PCR-based, cost-efficient and fast screening strategy for sgRNA inserts

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Introduction

Cloning plasmids is time-consuming and costly. Any effort to minimize cloning labor, time, and cost will improve plasmid production and support researchers. The amount of single-guide RNA (sgRNA) cloning in laboratories has increased exponentially since the CRISPR/Cas9 system was implemented into research laboratories around the world. Short sgRNAs are cloned into a plasmid with a promoter and trans-activating crispr RNA in a plasmid (tracrRNA). Cloning includes restriction enzyme-mediated plasmid cutting followed by plasmid gel extraction, and inserting sgRNA in front of the promoter. Following transformation, the efforts begin screening candidates for correct insertion, which necessitates restriction enzyme cutting and Sanger sequencing. Here we present a cost-effective and rapid screening protocol for identifying the right clones. Our optimized protocol, combined with a cost-cutting screening approach, reduces cloning time and overall cost to less than one day.

Results and Methods

Plasmid Cloning

As a starting template, we chose an empty sgRNA vector pRB1017. pRB1017 has the U6 promoter and tracrRNA with two BsaI recognition sites, a type II restriction endonuclease. BsaI cleaves DNA beyond their recognition site, producing 4 nucleotides (nt) overhangs in the plasmid that can be used for cloning. Engler et al 2008 took the advantage of type II restriction endonuclease and introduced a single step cloning method called Golden gate cloning. We first optimized the buffer for sgRNA cloning and found that the T4 ligase buffer works well together with a restriction enzyme and T4 ligase. We designed a 20-nt sgRNA sequence (https://www.benchling.com/) and ordered two sets of 25-nt oligonucleotide primers (Macrogen, South Korea) complementing each other by 21-nt and these oligonucleotides contain 4-nt

sequence overhangs at the 3' ends complementing plasmid overhangs. These two oligonucleotides (OK403; OK404 and 250 pmol/µl each) were placed into 0.2 ml PCR tubes containing 2 µl T4 ligase buffer (Thermo Scientific) and 6 µl ddH$_2$O, and the temperature in the thermocycler was set to 95 °C for 5 minutes, then reduced by 1 °C every 45 seconds until it reached 25 °C (70x) and was kept at 4 °C, allowing the complementary oligos to form double-strand DNA (dsDNA) at their annealing temperature. It is noteworthy that leaving oligonucleotides at room temperature to cool down from 95 °C tends to be less successful as it cools down very quickly.

In the next step, we added template plasmid DNA (1µg), annealed oligos (1µg or 5µg), 0.5 µl BsaI restriction enzyme (Thermo Scientific), 0.5 µl T4 ligase (Thermo Scientific) and 2 µl T4 ligase buffer (Thermo Scientific) and ddH$_2$O into 0.2 ml PCR tubes (total reaction 20 µl) and the reaction tube was held at 37 °C for 1 h, then incubated at 50 °C for 5 min and at 65 °C for 20 min. We later continued to perform a basic bacterial transformation using the final product. In brief, the whole product was immediately added to DH5α chemically competent cells (100 µl) and carefully mixed with DH5α. We added 900 µl of LB into the bacterial cells after heat shock at 42 °C for 2 min and shook them vigorously (230-250 rpm) for 0.30-1 h at 37 °C. The bacterial cells were finally centrifuged at 5000 rpm for 1 min to increase the bacterial density. The supernatant was later discarded up to 100 µl. The bacterial cells were distributed over the LB/Kan+ plate and grown overnight at 37 °C.

Screening strategy for identifying correct clones

The small 21-nt fragment insert in the sgRNA plasmid was screened using polymerase chain reaction (PCR). To test the insert, the readily available reverse oligonucleotide inserted into sgRNA (OK404) and M13 forward primer (OK129) were used instead of developing new sets of primers.

