Benchmarks

Effect of Growth Temperature and Vector Ends Phosphorylation in the Cloning Efficiency of Specific DNA Fragments

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Subcloning of DNA fragments into plasmid vectors is a routine procedure in molecular biology laboratories. However, occasionally DNA fragments are difficult to clone into specific vectors, resulting in low efficiency of isolation of individual constructs. During subcloning experiments for the study of regulation of expression of the mouse gamma-glutamyl transpeptidase gene (*GGT*) gene, we encountered difficulties in cloning a 416-bp DNA fragment that includes part of the untranslated region of the type II *GGT* RNA and 346 bp of the type II promoter region (4). In this study, we tested whether omitting the alkaline phosphatase treatment of the cloning vector 5' ends and lowering the growth temperature of transformed bacteria to 30°C would result in higher yields of plasmid-insert constructs.

The *GGT* type II 416-bp fragment was obtained by polymerase chain reaction (PCR) amplification of a plasmid containing a 6.0-kb insert of the *GGT* promoter region (3). The amplifying primers contained a *Xho*I restriction site to be used for subsequent subcloning. The PCR products were subcloned into the pT7Blue vector and transfected into NovaBlue® Competent Cells (both from Novagen, Madison, WI, USA). Individual clones of the pT7Blue vector containing the insert were digested with *Xho*I to release the *GGT* type II 416-bp fragment, which was then used for subcloning experiments into the promoterless vector pJFCAT (1). The pJFCAT vector was linearized with *Xho*I, followed by treatment with alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN, USA), at 37°C for 60 min, and inactivation of alkaline phosphatase by heating the reaction at 65°C for 10 min. The ligation of the *GGT* type II 416-bp *Xho*I fragment into pJFCAT was performed using equimolar amounts of

vector and insert; it was ligated overnight at 16°C, with T4 DNA Ligase (Promega, Madison, WI, USA) in a 10-μL final volume. The ligation products (1 μL) were used to chemically transform NovaBlue Competent Cells, as described by the manufacturer. Because this procedure did not yield positive clones containing the insert, and the overall number of bacterial clones was small, we also transformed Epicurian Coli® SURE® Electroporation-Competent Cells (Stratagene, La Jolla, CA, USA). After transformation, bacterial cells were grown for 1 h at 37°C, plated and grown overnight at 37°C onto ampicillin plates. All of the isolated clones invariably yielded a combination of the original vector (pJFCAT), possibly resulting from intramolecular ligation of non-dephosphorylated plasmid and a plasmid of abnormal size (Figure 1); however, no clones containing the *GGT* type II 416-bp *Xho*I fragment were identified. The clones were screened by digestion with *Aat*II (Boehringer Mannheim), because one recognition site for this enzyme is present in the cloning vector (pJFCAT), and another site is present within the *GGT* type II 416-bp *Xho*I fragment. The expected results after digestion of pJFCAT clones containing the *GGT* type II 416-bp *Xho*I fragment with *Aat*II include: (i) 5.6-kb plasmid, representing linear forms of the pJFCAT cloning vector, and (ii) clones contain-

ing the insert should release a 1.4-kb fragment after digestion with the enzyme (Figure 1). However, we could only identify linear forms of pJFCAT and plasmid forms with abnormal molecular weight. These results surprised us because we have previously cloned larger fragments of the *GGT* type II promoter region containing the 416 bp into the pJFCAT vector (3).

A previous report indicated that growing bacterial clones at 30°C, after transformation of bacteria with ligation products resulted in a higher level of recovery of inserts (2). Therefore we tried to grow the bacteria after transformation with the ligation products at either 37° or 30°C. In addition, we used pJFCAT with and without alkaline phosphatase treatment in the ligation reactions. The cloning vector (pJFCAT) was linearized with *Xho*I, either treated with alkaline phosphatase or not and was then isolated by gel purification to decrease the possibility of contamination with non-linearized vector. The *GGT* type II 416-bp region was released from pT7Blue after digestion with *Xho*I, and then the fragment was isolated by gel purification. Approximately equimolar amounts of vector and insert were ligated overnight at 16°C. Electroporation-competent SURE cells were transformed and, after shocking, were allowed to grow for 1h at either 37° or 30°C and then plated onto ampicillin plates at the same temperatures. Twenty

