Mini Review

Beyond pUC: Vectors for Cloning Unstable DNA *

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The foundation of genomic sequence analysis is large-scale cloning and sequencing from shotgun plasmid libraries. Phenomenal advances in the technology for obtaining and assembling sequence information from vast numbers of clones have made this approach practical and efficient. In stark contrast, there has been very little improvement in the vectors used for generating plasmid libraries for shotgun sequencing. Common vectors are typically maintained at high copy number and induce transcription and translation of inserted fragments, causing instability of certain classes of DNA sequences. This “unstable” DNA may result in sequence stacking, clone gaps, or severe difficulties in creating plasmid libraries, especially from DNA with a high percentage (>70%) of adenine and thymine bases (AT-rich DNA).

By far the most commonly used plasmid for constructing shotgun libraries is pUC18 or its closely related derivatives. These vectors have several features widely accepted as advantageous, such as their blue/white screening capacity, large multiple cloning site, and high copy number, as well as the ability to generate RNA transcripts from bacteriophage promoters and single-stranded DNA (ssDNA) from the M13 origin of replication (reviewed in 1). However, very little attention has been devoted to characterizing the potential disadvantages of these traits. For example, specific fragments or even large portions of genomes are recalcitrant to cloning in these vectors, hindering most sequencing efforts. As a result, despite extensive finishing efforts applied over the past years, gaps remain in the genomic sequence of nearly every multicellular organism studied, including those assemblies described as “complete” (2).

Drawbacks of pUC Vectors

Gaps in shotgun libraries and seemingly “unclonable” DNA fragments are quite common. Such DNA is typically characterized by high AT content, strong secondary structure, open reading frames, or *cis*-acting functions (e.g., transcriptional promoters or replication origins). In some cases, most notably AT-rich DNA, the reasons for difficulty in cloning are not well defined. In other instances, one or more features of pUC plasmids have been shown to be incompatible with cloning or stable maintenance of the inserts. Difficulties caused by each of these features are summarized in Table 1.

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Table 1. Advantages and disadvantages of pUC-type plasmids

Feature	Advantage	Disadvantage	Targets Selected Against
Blue/white screen or direct selection	Easy screening	Transcription/translation of inserts, false positives, false negatives, or loss of recombinant clones	Toxic ORFs, repeats, AT-rich DNA, short inserts, promoters
High copy number	High plasmid yield	Instability	Large inserts, AT-rich DNA
Ampicillin resistance	Common, inexpensive	Satellite colonies, loss of selection in liquid culture	Slow growers, unstable inserts
M13 origin	Generates ssDNA	Instability when transcribed	Promoters, cDNAs

The central feature of pUC plasmids, which has become ubiquitous among cloning vectors, is the "blue/white" colony screen to detect recombinant plasmids (3). This screen is based upon inactivation of the *lac*Za peptide of beta-galactosidase, which is expressed by the vector. A similar approach is used with direct selection vectors (e.g., pZeRO™; Invitrogen, Carlsbad, CA), except that a gene encoding a lethal product, such as *ccd*B or *sac*B, is used in place of, or in addition to, *lac*Za to select against non-recombinants. Although these screens provide a simple and powerful method to identify most recombinant colonies, they may in fact select against a relatively large number of clones. Both screens induce a high level of transcription and translation of the indicator gene, driven by a promoter in the vector region adjacent to the cloning site. Sequences inserted into the multiple cloning site are also subject to this transcription and translation, which may interfere with cloning of several classes of inserts

Recently, several alternative vectors have been introduced by Lucigen to reduce the cloning bias inherent to pUC plasmids. Dramatic improvements in library construction are possible with these new vectors that reduce the cloning bias. For example, telomeric repeats and other AT-rich fragments from *Pneumocystis carinii* (65% AT) were stable in a low copy, transcription-free vector but were unstable in pUC19. Likewise, the AT-rich genome of *Lactobacillus helveticus* (65% AT) was cloned with 25-fold greater efficiency and significantly less bias using a transcription-free vector. Toxic regions of the mouse hepatitis virus genome were readily cloned and stable in the new type of vector, but they were deleted, rearranged, or slow-growing in conventional vectors. Although successful genomic sequencing with pUC-based vectors has validated their utility and reinforces their continued use, employing alternative vectors may substantially reduce the effort required for genomic sequencing and assembly.

pSMART® Vectors

The pSMART series of vectors (Figure 1, next page) lack several characteristics of pUC18 that have been associated with cloning bias and deletion of inserts. The most notable feature of the pSMART vectors is the absence of transcription into or out of the inserted DNA.

