High Stability Vectors for Cloning Unstable DNA

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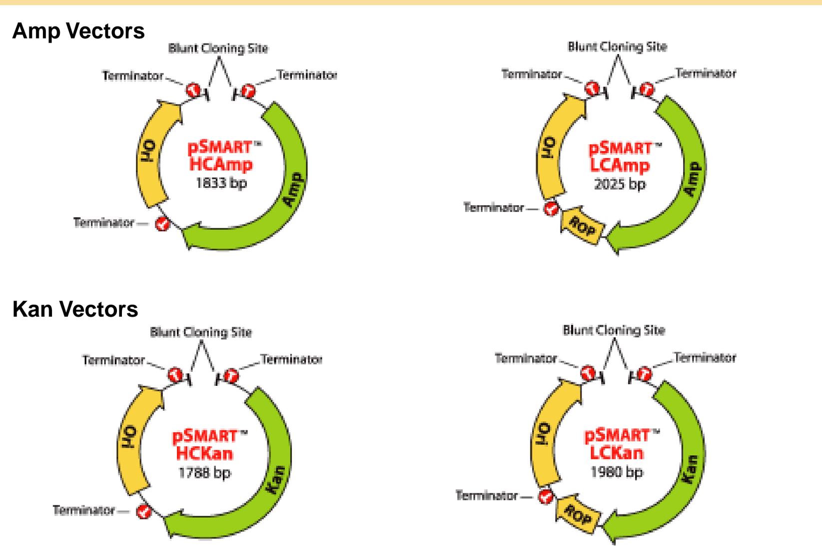
ABSTRACT

Large sections of eukaryotic, microbial, and viral genomes are refractory to cloning with conventional host-vector systems. Most plasmid vectors induce transcription and translation of inserted fragments, leading to instability of certain classes of DNA sequences or gene products. This apparently "unclonable" DNA results in sequence "stacking", clone gaps, or a complete inability to create genomic libraries, especially from AT-rich DNA. The pSMART series of transcription free cloning vectors were therefore developed to generate random shotgun libraries from difficult-to-clone DNAs. For example,

- Telomeric repeats and other AT-rich fragments from *Pneumocystis carinii* were 100% stable in pSMART but were very unstable in pUC19.
- Using pSMART rather than pUC19 to clone the AT-rich genome of *Lactobacillus helvecticus* was 25-fold more efficient and significantly less biased.
- Toxic regions of the mouse hepatitis virus genome were readily cloned and stable in pSMART, but they were deleted, rearranged, and slow-growing in TOPO and pGEM vectors.
- A low background of empty pSMART vector (< 1%) eliminated the need to screen for recombinants and facilitated cloning nanogram quantities of DNA.

Finally, a new system for multiplex cloning was developed to allow two independent DNA fragments to be cloned simultaneously into separate sites of a transcription free vector.

CloneSmart™ Vectors: No Background, No Transcription



The pSMART™ vectors eliminate vector-driven transcription of insert DNA and terminate transcription that initiates from promoters within the insert. Background of non-recombinant vector is < 0.1%. pSMART™-HCKan and -HCAmp are high-copy (300-500 copies/cell, similar to pUC19). pSMART™-LCKan and –LCAmp are low-copy (15-20 copies/cell, similar to pBR322).

CloneSmart Library Construction

Libraries of sheared, end-repaired DNA were constructed using the pre-cut pSMART vectors, buffer, ligase, and electrocompetent cells from the CloneSmart Kits.

Step 1. Ligate DNA to vector

Mix: 6.5 µl Insert DNA
2.5 µl CloneSmart 4X Vector Premix
1.0 µl CloneSmart T4 DNA Ligase
10.0 µl total reaction volume

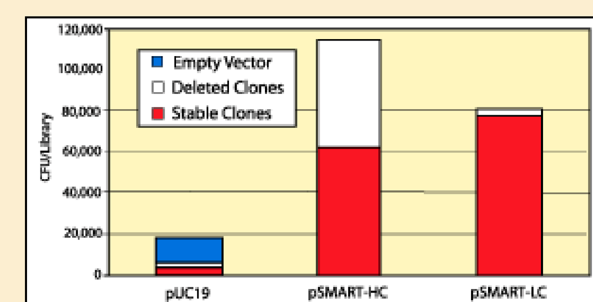
Step 2. Transform cells

Electroporate 1 µl ligation reaction into *E.cloni*™ Electrocompetent Cells provided with the Kits. Plate and grow overnight.

