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pKILLIN: a versatile positive-selection cloning vector based on the toxicity of Killin in *Escherichia coli*

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ABSTRACT

The invention of DNA cloning over 40 years ago marked the advent of molecular biology. The technique has now become a routine practice in any modern biomedical laboratory. Although positive-selection of recombinants in DNA cloning seems to be superior to blue/white selection based on the disruption of the *lacZ* gene, it is rarely practiced due to its high background, lack of multiple cloning sites, and inability to express the genes of interest or purify the protein products. Here we report the creation of a new positive-selection cloning vector dubbed pKILLIN, which overcomes all of the above pitfalls. The essence behind its high cloning efficiency is the extreme toxicity and small size of the toxic domain of *killin*, a recently discovered p53 target gene. Insertion inactivation of *killin* within the multiple cloning site via either blunt- or sticky-end ligation not only serves as a highly efficient cloning trap, but also may allow any cloned genes to be expressed as His-tagged fusion proteins for subsequent purification. Thus, pKILLIN is a versatile positive-selection vector ideal for cloning PCR products, making DNA libraries, as well as routine cloning and bacterial expression of genes.

1. Introduction

Since its invention in the early 1970s, gene cloning has become a routine practice in modern biomedical laboratories. DNA fragments or PCR products are often cloned into a plasmid vector for subsequent sequence analysis, expression or other molecular biological manipulations. A number of strategies developed over the years made the screening of the recombinant plasmids more efficient. Blue/white screening is one of the most widely used approaches for this purpose. When transformed into an *Escherichia coli* host harboring the *lacZ ΔM15* allele and plated on a LB plate with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), plasmids containing the *lacZα* gene produce blue colonies due to "α-complementation" of the truncated β-galactosidase encoded by *lacZ ΔM15*. White colonies are produced when a foreign DNA fragment is inserted into the *lacZα* gene, thereby disrupting its function (Slilaty and Lebel, 1998). Various vectors employing this mechanism have been reported (Gu and Ye, 2011; Oster and Phillips, 2011; Rabbani et al., 2009). However, the approach has the following drawbacks: false positives (white colonies without inserts) or false negative colonies (blue colonies with inserts) (Slilaty and Lebel, 1998); high background of blue colonies from vector self-ligation; substrates such as X-Gal and isopropyl-β-D-thiogalactopyranoside (IPTG) are necessary and costly; requirement for vector dephosphorylation to reduce colonies formed from self-ligation when a single restriction site is used; and time needed for the color development and hosts must contain *lacZ ΔM15* allele (Cheong et al., 2009). The same drawbacks from the lacZ-Xgal system remain for vectors based on other color selection schemes employing different metabolic enzymes and substrates (Cheong et al., 2009, 2012). Vectors using green fluorescent protein (GFP) gene as an indicator (Banerjee et al., 2010; Inouye et al., 1997; Tang et al., 2009) require cells to be exposed to toxic UV light (Cheong et al., 2009).

A cloning vector with positive selection is an alternative strategy that makes the selection of recombinant DNA simpler and more efficient. The approach often employs a plasmid with the expression of a lethal gene as an indicator to prevent growth of the host bacteria, whereas insertion of any foreign DNA fragments or PCR products disrupts the coding sequence of the toxic indicator gene, thus resulting in growth of colonies containing recombinant plasmids. A number of positive-selection cloning vectors have been described, but due to pitfalls listed below, positive-selection cloning vectors have yet to catch on, both in applications and popularity. These pitfalls include: high background

Abbreviations: X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; IPTG, isopropyl-β-D-thiogalactopyranoside; GFP, green fluorescent protein; UV, ultraviolet; LB, Luria–Bertani; Amp, ampicillin; *Adk*, *Adenylate kinase*; AK, adenylate kinase; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; OD, optical density; MCS, multiple cloning sites; ORF, open reading frame.

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resulting from either incomplete lethality of the indicator gene employed (Kim et al., 2004) or the incomplete inactivation of the indicator gene due to its large size (Lee and Park, 2012; Schneider et al., 1997); limited cloning sites available for insert or limited unique flanking sites for retrieving DNA fragments cloned (Kast, 1994; Matin and Hornby, 2000; Van Reeth et al., 1998; You et al., 2009); and requirement for special treatment or substrates on the LB agar plate in order to confer lethality of the indicator genes (Lee and Park, 2012; Liu et al., 2011). Moreover, few positive-selection cloning vectors reported, including those commercially available that employ the *ccdB* gene, could allow simultaneous expression of the recombinant gene of interest with or without a tag to assist in subsequent purification of the target protein (Bernard et al., 1994; Schlieper et al., 1998; Yazynin et al., 1996).

