Rapid Procedure for Cloning of PCR Product and Sequencing with Automatic DNA Sequencer

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A rapid and reliable procedure for molecular cloning and nucleotide sequencing of PCR product is described. PCR amplified fragment recovered from agarose gel by centrifugation through siliconized sterilized glass wool was further purified in sequential extraction with phenol, phenol/chloroform and chloroform. This was ligated into 'T'-hanged plasmid vector and transformed E. Coli was isolated. The plasmid recovered by the alkali denature method was purified by means of spun columns containing Sepharose CL-4B. This step is critical in obtaining a reading >500 bp with an automatic DNA sequencer.

Key words: cloning, horse, PCR, sequencing

J. Equine Sci.
Vol. 5, No. 1,
pp 33-36

Innovation of the polymerase chain reaction (PCR) has revolutionized many fields of molecular biology, in particular isolation of genes and nucleotide sequencing [2, 3, 4]. Molecular cloning of PCR product is usually carried out by blunt-end ligation after this product was filled with dNTPs and klenow fragment or digestion with appropriate restriction enzymes before ligation. Although faithful, these methods are both time-consuming and cumbersome.

T-hanged vector is a convenient plasmid vector that is able to omit fill-in reaction for blunt-end ligation or restriction enzyme digestion to generate the cohesive ends. The vector was developed based on the characteristics that almost all PCR products have the adenyl tails in the 3' end of both strands. The vector is added with deoxythymidine triphosphate after plasmid is linearized by restriction enzyme digestion with *Sma* I or *Eco* R V to generate the blunt ends. This treatment leads thymine-adenine bondage between vectors and PCR products. We have cloned many PCR products by means of this technique, however the efficiencies were lower than that expected (<60%).

So we refined the protocols to increase the efficiency. We examined the effects of purification of PCR products and the ratio of vector and insert in this study.

As automatic DNA sequencers became commercially available, the researchers anticipated that they were liberated from awkward procedures. Although automatic sequencers are less sensitive than conventional RI sequencing system, sometimes long range sequences are determined only with conventional RI protocols. The refinement of the sequencing protocols is needed for Automatic DNA sequencers as frequent operations. To this purpose, we also developed simple and reliable techniques to purify plasmid DNA for determination of long range sequences with an automatic DNA Sequencer (A.L.F. DNA Sequencer, Pharmacia LKB).

In the present study, we chose the equine interspersed element DNA as a target for PCR. One pair of primers (Primer a: 5'-CCAAACAGAGAGAATTTTCACAC-3' and Primer b: 5'-AAGAGTGCCCTGAACATAAAGA-3') was designed to amplify a 298 bp fragment from equine genome. Thirty ng of genomic DNA was used for PCR amplification in 30 μ l of 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (W/V)

gelatin, each deoxynucleoside triphosphate at 200 μ M, 60 pmol of each primer and 1.25 U of Taq polymerase (Perkin-Elmer Cetus). Amplification was carried out for 30 cycles in the GeneAmp PCR system 9600 (Perkin-Elmer Cetus) (1 min, denaturation at 95°C; 1 min, annealing at 62°C; 1 min, elongation at 72°C). After PCR products were electrophoresed in 2% agarose gel (SeaKem GTG or Metaphor agarose, FMC), DNA fragments from the gel slices were recovered by centrifugation through the siliconized sterilized glass wool. This technique was originally described by Heery *et al.* [1]. Briefly, the gel slice was transferred to a punctured sterilized microcentrifuge tube in a two-tiered system. This was placed on top of another tube and centrifuged at 7000 rpm for 15 min. The eluted samples were further purified by extraction of phenol, phenol/chloroform and chloroform, respectively. The optional steps for purification of PCR product did not alter the recovery of PCR product. We routinely recover over 30 ng of PCR product from 30 μ l of PCR reaction. The purified PCR product (more than 6 ng) was ligated with 25 ng of PCR II^{TM} vector

(Invitrogen) at 12°C for 4 h. We routinely used 20 ng of purified PCR product for a ligation. The ratio of vector and insert was estimated to 1: 10 in this example. JM109 strain of *E. Coli* was transformed. The recombinant clones were selected in ampicillin/X-gal/IPTG containing LB plates and plasmid DNA was isolated by the alkali denature method as described by Sambrook *et al.* [5]. It was effective to extend the efficiency for cloning of PCR product. For example, Fig. 1 shows the results for plasmid DNA digested with *Eco* RI. Twenty-seven clones out of thirty contained appropriate length (300 bp) of inserts (90%). The efficiency was thirty per cent higher than that of the previous protocol without purification of PCR products.

We have utilized conventional RI protocols to the nucleotide sequencing with an automatic DNA sequencer. In such experiments, we could not obtain the reproducible results. Because single strand phage DNA or ultra-centrifuged plasmid DNA were both good templates for the sequencing system, the impurity of template DNA was suspected for the major source of our failures. So we

Fig. 1. Transformed *E. Coli* colonies were randomly picked up and were cultured at 37°C over night. Plasmid DNAs recovered from the cultures were digested with *Eco* RI. Then the samples were electrophoresed in 2% agarose gel. M: molecular weight marker.

attempted to purify plasmid DNA with a simple application. Plasmid DNA prepared as above was further purified by passing through spun columns containing Sepharose CL-4B. The resin suspended in STE (10 mM NaCl, 1 mM EDTA and 10 mM Tris·Cl, pH 7.5) was poured into columns and centrifuged at 400 g for 2 min. After crude plasmid solutions were dipped onto the packed resins, the columns were centrifuged again at 400 g for 4 min. The eluted samples contained approximately 10 µg of pure plasmid. The solution could be used directly as sequencing templates with AutoCycle sequencing kit or AutoRead sequencing kit (Pharmacia). This step was critical for a reading over 500 bp (Fig. 2A-B). We routinely sequenced double strand DNA over 450 bp with a process. The protocols outlined here has some advantages to previous publications, e.g., (1) Cloning and sequencing steps are completed for just 3 days, (2) more sequence informations (450 to 500 base pairs routinely) are available from the process, (3) small cultures are enough to obtain template DNAs (<2 ml), (4) further purification of plasmid DNA such as CsCl centrifugation or PEG-precipitation is not required. It should contribute much to clinical and diagnostic investigation, such as in the detection of genetic disorders and the identification of viral strains in racing horses.

Acknowledgment

We are grateful to Ms. Murao and Ms. Yamakawa for technical assistance and to Dr. Mukoyama for critical reading.

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