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Simple and efficient method for cloning of large DNA fragments with identical ends into plasmid vectors

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Recloning of an inserted fragment or its part from a recombinant bacteriophage into a plasmid vector is almost always necessary. However, when the fragment of interest is long and has identical cohesive or blunt ends, conventional methods are not satisfactory(1). Here we introduce a novel efficient method based on controlled ligation, the insertion conditions being optimized independently of those for circularization. As the first step which favours generation of linear concatemers a linearized plasmid is ligated with a molar excess of the insert under high total DNA concentration. When the stage of dimer formation is reached the conditions are changed by dilution to favour circularization. This procedure yields high proportion of circular molecules capable of transformation. Most importantly, representation of recombinants is so abundant that plasmids with insert can be found directly by analysing minipreps from a few clones without any previous screening or selection.

The procedure which could alternatively be used consists in the ligation of the vector and the insert allowing the formation of long concatemers which are subsequently cleaved with a restriction endonuclease having a unique target site only in the vector; the resulting fragments are then circularized (2). This method is very simple and efficient, but requires preliminary mapping of the recombinant phage (the procedure one wants to avoid by recloning) and not always can a suitable enzyme for concatemer cleavage be found. Our method circumvents this limitation and is also quite simple. It has been successfully used for recloning a 9.8 kb fragment carrying MAV provirus (3) and a 10 kb fragment containing regulatory region of the c-myb gene (in preparation).

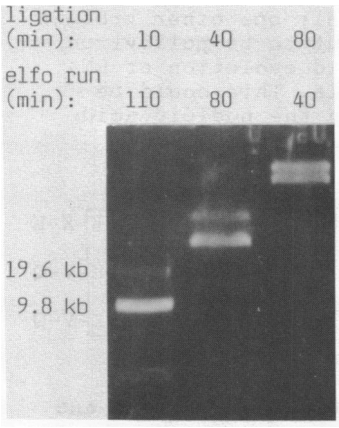

Fig. 1: Time course of the ligation reaction

Example of procedure: 50 ng of an EcoRI digested pAT 153 plus 500 ng of a 9.8 kb EcoRI fragment (2) were ligated in a 5 µl volume at a slow reaction rate (0.02 U T4 DNA ligase, 0° C). To check the degree of ligation 0.2 µl samples were analysed on an agarose minigel (length 3 cm, 0.4 % agarose, TBE, 5 V/cm) - Fig. 1. With respect to the DNA amount analysed, the degree of ligation could only be derived from the formation of insert oligomers (which in our case all comigrated). After 120 min incubation (this approximate time of optimal dimer representation was obtained by extrapolating 40 and 80 min reaction patterns) the mixture was diluted with 250 µl of the ligase buffer, supplemented with 2 U of ligase and incubated 20 h at 20° C. 1/5 of purified product (e.g. originating from 10 ng of vector and 100 ng of insert) was used for transformation and yielded approx. 2000 colonies (the same number as after parallel control transformation with 10 ng of circular pBR 322). 3 of 12 randomly chosen colonies contained the cloned 9.8 kb insert. Using conventional (1) one step ligation procedure (50 ng of linearized and dephosphorylated pAT 153 plus 150 ng of the 9.8 kb fragment was ligated with 0.5 U ligase in a volume of 50 ul at 20°C for 20 h) we obtained only about 100 colonies from the purified product. None of 24 colonies analysed contained the desired insert.

References: (1) Maniatis, T., Fritsch, E. F., Sambrook, F. (1982) Molecular Cloning: A Laboratory Manual, p. 14. Cold Spring Harbor Laboratory, New York; (2) Upcroft, P., Healey, A. (1987), Gene 51, 69; (3) Pečenka et al. (1987), submitted to Folia Biol. (Praha).