

# Rapid PCR<sup>1</sup> Sequencing of Plasmid DNA Directly from Colonies of *Saccharomyces cerevisiae*

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<sup>1</sup>The polymerase chain reaction (PCR) process for amplifying nucleic acid is covered by U.S. Patent Nos. 4,683,195 and 4,683,202, assigned to Hoffmann-La Roche. Patents pending or issued in other countries.

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*We describe a method to obtain sequence directly from DNA isolated from yeast transformants using the fmol™ DNA Sequencing System. This method decreases the time to determine sequence results from 3 days to approximately 24 hours. Critical parameters of this procedure are also discussed.*

## Introduction

Current methods of sequencing plasmid DNA harbored in yeast colonies involve isolation of yeast DNA from an overnight culture, subsequent transformation of that DNA into *E. coli*, and preparation of plasmid DNA suitable for sequencing from the *E. coli* transformants. This procedure requires several days to obtain sequence data. A method has been reported previously for the direct sequencing of amplified plasmid DNA from *E. coli* colonies (1). However, we have found this technique inapplicable to yeast colonies due to either: a) the rigidity of the yeast cell wall which prevents adequate lysis and subsequent access of primers to the DNA, or b) inhibition of PCR-mediated amplification of vector DNA by component(s) in yeast colony lysates. We report here a rapid method of amplifying and sequencing plasmid DNA directly from yeast transformants utilizing the polymerase chain reaction and Promega's *fmol* DNA Sequencing System.

## Preparation of DNA from yeast colonies for sequencing

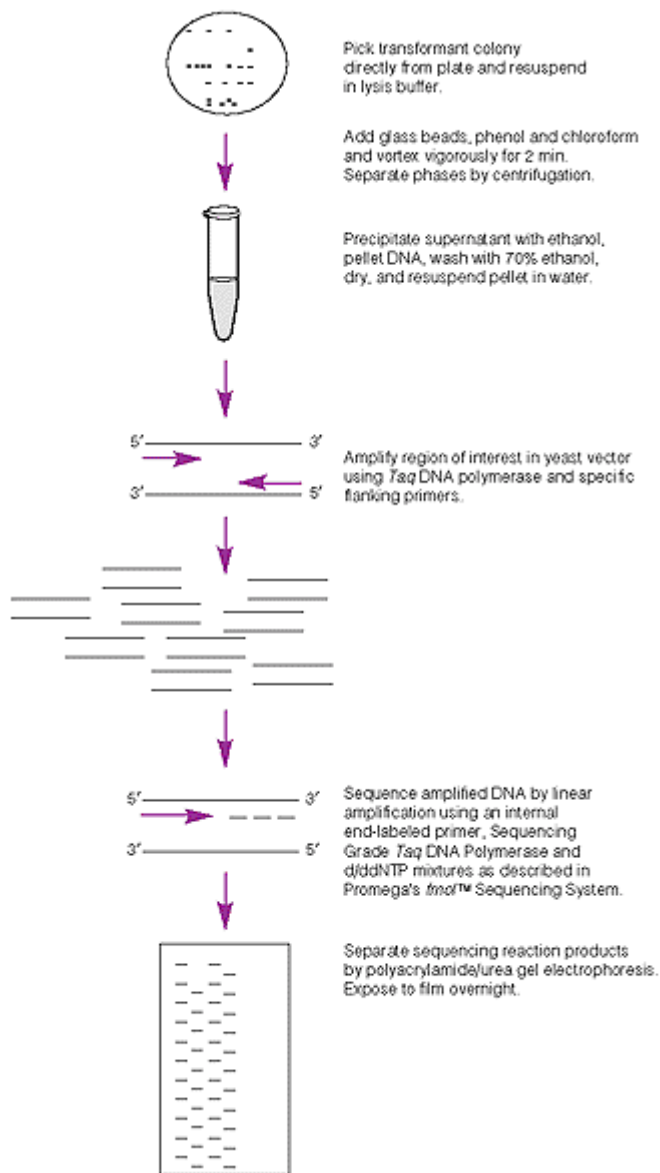
**Figure 1** summarizes the steps to isolate and sequence DNA directly from yeast colonies. We present the details of these reactions below which were used to screen oligo-directed mutants of the mating pheromone **a-factor** in yeast.

For each mutant screened, a single yeast colony containing plasmid DNA was picked directly from a plate, resuspended in 10µl of H<sub>2</sub>O, mixed well with a pipette and treated according to the following modification of the procedure of Ward (4). The yeast suspension was added to a microcentrifuge tube containing 100µl of 2.5M LiCl, 50mM Tris-HCl, pH 8.0, 4% Triton® X-100, 62.5mM EDTA. An equal volume of phenol:chloroform was added along with 0.2g of glass beads (0.45-0.50mm). This mixture was vortexed well for 2 minutes and separated by centrifugation for 1 minute at 12,000 x g in a microcentrifuge. The resulting supernatant was transferred to a fresh microcentrifuge tube, and the DNA

was precipitated with 2.5 volumes of ethanol at -20°C for 20 minutes. Following centrifugation for 15 minutes at 12,000 x g at 4°C, the nucleic acid pellet was washed with cold 70% ethanol, dried under a vacuum for 2 to 5 minutes, then resuspended in 20µl of H<sub>2</sub>O. We find that the 70% ethanol wash is essential to remove contaminants which interfere with the subsequent amplification reactions.

