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Construction of a T-Vector Using an Esterase Reporter for Direct Cloning of PCR Products

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We constructed an efficient T-vector, pTQEST216T that employed an engineered esterase as an indicator for direct cloning of PCR products. After ligation of the *Xcm*I-digested vector with PCR products, this cloning system could easily discriminate positive clones owing to insertional inactivation of the esterase reporter. Additionally, PCR products were efficiently cloned into this vector without the gel purification steps, owing to the well-designed multi-cloning site that was in-frame fused at the circularly permutated gap of the reporter.

Keywords: Esterase, reporter, TA cloning, T-vector, PCR product

The polymerase chain reaction (PCR) is recognized as a basic tool in molecular biology and biotechnology. Therefore, PCR-based gene probing, cloning, and then expression are widely used for gene manipulations. Most of these approaches require ligation of the targets genes, or DNA fragments, with various specialized vectors for subsequent applications. For this purpose, it is well known that TA cloning systems are the most effective methods for direct cloning of PCR products, because the method can avoid laborious and time-consuming steps of restriction enzyme digestion and ligation. This approach is based on the principle that non-proofreading polymerases such as Taq and Tth attach a nucleotide adenosine into the 3'-end in a template-independent manner. Thus, protrusion of a thymine at the terminal ends of the cloning vector can ligate PCR products. This TA cloning system was realized by some reports [9, 10], and further improved by the incorporation of recognition sites by *Xcm*I or *Eam1105*I into the vector as cloning sites, resulting in exclusion of the T-tailing step [7]. Additional attempts to inactivate the reporter proteins by using restriction sites could provide a method of simple selection owing to substitution of the intact ORF, encoding functional reporter, with PCR products in the recombinant plasmids [11] or destruction of α-complementation for the functional expression of reporter enzyme [5]. For this purpose, two reporters, green fluorescent protein (GFP) and β-galactosidase, are generally used as selection reporters for gene cloning [6, 8].

The blue-white screening system based on β-galactosidase activity for the selection of recombinants requires 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as a substrate and isopropyl-β-D-thiogalactopyranoside (IPTG) as an inducer for increased fidelity; therefore, it has a relatively high cost. Additionally, this system is also functional in a *lac*Z-mutation or -deficient host. The other reporter, GFP, shows autofluorescence without any substrates and is available for a range of host strains of any genotypes [6]. However, the time-consuming maturation of the fluorophore and the need to expose the clones to toxic UV to excite the reporter protein are issues that must be solved to enable GFP to be applicable for more general use.

Previously, some reports revealed that esterases have been used as a fusion partner or reporter for the *in vitro* probing and analyses of protein synthesis [1]. We also reported a novel cloning system using a mutant esterase cp216M as an indicator for gene cloning [2]. The activity of a mutant esterase cp216M containing inserted *Nhe*I sites at G216 was determined to be simple on solid plate within 5 min, using an overlaid soft agar (0.6%) supplemented with Fast Blue RR (1.5 μg/ml) and α-naphtylacetate (1.6 μg/ml) as substrates. We report here a novel TA cloning vector system using a mutant esterase that contains two *Xcm*I recognition sequences within the circularly permutated site G216.

To prepare the T-cloning vector, cp216M was used as a template for PCR. The esterase gene was amplified by PCR using the primer sets shown in Table 1. Specifically,

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Table 1. The list of primers used in this work.

Primer	Sequence	Recognition sites
ESTF1	5'-ATAGAATTCGTGCAGATTCAGGGTCATTAC-3'	*Eco*RI
ESTF2	5'-ATAGGATCCGTGCAGATTCAGGGTCATTAC-3'	*Bam*HI
ESTR	5'-ATAAAGCTTTTACAGACAACCGGCCAAT-3'	*Hin*dIII
Cp216F	5'-ATAGCTAGCGATGAAGCCGCACAACG-3'	*Nhe*I
Cp216R	5'-ATAGCTAGCGCCCATATTGCCTTTACTGC-3'	*Nhe*I
MCS	5'-CTAGCCAGAGCTCCGGTGGGCCCCAATGCAATGTTGGGG-3' 5'-GGTCTCGAGGCCACCCGGGGTTACGTTACAACCCCGATC-3'	*Nhe*I, *Xcm*I - *Xcm*I, *Nhe*I

