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Rapid modification of the pET-28 expression vector for ligation independent cloning using homologous recombination in *Saccharomyces cerevisiae*

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Abstract

The ability to rapidly customize an expression vector of choice is a valuable tool for any researcher involved in high-throughput molecular cloning for protein overexpression. Unfortunately, it is common practice to amend or neglect protein targets if the gene that encodes the protein of interest is incompatible with the multiple-cloning region of a preferred expression vector. To address this issue, a method was developed to quickly exchange the multiple-cloning region of the popular expression plasmid pET-28 with a ligation-independent cloning cassette, generating pGAY-28. This cassette contains dual inverted restriction sites that reduce false positive clones by generating a linearized plasmid incapable of self-annealing after a single restriction-enzyme digest. We also establish that progressively cooling the vector and insert leads to a significant increase in ligation-independent transformation efficiency, demonstrated by the incorporation of a 10.3 kb insert into the vector. The method reported to accomplish plasmid reconstruction is uniquely versatile yet simple, relying on the strategic placement of primers combined with homologous recombination of PCR products in yeast.

Keywords

Ligation-independent cloning; Plasmid reconstruction; Plasmid assembly; Homologous recombination

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DNA sequence accession numbers: The complete sequence for pGAY-28 is deposited under GenBank: KJ782405.

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Appendix: Supplementary material: Supplementary data to this article can be found online at doi:10.1016/j.plasmid.2014.09.005.

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1. Introduction

Originally derived from pBR322, the pET series of cloning plasmids has been an extremely popular choice for laboratory scale inducible protein production (Rosenberg et al., 1987; Studier and Moffatt, 1986; Studier et al., 1990). The system is currently marketed by Novagen® as the most powerful set of vectors yet developed for the subcloning and overexpression of recombinant proteins in *Escherichia coli*, with nearly 1000 scientific publications reporting the use of the pET-28 variant for protein expression in 2013.¹ Genes that encode a protein of interest are generally inserted into a restriction-enzyme based multiple cloning region downstream of a T7 promoter for IPTG-inducible transcription by the T7 RNA polymerase. While this gene insertion method remains a widely used technique, alternative procedures have recently gained attention for side-stepping several disadvantages inherent to restriction-enzyme based methods, including restriction-sites internal to the gene of interest and the requirement for ligase-catalyzed vector circularization.

One method for inserting genes into expression vectors that has become an increasingly popular substitute for restriction-enzyme based methods is ligation-independent cloning (LIC) (Aslanidis and Jong, 1990; Haun et al., 1992). The LIC method does not require ligase to covalently circularize the vector and insert, but instead relies on the affinity of sufficiently long base-pair overhangs that anneal *in vitro* to afford a stable circular product. Base-pair overhangs of a predetermined length are generated by treatment with T4 DNA polymerase, capitalizing on the potent 3'→5' exonuclease activity of this enzyme in the absence of free nucleotides. Primers for amplification of the gene of interest are designed to include overhangs complementary to those of the vector, avoiding complications associated with a multiple cloning region that may contain restriction sites internal to the gene. The LIC method has become especially popular among structural biologists (Luna-Vargas et al., 2011; Stols et al., 2002), who often screen multiple constructs of a single protein target to determine if modifying the location of N- and C-termini impacts the crystallizability of the macromolecule. The simplicity of LIC primer design combined with the inherent amenability to parallel processing makes this cloning strategy an attractive alternative to traditional restriction-enzyme based methods for high-throughput plasmid generation.

The ability to rapidly modify an expression vector is a powerful tool that can be implemented to increase the efficiency of standard molecular cloning prevalent throughout academic and industrial laboratories. Robust methods for plasmid assembly that employ *in vivo* homologous recombination techniques have been previously reported (Chino, et al., 2010; Ma, et al., 1987, Oldenburg, et al., 1997). We have applied these methods to construct pGAY-28, in which the multiple cloning region of the common pET-28 cloning vector was replaced with a custom LIC cassette. While Novagen® does market a limited number of pET vectors that contain LIC options for gene integration, the methods outlined here represent a rapid and uniquely versatile protocol that can be used to quickly modify any genetic element within an expression vector using homologous recombination in *Saccharomyces cerevisiae*. By strategically designing primers that are positioned to anneal upstream of a unique restriction-site within the parental vector, the final step of plasmid re-circularization is

¹http://scholar.google.com/scholar?q=pet28&hl=en&as_sdt=0%2C44&as_ylo=2013&as_yhi=2013 (accessed September 15, 2014).

greatly facilitated to enable the complete reconstruction of plasmids within a relatively short time frame (3 days). The robust nature of the new cloning vector is demonstrated through a comparison of cloning efficiencies when inserting a 10.3 kb insert into both pET-28b and pGAY-28.

