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Kinasing PCR Products for Efficient Blunt-End Cloning and Linker Addition

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In this report we provide evidence that inserting an additional step (kinase reaction) between the purification of polymerase chain reaction (PCR) products and the blunt-end cloning or restriction site creation by linker-addition greatly increases the efficiency of the PCR-product subcloning. The PCR-amplified DNA was purified first by chloroform extraction to remove the mineral oil and then ethanol precipitated. A brief kinase reaction was performed using T_4 Polynucleotide kinase and ATP before the "fill-in" reaction by Klenow enzyme to blunt end the DNA fragment. This intermediate kinase reaction was found to be the critical step that enhanced the blunt-end cloning of the PCR products and increased the efficiency of the linker-addition to a desired amplified DNA fragment.

PCR has become a routine tool for amplifying the desired piece of DNA from various sources of templates (8). We and others have experienced certain difficulties in subcloning the PCR products in desired vectors (9). One of the solutions to this problem is the treatment of the PCR product with proteinase K, thus providing access to the restriction endonuclease to efficiently cut and create the sticky ends that are inserted into the DNA fragment through predesigned primers flanking the amplified product (2). Another remedy is to add the restriction enzyme to the blunt-end ligation reaction to check the vector self-ligation (4). A number of reports have appeared to circumvent the undesired problems in subcloning the PCR-generated DNA fragments. In our attempt to subclone the extracellular ligand-binding domain of the murine guanylate cyclase coupled atrial natriuretic factor receptor (GC/ANF-R) (6,7), we have developed an efficient subcloning procedure to ligate the PCR fragments by adding desired linkers.

The extracellular ligand-binding domain of the murine GC/ANF-R was amplified from its cDNA clone (6). The PCR mixture was treated with 150 μl of chloroform, and the upper aqueous phase was recovered. The amplified product was ethanol precipitated following routine procedures (5). The 40 μl kinase reaction mixture contained 1–2 μg DNA, 66 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mg/ml bovine serum albumin (BSA), 20 mM ATP and 2.0 units T_4 polynucleotide kinase (Promega, Madison, WI). The reaction was allowed to proceed at 37°C for 1 h, and then the tubes were heated to 75°C for 10 min and cooled at 4°C. After the kinase reaction, the DNA was subjected to a fill-in reaction that contained 5 mM deoxyribonucleoside triphosphates (dNTPs) and one unit of the Klenow fragment of DNA Polymerase (Promega). After 30 min at room temperature, the reaction mixture was loaded onto a low melting point agarose gel to remove the primers. The blunt-ended DNA fragment was purified using the Geneclean® Kit (Bio 101, La Jolla,

CA), and then phosphorylated *Bam*HI linkers (GGCATCCC; Boehringer Mannheim, Indianapolis, IN) were added by the routine procedures (5).

The pGEX-3X plasmid vector was linearized by digestion with *Bam*HI and then dephosphorylated using calf intestinal phosphatase (Promega). The restriction endonuclease digestion of the linker-added DNA fragment (to generate the flanking *Bam*HI restriction sites) and ligation to the pGEX-3X plasmid vector (Pharmacia Biotech, Piscataway, NJ) were performed according to the published protocols (5).

We have had difficulties in subcloning the PCR fragment of extracellular ligand binding domain of GC/ANF-R into the *Sma*I site of the pGEX-3X prokaryotic expression vector in order to maintain the open reading frame for the synthesis of fusion protein in *Escherichia coli.* Treatment of the PCR product with Klenow enzyme did not make the DNA fragment compatible for the ligation. The products of the thermostable *Taq* DNA Polymerase (Perkin-Elmer, Norwalk, CT) reactions contain “ragged ends” that are caused by non-template, nucleotide-addition reactions and interfere with the blunt-end cloning of the DNA fragment and other amplimers (1). Consequently, cloning of these products is a universal problem and in most cases must be overcome by “building-in” flanking restriction enzyme sites or by filling in or cutting off the non-template nucleotide additions (3). We have made an attempt to circumvent this problem by adding the linkers and generating sticky ends so that the DNA fragment could easily be ligated to the *Bam*HI site of the plasmid vector. When we followed the original procedure, we faced the difficulty of adding the linkers to the Klenow-treated fragment, and thus both the blunt end ligation and the linker addition were unsuccessful. We assumed that the PCR product still remained ragged at its flanking sites. In PCR, the 5′ ends of the PCR products do not contain 5′ phosphate. The absence of this phosphate group probably hinders the addition of an extra base. Possibly for this reason, the Klenow fill-in reaction did not work and prohibited further ligation to the blunt end sites of the vector to the linkers. Because the Klenow fill-in reaction was not successful, we decided to add a kinase reaction. As shown in Table 1, approximately 33% of the clones were found to be the recombinants using our strategy of kinasing the PCR products for restriction site creation and/or blunt end cloning. Following this method, the subsequent cloning of the different PCR products to the blunt end sites of other vectors has also been found to be very successful.

It is evident that in particular circumstances like ours, using only the Klenow reaction does not anneal all the ragged ends of the PCR products. Because adding the kinase reaction increased both the efficiency of the ligation and of the linker-addition, we recommend kinasing the amplified DNA fragment prior to the Klenow fill-in reaction for efficient blunt end ligation and linker-addition during the PCR subcloning.

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Table 1

Ligation Efficiency of PCR-Amplified DNA to *Bam*HI Site of pGEX-3X Plasmid Vector

Treatment of PCR Products Before Linker Addition	Number of Clones Analyzed	Positive Clones	%of Recombinants
Without kinase reaction a	8	ND	ND
With kinase reaction b	6	2	33

Each ligation reaction constituted a 10 μl volume from which 1 μl was used to transform bacterial cells. Competent cells were prepared by $CaCl_2$ method (7).

Reaction a is average of 10 separate experiments.

Reaction b is average of 3 separate experiments.

NO = Not detectable.