Lucigen®
Simplifying Genomics

CloneSmart® Cloning Kits (pSMART Vectors)

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE

Lucigen Corporation 2905 Parmenter St, Middleton, WI 53562 USA
Toll Free: (888) 575-9695 | (608) 831-9011 | FAX: (608) 831-9012
lucigen@lucigen.com www.lucigen.com

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE
MA004 Rev. 01JAN2016

Table of Contents

Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the reagents supplied by the user, especially the DNA targets to be cloned, are of the highest quality. Please follow the manual carefully and contact our technical service representatives if additional information is necessary. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

Lucigen Technical Support

Email: techserv@lucigen.com

Phone: (888) 575-9695

Product Guarantee: Lucigen guarantees that this product will perform as specified for one year from the date of shipment. Please avoid using reagents for greater than one year from receipt**.**

Product Designations

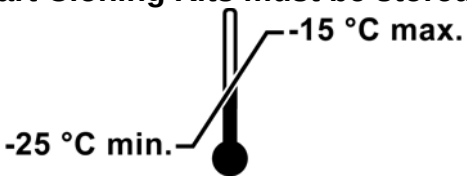
Several versions of the CloneSmart Blunt Cloning Kit are available. The kits differ in number of reactions and version of pSMART® vector (high or low copy; kanamycin or ampicillin resistance). Please refer to Appendix B: Application Guide for more information and recommended uses of the Kits.

Vector	20 Reactions	40 Reactions
pSMART-HCKan (High Copy)	40704-2	40704-4
pSMART-LCKan (Low Copy)	40821-2	40821-4
pSMART-HCAmp (High Copy)	40041-2	40041-4
pSMART-LCAmp (Low Copy)	40300-2	40300-4

Components & Storage Conditions

The Ligation Components of the CloneSmart Kits should be stored at **-20 ºC**. Additional CloneSmart Ligation Components and *E. cloni* Competent Cells may be purchased separately.

CloneSmart Cloning Kits must be stored at -20 °C



CloneSmart Ligation Components

	20 Reactions	40 Reactions
4X CloneSmart Vector Premix Includes Buffer, ATP, and ***one*** type of ligation-ready Vector: pSMART-HCKan Premix *or* pSMART-LCKan Premix *or* pSMART-HCAmp Premix *or* pSMART-LCAmp Premix	1 x 50 µL	2 x 50 µL
CloneSmart DNA Ligase (2 U/µL)	24 µL	2 x 24 µL
Positive Control Insert DNA (500 ng/µL lambda *Hinc*II)	5 µL	2 x 5 µL
CloneSmart Sequencing Primers (200 reactions each) SL1 Primer (3.2 pmol/µL) SR2 Primer (3.2 pmol/µL)	 200 µL 200 µL	 2 x 200 µL 2 x 200 µL

Competent cells are available separately from Lucigen (www. lucigen.com).

Abbreviated Protocol

Please see page 9 for a Detailed Protocol.

Insert DNA Preparation

1. Generate target DNA fragments by shearing, restriction digestion, or PCR.
2. If necessary, repair the DNA ends to make them blunt, with 5' phosphate groups.
3. Heat denature the repair reaction for 10 minutes at 70 °C.
4. Purify DNA by phenol/chloroform extraction or gel electrophoresis. **Do NOT use 256, 302, or 312 nm UV light to visualize the DNA.**

Ligation

1. Briefly centrifuge and gently mix the CloneSmart Vector Premix.
2. Combine the following components in a 1.5-mL tube. Add ligase last.

x µL Insert DNA (100-500 ng, 1 – 4 kb, blunt-ended, 5'-phosphorylated)
y µL H_2O
2.5 µL 4X CloneSmart Vector Premix (pSMART® vector, ligation buffer, ATP)
1.0 µL CloneSmart DNA Ligase (2 U/µL)
10.0 µL total reaction volume

3. Incubate 30 minutes at room temperature. (Incubate 2 hours for maximum number of clones.)
4. **Incubate the ligation reaction 15 minutes at 70 °C.**
5. Cool 15 seconds at room temperature and 15 seconds on ice.
6. Spin 1 minute at 12,000 rpm.

Transformation (USE ONLY ELECTROCOMPETENT CELLS FOR ELECTROPORATION AND CHEMICALLY COMPETENT CELLS FOR HEAT SHOCK TRANSFORMATION!)

1. Place Recovery Medium at room temperature.
2. Chill electroporation cuvettes, 1.5 mL microfuge tubes, and sterile culture tubes on ice.
3. Thaw *E. cloni* Electrocompetent Cells or Chemically Competent Cells on wet ice. Pipet 25 µL of Electrocompetent cells into a pre-chilled 1.5 mL tube on ice or 40 µL Chemically Competent cells into a pre-chilled 17 mm x 100 mm culture tube on ice.
4. Add 1 µL of heat-treated ligation reaction to an aliquot of chilled cells on ice.

