

High Stability Vectors for cDNA Cloning and Microarray Production

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ABSTRACT

DNA microarrays are a powerful tool for studying the complex genetic alterations that occur in cancer cells and other physiologically stressed cells. The production of microarrays often depends on amplification of cDNA or other appropriate libraries. However, large sections of viral, microbial, and eukaryotic genomes are refractory to cloning or unstable when cloned into conventional host-vector systems. This cloning bias leads to inaccurate and incomplete analyses. For example, approximately 30% of the clones obtained from a *Fundulus* heart cDNA library made in a pUC-type vector were not stable when re-grown in liquid culture. The loss of insert is due to three factors common to most plasmid vectors: 1) vector-driven expression of deleterious genes, 2) insert driven transcription interfering with vector stability, and 3) inappropriate antibiotic resistance. Most plasmid vectors induce strong transcription and translation of inserted fragments even when uninduced, which prevents cloning of numerous coding sequences. Standard vectors also allow transcription initiated by cloned promoters to interfere with plasmid replication or selection, causing additional bias. In addition, ampicillin resistance of common plasmids is mediated by a secreted enzyme, beta-lactamase. Its high catalytic activity rapidly degrades ampicillin in the medium. The rapid loss of antibiotic selection strongly favors cells without recombinant plasmids. The pSMART™-cDNA series of transcription-free cloning vectors were developed to alleviate these problems. This vector lacks an indicator gene and associated promoter, has termination signals on either side of the insertion site, and is kanamycin resistant. When used for production of cDNA libraries, >99% of pSMART-cDNA clones were stable vs. 83% of clones in a standard vector. To specifically compare pSMART-cDNA to standard pUC based vectors, the same 150 cDNAs were re-amplified from both types of plasmids. All of the pSMART-cDNA sequences were successfully amplified, whereas 17% of the same cDNAs failed in the pUC vector. For the production of 10,000 spot arrays, this could mean the loss of 1,700 genes. Other specific examples of increased stability in a transcription-free vector were noted. For example, toxic regions of the mouse hepatitis virus genome were readily cloned and stable in pSMART-cDNA, but they were deleted, rearranged, and slow-growing in TOPO and pGEM vectors. A lethal microbial nucleic acid gene was completely stable in the pSMART-cDNA vector, but unclonable in conventional plasmids. The efficiency, yield, and success rate of cloning in pSMART-cDNA libraries is a significant improvement over traditional vectors. These tools should improve the quality and quantity of data collected from microarrays, thereby increasing the likelihood of finding statistically significant differences in expression.

INCREASED STABILITY OF cDNA LIBRARIES

pUC vector

To examine gene expression in cardiac tissue of the teleost fish *Fundulus*, we constructed a microarray of relevant cDNAs. A cDNA library was constructed in a pUC based vector, and the inserts from approximately 5000 clones were amplified by PCR. Following sequence analysis of the PCR products, bacterial stocks from 1152 clones of interest (12 x 96-well plates) were re-grown to allow re-amplification of the inserts. However, only 786 of the clones (68%) yielded a PCR product after re-growth of the stock cultures (Figures 1 and 3).

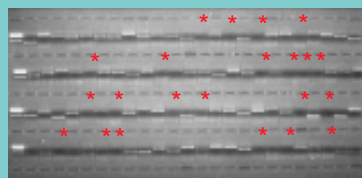


Figure 1. Amplification of cDNA clones from a pUC library. Of the clones from the pUC library, only 68% were successfully re-amplified. Failures are marked (*).

cDNA Clone Instability

Of 1152 pUC clones initially analyzed, only 788 (68%) yielded products on re-amplification. The pUC clones that appeared to be stable were tested in a third round of PCR amplification. However, an additional 15% of the "stable" clones failed to amplify (Figure 3). In contrast, >99% of randomly selected clones in the pSMART-cDNA vector were stable.

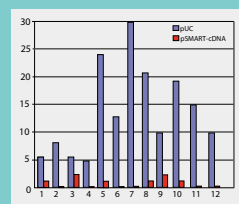


Figure 3. Instability of cDNA clones in pUC vs. pSMART-cDNA vectors. Distribution of failed clones among 12 96-well plates from cDNA libraries created in pUC (and pre-selected for stability) and the pSMART-cDNA vector (randomly chosen).

pSMART-cDNA vector

A new library from the same tissue was constructed in the vector pSMART-cDNA. Inserts from this library were similarly amplified by PCR and sequenced. Strikingly, upon re-growth and re-amplification, 1144 of 1152 pSMART-cDNA clones (>99%) resulted in successful PCR amplification (Figures 2 and 3).

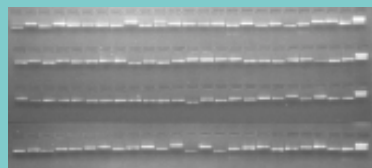
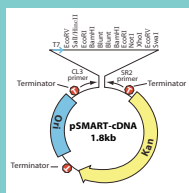


Figure 2. Amplification of cDNA clones from a pSMART-cDNA library. Over 99% of the cDNA clones in a pSMART-cDNA vector were stable upon re-growth, successfully producing PCR bands.

