Efficient cloning of PCR generated DNA containing terminal restriction endonuclease recognition sites

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DNA generated by the polymerase chain reaction (PCR) (1) containing terminal restriction endonuclease recognition sites to permit cloning usually relies on the use of unphosphorylated primers incorporating a restriction endonuclease recognition site of choice plus 3-4 extra 5' bases flanking that site. Various sites (*Not*I, *Xho*I and *Xba*I (2), for example) incorporated into the termini of PCR products have proven difficult to cut with their respective restriction endonucleases. Possible explanations are:

(1)Taq polymerase is inefficient for certain terminal sequences, producing frayed ends that cannot be cleaved by the restriction endonuclease.

(2)'Breathing' of terminal sequences prevents stable association of the restriction endonuclease with the terminal site.

(3)Blockage of restriction endonuclease activity by Taq polymerase binding to the ends of the PCR products.

(4)The extra 3-4 bases at the terminal restriction endonuclease recognition site are insufficient to allow for stable association with and cutting by certain restriction endonucleases.

The data of Table I exclude explanations 1-3 since various combinations of steps designed to deblock (proteinase K), to stabilize (spermidine), or to repair (Klenow or T4 polymerase) the ends failed to increase the cleavage efficiency and hence the cloning efficiency. Also, prolonged or overnight digestion had no significant effect. We speculated that the inability to cleave terminal sites might be reversed by concatamerization of the PCR generated DNA fragment to convert the terminal restriction endonuclease recognition site into an internal site that would be more readily cleaved. To test this hypothesis, the PCR product was generated with the use of 5'-phosphorylated primers and was then concatamerized with T4 DNA ligase. The concatamerized DNA can be readily digested with the restriction enzyme specific for the terminal recognition sites. The cleavage products can then be conveniently ligated to a vector of choice.

As shown in Table I, the 1000-fold increase in cloning efficiency of the PCR product by this method suggests that certain terminal restriction endonuclease sites are less susceptible to cleavage and that this inefficiency can be eliminated by converting the terminal sites into internal sites through concatamerization of the DNA. The procedure does not require additional nucleotides beyond the restriction endonuclease recognition site. Therefore, the oligodeoxynucleotide primers can contain fewer extraneous nucleotides that do not hybridize to the target DNA sequence. Moreover, for palindromic restriction endonuclease recognition sites, only half the recognition site need be incorporated at the end of each primer since concatamerization would reconstitute the site. To accomplish this we used oligonucleotide primers with only half the *Xho*I restriction endonuclease recognition site (GAG) at the 5' end of each primer. With the use of phosphorylated primers as noted above, the amplified PCR product was effectively ligated and the ligated product cut with *Xho*I restriction endonuclease. This technique is also applicable to primers with different restriction sites. In that case, only one-quarter of the reconstituted sites will be cleavable. The procedure, therefore, increases the efficiency, specificity and economy of the PCR used for cloning with flanking restriction endonuclease recognition sites.

REFERENCES

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Table I

Experimental Conditions	Cloning Efficiency
Control	≤0.05%
Proteinase K	≤0.05%
Klenow	≤0.05%
T4 DNA polymerase	≤0.05%
Spermidine	≤0.05%
Prolonged Digestion with Restriction Endonuclease *Xho*I	
4 hr	≤0.05%
overnight	≤0.05%
Concatamerization	50%

Enzymes were used according to company specifications. The primers contained a *Xho*I site with 3 extra bases (G or C) and, after digestion with *Xho*I, the product was used for cloning portions of a Hu-IFN-γ receptor cDNA into a vector derived from pVJ3 (3). The cloning efficiency is the ratio of the number of positive colonies determined by colony hybridization to the total number of colonies obtained. Combinations of proteinase K treatment of the PCR product, followed by phenol extraction and filling the ends with either the Klenow fragment of *E. coli* DNA polymerase I (Klenow) or with T4 DNA polymerase were also tried with results identical to the use of each alone.

Phosphorylated primers used instead of unphosphorylated primers to prepare the PCR product yielded the same results as the control. The data in the table reflect results with the use of unphosphorylated primers except for the concatamerization reactions.

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