A novel vector for positive selection of inserts harboring an open reading frame by translational coupling

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BioTechniques 43:751-754 (December 2007)
doi 10.2144/000112629

*We have developed a novel vector, pTCS, as a tool for efficient selection of open reading frame (ORF)-containing inserts. In pTCS clones containing an insert with an ORF, a downstream marker gene (*immE3*, conferring resistance to colicin) is activated via translational coupling with the insert, and transformed cells can then be selected by exposure to colicin E3. Our method differs from previous methods in that the marker gene is activated without protein fusion, and that selection occurs irrespective of the reading frame of the insert.*

Comprehensive analysis of gene function requires construction and screening of expression libraries containing DNA fragments; however, during construction of the libraries, control of the reading frame of the inserts is important for screening efficiency. To permit enrichment for authentic translation products, several open reading frame (ORF) selection vectors, which involve protein fusion between DNA inserts and selectable marker genes, have been developed (1,2). Fusion of a foreign protein to a reporter protein often causes significant loss of activity or inactivation of the protein of interest (3,4). In addition, the pKE-1 vector selects positively no more than two out of the three downstream reading frames of cDNAs, utilizing the phenomenon whereby translation can reinitiate without a ribosome binding site if an AUG codon exists within a few nucleotides of the stop codon of the preceding translated sequence (5).

In an effort to overcome the above limitations (protein fusion and reading frames) and design a more effective method of ORF-specific screening, we developed a novel vector, pTCS (GenBank accession no. AB353144), which functions via “translational coupling” of in-frame inserts and a

selectable marker gene. On plasmid ColE3, colicin E3 (*colE3*), which kills susceptible *Escherichia coli* cells (6) and the immunity E3 (*immE3*), which neutralizes the colicin E3, face the same direction with an intergenic space of nine nucleotides (7), and their expression can be “translationally coupled” (8). In the present study, we used *immE3* as a selectable marker gene and selected cells using colicin E3. The intention was that in-frame inserts would confer colicin E3 resistance

upon the host by activating expression of *immE3*, whereas the vector alone and inserts containing in-frame stop codon(s) would not (Figure 1).

To achieve this translational coupling of inserts and *immE3*, and to achieve efficient selection independent of the downstream reading frame of inserts, we designed a novel sequence, TGAATGAATGATG, which contains three termination codons (underlined) in different translational reading frames and an initiation codon (italicized) for *immE3* overlapping with the third termination codon. The intergenic spaces for colicin and the immunity genes in strains of *E. coli* producing classes E2, E3, E8, E9, and D of colicin are similar, ranging from -2 bp (partially overlapping) to 9 bp. Therefore, for TGAATGAATGATG, we hypothesized that distances of -1 bp, 3 bp, and 7 bp, respectively, would permit translational coupling. In order to confirm that the sequence functions as a sensor for detecting the translational read-through of an insert, six different inserts containing the influenza virus hemagglutinin (HA) epitope sequence (33 bp) were prepared to yield six possible translation outcomes. Fragments containing the HA epitope sequence, the novel sequence, and *immE3* were prepared from pSH350 (9) by PCR (Expand High-Fidelity PCR System; Roche Diagnostics, Basel, Switzerland), then cloned into pUC19

Table 1. Number of Colonies Obtained through Positive Selection

Fragment	Colicin	
	+	-
Avi-1	3 × 10 ⁴	5 × 10 ⁴
Avi-2	>10 ⁶	>10 ⁶
Avi-3	>10 ⁶	>10 ⁶
TAA-Avi1	9 × 10 ²	6 × 10 ⁵
TAA-Avi2	1 × 10 ³	>10 ⁶
TAA-Avi3	3 × 10 ²	2 × 10 ⁴

Six DNA fragments, Avi-1, Avi-2, Avi-3, TAA-Avi1, TAA-Avi2, and TAA-Avi3, containing the synthetic streptavidin-encoding gene were used as inserts. Avi series, which all had ORF-containing inserts, differed from each other by one nucleotide in length at the 3'-end of the synthetic streptavidin-encoding gene, and TAA-Avi series all had termination codons at their 5'-ends. These fragments were ligated at an approximately 1:1 (vector:insert) molar ratio with pTCS vector (10–20 fmoles), and transformed into XLI-Blue MRF' according to industrial protocol. A portion of the transformed cells (approximately 50 µl) suspended in SOC medium were cultivated in 400 µl LB broth containing 1 mM IPTG at 37°C for 6 h, divided in two, and plated on the LB/ampicillin/IPTG plates with [colicin (+)] or without [colicin (-)] addition of 10 µg/mL colicin E3, respectively. The numbers of colonies formed on each plate are indicated.

