

# Direct sequencing of PCR products with degenerate primers

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*Degenerate primers were used to amplify a Schizosaccharomyces pombe homolog of transcription elongation factor SII. This report describes the use of degenerate sequencing primers with the fmol™ DNA Sequencing System for sequence verification of the PCR amplification products.*

## Introduction

The polymerase chain reaction (PCR)\* is used by numerous investigators to amplify and clone DNA fragments. A popular use of PCR is the isolation and cloning of genes by their homology to related genes. To accomplish this, degenerate primers are synthesized which correspond to conserved amino acid sequences in the related genes. These primers are then used to amplify sequences from heterologous DNA with the goal of recovering a homologous gene of interest. One complication of this approach is that amplification with degenerate primers frequently yields multiple amplification products. These products must be separated and their sequence verified to identify the DNA fragment of interest. Sequencing also verifies that the correct product has been amplified.

\* The polymerase chain reaction (PCR) process for amplifying nucleic acid is covered by U.S. Pat. Nos. 4,683,195 and 4,683,202 assigned to Hoffmann-La Roche. Patents pending in other countries.

## Problems with sequencing cloned PCR products

A common approach to sequencing PCR products involves cloning into an appropriate vector. The cloning is complicated by the presence of ends which are incompatible for efficient ligation of the amplified fragment without using further manipulations or specialized cloning vectors (1-3). When multiple amplification products are generated, several products must be cloned and sequenced. In addition, multiple clones of each PCR product must be analyzed to obtain a consensus sequence, since misincorporation by *Taq* DNA polymerase can give rise to individual clones with varying sequences (4).

## Direct sequencing of PCR products

In contrast, a representative sequence is obtained from direct sequencing of a population of PCR products. Direct sequencing of PCR product by the Sanger method (5) is complicated due to reannealing of the linear double-stranded templates. This problem can be avoided by performing asymmetric PCR or strand capture using biotinylated oligonucleotides (6,7). While these approaches avoid reannealing problems by generating single-stranded template, they require additional optimization steps or customized DNA primers.

Direct sequencing of double-stranded PCR products with the *fmol* DNA Sequencing System offers several advantages. The elevated temperatures and multiple denaturation cycles reduce problems of template reannealing, secondary structure and false priming. False priming is commonly observed when using degenerate primers. Furthermore, the linear amplification procedure permits sequencing of small amounts of template DNA ( $\geq 4$ fmol). The reactions are quickly assembled and placed in a thermal cycler, eliminating the separate annealing, labeling and termination steps needed for conventional sequencing strategies.

## Methods

### PCR Amplification

Two degenerate primer pairs (Table 1) were used to amplify DNA segments from *Schizosaccharomyces pombe* genomic DNA which were potentially from the transcription elongation factor SII gene homolog. Amplification conditions consisted of 20mM Tris-HCl, pH 7.5, 50mM KCl, 3.5mM MgCl<sub>2</sub>, 0.1% Triton X-100, 0.8mM dNTPs, 100pmol of each primer and 2.5u *Taq* DNA polymerase (Perkin-Elmer Cetus). Thirty cycles were performed as follows: for the 440bp product, 94°C, 1 minute; 55°C, 2 minutes; 72°C, 3 minutes; and for the 108bp product, 94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute. The DNA amplification products were separated by electrophoresis on a low melting agarose gel, phenol extracted and ethanol precipitated.

**Table 1. Degenerate Primers Used for PCR Amplification and DNA Sequencing.**

Primer	Length	Sequence	Degeneracy	PCR Product
1	20-mer	5'- GCT/CGCT/CGCT/CTTT/CAGAACT/CGG -3'	32-fold	440bp
2	19-mer	5'- CA/GACA/GAAA/GGTA/GGTCATT/AGG -3'	32-fold	440bp
3	20-mer	5'- GCIGAT/CGAA/GCCTATGACA/TAC -3'	8-fold	108bp
4	20-mer	5'- AAT/CTTCCAICG/TA/GTTICCA/GCA -3'	16-fold	108bp

