RESEARCH NOTE

Engineered *Xcm*I cassette-containing vector for PCR-based phylogenetic analyses

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Introduction

A simple and general method is described to construct a new vector bearing a synthetic *Xcm*I cassette for direct cloning of PCR-amplified genes of interest. Cleavage of the vector with *Xcm*I generates a linearized molecule with a single thymidine (T) overhang at the 3′ ends (T-vector) that facilitates TA cloning of PCR products carrying complementary 3′ adenosine (A) residue, without modification. Once constructed, this newly designed circularized vector (pEMX) is reproducible in any molecular biology laboratory, and is a high performance and cost effective alternative to commercially available T-vectors.

PCR-based DNA analysis has become a widely used tool in investigating genetic relationships in naturally occurring and aquaculture populations of marine organisms. In general, genes of interest are amplified by PCR using thermostable DNA polymerase, and subsequently cloned into adequate vectors for sequencing and other manipulations. *Taq* polymerase, the most widely used PCR enzyme, adds a single adenosine at the 3′ ends of PCR products due to the terminal transferase activity (Clark 1988). PCR products derived using such polymerases can be directly cloned into vectors with complementary 3′ thymidine overhangs (T-vectors). T-vectors are routinely utilized for sequence analysis of 5S ribosomal RNA genes (5S rDNA) as a genetic marker to assess phylogenetic relationships in marine organisms (Martins and Galetti 2001). Multiple products encoding various sizes of multilocus 5S rDNA are amplified from a single fish species by PCR, and each product is separately cloned into a T-vector. This requires large numbers of T-vectors for constructing 5S rDNA library of even a single species. Moreover, commercially available T-vectors, such as pGEM-T (Promega) and pT7Blue (Novagen), are expensive and cannot be regenerated in the laboratory for further use. We describe here the development of a simple and general method for constructing T-vectors bearing an oligonucleotide cassette that produces complementary 3′ thymidine overhangs by restriction enzyme digestion.

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Materials and methods

A 36 bp double stranded oligonucleotide cassette containing two adjacent *Xcm*I recognition sites was phosphorylated with 1 mM ATP by T4 polynucleotide kinase (Invitrogen) at 37°C for 1 h, and the enzyme was then heat inactivated. Following digestion and dephosphorylation of 500 ng of pEMBL19 with 5 units of *Xba*I (Fermentas) and 0.5 units of shrimp alkaline phosphatase (Promega) at 37°C for 1 h, the reaction was terminated by keeping the reaction mixture at 65°C for 20 min. An aliquot containing the cassette and ATP was added directly to the dephosphorylation reaction mixture containing the linearized vector at a molar ratio of 1 : 5, and ligation was performed at the *Xba*I site (figure 1) by T4 DNA ligase (Invitrogen). The circularized vector, termed as pEMX, was transformed into EC100 competent cells (Epicentre) and inoculated onto LB/amp/IPTG/X-gal agar. Blue colonies were inoculated in LB/amp broth, and plasmids were extracted and sequenced, following Aranishi (2002). For direct cloning of PCR products, pEMX has been routinely used as follows. Five μg of purified uncut pEMX are digested with 10 units of *Xcm*I (New England Biolabs) at 37°C, and then the excised 15 bp fragment from the cassette is removed by ethanol precipitation.

Keywords. T-vector; direct cloning; *Xcm*I cassette; sequencing; PCR; marine population genetics.

Figure 1. Construction of direct cloning vector pEMX. A short oligonucleotide cassette containing two adjacent *Xcm*I restriction sites (underlined) was (a) inserted at the *Xba*I restriction site in the multiple cloning sites of pEMBL19, (b) digested with *Xcm*I to produce 3′ T overhang (c) for direct cloning of a PCR product carrying complementary 3′ A residues.

Results and discussion

In order to determine the cloning efficiency of PCR product into pEMX, 1235 bp of fish 5S rDNA was amplified in 10 µl of PCR buffer (10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, pH 8.3) containing 200 µM dNTPs, 0.25 units *Taq* polymerase (Sigma), 0.5 µM primers (5S21F: 5′-TACGCCCGATCTCGTCCGATC-3′ and 5S21R: 5′-CAGGCTGGTATGGCCGTAAGC-3′) and 50 ng genomic DNA prepared from boiled fish muscle by a modified urea-SDS Proteinase K method (Aranishi *et al*. 2002). PCR amplification was conducted in a Techgene Thermal Cycler (Techne) programmed as 2 min at 94°C, 35 cycles of 10 s at 94°C, 20 s at 62°C and 40 s at 72°C, and finally 7 min at 72°C. A 5 µl portion of PCR products was purified and then ligated with 25 ng linearized pEMX, followed by transformation into EC100 cells. Among a total of 108 ampicillin-resistant transformant colonies, 86 developed white. Colony-direct PCR was carried out on 12 randomly chosen white colonies, and all contained the expected size of insert.

Although pEMX is easy to construct in low-technology laboratories by a single restriction enzyme digestion, it can also be a high performance and cost effective alternative to commercially available T-vectors for direct cloning of PCR products. An important factor for efficient cloning is complete digestion of pEMX with *Xcm*I, as indicated previously (Schutte *et al*. 1997; Pitulle and Pace 1999), and this is easily achieved by leaving the *Xcm*I digestion mixture overnight in a 37°C incubator. We have already used pEMX successfully to construct a plasmid-based library of 5S rDNA from more than 35 marine fish species for population genetics studies.

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