Electroporation	Heat Shock Transformation
5. Pipet 25 µL of the cell/DNA mixture to a chilled electroporation cuvette.	5. Incubate 30 minutes on ice.
6. Electroporate. Immediately add 975 µL of room temperature Recovery Medium. Place in culture tube.	6. Incubate 45 seconds at 42 °C; then 2 minutes on ice. Add 960 µL of room temperature Recovery Medium to the culture tube.

7. Shake at 250 rpm for 1 hour at 37 °C.
8. Spread up to 100 µL per plate on bacterial medium agar plates containing the appropriate antibiotic. Incubate overnight at 37 °C.

Colony Growth

1. Pick colonies at random and grow in rich (e.g., LB) medium containing the appropriate antibiotic.

Product Description

Lucigen's CloneSmart kits are ideal for constructing shotgun libraries, for general purpose cloning, or for cloning blunt PCR products, especially when amounts of target DNA are limited. The CloneSmart Blunt Cloning Kits are convenient to use, containing pre-cut, dephosphorylated pSMART cloning vector premixed with buffer and ATP, as well as ligase, sequencing primers, and DNA controls. Competent cells are available separately.

Lucigen's CloneSmart transcription-free cloning technology (U.S. Pat. 6, 709, 861) is designed to eliminate cloning bias and maximize cloning efficiency. The ultra-low background of empty vector (< 0.5%) eliminates the need to screen for recombinants and enables library construction from nanogram amounts of DNA.

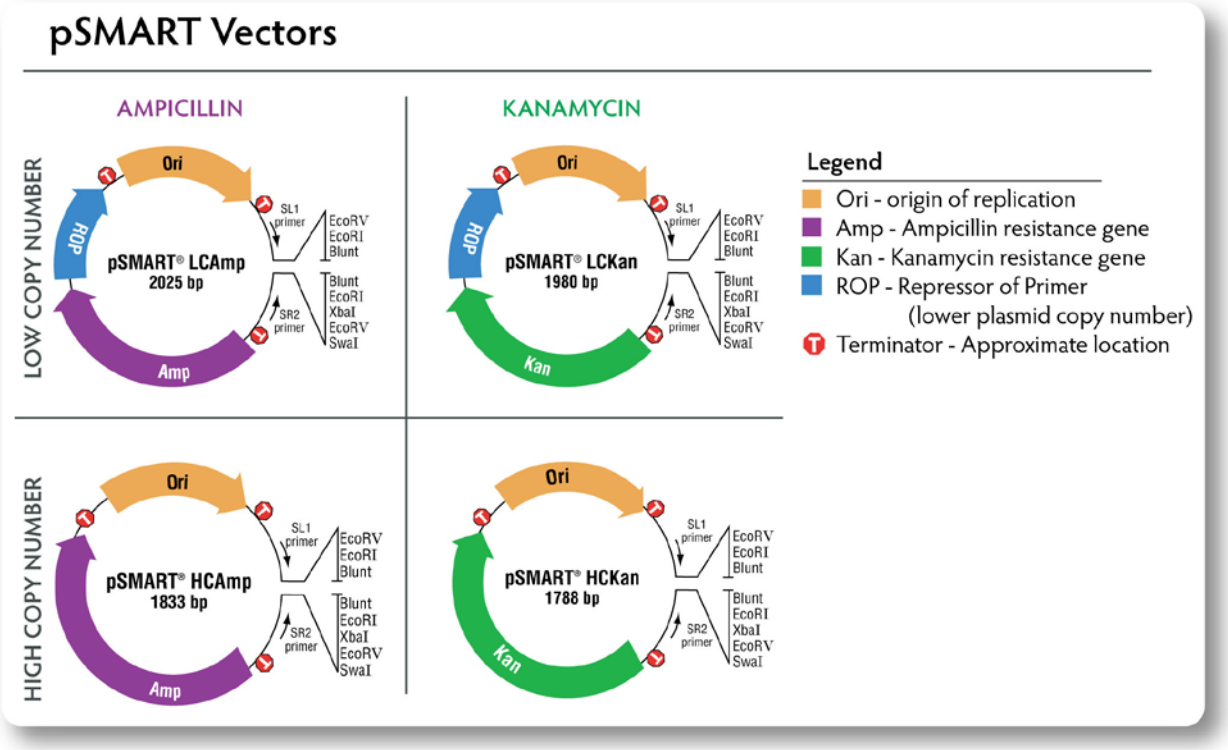


Figure 1. pSMART vectors. Ori, origin of replication; Amp, ampicillin resistance gene; Kan, Kanamycin resistance gene; ROP, Repressor of primer (lowers plasmid copy number). Approximate positions of sequencing primers and transcription terminators (T) are indicated.

