Solid-Phase Nested Deletion: A New Subcloning-less Method for Generating Nested Deletions

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Abstract

We have developed a new subcloning-less method for generating nested deletions which we have termed Solid-Phase Nested Deletion. The basic procedure for this method is as follows. The target DNA fragment is cloned in the multiple cloning site of a cloning vector, pUC or its derivatives, and amplified by PCR using a set of primers, one of which is 5'-biotinylated. The amplified DNA is partially digested by a restriction enzyme with a 4-base recognition sequence. The digested DNA is ligated with a synthetic adapter DNA. Monodiverse beads coupled with streptavidin (Dynabeads™ M-280 streptavidin) are added to the mixture and the biotinylated DNA fragments are separated by applying magnetic field. The unidirectionally deleted DNA fragments are recovered by PCR from the magnetic beads, and size-fractionated by agarose gel electrophoresis. The DNA fragments are amplified by PCR and used for sequencing. We demonstrate the potential of this method using a 4878-bp EcoRI fragment of λ phage DNA.

Key words: deletion; partial digestion; PCR, sequencing; solid-phase

1. Introduction

There are three types of strategies for sequencing long DNA fragments: shotgun methods,1 primer-walking methods2 and nested deletion methods.3 Among them, nested deletion is the most economical method. The sequence redundancy required for this method is much smaller than the shotgun approach and synthesis of oligonucleotide sequencing primers is not necessary.

The commonly used method for generating nested deletions depends on digestion of the cloned insert by Exonuclease III or BAL31 nuclease.3'4 Hattori et al. have developed a new strategy based on random digestion by ultrasonication and applied it to a large-scale sequencing project.5 One of the problems with these methods is that preparation of many template DNAs is necessary to obtain a complete set of deleted DNA fragments, which is an expensive and time-consuming process. Wright has developed a technique called partial-digest DNA sequencing that permits DNA sequencing without subcloning.6 No redundant template preparation is necessary for making nested deletions by this method. However, it is difficult to separate and obtain correctly deleted DNA fragments by this method because many artificial PCR products are formed.

We report here a new subcloning-less procedure for generating nested deletions, designated Solid-Phase Nested Deletion. By this method, nested deletions of DNA fragments of up to several kilobases can be made with ease.

2. Materials and Methods

2.1. DNA

An EcoRI-digested fragment of λ phage DNA (4878 bp) subcloned into the EcoRI site of pUC18 was used as a target DNA.

2.2. Enzymes, oligonucleotides and kits

Sau3AI, bacterial alkaline phosphatase, T4 polynucleotide kinase and a Ligation kit (Ver. 2)™ were purchased from Takara Shuzo Co., Ltd. Ex Taq™ polymerase and 10 x Ex Taq™ buffer were also obtained from Takara Shuzo Co., Ltd. DNA was recovered from agarose gels using Geneclean II™ (Bio101). AutoCycle™ sequencing kit and FITC-labeled T7 primer were purchased from Pharmacia Biotech. The oligonucleotide primers used for amplification and biotinylation of the target DNA were purchased from Dynal AS. All other oligonucleotides were synthesized by Sawady Technology.
Figure 1. Schematic diagram of Solid-Phase Nested Deletion.
2.3. Magnetic beads and other materials

Magnetic beads containing covalently coupled streptavidin, Dynabeads™ M280-streptavidin, were obtained from Dynal AS. A 1-kb DNA ladder was used as a molecular size marker (Gibco BRL). Excess primers or salts were removed using Microcon™ 100 (Amicon).

