Benchmark

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pET expression vector customized for efficient seamless cloning

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ABSTRACT

Here we present a modification of the widely used pET29 expression vector for use in rapid and straightforward parallel cloning via a gene replacement and Golden Gate strategy. The modification can be applied to other expression vectors for Gram-negative bacteria. We have used the modified vectors to clone large numbers of bacterial natural enzyme variants from genomic and metagenomic sources for applications in biocatalysis.

METHOD SUMMARY

The pET29 vector was modified to contain, instead of the multiple cloning site, a negative selection marker (*sacB*) flanked by Type IIs restriction sites. One-pot restriction-ligation reaction, transformation into *Escherichia coli* and plating on selective media yields >98% recombinant plasmids containing the gene of interest fused to the His6-tag at the C-terminus or, if required, genes that do not contain any vector-encoded sequences.

KEYWORDS:

expression in *E. coli* ● Golden Gate ● pET vectors ● *sacB* ● seamless cloning

Rapid advances in next-generation sequencing technologies have generated vast amounts of genomic sequence data that represent a rich source for bioprospecting for enzymes for industrial biotechnology applications. In order to speed up enzyme discovery and functional analysis, tools and protocols for high-throughput cloning are necessary. A variety of cloning strategies have been developed for creating protein expression constructs for structural and functional studies [1]. The Golden Gate cloning strategy has been shown to be incredibly powerful in cloning DNA fragments with high efficiency [2–5]. It relies on the use of Type IIs restriction enzymes that cleave DNA outside of their recognition site, providing unique cohesive ends that enable directional and seamless cloning of the gene of interest. The Golden Gate protocol allows for convenient and rapid cloning in a single-tube, one-step coupling restriction digestion and ligation [2].

Vectors from the pET System (Novagen) are a popular choice for the heterologous expression of genes in *Escherichia coli* under control of the T7*lac* promoter for applications that require high protein yields. Here we present the modification of one of these vectors, *pET29a*(+), for use with the Golden Gate cloning strategy. Two vectors were generated and designated pET29:*SacB-Bsa*I and pET29:*SacB-Sap*I. Both contain a negative selection marker in place of the multiple cloning site of pET29: the *Bacillus subtilis sacB* gene, expressed from its native promoter and flanked by *BsaI* or *Sap*I restriction sites (Figure 1A).

The *sacB* gene from *B. subtilis* encodes levansucrase, an enzyme secreted in the culture medium after induction by sucrose. Levansucrase catalyzes the hydrolysis of sucrose and synthesis of the branched fructose polymer known as levan. Expression of *sacB* in *E. coli* and other Gram-negative bacteria is lethal in the presence of sucrose, probably due to the accumulation of levans in the periplasm [6–8]. In *B. subtilis*, the upstream region of the *sacB* gene contains the promoter and the regulatory sequence *sacR* which, when cloned together with *sacB*, promote its efficient expression in *E. coli* [9]. We thus amplified the 1903-bp region from *B. subtilis* containing 445 bp of upstream regulatory sequences, the *sacB* gene and the terminator sequence, and inserted it in the pET29, where it replaced the multiple cloning site in the opposite orientation to the *T7lac* promoter [10]. We found that this orientation resulted in efficient *sacB* expression and counterselection.

All molecular biology reagents were obtained from New England Biolabs and used following the manufacturer's protocols, unless stated otherwise. All PCR reactions were performed with the Phusion® High-Fidelity PCR Master Mix with HF Buffer. PCR products were gel-purified or PCR-purified with Monarch® PCR & DNA Cleanup Kit. All ligation reactions were performed with the high-concentration T4 DNA ligase. Restriction digestion protocols were performed with restriction enzymes: *Nde*I, *Xho*I, *Dpn*I, *Sap*I and *Bsa*I-HFv2. QiaQuick Plasmid Kit (Qiagen) was used for plasmid DNA preparation. All primers were synthesized by Eurofins Genomics and are listed in Table 1.

