A method for the cloning of unpurified single-stranded oligonucleotides

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Oligonucleotides can now be synthesized with a repetitive yield per added base of greater than 98%. This has made gene synthesis and replacement experiments very attractive as an alternative to cloning naturally occurring sequences. However, at lengths of greater than 100 bases, the single-stranded molecules become exceedingly difficult to purify by current methods, mainly polyacrylamide gel electrophoresis (PAGE) and high performance liquid chromatography (HPLC). To overcome this constraint, we have tested the ability of the in vivo gap repair method (1) to clone large oligonucleotides directly from unpurified, deprotected synthesis–reaction products. The method relies on the inability of E. coli to be transformed efficiently with linear DNA plasmid molecules. The long oligonucleotide is annealed to a suitably adapted vector DNA. The 5' and 3' ends of the vector have been modified to act as affinity groups for the complementary ends found at the 3' and 5' ends of a completed long oligonucleotide. The method is diagrammed in Figure 1. Using this method we have successfully cloned oligonucleotides with lengths greater than 150 bases.

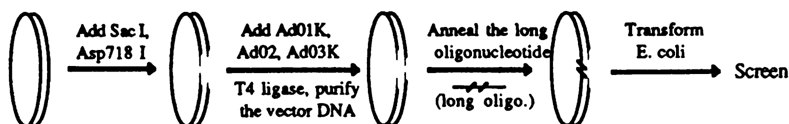

Figure 1. Schematic diagram of the method. Twenty μg of pBlueScript vector DNA (pBS; Stratagene, Inc.) was digested to completion with Sac I and Asp718 I. The 4-base recessed 5' phosphate of Sac I and the 4-base protruding 5' phosphate of Asp718 I were modified to 9-base recessed and protruding 5' ends, respectively, using the adapters Ad01K (5'–pGCCGCT–3'), Ad02 (5'–AAGCTTGGGAGCGGCAGCT–3') and Ad03K (5'–pGTACAGCGGCGAATTCGGT–3') at a molar ratio of 200:100:100:1 (Ad01K:Ad02:Ad03K:pBS). Ad01K and Ad03K were treated with T4 kinase (Stratagene, Inc.; 1 unit of enzyme per μg oligonucleotide, 37 °C, 12 hrs.) prior to ligation. Conditions for ligating the adaptors to the vector can be found in Seth (2). The unligated adaptors were separated from the modified vector by HPLC (Gen–Pak Fax column; Waters, Inc.) using conditions specified by the manufacturer. The column fraction containing the modified vector (as determined by agarose gel electrophoresis) was diluted to a concentration of 0.02 μg μl^{-1} and an aliquot of it divided into four tubes containing the unpurified, extensively deprotected (25.7 M ammonium hydroxide, 55 °C, 36 h.) long oligonucleotide at final concentrations of 100, 10, 1 and 0.1 ng μl^{-1}. These dilutions were derived from pilot experiments showing that a high concentration of oligonucleotide inhibited the recirularization of the vector, presumably by binding at both the 3' and 5' ends of the vector. Treating the long oligonucleotide with T4 kinase and the annealed oligonucleotides with T4 ligase or DNA polymerases was unnecessary. The annealing reactions were carried out by heating the four tubes to 65 °C for 10 minutes and then allowing them to cool to 22 °C. The annealed mixture was transformed into E. coli. Successful cloning of the long oligonucleotides was confirmed initially by colony hybridization using the crude long oligonucleotide as a probe and subsequent plasmid DNA sequencing of hybridization–positive colonies. We believe that the increased deprotection time decreases the mutagenic events in cloned sequences; of the over 1000 bases that we have cloned with this method we have not detected any mutations.

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