Here we describe a highly efficient positive-selection cloning vector dubbed pKILLIN. It is based on the toxicity of the *killin* gene we recently discovered (Cho and Liang, 2008), which alleviates most of the above pitfalls. *Killin* is a p53 target gene that encodes a high-affinity single-stranded DNA-binding protein required for S-phase arrest and apoptosis in mammalian cells (Cho and Liang, 2008). When expressed in *E. coli*, Killin is highly toxic and causes total lethality of the host cells. Systematic deletion analysis revealed that the lethality resided in the N-terminus of Killin, with only 42 amino acid residues (N8-49) necessary for DNA binding (Cho and Liang, 2008). The small size of the N8-49 Killin peptide required for host cell lethality, and the incorporation of multiple cloning sites within its coding sequence through silent mutations, allow for convenient and highly efficient cloning of any foreign DNA fragments, including PCR products using blunt-end ligation. The strong T5 promoter under the *lac* operator control located upstream of the toxic His-tagged N8-49 Killin peptide also enables bacterial expression and convenient purification of any target gene cloned. This makes pKILLIN a truly versatile positive-selection cloning vector.

2. Material and methods

2.1. Construction of pKILLIN

To incorporate multiple cloning sites into a 150 bp DNA template encoding Killin/N8-49 peptide, silent mutations were introduced via gene synthesis (GenScript, Nanjing). The resulting Killin cassette was first cloned into pUC18 as an *EcoRI–HindIII* fragment. To create a His-tagged Killin/N8-49 cassette with multiple cloning sites, the above plasmid was used as a template for PCR amplification using primers L-Killin-BamHI and R-Killin-HindIII (Table 1) under the following PCR condition: 94 °C for 30 s, 57 °C for 20 s and 72 °C for 40 s, for 30 cycles, with Taq polymerase (Takara). After gel purification, the Killin/N8-49 encoding fragment was digested with *BamHI* and *HindIII*, purified by phenol/chloroform (3:1) extraction and ethanol precipitation, and ligated into the corresponding restriction sites in pQE32 (QIAGEN) using T4 DNA ligase (Takara). The resulting plasmid containing the His-tagged Killin/N8-49 with multiple cloning sites was transformed into XL1-Blue host cells, verified by DNA sequence analysis and named as pKILLIN.

2.2. Toxicity test of pKILLIN

To test the toxicity of pKILLIN in *E. coli*, when induced by IPTG in the *E. coli*, 100 μL of XL1-Blue competent cells was mixed with either 100 ng of pKILLIN or pQE32 vectors. These cells were then incubated on the ice for 30 min, heat-shocked at 42 °C for 90 s, immediately incubated on ice for 3 min, 900 μL of Luria–Bertani (LB) medium without any antibiotics was added, and then incubated at 37 °C with shaking at 220 rpm for 1 h. 100 μL of the culture was then plated on LB-ampicillin (50 μg/mL) agar plates with or without IPTG (1 mM) respectively and incubated at 37 °C overnight. Similarly, pKILLIN or pQE32 was transformed into DH5α competent cells as described before, and plated on LB-ampicillin (LB-Amp) agar plates without IPTG.

Table 1
Primers used in this study.