Step 3. Pick random colonies for sequence analysis

Colonies were picked without screening.

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. Ligations were transformed into Lucigen's *E. cloni* 10G bacteria. 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:

A) The low-copy number of pSMART-LC and its lack of transcription into or out of the insert DNA appeared to cause a 30-fold increase in the cloning efficiency of AT-Rich DNA.

B) The pSMART-LC vector reduced clone "stacking" with an AT-Rich genome. Sequencing 19,000 pUC clones provided 70% coverage of the *L. helveticus* genome. The addition of 7000 pSmart clones increased coverage to 98% (data not shown).

C) 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%.

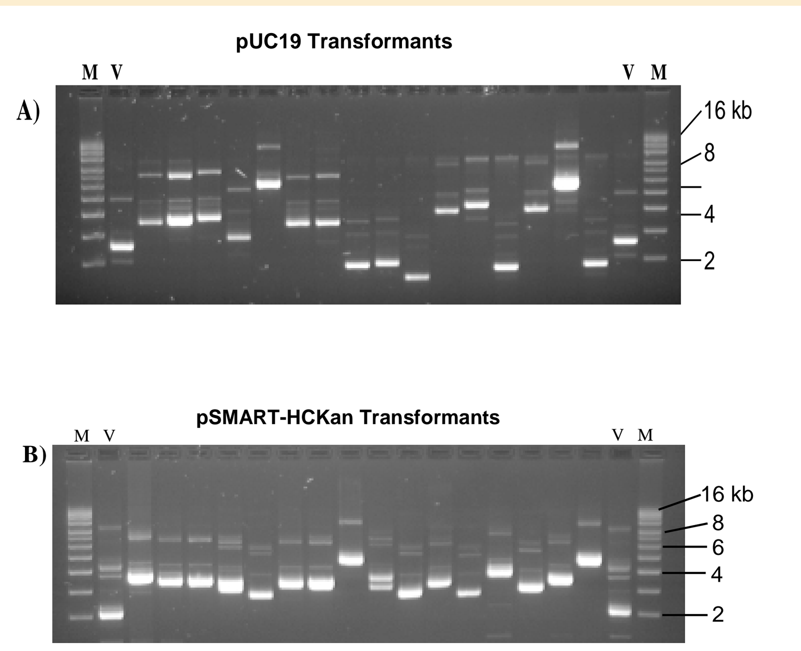
-In association with: Jim Steele, University of Wisconsin
Jeff Broadbent, Utah State University

Cloning AT-rich DNA

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

Panel A) Over 25% of the pUC19 transformants were unstable, yielding plasmids smaller than the parent vector.

Panel B) Plasmids from randomly picked pSMART transformants were all within the expected size range, demonstrating enhanced stability. M, supercoiled plasmid ladder; V, empty vector control.

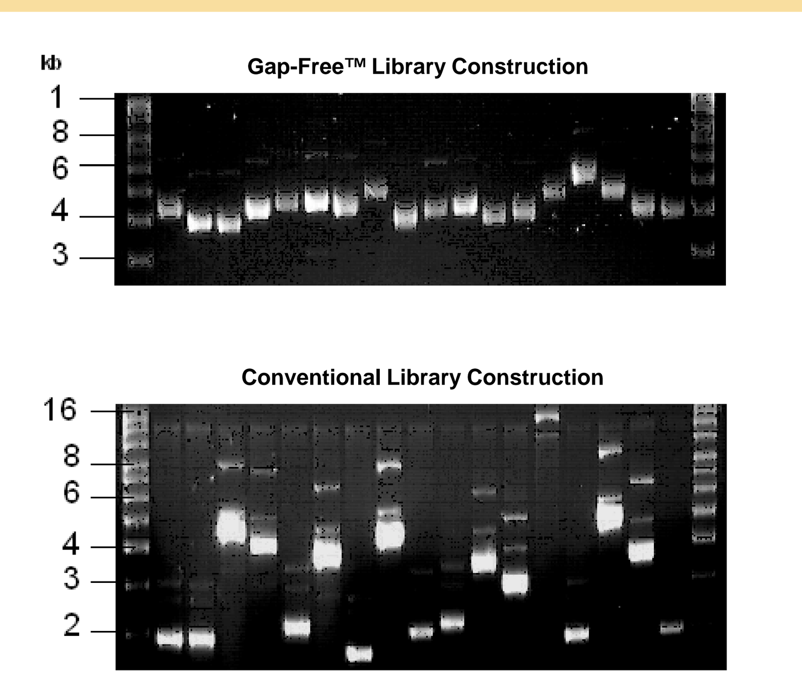


Conclusion: For cloning AT-rich DNA, maximum stability is provided by the vector pSMART-HCKan, which is transcription-free, low-copy number, and kanamycin resistant.