Figure 1. Design and function of the pTCS vector. (A) pTCS was derived from pUC19. It contains a short amino-terminal fragment of *LacZα* under the control of the inducible *lac* promoter; two *Sfi*I sites generating sticky ends of different sequences from each other to allow directional cloning; a novel sequence, TGAATGAATGATG; and the *immE3* coding region. (B) Sequence between the short amino-terminal fragment of *LacZα* and the *immE3* ATG start codon. The two *Sfi*I sites and the novel sequence are indicated in italic and bold letters, respectively. The arrow indicates the translation path, and nucleotide triplets show the reading frame. *Escherichia coli* cells containing pTCS alone are colicin E3-sensitive (colE3^s), because a stop codon between the two *Sfi*I sites interrupts translation from the *LacZα* start codon and *immE3* is not translated. (C–F) Inserts are represented by X's. (C) A fragment containing in-frame stop codon(s). (STP) indicates the stop codon(s). Transformants containing plasmids with this insert were also colicin E3-sensitive. Fragments with (D) a multiple of three, (E) a multiple of three plus one, (F) a multiple of three plus two in length. In these cases, *immE3* is translated by complete translation of fragments up to the in-frame TGA stop codon of the novel sequence. Thus, the host cells are colicin E3-resistant (colE3^r).

(Takara, Otsu, Shiga, Japan) for expression under the control of the *lac* promoter, and transformed into *E. coli* XLI-Blue MRF' (Stratagene, La Jolla, CA, USA) (see Supplementary Figure S1 available online at www.BioTechniques.com). HAimm-1, HAimm-2, and HAimm-3, which all had ORF-containing inserts, differed from each other by one nucleotide in length at the 3'-end of the HA epitope sequence. HAimm-t1, HAimm-t2, and HAimm-t3 all had termination codons at their 5'-ends. Colicin E3 was prepared from W3110Str^r (ColE3-CA38) and purified using the method of Herschman and Helinski (10), except that 0.5 M guanidine

hydrochloride was used instead of 1 M NaCl for extracting E3 (11). Transformant sensitivity to colicin E3 was tested on plates containing 100 µg ampicillin/mL plus 200 µM IPTG using the cross-streak test described by Takagaki et al. in Reference 12. The HAimm-t1, HAimm-t2, and HAimm-t3 transformants were all sensitive to colicin E3, whereas the HAimm-1, HAimm-2, and HAimm-3 transformants were not. We therefore surmised that *immE3* was translated only if translation of the insert was not terminated before the novel sequence was reached. Using this mechanism, other marker genes could be used instead of *immE3*,

including other genes conferring drug tolerance, and genes with other functions. Given the above results, we then constructed the pTCS vector, which contained *immE3* downstream of the novel sequence. Upstream of the novel sequence contained two *Sfi*I sites (13), which had different sequences at the three-base cohesive ends to allow directional cloning with single enzyme digestion (Figure 1) (see Supplementary Figure S2).

In order to evaluate the efficiency of selection using the pTCS vector, six DNA fragments containing the synthetic streptavidin-encoding gene (387 bp) (14), each with an *Sfi*I site at both ends, were prepared by PCR in the same manner as the HAimm fragments. The fragments were cloned into the *Sfi*I site of the pTCS vector, and the vector was transformed into *E. coli* cells. After induction of the *lac* promoter using 1 mM IPTG (at 37°C for approximately 6 h with shaking), colicin E3 protein was added at a final concentration of 10 µg/mL and the transformants were immediately plated onto LB plates containing 100 µg ampicillin/mL plus 200 µM IPTG. Comparing colony formation of six transformed cells between those with and without colicin E3 protein, three transformed cells containing clones with an insert cloned in frame (Avi1, Avi2, and Avi3 fragments) formed similar number of colonies, respectively. In contrast, the colony numbers of three transformed cells containing clones with an insert cloned out of frame (TAA-Avi1, TAA-Avi2, and TAA-Avi3) were dramatically decreased upon addition of colicin E3 protein (Table 1). Sequencing of some clones obtained from these transformants revealed mutations that were probably introduced during PCR amplification or during cultivation, which allowed translational read-through. (see Supplementary Table S1). It is possible that the long duration of IPTG induction before selection (6 h) may have permitted significant growth of *E. coli* cells in the absence of selection pressure. Subsequent experiments showed that 2 h of IPTG induction and 3 µg/mL colicin E3 for subsequent growth were optimal (data not shown).