2.4. Solid-phase nested deletion

2.4.1. Amplification and biotinylation of target DNA

About 1 ng of plasmid containing the target DNA was used for PCR amplification performed with two oligonucleotide primers, 5'-GCT-TCC-GGC-TCG-TA-TGT-GTG-TG-3' and 5'-AAA-GGG-GGA-TGT-GCT-GCA-AGG-CG-3', which are complementary to regions upstream and downstream of the multiple cloning site of pUC18, respectively. The upstream primer was biotinylated at the 5' end. The reaction mixture (100 μl) consisted of 1 ng of plasmid DNA, 1× EX Taq™ buffer, 0.2 mM dNTP, 0.025 μM each primer and 2.5 units of EX Taq™ polymerase. The thermal profile consisted of heating at 96°C for 3 min, followed by 30 cycles of template denaturation at 96°C for 30 s, primer annealing at 65°C for 1 min, and extension at 72°C for 3 min.

2.4.2. Partial digestion by Sau3AI

The PCR product was purified and concentrated by ultrafiltration using Microcon™ 100. The DNA was dissolved in 100 μl of H buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 100 mM NaCl). Forty microliters of the DNA solution was transferred to a microfuge tube, and the remaining 60 μl was divided equally into 3 tubes. Sau3AI (0.2 units) was added to the first tube and the reaction mixture was mixed well. A 20 μl aliquot of the reaction mixture was transferred to the third tube. The last 20 μl was discarded. The reaction mixtures were incubated for 1 hour at 37°C, and the reaction was stopped by the addition of 1 μl of 25 mM EDTA. Three microliter aliquots of the reaction products were subjected to agarose gel electrophoresis. The remaining reaction products were ethanol-precipitated after phenol/chloroform extraction.

2.4.3. Dephosphorylation of the digested target DNA and phosphorylation of the adapter DNA

The appropriately digested DNA was dissolved in 150 μl of alkaline phosphatase reaction buffer (50 mM Tris-Cl, pH 8.0, 10 mM MgCl₂) containing 1.2 units of bacterial alkaline phosphatase, and the reaction mixture was incubated for 1 hour at 37°C. The alkaline phosphatase was removed by phenol extraction. The dephosphorylated DNA was recovered by ethanol precipitation and dissolved in 10 μl of TE (Tris-HCl, pH 8.0, 1 mM EDTA). Fifty microliters of 1 μM Sau3AI cassette adapter (equimolar mixture of 5'-GTA-CAT-ATT-GTC-GTT-AGA-ACG-CGT-ATT-CCT-ATA-GTG-AGT-CGT-ATT-ACG-CG-T-TCT-AAC-GAC-AAT-ATG-TAC-3' and 5'-GAT-CTC-CCT-ATA-GTG-AGT-CGT-ATT-ACG-CG-T-TCT-AAC-GAC-AAT-ATG-TAC-3') was mixed with 10 μl of 10× kinase buffer (500 mM Tris-HCl, pH 8.0, 100 mM MgCl₂, 50 mM DTT), 38 μl of H₂O and 1 μl of 5 mM ATP. Phosphorylation was started by the addition of 1 μl of T4 polynucleotide kinase (10 units/μl). After incubation for 1 hour at 37°C, the reaction was stopped by heating for 10 min at 70°C. There was a T7 sequencing primer site in the sequences of this adapter DNA.

Figure 2. Schematic diagram of the target DNA, and the predicted fragment lengths of unidirectionally deleted DNAs. An EcoRI-digested fragment of λ DNA (4878 bp) was used as a target DNA. This fragment was subcloned in the EcoRI site of pUC18. Sau3AI sites are shown by arrowheads. The predicted unidirectionally deleted DNA fragments are shown with their lengths.
Figure 3. Recovery and size-fractionation of the unidirectionally deleted DNA. DNA fragments bound to the magnetic beads were recovered by PCR and size-fractionated on a 1% agarose gel. Lane 1, molecular size marker. Lane 2, DNA fragments recovered from the magnetic beads.

2.4.4. Adapter ligation

An aliquot of 2.5 µl of dephosphorylated digested DNA was mixed with 2.5 µl of phosphorylated adapter DNA, followed by ligation reaction using a Ligation kit (Ver. 2)™, according to the manufacturer’s instructions. Following incubation at 16°C for 30 min, the reaction was stopped by heating at 70°C for 10 min. The ligated DNA was purified and concentrated using Microcon™ 100.

