Simplified Method for Ligase-Free Cloning of PCR Products

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Cloning of polymerase chain reaction (PCR) products into plasmid vectors by conventional methods, relying either on recognition sequences built into the primers or taking advantage of the template-independent terminal transferase activity of *Taq* DNA polymerase to add an extra adenine to the 3′ end of the product, are often difficult (1,10,12). In recent years, numerous cloning strategies (10,13) have been developed to overcome these problems. We were using one such technique, the ligase-free subcloning method (13), to clone PCR products into plasmid vectors. However, we found that the method is still time-consuming and, above all, leads to a considerable number of wrong products. On the average, 30% of the recombinant plasmid products were found to be wrong. These were probably formed mostly during the long heterologous reannealing step of the method. Avoiding this step and transforming cells directly with a single second PCR product were attempted (13), but the structure of the few plasmids obtained in this way was not examined. There are many reports in the literature of transformations with linear plasmid DNA (3,11,14). Depending on both the DNA and the type of host cell used, a low yield of transformants and frequent modifications of the transformed DNA by exonucleases or recombination events, were observed after in vivo ligation (14).

We have applied a simplified ligase-free cloning method, which requires only three primers and rests essentially on a direct transformation of *E. coli* cells with linear DNA produced in a second PCR. Plasmids containing cDNA fragments from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and liver, muscle and heart isozymes of rat 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/F2,6Pase) (Figure 1) were constructed. Sequences of the primers used are given in Table 1. Primer b was used for the synthesis of first-strand cDNA from total RNA by reverse transcription (Figure 1). Primers a and b were used for the first PCR. Primer b contains, in addition to specific sequences of about 25 nucleotides at its 3′ ends, additional 5′-end sequences of 24 nucleotides, which are identical to the 3′ end of linearized pT3T7BM (digested with *Hin*cII). In the second PCR, the initial amplified fragment and linearized pT3T7BM were annealed and amplified using primers a and c (Figure 1). Primer c, 5′-TTTAGTGAGGGTTAATTTCGAG-3′, a downstream primer and identical in all cloning reactions, is complementary to the (+)-strand of the plasmid. The linear plasmid produced was directly used to transform competent *E. coli* TG2 cells (7). Typically, about 250

