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T-Cloning Vector for Plasmid-Based 16S rDNA Analysis

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Certain DNA polymerases, including the thermostable *Taq* DNA polymerase, add a single deoxyribonucleotide, predominantly adenylate, to the 3'-termini of blunt-ended DNA (1). Polymerase chain reaction (PCR) products derived with such polymerases can be cloned directly into T vectors containing single 3'-terminal thymidylate overhangs (2,4,6).

T vectors are used extensively to construct recombinant libraries by direct cloning of PCR-derived DNA. We use sequence analysis of 16S ribosomal RNA genes (rDNA) as a tool to determine phylogenetic relationships among organisms and to study microbial diversity in the environment (see Reference 3 for review). rDNAs are amplified by PCR using rDNA-specific primers, then cloned inserts of the rDNA libraries are again amplified by PCR for sorting by restriction fragment-length

polymorphism (RFLP), and representative RFLP types are sequenced. Selected clones also can be used to make RNA probes of the rDNA insert by in vitro transcription. Ideally, plasmid encoded primer-binding sites and promoter regions for RNA polymerases are used for this purpose. Unfortunately, commercially available T vectors such as pGEM®-T (Promega, Madison, WI, USA) or pCR® 2.1 (Invitrogen, Carlsbad, CA, USA) include lengthy sequences between these sites and the TA cloning sites, which results in large extensions of cloned inserts during PCR, sequencing and in vitro transcription. This compromises the utility of these vectors for plasmid-based 16S rDNA analysis for several reasons: (i) PCR products derived from different clones with identical 16S rDNAs, but in the opposite orientations, often show different RFLP patterns; (ii) when sequencing PCR products from plasmid universal priming sites, a significant portion of the sequence data is plasmid-derived; and (iii) 16S rRNA probes made by in vitro transcription carry significant plasmid-encoded sequence that can interfere in hybridization experiments with plasmid-containing cells.

To circumvent these limitations, we have constructed the plasmid pCPT9, an efficient alternative to commercially available T vectors. For TA cloning (2,6) two recognition sites for the endonuclease *Xcm*I (7) and for regular cloning unique recognition sites for the endonucleases *Bam*HI, *Bst*EII (5), *Eco*0109I, *Eco*RI, *Nde*I, *Nsi*I and *Ppu*10I were introduced into the multiple cloning site (MCS) of pCPT9. Relative to the TA cloning sites, the promoter regions for T7 and SP6 RNA polymerases are located only 19-bp upstream and 21-bp downstream, respectively. This limits the size of plasmid-encoded regions during PCR, sequencing and in vitro transcription. Consequently, RFLP patterns of PCR products that carry identical 16S rDNA fragments are not influenced by large plasmid-encoded regions and therefore are easy to identify as identical even when cloned in opposite orientations. Sequence data represent the cloned insert only, and RNA probes derived from in vitro transcriptions contain only short non-insert regions.

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Figure 1. Schematic overview of the construction of pCPT9. (A) Partial multiple cloning site (MCS) of pGEM-5Zf(+) (Promega). (B) DNA fragment I (bold letters) was constructed by annealing the oligonucleotides 5'-CCAGAATTCATATGGATCCAGGTAACCAATGG-3' and 5'-CATGCCATTGGTTACCTGGATCCATATGAATTCTGGGGCC-3' and used to replace the *ApaI/NcoI* fragment of pGEM-5Zf(+). The resulting plasmid was designated pCPT8. (C) DNA fragment II was constructed by annealing the synthetic oligonucleotides 5'-TCCCATG-3' and 5'-GTAACCAATGGGATGCA-3' (outline letters) and was used to replace the *BstEII/NsiI* fragment of pCPT8. The resulting plasmid was designated pCPT9. The cleavage sites for *XcmI* are indicated by ▼. Restriction enzymes with only one recognition site in pCPT9 are underlined. Positions are relative to the T7 RNA polymerase transcription initiation site, which is position 1.

The derived plasmid was constructed to retain blue-white selection on 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). Figure 1 describes the construction of the plasmid pCPT9. The correctness of all constructs was verified by restriction digests and sequencing.

The vector was tested and found to be a competent template with both SP6 and T7 RNA polymerases. Sequences were deposited in GenBank® under the Accession Nos. AF092546 (pCPT8) and AF092940 (pCPT9). Plasmid DNA can be requested from <http://crab2.berkeley.edu/~pacelab/>.

In different experiments, we have used pCPT9 as a T vector under the following conditions: 2 pmol pCPT9 were incubated for 1–4 h in 50 μ L of 1 $\times$ NEBuffer™ 4 containing 10 U *XcmI* (New England Biolabs, Beverly, MA, USA). Reactions were purified directly over CHROMASPIN™-200 Columns (CLONTECH Laboratories, Palo Alto, CA, USA). Digestions of pCPT9 with *XcmI* under different buffer conditions for up to 24 h did not cause any non-specific cleavage products. PCR products of partial 16S rDNA (ca. 1485 bp) were purified using the QIAquick™

PCR Purification Kit (Qiagen, Chatsworth, CA, USA). Each ligation was performed in 10 μ L of 1 $\times$ Ligase Buffer (Promega) containing 50 ng of *XcmI* cut pCPT9 at a molar ratio of 4:1 PCR products to pCPT9, and 3 U of DNA ligase (Promega) at 14°C for 4 h. The ratio of white to blue colonies was directly dependent on the time for the *XcmI* digest of pCPT9. In general, after 2 h of digestion, the amount of white colonies was >95%. All plasmids tested carried inserts of interest.

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