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A Simple PCR-based Strategy for the Introduction of Point Mutations in the Yeast *Saccharomyces cerevisiae* via CRISPR/Cas9

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Abstract

The methods currently employed for *in vivo* site-directed mutagenesis in yeast are laborious and/or inefficient. Recent developments of the CRISPR-based approaches hold great promise for genome editing, but its application in the yeast *S. cerevisiae* remains a time-consuming affair. The rate-limiting step in CRISPR-mediated genetic engineering in yeast is the incorporation of the guide sequences, which target Cas9 to relevant chromosomal locus, into the relevant yeast vectors. Here we present a PCR-based strategy to introduce specific point mutation into the yeast CDC48 gene via CRISPR. Our method eliminates the need for special dam- strain and markedly shortens the elaborate multi-step cloning process, leading to significant savings in time, labor and cost.

Keywords: Yeast; Mutagenesis; Integration; CRISPR; Genome editing

Introduction

The budding yeast *Saccharomyces cerevisiae* serves as a powerful model system with its facile genetics, well-characterized genomics, and a vast array of biochemical assays and tools [1-4]. Yeast research has provided key insights into the mechanisms underlying fundamental cellular processes (e.g., gene expression, DNA repair and proteolysis) and facilitated the development of numerous techniques including DNA transformation, genome manipulation and synthetic biology [1,3,4]. Nevertheless, existing approaches for the incorporation of specific mutations in the yeast genome remain tedious, laborious and inefficient [5-7] as they often require specialized systems (e.g., plasmids or conditions) and/or manipulating selectable markers, which introduces additional sequences that may have unintended effects [6,7].

