A Highly Efficient CRISPR Knock-Out / Knock-In Tool

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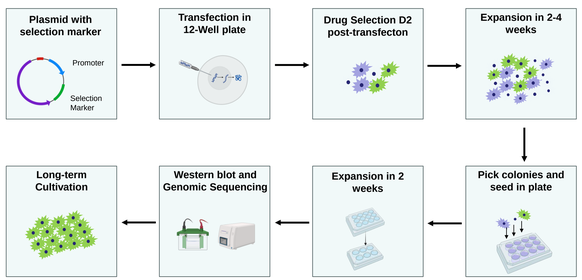
Introduction

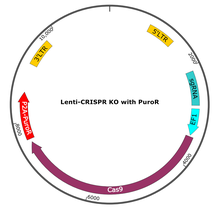
CRISPR is a potent tool widely used for genome editing. While CRISPR knock-out, achieved through in-del mutations via cellular repair mechanisms, has proven remarkably effective, the knock-in system for exogenous fragment insertion encounters challenges due to limited specificity and efficiency. Two primary methods for exogenous fragment insertion, namely NHEJ (Non-Homologous End Joining) and HR (Homologous Recombination), exist. HR allows for the construction of DNA insertions with precise junctions but is comparatively less efficient than NHEJ. To address these limitations, our system focuses on enhancing the efficiency of HR-based genome editing.

We have optimized the Lenti All-In-One CRISPR system to enhance the delivery and targeting efficiency of the Cas9-gRNA complex. This system has been successfully utilized to knock out Glutamine Synthetase (GLUL) in HEK293T cells, and MAP2K3 in MDA-MB-468 cells, demonstrating superior efficiency compared to commercially available tools. Additionally, we combined the All-In-One system with the donor vector to facilitate their knock-in effects and adopt integration of GFP into AAVS1 sites to quickly examine the efficacy. The results successfully demonstrated that our system is a powerful tool for CRISPR knock-out and knock-in functionality.

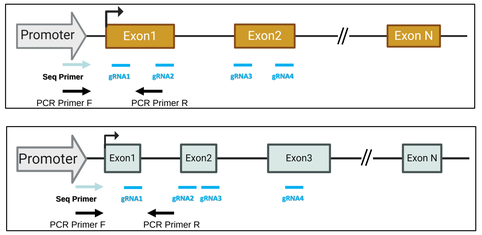
Procedure and Results



A. CRISPR Knock-Out against GLUL



Lenti-CRISPR Knock-Out Vector with Puro-R



GLUL Knock-Out in HEK293T cells

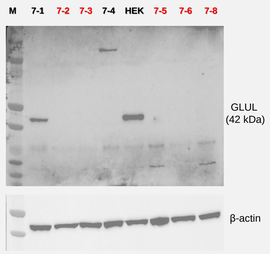
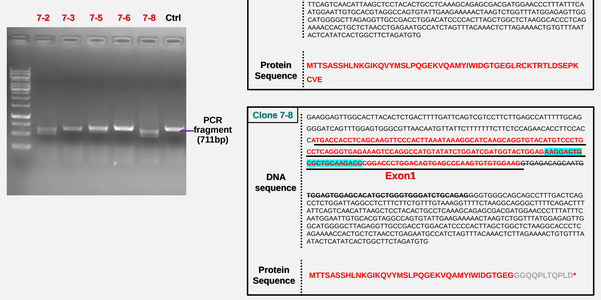


Fig 1. The GLUL gene expression was eliminated through CRISPR Knock-out. Four distinct gRNAs were constructed in All-In-One CRISPR plasmids to target the GLUL gene (in the Top Box). These plasmids were packaged into virus and transduced into HEK293T cells. Single stable colonies were established after 4 weeks of puromycin selection at 0.8 μg/mL. Western blot analysis was conducted on seven clones, with five of them showing complete abolition of GLUL expression. The remaining clones exhibited either reduced target gene expression or changes in the size of the target protein.


7-2
7-3
7-5
7-6
7-8
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Protein Sequence
MTTSASSHLNKGIKQVYMSLPQGEKVQAMYIWIDGTGEGGGQQPLTQPLD*

Fig 2. The GLUL gene deletion was analyzed by genomic DNA sequencing. Genomic DNA from five positive clones and wildtype cells was extracted for electrophoresis and DNA sequencing analysis. The gel image of the PCR products is presented in the left panel, and the results of nucleotide analysis are shown in the right panel. The upper text-line of each box displays DNA sequences, with red indicating the first exon, blue representing the gRNA target site, and a deletion line denoting the deleted nucleotides. The translated protein is also indicated in the lower line of each box, with gray highlighting alterations in gene expression following the CRISPR knock-out experiment.

