CSMART™ CDNA CLONING KITS

IMPORTANT!
-86°C and -20°C Storage Required Immediately Upon Receipt

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Technical Support

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cSMART™ Kit Designations

Several versions of the cSMART™ cDNA Cloning Kit are available. The kits differ in number of reactions, version of pSMART® vector, and cells that are included. The catalog numbers are listed below. Please refer to Appendix B: Application Guide for more information and recommended uses of the kits.

Catalog numbers of vector and cell combinations

Vector	Reactions	10G Elite Electrocompetent Cells	10G Supreme Electrocompetent Cells	No Cells
pSMART-cDNA-Blunt	10	41047-1	41049-1	---
	20	41047-2	41049-2	41045-2
	40	---	---	41045-4
pSMART-cDNA-BluntN	10	41036-1	41038-1	---
	20	41036-2	41038-2	41034-2
	40	---	---	41034-4
pSMART-cDNA-RN	10	41015-1	41018-1	---
	20	41015-2	41018-2	41012-2
	40	---	---	41012-4

Components & Storage Conditions

The Ligation Components of the cSMART Kits, including 4X Vector Premix, Ligase, Controls, and sequencing primers, are shipped in Container 1, which should be stored at **–20°C**. If *E. cloni*® Cells are ordered with the Kit, they are shipped in Container 2, which must be stored at **-86°C**. Additional cSMART Ligation Components and *E. cloni* Electrocompetent Cells may be purchased separately.

Container 1: cSMART™ Ligation Components

Store at -20°C

	10 Reactions	20 Reactions	40 Reactions
4X cSMART Vector Premix Includes Buffer, ATP, and ligation-ready pSMART-cDNA Vector	25 µl	50 µl	2 x 50 µl
CloneSmart™ DNA Ligase (2 U/µl)	10 µl	1 x 20 µl	1 x 40 µl
Positive Control Insert DNA (50 ng/µl AmpR fragment)	5 µl	5 µl	5 µl
cSMART Sequencing Primers (200 reactions each) CL3 Primer (3.2 pmol/µl) SR2 Primer (3.2 pmol/µl)	 200 µl 200 µl	 200 µl 200 µl	 200 µl 200 µl

Container 2: *E. cloni* ® Competent Cells

Store at -86°C

	Catalog #	Reactions
E. cloni 10G Elite Electrocompetent Cells	60052-1 60052-2	12 (6 x 50 µl) 24 (12 x 50 µl)
or *E. cloni* 10G Supreme Electrocompetent Cells	60080-1 60080-2	12 (6 x 50 µl) 24 (12 x 50 µl)

Control pUC19 DNA (1 ng/μl)	store at **-20°C** or **-86°C**	----	(1 x 5 μl)
Recovery Medium	store at **-20°C** or **-86°C**	----	12 (1 x 12 ml) 24 (1 x 24 ml)

cSMART™ cDNA Cloning Kit Description

The cSMART™ family of cloning kits are designed to eliminate cloning bias and maximize cloning efficiency. When used with Lucigen's *E. cloni*® cells, the cSMART cDNA Cloning Kits routinely yield up to a million recombinant clones from 500 ng of insert DNA, with no vector preparation or colony screening required. Less than 10 ng of insert DNA is sufficient to yield thousands of recombinant clones. The kits are ideal for general purpose cloning, cloning cDNAs, constructing cDNA or shotgun libraries, or cloning PCR products, especially when amounts of target DNA are limited. The cSMART cDNA 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. Kits containing high-efficiency *E. cloni* Electrocompetent Cells are also available.

The pSMART-cDNA vectors contain a high-copy replication origin and encode kanamycin resistance (Figure 1). The unique design of these vectors eliminates transcription both into and out of the insert DNA, reducing the cloning bias commonly found with standard plasmids. In conventional plasmids, strong promoters are used to transcribe an indicator gene such as *lac*Zα or a negative selection gene such as *ccd*B. DNA cloned into these vectors can be lost due to plasmid instability caused by transcription into toxic coding sequences, strong secondary structure, or other deleterious features. The pSMART-cDNA vectors do not use an indicator gene, so transcription across the insert is avoided. Conventional plasmids can also be lost due to fortuitous transcription from inserts containing *E. coli*-like promoters, which can cause instability by transcribing into essential regions of the vector. In pSMART vectors, strong transcription terminators flank the cloning site to block this transcription (Figure 1), eliminating another source of cloning bias and sequencing gaps.

