Allele-Specific Gene Deletion using High Fidelity Overlap PCR Technique

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INTRODUCTION

The completed genome sequence for the human fungal pathogen *Cryptococcus neoformans* (Duke University *Cryptococcus neoformans* H99 genome sequencing project) has provided a powerful resource for identifying genes involved in pathogenesis. To take full advantage of the genomic sequence, new techniques have been sought to develop allele-specific gene deletion constructs, which previously had to be created using tedious and time consuming cloning techniques.

The polymerase chain reaction overlap technique (PCR-OL), which has been used extensively to create allele-specific mutants in *Saccharomyces cerevisiae* and the human pathogen *Candida albicans*, was adapted for use in *C. neoformans* and has proven to be an effective technique for rapidly developing allele-specific deletion constructs (Davidson *et al*.). A limitation of the PCR-OL technique, with regard to its application to *C. neoformans*, was that a limited number of polymerases were available that could amplify high yields of the final fusion product. We evaluated the ABgene® Extensor Hi-Fidelity PCR Enzyme Mix for use in the construction of allele-specific *C. neoformans* deletion constructs, using PCR-OL, and found that the Extensor Hi-Fidelity PCR Enzyme Mix provided excellent amplification yields with the added benefit of proof-reading activity.

PCR-OL METHOD

PCR-OL was performed using the Extensor Hi-Fidelity PCR Enzyme Mix to create a *C. neoformans CCP1* gene deletion construct. The first step of PCR-OL involved the amplification of two ~1kb products, homologous to regions flanking the 5' and 3' region of the *CCP1* allele, plus a selectable marker, in this case *NAT*. PCR reactions were performed according to the manufacturer's instructions using *C. neoformans* H99 genomic DNA as the template and the primer pairs OL-1 and OL-2 to generate the left overlap arm and OL-3 and OL-4 to amplify the right overlap arm. Primers OL-2 and OL-3 contained 20 nucleotides at the 5' end of each oligo that were homologous to the 5' and 3' sequence of *NAT*, respectively (Figure 1, Step 1).

The selectable marker *NAT* was amplified using the primer pair OL-5 and OL-6. Primer OL-5 contained 20 nucleotides at the 5' end of the oligo that were homologous to the 3' sequence of the left arm, and primer OL-6 contained 20 nucleotides at the 3' end that were homologous to the 5' sequence of the right arm (Figure 1). The amplified products were separated on a 0.8% agarose gel and purified using the Qlinquick gel purification kit (Figure 2A). The fusion reaction, or the second step of PCR-OL, was performed using the purified amplification products from step 1 as template and the primer pair OL-1 and OL-4. PCR was performed using the Extensor Hi-Fidelity PCR Enzyme Mix according to the manufacturer's instructions with the following exception: the time required for template amplification was determined by multiplying the length of the final product by 1.5 minutes.

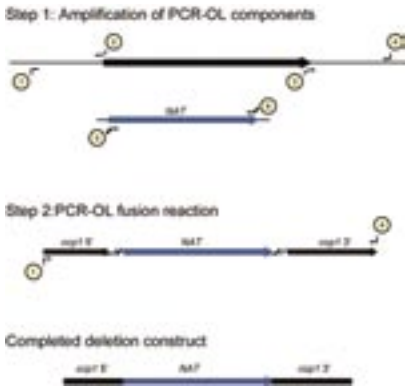

Figure 1: PCR-OL is a two-step technique that can be used to rapidly create allele-specific deletion constructs. In step 1, the selectable marker (*NAT*) and regions of genomic DNA with homology to the gene of interest were amplified. In step 2, the three components were fused using the primer pair OL-1 and OL-4.

RESULTS

The Extensor Hi-Fidelity PCR Enzyme Mix provided high yields of the individual PCR-OL components as well as the desired fusion product (*ccp1::NAT*). Figure 2A illustrates the successful amplification of the individual PCR-OL components. The individual components (Step 1) were gel purified and 2.5µl of the combined amplified products were used as template for the PCR-OL fusion reaction (Step 2). Lanes in figure 2B were loaded with 25µl of amplified product from two separate fusion reactions. The desired fusion product *ccp1::NAT* (3.4 kb) was then gel purified, the DNA was concentrated by speed-vac, and the construct was delivered to *C. neoformans* strain H99 by biolistic transformation, as described by Toffaletti *et al*.

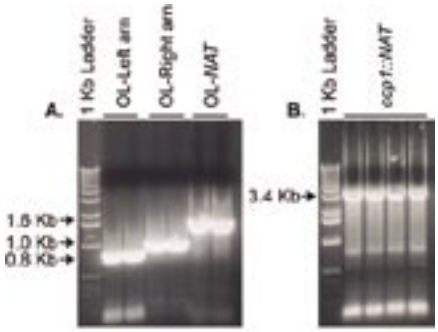

Figure 2: PCR-OL construction of *ccp1::NAT*. (A) Step 1, amplification of the three PCR-OL components. (B) Step 2, amplification of the *ccp1::NAT* construct.

CONCLUSIONS

The Extensor Hi-Fidelity PCR Enzyme Mix provided excellent amplification yields of the desired PCR-OL deletion construct (*ccp1::NAT*), which was used to successfully delete the *C. neoformans CCP1* allele.

To illustrate the impact of this technique on time to completion, steps 1 and 2 of the PCR-OL reaction were completed within one work day and the deletion construct was delivered by biolistic transformation into *C. neoformans* wild-type stain H99 cells the following day. Transformants were observed after 4 days of incubation, which is standard, and mutants were identified genotypically on the 7th day by performing colony PCR. Thus, we were able to go from the creation of an allele-specific deletion construct to the identification of a *ccp1* mutant in only 7 days.

REFERENCES

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Toffaletti, D. L., T. H. Rude, S. A. Johnston, D. T. Durack, and J. R. Perfect. 1993. Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. J Bacteriol 175:1405-11.

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