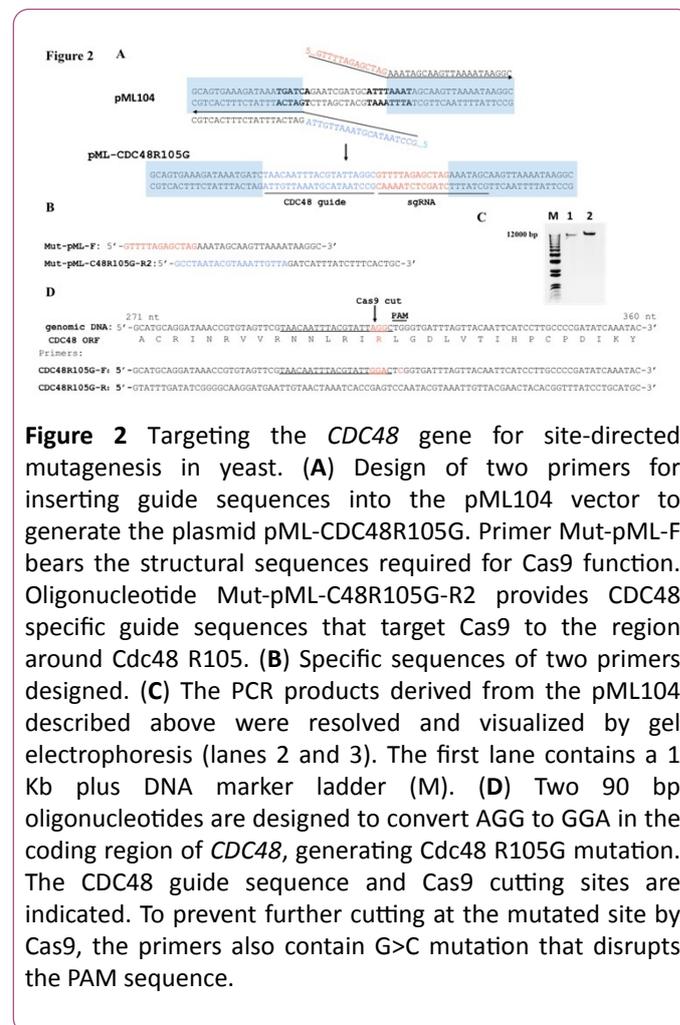
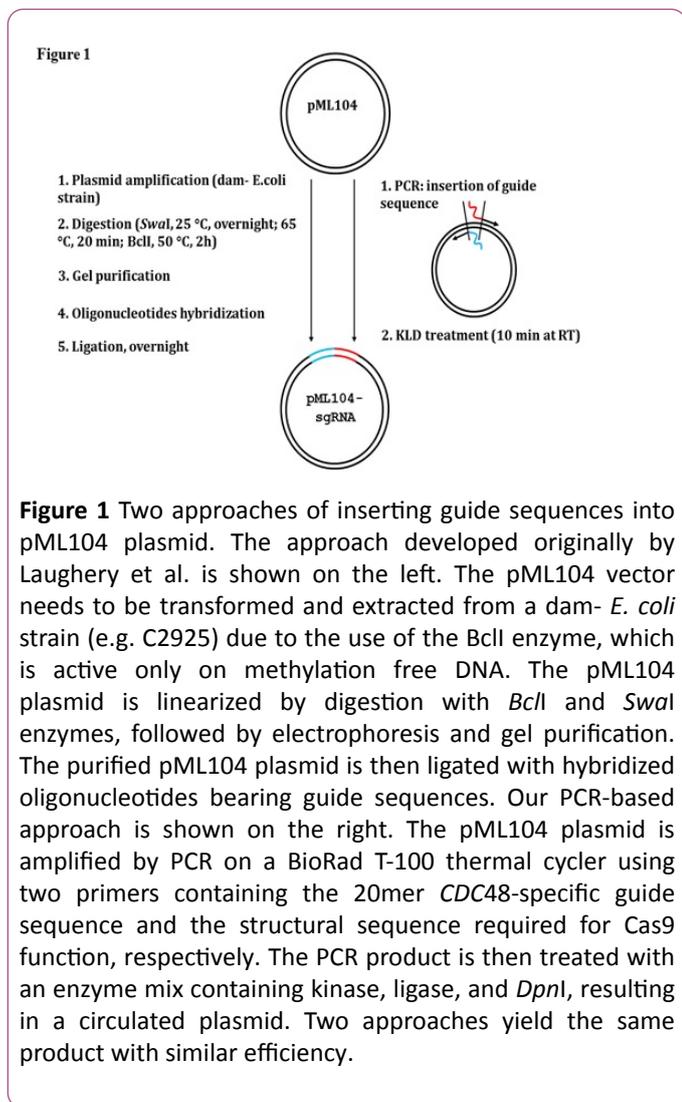
The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system is emerging as a powerful gene-editing tool in various organisms from yeast to human [8-10]. The RNA-guided endonuclease Cas9 can be targeted to specific genomic locus to generate a double-strand break, which allows the introduction of precise mutations by providing a DNA template for homologous repair. With its high efficacy and marker-free exercise, the CRISPR-Cas9 system overcomes many existing limitations and is becoming a preferred method for *in vivo* genome manipulation [9,11-13]. Among several yeast CRISPR approaches [11-13] derived from the work of DiCarlo et al. which first demonstrated the utility of CRISPR in the yeast [9], the protocol developed by Laughery et al. stood out with its higher efficiency and relative simplicity [11]. Nevertheless, one time-consuming step in Laughery's approach involves the cloning of the single-guide RNA (sgRNA), which target Cas9 to relevant chromosomal loci [11], into the vectors (e.g., pML104, pML107) with unique restriction sites (*SwaI* and *BclI*). The strategy developed appeared to be faster, more convenient and efficient than several other CRISPR approaches in yeast [9,11-15], but still requires ~3-4 days to obtain the plasmids with right guide sequences (Figure 1) [11]. As previously described, the vector plasmids need to be transformed first into a dam- *E. coli* strain so that the methylation free plasmid can be digested by *BclI*; and overnight incubation is suggested for subsequent *SwaI* digestion; then the linearized plasmid is purified after gel electrophoresis and ligated overnight with oligonucleotides containing guide sequences (Figure 1) [11]. Then the plasmids constructed can be introduced to yeast for efficient genome editing [9,11].