pSMART® Vectors

All pSMART vectors are supplied pre-cut with blunt, dephosphorylated ends (Figure 1). The unique design of these vectors eliminates transcription into and out of the insert DNA, reducing cloning bias. Strong promoters driving indicator genes or negative selection genes (*lac*Zα or *ccd*B) can cause plasmid instability with their strong secondary structure, and by transcription of toxic inserts. pSMART vectors do not contain a promoter or indicator gene, so transcription across the insert is avoided. Strong transcription terminators flank the insert cloning site to eliminate fortuitous transcription from cloned inserts.

The copy number of the pSMART-HCKan and -HCAmp vectors is similar to that of pUC plasmids (~300 copies/cell) and that of the pSMART-LCKan and -LCAmp vectors is similar to pBR322 (~20 copies/cell). The low copy versions contain ROP, which inhibits plasmid replication.

The pSMART vectors contain kanamycin or ampicillin resistance. Ampicillin-resistant pSMART vectors have been designed to reduce the growth of satellite colonies, which complicate colony picking and can contaminate cultures. Satellite colonies are eliminated with the kanamycin-resistant pSMART vectors.

pSMART transformants do not require screening to avoid colonies containing empty vector. The pSMART® vectors produce 99.5% recombinant clones in typical experiments. Vectors utilizing *lac*Zα blue/white screening can generate blue colonies and ambiguous "light blue" colonies, both of which may contain inserts but are often discarded. The DNA contained in such clones is lost, leading to gaps in sequence assemblies. The high efficiency of CloneSmart technology eliminates the need for XGAL/IPTG and removes the uncertainty of false negatives/false positives.

The GenBank accession numbers for the vectors are as follows: pSMART-HC, AF399742; pSMART-LC, AY090111; pSMART-HCKan, AF532107; and pSMART-LCKan, AF532106.

E. cloni® Competent Cells

Competent cells are available separately from Lucigen (www. lucigen.com).

For maximum cloning efficiency, we strongly recommend the use of Lucigen's *E. cloni*® 10G ELITE or 10G SUPREME Electrocompetent Cells. For less demanding applications, *E. cloni* Chemically Competent cells may be used. The number of clones will decrease in proportion to the competency of the cells.

E. cloni 10G Competent Cells are optimized for high efficiency transformation. They are ideal for cloning and propagation of BAC, cosmid, or plasmid clones. They give high yield and high quality plasmid DNA due to the *rec*A1 and endA1 mutations, and are phage T1-resistant (*ton*A mutation). *E. cloni* 10G strains contain the inactive *mcr* and *mrr* mutations, allowing methylated genomic DNA that has been isolated directly from mammalian or plant cells to be cloned without deletions or rearrangements. The *rps*L mutation confers resistance to streptomycin.

Cell Preparation	24 Reactions		Transformation Efficiency
	SOLO*	DUO*	
***E. cloni* 10G SUPREME Electrocompetent Cells**	60081-2	60080-2	$\geq 4 \times 10^{10}$ cfu/µg
E. cloni* 10G ELITE Electrocompetent Cells*	60051-2	60052-2	$\geq 2 \times 10^{10}$ cfu/µg
***E. cloni* 10G Chemically Competent Cells**	60106-2	60107-2	$\geq 1 \times 10^{9}$ cfu/µg

*SOLO and DUO formats contain 1 or 2 transformations per vial.

**E. cloni 10G ELITES are also available in 150 µL aliquots (SixPacks), sufficient for six transformation reactions of 25 µL each.

Genotype of *E. cloni* 10G: F- mcrA Δ(mrr-hsdRMS-mcrBC) endA1 recA1 Φ80dlacZΔM15 ΔlacX74 araD139 Δ(ara,leu)7697galU galK rpsL nupG λ- tonA (StrR)

Considerations For Use

Please read the entire manual and prepare the necessary equipment and materials before starting.

Preparation and Purification of Insert DNA

pSMART® Blunt Vectors require a blunt-ended DNA insert with 5' phosphate groups.

PCR Products

PCR products with 3' single base overhangs can be treated with Lucigen's DNATerminator® End Repair Kit to generate blunt phosphorylated ends. Alternatively, phosphorylated PCR primers and a proofreading thermostable polymerase can be used to generate blunt phosphorylated DNA. PCR primers may be synthesized with 5' terminal phosphate groups or treated with T4 polynucleotide kinase prior to PCR.

