Limited T4 Exonuclease Activity and Partial Fill-in Expand Insertion Site Options for PCR Subcloning

BioTechniques 27:914-916 (November 1999)

We have derived a method for sticky-end subcloning of a polymerase chain reaction (PCR) fragment into almost any unique restriction site in a vector, whether or not that same site is present in the sequence of the PCR fragment itself. The approach avoids the frequent low efficiency of blunt-end ligation. It does not require the use of special vector systems and completely eliminates the need to perform restriction enzyme digestions on the PCR product itself. Both the restriction enzyme-digested vector and the PCR fragment are subjected to limited T4 polymerase/3′ exonuclease activity. To minimize self-ligation, the digested vector is treated with T4 polymerase and a limited choice of nucleotides to give 3′ recessed ends that are noncomplementary (9). PCR primers are designed so that treatment of the PCR product with T4 polymerase and a limited selection of nucleotides results in ends complementary to the modified vector ends.

Our goal was to insert the promoter for the mouse skeletal muscle α-actin gene (Reference 3 and unpublished sequence) into the single insertion site, HindIII, upstream of the chloramphenicol acetyltransferase (CAT) gene in the reporter vector pHCA0CAT (7). Engineering HindIII sites into our PCR primers (8) was not an option because the promoter contains an internal HindIII site. Figure 1 outlines our strategy. The pHCA0CAT promoterless vector was digested with HindIII. T4 DNA polymerase, dATP and dGTP were used to modify the ends so that they were no longer complementary. The 40-µL reaction consisted of 5 µg digested vector in 1× T4 Polymerase buffer (MBI Fermentas, Amherst, NY, USA) containing excess dATP and dGTP (1 mM each) and 10 U T4 Polymerase (MBI Fermentas). The reaction mixture was incubated at 14°C for 20 min, heat inactivated at 75°C for 15 min, stored at -20°C overnight and then heat-treated again at 75°C for 10 min.

To generate the mouse skeletal muscle actin promoter insert, we used as template a plasmid consisting of a 3.9-kbp BamHI/EcoRI skeletal muscle actin genomic fragment inserted into pSP62-PL (3). The primers were designed to amplify the 1647-bp region from the EcoRI site at -1538 to +109 in

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**Figure 1.** Schematic of plasmid construction using partial fill-in on the vector and limited T4 exonuclease/polymerase activity on the insert. Color legend for construct diagram: white = pBR322 sequence from HindIII to BamHI, including the ampicillin resistance gene; light grey = CAT gene/simian virus 40 (SV40) splice and polyadenylation sequences; dark grey = mouse skeletal muscle α-actin promoter. Mskma = mouse skeletal muscle actin.
the first intron of the transcribed region. Each primer sequence also included one of two unique restriction enzyme sites, *Mlu*I or *Xho*I, to allow for later construction of deletion mutations. The forward primer contained the *Mlu*I site and an *Eco*RI site corresponding to the site in the starting template. The reverse primer included the *Xho*I site. The template plasmid was linearized using *Eco*RI to maximize the efficiency of denaturation during thermal cycling. The amplification was carried out in 100-µL reactions, each containing 100 ng of template, 400 ng of each primer, 250 µM of each deoxynucleotide triphosphate and 2.5 U of *PfuTurbo™* DNA Polymerase (Stratagene, La Jolla, CA, USA) in 1× *PfuTurbo™* buffer.

Following amplification, five reactions were combined and the product was isolated using the QIAquick™ PCR Purification Kit (Qiagen, Valencia, CA, USA). A limited T4 exonuclease/polymerase reaction was carried out on 1 µg of the purified PCR product, as described above for the vector, but in a 50-µL final volume and in the presence of only dCTP and dTTP. This reaction modified the blunt-ended PCR product, leaving a 2-bp overhang complementary to the modified vector ends. Ligation reactions were set up using both a 1.25:1 and a 2.5:1 molar ratio of insert to vector using 250 ng of vector and 2–3 U of T4 ligase per 10–15 µL reaction volume. The reactions were incubated at 14°C overnight and heat-inactivated at 65°C for 10 min. *Escherichia coli* DH5α competent cells were then transformed with 10 µL of ligation reaction.