Materials and Methods

Yeast cultures were grown in rich (YPD) or synthetic media containing standard ingredients. The pML104 derived plasmids were transformed to yeast strain BY4741 (*Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) by standard LiAc based protocol [1,11]. The transformants were spread onto synthetic plates lacking uracil and grown for 3 days at 30°C. Colonies were

inoculated in 3 ml YPD medium and grown overnight. The genomic DNA was extracted for PCR amplification of the region surrounding *CDC48*, which was then analyzed by sequencing to ascertain the incorporation of R105G mutation. Oligonucleotides were obtained from the Integrated DNA Technologies (Skokie, IL). PCR conditions are detailed below.

mix. The PCR reaction was performed as follows: an initial denaturation step at 98 for 1 min, followed by 25 cycles of denaturation at 98 for 10 s, annealing at 55 for 30s, and extension at 72 for 8 min; a final extension step of 5 min at 72. PCR products were resolved by gel electrophoresis to confirm the amplification (**Figures 2C and 2D**).



Results and Discussion

To generate *Cdc48* R105G mutant, two primers were designed for inserting relevant guide sequences to target Cas9 specifically to the region around *CDC48* R105. Primer Mut-pML-F (i.e. 5'-GTTTATAGAGCTAGAAATAGCAAGTTAAAATAAGGC-3') contains the 20 nucleotides (unlined) of the structural element required for Cas9 function (**Figures 2A and 2B**). Primer Mut-pML-C48R105G-R2 (i.e. 5'-GCCTAATACGTAATTGTTAGATCATTATCTTCACTGC-3') bears the 20mer guide sequence of *CDC48* (**Figures 2A and 2B**). These two oligonucleotides were used to PCR-amplify the plasmid pML104 using the Q5 site-directed mutagenesis protocol (New England Biolabs) with minor modifications. Specifically, in a 25 l PCR reaction, 8 ng of pML104 plasmid was mixed with 15 M of each primer and 12.5 l of 2x Q5 master

The PCR products were then incubated with the mixture containing kinase, ligase and *Dpn*I enzymes provided in the Q5 site-directed mutagenesis kit (**Figure 1**), which promotes rapid circularization of the PCR products and removal of the template DNA. The reaction was performed at room temperature for 10 min and contained 2 l PCR products, 5 l 2x KLD reaction buffer, 1 l KLD enzyme mix, and 2 l nuclease-free water. Then it was transformed into competent *E. coli* cells C2987. Plasmid DNA was extracted from the resulting colonies and sequenced by T3 primer. Typically >60% colonies contained right inserts.

The resulting plasmid pML104-CDC48R105G could bring the Cas9 nuclease to the region surrounding R105 of *Cdc48* to make a cut (**Figure 2D**). To generate *Cdc48* R105G substitution, two 90 bp oligonucleotides were designed to replace AGG with GGA, and the protospacer-adjacent motif (PAM) was also disrupted by replacing TGG with TCG without changing amino acid (**Figure 2D**), which prevents subsequent Cas9 cutting in newly generated mutant cells. The plasmid pML104-

CDC48R105G and hybridized double-stranded oligonucleotides CDC48R105G-F/CDC48R105G-R were co-transformed into yeast strain BY4741 and selected on plates containing synthetic complete media lacking uracil (SD-URA) [11]. Genomic DNA was then extracted from the resulting colonies and sequenced. Over 50% of colonies harbored CDC48 R105G mutation.

Conclusion

Here we have demonstrated an efficient PCR-based procedure for precise genome editing of yeast cells. This revised method retains high efficiency but eliminates the need of the dam- strain and markedly shortens the elaborate multi-step cloning process, which includes overnight restriction digest and ligation, in creating the sgRNA targeting plasmid [11]. To incorporate sgRNA sequences to the pML104 plasmid, it takes ~5 hours with our modified method instead of ~3-4 days with the approach originally developed by Laughery et al. (Figure 1) [11]. The modifications introduced lead to significant improvements in substantial time, labor and cost savings. Our approach can be easily adapted for other organisms and may facilitate high-throughput generation of CRISPR-based genome engineering.

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Author Contributions

G.H., H.C. and X.G. carried out the experiments. All authors analyzed the data. H.C. conceived and designed the experiments with inputs from H.R. H.C. and H.R. wrote the paper with the input from all authors.

Competing Interests Statement

The authors declare no competing interests.

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