Cas9 driven by an optimal promoter improves gene editing in eukaryotic cell lines when paired with synthetic crRNA and tracrRNA

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Introduction

Interest in genome engineering of mammalian cells has been increasing in the past few years with the development of new tools to create DNA breaks at specific locations on the cell genome. Among these tools, the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR associated protein 9) system has gained significant interest due to its relative simplicity and ease of use compared to other genome engineering technologies. The CRISPR-Cas9 system requires a complex of the Cas9 protein with a trans-activating RNA (tracrRNA) and a gene-targeting CRISPR RNA (crRNA) **(Figure 1)**, or a single guide RNA (sgRNA, a chimeric form of tracrRNA with a crRNA).

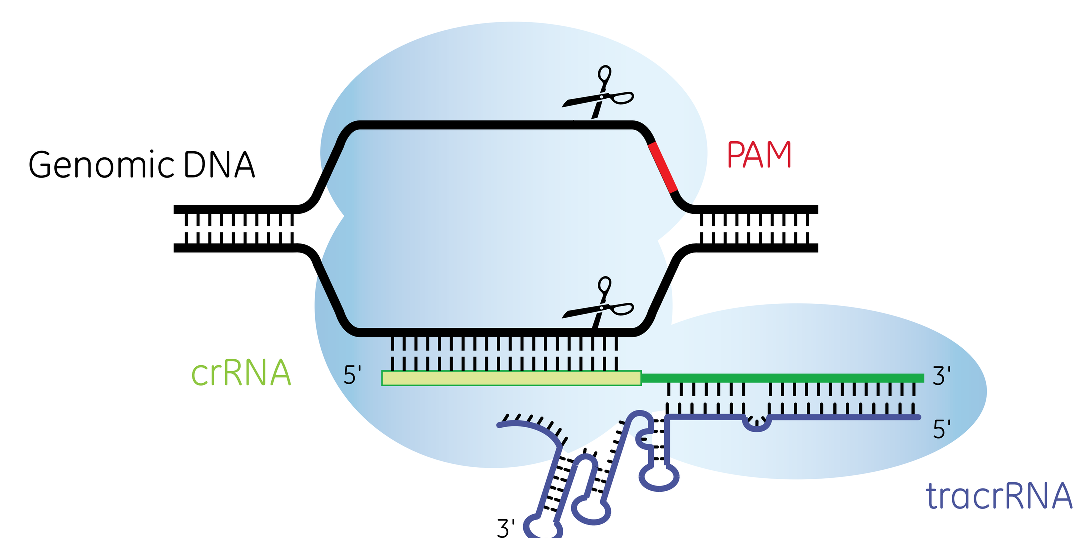

Figure 1. Gene editing with a three-component system: Cas9 nuclease (light blue), programmed by the tracrRNA:crRNA complex (blue and green, respectively) cutting both strands of genomic DNA 5' of the PAM (red). A synthetic approach to tracrRNA:crRNA complex enables fast assessment of multiple target sites per gene or for multiple genes without the requirement of any cloning steps.

Presented here are results on the efficiency of using synthetic crRNA and tracrRNA to introduce gene editing events when co-transfected with a plasmid expressing Cas9. We explored the use of antibiotic and fluorescence-activated cell sorting (FACS) methods for enrichment of cells that have undergone gene editing, and the use of multiple promoters to increase efficiency of gene editing with Cas9 and synthetic tracrRNA and crRNAs.

Optimal promoter for Cas9 expression improves gene editing

The strength of commonly used constitutive mammalian promoters varies among different cell types and cellular contexts. It has been previously shown that the likelihood of a gene editing event is dependent on the nuclease expression level (Certo *et al.*, 2011, Fu *et al.*, 2013). Therefore, to achieve efficient gene knockout one must assess the most suitable promoter to express Cas9 in the cell line of choice. In addition, the ability to enrich for cells transfected with CRISPR-Cas9 system may be advantageous for one particular experimental condition. To evaluate the effect of promoter choice on expression of Cas9 in multiple cell lines we constructed twelve different vectors expressing Cas9 from six promoters allowing for enrichment either by antibiotic selection or FACS analysis **(Figure 2)**.