Expression of inserts introduced between the *Sfi*I sites is under the control of the *lac* promoter and positive selection of transformants containing pTCS with ORF-containing DNA fragments is carried out in the presence of colicin E3. Introduction of fragments that contain the only one termination codon (TAA) in frame suppresses the expression of *immE3*. This process enables selection for DNA fragments derived from structural genes, including those prepared from cDNA and fragmented genomic DNA. However, inserts containing coding sequences with the native stop codon may be missed if there is too much distance between the native stop codon and the start of the immunity gene so as to prevent translational coupling. Except in such circumstances, notably, pTCS is able to activate the marker gene without protein fusion, and permits selection irrespective of the downstream reading frame of an insert. Use of the pTCS plasmid for efficient selection of any insert without an in-frame stop codon, regardless of the downstream reading frame, is likely to be a useful technique in comprehensive gene function analysis.

ACKNOWLEDGMENTS

The research was supported by a grant from the New Energy and Industrial Technology Development Organization (NEDO), Japan.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. **Daugelat, S. and W.R. Jacobs, Jr.** 1999. The *Mycobacterium tuberculosis* recA intein can be used in an ORFTRAP to select for open reading frames. *Protein Sci.* 8:644-653.
2. **Rombel, I.T., K.F. Sykes, S. Rayner, and S.A. Johnston.** 2002. ORF-FINDER: a vector for high-throughput gene identification. *Gene* 282:33-41.
3. **Ladner, R.C.** 1996. Display and selection of proteins on genetic packages, p. 151-194. *In* B.K. Kay, J. Winter, and J. McCafferty (Eds.), *Phage Display of Peptides and Proteins: A*

Laboratory Manual. Academic Press, San Diego, CA.

4. **Das, A.** 1990. Optimization of starting materials, p. 93-146. *In* M.P. Deutscher (Ed.), *Guide to Protein Purification.* Academic Press, San Diego, CA.
5. **Davis, C.A. and S. Benzer.** 1997. Generation of cDNA expression libraries enriched for in-frame sequences. *Proc. Natl. Acad. Sci. USA* 94:2128-2132.
6. **Pugsley, A.P. and B. Oudega.** 1987. Methods for studying colicins and their plasmids, p. 105-161. *In* K.G. Hardy (Ed.), *Plasmids: A Practical Approach.* IRL Press, Oxford, UK.
7. **Masaki, H. and T. Ohta.** 1982. A plasmid region encoding the active fragment and the inhibitor protein of colicin E3-CA38. *FEBS Lett.* 149:129-132.
8. **Lau, P.C., R.W. Rowsome, M. Zuker, and L.P. Visentin.** 1984. Comparative nucleotide sequences encoding the immunity proteins and the carboxyl-terminal peptides of colicins E2 and E3. *Nucleic Acids Res.* 12:8733-8745.
9. **Toba, M., H. Masaki, and T. Ohta.** 1986. Primary structures of the ColE2-P9 and ColE3-CA38 lysis genes. *J. Biochem. (Tokyo)* 99:591-596.
10. **Herschman, H.R. and D.R. Helinski.** 1967. Purification and characterization of colicin E2 and colicin E3. *J. Biol. Chem.* 242:5360-5368.
11. **Masaki, H. and T. Ohta.** 1985. Colicin E3 and its immunity genes. *J. Mol. Biol.* 182:217-227.
12. **Takagaki, Y., K. Kunugita, and M. Matsushashi.** 1973. Evidence for the direct action of colicin K on aerobic $^{32}\text{P}_i$ uptake in *Escherichia coli* in vivo and in vitro. *J. Bacteriol.* 113:42-50.
13. **Christian, R.B., R.N. Zuckermann, J.M. Kerr, L. Wang, and B.A. Malcolm.** 1992. Simplified methods for construction, assessment and rapid screening of peptide libraries in bacteriophage. *J. Mol. Biol.* 227:711-718.
14. **Thompson, L.D. and P.C. Weber.** 1993. Construction and expression of a synthetic streptavidin-encoding gene in *Escherichia coli*. *Gene* 136:243-246.

Received 28 May 2007; accepted 1 October 2007.

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