ABSTRACT

The isolation of a single DNA molecule for cloning is technically difficult, and the subsequent screening of colonies for recombinant DNA clones is time consuming. Ion-pair-reversed phase HPLC (IP-RP-HPLC) analysis of nucleic acids improves the resolution and isolation of PCR products to be cloned. We demonstrate that PCR products analyzed and collected using the IP-RP-HPLC approach (WAVE™ DNA Fragment Analysis System) can be cloned directly into a plasmid vector. In addition, we demonstrate that when IP-RP-HPLC analysis is extended to the colony screening process, the time required for these procedures is reduced.

INTRODUCTION

PCR products are frequently used for cloning, yet the isolation of a single DNA molecule for ligation into a plasmid vector is both technically difficult and time consuming. PCR products are routinely subjected to agarose or polyacrylamide gel electrophoresis (PAGE), whereby a single DNA molecule to be cloned is separated from multiple nonspecific products and primer dimers. The DNA fragments must then be isolated from the gels. There are a number of approaches for isolating DNA fragments from agarose gels including electroelution (6), low melting temperature agarose (1) and glass beads or silica gel (5).

Common methods for the elution of DNA fragments from polyacrylamide gels include passive diffusion and electroelution (5). Unfortunately, gel separation methods and their associated fragment isolation protocols do not offer optimal purity of fragments to be cloned. The limited resolution capabilities of gel separation and the technical difficulties of removing single-size fragments following gel analysis have been demonstrated to result in inadvertent cloning of multiple fragments (2). Therefore, to properly identify the appropriate clone, more clones must be screened and sequenced. Colony screening for recombinant DNA clones is time consuming and laborious as well as costly. Screening methods for recombinants, which include colony hybridization/autoradiography and the analysis of plasmid DNA by restriction endonucleases, may take up to several days to complete (1). Although PCR-based colony screening strategies may reduce screening time (4,7), improved fragment separation and purification approaches that allow for the subcloning of only the single fragment of interest offer the biggest time-saving potential. In this regard, ion-pair-reversed phase HPLC (IP-RP-HPLC) analysis of nucleic acids offers significant improvements in nucleic acid fragment resolution (3).

The ability of IP-RP-HPLC to potentially contribute to the efficacy of cloning is exemplified by Hecker et al. (2). Extensive and costly screening lead to the identification of multiple subclones from the ligation reaction of a PCR product that has been separated by PAGE and isolated as a single DNA fragment. However, when IP-RP-HPLC analysis was performed on the PAGE-separated fragment, multiple similarly sized fragments were resolved. The fragment sizes corresponded exactly to the insert sizes that were obtained in the colony screening and sequencing analyses (2). These studies clearly demonstrated the improved resolution of PCR products by IP-RP-HPLC analysis. It remained to be determined whether the eluent from IP-RP-HPLC analysis would interfere with cloning.
Here, we extend those studies and demonstrate that PCR products analyzed and isolated using the IP-RP-HPLC approach (WAVE™ DNA fragment analysis system; Transgenic, Omaha, NE, USA) can be cloned directly into a linearized plasmid vector, without additional processing before performing the ligation reaction. Furthermore, no cross-contamination between adjacentely eluting peaks or carryover between injections is detected in the cloning analysis of the appropriate collected controls. We also demonstrate that when IP-RP-HPLC analysis is extended to the colony screening process, the overall time required for these procedures, from fragment amplification and isolation to analysis of recombinants and large-scale plasmid preparations, is reduced to 1.5–2 days.

