HPLC purification of PCR products for direct sequencing

The usefulness of sequence data derived from cloned PCR products may be limited by cloning artefacts and the cloning of minor sequence variants (e.g. *ex vivo* HIV). Direct sequencing of PCR-amplified fragments avoids these problems, and can also clarify where artefacts may have been introduced by Taq polymerase. However, PCR products larger than 200 bp cannot usually be sequenced directly without further purification.

Such PCR products may be purified by gel electrophoresis. Alternatively, products can be purified by ethanol precipitation or by using microconcentrator columns (e.g. Centricon™) which, although fast and very popular, may not yield consistent results; moreover, neither of these methods can separate individual PCR products.

It is possible to purify PCR products by HPLC[1]. We have adapted this technique to give a complete strategy for direct sequencing of PCR products that yields reproducible high-quality sequence data and uses the same primers as the PCR amplification. The method is fast (25 min), can purify several PCR products simultaneously and allows direct sequencing of products up to 500 bp. Standard PCR protocols can be used, and both radioactively or fluorescently labeled nucleotides are suitable for use during sequencing.

We use a HPLC system (ABI) consisting of two pumps, an injector/mixer and a diode array (ABI-1000s). The column used was a Perkin-Elmer PE TSK DEAE-NPR. Solutions A and B were prepared as described[1], but were filtered through a 0.22 μm membrane and then sterilized. We used a slightly modified gradient: 25% A, 0–0.1 min; 25–45% A, 0.1–1.0 min; 45–55% A, 1–10 min; 55–75% A, 10–25 min. The column was cleaned afterwards using 75–100% A, 25–26 min and kept at 100% A 26–36 min, then A was reduced to 25%, 36–37 min and kept at 25%, 37–47 min. The PCR product was applied to the column in MilliQ™-quality water. For peak resolution, it is important that during the separation the temperature of the column should be 25–35°C. Using this protocol, products up to 1.2 kb can be separated. After HPLC purification, products were either concentrated using Centricon™ columns or ethanol precipitated.

Sequencing was carried out according to the protocol of the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems), using 10 pM primer and about 1/40 of the PCR product. The sequencing reaction was performed on a Perkin-Elmer DNA Thermal Cycler 9600, using 25 cycles under these conditions: 96°C for 15 s, 50°C for 15 s, 60°C for 4 min. Samples were extracted twice with phenol:chloroform:H_2O and concentrated by precipitation with sodium acetate and ethanol at room temperature. Samples were then denatured at 96°C for 2 min and analysed on an ABI 373A DNA Sequencer (6% SequaGel™, National Diagnostics).

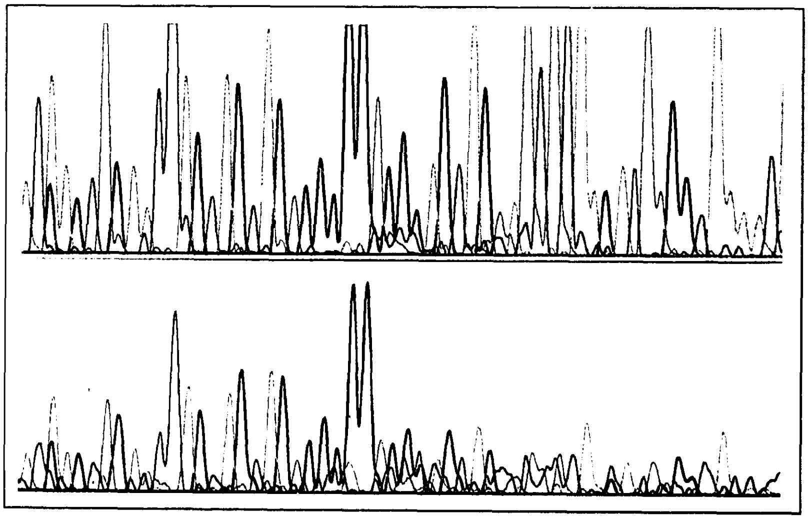

FIG 1

Top: sequence of a HPLC-purified fragment. Bottom: sequence of an identical portion of the same fragment after purification using a Centricon™ column. The nucleotides are represented by different colors: A, green; C, blue; G, black; T, red.

Figure 1 shows parallel sequencing of a PCR-amplified fragment purified from the same reaction, using either our HPLC protocol (top) or a Centricon™ column alone. Sequencing was performed simultaneously and the products analysed on the same gel; Fig. 1 shows the sequence data obtained for nucleotides 145–200. While sequence data were obtained for 500 bp of the HPLC-purified fragment, that purified using the Centricon™ column yielded less than 200 bp of sequence. The HPLC-purified PCR products were of consistently high grade, while the other methods yielded sequencing templates of variable quality. Most amplification primers used consistently yielded longer sequences of much better quality (as judged by the number of unidentified nucleotides, N, in each sequence) when using PCR fragments purified by HPLC (about 450–500 nucleotides, 3–5% N) rather than by the other methods tested (300–400 nucleotides, 10% or more N). Exceptions were primers located inside the PCR fragment, which gave comparable results for fragments purified using either HPLC or Centricon columns.

HPLC purification is therefore recommended as a fast, reproducible and simple method for preparing PCR products for direct sequencing.

REFERENCE

1 Katz, E.D., Haff, L.A. and Eksteen, R. (1990) *J. Chromatography* 512, 433–444

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