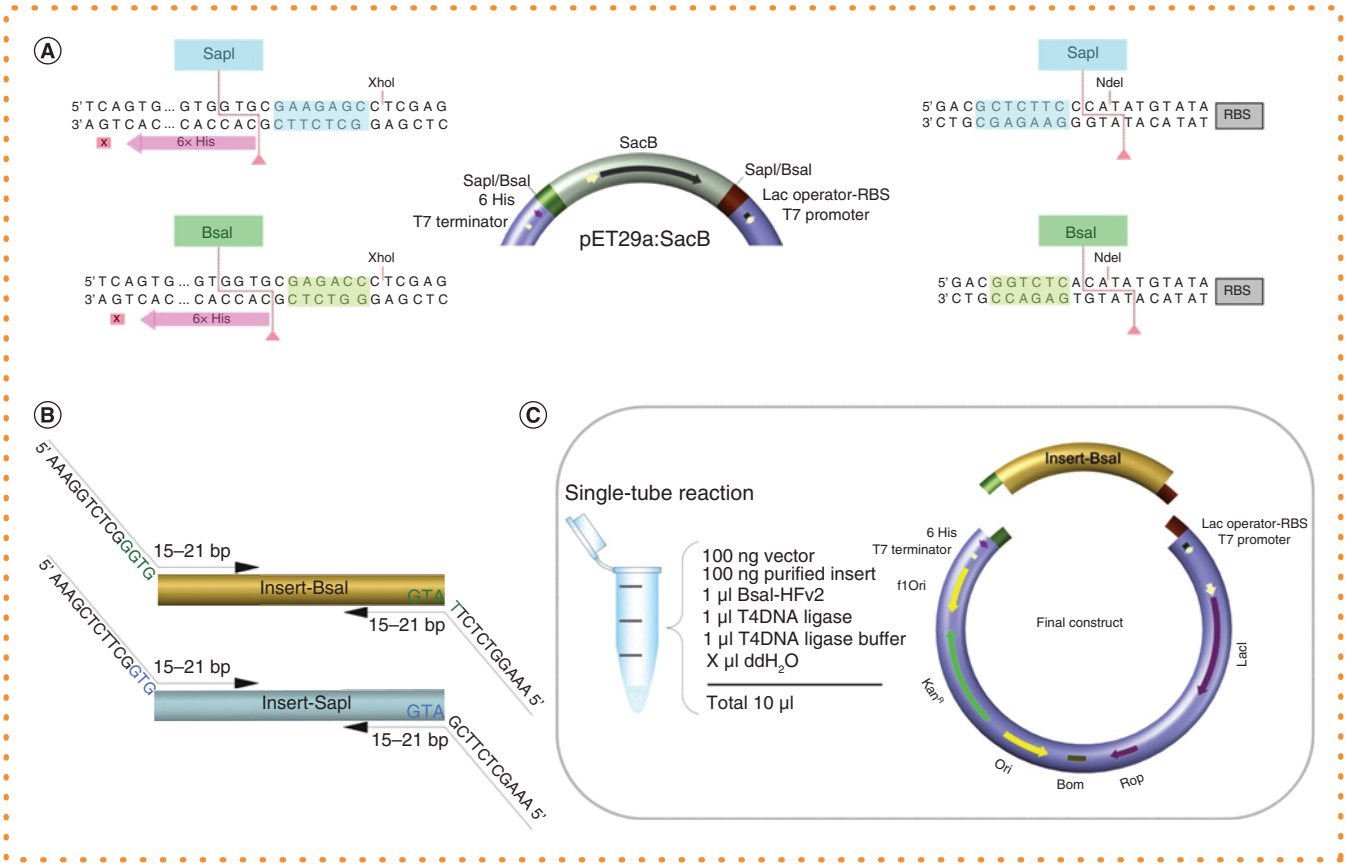

Figure 1. One-pot cloning reaction with modified pET vector and a single insert. (A) *Bsa*I and *Sap*I restriction sites allow excision of *sacB* and result in 4-base and 3-base overhangs, respectively, that direct the assembly. **(B)** Recognition sequences for *SapI* and *Bsa*I are added via the PCR to the gene of interest. Complementary overhangs in the insert that direct the assembly are in bold, green and blue. **(C)** In the single-tube reaction, the *sacB* in the destination vector is replaced with the gene of interest so that the ATG within the *NdeI* site (CATATG) is used as a start codon, putting the gene of interest in-frame with vector-encoded C-terminal His6-tag. In the final construct no amino acids are present between the protein of interest and the His6-tag.

Table 1. Primers used for vector modification.

Primer	Sequence (5′–3′)
*pET29-Bsa*I-F	AAA**CATATG**TATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAG
*pET29-Bsa*I-R	AAAA**CTCGAG**GGTCTCGCACCACCACCACCACCACTGAG
*sacB-Bsa*I-F	AAA**CATATG**TGAGACCGTCAATGCCAATAGGATATCGGCATTTTC
*sacB-Bsa*I-R	AAAA**CTCGAG**ACATATACCTGCCGTTCACTATTATTTAGTG
P3-F	**P-**GCTTCCTCGCTCACTGACTCGCTG
P3-R	**P-**GGAAGTGCGCCTGATGCGGTATTTTCTCCTTACG
*pET29-Sap*I-F	AAA**CATATG**TATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAG
*pET29-Sap*I-R	AAAA**CTCGAG**GCTCTTCGCACCACCACCACCACCACTGAG
*sacB-Sap*I-F	AAAC**ATATGG**GAAGAGCGTCAATGCCAATAGGATATCGGCATTTTC
*sacB-Sap*I-R	AAAA**CTCGAG**ACATATACCTGCCGTTCACTATTATTTAGTG

NdeI and *XhoI* restriction sites are in bold; *BsaI* and *SapI* restriction sites are underlined. Additional nucleotides introduced during the cloning procedure are in gray.

E. coli Nova Blue cells from Novagen were made chemically competent using the standard calcium chloride protocol. The working concentration for kanamycin was 50 µg/ml.