two primers (EstF1 and cp216R) were used for amplification of the N-terminal fragments, whereas another two primers (cp216F and EstR) were used for amplification of the C-terminal fragments under general conditions [2]. Each DNA fragment was digested with *Eco*RI/NheI and NheI/*Hin*dIII, after which it was subcloned into the *Eco*RI and *Hin*dIII sites on pTrc99A (Amersham Pharmacia). Subsequently, the synthetic multicloning site (MCS) was inserted into this construct by digestion with *Nhe*I. The *Xcm*I sites, intentionally in-frame incorporated into the synthetic MCS as cloning sites, were naturally present in pTrc99A. Therefore, the resulting gene was subsequently amplified by PCR using two primers (EstF2 and EstR) and then subcloned into the *Bam*HI and *Hin*dIII sites of pQE30 (Qiagen). The expression of the reporter esterase in pQE30 had also taken advantage of promoter strength to avoid unintended toxicity and false-positivity. The resulting construct, pQEST216T, contained an esterase reporter that included two *Xcm*I-recognition sequences in-frame as cleavage sites for the T overhang (Fig. 1). The synthetic MCS was designated based on analyses of the hydropath profile and secondary structure prediction to avoid distortion of local sequences and structure [3]. As expected, the engineered reporter was fully functional (Fig. 2A) and determined to have a similar activity (85–90%) to wild-type enzymes when compared by enzyme assay. Thus, it was anticipated that this construct was suitable for use as an indicator for gene cloning if the reporter was inactivated

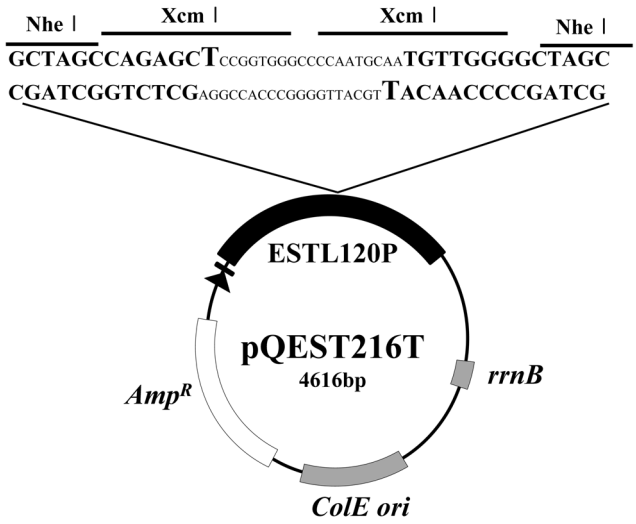

Fig. 1. Genetic organization map and MCS sequence of the novel T-cloning vector pQEST216T.