PCR products with blunt, phosphorylated ends and DNA fragments created by digestion with blunt-cutting restriction enzymes (e.g., *Eco*RV or *Hin*cII) can be used with the CloneSmart Blunt Cloning Kits without further processing.

Generation of Sheared DNA Fragments

For shotgun library construction, blunt phosphorylated DNA is typically generated by randomly shearing the DNA followed by end repair to generate blunt ends, and a final fractionation step to size-select the fragments. Lucigen recommends the following methods to generate DNA fragments.

Desired Fragment Size	Recommended Method	Recommended Instrument	Manufacturer
2kb-50kb	Physical Shearing	Megaruptor	Diagenode
50-5000bp	Sonication	Covaris	Covaris
50-1000bp	Sonication	Bioruptor	Diagenode

Mechanical methods of DNA fragmentation recommended in the table above are preferred over enzymatic methods, as they are more random and reduce the bias of sequencing projects (1).However, mechanical fragmentation results in a heterogeneous mix of blunt and 3'- and 5'-overhanging ends that may not ligate efficiently. Successful library construction requires end repair to convert these ragged ends to blunt ends.

End Repair

For repairing mechanically sheared DNA, we recommend using Lucigen's DNATerminator® End Repair Kit. The DNATerminator End Repair Kit also efficiently removes 3' or 5' overhanging ends created by restriction digestion.

Note that genomic DNA fragments must be relatively free of RNA before end repair. Large amounts of contaminating RNA will severely impair the efficiency of the end repair reaction, resulting in DNA with poor cloning capability. We recommend RNAse I (an exonuclease that breaks RNA down into nucleosides) to remove residual RNA associated with DNA purification protocols. RNAse A (a site specific endonuclease) will not sufficiently degrade the RNA and is not recommended.

Purification and Size Fractionation of DNA

DNA must be purified from restriction or repair enzymes before ligation to pSMART vectors. If end repaired or kinased fragments are subsequently fractionated by gel electrophoresis, no further purification is necessary. After electrophoresis, DNA may be isolated using your method of choice.

If the DNA is *not* size fractionated, it must be purified by phenol/chloroform extraction or binding to a purification column. Heat denaturation is NOT sufficient to inactivate the enzymes. Failure to completely remove residual enzymes may lead to inefficient ligation or high background of non-recombinant clones.

Avoid exposing the DNA to short wave UV radiation

Exposure to short wavelength ultraviolet light (e.g., 254, 302, or 312 nm) can reduce cloning efficiencies by several orders of magnitude (Figure 2).

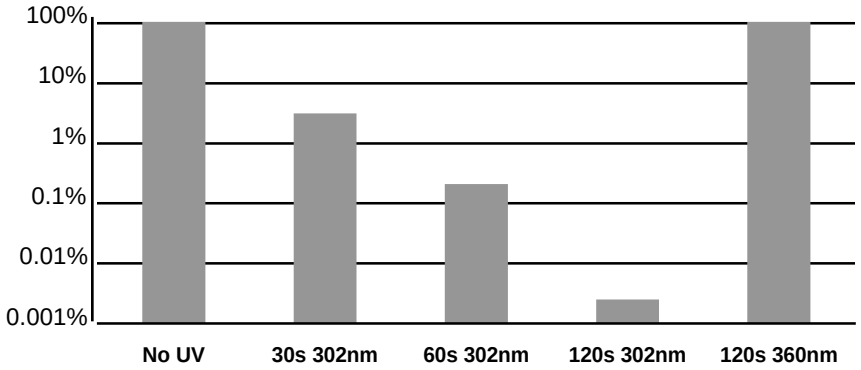


Figure 2. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 90 seconds ("30 s 302 nm, 60s 302 nm,120 s 302 nm") or to 360 nm UV light for 120 seconds ("120 s 360 nm"). Cloning efficiencies were calculated relative to un-irradiated pUC19 DNA.

Use a long wavelength (e.g., 360 nm) low intensity UV lamp and short exposure times when isolating DNA fragments from agarose gels. Alternatively, fluorescent DNA stains with optimum illumination wavelengths outside the UV range can be used for DNA visualization.

Materials and Equipment Needed

Please read the entire manual and prepare the necessary equipment and materials before starting. Following ligation, the following items are required for transformation:

- **Chemically or electrically competent *E. coli*** (see recommendations on page 6).
- Electroporation apparatus and 0.1 cm cuvettes (for electrocompetent cells). Successful results are obtained with cuvettes from BTX (Model 610) or BioRad (Cat. #165-2089). Users have reported difficulties using *E. cloni* cells with Invitrogen cuvettes (Cat. # 65-0030).
- Water bath at 42 °C (for chemically competent cells).
- Wet ice.
- Sterile 17 x 100 mm culture tubes.
- Bacterial medium agar plates containing ampicillin or kanamycin.