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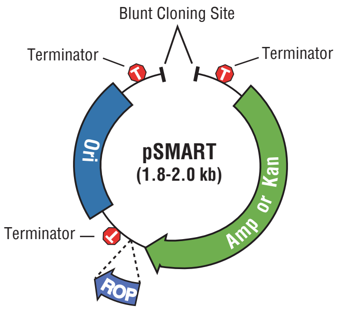


Figure 1. Schematic diagram of pSMART vectors. The pSMART vectors (Lucigen) contain either ampicillin or kanamycin selection. The ROP gene (Repressor of Primer) is present in the low copy versions to decrease copy number. Transcriptional terminators flank the cloning site to prevent insert-driven transcripts from entering the vector. Another terminator follows the drug selection marker to prevent vector-driven transcription of inserts. Vector sizes are 1.8 to 2.0 kb. The pSMART vectors accommodate inserts up to 20 kb.

Vector driven transcription into the insert is eliminated by removal of the *lac*Z promoter and the *lac*Za gene. In addition, a transcriptional terminator downstream of the drug resistance gene prevents read-through of insert sequences from this promoter. Because there is no marker gene to indicate which clones have inserts, the pSMART vectors are supplied by Lucigen pre-cut and dephosphorylated, such that the background of self-ligation is less than 0.1% in most applications. With this low level of background, screening for recombinants is typically not required. The low level of background also facilitates cloning nanogram amounts of sample or cloning samples that produce few colonies for other reasons. Variants of pSMART employ either kanamycin or ampicillin selection, and they contain high or low copy origins of replication.

Extremely efficient cloning in the absence of a visual screen has been verified by sequence analysis of numerous libraries constructed with pSMART. For example, sequencing 32,000 randomly picked clones from a library of 2.5 to 4.5 kb inserts revealed only 40 empty vector clones, or 99.9% recombinants (P. Wilson, Australian Genome Research Facility; personal communication). This level of efficiency is much higher than that obtained with plasmids employing the blue/white screen or *ccd*B selection, which may yield 5% to 10% empty vector clones (i.e., false positives) after screening or selection for recombinants (B. Chiapelli, Washington University Genome Sequencing Center; personal communication).

As expected, the lack of vector-driven expression of insert DNA alleviates the selection against cloning toxic coding sequences, strong promoters, and other sequences that are unstable in pUC-based vectors. A 350-bp fragment encoding a prokaryotic RNase was recovered in either orientation in pSMART, whereas it could be obtained only in the "reverse" orientation in pUC19 (Table 2). Transcription initiated by promoters within the insert is blocked by terminators on either side of the cloning site in pSMART (Figure 1), thereby insulating essential portions of the vector (i.e., the replication origin and drug resistance gene) from transcriptional interference. The efficacy of this arrangement was demonstrated by cloning a 450-bp fragment containing the strong lambda P_R promoter into pSMART and pUC19. Clones containing this promoter were isolated readily in pSMART, whereas they were rare and nearly obscured by empty vector clones when ligated into a dephosphorylated preparation of pUC19 (Table 2).

Table 2. Cloning efficiency of a toxic gene or strong promoter in pSMART or pUC19.

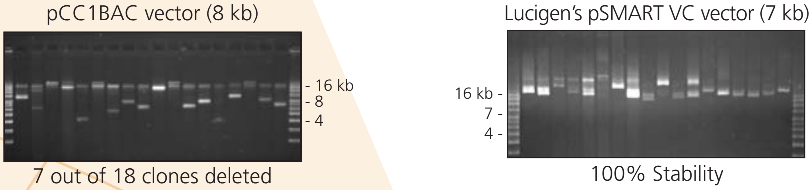
Vector	**Target: RNase Gene** Total cfu (% Forward)	**Target: Lambda P_R** Total cfu (% Intact)
pSMART-HC	45,000 (38%)	8,500 (75%)
pSMART-LC	20,000 (33%)	3,500 (75%)
pUC19	50,000 (0%)	Blue 100,000 (n.d.) White 100 (25%)