2.4.5. Solid-phase separation

The ligated DNA was dissolved in 40 µl of binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 M NaCl), and mixed with 40 µl of Dynabeads™ M-280 streptavidin (5 µg/µl) prewashed and suspended with binding buffer. The biotinylated DNA was adsorbed onto magnetic beads by incubation for 20 min at room temperature. To remove the non-biotinylated DNA fragments, the magnetic beads were washed with 40 µl of binding buffer and then with 40 µl of H2O. Finally, the magnetic beads were suspended in 15 µl H2O.

2.4.6. Recovery of DNA from magnetic beads by PCR

The magnetic bead suspension was diluted 50 times and used for PCR recovery. The reaction mixture (100 µl) consisted of 2 µl of diluted magnetic bead suspension, 1× EX Taq™ buffer, 0.2 mM dNTP, 0.1 µM of each primer (5'-GCT-TCC-GGC-TCG-TAT-GTT-GTG-TG-3' and 5'-GTA-CAT-ATT-GTC-GTT-AGA-ACG-CG-3', complementary to the biotinylated end and adapter end of the immobilized DNA, respectively) and 2.5 units of EX Taq™ polymerase. The thermal profile consisted of 25 cycles of template denaturation at 96°C for 30 s, primer annealing at 55°C for 1 min, and extension at 72°C for 3 min.

2.4.7. Size-fractionation and amplification of deleted DNA fragments

Ten microliter aliquots of the amplified DNA were applied to agarose gel electrophoresis. Each DNA fragment was extracted and purified by Geneclean II™, according to the manufacturer’s instructions. About 10 ng of the extracted DNA was subjected to PCR amplification. The reaction conditions were the same as those described above except that the cycle number was reduced to 20. Three microliter aliquots of the reaction solutions
Analyzed Sequence Length

| Fragment   | Sequence Length (bp) | Length (bp) |
|------------|----------------------|-------------|
| 1          | 3888                 | 409         |
| 2          | 3364                 | 307         |
| 3          | 2967                 | 422         |
| 4          | 2551                 | 470         |
| 5          | 1879                 | 399         |
| 6          | 1278                 | 476         |
| 7          | 1199                 | 302         |

Figure 5. Schematic diagram of sequence results of the deleted fragments. The analyzed sequence results of deleted fragments are schematically shown with their lengths. The target DNA is shown as a shaded bar. Sau3AI sites of the target DNA are shown by arrowheads.

were applied to agarose gel electrophoresis. The remaining amplified DNAs were purified and concentrated by Microcon™ 100.

2.4.8. Sequencing

The concentrated DNA fragments were used for sequencing. Sequence reactions were carried out using AutoCycle™ sequencing kit, according to Pharmacia’s specifications with the following exceptions. FITC-labelled T7 primer was used and the annealing temperature of the temperature cycle was changed to 45°C.

3. Results and Discussion

The basic Solid-Phase Nested Deletion procedure is outlined schematically in Fig. 1. The target DNA fragment is cloned into the multiple cloning site of a vector such as pUC or its derivatives, and amplified by PCR using a set of primers, one of which is 5'-biotinylated. Thus, the obtained DNA fragment is 5'-biotinylated at one end. The target DNA can also be biotinylated by filling in a 5'-overhanging restriction digestion site with biotin-11-dUTP and appropriate dNTPs using Klenow polymerase.7 If the sequences of both ends of the target DNA are known, the target DNA can be amplified and biotinylated directly from genomic DNA. By biotinylating another 5' end, the nested deletion of another strand can be made.

The amplified DNA is partially digested by a restriction enzyme with a 4-base recognition sequence (4-base-cutter). There is, on average, one digestion site of a 4-base-cutter in every 256 bases. Since it is possible to analyze several hundred bases at once using a fluorescence DNA sequencer, 4-base-cutters are suitable for generation of nested deletions. Several different 4-base-cutters will have to be used to obtain a complete set of nested deletions because of the random distribution of digestion sites in the sequences. The reaction conditions should be set such that, at most, one digestion site of one target DNA molecule is cut by the 4-base-cutter used. Under such conditions, the amounts of each deleted fragment will be almost equivalent. If the DNA is treated by the enzyme for a longer period, the amounts of long fragments will decrease.

