A step by step protocol for genomic knock-in of long sequences using dCas9-SSAP editor

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Method Article

**Keywords:** CRISPR, dCas9, Single-strand annealing protein, genomic knock-in, long sequence

**Posted Date:** May 19th, 2022

**DOI:** https://doi.org/10.21203/rs.3.pex-1711/v1

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Abstract

Gene-editing is a powerful tool for probing the mechanisms of human health and diseases, and holds promise as a durable therapeutic approach. Exemplified by CRISPR-Cas systems, gene-editing tools often cause DNA damage at on- and off-target sites, and thus trigger diverse endogenous DNA repair processes that are error-prone. Such unwanted mutations and safety concerns can be exacerbated when altering long sequences. Here, in this protocol, we present a method that couples microbial single-strand annealing proteins (SSAPs) with dCas9-guideRNA complex to stimulate DNA strand exchange for gene-editing. The dCas9-SSAP editor had low editing errors at target loci, minimal detectable off-target effect, and higher accuracy than canonical Cas9 methods. This method was effective for inserting sequences up to kilobase-scale, with up to ~20% knock-in efficiencies and robust performances across donor designs and cell types, including human stem cells.

Introduction

Since the initial demonstration of CRISPR-Cas9 genome engineering, gene-editing technologies have gained broad applications in basic and translational settings\(^1\)\(^{-}\)\(^{10}\). New generations of tools substantially improved the efficiency and fidelity of gene editing and are powerful for altering relatively short sequences\(^11\). Most gene-editing tools work by cleaving genome DNA to induce single-strand nicks (SSNs) or double-stranded breaks (DSBs) that facilitate targeted editing\(^12\),\(^{13}\). These DNA modifications could be repaired by error-prone endogenous pathways such as non-homologous end-joining (NHEJ)\(^12\). This often leads to unwanted mutations and off-target effects, which could result in toxicity and raise safety concerns\(^14\)\(^{-}\)\(^{18}\). While recent advance in using mRNA/protein with optimized donors enhanced efficiencies\(^12\), editing errors and off-target effects would become increasingly severe when engineering long sequences (\(\geq100\text{bp}\)). These unwanted effects limit the application of gene-editing for genomic knock-in or \textit{in vivo} gene therapy\(^19\)\(^{-}\)\(^{21}\).

Available methods for long-sequence editing, such as homology-directed repair (HDR) or microhomology-mediated end-joining (MMEJ), rely on DNA cutting and often trigger random indel formation\(^12\),\(^{13}\),\(^{20}\). Recent efforts have enhanced long-sequence editing precision, using chemical enhancers, fusion of enhancement domains, or modified donors\(^22\)\(^{-}\)\(^{24}\). Nicking-based HDR has been shown to reduce errors but could lead to lower efficiency\(^13\). Thus, there remains a need for efficient, safer CRISPR editing tools for long-sequence alterations\(^19\),\(^{21}\).

Bacteriophages evolved enzymes that perform precise recombination\(^25\)\(^{-}\)\(^{30}\). We reasoned that the key enzyme for microbial recombination, the single-strand annealing protein (SSAP), could be useful for cleavage-free gene-editing in mammalian cells. Notably, it does not have DNA cleavage activity\(^26\),\(^27\), thus may not trigger error-prone pathways needed by Cas9 editing. Motivated by this hypothesis and our prior work showing its ability to stimulate genomic recombination, we developed a gene-editing tool using the deactivated Cas9 (dCas9, or catalytically inactive Cas9) and microbial SSAPs\(^31\)\(^{-}\)\(^{38}\) (Figure 1). This dCas9
editor uses the SSAP for knock-in editing with a donor DNA without the need for DNA cleavage. We termed it dCas9-SSAP editor (dCas9-SSAP).

Reagents

SpCas9 Plasmids (to be added in Addgene)

pCas9-MS2-BB_BbsI: pU6-MS2-gRNA-backbone(BbsI)-CBH-SpCas9-T2A-EBFP

pMCP-RecT: pLenti-EF1A-MCP-EXTEN-RecT-NLS

dCas9-SSAP Plasmids (to be added in Addgene)

p-dCas9-SSAP-MS2-BB_BbsI: pU6-MS2-gRNA-backbone(BbsI)-CBH-dSpCas9-T2A-EBFP

pMCP-RecT: Same as above

dSaCas9-SSAP plasmids (to be added in Addgene)

p-dSaCas9-SSAP-boxB-BB_BsaI: pU1a-N22-miniRecT-p2A-dSaCas9

p-dSa-SSAP-template: pU6-boxB-BB-H1-boxB-BB-Right HA-mKate-Left HA

Reagents

T4 Polynucleotide Kinase (New England BioLabs, M0201L)

T4 DNA Ligase Reaction Buffer (New England BioLabs, B0202S)

BbsI-HF (New England BioLabs, R3539L)

T4 DNA Ligase (New England BioLabs, M0202M)

Adenosine 5'-Triphosphate (New England BioLabs, P0756L)

Lipofectamine™ 3000 Transfection Reagent (ThermoFisher Scientific, L3000001)

Equipment
Procedure

A. Design guideRNA sequences at target genomic loci

This step is the same as standard Cas9 experiments. Briefly, based on the Cas9 enzyme used, target sequence (usually 20-bp) near the knock-in or editing sites can be selected next to the protospacer adjacent motif (PAM). For SpCas9 use “NGG” and for SaCas9 use “NNGRRT”. We usually append extra “G” base to the beginning of the guide sequence to facilitate U6/PoI-III transcription initiation if the first base of the guide sequence is not “G”. Two DNA oligos could be ordered based on selected guides, with golden gate cloning overhangs, as shown below.

\[ 5' - {\text{CACCGNNNNNNNNNNNNNNNNN}} - 3' \]
\[ 3' - {\text{CNNNNNNNNNNNNNNNNNNNNNC}} - 5' \]

N denotes the guide sequences. Standard desalting oligos are sufficient for this cloning. The two oligos above will be annealed to form the insert fragments in the next step.