The *B. subtilis sacB* coding sequence, complete with its signal peptide and regulatory sequences (GenBank CP051860.1: 1746670–1748572, 1903 bp), was previously amplified from strain 168 genomic DNA and cloned into a customized vector. To construct pET29:*SacB-Bsa*I, *sacB* and pET29a(+) were first PCR amplified using the primers sacB-BsaI-F/sacB-BsaI-R and pET29-BsaI-F/pET29-BsaI-R, re-

spectively. PCR fragments were then digested with *Nde*I and *Xho*I restriction enzymes and ligated to form the final vector. To construct pET29:*SacB-BsaI*, the *Sap*I restriction site was deleted in the pET29a(+) by changing the sequence from GC**T**CTTC to GC**A**CTTC. 5′ phosphorylated primers P3-F and P3-R were used to do this in a routine PCR reaction with 20 cycles. The PCR reaction product was digested with *Dpn*I to remove the methylated template vector and thus reduce the background. PCR products were gel-purified, ligated to form circular vectors and transformed into chemically competent *E. coli* Nova Blue cells. The vector with deleted *Sap*I restriction site was PCR amplified using the primers pET29-SapI-F and pET29-SapI-R. The PCR product was digested with *Nde*I and *Xho*I and ligated to *sacB* that was amplified with sacB-SapI-F and sacB-SapI-R and digested with the same restriction enzymes.

A functional *sacB* gene is a prerequisite for efficient counter selection; therefore after ligation and transformation, individual colonies were tested for their growth on Luria–Bertani medium supplemented with kanamycin, in the presence or the absence of 10% sucrose. Vectors were isolated from transformants that were resistant to kanamycin and sensitive to sucrose. Both constructed vectors, pET29:*SacB-Bsa*I and pET29:*SacB-Sap*I, were further verified by DNA sequencing and their functionality was initially tested by cloning and expression of *eGFP* in *E.coli* BL21 (DE3) (data not shown).

To clone the genes of interest in the modified vectors, primers generally contained a minimum of 15 (preferably 18–21) nucleotides complementary to the sequence of interest with, when possible, a GC content of about 50%. The following overhangs were added to the primers: Forward 5′-AAAGGTCTCTTATG-3′ and Reverse 5′-AAAGGTCTCGGGTG-3′ for cloning in pET29:*SacB-Bsa*I; and Forward 5′-AAAGCTCTTCGATG-3′ and Reverse 5′- AAAGCTCTTCGGTG-3′ for cloning with pET29:*SacB-Sap*I. Overhangs contained restriction sites and were flanked by three 'spacer' nucleotides at the 5′ end to allow for efficient digestion (Figure 1B). In our case, the choice of the three- and four-base overhangs was restricted by the vector sequence, whereas in multipart assemblies junctions can often be arbitrarily chosen [11,12]. To avoid the vector-encoded C-terminal Hisx6 fusion, a translation stop codon could be included in the insert via the primer. Genes were amplified using the standard PCR procedure and gel-purified before being used in the one-pot cloning reaction.

The one-pot cloning reaction mixture consisted of 100 ng vector, 100 ng amplified DNA fragments, 1 μl restriction enzyme, 1 μl T4 DNA ligase and 1 μl 10× T4 DNA ligase buffer in a total reaction volume of 10 μl (Figure 1C). The mixture was incubated at 37°C for 30 min. 2 μl of each reaction was added to 20 μl of Nova Blue *E. coli* chemically competent cells and incubated on ice for 30 min. The cells were heat shocked at 42°C for 60 s, chilled on ice for 5 min, then recovered in 200 μl of SOC for 1 h at 37°C. Cells were plated on Luria–Bertani agar supplemented with kanamycin and 10% v/v sucrose (sterile sucrose solution was prepared by filtering and added to warm Luria–Bertani agar before pouring into plates). Typically >98% of clones positive for the desired recombinant vector were obtained after transformation. This high efficiency eliminated the need for colony screening and allowed for a single colony per construct to be picked and verified by DNA sequencing.

The method described has been extensively used in our laboratory to construct and expand our biocatalysis enzyme toolbox with a wide range of enzymes. It does not require very long primers or commercial kits. Cloning is directional and the protocol has proved to be fast, reliable and efficient; in a great majority of the clones, only the desired product is present. One-pot restriction digestion/ligation is carried out in a short time (30 mins) and the ligated DNA is immediately transformed. Expression-ready clones are available the next day after growth of the transformed cells. To date, using the adapted vectors, we have effectively cloned and expressed in *E. coli* hundreds of genes with diverse origins (from metagenomic DNA and individual genomes) and complexity, with GC content ranging from 35 to 73% and gene lengths between 0.3 and 2.5 kb. Examples of panels of recombinant enzymes constructed in this way include enzyme classes such as transaminases from a drain metagenome [13], ene-reductases also from a drain metagenome [14] and epoxide hydrolases mined from sequenced bacterial genomes [15], demonstrating the versatility of the protocol.

Author contributions

D Dobrijevic and L Nematollahi conceived the experiments; D Dobrijevic designed and performed the experiments and analyzed the data; D Dobrijevic and L Nematollahi wrote the manuscript. All authors edited and approved the final manuscript. Funding was from grants to D Dobrijevic, J Ward and H Hailes.

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Financial & competing interests disclosure

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