The digested DNA is ligated with a synthetic adapter DNA which has a cohesive end complementary to that of the digested DNA. The sequences of the adapter DNAs will be used as the primer sequences for PCR amplification. There is also a sequencing primer site in the adapter sequence. Digested DNA is dephosphorylated to avoid dimerization of biotinylated DNA fragments, and the adapter DNA is 5'-phosphorylated prior to the ligation reaction.

Monodiverse beads coupled with streptavidin (Dynabeads™ M-280 streptavidin) are added to the mixture. The biotinylated DNA fragments are adsorbed to the magnetic beads and separated by applying magnetic field. Since one end of the fragment is biotinylated, only the unidirectionally deleted fragments can be separated by this procedure. The DNA fragments are recovered by PCR from the magnetic beads. The amplified DNAs are size-fractionated by agarose gel electrophoresis. Appropriately deleted DNA fragments can be recovered from the gel by any of various standard methods and amplified by PCR. The fragments may be picked up using tooth picks and subjected to PCR amplification. The amplified DNA can be sequenced using a fluorescence-labeled primer corresponding to the adapter DNA sequence after some purification procedures. If a biotinylated primer is used for amplification, the fragment can be sequenced by the solid-phase sequencing method.8

To evaluate the efficiency of this method, an EcoRI fragment of λ DNA was used to generate nested deletions. The fragment was subcloned in pUC18, and Sau3AI was used as a 4-base-cutter. The predicted Sau3AI digestion
sites and the lengths of deleted products are shown in Fig. 2. Figure 3 shows the results of PCR recovery of immobilized deleted DNA fragments. Eight DNA fragments corresponding to deleted fragments were amplified. The smallest deleted product (105 bp) was too small to analyze by the gel. The size of each fragment was a little larger than the predicted size since the amplified DNA included adapter and primer sequences. No artifactual bands were found. The intensities of all bands were almost equivalent. Taking account of the differences in fragment lengths, the smaller fragments were amplified preferentially. Each fragment except the largest one, which would give the same sequence as the target DNA, was cut out of the gel and extracted by Geneclean II™. The purified DNA was used for PCR amplification. As shown in Fig. 4, each fragment was amplified successfully. Figure 5 shows sequence results of the deleted fragments. The average sequence length was about 400 bases. Figure 6 shows the unprocessed sequence raw data of fragment 6. This result clearly shows that the amplified fragment was not contaminated with other fragments or artifactual PCR fragments.

To make nested deletions by partial-digest DNA sequencing, the optimization of the reaction conditions of partial digestion and ligation is critical. If the reaction conditions are not optimized, many artifactual PCR products will be formed by the double digestion with the 4-base cutter or multimerization of the digested fragments. Solid-Phase Nested Deletion is free from such problems because double digested fragments were removed by magnetic separation, and multimerization of the deleted fragments was prevented by dephosphorylation.

Errors might be introduced during PCR amplification procedures, but these will only affect the sequence results determined by this method slightly as there is no subcloning procedure. Errors in the very early stages of amplification affect the sequence results in causing mixing peaks at the bases.

The performance of this method is limited by the efficiency of PCR amplification and the resolution of agarose gel electrophoresis. With the advent of long PCR techniques, we can amplify DNA fragments longer than 10 kb. Agarose gel electrophoresis required for this method must be able to separate fragments which differ from each other by about 2-300 bases. At present, the resolution of fragment separation is limiting the performance of this method.

Solid-Phase Nested Deletion can be used for sequencing DNA fragments up to about several kilobases. We think that this method will be a useful tool in both large scale sequencing projects and cDNA analysis.
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