The sequences of the original PCR products from the 1152 pUC and 1152 pSMART-cDNA clones were compared, showing that these two groups had 157 cDNA clones in common. Successful re-growth and re-amplification was achieved for all 157 clones in the pSMART-cDNA vector, but 24 of the pUC clones (15%) failed to produce a PCR band. Thus, analysis of identical groups of inserts confirmed the increased stability in the pSMART-cDNA vector.

Transcription-free pSMART™-cDNA



The pSMART vectors eliminate vector-driven transcription of insert DNA and terminate transcription that initiates from promoters within the insert. The background of non-recombinant vector is < 0.1%.

In addition to pSMART-cDNA, members of the pSMART series include: pSMART-HCKan and -HCamp (high copy number), and pSMART-LCKan and -LCamp (low copy number).

Cloning Toxic Genes and Promoters

Unlike most vectors, pSMART™ vectors do NOT transcribe inserted DNA, allowing one to clone toxic coding sequences. Terminators at the cloning site also allow maintenance of cloned promoters. Examples include:

- Toxic regions of the Mouse Hepatitis Virus genome: These sequences are highly resistant to cloning in standard vectors (e.g. pGEM, pTOPO), but they were very stable in pSMART vectors.

-Ralph Baric, University of North Carolina Genome, J. Virol. 76:11065 (2002)
- Novel enzymes: A pSMART library contained multiple intact copies of a 3 kb gene encoding a saccharolytic enzyme. In pUC based plasmids, only deleted or rearranged versions of the gene were detected.
- Lethal genes: Many intact clones encoding a prokaryotic RNase, inserted in either orientation, were obtained in pSMART. In pUC19, the gene was only recovered in the "reverse" orientation.
- Strong promoters: The strong PR promoter of phage lambda (400 bp, 25 pg) was cloned into the pSMART and pUC19 vectors. This promoter was easily cloned and maintained in the pSMART vectors, whereas intact clones were very rare in pUC19.

Vector	Total cfu per plate	Intact λPR clones
pSMART-HC	170	75%
pSMART-LC	72	75%
pUC19	2000 Blue 20 White	---

Conclusion: Intact toxic genes, coding sequences, or strong promoters can be cloned with high efficiency in either orientation with the transcription-free pSMART vectors.

INCREASED STABILITY OF GENOMIC DNA LIBRARIES

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AT-rich DNA

A cosmid containing genomic DNA from *Pneumocystis carinii* (70% AT) was sheared to 1.5-2.5 kb, size selected, and cloned into pUC19 or pSMART™-HCKan. Plasmid DNA from transformants was analyzed by agarose gel electrophoresis.

Panel A) Plasmids from randomly picked pSMART transformants were all within the expected size range, demonstrating enhanced stability.

Panel B) Over 25% of the pUC19 transformants were unstable, yielding plasmids smaller than the parent vector. M, supercoiled plasmid ladder; V, empty vector control.

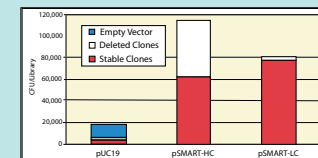


Conclusion: For cloning AT-rich DNA, increased stability is provided by the vector pSMART-HCKan, which is transcription-free and kanamycin resistant.

-In association with James Stringer and Melanie Cushion, University of Cincinnati

Creating libraries with AT-rich genomic DNA

Genomic DNA from *Lactobacillus helveticus* (65% AT) was sheared to 2-4 kb, end-repaired, and cloned into pUC19, pSMART-HC, and pSMART-LC. Plasmid DNA from transformants was analyzed by gel electrophoresis to determine whether inserts were stable (size of 2-4 kb) or deleted (<1.5 kb).



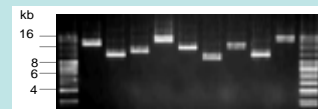
Conclusions:

- The low-copy pSMART-LC allowed a 30-fold increase in the cloning efficiency of AT-Rich DNA.
- pSMART-LC reduced clone "stacking" with an AT-rich genome. Sequencing 19,000 pUC clones provided 70% coverage of the *L. helveticus* genome. The addition of 7,000 pSMART clones increased coverage to 88% (data not shown).
- The pSMART vectors increased the sequencing efficiency of AT-rich DNA. 96% of the pSMART-LC clones yielded successful sequence reactions, compared to 80% for pUC19 (data not shown). As a result, sequencing expenses were reduced by 10-15%.

-In association with Jim Steele, University of Wisconsin
Jeff Broadbent, Utah State University

Large fragments (>10 kb)

Genomic DNA from *Shigella dysenteriae* (10 µg) was sheared, size-selected to 8-14 kb, end-repaired, and cloned into pSMART-LC. Over 20,000 clones of 8-14 kb were obtained. Many new genes with no significant homology to known genes were recovered.



Conclusion: Complex genomic libraries containing stable inserts of >10 kb inserts can be readily constructed with pSMART-LC.

-In association with Thomas Whittam, Michigan State University

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