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Efficient cloning of fragments of the polymerase chain reaction directly into the single stranded bacteriophage M13mp18

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Attempts to clone non-modified PCR-fragments into blunt-ended digested vectors have been inefficient, due to the template-independent terminal transferase activity of the Taq-polymerase which results in the addition of a single adenine nucleotide at the 3′ end of the fragment (1, 2). An alternative method has been described using a blunt-ended vector to which a single thymidine has been transferred by the terminal transferase activity of the Taq-polymerase (3). However, the activity of the Taq-polymerase does not result in 100% overhanging 3′ thymidines, and some of the vectors remain blunt-ended. Ligation leads to a high percentage of insertless vectors. A commercially available cloning system (TA Cloning™ System, Invitrogen, San Diego, USA) contains the vector PCR™II which already has overhanging 3′ thymidines. However, the vector is described as being unstable in solution, resulting in less efficient cloning of the PCR-fragments when it has been dissolved for at least two months (instructions to the 'TA Cloning™ System', version 1.1). In this case a high percentage of plasmids without insert is obtained and these can only be distinguished from the positive ones by restriction analyses. Moreover, in cases where single stranded DNA is desired from PCR™II, the host cells must be infected by a helper phage and the yield of a single stranded DNA preparation is much lower than that from filamentous bacteriophage vectors carrying the same segment of foreign DNA (4). In addition, the best results in sequencing DNA with a high G+C content of about 70%, as in *Azospirillum*, were obtained with single stranded sequence templates.

A complete new method was constructed to stabilize PCR-fragments with their overhanging 3′ adenosine residues directly in single stranded phage M13mp18, for use in deletions, site directed mutagenesis and for quick and efficient preparation of single stranded DNA e.g. for DNA sequencing. Three oligonucleotides (Figure 1) and single stranded M13mp18 DNA were necessary for this method, synthesized by a Pharmacia LKB Gene Assembler Plus (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and purified by separation on a 1.5 ml NAP™-10 column (Pharmacia LKB). 0.5–5 pmol of the first oligonucleotide (Figure 1) were annealed at the polylinker of single stranded M13mp18 (400 ng M13mp18 DNA, Gibco BRL, Life Technologies GmbH, Berlin, FRG) by an incubation of 2 min at 70°C in a total volume of 100 µl buffered with 1×REACT2 (Gibco BRL). After slowly cooling down to 37°C a complete restriction was performed with *Xba*I (2 h, 5 U, Gibco BRL). The reaction was stopped (70°C, 10 min), the enzyme removed by phenol/chloroform extraction, the DNA precipitated, dried and resuspended in sterilized water. For the generation of a thymidine overhang at both ends of the restriction site, 5 pmol oligonucleotide 2 (Figure 1) and 5 pmol phosphorylated oligonucleotide 3 (Figure 1) were annealed (10 min, 70°C, slow cooling down to 37°C) to 50 ng of the *Xba*I digested single stranded M13mp18 in a final volume of 8 µl containing 1 µl 10×ligation buffer (Boehringer, Mannheim, FRG). Subsequently, 1 µl of the PCR-assay with the amplified fragment (10 ng DNA of 0.5 to 1 kb size) and 2 U T4 DNA ligase (Boehringer, Mannheim) were added and ligation was performed at 12°C for 16 h. The complete ligation mix was transformed into 100 µl of freshly prepared competent cells of *E.coli* TG1 (5) and incubated in LB topagar (4) containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and isopropylthio-β-D-galactoside (IPTG) at 37°C for 16 h.

Recircularization of the vector without insert was only possible in case of a T:T mismatch. As the M13mp18 with the overhanging thymidine could easily be prepared freshly, the chance for loosing the overhang and blunt-end ligation was low. In addition the losses could be screened as blue plaques on the X-Gal containing LB petri dishes. Excess of oligonucleotide 1 led to recircularization of the original vector whereas insufficient quantities resulted in incomplete digestion. The best results, of 30 to 40% insert carrying, β-galactosidase negative white plaques (amoung the 50 to 100 plaques per petri dish), were obtained

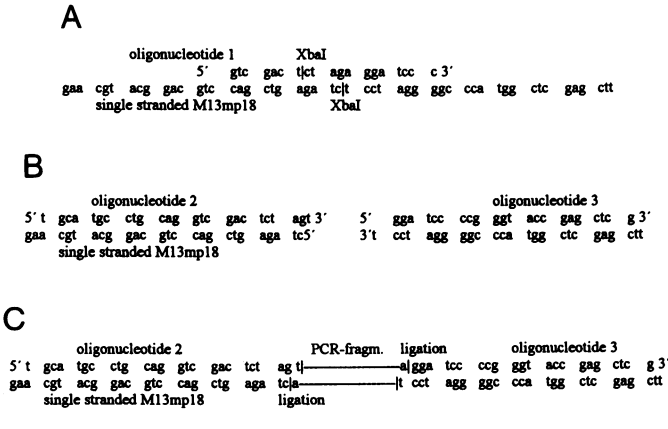

Figure 1: Scheme of the cloning procedure. 1. Annealing of oligonucleotide 1 and restriction by *Xba*I (**A**). 2. Detaching of digested oligonucleotide, annealing of oligonucleotide 2 and oligonucleotide 3 (**B**). 3. Ligation with PCR-fragment (**C**). 4. Transformation.

with 0.5 to 5 pmol oligonucleotide 1 per 400 ng of single stranded M13mp18 DNA. Lower and higher concentrations finally led to increased numbers of blue insertless plaques on the selection plates.

This new method was exemplified to clone PCR-fragments obtained from a DNA-region about 8.4 kb upstream of the *trpGDC*-cluster of *Azospirillum brasilense* Sp7 leading to the identification of *pyrG* which encodes a CTP-synthase (6). In this case, ten β-galactosidase negative plaques were selected after the ligation of the PCR-fragments (6) into M13mp18 and transformation into *E.coli* TG1. The sizes of the inserts were determined by restriction analyses. 60 to 90% of the tested phages contained a fragment of the same size as the original PCR-fragment. Sequencing of these inserts resulted in a recovery of the oligonucleotids in the fragments in all cases (6). The sequences at the ligation-sites between oligonucleotide 2 and the oligonucleotides used for the PCR-amplification were exactly as predicted. This was also true for oligonucleotide 3. Moreover, the method was successfully used to clone a PCR-fragment of the dissimilatory nitrite reductase gene of *Azospirillum brasilense* Sp7 (7).

After the synthesis of one set of the 3 oligonucleotides the described method allows an economically favourable cloning of PCR-fragments in some 10 000 different assays. It was adopted and successfully applied to clone PCR-fragments into the polylinker of the phage M13mp18, but it can be extended to phagemids. The same three oligonucleotides can be used for the phagemid pUC118 where single stranded DNA can be obtained by infecting the host cells with the helper virus M13KO7 (8). For other phagemids like pBluescript (9) the use of analogous oligonucleotides would allow the generation of overhanging thymidines at an *Xba*I site.

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