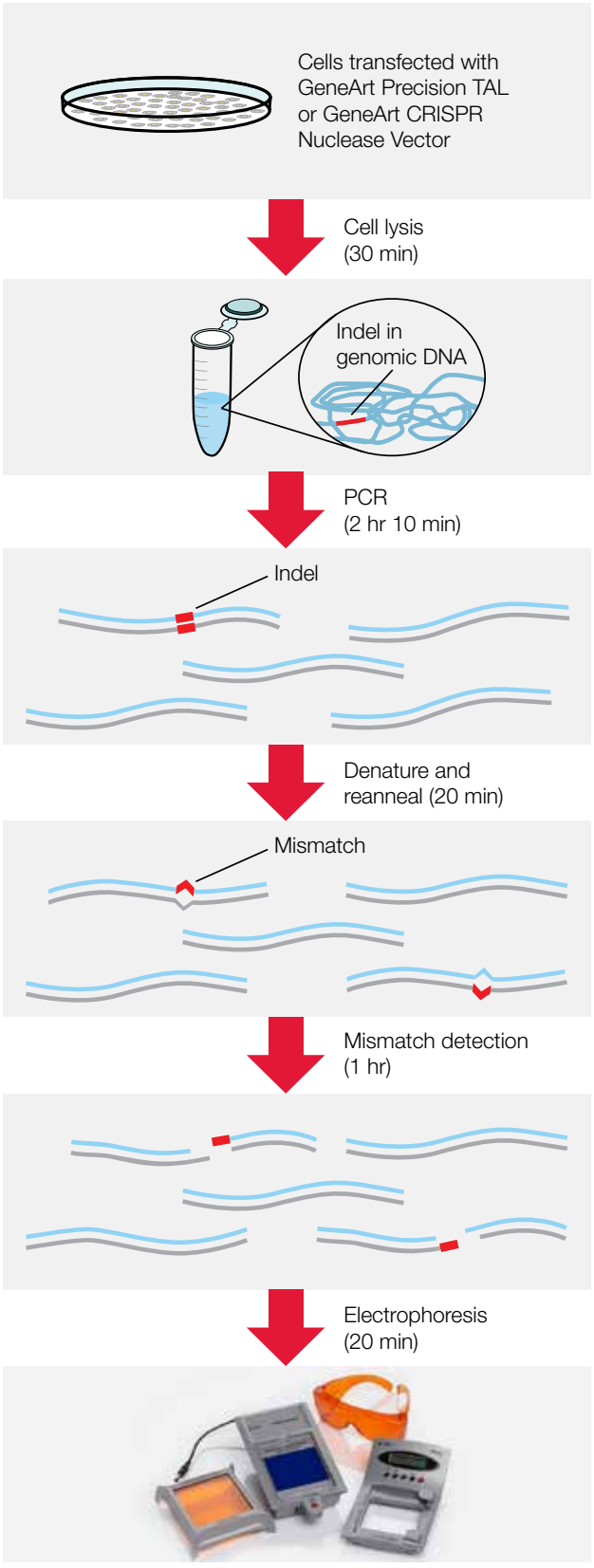

Figure 2. GeneArt Genomic Cleavage Detection Kit workflow.