Fragments containing a prokaryotic RNase gene or the lambda P_R promoter were amplified with Vent® DNA polymerase (New England Biolabs) and treated with T4 polynucleotide kinase (New England Biolabs), according to the manufacturer's recommendations. Fifty nanograms of each fragment was ligated to blunt, dephosphorylated vector preparations and transformed into electrocompetent *E. coli* cells, using standard molecular biology techniques. Values represent total number of colonies per ligation. PCR was used to determine insert size and percentage of clones in the each orientation.

Several fragments encoding "toxic" regions of the mouse hepatitis genome also were readily cloned and highly stable in pSMART. These same fragments were very susceptible to rearrangement in standard vectors (4). The lack of transcription is likely responsible for producing more representative cDNA libraries as well (R. Drinkwater, Xenome Corp.; personal communication). Cloning into pSMART vectors is expected to increase the stability of trinucleotide repeats, based on their stability in a transcription-free derivative of pUC (5). Libraries from AT-rich genomic DNA tend to be much larger and more representative when constructed in pSMART rather than pUC19. For example, a cosmid containing genomic DNA from *Pneumocystis carinii* (60%–65% AT) was sheared, end-repaired, and size-selected to 2 to 4 kb. Aliquots of this material were cloned into pSMART HCKan or pUC19. No deleted inserts or empty vectors were detected among 151 pSMART clones analyzed. In contrast, over 25% of the clones in the pUC19 library suffered obvious deletions, resulting in plasmids smaller than the parental pUC19 (Figure 2).

pSMART® VC Vectors

The pSMART VC vector (Lucigen) appears to be the most stable vector for cloning extremely recalcitrant DNA fragments, especially those that are over 10 kb in length. This vector incorporates the transcription-free aspects of pSMART into a minimized derivative of pBELOBAC (Figure 3). The pSMART VC vector is single copy for increased cloning stability, but it contains the inducible OriV origin (6) to increase the copy number to 20–100 per cell for DNA isolation. This vector facilitated construction of a stable genomic library of 10–20 kb inserts from *Tetrahymena thermophila* (~75% AT), an accomplishment that has not been reported in any other vector (E. Orias, personal communication). Importantly, this library was not stable in a similar BAC vector containing the *lac*Z-based blue/white screening (Figure 4).


pCC1BAC vector (8 kb)

Lucigen's pSMART VC vector (7 kb)

7 out of 18 clones deleted

100% Stability

Figure 4. Genomic DNA from *Tetrahymena thermophila* (75% AT) was sheared, size-selected to 10-20 kb, and cloned into pSMART VC or a leading BAC/large insert vector. Uncut mini-prep DNA shows only large plasmids in the pSMART VC library, but many small, deleted clones in the conventional BAC vector.

Conclusions

Although pUC18 and its close relatives stably maintain most inserts, their limitations are becoming increasingly important with cloning of targets that may be unstable (e.g., AT-rich, highly repetitive, trace amounts, etc.). Numerous types of inserts are rendered "unclonable" by the high level of transcription and translation caused by the blue/white colony screen. In addition, transcription proceeding through inserts, or initiated within them, may lead to instability of the plasmid or failure of the blue/white screen. The pSMART vectors, designed specifically to circumvent many of the limitations of

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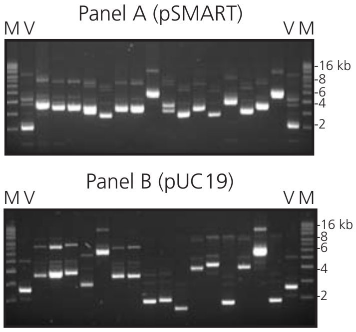
Panel A (pSMART)

Panel B (pUC19)

Figure 2. Increased stability of AT-rich genomic clones in pSMART. A cosmid containing genomic DNA from *P. carinii* was hydrodynamically sheared, end repaired (DNATerminator® Kit; Lucigen) and shotgun-cloned into pSMART-HCKan or pUC19 (Lucigen). Plasmid DNA was purified from transformants by a standard alkaline lysis protocol. Uncut DNA from each clone was analyzed by gel electrophoresis (approximately 200 ng/lane, 1% agarose, TAE buffer). (Upper panel) Plasmids from randomly picked pSMART transformants were all within the expected size range, demonstrating high stability. (lower panel) Over 25% of the pUC19 transformants were unstable, yielding plasmids smaller than the parent vector. M, supercoiled plasmid ladder (Invitrogen); V, empty vector control.