Initially, a 500 bp Lambda fragment was PCR-amplified and size-eluted using the IP-RP-HPLC approach of the WAVE system. The 100 µL final PCR mixture consisted of 1 ng whole bacteriophage Lambda DNA (PE Biosystems, Foster City, CA, USA), 2.5 U Pfu polymerase (Stratagene, La Jolla, CA, USA) and primers (PE Biosystems) at a concentration of 0.2 µM each. The sequences of the sense and antisense primers used for the amplification of the Lambda fragment to be cloned were as follows: 5'-GATGAGTGTCGTCCGTA-CAACTGG-3' and 5'-GGTTATCGAAATCAGCCACAGG-C-3'. Amplification was performed for 25 cycles at 94°C for 15 s, 68°C for 1 min and with a final extension of 7 min at 72°C. A single 20 µL aliquot of the 100 µL PCR was injected on the WAVE system and analyzed by IP-RP-HPLC using the high resolution DNASEP® polymer matrix (Transgenic). The elution profile for this injection of the Lambda PCR product is depicted in Figure 1A.

Primer dimers and nonspecific PCR products were separated from the 500 bp amplified fragment, and the purified fragment was collected automatically by an inline fragment collector (Transgenic). A 30 µL aliquot of the collected fragment was reanalyzed on the WAVE system to demonstrate successful isolation of the 500 bp fragment (Figure 1B). A 2 µL aliquot of the 500 bp fragment collected from the WAVE system was ligated into the pPCR-Script™ Amp SK(+) cloning vector (Stratagene). Ligation reactions were performed according to manufacturers’ instructions. Two microliters of the ligation mixture were used for transformation of XL10-Gold® Kan ultracompetent cells (Stratagene). The transformation procedure was as suggested by the supplier with 50 µL of the final cell suspension spread on the agar plate.

Two PCR screening approaches for the detection of recombinant colonies were compared. First, a single bacterial colony was touched using a sterile pipet tip and inoculated into 45 µL of distilled water. The colonies were lysed by heating at 98°C for 10 min. This mixture was then used directly in PCRs. Second, plasmid DNA was isolated from 3 mL overnight cultures of the same colonies using QIAGEN® plasmid mini kit (Qiagen, Valencia, CA, USA). The 25 µL PCR mixtures contained either 17 µL of the lysed colony or 0.5 µL of plasmid, 1.25 U AmpliTaq® DNA polymerase (PE Biosystems) and 1.0 µM T3/T7 primer set (Stratagene). Amplification was performed for 35 cycles at 94°C for 1 min 10 s, 55°C for 1 min 10 s, 72°C for 1 min 55 s and with a final extension of 10 min at 72°C.

Four bacterial colonies were selected from the test plates for screening by IP-RP-HPLC analysis. T3 and T7 primers that are located 63 bp and 102 bp from the cloning site in the pPCR-Script Amp SK(+) vector, were used for PCR amplification of DNA fragments to be analyzed. Following PCR by either approach, a 665 bp fragment is indicative of a recombinant clone, and a 165 bp fragment indicates a colony with no insert. Detection and resolution of these fragments by IP-RP-HPLC can be achieved in less than 20 min. Five microliters of each of the PCRs from the lysed colonies were injected on the WAVE system to determine the size of the amplified fragment, based on separation of the ladder generated by HaeIII digestion of pUC18 plasmid (Figure 2A).

Three of the four colonies screened contained an insert resulting in a 665 bp PCR product (Figure 2B). The fourth colony screened contained no insert (Figure 2C) resulting in a 165 bp PCR product. In each case, the results obtained by PCR amplification of the lysed colony (Figure 2, B and C) were identical to those obtained by PCR amplification of the purified plasmid (data not shown). Furthermore, these data were confirmed by DNA sequence analysis of the plasmid DNA prepared from each of the colonies. The plasmids were subjected to standard dideoxy chain termination sequence analysis and were analyzed on an Applied Biosystems Model

![Figure 1. IP-RP-HPLC analysis of a 500 bp PCR product for collection.](image)

