Full Spectrum of In Vitro Disease Models with Optimized CRISPR/Cas9 Gene Editing in Difficult-to-Transfect Blood-Lineage Cells

Huanyu Jin, Zhongsheng Yu, Vladimir Pak, Yin Zhang, Padmaja Tummala, Pavithra Rajeswaran, Diana Nguyen, Monika Maleszewska, Jinling Li, Charles Cao, Ruby Yanru Chen-Tsai

Applied StemCell, Inc., Milpitas, CA

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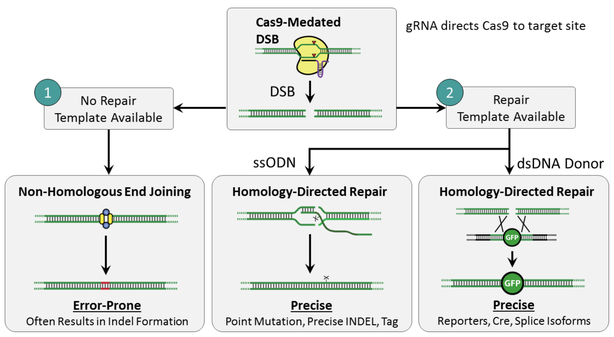
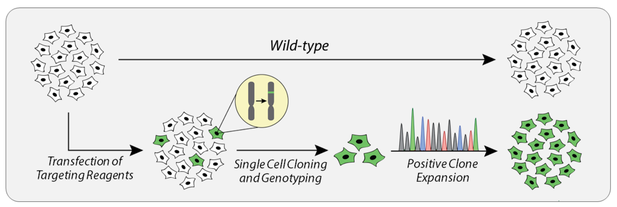
INTRODUCTION

- CRISPR gene editing in cell lines, the workhorse of preclinical and biomedical research, enables the generation of unlimited *in vitro* models with precise gene modifications and advanced gene expression design that are physiologically relevant.
- CRISPR genome editing in certain types of cells, such as suspension cells and several blood lineage cell lines is very inefficient and problematic.
- Contributing factors include low transfection efficiency of CRISPR reagents, cytotoxicity, and low or undesired gRNA and Cas9 activities in the cell lines.
- In this poster, we describe techniques optimized for CRISPR genome editing in some difficult-to-transfect blood-lineage cell lines, the factors influencing efficiency such as cytotoxicity, and the types of modifications achieved (double gene knockout, large fragment knock-in, point mutation).
- These results demonstrate that different cell lines may require different approaches or modified protocols to deliver CRISPR components for efficient and successful modifications of the targeted gene.

CRISPR/Cas9 Cell Line Engineering





Efficient HDR-Mediated Homozygous Point Mutations in K562 Cells

Using a DNA-dependent protein kinase inhibitor to improve efficiency of HDR mediated sequence insertion:

A

CACGCAAGTTAATAAAAGAGAATGGATTAAATGCAGGCC
TGGCATTTCCTACTGGATGTTCTCTCAATAATTGTGCTG
CCAACTATACTCCCAATGCAGGTGACACAACAGTATTAC
AGTATGATGACATCTGTAAAATAGACTTTGGAACACATA
TAAGTGGT

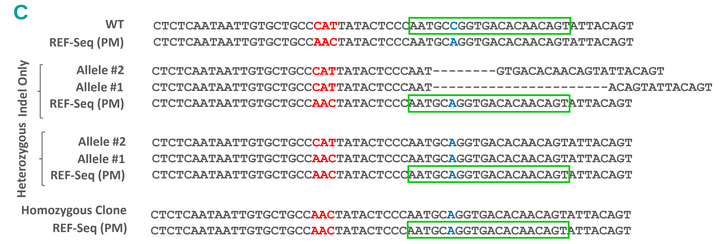
CAT > AAC | PAM | gRNA | Silent mutation

B

Table. Effect of DNA-dependent protein kinase on CRISPR-mediated HDR in K562 cells