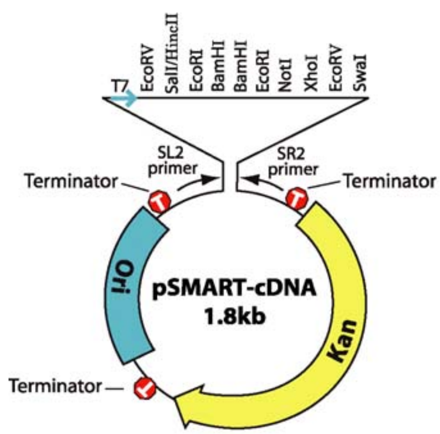


Figure 1. Schematic diagram of the pSMART® vectors. Ori, origin of replication; Kan, Kanamycin resistance gene; T7, phage T7 promoter. Approximate positions of sequencing primers and transcriptional terminators are indicated.

The ultra-low background of the cSMART system is unparalleled. pSMART vectors undergo a proprietary processing method to assure completely digested and dephosphorylated ends. As a result, > 99.9% of clones will have an insert, so there is no need for blue/white screening or direct selection schemes – nearly all colonies will have an insert. In contrast, conventional vectors utilizing the blue/white screen can generate a dense background of blue colonies and many ambiguous "light blue" colonies, both of which may contain inserts but are often discarded. The DNA contained in such clones is lost and consequently thought to be "unclonable", leading to gaps in sequence assemblies.

Conventional ampicillin resistant plasmids are often surrounded by non-transformed "satellite" colonies, which complicate colony picking and contaminate cultures. Growth of satellite colonies is completely eliminated with the kanamycin resistant pSMART vectors.

pSMART-cDNA Vectors

All pSMART-cDNA vectors are supplied pre-cut with dephosphorylated ends (Figure 1). The copy number is similar to that of pUC plasmids (~300 copies/cell). Blunt or cohesive-end insert DNA with 5'-phosphates is ligated to a pSMART vector, transformed into competent cells, and spread on plates containing kanamycin. pSMART transformants do NOT require additional screening against colonies containing empty vector, as they typically are not present at detectable levels. The vector sequence is shown in Appendix F.

E. cloni® 10G Electrocompetent Cells

For maximum cloning efficiency, use of Lucigen's *E. cloni* 10G Electrocompetent Cells is strongly recommended. *E. cloni* 10G is an *E. coli* strain optimized for high efficiency transformation by electroporation. *E. cloni* 10G Elite cells yield > 2 x 10^{10} cfu/µg supercoiled pUC19 DNA. An enhanced preparation of cells, designated *E. cloni* 10G Supreme, produces ≥ 4 x 10^{10} cfu/µg.

E. cloni 10G cells are ideal for cloning and propagation of BAC, cosmid, or plasmid clones. They give high yield and high quality plasmid DNA due to the *end*A1 mutation. They also contain the inactive *mcr* and *mrr* mutations, allowing methylated genomic DNA isolated directly from mammalian or plant cells to be cloned without deletions or rearrangements. These cells can be used to generate unbiased and complete plasmid or Bacterial Artificial Chromosome (BAC) libraries. They do not contain the F plasmid.

> *E. cloni*™ 10G Genotype: F^- *mcr*A Δ(*mrr*-*hsd*RMS-*mcr*BC) *end*A1 *rec*A1 φ80dlacZΔM15 ΔlacX74 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K *rps*L *nup*G λ^- tonA

The plasmid pUC19 (ampicillin resistant) is provided as a control for transformation.

Materials and Equipment Needed

The cSMART cDNA Cloning Kits supply most of the items needed to efficiently generate recombinant clones. While simple and convenient, successful use of the cSMART Kit requires proper planning for each step. Please read the entire manual and prepare the necessary equipment and materials before starting. Following ligation, the following items are required for transformation:

- Electroporation apparatus with 0.1 cm cuvettes (for electrocompetent cells). Successful results are obtained with cuvettes from Eppendorf (Model 940001005), BTX (Model 610) or BioRad (Cat. #165-2089). Users have reported difficulties using *E. cloni* cells with Invitrogen cuvettes (Cat. # 65-0030).
  or
- Water bath at 42 °C (for chemically competent cells).
- Wet ice.
- Sterile 17 x 100 mm culture tubes.
- Terrific Broth (see Appendix for recipe).
- TY agar plates containing ampicillin or kanamycin (see Appendix for recipes).

