Inverse polymerase chain reaction (PCR) is a powerful approach for cloning the DNA sequences lying outside the boundaries of known sequences (7). This approach can be used to determine the integration site of a virus or a transposon (2,4). The procedure described by Triglia (7) can be directly used to amplify the flanking sequence in a plasmid or in a bacterial genome. However, it has been difficult to apply this method in cloning the flanking sequence in a mammalian genome. Since the target sequence is usually rare, the nonspecific amplification often masks the target products. Several groups overcame the difficulty by enriching the target sequence, in which the size of the restriction fragment has to be determined by Southern blot (3), or by using oligonucleotide hybridization to determine the target products containing the known sequence (2,3).

Here, we modified the classical inverse PCR procedure and successfully applied it in cloning the flanking sequence of a known sequence integrated in the human genome in two days. Four primers were designed in a 130-bp known sequence (2,3). Underlined and in italic is the primer (3 and 4) sequences or complement to the primer (1 and 2) sequences. Underlined is the primer (1 and 4) sequences. Underlined and in italic is the TaqI recognition site (TCGA).

<table>
<thead>
<tr>
<th>Sequences</th>
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<tr>
<td>Left arm of the Tc1 transposon</td>
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<tr>
<td>Primer 1</td>
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<td>Primer 2</td>
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<tr>
<td>Primer 3</td>
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<td>Primer 4</td>
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Table 1. Sequences of the Left Arm of Tc1 Transposon and Primers for Inverse PCR

**Taql** at 65°C and with *SspI* at 37°C, then extracted with phenol/chloroform and chloroform, precipitated in ethanol and washed with 70% ethanol. Approximately 10 ng digested DNA were ligated in a total volume of 50 µL, containing 1 U T4 DNA Ligase (Promega, Madison, WI, USA) at 12°C overnight, and 1 µL of the ligated product was used as the template in the first-round PCR on an UNO II Thermal Cycler (Biometra, Gottingen, Germany). After 5 min denaturing at 94°C, the PCR was performed in a total volume of 50 µL, containing 10 mM Tris, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 µM dNTPs, 1 U *Taq* DNA polymerase, 5% formamide and 30 pmol primers 2 and 3, for 27 cycles of 30 s at 94°C, 30 s at 55°C and 50 s at 72°C. Then, 1 µL PCR product was diluted in 1000 µL double-distilled (dd)H₂O, and 1 µL was used in the second round PCR amplification, in which the reaction condition was the same as that of the first round except that the primers 1 and 4 were used. The result (Figure 1A) indicated that the modification was effective and that the reactions were very efficient and highly specific. In most cases, only one band could be detected after the nested PCR, while many bands were observed if no 5% formamide were added in the PCR. The fragment sizes were the same as predicted, and the sequencing result indicated that the cloned fragments were correct.

In one reaction, the sequence of a cloned fragment (Figure 1A, lane 2) showed that there was a 53-bp TaqI fragment inserted in the flanking sequence.
quences, indicating that the target fragment was not self-ligated but trapped a small sequence (TGGTAAGGATCATCTTAAAGGAATGGGAATCATCAGAATGCT) that belongs to a known gene (GenBank® Accession No. 56662). It is very unlikely that the same fragment can be ligated into the transposon ends more than once, suggesting that this protocol is so efficient that a single-ligated target sequence can be cloned out of a complex genome. We then tested whether or not different flanking sequences can be cloned from cell pools. The two plasmids, pRP466-NEO and pCEP4, were delivered into human KB cells by electroporation. If the transposon jumps in human cells, integrating in the genome, it will have different flanking sequences depending on the transposition sites (6). The cells containing various G418-resistant clones were counted and harvested. The genomic DNA was extracted and digested by TaqI. After the regular phenol/ethanol precipitation procedure, the digested DNA from approximately 1000 cells was ligated at 12°C overnight in a 50-µL volume. The nested PCR described above was performed in 10–20 parallel reactions each containing 0.05–2 µL ligated product. As shown in Figure 1B, in most reactions in which the template DNA was from 1–4 cells, only one band was detected, and a few tubes gave no signal after electrophoresis on 2% agarose gel and visualization by UV. The amplified fragments were recovered from the gel and directly sequenced with primer 4. The primers were then designed, and all of the 8 amplified fragments we sequenced proved to be the flanking sequences of the Tc1 transposon integrated or transposed into the human genome. When the amount of the template was increased, several expected fragments can be amplified in one reaction. The 2 fragments of approximately 200 and 90 bp, respectively, were amplified with high frequency, indicating that these cells represent the major groups in cell pools. This result is consistent with the clonal analysis.

In this paper, we report a protocol to clone the flanks of a known sequence from cell pools. This procedure eliminates many tedious works, including the amplification of a large number of cell clones, extraction and ligation of a large number of DNA samples, as well as the use of an isotope. It is also particularly useful to clone the various flanking sequences of a transposon or a virus when there are multiple copies of either one in a cell. Compared to the classical method of cloning the flanking sequences, this modified procedure is easy, economic and fast. This method can be used to identify random integration sites in different clones or a population of clones. It is also a quick method to identify either transposition frequency or a preferential site.

REFERENCES


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Detection of Frame-Shifts within Homopolymeric DNA Tracts Using the Amplification Refractory Mutation System (ARMS)

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Analysis of point mutations is routinely performed following site-directed mutagenesis for the purpose of selecting mutated clones or when screening natural alleles for the presence of specific mutations. Several different techniques have been developed for such screens, e.g., single-strand conformational polymorphism (SSCP) and heteroduplex analysis (4), denaturing gradient gel electrophoresis (DGGE) (6) and constant denaturant gel electrophoresis (CDGE) (3). Among the polymerase chain reaction (PCR)-based methods, the amplification refractory mutation system (ARMS) is easiest to perform and does not need specific PCR materials or special detection equipment (1,2). ARMS is based on the principle that a perfect match at the 3′ end of PCR primers is needed for the efficient extension of the primers by Taq DNA polymerase. Primers used in ARMS cover the mutated nucleotide on their respective 3′ ends. Primer pairs representing either the wild-type or the mutated nucleotide are applied to distinguish both versions by quantitative and qualitative differences in the performance of the PCRs (5).

This standard method is inadequate for the detection of single-nucleotide