B. CRISPR Knock-Out against MAP2K3

MAP2K3 Knock-Out in MDA-MB-468 cells

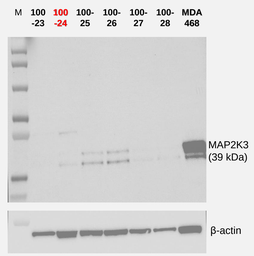


Fig 3. The expression of MAP2K3 genes was examined in CRISPR knock-out cells. Four distinct gRNAs were constructed in All-In-One CRISPR plasmids to target the MAP2K3 gene (in Top Box). The plasmids were packaged into virus and transduced into MDA-MB-468 cells. Single stable colonies were established after 4 weeks of puromycin selection at 0.4 μg/mL. Western blot analysis was performed on six clones, revealing that four out of the six exhibited complete abolishment of MAP2K3, while the remaining two showed a reduction in the expression level of the target gene.

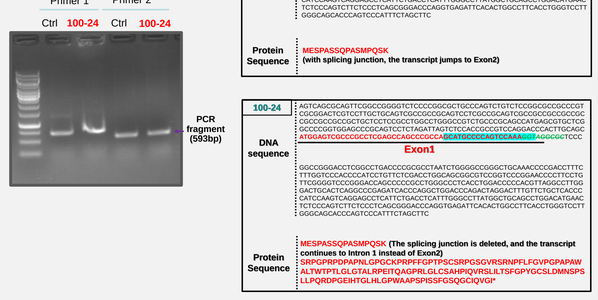
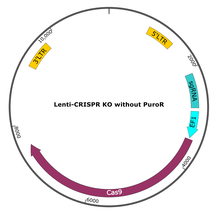
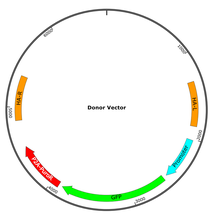


Fig.4. **The MAP2K3 gene deletion was analyzed by genomic DNA sequencing.** The genomic DNA from a positive knockout clone (100-24) and wildtype cells underwent electrophoresis and DNA sequencing analysis. The gel image of the PCR products is presented in the left panel, and the results of nucleotide analysis are shown in the right panel. The upper text-line of each box displays DNA sequences, with red indicating the first exon, blue representing the gRNA target site, and a deletion line denoting the deleted nucleotides. The translated protein is also depicted in the lower text-line of each box, with gray indicating the alteration of gene expression due to CRISPR knockout.

C. CRISPR Knock-In at AAVS1



Lenti-CRISPR Knock-Out Vector without Puro-R



Donor Vector with GFP-P2A-Puro

GFP Integration at AAVS1 site

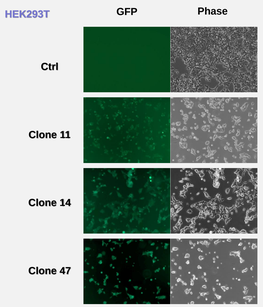


Fig.5. **The integration of GFP was evaluated through fluorescence imaging.** The donor vector, including Puro for cell enrichment, GFP for target cell screening, as well as homology arms (HA-L and HA-R), was integrated at the AAVS1 sites in HEK293T cells with the introduction of the All-In-One Lenti-CRISPR vector. Fluorescence and phase images were captured to preliminarily screen the positive clones. The pictures of three positive clones and wildtype cells are shown in the left panels.

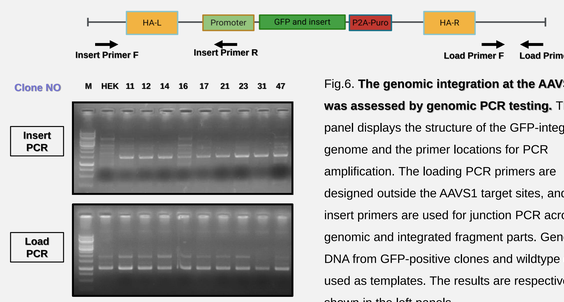


Fig.6. **The genomic integration at the AAVS1 site was assessed by genomic PCR testing.** The top panel displays the structure of the GFP-integrated genome and the primer locations for PCR amplification. The loading PCR primers are designed outside the AAVS1 target sites, and the insert primers are used for junction PCR across the genomic and integrated fragment parts. Genomic DNA from GFP-positive clones and wildtype cells are used as templates. The results are respectively shown in the left panels.

Conclusions

1. Our All-In-One Lenti-CRISPR system is an effective tool for gene knockout in mammalian cells, featuring a compact structure to enhance virus packaging and a puro-R element for cell selection and enrichment.

2. Our All-In-One Lenti-CRISPR vector can be paired with a donor vector containing different selection markers as a CRISPR knock-in system, which facilitate rapid and efficient fragment integration in an HR-dependent manner.