In brief, we performed colony PCRs to identify the colonies that had inserts. A total of 10-12 bacterial colonies were singled out and diluted with 20 µl of ddH$_2$O. Of these, 2 µl were utilized for colony PCRs. Here is the reaction of 25 µl colony PCRs: 1-2µl diluted bacterial colonies, 0.5 µl dNTP, 0.5 µl M13 reverse primer, 0.5 µl forward oligonucleotide, 0.25 µl *Taq* polymerase (Transbio Easy *Taq*), 2.5 µl *Taq* buffer (10X Transbio Easy *Taq* buffer) and 19.75 µl ddH$_2$O. The lid of the thermocycler was set at 105 °C followed by 95 °C for 2 min (1X) and 34X, 95 °C, for

30s, 57 °C for 30 s, 72 °C for 1 min, and 1X 72 °C for 10 min. As a control, an empty sgRNA vector pRB1017 that did not contain the 21-nt insert and cloned sgRNA (OK132) that has different sgRNA sequence were included, and the empty sgRNA vector and cloned sgRNA produced no PCR band around 580 bp, whereas plasmids with the 21-nt insert produced a single band around 580 bp, indicating the insertion of 21 (**Figure 1**). Our experience with this technique has shown that almost all bacterial colonies contain the insert and went on generating a null mutant of *C40H1.3* (an orthologue of human Joubert syndrome associated CEP104) (**Figure 2**). Bacterial colonies with positive bands were cultured and plasmids isolated later on. Our reuse of forward and reverse oligonucleotides for screening lowers total costs and would be helpful to the community.