Preparation of Insert DNA

The pSMART-cDNA vectors are pre-cut at various restriction sites, and they lack 5' phosphates. Therefore it is *critical* that fragments to be inserted contain compatible ends *and* have 5' terminal phosphate groups on both ends. Inserts digested on both ends by appropriate restriction enzymes are suitable for cloning directly into pSMART-cDNA vectors.

Blunt fragments generated from some cDNA synthesis protocols are not phosphorylated. These fragments can be phosphorylated by use of Lucigen's DNATerminator® Kit or by T4 polynucleotide kinase plus ATP. The DNATerminator Kit can also be used for blunt-end cloning of any fragments generated by physical shearing or fragments that have 3' or 5' extensions.

To generate blunt phosphorylated ends on PCR products that have single nucleotide overhangs, Lucigen has developed the PCRTerminator® End Repair Kit. For more information on these kits, please see our web site (www.lucigen.com).

After the kinase reaction or end repair reaction is complete, the enzymes must be removed from the DNA (e.g., by gel electrophoresis, extraction, or binding to a purification column). The presence of repair enzymes in the pSMART ligation reaction will lead to a very high background of empty vector clones.

Sensitivity of DNA to Short Wavelength UV Light

DNA resolved on agarose gels is generally stained with ethidium bromide and visualized by illumination with ultraviolet light. Exposure to short wavelength ultraviolet light (e.g., 254, 302, or 312 nm) can reduce cloning efficiencies by several orders of magnitude (Figure 2). Note that the wavelength of most UV transilluminators, even those designated specifically for DNA visualization, is typically 302 nm or 312 nm, and can cause significant damage to DNA.

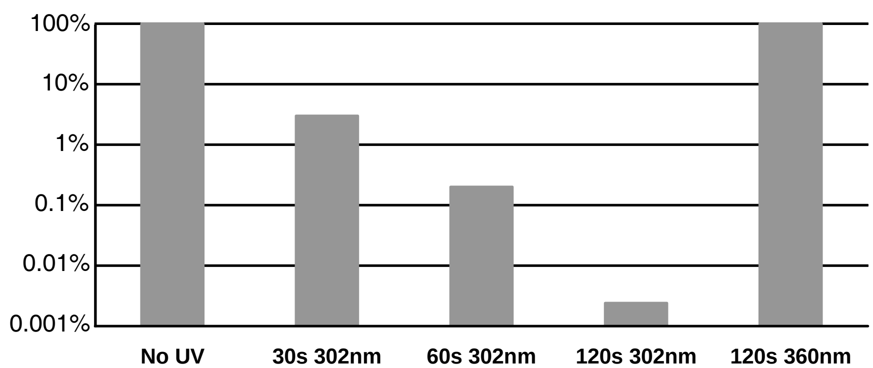


Figure 2. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 90 seconds ("30s 302nm, 60s 302nm,120s 302nm") or to 360 nm UV light for 120 seconds ("120s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

IMPORTANT: Avoid exposure to genotoxic short wavelength UV light (e.g., 254, 302, or 312 nm) when preparing samples for cloning. Use a long UV wavelength (e.g., 360 nm) low intensity lamp and short exposure times.

Detailed Protocol

Ligation to the pSMART® Vector

In the cSMART™ ligation reaction, the pre-processed pSMART vector is ligated with phosphorylated insert in a total volume of 10 µl. For library construction, we recommend using 50-500 ng of insert DNA in the size range of 500 to 4000 bp. For cloning a single DNA species, 50 ng of insert is sufficient. Successful cloning can be achieved routinely with less than 50 ng of insert, but use of low amounts of insert will result in significantly fewer transformants. The ligation is performed as follows:

1. Briefly centrifuge the cSMART Vector Premix before use. Mix by gently pipeting up and down .

2. Combine the following components in a 1.5-ml tube, adding the ligase last:

   x µl Insert DNA (50-500 ng, with 5' phosphates, compatible with vector)
   y µl H_2O
   2.5 µl 4X cSMART Vector Premix (pSMART-cDNA vector, ATP, buffer)
   1.0 µl CloneSmart® DNA Ligase (2 U/µl)
   10.0 µl total reaction volume

3. Mix by gently pipeting the reaction mixture 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:

Vector Background	To determine the background of empty vector, omit Insert DNA in the above reaction.
Positive Control Insert DNA	To determine the ligation and transformation efficiency with a known insert, use 1 µl (50 ng) of the supplied control DNA.