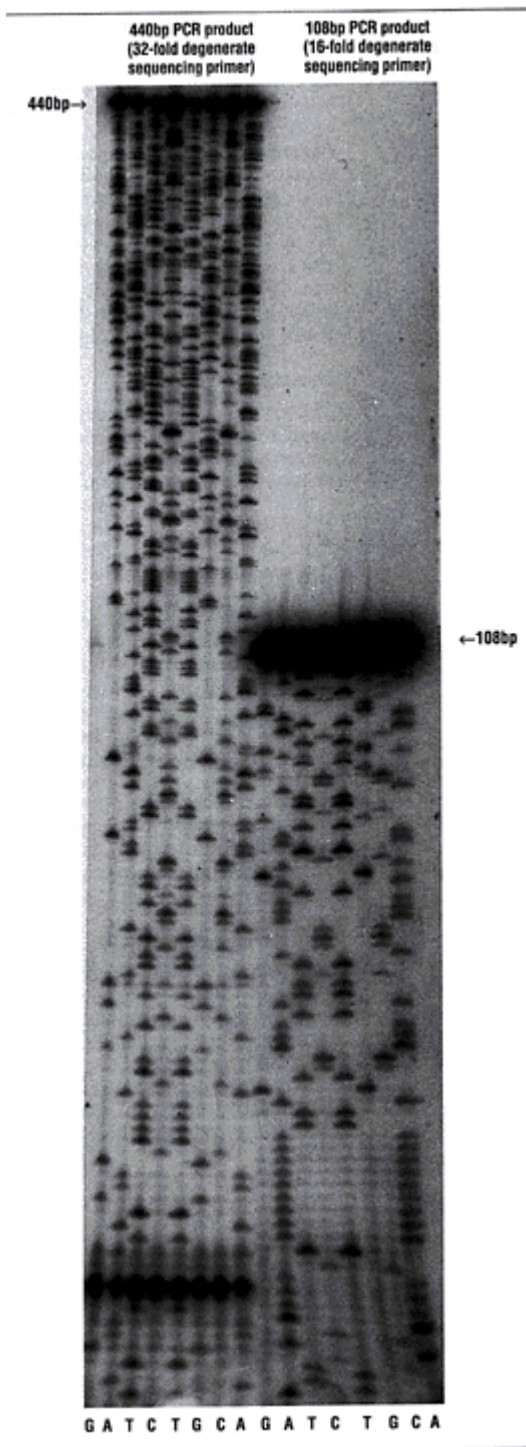
I = Inosine.

### *fmol* DNA Sequencing

DNA sequencing was performed as described in the *fmol* DNA Sequencing System Technical Manual (8), but using degenerate [ $\gamma$ -<sup>32</sup>P]ATP end-labeled, primers. The cycling profile consisted of an initial 95°C denaturation for 2 minutes followed by 30 cycles of 95°C, 30 seconds (denaturation); 55°C, 30 seconds (primer annealing); and 70°C, 60 seconds (primer extension). The sequencing ladder was separated on a 6% polyacrylamide/7M urea sequencing gel, dried and exposed to X-ray film for 6.5 hours (Figure 1).

## Sequence data quality with degenerate primers

As seen in [Figure 1](#), high quality sequence data was obtained from direct sequencing of the PCR products using 16- and 32-fold degenerate primers. Sequence data was observed from bases adjacent to the primer and extended to the end of the template. In this experiment, a single product was amplified by each of the degenerate primer pairs. However, the DNA sequence of only the 108bp product indicated that it was amplified from the gene of interest, transcription elongation factor SII.



**Figure 1. *fmol* DNA Sequencing reactions using end-labeled, degenerate primers.** The 440bp PCR product was amplified from *S. pombe* genomic DNA with primers 1 and 2 (Table 1), as described in the text. The 108bp PCR product was amplified with primers 3 and 4. For convenience, a 1ml volume (50pmol) of each product was used for sequencing. Left panel, 440bp product sequenced with <sup>32</sup>P-labeled primer 1; right panel, 108bp product sequenced with <sup>32</sup>P-labeled primer 4. Arrows indicate the ends of the PCR templates.

## Annealing temperature

The annealing temperature is the most important consideration for thermal cycle sequencing of PCR products made with degenerate primers. Because *Taq* DNA polymerase extends primer sequences at low annealing temperatures (9), mismatched primers are stabilized prior to reaching the dissociation temperatures in the primer extension step. Therefore, the highest possible annealing temperature should be used to prevent nonspecific primer annealing. As a general guideline, primers <24 bases, or with a GC-content ≤50%, require annealing temperatures of 42°-70°C. A combined annealing/extension step at 70°C may be used for primers ≥24 bases. When the appropriate annealing temperature is not known, first try the annealing temperature used in the amplification reaction. In subsequent experiments, the annealing temperature may be increased to improve the quality of the sequence data.

## Sequencing template purity

Sequence quality also is dependent upon the purity of the amplification product. Degenerate primers commonly yield multiple PCR products. Multiple templates result in superimposed sequencing ladders, preventing accurate base calling. Therefore, it is essential to purify the products of interest prior to DNA sequencing.

## Primer degeneracy

An additional consideration is the level of primer degeneracy. Highly degenerate primers increase the likelihood of nonspecific primer annealing and reduce the molar concentration of the correct primer. The reduced molar concentration may be partially overcome by increasing the primer concentration. However, excessively high primer concentrations frequently increase the level of nonspecific annealing.

## Summary

The *fmol* DNA Sequencing System offers several advantages for sequencing PCR amplification products using degenerate primers. The high temperatures employed during the sequencing reaction increase the stringency of primer annealing, minimizing problems of false priming. The elevated thermal cycling temperatures also reduce secondary structure and strand reannealing. These advantages permit the direct sequencing of PCR products without the requirement for fragment cloning.

## References

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## Ordering Information

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

The system contains sufficient reagents for 100 sets of sequencing reactions.

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