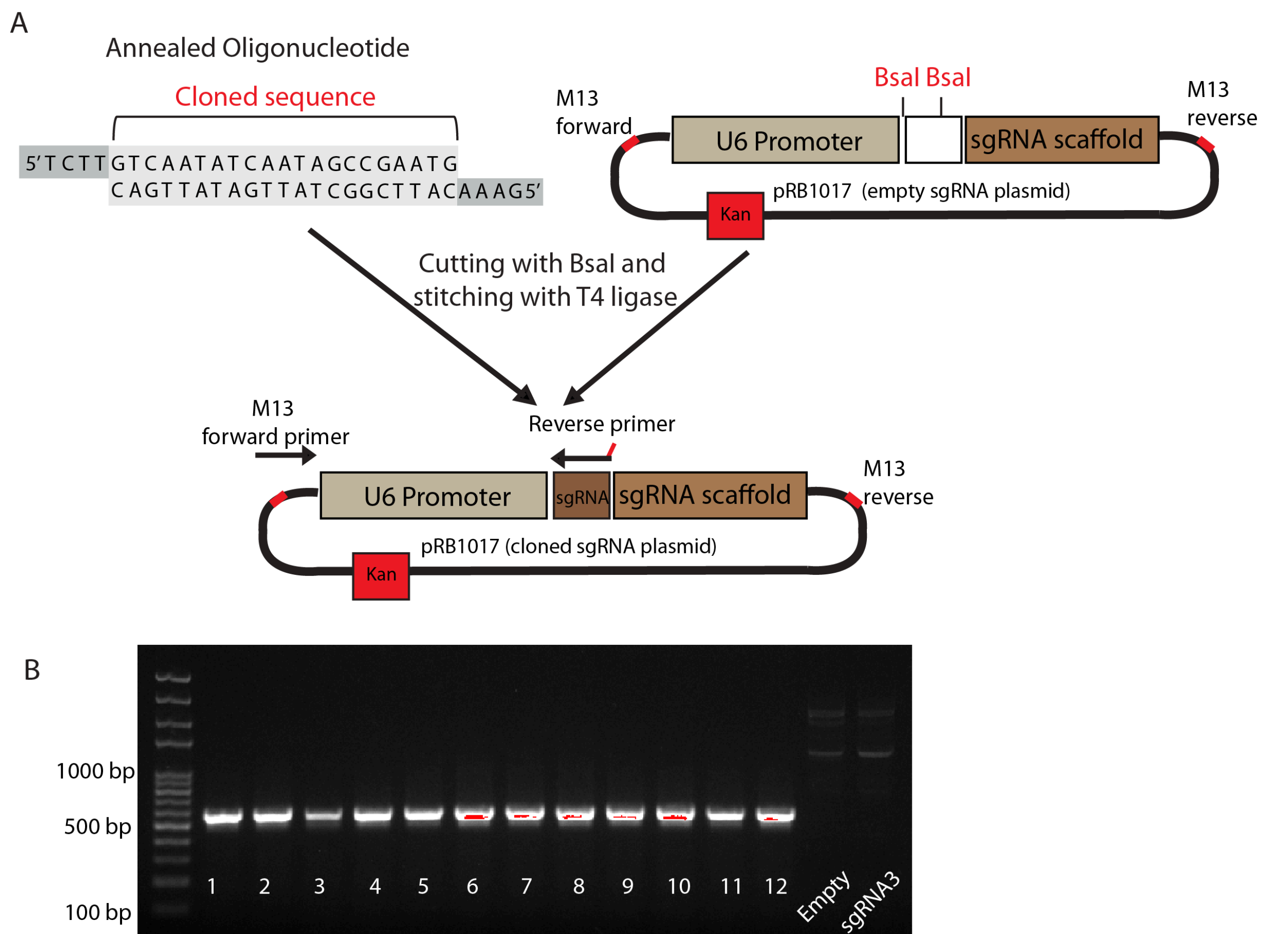

Figure 1: (A) Shown is the schematic workflow of sgRNA cloning and screening strategy. Annealed oligos with overhangs and empty sgRNA vector (pRB1017) containing U6 promoter followed by two BsaI recognition sites and sgRNA scaffold are placed into the cloning reaction buffer containing BsaI and T4 ligase. pRB1017 includes the kanamycin resistance gene (Kan)

and the forward and reverse sites of universal M13. Following transformation, colony PCR was performed using the universal M13 forward and reverse sgRNA oligonucleotide with 4 nt overhang (red). (B) A total of 12 random colonies was chosen, and PCR reactions were carried out. The control included an empty sgRNA expression vector and a cloned sgRNA (OK132: sgRNA3) containing different sgRNA sequences. Each sample was placed into separate wells followed by separation of DNA molecules in 1 % agarose gels. If the 21-nt was successfully cloned into pRB1017, the predicted band size was 580 bp. A strong single band near 600 bp was observed for all randomly selected colonies (1-12) while controls (empty sgRNA and OK132) showed no band around 600 bp, indicating successful cloning of 12 sgRNAs into the plasmid.

