Increasing gene editing efficiencies in eukaryotic cell lines by selection of appropriate CRISPR-Cas9 reagents

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Abstract

Genetic engineering of living cells is critical for understanding gene function in normal and diseased states. The CRISPR-Cas9 system is widely utilized because of its ease-of-use compared to other gene editing methods. This system requires a complex of Cas9 protein with tracrRNA and a gene-targeting crRNA to introduce double-strand DNA breaks at specific locations in the genome to disrupt protein translation and knockout gene function. To achieve high gene editing efficiencies, it is essential to choose the best CRISPR-Cas9 reagents for delivery and expression in the cells of interest.

Depending on the transfectablity of specific cells, CRISPR components can be delivered using plasmid transfection or lentiviral transduction. Plasmid-expressed Cas9 can be co-transfected with synthetic crRNA and tracrRNA for efficient gene editing in cells amenable to lipid delivery. Cas9 that is packaged into lentiviral particles can be transduced into cells that are refractory to transfection. Lentiviral Cas9 can also be used to generate stable cell lines, which can then be transfected or transduced by synthetic or lentiviral-based CRISPR RNA components; this is particularly useful for screening applications. Importantly, expressing humanized Cas9 from different promoters (*e.g.*, human and mouse CMV and EF1α, PGK, CAG) in different cell types results in varying levels of Cas9 expression and consequently, varying efficiencies of gene editing. In addition, cells transfected or transduced with gene editing reagents can be enriched by antibiotic selection or FACS using reagents in which the Cas9 gene is co-expressed with either an antibiotic resistance marker or a fluorescent protein reporter. This enrichment facilitates the isolation of clonal cells containing the desired mutation. Presented here are data demonstrating improvement of gene editing efficiencies in cells of interest by using the most effective delivery and selection approaches with the optimal CRISPR-Cas9 reagents.

Choosing a CRISPR-Cas9 system for efficient gene editing

CRISPR components can be delivered using transfection or viral transduction. Plasmid-expressed Cas9 can be co-transfected with synthetic crRNA:tracrRNA for efficient gene editing in cells amenable to lipid delivery, followed by isolation of knockout clonal lines (**Figure 1**). Cas9 vectors packaged into lentiviral particles can be used to generate a Cas9 stable cell line for transfections with crRNA:tracrRNA to evaluate gene knockout phenotypes in a population (**Figure 4**).

Co-transfection of Cas9 plasmid and synthetic crRNA:tracrRNA

A synthetic approach to crRNA:tracrRNA complex enables fast assessment of multiple target sites per gene, or for multiple genes, without the requirement of any cloning steps or *in vitro* transcription (**Figures 2 and 3**).

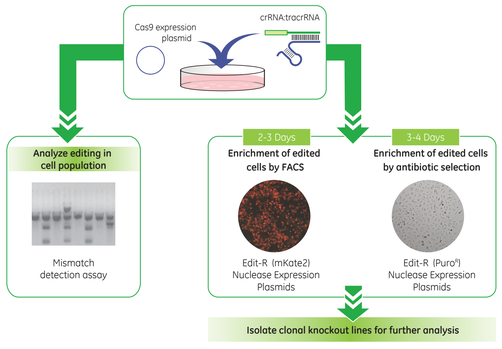

Figure 1. Plasmid Cas9 and synthetic crRNA:tracrRNA co-transfection and enrichment workflow.

Rapid assessment of multiple crRNAs with Dharmacon™ Edit-R™ CRISPR-Cas9 Gene Engineering system

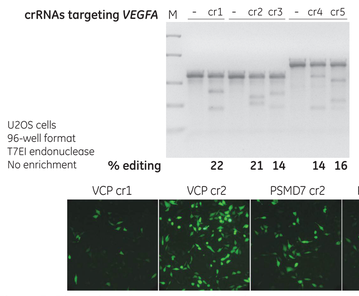

Figure 2. Edit-R Cas9-mKate2 plasmid, synthetic tracrRNA and five different synthetic crRNAs targeting *VEGFA* were co-transfected into U2OS cells. Various levels of gene editing were achieved as assessed by a mismatch detection assay using T7 Endonuclease I (T7EI).

Figure 3. Disruption of proteasome function by crRNAs targeting components of the 26S proteasome pathway: *VCP*, *PSMD7* and *PSMD14*. A recombinant U2OS ubiquitin-EGFP proteasome cell line was co-transfected with Cas9 plasmid and crRNA:tracrRNA. The accumulation of ubiquitin-EGFP was measured 72 hours post-transfection demonstrating disruption of proteasome function by knockout of functional proteins.

Lentiviral Cas9 combined with synthetic crRNA:tracrRNA

Edit-R Lentiviral Cas9 Nuclease Expression vectors allow generation of a Cas9 stable cell population for evaluating knockout phenotypes in a population (**Figure 4**). This approach can be especially beneficial for experiments where reproducibility and wider dynamic range of the phenotypic assay are highly desired, such as screening applications. Additionally, integrated Cas9 and synthetic crRNA:tracrRNA results in higher editing efficiency without the need to perform enrichment of plasmid-transfected cells using FACS or puromycin selection (**Figures 5 and 6**).