B. Anneal two DNA oligos for each guideRNA target.

Perform phosphorylation and annealing of each pair of oligos via reaction setup below.

oligo1 Top (100 uM): 1 ul
oligo2 Bottom (100 uM): 1 ul
10X T4 ligation Buffer(NEB): 1 ul
ddH₂O: 6.5 ul
T4 PNK (NEB): 0.5 ul
Total: 10 ul

Anneal in a thermocycler using the following parameters:

37 °C 30 min
95 °C 5 min and then ramp down to 25C at 5C/min
C1. Golden Gate Cloning of annealed oligos into sgRNA/spCas9 plasmid

The wild-type spCas9 cloning was similar as our previous protocol\(^{31}\), one guide RNA is needed and the backbone vectors for the cloning will bear BbsI cloning sites matching the annealed oligos from Step B.

The wild-type Cas9 plasmids for this step will be: \texttt{pCas9-MS2-BB\_BbsI} \\

C2. Golden Gate Cloning of annealed oligos into sgRNA/dspCas9 (dCas9-SSAP) plasmid

For dCas9-SSAP using dSpCas9, one or two guide RNAs can be used with double guideRNAs providing slightly better efficiency of editing. The backbone vectors for the cloning will bear BbsI cloning sites matching the annealed oligos from Step B.

The dCas9-SSAP plasmids for this step will be: \texttt{pdCas9-SSAP-MS2-BB\_BbsI} \\

\begin{itemize}
  \item \textbf{Item} \textbf{Volume} \textbf{Note}
  \item Water: 4.3 ul: Add first
  \item Cutsmart Buffer: 0.8 ul
  \item T4 DNA ligase: 0.2 ul
  \item BbsI-HF: 0.4 ul
  \item ATP (25mM): 0.3 ul: ~ final 1mM
  \item dCas9-SSAP-MS2-BB plasmid/vector: 1 ul: ~ 50ng total p-dCas9-SSAP-MS2-BB plasmid
  \item Annealed Oligo (1:10 diluted): 1 ul: diluted 10ul into 100ul
  \item Total: 8 ul
\end{itemize}

After setting up the golden gate reaction (on ice), immediately move the reaction into Thermocycler and perform the Golden Gate reaction using the following parameters:

37 °C 5 min
16 °C 5 min
cycle for ~25 cycles
65 °C 5 min
4 °C hold

After the reaction, perform bacterial transformation as per standard protocol of the competent cells used in the lab immediately.

D. Prepare donor DNA (HDR templates)

Please refer to the associated publication and Figure 2 for examples of template designs. We recommend using a dsDNA template with at least 200bp of homology arms on each end of the insertion/replacement sequences (the edited portion of the template). We suggest cloning the template into simple plasmids such as pUC19, then, restriction digestion of plasmids or standard PCR could be employed for generating large amounts of dsDNA templates.

E. Perform gene-editing via delivery of dCas9-SSAP plasmids and donor DNA

With previous steps, the 3 components of dCas9-SSAP editing method are ready for experiments: the guideRNA/Cas9 plasmid (cloned in step A-C), the donor DNA (from step D), and the SSAP plasmid (pMCP-RecT, can be obtained from Addgene). For delivery into cells in vitro, routine transfection or electroporation could be performed following the recommended conditions by the reagent or equipment manufacturer and selected based on the cell types. For HEK293T cells as an example, a typical transfection condition is described below:

1. One day before transfection, 3E4/well HEK293T cells were seeded on 96-well plates, the cell density should be around 70% on the next day at the time of transfection.

2. For lipofectamine 3000 transfection reagent, use a total of 250 ng DNA + 0.4 ul Lip3000 reagents (ea.) and mixed in 10 ul/well of Opti-MEM, set up the transfection following the manufacturer’s protocol.

3. Transfection material (before the dosage optimization):

dCas9-SSAP guideRNA plasmids, 160 ng (for double sgRNAad design, use equal amount of the two guideRNA plasmids, i.e. 80 ng each); pMCP-RecT or GFP control plasmid, 60 ng;

donor DNA, up to 30 ng, we found 10ng should be sufficient for cell lines.
4. 12-24 hours after transfection, if applicable, could switch to fresh culture media.

5. 3 days post transfection, cells could be harvested or proceed to downstream analysis.

Troubleshooting

Time Taken

Anticipated Results

References


**Acknowledgements**

We thank Dr. Andrew Z. Fire and Dr. Ravi K. Dinesh for their insightful comments and suggestions, the entire Cong and Cleary laboratory for their support and help with proof-reading.
Figures

Figure 1

Schematic model of dCas9 editor with single-strand annealing proteins (SSAP)

Figure 2

Design of knock-in donor with different lengths of transgenes.