-In association with J. Stringer and M. Cushion, Univ. Cincinnati

Creating libraries from modified DNA

Genomic DNA from *Streptococcus thermophilus* was Hydroshear™ fragmented to 2-3 kb and end-repaired. It was cloned into pSMART-LC using Lucigen's Gap-Free™ library construction methods (Upper Panel) or with standard methods (Lower Panel).



Conclusion: Using Lucigen's Gap-Free library construction techniques for cloning *S. thermophilus* DNA resulted in approximately 1000-fold more colonies, with a dramatic increase in clone stability.

–In association with P.Richardson, JGI

Creating libraries from trace amounts of DNA

Lucigen's Gap-Free and NanoClone™ library construction processes, which employ pSMART vectors and high-efficiency *E. cloni* electrocompetent cells, were used to construct complex libraries from trace amounts of DNA. The following libraries of 10^5 to 10^6 clones have been constructed at Lucigen:

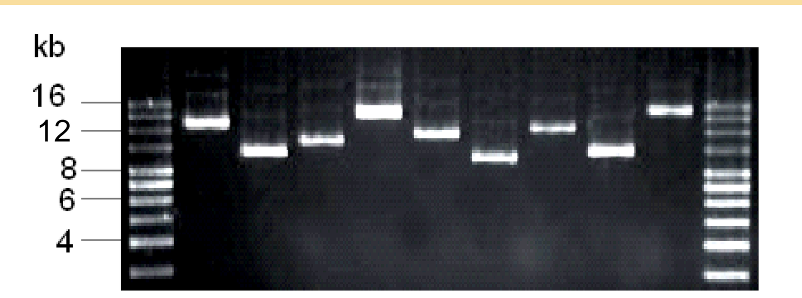
- Bacterial genomic libraries from 100 ng DNA.
- Chromosomal libraries from isolated gel plugs.
- Phage genomic libraries from 10 ng DNA.
- Libraries from 10 ng of DNA from numerous hyperthermal environmental sources.
- 1000's of colonies from 5 ng of an individual DNA fragment.

Conclusion: Lucigen's proprietary Gap-Free and NanoClone library construction technologies allowed construction of complex libraries from minimal amounts of genomic or cloned DNA. These techniques also facilitated cloning of genomic DNA that contains undefined base modifications.

Creating libraries of large fragments (>10 kb)

Genomic DNA from *Shigella dysenteriae* (10 µg) was sheared, size-selected to 8–14 kb, and end-repaired. Fragments were cloned into pSMART-LCAmp and transformed into Lucigen's *E. cloni* 10G cells. Plasmid DNA from transformants was analyzed by gel electrophoresis.

Over 20,000 clones of 8-14 kb were obtained from the *Shigella* genomic DNA. Over 29% of the clones were more than 8 kb, and an additional 26% were over 4 kb. This library contained a number of new genes with no significant homology to known genes.



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

-In association with T. Whittam, Michigan State University

Cloning Toxic Genes

A) The Mouse Hepatitis Virus genome contains several toxic regions that are highly resistant to cloning in standard microbial vectors (e.g. pGEM, pTOPO). Breaking the toxic domains into two separate components allowed them to be cloned in standard vectors, but the bacterial were slow growing and the inserts were rapidly rearranged. Under the same conditions, these inserts were highly stable in pSMART vectors.

-Ralph Baric, University of North Carolina Genomic, J. Vir. 2002 (in press)

B) DNA from a proprietary bacterial strain was sheared to 4-8 kb and cloned into pSMART-LCAmp. The library was enriched by hybridization and screened for the gene encoding a novel saccharolytic enzyme. Numerous clones in the pSMART library contained the intact 3 kb gene. In pUC based plasmids, only deleted or rearranged versions of the gene were detected.

C) A lethal prokaryotic RNase gene was cloned into pSMART vectors or into pUC19. Many intact clones, inserted in either orientation, were obtained in pSMART, but the gene was only recovered in the "reverse" orientation in pUC19.

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

Cloning Strong Promoters

A 400 bp fragment containing the PR promoter of phage lambda was amplified by PCR and cloned into the pSMART and pUC19 vectors. Ligation reactions were transformed into Lucigen's high efficiency *E. cloni* cells.