2. Materials and Methods

2.1 Strains and plasmids

E. coli TOP10 (Invitrogen) and BL21 (DE3) were used as the host strains for subcloning and protein production from the pGAY-28 vector, respectively. The pET-28b(+) expression vector used as the template for plasmid modification was obtained from Novagen. *S. cerevisiae* strain BJ5464-NpgA (*MATa ura3-52 his3- 200 leu2- 1 trp1 pep4: HIS3 prb1 1.6R can1 GAL*) was used for the recombinatorial assembly of PCR products (Ma et al., 2009). The YEpADH2p plasmid used for colony selection in *S. cerevisiae* is a 2μ YEpIac195-based shuttle vector with a *URA3* selection marker (Gietz and Akio, 1989; Ma et al., 2009). Genomic DNA was extracted from *Bacillus subtilis* ssp. 168 as a template for the amplification of the 10.3 kb fragment from the *pksJ* gene.

2.2 PCR amplification of pET-28b and yeast shuttle vector integration

Unless otherwise mentioned, all DNA samples were purified by agarose gel excision using the QIAquick Gel Extraction Kit (Qiagen) and eluted into deionized water before use. Amplification of pET-28 for integration into the YEpADH2p vector was performed using standard PCR methods with KAPA HiFi polymerase (KAPA Biosystems). The vector was divided into three pieces for amplification (A, B, and C; Fig. 1) using the following primers: a₁: 5'-
CAAAAAGCATACAATCAACTATCAACTATTAATACTATATCGTAATACCATATGTGTTTT
CCCGGGGATCGCAG-3', a₂: 5'-CGCATCCATACCGCCAGTTGTTTAC-3', b₁: 5'-
GAGGATGCTCACGATACGGGTTACTG-3', b₂: 5'-
GTGTTGTGCATGACTCCTCGTCTAGCGACGTGCAGAGGAGCTGACTGCAAAGAG
CCGCGCGGCACCAG-3', c₁: 5'-
TTTGACGTCAGCTCCTCTGCACGTCGCTAGACGAGGAGTCATGCACAACACCAC
CACCACCACCACTGAGATC-3', and c₂: 5'-
CGCACAATTTGTCATTAAATTAGTGATGGTGATGGTGATGCACGTGATGCATGG
TTACTCACCCTGCGATC-3' (BseRI sites in bold, LIC cassette underlined, and
YEpADH2p homologous recombination sites (Y-3' and Y-5') are italicized). YEpADH2p
was restricted with NdeI and PmlI to form a linearized vector prepared for homologous
recombination. The three pET-28b amplicons and linearized YEpADH2p vector were mixed
to molar equivalency, and transformed into *S. cerevisiae* strain BJ5464-NpgA using the S. C.
EasyComp Transformation Kit (Life Technologies). Cells were plated on uracil deficient
media for selection. Many colonies (>200) appeared within 2-3 days, and six out of six
colonies screened positive as successful transformants.

2.3. PCR amplification of pGAY-28 from recombined shuttle vector

Several transformed *S. cerevisiae* colonies (5–10) were transferred into 100 μl of water, and incubated at 100 °C for 5 minutes to induce cell lysis. The cell suspension was then

centrifuged at 21,000 rcf for 3 minutes, and 5 µl of the supernatant was used as the template DNA for the subsequent PCR amplification (50 µl total reaction volume), in which the primers used were a₁ and c₂ (see Section 2.2). The 5' and 3' ends of this amplicon contained the XmaI restriction site native to the original pET-28b(+) vector, and therefore restriction with XmaI followed by ligation with T4 DNA ligase (NEB) yielded the complete pGAY-28 expression vector that was transformed into competent *E. coli* TOP10 cells. Due to the use of a single restriction site, the number of colonies from this transformation was greater than 500. False positive clones are extremely unlikely using this method since the plasmid was generated by PCR amplification, reducing the background often observed with the transformation of doubly-digested vectors. Therefore, only two colonies were screened by DNA sequencing, and both revealed the successful construction of pGAY-28.