Preparation for Transformation

1. Heat denature the ligation reaction at 70°C for 15 minutes.
2. Cool to room temperature for 15 seconds followed by 0-4 °C for 15 seconds to condense water vapor inside the tube.
3. Spin 1 minute at 12000 rpm to collect condensation and pellet precipitated material.
4. The sample is ready for transformation; precipitating the DNA is not necessary.

Transformation

Most laboratory strains of *E. coli* (e.g., DH10B, DH5α, etc.) can be effectively transformed with pSMART ligation reactions. However, to ensure optimal cloning results, we strongly recommend the use of Lucigen's *E. cloni*® 10G Elite or 10G Supreme Electrocompetent Cells. These cells yield > 2 X 10^{10} or ≥ 4 X 10^{10} cfu/ug of pUC19, respectively, to maximize the number of transformants. For less demanding applications, other electrocompetent or chemically competent cells can be used. The following protocols are provided for transformation of *E. cloni* Electrocompetent Cells.

ELECTROPORATION OF *E. Cloni* ELECTROCOMPETENT CELLS

Lucigen's *E. cloni* Electrocompetent Cells are provided in 50-µl aliquots, which is sufficient for two transformation reactions. Optimal settings for electroporation are listed in the table below. Typical time constants are 3.5 to 4.5 msec.

Optimal Setting		Alternate Settings (~ 20-50% lower efficiencies)
1.0 mm cuvette 10 µF 600 Ohms 1800 Volts		1.0 mm cuvette 25 µF 200 Ohms 1400 – 1600 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 Electroporator 2510.

An optional transformation control reaction is electroporation with 1 µl (10 pg) of supercoiled pUC19 DNA (1µl of a 1:100 dilution of the 1 ng/µl stock provided.

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

- ***ESSENTIAL:* After ligation, the reaction must be heat killed at 70ºC for 15 minutes!**
- Microcentrifuge tubes and electroporation cuvettes must be thoroughly pre-chilled on ice before use. 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).
- The cells must be completely thawed **on ice** before use.

1. Have room temperature Recovery Medium and 17 x 100 mm sterile culture tubes readily available (one tube for each transformation reaction). Do not use SOC or other media, as the transformation efficiency may noticeably decrease.
2. Place electroporation cuvettes (0.1 cm gap, BTX or BioRad brand) and microcentrifuge tubes on wet ice (one cuvette and one tube for each transformation reaction).
3. Remove *E. cloni* cells from the -86°C freezer and thaw completely on wet ice (20-30 minutes).
4. Add 25 µl of *E. cloni* cells to the chilled microcentrifuge tube.
5. Add 1 µl of pSMART ligation reaction to the 25 µl of cells on ice. (Note: adding the DNA to the tube before adding cells may decrease transformation efficiency by 2-fold.) Do not use DNA samples containing salts, as they may cause electrical arcing during electroporation. Stir briefly with pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells.
6. As a positive control for transformation, dilute the supplied pUC 19 plasmid 1:100 to a final concentration of 10 pg/µl using either sterile water or TE. Use 1 µl of the diluted control for transformation.
7. 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 on the bottom of the well. Electroporate according to the instructions provided by the instrument's manufacturer.
8. 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.
9. Place the tube in a shaking incubator at 250 rpm for 1 hour at 37°C.

10. Spread the following amounts of experimental reactions on kanamycin plates. For the pUC19 positive control transformation, dilute 10 µl of the transformed cells into 990 µl of Recovery Medium and spread 100 µl on a YT+amp agar plate.