The yeast nucleic acid solution was heated at 94°C for 10 minutes and then immediately placed on ice to facilitate the dissociation of DNA strands and aggregates, providing the amplification primers better access to the target DNA sequence. Amplification of target sequences was performed by mixing 5µl of a 10X reaction buffer (500mM KCl, 100mM Tris-HCl, pH 9.0, 1% Triton X-100) with 1µl of the yeast nucleic acid preparation. This solution was overlaid with 2 drops of mineral oil, placed in a thermocycler and held at 94°C for 15 minutes. The remaining amplification reagents were combined and then directly added through the oil overlay: 0.5µM of each primer (designed to flank the region of interest), 50µM dNTPs, 1.5mM MgCl<sub>2</sub>, 2.5 units AmpliTaq® DNA Polymerase and sufficient H<sub>2</sub>O to bring the final volume of the reaction to 50µl. The samples were subjected to 30 cycles: 94°C for 1 minute, 50°C for 1 minute (annealing) and 72°C for 2 minutes (extension). The annealing temperature will vary accordingly with different primer sets.

We have observed that using larger amounts of the yeast nucleic acid in these reactions inhibits amplification. The procedure also incorporates a "hot start." This "hot start" method prevents false priming in the initial stages of amplification, reducing backgrounds observed in direct sequencing reactions that follow.



1.

**Figure 1. Rapid sequencing of plasmid DNA from yeast colonies.**

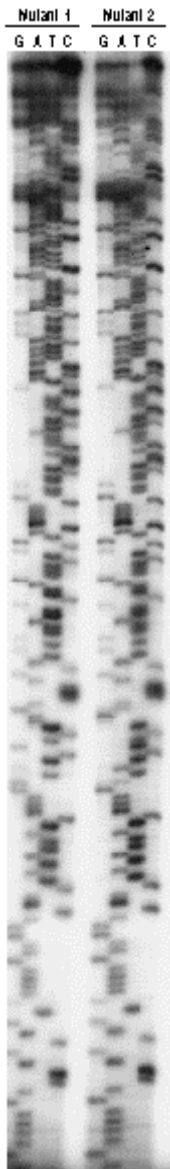
## Sequencing amplified yeast DNA with fmol DNA Sequencing System

Ten picomoles of a third primer, internal to those used for amplification, were end-labeled with  $^{32}\text{P}$  using T4 polynucleotide kinase according to Sambrook *et al.* (3) and were used in the linear amplification of template DNA by dideoxy sequencing using Promega's *fmol* DNA Sequencing System and Sequencing Grade *Taq* DNA Polymerase<sup>2</sup> (supplied). Sequencing reactions were performed according to the manufacturer's instructions (2) using only 1  $\mu\text{l}$  of the amplified yeast template DNA. Usually no further purification of the amplified nucleic acid is required.

<sup>2</sup> U.S. Patent No. 5,108,892 has been issued to Promega Corporation for the use of a modified *Taq* DNA Polymerase to determine DNA sequence and amplify DNA sequence.

A number of sequencing reaction parameters are critical to success. First, increased amounts of the amplified yeast template tend to elevate background detected in the autoradiograms. In addition, a heterogeneous amplification product resulting from the initial amplification also may produce high backgrounds. If this situation arises, sequencing must be performed following gel purification of the amplified yeast sequences. We also do not recommend use of the initial amplification primers as the end-labeled sequencing primer without gel purification of the amplified yeast sequences. Unlabeled primers compete in the sequencing reaction resulting in decreased signal.

**Figure 2** presents the sequencing results of two yeast **a**-factor mutants using this method and demonstrates the quality of the sequence data obtained using the described procedure. Following the above protocol on a homogeneous amplification product, the quality of sequence data is excellent and can be obtained in a single day.



**Figure 2. Results from two yeast mutants using the rapid direct sequencing method.** Individual mutants generated by oligo-directed mutagenesis of the **a**-factor gene were sequenced according to procedures detailed in this article.

## Summary

Direct sequencing of plasmid DNA from yeast transformants using this method allows for rapid screening at the sequence level. The complete time for such a procedure has been decreased from several days to one day, and the method is now amenable to processing of many samples simultaneously. We have successfully employed this method for the analysis of random oligonucleotide-targeted mutations in the gene encoding the yeast mating pheromone **a**-factor to study the functional consequences of structural alterations in this peptide. This technique facilitates the direct analysis of colonies containing vectors expressing mutated DNA fragments identified by phenotypic screens, as well as library inserts, gene disruptions and other yeast-related vector constructs.

## References

1. Hofmann, M.A. and Brian, D.A (1991) *BioTechniques* **11**, 16.
2. *fmol DNA Sequencing System Technical Manual*, TM022, Promega Corporation
3. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY p.10.66.
4. Ward, A.C. (1990) *Nucleic Acids Res.* **18**, 5319

## Acknowledgments

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## Ordering Information

Product	Cat.#
----- <i>fmol</i> <sup>™</sup> DNA Sequencing System -----	Q4100

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