2.4. Standard protocol for subcloning into pGAY-28 for overexpression in *E. coli*

The reader is encouraged to view the AudioSlides content associated with this manuscript for a brief video describing how to use the pGAY-28 vector. The gene of interest is amplified using the sense primer 5'-GCGGCCTGGTGCCGCGCGGCTCTAGC(**X**)-3' and anti-sense primer 5'-GTGGTGGTGGTGGTGGTATG(**Z**)-3', where (**X**) represents the sequence designed to anneal to the 5' end of the target gene, and (**Z**) represents the reversed complementary sequence designed to anneal to the 3' end of the target gene. The LIC cassette of pGAY-28 contains dual histidine tags for purification of proteins by immobilized metal affinity chromatography, with a mandatory N-terminal hexahistidine tag and an optional C-terminal heptahistidine tag. The optional C-terminal tag can be avoided by incorporating a stop codon 5' of the (**Z**) sequence during primer design (e.g., 5'-GTGGTGGTGGTGGTGGTATGTTA(**Z**)-3', stop codon underlined). Amplification of the desired gene is followed by agarose gel purification, and eluted into 50 µl of water. Two micrograms of the pGAY-28 vector are restricted with BseRI (NEB), agarose gel purified, and eluted into 50 µl of water. The A₂₆₀ for both the insert and vector are recorded. Independently, both the linearized pGAY-28 and the target amplicon are incubated with nucleoside triphosphates in the following manner: 2 µl of 100 mM dATP is added to 50 µl of the purified vector, and 2 µl of 100 mM dTTP is added to 50 µl of the purified insert. To both (but separately), the following are then added: 11 µl water, 1 µl 0.5 M DTT, 8 µl NEB Buffer 2, 8 µl of 1 mg/ml BSA, and 2 µl T4 DNA polymerase (NEB). Both reactions are incubated at 22 °C for 30 minutes, followed by polymerase inactivation by incubation at 75 °C for 20 minutes. The vector and insert are then mixed in a standard 200 µl PCR tube to a total volume of 9 µl according to the following calculation:

$$\frac{V}{I} = \frac{1.9 \times A_{260_{\text{Insert}}}}{kB_{\text{Insert}} \times A_{260_{\text{Vector}}}}$$

where $\frac{V}{I}$ is the ratio of vector to insert in µl (e.g., $\frac{V}{I}=2$ would indicate that 6 µl of T4 DNA polymerase treated vector should be added to 3 µl of T4 DNA polymerase treated insert), $A_{260_{\text{insert}}}$ and $A_{260_{\text{vector}}}$ are the A₂₆₀ values measured before T4 DNA polymerase treatment, and is equal to the kilobase pairs of the gene to be inserted into the vector. To

promote the annealing of base-pair overhangs between the vector and insert, the reaction is subjected to progressive cooling using the following thermal cycler protocol: 98 °C for 30 s, followed by 60 cycles (3 s each) where the reaction temperature is reduced by 1 °C each cycle. While not critical for successful gene integration, this step has been observed to increase transformation efficiency of positive clones ~5 fold. After the thermal cycler reaches 38 °C, 1 µl of 50 mM EDTA (pH 7.0) is added to the reaction mixture, and it is incubated at 22 °C for 5 minutes. Ultimately, 1 – 5 µl of the reaction mixture can be transformed into competent *E. coli*, and plated on solid media containing kanamycin for selection of positive clones.

2.5. Comparison of cloning efficiencies between pET-28b and pGAY-28

[illegible]

3. Results and discussion

The pGAY-28 vector was generated to provide a LIC alternative to restriction-enzyme based cloning predominant within the pET system, while retaining all the other features found within the the pET-28 subtype that has made it such a popular choice for protein overexpression in *E. coli*. The methods outlined here provide a simple and general procedure that can be adapted to reconstruct a multiple cloning region, generate a LIC cassette, or incorporate any genetic element of choice into an expression vector within several days.