The number of colonies per plate and the proportion of clones containing the intact promoter are shown below. The transformants on each plate were derived from ligation of 25 pg of vector and 30 pg of insert.

Vector	Total cfu per plate	Intact λ P_R clones
pSMART-HC	170	75%
pSMART-LC	72	75%
pUC19	2000 Blue 20 White	--- 25%

Conclusion: The strong LambdaPR promoter was easily cloned and maintained in the pSMART vectors, whereas intact clones were very rare in pUC19. The high transformation efficiency of the *E. cloni* cells provided many colonies from minimal amounts of DNA.

High Throughput Sequencing with CloneSmart Kits

A) DNA from an organism with an AT content of 64% was sheared to 2-4 kb and 8-10 kb and cloned into pSMART-HCAmp. Sequencing 32,000 clones revealed 40 empty vector clones (99.9% recombinants).

Clones sequenced	Clones with inserts	Empty Vector Clones	Cloning Efficiency
32,000	31,960	40	99.9%

-Peter Wilson, Australian Genome Research Facility.

B) BACs containing mouse genomic DNA were sheared to 1.5 – 4 kb, end-repaired, and used for construction of libraries in pSMART-HCAmp.

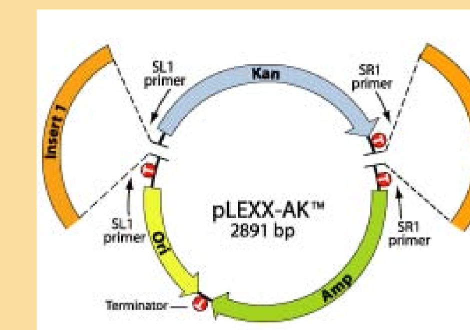
Number of Libraries (# successful/# attempts)	Clones with Inserts*	Clones/Library	Read Length
33 / 33	~ 2400 / 2400	2-4 x 10^5	> 500 bp

*96 clones from each of 24 libraries were sequenced to determine frequency of inserts. No empty vector was detected. Similar results have been obtained with 22 additional libraries.

-Kate Montgomery, Cecilia Shim, Jeremy Decker, Wendy Zencheck, Li Li, George Grills, Raju Kucherlapati. Harvard Partners Genome Center, Cambridge, MA.

Conclusion: The CloneSmart Kit reliably gives near zero background without screening for recombinants, avoiding the uncertainties and potential bias inherent to the blue-white screen.

Multiplex Cloning Accelerates High-Throughput Sequencing



Two blunt DNA fragments are inserted simultaneously and independently into the blunt cloning sites of pLEXX-AK™. Like the pSMART vectors, pLEXX-AK is transcription-free and produces <0.1 % non-recombinant clones. The pLEXX-AK vector is maintained at high copy number; pLEXX-AKL is maintained at low copy number.

ClonePlex™ libraries generate 100% recombinant clones

Lambda DNA Inserts		CFU per ml transformed cells	
Fragmentation Method	Insert Size (kb)	Vector plus Insert	Vector w/o Insert
HydroShear™	2 - 4	36,000	0
Sonication	2 - 4	27,000	0
RsaI Digestion	0.1 - 2	90,000	0

SUMMARY

- **Transcription-free pSMART vectors allow cloning of unstable DNA.**
  Use of pSMART vectors facilitated cloning of AT-rich DNA, toxic coding sequences, and strong promoters. In some cases, the low copy number and kanamycin resistant versions were essential for stable maintenance of genomic libraries.
- **Gap-Free library construction allows cloning of modified DNA.**
  Libraries were successfully constructed from nanogram amounts of modified phage or bacterial genomes.
- **Zero background eliminates colony screening.**
  With the CloneSmart and ClonePlex systems, the background of empty vector was nearly undetectable. Therefore, the error-prone process of colony screening was not necessary.
- **Rapid and simple protocol simplifies library construction.**
  Shotgun libraries were constructed simply by adding insert DNA to the components of the CloneSmart or ClonePlex Kits. The entire process of ligation, transformation, and plating was completed in less than 3.5 hours, involving less than 30 minutes of hands-on time.
- **The ClonePlex duplex cloning system yields two inserts per plasmid.**
  In a single ligation reaction, each of two inserts could be directed to either of two cloning sites in the ClonePlex vector. Thus, 4 sequence reactions could be generated from each template preparation. Dual insert clones may also be useful in other applications, such as analyzing interacting genes or creating vectors for gene knock-out or replacement.

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