Drug Conc.	0 μM	10 μM	20 μM
# Indel only clones (%)	18 (90.0%)	36 (78.3%)	3 (17.6%)
# Heterozygous clones (%)	2 (10.0%)	7 (15.2%)	8 (47.1%)	
# Homozygous clones (%)	0 (0.0%)	3 (6.5%)	6 (35.3%)
Total # clones	20	46	17

C

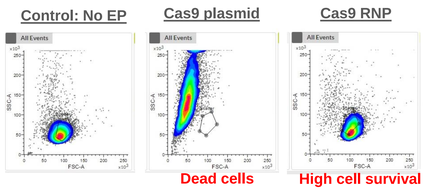


(A) Schematic representation of the donor sequence (REF-seq) that will serve as the HDR template to introduce the CAT > AAC point mutation in the gene of interest in K562 cell lines. The gRNA, PAM and silent mutation targeting designs are represented accordingly. A silent mutation was introduced to prevent re-cleavage by Cas9. (B) CRISPR/Cas9-mediated HDR efficiency is improved upon co-treatment with a DNA-dependent protein kinase inhibitor for 24h. (C) Sequence alignment of representative clones indicate required point mutation in the targeted region. Treatment with the drug generated indels in both alleles of the targeted region in the cell line. Representative heterozygous and homozygous clones are also aligned with reference sequence to indicate required mutation in the targeted locus. WT: wild type sequence; REF-seq: reference sequence/ donor sequence with silent mutation.

Gene Knockout in Leukemia Cell Lines, KG-1 Cells

Using Cas9-RNP:

A



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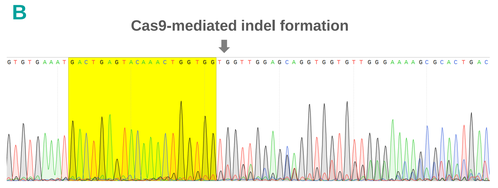

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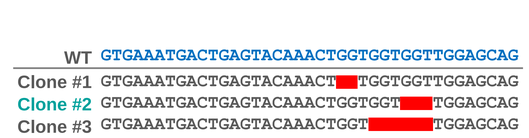


(A) FACS analysis reveals cell death upon Cas9-plasmid electroporation into KG-1 cell line, but not electroporation with Cas9-RNP. (B) Cas9-RNP can generate INDELs (insertion or deletion) in a mixed population of KG-1 cells. The yellow highlighted region indicates the gRNA target for the Cas9-RNP complex. (C) Sequence alignment for selected clones generated by RNP-mediated NHEJ repair showed Clone #1 to have 2bp insertion which will cause a frame shift mutation in the coding region of the gene of interest, resulting in gene knockout.

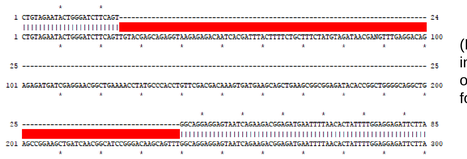
Double Gene Knockout in Jurkat Cell Lines

Using Cas9-plasmid:

A

(A) A sequential targeting strategy was used to disrupt two genes of interest in Jurkat (suspension) cells. First, exon 2 of gene #1 was disrupted using 2 gRNAs (g1.1 and g1.2) and single cell clones were identified. The identified gene #1 knockout (KO) clones were re-targeted with two different gRNAs (g2.1 and g2.2) for gene #2. Single cell clones were isolated and confirmed to have double KO deletion by PCR and sequencing.

B



(B) Representative Gene #1 KO clone has a 215 bp insertion that causes a frame shift mutation. Three out of 40 clones were identified as gene #1 KO and used for gene #2 KO.

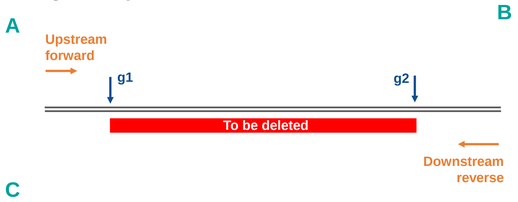
C

(C) After second targeting experiment, one clone was identified as a gene #2 KO. This clone contained a 115 bp insertion at targeted locus that causes a frame shift mutation and disruption of gene expression.

