Adaptor Polymerase Chain Reaction for Single Molecule Amplification

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The adaptor polymerase chain reaction (PCR) permits the amplification of DNA fragments with arbitrary sequences. In this paper, we describe the successful amplification of plasmid-derived single molecule DNAs digested by a restriction enzyme. By using adaptors made of short and long oligonucleotides, nonspecific interactions during PCR were suppressed. The method will be applicable to the detection of single molecule DNA fragments even if their sequence is unknown.

[Key words: polymerase chain reaction, adaptor polymerase chain reaction, sequence-independent single-primer amplification, single molecule amplification]

Polymerase chain reaction (PCR) (1) is a very useful technique in genetic engineering. Unknown DNA fragments, however, cannot be amplified by the standard PCR method because it requires known sequences at both termini of the template DNA. To amplify unknown DNA fragments, therefore, a short DNA adaptor should be attached. This method, called adaptor PCR or sequence-independent, single-primer amplification (SISPA), has been investigated elsewhere (2–7). This method is, in particular, effective in preparing a cDNA library (8, 9). Although PCR can amplify a single DNA molecule in theory, actual amplification of a single DNA molecule is difficult due to nonspecific interactions, for instance, primer-dimer formation. Because amplification of single DNA molecule is very useful for various techniques of molecular biology, many researchers have been developing single-molecule PCR and the results are applied in single-molecule biological methods such as combinatorial protein engineering and highly sensitive human immunodeficiency virus (HIV) detection (10–12). The single-primer method is widely used for single-molecule PCR because the formation of nonspecific products can be suppressed when unique primers are used (10).

In this paper, we demonstrate adaptor PCR for a single DNA molecule. In this demonstration, we used restriction fragments of pUC19 DNA as unknown DNA fragments, which were used as model fragments for those obtained from genomic DNA by restriction digestion. In adaptor PCR, the termini are determined by restriction enzyme digestion and suitable primers are designed on the basis of the recognition site of the restriction enzyme. In this study, the adaptor PCR was modified using a single-primer and specialized adaptor DNA. PCR using single-primers involves the use of only one primer in the amplification reaction mixture, whereas in conventional PCR two primers are used. The adaptor DNA consisted of long and short oligonucleotides as illustrated in Fig. 1. This combination was effec-

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FIG. 1. Dissociation of short adaptor DNA. Ligation between template DNA and adaptor DNA (I), dissociation of short adaptor DNA (II), extension to fill cohesive end (III), and standard PCR (IV).
tive for carrying out single-molecule PCR.

Template DNA was prepared as follows: Fragments were prepared from pUC19 DNA (Takara Bio, Shiga) by digesting it with the restriction enzyme *Hind*III (Takara Bio), followed by separation by agarose gel electrophoresis. A band corresponding to 517 bp was recovered from the gel and the fragment was then extracted from the gel using a GENECLEAN Turbo kit (Bio 101, Burlington, CA, USA). The concentration of the fragment was estimated by comparing the intensity with that of standard pUC19 DNA (Takara Bio) by gel electrophoresis. Adaptor DNA was prepared by hybridizing a long oligonucleotide (5'-GCCGATT CATTAATGCAGATCTCGATCCCG-3'), and a short oligonucleotide (5'-ACTCGGGATCGA-3'). The sequence of the single primer was 5'-CGATTCATTAAAGCAGATCTCG ATCC-3'. These oligonucleotides were synthesized and supplied by Funakoshi (Tokyo).