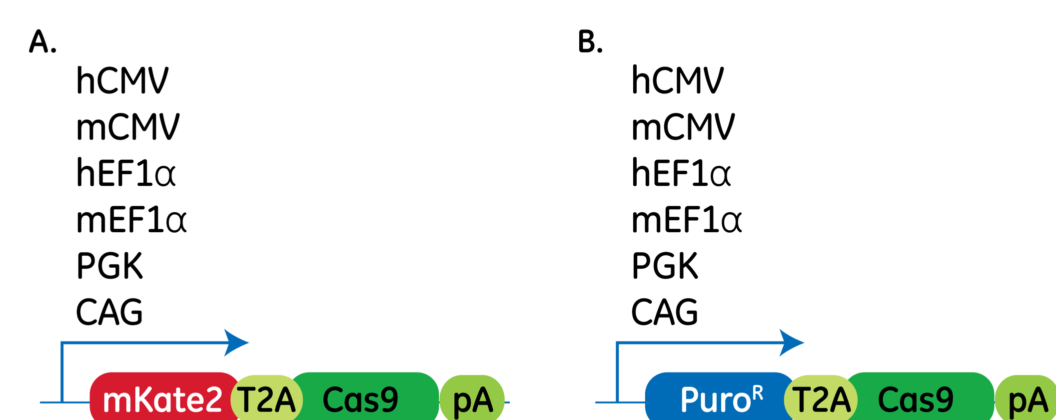

Figure 2. Plasmid variants for Cas9 expression under control of a collection of constitutive promoters. Cas9 protein is expressed as a bicistronic transcript using a 2A "self-cleaving" peptide linking Cas9 to either **(A)** mKate2 (far-red fluorescent protein reporter) or **(B)** the puromycin resistance gene ($Puro^R$) followed by the bovine growth hormone polyadenylation signal (pA).

Optimal promoter for Cas9 expression improves gene editing

Expression of Cas9 by stronger promoters yields higher percentages of gene knockout in the same cell line when multiple promoters are compared. In mouse embryonic stem cells, Cas9 expression driven by mEF1α, followed by the hEF1α, showed higher frequency of mutations (indel) compared to the more commonly used hCMV promoter.

