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EMERGING TECHNIQUES

Direct Sequencing of PCR Products Using the Maxam–Gilbert Method

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Direct sequencing of polymerase chain reaction (PCR) products by using the Maxam–Gilbert method is described. In this method, one of the primers is end labeled. Thus it is possible to sequence the reaction product directly following purification using this chemical method.

Introduction

Recent methods for direct sequencing of PCR products have used the Sanger dideoxy sequencing method. Several protocols for double-stranded sequencing have been reported [1], as well as methods for generating single-stranded templates using enzymatic modification [2] or unequal forward to reverse primer ratios (asymmetric PCR) [3]. Using PCR to investigate processing of clathrin light chain mRNAs, we routinely see multiple PCR products, reflecting different alternatively spliced forms. Therefore, asymmetric PCR prior to sequencing necessitates separation and reamplification of the different DNA fragments. In order to facilitate discrimination of different PCR products on agarose gels we have chosen primers that yield various small products (150–220 bp) and thereby increase their relative size differences. However, we found that direct double-stranded enzymatic sequencing gives unsatisfactory results with these small fragments, possibly because template reannealing may be favored over primer/template annealing. Another application where direct sequencing using the Maxam–Gilbert method is advantageous involves PCR products made with degenerate primers. Often, poor results are obtained if degenerate primers are used in dideoxy sequencing, due to mispriming. Therefore, we have devised a simple method that does not require an additional enzymatic step (lambda exonuclease [2]), reamplification, or an exact primer match to generate DNA fragments for Maxam–Gilbert sequencing.

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Methods

Primer Preparation

It is convenient to carry out primer labeling and the PCR reaction in the same 0.5 ml microcentrifuge tube. One of the primers is labeled with γ-^{32}P-ATP in a 30 µl reaction using the amount of primer (2.5–25 pmol) for one "cold" reaction, 10 U T4 polynucleotide kinase, 15 µl γ-^{32}P-ATP (3000 Ci/mmol, 10 mCi/ml), and 3 µl 10× kinase buffer (700 mM Tris–HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT). The reaction is carried out at 37°C for 30 minutes. The primer is precipitated by adding 6 µl of 10 M NH_4Ac, 350 ng tRNA (Boehringer), and 75 µl ethanol. After 30 minutes at −80°C the primer is centrifuged for half an hour in a microcentrifuge, washed with 80% ethanol, and dried. Glycogen (Boehringer) may alternatively be used as a precipitation aid for the labeled primer. It is crucial to use NH_4Ac instead of NaAc since NaAc seems to interfere with the PCR reaction.

PCR Reaction

To perform the PCR reaction a mix containing the second cold primer is added to the tube and the reaction is carried out under the same conditions used in the "cold" reaction (the conditions when two unlabeled primers are used).

Separation and Sequencing

The PCR products are separated on a 2% agarose gel (SeaKem in 50 mM Tris, 50 mM Boric acid, and 1 mM EDTA [TBE]), and the bands are excised and electroeluted using an elutrap (Schleicher and Schuell) according to the manufacturer's instructions. This labeled DNA (5000 cpm/µl) can be used for Maxam–Gilbert sequencing [4]. The chemical modification and the cleavage is performed as described [5], except that removal of the piperidine is done by *n*-bu-

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Figure 1. Sequence ladder generated by direct sequencing of a DNA fragment generated with a 68-fold degenerate 20-mer and a nondegenerate 17-mer T7 primer. The degenerate primer was labeled. One million plaque forming units of a mouse brain cDNA library were without further treatment amplified in a 50 µl PCR reaction containing 2.4 mM $MgCl_2$, 10 mM Tris pH 8.3, 50 mM KCl, 0.01% w/v gelatin and 10 pmol of each primer. Thirty-five cycles were used; each cycle was: 30 seconds at 94°C, 1 minute at 55°C, 2 minutes at 72°C. One of the main bands was eluted and sequenced. The 10% sequencing gel was dried and exposed for 3 days to Kodak X-AR5 using an intensifying screen.

tanol concentration [6]. The labeled PCR fragments are sufficient for three to four sets of chemical reactions.

Summary

This method describes rapid direct sequencing of small PCR products that might be difficult to sequence with previously described methods. It was used for a number of primer combinations and PCR buffer conditions, for example, to analyze differentially spliced clathrin light chain forms and to screen a brain cDNA library for nerve growth factor related cDNAs using degenerate primers (Figure 1).

While this paper was under review a similar paper describing direct chemical sequencing of PCR products was published [7]; however, the experimental details are different.

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