Plating Transformed Cells

Reaction Plate	µl/Plate
Experimental Insert (50-500 ng per ligation)	2, 20, & 100
Control Insert (Positive Control)	5
No-Insert Control (Vector Background)	50
Supercoiled pUC19 Control (10 pg; Amp^R)	100 (@ 1:100 dilution)

11. Incubate the plates overnight at 37°C.

Expected Results

The results presented below are expected when cloning 50 ng of intact, purified DNA fragments, with appropriately digested ends and 5' phosphate groups, into Lucigen's *E. cloni* 10G Electrocompetent Cells. 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 constant (< 25 colonies per 50 µl of cells plated), unless kinase is introduced as a contaminant. However, use of too little insert DNA, or insert DNA that is improperly end-repaired, or modified DNA that is not repairable yields significantly lower recombinant 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 from 50 µl of cells plated, then the 25 colonies obtained from 50 µl of the No-Insert Control ligation will represent a background of 10%.

Expected Transformation Results from Electroporation

Reaction	CFU/Plate	Efficiency
pSMART-cDNA vector plus 50 ng Insert	> 200	> 99.9% inserts
No-Insert Control (Vector Background)	< 25	< 0.1% background
Supercoiled pUC19 Control (10 pg; Amp^R)	> 200	> 1 x 10^{10} cfu/ug plasmid

1. Using Lucigen's *E. cloni* Electrocompetent Cells, a pSMART-cDNA ligation reaction with 50 ng of positive control insert DNA is expected to yield >200 colonies from a 5 µl aliquot of transformed cells, with >99.9% inserts. Results with experimental DNA may vary significantly, particularly with larger insert sizes, skewed base composition, encoded peptides, etc. To compensate for uncertainty in the nature or quantitation of the experimental DNA, we recommend plating 2, 20, and 100 µl of transformed cells to obtain a suitable number of clones.
2. A 50 µl aliquot of the empty vector control reaction should produce < 25 colonies, representing less than 0.1% background.
3. A 100-µl aliquot of diluted cells from the supercoiled pUC19 transformation should yield > 200 colonies, or > 1 x 10^{10} colonies per µg plasmid.

HEAT SHOCK TRANSFORMATION OF CHEMICALLY COMPETENT CELLS

1. Chill sterile culture tubes on ice (17 mm x 100 mm tubes, one tube for each transformation reaction).
2. Remove *E. cloni* cells from the -86°C freezer and thaw completely on wet ice (10-20 minutes).
3. Add 40 µl of *E. cloni* cells to the chilled culture tube.
4. Add 1 µl of the heat-denatured pcrSmart ligation reaction to the 40 µl of cells on ice. (Failure to heat-inactivate the ligation reaction will prevent transformation.) Stir briefly with pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells.
5. As a positive control for transformation, dilute the supplied pUC19 by 1:100 to a final concentration of 10 pg/µl using sterile water or TE. Use 1 µl of the diluted control for transformation.
6. Incubate on ice for 30 minutes.
7. Heat shock cells by placing them in a 42 °C water bath for 45 seconds.
8. Return the cells to ice for 2 minutes.
9. Add 960 µl of room temperature Recovery Medium to the cells in the culture tube.
10. Place the tubes in a shaking incubator at 250 rpm for 1 hour at 37 °C.

11. Plate up to 100 µl of transformed cells on YT+kan agar plates. Plate 100 µl of the pCU19 transformation on a YT+amp plate.

11. Incubate the plates overnight at 37°C.

12. Transformed clones can be further grown in LB or any other rich culture medium.

Expected Results

The results presented in the previous table are expected for cells with a transformation efficiency of > 1 x 10^{10} cfu/µg with pUC19. Since chemically competent cells may be 10-100 fold less competent than electrocompetent cells, they will produce 10-100 fold fewer colonies. The relative background of empty vector will remain <0.1% for chemically competent cells.

No Screening Required

For most applications, no additional screening for recombinant colonies is required, as the cSMART™ system typically delivers >99.9% recombinant clones. Because the background of empty vector transformants is extremely low, colonies can usually be picked at random for growth and plasmid purification. However, some insert DNAs may produce very few colonies (e.g., those that are large or have unusual base composition), 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 TB medium plus 30 µg/ml kanamycin. Use standard methods to isolate plasmid DNA suitable for sequencing. The pSMART plasmid contains the high copy number pUC origin of replication, yielding 20-80 ug of plasmid DNA per ml of culture. The *E. cloni* 10G Electrocompetent cells are *recA endA* deficient and will provide high quality plasmid DNA. The cSMART Kit is provided with the sequencing primers CL3 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. Thorstenson YR, Hunicke-Smith SP, Oefner PJ, Davis RW. 1998. An automated hydrodynamic process for controlled, unbiased DNA shearing. Genome Res 8: 848-55.