Figure 1. (S) represents the supercoiled plasmid forms, which were run in parallel to demonstrate the differences in product sizes after digestion of the same clone by *Aat*II (A). Clone 1 is the cloning vector pJFCAT. Clones 2 and 3 (insert) represent examples of subclones containing the 1.4-kb fragment released by digestion with *Aat*II, expected in clones containing the *GGT* fragment. Clones 4 and 5 represent abnormal clones recovered during subcloning, including a plasmid with smaller molecular size (AC5) and a plasmid that did not digest with *Aat*II (AC4).

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Table 1. Effect of Temperature (37° or 30°C) and of Alkaline Phosphatase (AP) Treatment of Cloning Vector Ends on the Recovery of Insert-Containing Subclones

Ligations	Temperature	AP ^a	pJFCAT-GGT416 ^b	pJFCAT ^c	Abnormal Clones
pJFCAT + 416 bp	30°C	N	40% (8)	30% (6)	30% (6)
pJFCAT + 416 bp	37°C	N	15% (3)	25% (5)	60% (12)
pJFCAT + 416 bp	30°C	Y	0% (0)	15% (3)	85% (17)
pJFCAT + 416 bp	37°C	Y	0% (0)	45% (9)	55% (11)
pJFCAT	30°C	N	N/A ^d	100% (10)	0% (0)
pJFCAT	37°C	N	N/A	100% (10)	0% (0)

^aTreatment of vector ends with alkaline phosphatase (Y) or absence of treatment (N).
^bTotal number of pJFCAT + GGT 416-bp clones tested (20).
^cTotal number of pJFCAT clones tested (10).
^dN/A, not applicable.

clones were randomly picked from each plate and grown overnight at the indicated temperature. Plasmid was isolated and screened by digestion with *AatII*.

The results are indicated on Table 1. No clones containing the *GGT* type II 416-bp inserts were identified when the linear pJFCAT vector was treated with alkaline phosphatase and therefore contained dephosphorylated ends, either growing the clones at 30° or 37°C. Under these conditions, we could only identify the original pJFCAT or plasmids that migrated with an abnormal molecular weight (Figure 1). These abnormal molecular-size forms of the pJFCAT plasmid were extremely frequent when the cloning vector was treated with alkaline phosphatase, representing 17 (85%) of the clones grown at 30°C and 11 (55%) of the clones grown at 37°C. In contrast, when pJFCAT plasmid ends were not treated with alkaline phosphatase, positive clones (vector containing the *GGT* 416-bp insert) were obtained at both 37° and 30°C; however, a higher number of positive clones was obtained when the bacteria were grown at 30°C, resulting in eight pJFCAT-GGT416 clones (40%). As control, we used ten clones of the pJFCAT vector alone after digestion with *AatII*, followed by ligation and growth of the transformed bacteria at either 30° or 37°C. In both instances, all the re-annealed vectors had the expected pattern of migration (Table 1).

These data show that both the tem-

perature used to grow competent bacteria after transformation with ligation products and treatment of the cloning vector with alkaline phosphatase can affect cloning efficiency. Growing the bacteria at lower temperature (30°C) resulted in higher yield of positive clones when the cloning vector was not treated by alkaline phosphatase. In our experiments, we could not isolate any vectors with the *GGT* 416-bp insert when the vector ends had been treated with alkaline phosphatase. In addition, the inability to obtain positive clones was associated with the identification of abnormal forms of the pJFCAT plasmid. We interpreted those abnormal plasmid forms as possibly resulting from recombinational events, although we used Epicurian Coli SURE competent cells, which are known to have low levels of recombinational events. It is possible that specific structural constraints or sequence composition of our insert in the context of this particular cloning vector resulted in the cloning difficulties encountered. It is noteworthy that the same *GGT* 416-bp fragment was successfully cloned into the pT7Blue vector with the expected efficiency.

In conclusion, in this study, we show that unpredictable difficulties in cloning specific DNA fragments can be corrected by simple procedures such as avoiding treatment of the cloning vector ends with alkaline phosphatase and by growing the products of ligation at lower temperature (30°C).

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