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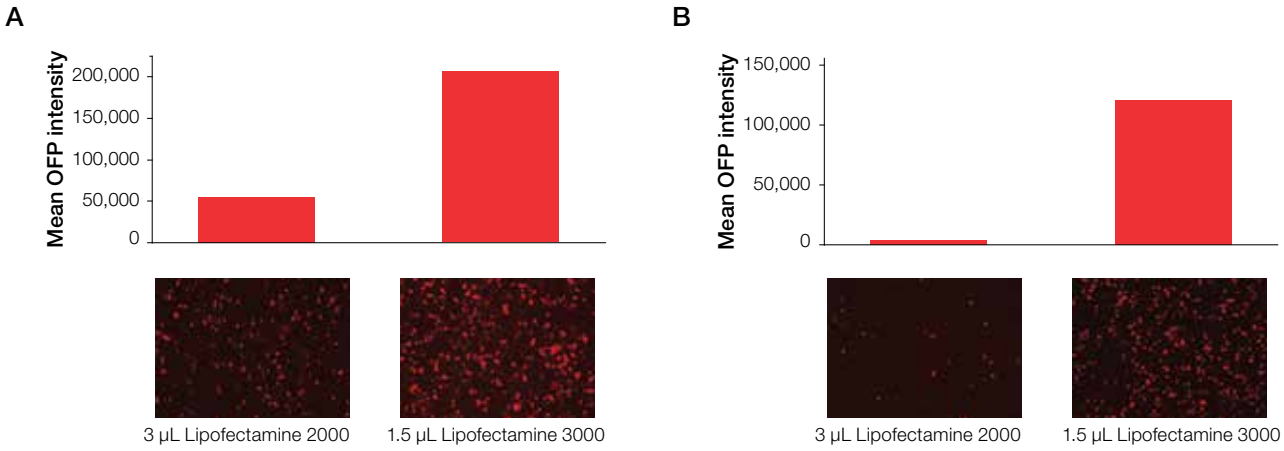

Figure 3. Transfection efficiency and protein expression using a CRISPR vector. The vector contained an OFP reporter gene and was transfected with Lipofectamine 2000 or Lipofectamine 3000 reagent into **(A)** U2OS and **(B)** HepG2 cell lines. Bar graphs show reporter gene expression; images show fluorescence of corresponding cells expressing OFP.

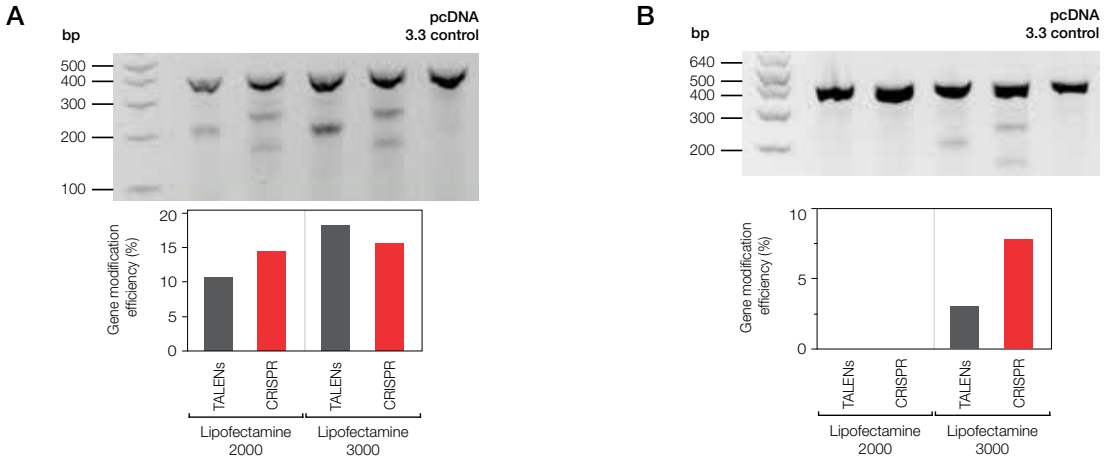

Figure 4. Cleavage efficiency of GeneArt TALENs and CRISPR nucleases. The TALENs and CRISPR nucleases targeted the *AAVS1* locus in **(A)** U2OS and **(B)** HepG2 cell lines. Cleavage was assayed using a GeneArt Genomic Cleavage Detection Kit.

Conclusion

Lipofectamine 3000 reagent was developed to improve transfection efficiency in difficult-to-transfect cell lines and to minimize painstaking downstream processes involved in genetic engineering protocols. In this study, we demonstrated that using a better transfection reagent such as Lipofectamine 3000 reagent will improve the cleavage efficiency with TALENs or CRISPR nucleases, ultimately maximizing the efficiency of genetic modifications and simplifying the downstream processes. Clonal selection is one example of a protocol that would benefit from an improved transfection reagent. Therefore, this study confirms the hypothesis that improving transfection will improve cleavage and recombination efficiency when using genome editing tools such as GeneArt Precision TALs and CRISPR nucleases.

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