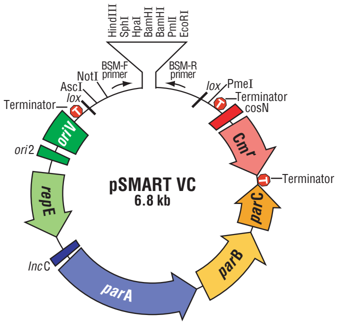

Figure 3. Diagram of the CopyRight™ Vector. The pSMART VC vector (transcription-free) is illustrated, showing positions of terminators (T), chloramphenicol-resistance gene (Cm^r), *cos* site, partition genes (*par*A, *par* B, *par*C), single copy origin (*ori*2, *inc*C, *rep*E), inducible origin (*ori*V), cloning site, and primer binding sites.

pUC plasmids, completely eliminate the blue/white screening and also incorporate transcriptional terminators, lower copy numbers, and resistance to antibiotics other than ampicillin. Such dedicated cloning vectors substantially reduced the bias against cloning a variety of difficult targets, allowing stable maintenance of strong promoter fragments or toxic coding sequences that are refractory to cloning in pUC-type vectors. Cloning into transcription-free vectors rather than pUC derivatives appears to substantially decrease the bias in shotgun libraries, facilitating more rapid assembly of genomic sequences.

References

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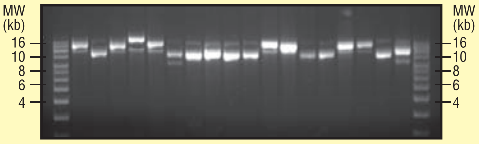

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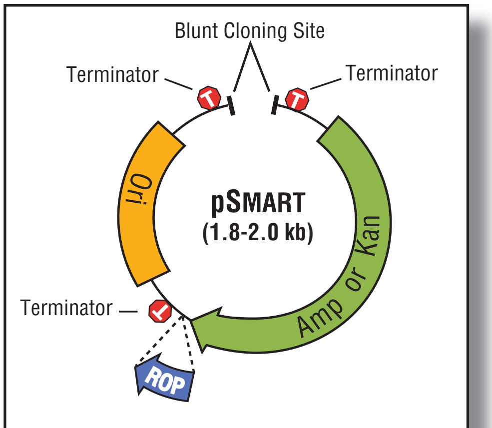

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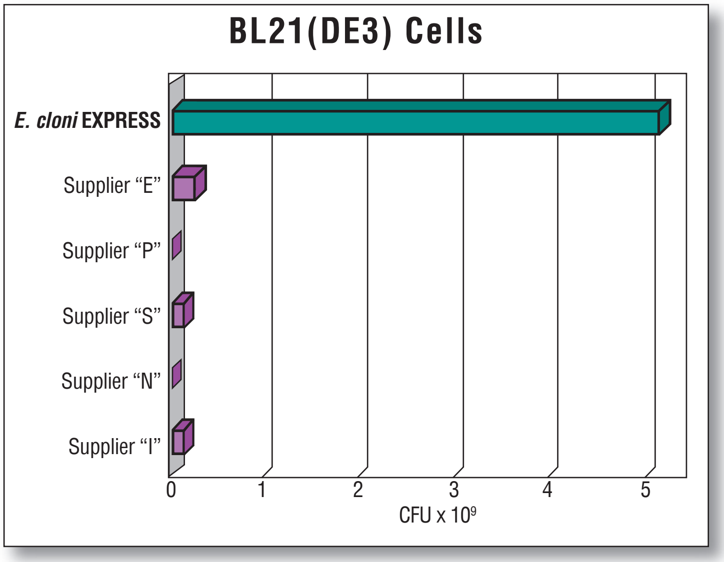

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