Name	Sequence
L-Killin-BamHI	5′-CG**GGATCC**ATCGCCCGGGGCCAGGCTCC-3′
R-Killin-HindIII	5′-GC**AAGCTT**TTACTTCCACCTCCTTTTG-3′
L-T4	5′-GGGGTACCCTTAAAATTCTGAACG-3′
R-T4	5′-CGGGATCCATAGACCAGTTACCTC-3′
L-Taq	5′-GAAGATCTTGGAATTCGGGATGCTG-3′
R-Taq	5′-GAAGATCTCCTTGGCGGAGAGCCAG-3′
L-endo180	5′-TCCAAGCTTCCACTGAGCGCCGCGCTCGGGGATGG-3′
R-endo180	5′-CATGCTCGAGTTTTTTTTTTTTTTTTTTTTTTTTC-3′
L-lac	5′-GCCCAATACGCAAACCG-3′
R-lac	5′-CGCCATTCGCCATTCAG-3′
L-lac-BamHI	5′-CG**GGATCC**GCCCAATACGCAAACCG-3′
R-lac-BamHI	5′-CG**GGATCC**CGCCATTCGCCATTCAG-3′
L-lac-KpnI	5′-GG**GGTACC**GCCCAATACGCAAACCG-3′
R-lac-KpnI	5′-GG**GGTACC**CGCCATTCGCCATTCAG-3′
L-lac-PstI	5′-AA**CTGCAG**GCCCAATACGCAAACCG-3′
R-lac-PstI	5′-AA**CTGCAG**CGCCATTCGCCATTCAG-3′
L-lac-BglII	5′-GA**AGATCT**GCCCAATACGCAAAC-3′
R-lac-BglII	5′-GA**AGATCT**CTGGCTTAACTATGC-3′
R-lac-HindIII	5′-CCC**AAGCTT**CGCCATTCGCCATTCAG
L-AK-BamHI	5′-CG**GGATCC**TGCGTATCATTCTG-3′
R-AK-HindIII	5′-CCC**AAGCTT**CGAATTAGCCGAGG-3′
His-F	5′-GAGGATCTCACCATCACCATCAC-3′
R-QE	5′-CGGCAACCGAGCGTTCTGAAC-3′

2.3. Positive-selection cloning of PCR amplified DNA fragments into pKILLIN

The *lacZα* fragment (446 bp) from pUC18 was PCR amplified with L-lac and R-lac primers (Table 1) under the following PCR conditions: 94 °C for 30 s, 55 °C for 20 s, 72 °C for 1 min, for 30 cycles, and directly ligated into *SmaI*-digested pKILLIN without purification followed by transformation into DH5α competent cells. Transformants were plated on LB-ampicillin (50 μg/mL) plates containing IPTG (1 mM) and 50 μL of X-gal (20 mg/mL). Blue colonies containing the correct 446 bp *lacZα* fragments were verified by colony PCR with primers His-F and R-QE (Table 1) with the same PCR conditions described above. To further confirm that *lacZα* was indeed inserted into *SmaI* site, recombinant plasmids were digested with *BamHI* and *KpnI*, which flank the *SmaI* cloning site in pKILLIN.

Larger DNA inserts were prepared by PCR using corresponding plasmid DNA templates and cloned into pKILLIN as follows. DNA template encoding *T4* DNA ligase (1.5 kb) and *Taq* DNA polymerase (2.5 kb) were amplified with Q5 high-fidelity DNA polymerase (NEB) with L-T4/R-T4 and L-Taq/R-Taq primers (Table 1), respectively under the following PCR conditions: 98 °C for 10 s, 57 °C for 20 s, 72 °C for 2 min, for 30 cycles. After gel purification, the PCR products were ligated with *SmaI*-digested pKILLIN and transformed into DH5α competent cells. Colonies from each plate were randomly picked and verified by colony PCR with His-F and R-QE primers (Table 1) under the PCR conditions: 94 °C for 30 s, 55 °C for 20 s, 72 °C for 3 min, for 30 cycles. *Endo180* (5.6 kb) fragment was amplified with L-endo180 and R-endo180 primers (Table 1) under the conditions: 98 °C for 10 s, 57 °C for 20 s, 72 °C for 5 min, for 30 cycles. After gel purification, the *Endo180* fragments were ligated with pKILLIN and transformed into DH5α competent cells. Randomly picked colonies were restreaked on the LB-ampicillin plates. Plasmids were then isolated and used as templates after 1:500 dilution in TE buffer for PCR analysis with His-F and R-QE primers and Q5 high-fidelity DNA polymerase under the same PCR conditions for template amplification.

2.4. Positive-selection cloning of DNA fragments at the multiple cloning sites in pKILLIN via single or double restriction digestions

To test the multiple cloning sites inside the (N8-49) gene, primers were designed to amplify the *lacZα* fragments with different restriction sites located at the ends of the PCR products (Table 1). After gel purification, the PCR products were digested with either single (*BamHI*,