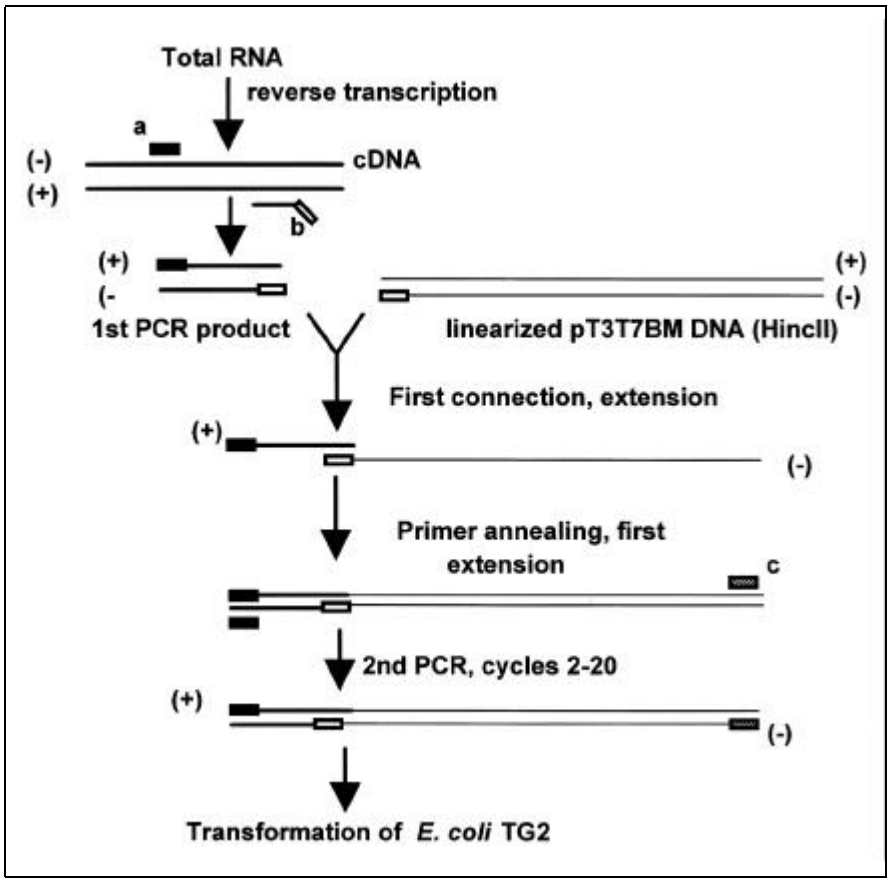

Figure 1. Construction of vectors by ligase-free subcloning and transformation of bacterial cells with linear DNA. cDNA fragments are obtained from total RNA by reverse transcription using a specific reverse primer (primer b). Primers a and b were used for the first PCR. In addition to target sequences at its 3′ ends, primer b contains an additional 5′-end sequences of 24 nucleotides, which are identical to the 3′ ends of pT3T7BM linearized by *Hin*cII digestion. First PCR: initial denaturation (95°C, 1 min), 25 cycles (1-min denaturation at 95°C, 1-min annealing at 50°C, 1-min and 30-s extension at 72°C). The amplified fragment was purified by electrophoresis on low-melting-point agarose to eliminate unused primers and then assayed by fluorescence (8). The second PCR with primers a and c included the purified fragment and linearized pT3T7BM (each 15 ng). Primer c, a downstream primer, is complementary to the (+)-strand of the plasmid pT3T7BM. 20 cycles of PCR were performed (1-min denaturation at 95°C, 1-min annealing at 55°C and 4-min extension at 72°C). In all cases, PCR was done in a final volume of 25 µL in 10 mM Tris-HCl, pH 8.3 at 25°C, 50 mM KCl (GeneAmp® PCR buffer II; Perkin-Elmer, Norwalk, CT, USA) supplemented by 1.5 mM $MgCl_2$, 50 µM each dNTP and 25 pmol of each primer using a GeneAmp DNA Thermal Cycler 480 (Perkin-Elmer). 1.0 U AmpliTaq® DNA polymerase (Perkin-Elmer) was added after heating the reaction mixture to 85°C (hot start). Note: As described above, the sequence of each inserted cDNA fragment was found to be correct. Nevertheless, the risk of error formation during *Taq* DNA polymerization (2) of such long plasmid can still be minimized by decreasing further the concentrations of $MgCl_2$, dNTPs, enzyme and the cycle number. 10 µL of the product of the second PCR were directly used to transform competent *E. coli* cells (9). *E. coli* TG2, a rec A-version of TG1 [(lac-pro), thi, supE, hsdR-, hsdM-, F′(traD36 proA+B+, lacIq, lacZ M15)] (7) was used for cloning.

Table 1. Sequences of the Oligodeoxyribonucleotide Primers Used for Reverse Transcription and PCR of Rat L-, M- and H-Types of PFK-2/F2, 6Pase and GAPDH

Gene	Primer	Sequences (5′——3′)
L-PFK-2/	a	AAAGAGGGAGCTGGAGATAATCTGT
F2, 6Pase	b	TGCGGCCGCGGTACCGGGCCCGTCTACTGCTGCTGTGTGGAATCCAGAT
M-PFK-2/	a	CCAACGGATGTCTCCGAGTTTTCTA
F2, 6Pase	b	TGCGGCCGCGGTACCGGGCCCGTCTCTCTTAGAGGCTTTTTCTTCCATC
H-PFK-2/	a	GAGCACTTGCCTCTTTTCATGAGAA
F2, 6Pase	b	TGCGGCCGCGGTACCGGGCCCGTCAGAAACATGTACCAGAGTGGGCTGG
GAPDH	a	AGCGAGATCCCGTCAAGATCAAATG
	b	TGCGGCCGCGGTACCGGGCCCGTCATCCACAACGGATACATTGGGGGTA

Oligodeoxyribonucleotides were synthesized on a Gene Assembler® Plus (Pharmacia Biotech, Piscataway, NJ, USA) by β-cyanoethyl phosphoramidite chemistry and purified by gel filtration on Sephadex G-25.

colonies of transformants were obtained per PCR product. This yield, though being about four orders of magnitude smaller than that obtained after transformation with circular DNA, is sufficient. When plasmids, purified from transformed cells, were analyzed by restriction mapping, no background of original pT3T7BM was observed. The yield of plasmids with correct inserts was found to depend on the annealing temperature of the second PCR. This is shown for plasmid pLPFK-2 containing a cDNA fragment specific for the liver-type of PFK-2/F2,6Pase (Table 2). An optimal annealing temperature was applied in experiment 1, a lower annealing temperature in experi-

Table 2. Direct Transformation of Competent *E. coli* TG2 Cells with Different Preparations of Plasmid pLPFK-2

Experiment	Experimental Condition	No. of Total Colonies	Colonies with Correct Insert[a]
1	2nd PCR product (annealing temp. = 55°C)	240	100%
2a	2nd PCR product (annealing temp. = 50°C)	180	70%
2b	Product from 2a and Klenow + dNTPs	192	60%
2c	Product from 2a and T4 DNA ligase	155	60%
2d	Product from 2a and Klenow + dNTPs and T4-Ligase	>>1000	n.d.

The plasmid pLPFK-2 contains a 243-bp cDNA fragment of exon 1b (4) of rat liver PFK-2/F2, 6Pase.
[a]As determined from 20 randomly selected independent colonies.
n.d. = not determined.

ment 2a. Neither treatment of the second PCR product from experiment 2a by either Klenow fragment of DNA polymerase I plus dNTPs or by T4 DNA ligase, before transformation increased the total number of colonies nor the yield of correctly assembled plasmids (Table 2). In a control experiment, ligation by T4 DNA ligase after Klenow treatment increased the number of transformants substantially (Table 2).

Our procedure was further applied to create plasmids pMPFK-2 containing exon 1a (115 bp) of M-type PFK-2/F2,6Pase (4), pHPFK-2 containing exon 15 (620 bp) of H-type PFK-2/F2,6Pase (5) and pGAP containing the sequence between bases 300 and 800 of the cDNA of GAPDH (6). In each case, the total yield of transformants and the yield of correctly assembled plasmids were comparable to those observed for the construction of pLPFK-2 (Table 2, experiment 1). Plasmids prepared from randomly selected colonies were analyzed by DNA sequencing between the T3 and T7 promoters. In each case, the sequence of the inserted cDNA fragment was found to be correct. Insertions or deletions at the cloning sites were never observed. The above mentioned plasmids were successfully used to synthesize labeled RNA probes by in vitro transcription in a study of gene expression of PFK-2/F2,6Pase in different tissues of normal and glutamate obese rats (15).

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