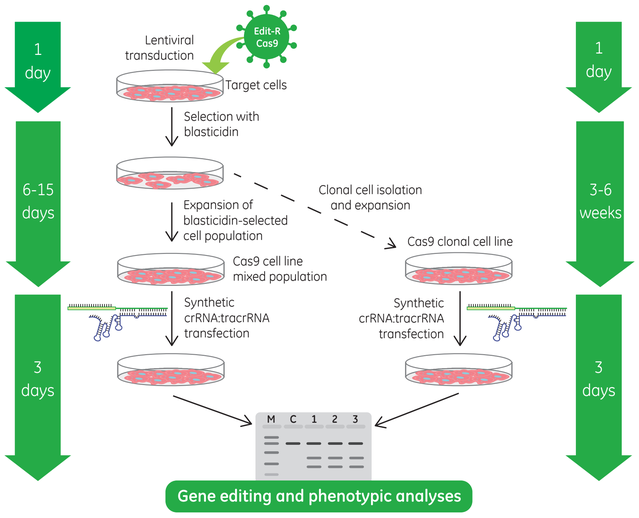

Figure 4. Lentiviral Cas9 and synthetic crRNA:tracrRNA workflow.

The optimal promoter for Cas9 expression results in greater gene editing efficiency

To achieve the highest editing efficiency in a cell line of interest, it is important to select the promoter most suitable for the cells of interest. Cas9 expression levels differ among promoters, leading to varying levels of gene editing efficiency (**Figures 5 and 6**).

Highly efficient gene editing in Cas9-expressing mouse NIH/3T3 cells using synthetic PPIB crRNA:tracrRNA

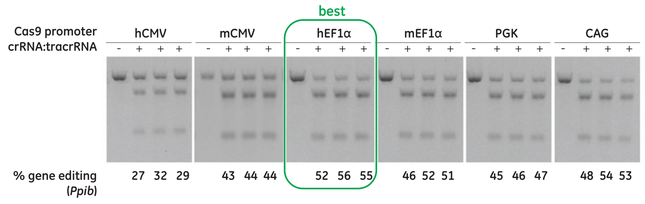

Figure 5. NIH/3T3 cells were transduced with Edit-R Lentiviral Cas9 Nuclease Expression particles for stable expression of Cas9 and a blasticidin resistance gene driven by the indicated promoters. The cell populations were selected with blasticidin for a minimum of 10 days before crRNA;tracrRNA transfections. Cells were transfected with 50 nM synthetic crRNA:tracrRNA targeting *Ppib* using DharmaFECT 1 Transfection Reagent. After 72 hours, the relative frequency of gene editing was calculated from a mismatch detection assay using T7EI.

Efficient gene editing in U2OS cells expressing Cas9 from different promoters

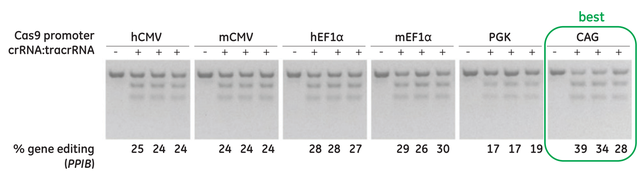

Figure 6. A recombinant U2OS ubiquitin-EGFP proteasome cell line with stably integrated Cas9 was transfected with 50 nM synthetic crRNA:tracrRNA using DharmaFECT 3 Transfection Reagent to target *PPIB*. After 72 hours, the relative frequency of gene editing was calculated from a mismatch detection assay using T7EI.

Enrichment of cell populations for increased gene editing events

Puromycin resistance marker allows rapid selection and enrichment of edited cells

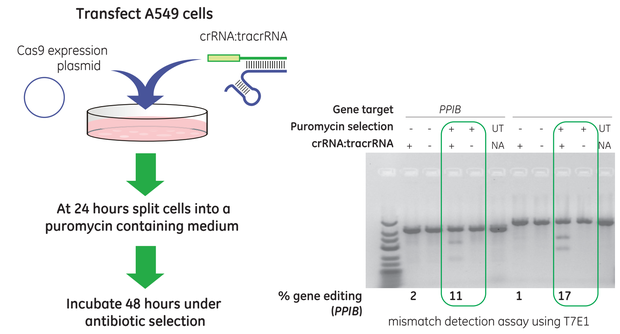

Figure 7. A549 cells were co-transfected with Edit-R Cas9-Puro^R plasmid, tracrRNA and crRNAs targeting *PPIB* or *CDKN1* and selected with puromycin for 48 hours. A mismatch detection assay using T7EI show increased editing in A549 cells after puromycin selection.

Enrichment of edited cells by FACS analysis

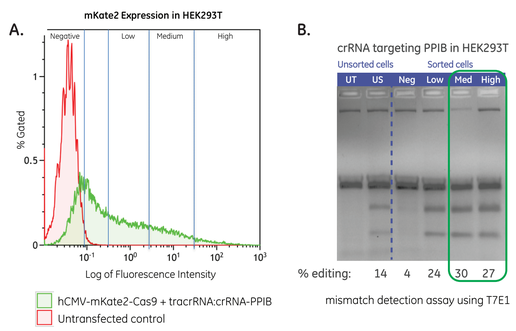

Figure 8. Edit-R mKate2-Cas9 bicistronic plasmids allow enrichment of gene edited cells by FACS analysis. HEK293T cells were transiently co-transfected with mKate2-Cas9 expressing plasmids, synthetic crRNA targeting *PPIB*, and synthetic tracrRNA. Cells were sorted 72 hours post-transfection. Percent editing was determined with a mismatch detection assay using T7EI.

Conclusions

- Synthetic crRNA:tracrRNA with a Cas9 nuclease allows fast, high efficiency gene editing without cloning steps.
- The ability to quickly evaluate multiple crRNAs leads to the selection of the best target sequence and, therefore, most effective knockout of the gene of interest.
- Antibiotic resistance gene or fluorescent marker on the plasmid expressing Cas9 enable enrichment of edited cells, thus improving phenotypic readout and facilitating clonal selection.
- Applying a highly efficient lentiviral Cas9 nuclease approach allows researchers to establish stable cell populations or clonal lines for improved reproducibility and a further increase in editing frequency.
- Choosing the best promoter is recommended to achieve maximum Cas9 expression in the cell line of interest and therefore better editing efficiency.

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