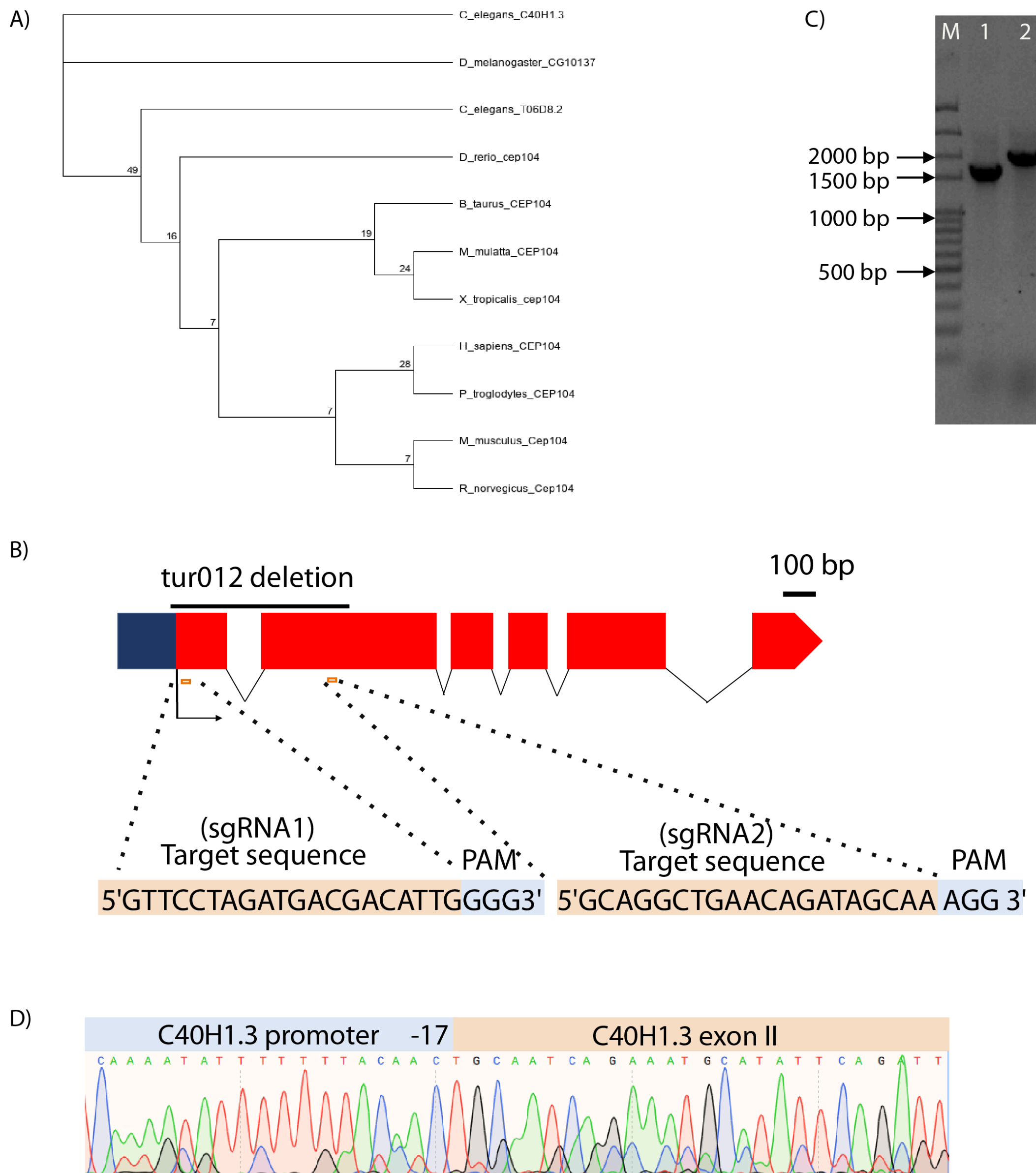

Figure 2. **Generation of a *C40H1.3* (an orthologue of human Joubert syndrome associated CEP104) mutant mediated by CRISPR/Cas9**. A) ConVarT search using human CEP104 protein sequence revealed that *C. elegans* T06D8.2 and C40H1.3 are likely orthologues of human Joubert syndrome associated CEP104. CEP104 amino sequences from *C. elegans* C40H1.3 (NP_499053.1), *C. elegans* T06D8.2 (NP_496400.3), *H. sapiens* CEP104 (NP_055519.1), P troglodytes CEP104 (XP_001152852.1), *M. mulatta* CEP104 (XP_001084851.1), *B. taurus* CEP104 (XP_002694164.1) *M. musculus* Cep104 (NP_808341.1), *R. norvegicus* Cep104 (NP_659550.2), *X. tropicalis*_CEP104 (XP_002937508.2), *D. rerio* cep104 (XP_005167010.1), *D. melanogaster* CG10137

(NP_609981.1) were obtained followed by generation of a phylogenetic tree. Shown is the phylogenetic tree for CEP104, with bootstrap values shown at nodes (high percentage represents support for them). **B)** sgRNA sequences (sgRNA1 and sgRNA2) for CRISPR/Cas9 targeting C40H1.3 are displayed. These two sgRNA were cloned into vectors followed by an introduction into *C. elegans*. F1 screening was performed with PCRs. Homozygous F2 containing 585 bp deletion (some sequences in the promoter and exon I and some sequences in Exon II). The tur012 deletion in C40H1.3 likely results in a null allele of C40H1.3 because of a frameshift. **C)** PCR amplification products of wild type and C40H1.3(tur012) on a 1% (weight/vol) DNA agarose gel; lane L: DNA marker (100 bp plus); lane 1: C40H1.3(tur012); lane 2: wild type **D)** Shown is the Sanger sequencing of a PCR product from C40H1.3(tur012). Sanger analysis revealed a 585-bps deletion that included exon I, intron I, and some exon II sequences, as well as the first 16 base pairs of the C40H1.3 promoter.

Reagents

pRB1017 (empty sgRNA plasmid)

sgRNA3 plasmid (OK132): 5' GCTGTATTTGGAGGTCGTCTG 3' (cloned sgRNA3 sequence)

Primer sequences:

OK129 (M13 forward) : 5' TGTAAAACGACGGCCAGT 3';

OK403 (sgRNA1 forward) : 5'TCTTGTCAATATCAATAGCCGAATG 3';

OK404 (sgRNA1 reverse) : 5' AAACCATTCGGCTATTGATATTGAC 3'

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Author Contributions

Ferhan Yenisert: Conceptualization, Investigation, Methodology, Formal analysis, Writing
Merve Gül Turan. Conceptualization, Investigation, Methodology, Formal analysis, Writing
Sebiha Cevik&Oktay I. Kaplan: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing - original draft.

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