Detailed Protocol

An Abbreviated Protocol for experienced users can be found on page 4.

Ligation to the pSMART® Vector

For library construction, we recommend using 300-500 ng of insert DNA in the size range of 1000 to 4000 bp. For cloning a single DNA species, 100-200 ng of insert is sufficient. Successful cloning can be achieved routinely with less than 100 ng of insert, but use of low amounts of insert will result in significantly fewer transformants.

1. Briefly centrifuge the CloneSmart Vector Premix before use. Mix by gently pipeting up and down several times.
2. Combine in a 1.5 mL Eppendorf tube or 0.2 mL PCR tube, in the following order:

   x µL Insert DNA (100-500 ng, blunt-ended, 5'-phosphorylated)
   y µL H_2O
   2.5 µL 4X CloneSmart Vector Premix (pSMART vector, ATP, buffer)
   1 µL CloneSmart DNA Ligase (2 U/ µ L)
   10.0 µL total reaction volume

1. Mix by gently pipeting up and down. Incubate at room temperature (21-25 °C) for 30 minutes. To obtain the maximum number of clones, ligation time can be extended to 2 hours. Optional control reactions include the following:

Positive Control Insert DNA (to determine ligation and transformation efficiency)	Use 1 µL (500 ng) of the supplied Positive Control DNA (*Hinc*II-digested lambda DNA)
Vector Background (to determine background of empty vector)	Omit Insert DNA in the above reaction

Preparation for Transformation

***ESSENTIAL:* The ligation reaction must be heat inactivated!**

1. Incubate ligation reaction at 70°C for 15 minutes.
2. Cool to room temperature for 15 seconds, then cool to 4°C on ice for 15 seconds.
3. Centrifuge briefly to collect the mixture at the bottom of the tube and proceed to transformation.

The following protocols are provided for transformation of Lucigen *E. cloni* Competent Cells.

Electroporation of *E. cloni* Electrocompetent Cells

Competent cells are available separately from Lucigen (www.lucigen.com).

Transformation is carried out in a 0.1 cm gap cuvette. Typical time constants are 3.5 to 4.5 msec. Optimal settings for electroporation are listed in the table below.

Optimal Setting	Alternate Settings (~ 20-50% lower efficiencies)
1.0 mm cuvette	1.0 mm cuvette
10 µF	25 µF
600 Ohms	200 Ohms
1800 Volts	1400 – 2000 Volts

Suggested Electroporation Systems:

Bio-Rad Micro Pulser #165-2100; Bio-Rad E. coli Pulser #165-2102; Bio-Rad Gene Pulser II #165-2105; BTX ECM630 Electroporation System; Eppendorf Model 2510.

Suggested Electroporation Cuvettes:

BTX (Model 610) or BioRad (Cat. #165-2089)

To ensure successful transformation, the following precautions must be taken:

- ✓ ***ESSENTIAL:* the ligation reaction must be inactivated at 70 °C for 15 minutes!**
- ✓ Microcentrifuge tubes and electroporation cuvettes must be thoroughly pre-chilled on ice.
- ✓ The cells must be completely thawed **on ice** before use.
- ✓ Recovery Medium should be used after electroporation. Use of SOC or other media may decrease transformation efficiency.

Transformation Protocol (Electrocompetent cells)

1. Prepare at room temperature:
   - 17 mm x 100 mm sterile culture tubes (one tube for each transformation)
   - Recovery Medium
2. Prepare on wet ice:
   - Electroporation cuvettes with 0.1 cm gap (1 cuvette for each transformation)
   - Microcentrifuge tubes on ice (one tube for each transformation)
3. Remove *E. cloni* cells from the -80 °C freezer and place on wet ice until they thaw **completely** (10-20 minutes).
4. When cells are thawed, mix them by tapping gently. Add 25 µL of *E. cloni*® cells to the chilled microcentrifuge tube on ice.
5. Add 1 µL of the **heat-inactivated** CloneSmart Ligation reaction to the 25 µL of cells on ice. Stir briefly with pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells. Use of more than 2 µL of ligation mix may cause electrical arcing during electroporation.

   For transformation control, use 1 µL (10 pg) of supercoiled pUC19 DNA.
6. Carefully pipet 25 µL of the cell/DNA mixture into a chilled electroporation cuvette without introducing bubbles. Quickly flick the cuvette downward with your wrist to deposit the cells across the bottom of the well. Electroporate according to the conditions recommended above.

7. Within 10 seconds of the pulse, add 975 µL of Recovery Medium to the cuvette and pipet up and down three times to resuspend the cells. Transfer the cells and Recovery Medium to a culture tube.
8. Place the tube in a shaking incubator at 250 rpm for 1 hour at 37 °C.
9. Spread up to 100 µL of transformed cells on bacterial medium agar plates containing the appropriate antibiotic.