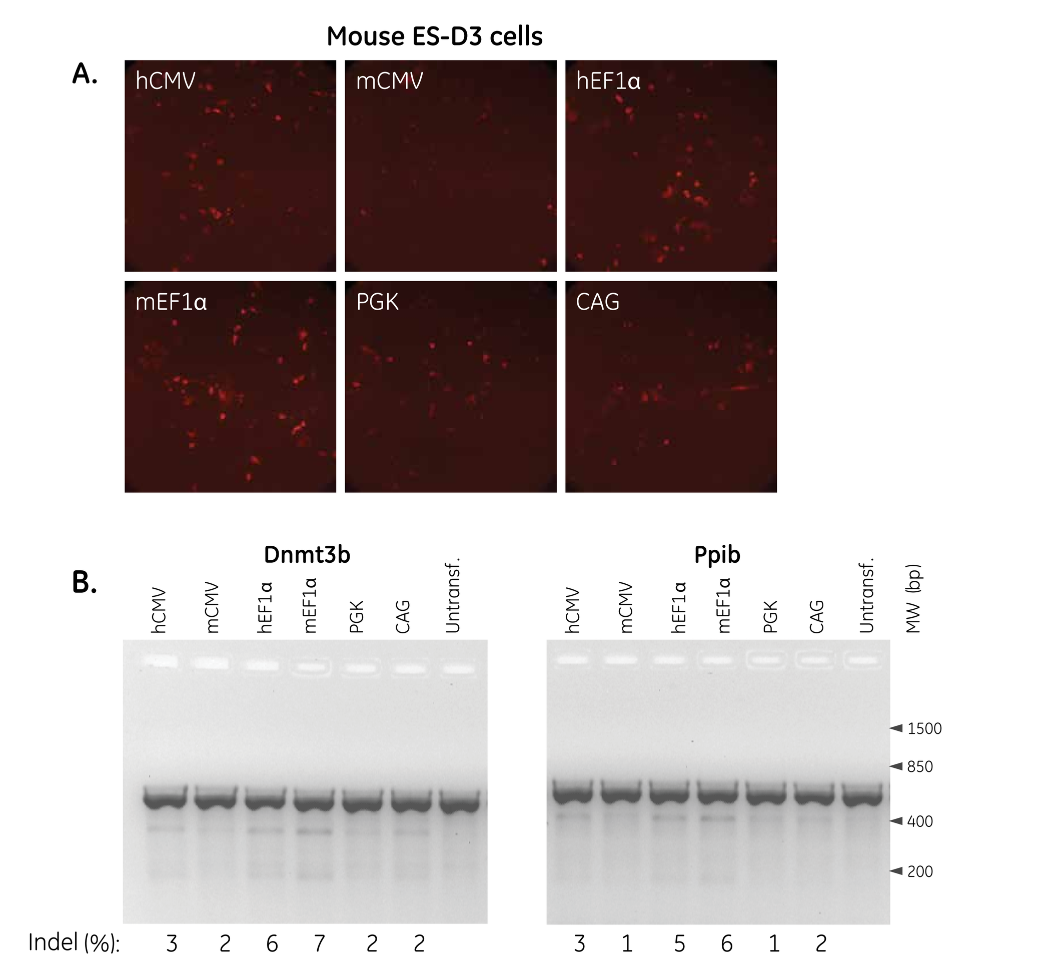

Figure 3. mKate2-Cas9 expression from different promoters in mouse ES-D3 cells. mEF1α drives the best Cas9 expression and gene editing in the murine embryonic cell line ES-D3. Cells were transiently co-transfected with mKate2-Cas9 expressing plasmids, synthetic tracrRNA and crRNA using Dharmacon Dharmafect™ Duo as the transfection reagent. **A.** Cells were imaged at 48 h post-transfection with 20x magnification, 2 s exposure and 2 Gain. **B.** Percentage of mutations (indel) were determined by mismatch detection assay (SURVEYOR™, Transgenomic) and calculated by densitometry as previously described (Cong *et al.*, 2013).

Enrichment of edited cells by FACS

FACS or antibiotic selection can be used to obtain cell populations with increased likelihood of Cas9-induced gene engineering events. We observed up to two-fold increase of mutations in a population of HEK293T or U2OS cells after enrichment by FACS compared with unsorted cells after transient co-transfection with the hCMV::mKate2-Cas9 plasmid and chemically synthesized tracrRNA and crRNA targeting PPIB **(Figure 4)**.