Partial fill-in of the *Hind*III ends before ligation reduced the number of colonies resulting from transformations with products from ligation reactions containing *Hind*III digested vector but no insert, as expected. Minipreparations of plasmid DNA were made from randomly chosen colonies resulting from transformations with products from ligation reactions containing both the vector and the insert. Transformations from either of the two ratios of insert to vector gave similar results, with 25%–30% of the colonies yielding plasmids carrying the insert, as reflected by the reduced mobility of intact plasmid DNA in an agarose gel. Plasmids not containing the insert were assumed to result from transformation by an undigested vector or a vector in which the *Hind*III sites remained unmodified, as had been observed with

![Image of agarose gel](image-url)

**Figure 2.** Restriction enzyme digests revealing orientation of insert and confirming destruction of the *Hind*III site in the vector. Results shown are from digests on pMskmaCAT, a plasmid found to have the insert in the desired orientation. Lane 1, 1 kb marker; numerals = bp. Lane 2, pHCA0CAT vector × *Hind*III; single band indicates single restriction site. Lane 3, pMskmaCAT × *Hind*III; single band larger than that in lane 2, consistent with loss of vector *Hind*III site and presence of single *Hind*III site within an insert. Lane 4, pMskmaCAT × *Xho*I and lane 5, pMskmaCAT × *Mlu*I; single bands confirm presence of unique sites engineered into primers. Lane 6, pMskmaCAT × *Xho*I and *Mlu*I and lane 7, pMskmaCAT × *Xho*, *Mlu* and *Hind*III; confirm release of insert containing *Hind*III site. Lane 8, pMskmaCAT × *Eco*RI; confirms the orientation of the insert. A 40-bp fragment spanning the 5′ end of the insert is not visible. Lane 9, λ-*Hind*III/*Eco*RI marker; numerals = bp.
control vector-only ligations and transformations, and were not further investigated. Plasmid DNA from presumed insert-containing transformants was subjected to a series of digests to determine orientation of the insert and to confirm the destruction of the vector HindIII site and the presence of the two newly engineered restriction enzyme sites (Figure 2).

Figure 3 shows that the approach we used can be adapted to accommodate almost any restriction enzyme site, whether the cut ends are 3′-recessed, blunt, or 5′-recessed. The length of the resultant 5′ overhang on the T4 polymerase-treated vector is determined by the sequence of the site and the choice of nucleotides. With some restriction enzyme activities, such as those represented by PstI or SmaI, it is necessary to know the sequence that flanks the recognition site. Because vector sequence is almost always known, this should not pose a problem. Note that it is not possible to use T4 polymerase to generate an overhang containing all four different nucleotides, as would be required to be complementary to an untreated HindIII site. Thus, in such cases, partial fill-in of the vector site is not simply an advantage, it is also a requirement. Like the vector ends, the ends of the insert PCR fragment can be modified with T4 polymerase whether they are blunt or have a 3′ overhang. Therefore, there is no restriction on choice of thermostable polymerase for the PCR amplification.

The approach we have described makes it possible to achieve the efficiency of sticky-end subcloning without the necessity of using a restriction enzyme digestion on the PCR product to render compatible ends. This is especially important when available vector sites are also located within the insert or when the sequence of the fragment is not known. Furthermore, even when the use of a restriction enzyme on a fragment is not prohibited by the presence of internal sites, one may confront inefficient digestion at a site located close to the end of a fragment. Built into our technique is the partial fill-in method for minimization of either insert or vector self-ligation.

Two other cohesive-end methods for PCR subcloning, sticky-end PCR (10) and A/T cloning (1,5,6), also avoid the need to use a restriction enzyme on the insert. However, sticky-end PCR requires three or four PCR primers rather than two and, as described by Zeng (10), uses a phosphatase treatment to minimize self-ligation. A/T cloning requires that the vector be modified by addition of a 3′ T overhang and, when Taq is not present in the PCR, also requires that the PCR product be modified by addition of a 3′ A overhang. Furthermore, to achieve cloning efficiency even as high as we observed, it is recommended that the modified vector be gel-isolated from unmodified vector (2). In contrast to the A/T method, limited T4 modification can be used to introduce ends with greater cohesiveness than that of a single A/T pair. For these reasons, the features of the limited T4 approach should combine to make it attractive for a broad variety of PCR-based subcloning projects.

REFERENCES


This work was supported by National Science Foundation Research at Undergraduate Institutions Grant Nos. MCB930-5351 and MCB9604289 to S.B.S. Address correspondence to Dr. Sandra B. Sharp, Biology and Microbiology, California State University, Los Angeles, 5151 State University Drive, Los Angeles, CA 90032, USA. Internet: ssharp@calstatela.edu

Received 3 June 1999; accepted 11 August 1999.

Nicole Green, Son Vu, Shadi Farahmand and Sandra B. Sharp
California State University, Los Angeles
Los Angeles, CA, USA