   Note: When transforming pUC19 control, plate cells on agar containing ampicillin.
10. Incubate the plates overnight at 37 °C.
11. Transformed clones can be further grown in any rich culture medium (e.g., LB).

Heat Shock Transformation of Chemically Competent cells

Competent cells are available separately from Lucigen (www.lucigen.com).

To ensure successful transformation, the following precautions must be taken:

- ✓ ***ESSENTIAL:* the ligation reaction must be heat inactivated at 70 ºC for 15 minutes!**
- ✓ Microcentrifuge tubes must be thoroughly pre-chilled on ice before use.
- ✓ The cells must be completely thawed **on ice** before use.
- ✓ Recovery Medium should be used after electroporation. Use of SOC or other media may decrease transformation efficiency.

Transformation Protocol (Chemically Competent cells)

1. Chill 17 mm x 100 mm sterile culture tubes on ice (one tube for each transformation reaction).
2. Remove *E. cloni* cells from the -80 °C freezer and place on wet ice until they thaw **completely** (10-20 minutes).
3. Add 40 µL of *E. cloni* cells to the chilled culture tube.
4. Add 1 µL of the heat-inactivated CloneSmart Ligation reaction to the 40 µL of cells on ice. Stir briefly with pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells.

   For transformation control, use 1 µL (10 pg) of supercoiled pUC19 DNA.
5. Incubate on ice for 30 minutes.
6. Heat shock cells by placing them in a 42 ºC water bath for 45 seconds.
7. Return the cells to ice for 2 minutes.
8. Add 960 µL of room temperature Recovery Medium to the cells in the culture tube.
9. Place the tubes in a shaking incubator at 250 rpm for 1 hour at 37 °C.
10. Plate up to 100 µL of transformed cells on bacterial medium agar plates containing the appropriate antibiotic.

    Note: When transforming pUC19 control, plate cells on agar containing ampicillin.
11. Incubate the plates overnight at 37 °C.
12. Transformed clones can be further grown in any rich culture medium (e.g., LB).

Expected Results

The results presented below are expected when cloning 500 ng of intact, purified DNA fragments, with blunt ends and 5' phosphate groups, into Lucigen's *E. cloni* 10G ELITE Electrocompetent Cells (transformation efficiency $\geq$ 2 x 10^{10} cfu/µg pUC19 DNA).

Plating Transformed Cells

Reaction Plate	µL /Plate
Experimental Insert (500 ng per ligation)	5, 20, & 100
Lambda *Hinc*II Insert (Positive Control)	5
No-Insert Control (Vector Background)	50
Supercoiled pUC19 Transformation Control Plasmid (10 pg, **Amp^R**)	2*

*Dilute 2 µL of transformed cells into 90 µl of medium.

The number of recombinant clones is typically 1000-fold greater than the background of self-ligated pSMART vector. The background number of empty pSMART vectors is less than 0.1% and is constant (< 25 colonies per 50 µL of cells plated) unless a contaminating kinase is present.

Use of too little insert DNA, insert DNA with improperly repaired ends, or modified DNA that is not repairable yields significantly lower cloning efficiencies. Cloning AT-rich DNA and other recalcitrant sequences may also lead to fewer colonies. With relatively few recombinant clones, the number of empty vector colonies becomes noticeable. For example, if the Experimental Insert ligation reaction produces only 250 colonies, then 25 colonies obtained from the No-Insert Control ligation represents a background of 10%.

Use of competent cells with a transformation efficiency of less than 1 x 10^9 will severely compromise the desired results, yielding less than 10% of the number of cfus listed below.

Expected Efficiencies with Lucigen's *E. cloni* 10G ELITE Electrocompetent Cells

Reaction	CFU/Plate	Efficiency
pSmart-HC plus 500 ng lambda/HcII Insert	> 200	> 99.9% inserts
pSmart-LC plus 500 ng lambda/HcII Insert	> 50	> 99.9% inserts
No-Insert Control (Vector Background)	< 25	< 0.1% background
pUC19 Supercoiled Plasmid Control (10 pg)	> 200**	> 2 x 10^{10} cfu/µg plasmid

** 2 µL of transformed cells from the supercoiled pUC19 reaction (diluted into 90 µL of medium) should yield > 200 colonies, or > 1 x 10^{10} colonies per µg plasmid.

No Screening Required

For most applications, no additional screening for recombinant colonies is required, as the CloneSmart system typically delivers >99.9% recombinant clones. Because the background is extremely low, colonies can usually be picked at random for growth and plasmid purification. However, some insert DNAs (e.g., those that are large or have unusual base composition) may produce very few colonies, in which case screening by insert size may be necessary to detect the relatively few recombinant plasmids among the small number of empty vector clones.