The pGAY-28 expression vector was generated by amplifying the parent pET-28b(+) vector in three parts (A, B, and C), using primer pairs a₁:a₂, b₁:b₂, and c₁:c₂ (Fig. 1). A key feature that permitted the rapid reconstitution of the final plasmid product involved exploiting the single XmaI site within the original pET-28 sequence. The primers a₁ and c₂ were designed

to anneal upstream of the XmaI site of pET-28, so that the recombined product contained two XmaI sites adjacent to the sequences for yeast shuttle vector integration. The recombined vector (YE_pADH2p/pGAY-28) becomes the DNA template for the following PCR amplification, such that primers a₁ and c₂ are used in conjunction to amplify only the pGAY-28 portion of the plasmid. The incorporation of identical restriction sites at the 5' and 3' ends of this amplicon facilitates the circularization of the final product that can be restricted and ligated to itself. XmaI was chosen for pGAY-28 construction to completely preserve the sequence of pET-28b(+) outside of the multiple cloning region, but any restriction site that does not exist in the parent vector can be incorporated into the primers for the same purpose. The likelihood of selecting false positive yeast colonies is sufficiently low, and therefore the second round of PCR amplification can simply be a "colony PCR", avoiding purification and sequencing of the recombined dual vector. In this step, several yeast colonies that likely contain the recombined construct (e.g., YE_pADH2p/pGAY-28) can be combined and lysed to provide template DNA for the subsequent amplification. Barring unusual recombination events, amplified DNA of the correct molecular weight in the second round of PCR is highly indicative that the recombination was successful.

The LIC cassette of pGAY-28 that replaces the multiple cloning region of pET-28 has several unique features (Fig. 2). Two BseRI restriction sites within the cassette provide a single-step linearization of the vector using one restriction enzyme, generating short sticky ends that are incapable of self-annealing and susceptible to T4 DNA polymerase digestion. T4 DNA polymerase exhibits potent 3'→5' exonuclease activity toward linear DNA, but this activity is significantly attenuated toward specific bases if high concentrations of the nucleoside triphosphate of that base exist in the reaction condition (Aslanidis and Jong, 1990). In the presence of millimolar concentrations of a specific nucleoside triphosphate, the T4 DNA polymerase will stall when it reaches that specific base in the sequence, generating considerably longer sticky ends than can be achieved with restriction enzyme digests. If BseRI linearized pGAY-28 is treated with T4 DNA polymerase in the presence of dATP, the polymerase removes nucleotides 3'→5' from both ends of the vector until it reaches an adenine, exposing lengthy segments of single-stranded DNA. Similarly, if the amplified gene to be inserted into the LIC cassette is treated with T4 DNA polymerase in the presence of dTTP, complementary sticky ends are generated that can anneal to the vector. Simply mixing the T4 DNA polymerase treated vector and insert before transformation is sufficient to achieve acceptable transformation efficiency of positive clones. To increase transformation efficiency further, the vector and insert should be subjected to progressive cooling, easily programmed into modern thermal cyclers. It is suspected that slowly cooling the mixture from 98 °C to 38 °C increases the population of complementary sticky ends that find and anneal to one another properly.

Using restriction-based methods, it has been generally observed that the size of a gene insert corresponds inversely to the number of positive clones. Our laboratory has observed a precipitous decrease in cloning efficiency with pET-28b(+) after the size of the insert exceeds 6-7 kb. To compare the cloning efficiencies of pET-28b(+) and pGAY-28, a 10.3 kb fragment (corresponding to 12 polyketide synthase domains housed within the *pksJ* gene from *B. subtilis*) was selected to challenge both systems. The gene fragment was amplified using a primer set encoding NotI and XhoI restriction sites for insertion into pET-28b(+), in

addition to amplification using a primer set encoding the LIC annealing sequences for insertion into pGAY-28. Highly competent *E. coli* Top10 cells ($\text{cfu} > 1 \times 10^7/\mu\text{g}$) were used to transform the constructs by electroporation, and transformants were plated on selective media. The pET-28b(+) construct yielded 10 colonies; however, five randomly selected colonies were screened and only contained the pET-28b(+) vector without insert. The pGAY-28 construct yielded 40 colonies, and 11 of these were selected and screened. Of these, two were positive clones and nine were the pGAY-28 vector without insert, indicating an approximate 18% success rate. The false positive colonies for the pET-28b(+) construct are attributed to incomplete digestion with either NotI or XhoI, and the false positive colonies for the pGAY-28 construct are attributed to incomplete digestion with BseRI. Although 18% is a relatively low success rate, it must also be considered that the pET-28b(+) vector produced no positive clones, and that the transformation of a ~16 kb nicked vector was at least possible with pGAY-28. When smaller inserts are used to generate pGAY-28 constructs (1-2 kb), it is exceptionally rare to encounter false positive colonies due to the abundance of positive clones.

