An improved ligase-free method for directional subcloning of PCR amplified DNA

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DNA cloning has become one of the most utilized techniques in scientific research. A limiting step in this process is the directional insertion of the target DNA into a cloning vector through the use of restriction endonucleases and DNA ligase. This procedure suffers from several limitations including poor ligation as well as the availability of unique restriction sites in both insert and vector. As a result, a large body of literature describing alternative methods for directional subcloning has come out in recent years. These methods (1-3) include using uracil DNA *N*-glycosylase, T4 DNA polymerase or exonuclease III to generate long compatible cohesive ends between the DNA insert and the cloning vector. The length of the cohesive ends, usually 10-15 nt as compared with 2-4 nt generated by restriction endonucleases, increases the probability of proper directional annealing of the insert and vector DNAs which in turn makes the use of DNA ligase unnecessary. The exonuclease III method (3) has advantages over the other two methods since using T4 DNA polymerase requires the construction of special cloning vectors and using uracil DNA *N*-glycosylase involves multiple steps including incubations at high temperatures. Exonuclease III, however, has its own limitations. The 3'→5' exonuclease activity acts on DNA with blunt or recessive but not protruding 3' ends (4) therefore requiring a unique restriction site in the cloning vector which results in such ends. Also, the reactions have to be performed on ice for very short times (30-90 s) to prevent excessive degradation of the DNA. Since exonuclease III is processive at low temperatures (5), we found it difficult to control the length of the generated cohesive ends; this becomes critical when the cloning of relatively small inserts is desired.

Here, we describe a method for directional subcloning of PCR amplified DNA that improves and expands previously described methods by using phage T7 Gene6 exonuclease in conjunction with primers containing internal phosphorothioate bonds. T7 Gene6 is a 5'→3' exonuclease which acts distributively on double stranded DNA with 5'-phosphoryl or 5'-hydroxyl ends (6). The enzyme is available commercially (US Biochemicals) and is inexpensive. As with other prokaryotic exonucleases, T7 Gene6

exonuclease hydrolyzes phosphorothioate bonds at a much reduced rate compared to phosphodiester bonds (7). We took advantage of this property in designing PCR primers which consisted of two domains (Fig. 1). The 3' domains of the primers were standard PCR primers while the 5' domains were complementary to sequences on the (+) and (-) strands of the cloning vector which straddle a unique restriction site such as the *EcoRV* site in pBluescript KS which we used here (Fig. 1 and ref. 3). Two phosphorothioate bonds were incorporated between the two domains. After PCR amplification of the target DNA, the product was digested with Gene6 exonuclease to generate 3' protruding ends as described in Figure 2. Although purification of the PCR product is generally advisable, in cases where a unique product is obtained with no contaminants, Gene6 exonuclease can be added directly to the reaction mix following PCR since its buffer requirements are compatible with those of *Taq* DNA polymerase. As can be seen in Figure 2, the presence of phosphorothioate bonds was very effective in controlling the extent of exonucleolytic digestion even when the reaction was carried out for 15 min at 22°C. It should be noted that the PCR product presented in Figure 2 was only 76 nt long and the phosphorothioate bonds were positioned 16 nt from the 5' ends, explaining the reduction in ethidium bromide staining intensity of the DNA after nuclease digestion. *EcoRV* restricted pBluescript KS⁺ was digested similarly with Gene6 exonuclease and mixed with the digested PCR product before transformation into competent *E.coli* cells using procedures similar to those described in reference 3. Table 1 lists the transformation efficiency of plasmid DNAs prepared as described here and containing one of four different inserts. As can be seen, high transformant numbers were obtained reproducibly. We found this method to be extremely useful in subcloning libraries of short DNA molecules containing regions of randomized sequences. Use of restriction endonucleases to subclone such DNAs would bias against molecules containing the recognition sequence of the restriction enzyme used.

Table 1. Transformation efficiency of DNA subcloned using the method described in this report

	Transformants number			Amount of	Transformants/
	Experiment 1	Experiment 2	Average	insert DNA (ng)	µg DNA
Insert 1	168	192	180	10	1.8×10^4
Insert 2	1280	1090	1185	25	4.7×10^4
Insert 3	288	272	280	25	1.1×10^4
Insert 4	304	324	314	60	5.2×10^3

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Figure 1. Schematic of ligase-free directional subcloning of PCR amplified DNA using T7 Gene6 exonuclease in conjunction with PCR primers containing internal phosphorothioate bonds.

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Figure 2. A 52mer oligonucleotide was PCR amplified with two 30 nt long primers which contained either phosphodiester or phosphorothioate bonds at the 13th and 14th position from the 3' ends. Approximately 400 ng of the PCR products (76mers) were then digested with 1 U of T7 Gene6 exonuclease for 5, 10 or 15 min at 22°C in 40 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 50 mM NaCl followed by electrophoresis on 2.5% agarose.

REFERENCES

- 1 Rashtchian, A., Buchman, G.W., Schuster, D.M., and Berninger, M.S. (1992) *Anal. Biochem.* **206**, 91-97.
- 2 Kuijper, J.L., Wiren, K.M., Mathies, L.D., Gray, C.L., and Hagen, F.S. (1992) *Gene* **112**, 147-155.
- 3 Hsiao, K. (1993) *Nucleic Acids Res.* **21**, 5528-5529.
- 4 Henikoff, S. (1986) *Gene* **28**, 351-359.
- 5 Wu, R., Ruben, G., Siegel, B., Jay, E., Spielman, P., and Tu, C.D. (1976) *Biochemistry* **15**, 734-739.
- 6 Kerr, C., and Sadowski, P.D. (1972) *J. Biol. Chem.* **247**, 311-318.
- 7 Nikiforov, T.T., Rendle, R.B., Kotewicz, M.L., and Rogers, Y.H. (1994) *PCR Methods Appl.* **3**, 285-291.