(A) DNA elution profile of a PCR-amplified 500 bp Lambda fragment. Twenty microliters of the 100 µL PCR were analyzed by IP-RP-HPLC using the WAVE DNA fragment analysis system and collected using the inline fragment collector. Intensity (mV) is plotted vs. retention time in minutes. Analysis conditions were as follows: column temperature was 50°C with a flow rate 0.9 mL/min. Buffer A: 0.1 M triethylammoniumacetate (TEA) in water and Buffer B: 0.1 M TEA and 25% acetonitrile in water. Gradient: 0 min: 45% A, 55% B; 0.5 min: 40% A, 60% B; 4.0 min: 20% A, 80% B; 5.5 min: 0% A, 100% B; 6.5 min: 45% A, 55% B; 8.5 min: 45% A, 55% B. (B) Elution profile of the collected Lambda fragment. Thirty microliters of the collected fragment were analyzed using the above gradient.
automated sequencer by HDZ Genetics (Omaha, NE, USA). Sequence analysis using T3 and T7 primers determined that three of the clones contained the 500 bp Lambda fragment, while the fourth clone contained no insert as predicted by WAVE system analysis (data not shown).

To examine potential effects of adjacent peaks on cloning efficiency, the same 500 bp Lambda fragment was mixed with a 719 bp TGFB1 PCR product and size-eluted using the WAVE system. A single 20 µL aliquot was injected on the WAVE system and analyzed by IP-RP-HPLC (Figure 3). The Lambda fragment was isolated away from the TGFB1 fragment and was collected with an inline fragment collector. Immediately following this collection, a blank (0 µL) injection was made, and the equivalent retention time was collected to demonstrate the absence of carryover between injections. A 2 µL aliquot of each collection from the WAVE system was ligated into the pT-Adv cloning vector (Clontech Laboratories, Palo Alto, CA, USA). Ligation reactions were performed according to the manufacturer’s instructions. Transformation and plating were as described earlier.

The resulting colonies were lysed in water and screened using the PCR-based approach already outlined to obtain cloning efficiency and carryover data. M13 primers (Clontech Laboratories) that are located 87 bp and 111 bp from the cloning site in the pT-Adv vector were used for PCR amplification of DNA fragments to be analyzed. Following PCR, a 698 bp fragment is indicative of a recombinant clone, while a 198 bp fragment indicates a colony with no Lambda fragment inserted. Nearly all (95%) of the colonies that were screened contained an insert resulting in a 698 bp PCR product (data not shown). The remaining 5% of the colonies contained empty vector. Of the colonies that contained insert, 100% contained the Lambda fragment as determined by PCR screening with Lambda primers (data not shown). When the same colonies were PCR-screened with TGFB1 primers, no amplifiable TGFB1 product was detected (data not shown). Together these data demonstrate the absence of cross-contamination between adjacently eluting peaks. M13 vector-specific primers were also used to screen colonies generated from ligation reactions of the 0 µL injection. All (100%) of the colonies that were screened resulted in a 198 bp fragment, indicating a colony with no insert (data not shown). This demonstrates the lack of carryover between injections.

In summary, IP-RP-HPLC offers high-resolution separation of PCR products and allows for optimal purification of a single-size fragment to be cloned into a linearized vector. We have demonstrated that a 500 bp PCR product, which has been separated from primer dimers, nonspecific products and adjacently eluting peaks by IP-RP-HPLC and has been...
collected by an inline automated fragment collector, can be cloned directly into a linearized plasmid vector. The eluent in which the fragment is collected does not affect either the ligation reaction or the transformation process. Furthermore, 100% of the recombinant colonies contained the correct size insert. By optimally isolating the fragment to be cloned using IP-RP-HPLC, the probability of obtaining a heterogeneous population of clones is decreased. Consequently, the analysis time for screening of recombinants is reduced.

Applying IP-RP-HPLC to the colony screening process can also decrease the time and cost of colony screening. The presence of insert for a given colony can be determined in less than 20 min when the IP-RP-HPLC is performed on PCR products from selected colonies. The entire process of colony screening can be reduced to a single day by eliminating plasmid isolation before the PCR amplification. The colonies that are screened can also be inoculated for large-scale plasmid preparations at the same time as the inoculation for PCR, thereby saving more time. In addition to the time-saving advantages offered by extending IP-RP-HPLC to the screening process, some of the considerable expense in sequencing empty vectors can also be eliminated.

REFERENCES


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