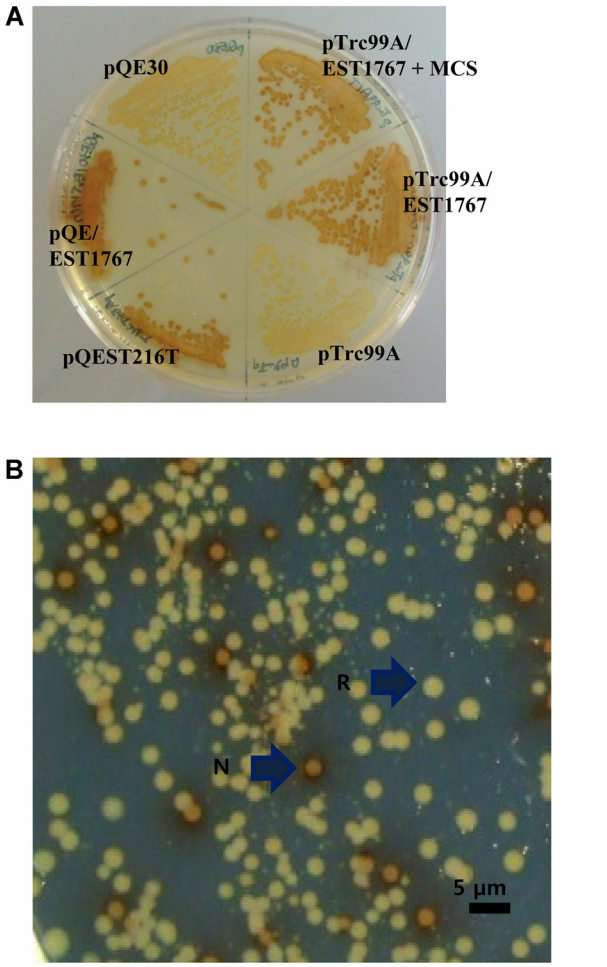

Fig. 2. Activity staining of various clones on the solid medium. All clones were cultivated on LB medium without any supplements. **A**. Comparison of the active clone harboring pQEST216T with other parent and subclones. EST1767 is the wild-type enzyme of engineered esterase. **B**. Discrimination of the recombinant (R) from the nonrecombinant (N) clone by activity staining.

by insertion of PCR products.

To evaluate its performance for practical use, various 16S rRNA genes from a metagenome resource were amplified by PCR using a set of universal primers (forward, 5'-AGAGTTTGATCMTGGCTCAG-3'; reverse, 5'-ACGGCTACCTTGTTACGACTT-3') and Taq polymerase under general conditions [4]. The amplified genes were ligated with *Xcm*I-digested pQEST216T using a DNA ligase for 2 h at 25°C. The ligation was carried out in a 10-μl reaction mixture containing 1 μl of ligation buffer, 20 ng of vector, 33 ng of insert, and 3 units of ligase. The resulting constructs were transformed into *E. coli* XL1-Blue and spread on typical LB medium (100 μg/ml of ampicillin) without any supplement. After cultivating at 37°C for 15 h under noninduced conditions, these cells were analyzed by activity staining on solid LB medium using Fast Blue RR and α-naphtylacetate as substrates (Fig. 2B). As expected, all of the clones showing no activity (white clones) were determined to have no inserts. The active clones indicated that these clones harbored self- or relegated vectors, because the purification step, after digestion with enzyme, was omitted for ease of manipulation. As a control, the same inserts were also cloned into a commercially available pGEM-T vector that selected positive clones by using X-gal as a substrate, according to the procedures recommended by the supplier. The resulting transformants were spread on the same medium supplemented with 40 μl of 2% X-Gal and 7 μl of 20% IPTG and its discriminating ability was compared with that of pTQEST216T.

Although both reporters were actively expressed and could discriminate the positive clones from nonrecombinants, the correlation between the color of the clone and the existence of the insert was about 35–60% higher in pTQEST216T than in the pGEM-T vector system. Furthermore, the esterase activity of our system was clearly detected only after cultivation at 37°C for 12 h. Under the same conditions, the blue/white color of β-galactosidase in pGEM-T was ambiguous, even in the presence of the inducer. It is well known that, when cultured without the inducer, the reporter activity of pGEM-T is not sufficient to discriminate positive clones after 20 h or more.

As more promising results, there were no distinct differences in the cloning efficiency after alteration of the ligation temperature (2 h at 25°C or 16 h at 4°C), and no growth retardation of clones harboring the recombinant pQEST216T were observed. The vector was highly reproducible and easy to use for direct cloning of PCR products in general hosts. Owing to the well-designed cloning sites that were in-frame fused at the circularly permutated gap, various negative (self-ligated and non-ligated) and religated (when used without purification) constructs were easily discriminated, because only positive constructs had no reporter activity due to the insertional inactivation. Moreover, further improvement in the sequence of the synthetic MCS by incorporation of promoter elements will provide an alternative way of expression cloning, owing to the tolerance of permutated site G216 against the insertion of larger inserts (upto 200 bp) and various sequences.

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