DNA Isolation & Sequencing

Grow transformants in rich medium plus appropriate antibiotic (100 µg/ml ampicillin or carbenicillin for pSMART-HCAmp or -LCAmp; or 30 µg/mL kanamycin for pSMART-HCKan or -LCKan.). Use standard methods to isolate plasmid DNA suitable for sequencing. The pSMART-HC plasmids contain the high copy number pUC origin of replication, yielding 20 – 80 µg of plasmid DNA per mL of culture. The pSMART-LC plasmids contain the low copy number pBR322 origin of replication,

reducing plasmid yields to 0.5 – 1.0 µg per mL of cells. The *E. cloni* 10G Competent Cells are *recA endA* deficient and provide high quality plasmid DNA. The CloneSmart Kit is provided with the sequencing primers SL1 and SR2. The sequence of the primers and their orientation relative to the pSMART plasmid is shown in Appendix D.

References

1. Sambrook, J. and Russell, DW. Molecular Cloning: A Laboratory Manual (Third Edition). 2001. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

2. Godiska R, Patterson M, Schoenfeld T, Mead DA. 2005. "Beyond pUC: Vectors for Cloning Unstable DNA." *In* DNA Sequencing: Optimizing the Process and Analysis. (J. Kieleczawa, ed.), Jones and Bartlett Publishers, Sudbury, MA.

Appendix A: Media Recommendations

Growing Transformed Cultures. Colonies obtained from a pSMART transformation can be further grown in rich (e.g., LB or TB) culture medium, containing the appropriate antibiotic (100 µg/mL ampicillin or carbenicillin for pSMART-HCAmp or -LCAmp; 30 µg/mL kanamycin for pSMART-HCKan or -LCKan.). Transformed cultures can be stored by adding sterile glycerol to 20% (final concentration) and freezing at –70ºC. Unused portions of the ligation reactions may be stored at –20ºC.

Appendix B: CloneSmart Application Guide

CloneSmart Blunt Cloning Kits accommodate any cloning situation. Use of the kanamycin-resistant pSMART vectors may be preferred, because ampicillin is more easily degraded by resistant clones. For routine applications, we recommend using the CloneSmart HCKan Blunt Cloning Kit, containing the high copy number pSMART-HCKan vector.

For the most challenging inserts of up to 30 kb, including AT-rich DNA, repeated sequences, or other regions with strong secondary structure, the pJAZZ® Linear vector in Lucigen's BigEasy® v2.0 Linear Cloning Kits provides unprecedented stability.

Use of the *E. cloni*® 10G strain is essential for cloning inserts that may be methylated, such as genomic DNA isolated directly from plant or mammalian cells, as this strain contains the inactive *mcr* and *mrr* alleles [*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)]. The 10G SUPREME preparation of these cells is recommended for cloning difficult or very small quantities of insert DNA.

Vector		Insert DNA Source			Desired Use	
Vector Name	Copy #	Cosmid, Plasmid, BAC, etc.	Genomic or cDNA	AT-Rich, "Difficult"	Digestion, Subcloning, Sequencing	PCR, etc.
pSMART-HC Kan	High	+	+	+	++	+
pSMART-LC Kan	Low	+	+	++	+	+
pSMART-HC Amp	High	+	+	+	++	+
pSMART-LC Amp	Low	+	+	+	+	+
pJAZZ®-OC	Low-Mid (Inducible)	+	+	+++	+	+

Appendix C: Vector Map, Cloning Site, and Sequencing Primers

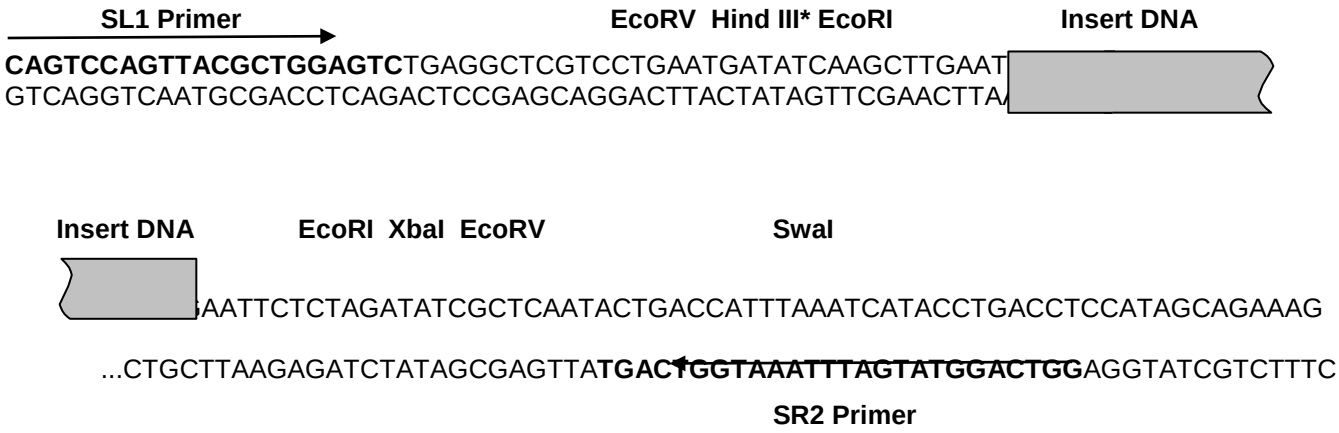
The pSMART® vectors are supplied predigested, with blunt, dephosphorylated ends. Transcriptional terminators border the cloning site to prevent transcription from the insert into the vector. Another terminator at the 3' end of the ampicillin or kanamycin resistance gene prevents this transcript from reading into the insert DNA.