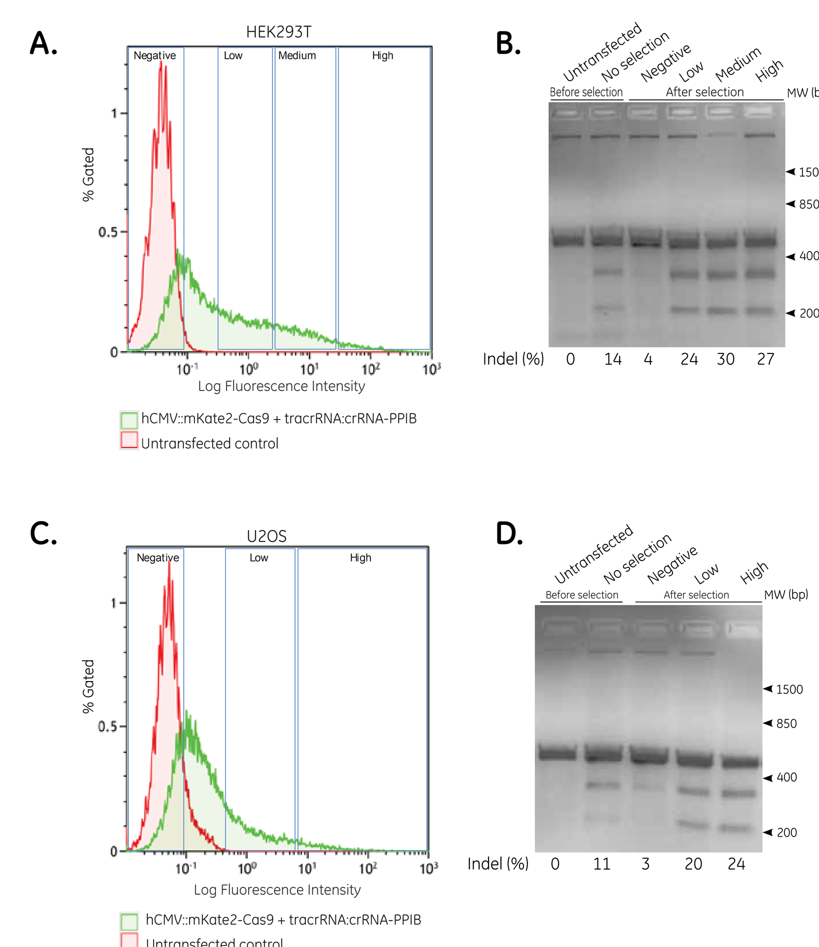

Figure 4. mKate2-Cas9 bicistronic plasmids allow enrichment of gene edited cells by FACS. HEK293T and U2OS cells were transiently co-transfected with mKate2-Cas9 expressing plasmids and synthetic crRNA and tracrRNA as described previously. Cells were sorted 72 h post-transfection. Percentage indel for the PPIB gene target was determined as described in **Figure 3**. Cells populations were sorted by their relative red fluorescence intensity as background (negative), low, medium or high.

Enrichment of edited cells by antibiotic selection

Cell populations were selected with puromycin for 48 h and an increase in the frequency of detected indels was observed in A549 and U2OS cells compared to unselected populations **(Figure 5)**.

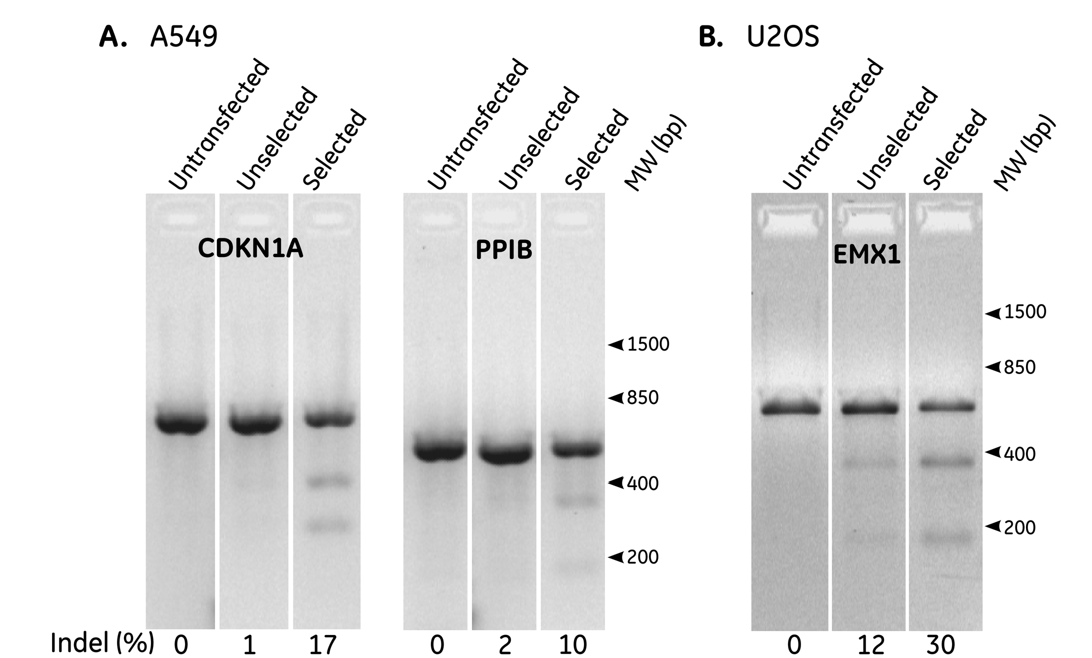

Figure 5. $Puro^R$-Cas9 bicistronic plasmids allow enrichment of gene edited cells by quick selection with puromycin. A549 and U2OS cells were transiently co-transfected with $Puro^R$-Cas9 expressing plasmids, synthetic crRNA and tracrRNA as described in **Figure 3.** Cells were treated with **(A)** 1.5 or **(B)** 2 μg/mL puromycin 24 h post-transfection and selected for 48 h. Percentage of mutations (indel) were determined by mismatch detection assay (T7Endonuclease I, NEB) and calculated by densitometry as described in **Figure 3**.

Conclusion

- Utilizing a highly active promoter for Cas9 expression enables better editing in specific cell lines.
- Enrichment of transiently transfected cells either by FACS or puromycin selection can further improve the yield of edited cells.
- Efficient gene editing can be achieved with a three-component system: plasmid Cas9 and synthetic tracrRNA and crRNAs.
- Use of synthetic tracrRNA and crRNAs is a simplified method for gene editing of one or more genes without requiring any cloning steps.
- By virtue of its simplicity, this three-component CRISPR-Cas9 system is amenable to high-throughput genome editing applications.

References

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