The procedure for the adaptor PCR is illustrated in Fig. 1. First, a ligation mixture was prepared, which included the template DNA, the adaptor DNAs and the ligation buffer (10 mM of the adaptor DNA, 17.5 U of T4 DNA ligase, 66 mM Tris–HCl (pH 7.6), 6.6 mM MgCl2, 10 mM dithiothreitol, and 0.1 mM ATP). In this process, the expected number of template DNA molecules in the solution was adjusted to unity by dilution. The ligation reaction in a 5-μl volume was carried out at 16°C for 2 h, followed by the inactivation of ligase for 5 min at 65°C (Fig. 1-I). The ligated sample was mixed with 9 μl of PCR master mix (16.7 mM (NH4)2SO4, 16.7 mM KCl, 0.17 mg/ml of bovine serum albumin [Takara Bio] and 0.17% of TritonX-100 [Sigma-Aldrich Japan, Tokyo], 0.2 mM each of dNTPs, 0.83 μM of primer) and 1 μl of Enzyme mix (0.5 unit of PfuTurbo DNA polymerase [Toyobo, Tokyo] in 10 mM Tris–HCl [pH 8.8], 1 mM MgSO4, 10 mM (NH4)2SO4, 10 mM KCl, 0.1 mg/ml of BSA and 10% of TritonX-100). The 15-μl reaction mixture was preheated to dissociate the short adaptor DNA (Fig. 1-II) and to fill in the sticky end by a polymerase reaction (Fig. 1-III). Finally, standard PCR was carried out (Fig. 1-IV). Thermal cycling was performed using an iCycler (Bio-Rad Japan, Tokyo) as follows: pretreatment at 72°C for 3 min; first denaturing at 95°C for 3 min; 60 cycles consisting of 95°C for 20 s, 60°C for 20 s and 72°C for 1 min; additional extension at 72°C for 7 min. The amplified products were separated by 1.2% agarose gel electrophoresis.

Figure 2 shows the results of the adaptor PCR amplification starting from a single molecule on average. The amplified products were observed in six of eight lanes. When the number of template DNA molecules was adjusted to unity by dilution, the number of template DNA molecules in an aliquot followed the Poisson distribution (11). According to the Poisson distribution, when the expected number of template DNAs is adjusted to unity in each tube by dilution, the probability of containing n molecules in the PCR tube can be deduced as follows: n=0, 36.8%; n=1, 36.8%; n=2, 26.4%. Table 1 shows the result of the χ²-test (level of significance: 5%) of amplified products compared with the probability given by the Poisson distribution. The results of the test agreed with the Poisson distribution, and strongly suggested that a single DNA molecule was successfully amplified by this method.

Another DNA fragment, a 585-bp fragment of pUC19/Sau3AI, was also used. This fragment has different cohesive ends and a different GC content. The GC contents of the 585-bp pUC19/Sau3AI and 571-bp pUC19/HindII fragments were 48.2% and 43.7%, respectively. The 585-bp fragment was also amplified successfully at the single molecule level as shown in Table 1.

Adaptor DNA consisted of short (12 nt) and the long (30 nt) oligonucleotides. Because the oligonucleotides were not modified with a phosphate group at the 5' end, only the 3' end of the long oligonucleotide was ligated to the 5' end of the template DNA. In the preheating process, the short oligonucleotide was dissociated from the ligated template DNA. Although this short oligonucleotide was present during the PCR, it did not affect the PCR because of its very low concentration and small size. The long oligonucleotide also did not affect the PCR for the same reason. In addition, the long oligonucleotide could be used as a primer because the same sequence of the primer was included, similar to the single-primer PCR method (10).

In this work, adaptor PCR for a single-molecule template

![FIG. 2. Results of adaptor PCR. Lane M indicates DNA molecular markers λ/HindIII. Lanes 1–8 indicate the products of single-molecule amplification.](image)

### Table 1. χ²-test of produced product

<table>
<thead>
<tr>
<th>Restriction enzyme used</th>
<th>Size of template DNA fragment (bp)</th>
<th>Number of samples</th>
<th>Number of amplified products</th>
<th>Probability of amplified products (%)</th>
<th>Probability given by Poisson distribution (%)</th>
<th>χ²-test (level of significance: 5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hind</em>III</td>
<td>517</td>
<td>21</td>
<td>15</td>
<td>71.4</td>
<td>63.2</td>
<td>0.22</td>
</tr>
<tr>
<td><em>Sau3AI</em></td>
<td>585</td>
<td>16</td>
<td>12</td>
<td>75</td>
<td>63.2</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Critical number

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was carried out. We believe that this adaptor PCR will contribute to genome analysis. We have been developing a new method for high-speed genome analysis using single-DNA-molecule manipulation (13–15). This method consists of five unit operations; (i) fixing DNA molecule with a stretched shape onto a solid substrate for optical mapping, (ii) site-specific cutting by a locally activated restriction enzyme, (iii) recovering target fragments from the stretched DNA, (iv) amplifying the recovered DNA fragments, and (v) sequencing. If DNA is digested and recovered successively in order from one terminus, alignment of sequence-determined fragments can be omitted. Because the alignment is a highly laborious process, our method may permit rapid and simple determination of long genomic sequences.

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