The cloning sites and sequencing primers are identical for pSMART-HCAmp, -LCAmp, -HCKan, and –LCKan. The sequences of the SL1 and SR2 primers are as follows:

SL1: 5'–CAG TCC AGT TAC GCT GGA GTC–3'
SR2: 5'–GGT CAG GTA TGA TTT AAA TGG TCA GT–3'

The GenBank accession numbers of the pSMART vectors are as follows:

pSMART-HCKan: AF532107	pSMART-LCKan: AF532106
pSMART-HCAmp: AF399742	pSMART-LCAmp: AY090111



***The Hind III site is NOT unique in the pSMART-HCKan and -LCKan vectors. Another Hind III site is present in the kanamycin resistance gene.**

Appendix D: Troubleshooting Guide

Problem	Probable Cause	Solution
Very few or no transformants	Inefficient end repair.	Check the insert DNA for self-ligation by gel electrophoresis. Repeat end repair if necessary.
	Contaminating enzymes in ligation reaction.	Heat-denature end repair reaction or restriction digest 10 minutes at 70 °C. Purify DNA by extraction or adsorption to matrix.
	No DNA, degraded DNA, or insufficient amount of DNA.	Check insert DNA by gel electrophoresis. Determine concentration of insert and add the correct amount. Use the supplied control insert to test the system.
	Ligation reaction failed.	Check the insert DNA for self-ligation by gel electrophoresis. Repeat end repair if necessary. Be sure insert DNA is phosphorylated. Use the supplied control insert to test ligation reaction.
	Inadequate heat denaturation of ligation reaction.	Be certain to heat denature for 15 min at 70 °C. Skipping this step may lower the number of transformants by 2-3 orders of magnitude.
	Loss of DNA during precipitation.	DO NOT precipitate DNA after ligation reaction. It is not necessary with this protocol and these cells.
	Incorrect recovery media.	Use Recovery Medium provided after transformation of electrocompetent or chemically competent cells. Use of other media (e.g., SOC) will result in lower yield.
	Improper electroporation conditions.	Use BTX or BioRad electroporation cuvettes with a gap of 0.1 cm. Pre-chill cuvettes on ice. Add the 1 µL of DNA to 25 µL of pre-aliquotted cells on wet ice; DO NOT add the cells to the DNA.
	Addition of XGAL/DMSO to competent cells.	DO NOT add additional compounds to competent cells, as they are fragile.
	Incorrect amounts of antibiotic in agar plates. Wrong antibiotic used.	Add the correct amount of Ampicillin/Carbenicillin or Kanamycin to molten agar at 55 °C before pouring plates (see Appendix A). DO NOT spread antibiotic onto the surface of agar plates.
High background of transformants that do not contain inserts.	Contaminating oligonucleotides in the ligation reaction.	Gel purify inserts before cloning. Use multiple methods of DNA purification to remove small fragments.
	Contaminating enzymes in ligation reaction.	Purify DNA after DNA End Repair reaction. DO NOT add T4 DNA Kinase to the ligation reaction.
	Incorrect amount of antibiotic in agar plates.	DO NOT spread antibiotic onto the surface of agar plates. Add the correct amount of kanamycin or ampicillin/carbenicillin to molten agar at 55 °C before pouring plates (see Appendix A).
	Unstable DNA Inserts	Use the BigEasy® v2.0 Linear Cloning Kit for maximum clone stability.

Notice of Limited Label License, Copyright, Patents, Warranties, Disclaimers and Trademarks

A complete list of trademarks, registered trademarks, limited label license, etc. held by Lucigen Corp. can be found at http://www.lucigen.com/Legal-Information.html