As no other features of pET-28 were modified through the incorporation of the LIC cassette, the T7 promoter, *lac* operator, ribosomal binding site, N-terminal hexahistidine tag, and thrombin cleavage site still precede the gene of interest. Translated protein products expressed from pGAY-28 will read: MGSSHHHHHHSSGLVPRGSS(#)HHHHHHH (stop), where (#) corresponds to the translation of the gene cloned into pGAY-28 for overexpression. The serine that immediately precedes (#) is the only N-terminal residue inconsistent with expression of proteins cloned into the NdeI site of pET-28b(+). While the N-terminal hexahistidine tag is mandatory and can be removed with thrombin digestion, the C-terminal heptahistidine tag is optional, and can be avoided by the incorporation of a stop codon in the reverse primer for the gene of interest (see Section 2.4).

The protocol described here can be easily adapted to modify any expression vector of choice via homologous recombination in *S. cerevisiae*. In the manner by which primers b₂ and c₁ harbor the LIC cassette used to generate pGAY-28, any sequence (and its reverse complement) can be designed into the primers to rapidly delete, amend, or add to any region of the parent plasmid. Although the pET-28b(+) multiple cloning region that was replaced with the LIC site is relatively small, the strategy is clearly amenable to large-scale modifications, such as the exchange of drug-resistance cassettes. If longer regions (greater than 50 nucleotides) are to be incorporated, an additional set of primers will be required to first amplify the genetic material of interest. As long as the primers used for this amplification contain ends that can recombine with homologous regions of adjacent amplicons (or linearized plasmid), there is no limit to the modifications that can be achieved within a relatively short time frame. It is conceivable that the 10.3 kb insert described in this study could have been amplified with regions designed to recombine with pET-28b(+) (i.e., the annealing sequences for primers b₂ and c₁, Fig. 1), transformed into yeast with the shuttle vector for recombination, followed by colony PCR of the entire 15.7 kb fragment corresponding to pET-28b(+) containing the appropriately placed insert. This fragment could subsequently be restricted with XmaI and ligated to itself to yield a construct exceptionally challenging to generate with traditional restriction-based cloning methods. Our laboratory has observed a significant increase in transformation efficiency using pGAY-28 in place of

pET-28, and recently solved several crystal structures of proteins expressed from this vector (Gay et al., 2014; in press). We encourage labs engaged in high-frequency cloning coupled to protein overexpression to explore possible modifications to their vector of choice in order to increase throughput and efficiency, and recommend assembly via the protocol described herein as the most rapid and cost-effective method for this procedure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Method for rapid customization of bacterial expression vectors.
- Large- or small-scale modifications accomplished in several days.
- Generation of a ligation independent expression vector based on pET-28.
- Successful incorporation of a 10.3 kb insert into modified vector.

Figure 1.

Construction of pGAY-28. The modification of pET-28 to replace the multiple cloning region (MCR) with a LIC cassette was accomplished in five steps. In step (1), the parent pET-28 vector is amplified in three segments: A, B, and C. Segment A contains a region homologous to the 3'-end of the linearized yeast shuttle vector YEpADH2p (Y-3'). Segment B contains the LIC cassette at its 3'-end. Segment C contains the LIC cassette at its 5'-end, and a region homologous to the 5'-end of YEpADH2p (Y-5'). In step (2), transformation of linearized YEpADH2p and the three amplified segments into competent *S. cerevisiae* leads to step (3), where the overlapping segments undergo homologous recombination *in vivo*. In step (4), two of the original primers from step (1) are used again to amplify the modified expression vector using "colony PCR". Since these primers were originally designed to anneal upstream of a single XmaI restriction site, step (5) involves digestion of the amplicon with XmaI followed by treatment with DNA ligase, yielding the complete pGAY-28 expression vector.

Figure 2.

Cloning into pGAY-28 for heterologous overexpression in *E. coli*. To insert a target gene into pGAY-28, the vector is first digested with the restriction-enzyme BseRI. Two inverted BseRI sites ensure that the short sticky ends of the linearized vector are incapable of annealing. The gene that encodes the protein of interest (#) is PCR amplified using primers that include tails specific to the LIC integration sequence of pGAY-28. Both the linearized vector and gene insert are then digested with T4 DNA polymerase (in the presence of dATP and dTTP, respectively), generating lengthy single-stranded overhangs. The vector and insert are then combined, heated, and progressively cooled to increase the fidelity of successful gene integration (triangles on single-stranded overhangs symbolically indicate base complementarity between vector and insert).