Large Fragment Deletion in Jurkat Cell Lines

Using Cas9-plasmid:

A



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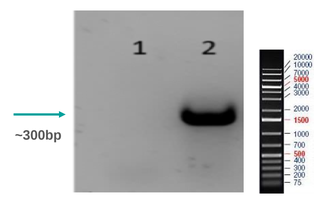


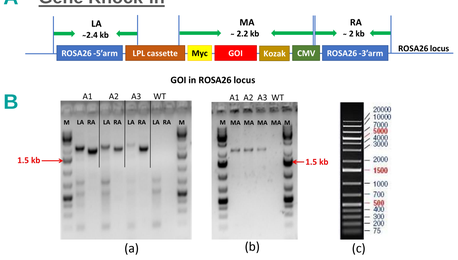
C

(A) Schematic representation of ~5kb targeted deletion for enhancer region of the GOI (gene of interest) generated by co-delivery of two gRNAs (g1 and g2). The primer design for detection of region of deletion is indicated by orange arrows. (B) Two single clones were examined by PCR and gel electrophoresis, followed by Sanger sequencing. Clone #2 showed a shorter band at the size of 300bp and was confirmed to be positive for the deletion of the ~5 kb fragment in the gene of interest. (C) Sequence alignment of target region between positive clone (#2) and wild type (WT) indicates deletion of required sequence. DNA ladder: GeneRuler 1kb plus DNA ladder.

Knock-in & Knockout in T2 Lymphoblastic Cell Line

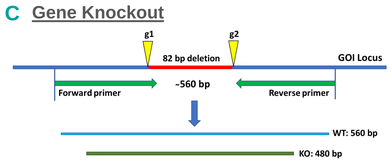
Using DNA-based CRISPR reagents:

A Gene Knock-in

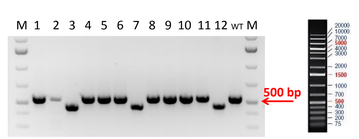


(A) Schematic representation of CRISPR/Cas9 targeting strategy for transgene (gene of interest; GOI) knock-in into a safe harbor locus in Lymphoblastic cell line, T2. (B) PCR gel electrophoresis of single cell clones indicate clones A1, A2, A3 are positive for knock-in of GOI at targeted ROSA26 locus as compared to wild type (WT). PCR with flanking primers to confirm insertion at ROSA26 locus (a) and (b) show fragment of required sizes: Left arm (LA): ~2.4 kb, Right arm (RA): ~2.0kb. PCR to confirm transgene knock-in, MA also shows fragment of required size:~2.2kb. Lane M: GeneRuler 1kb plus DNA ladder.

C Gene Knockout



D



(C) Schematic representation of CRISPR/Cas9 targeting strategy for fragment deletion in a gene of interest. (D) PCR gel electrophoresis of single cell clones indicate clones 3, 7, and 12 contained deletion of the desired fragment as compared to wild type (WT). Lane M: GeneRuler 1kb plus DNA ladder.

CONCLUSION

- CRISPR/Cas9 is a powerful tool for engineering many different cell lines from several species.
- CRISPR/Cas9 enables the generation of knockout, knock-in and point mutation lines with precise genetic modifications.
- CRISPR efficiency can be limited by the type or cell line being engineered, especially difficult-to-transfect cell lines such as blood-lineage cell.
- A variety of factors such as cell growth parameters, cell line sensitivity and cytotoxicity to transfected CRISPR reagents, can impact efficiency and reliability in genome editing.
- Optimizing protocols and transfection conditions for these blood lineage cells can contribute to higher cell survival, efficiency and success rate.
- Our results demonstrate that difference cell lines may require different approaches or modified protocols to deliver CRISPR/Cas9 components for efficiency and success modifications of targeted genes.