Appendix A: Media Recipes

YT+kan Agar Medium for Plating of Transformants
Per liter: 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, plus antibiotic. Mix all components except antibiotic; autoclave and cool to 55°C. Prepare YT+kan30 agar medium by adding kanamycin to a final concentration of 30 mg/l (equal to 30 µg/ml). Pour into petri plates.

TB Culture Medium
Per liter: 11.8 g Bacto-tryptone, 23.6 g yeast extract, 9.4 g dipotassium hydrogen phosphate (K_2HPO_4; anhydrous), 2.2 g potassium dihydrogen phosphate (KH_2PO_4; anhydrous), 0.4% glycerol. Mix all components except glycerol; autoclave and cool to 55°C. Add 8 ml filter-sterilized 50% glycerol per liter prior to using.

Growing Transformed Cultures
Colonies obtained from a pSMART transformation can be further grown in TB or LB culture medium, containing 30 µg/ml kanamycin. Transformed cultures can be stored by adding sterile glycerol to 20% (final concentration) and freezing at –70°C. Unused portions of the ligation reactions may be stored at –20 °C.

Appendix B: cSMART™ Application Guide

A variety of CloneSmart Cloning Kits is available to accommodate any cloning situation. For routine cDNA cloning applications, we recommend using cSMART cDNA Cloning Kit, as this kit contains a high copy number pSMART vector. For cloning cDNAs containing toxic genes or particularly difficult sequences, we recommend using a CloneSmart LCKan Blunt Cloning Kit containing the low copy number pSMART-LC vector. See our web site for more information on the applications of Lucigen vectors.

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.

Appendix C: Abbreviated Protocol (Please see Manual for detailed instructions.)

Insert DNA Preparation

1. Generate target DNA fragments by cDNA synthesis, shearing, restriction digestion, or PCR.
2. If necessary, cut or repair the DNA ends to generate appropriate ends, with 5' phosphates.
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 pSMART Vector Premix.
2. Combine the following components in a 1.5-ml tube. Add ligase last.

x µl	Insert DNA (50-500 ng, appropriate ends, 5'-phosphorylated)
y µl	H_2O
2.5 µl	4X pSMART 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. Heat denature 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. Have Recovery Medium at room temperature for electroporation and/or heat shock transformations.
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. 6. Electroporate. Immediately add 975 µl of room temperature Recovery Medium. Place in culture tube.	5. Incubate 30 minutes on ice. 6. Incubate 45 seconds at 42 °C; then 2 minutes on ice. Add 960 µl of room temperature Recovery Medium to the culture tube.

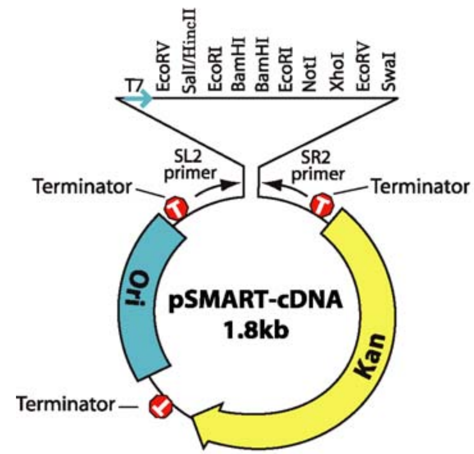
7. Shake at 250 rpm for 1 hour at 37°C.
8. Spread up to 100 µl per plate on YT agar plates containing the appropriate antibiotic. Incubate overnight at 37°C.

Colony Growth

1. Pick colonies at random and grow in TB medium containing the appropriate antibiotic.

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

The pSMART-cDNA vector is supplied predigested at various restriction sites, with 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 backbone of the pSMART-cDNA vector is identical to that of pSMART-HCKan. The cloning site and T7 promoter of pSMART-cDNA is unique to this vector. The sequences of the CL3 and SR2 primers are as follows:

CL3: 5'–GTT GAT TGC AGT CCA GTT ACG CT–3'

SR2: 5'–GGT CAG GTA TGA TTT AAA TGG TCA GT–3'

The sequence of pSMART-cDNA vector is shown on page 18.

CL3 Primer → **T7 Promoter** → **EcoRV SalI/HincII EcoRI BamHI (blunt)**

GTTGATTGCAGTCCAGTTACGCTAAAT*TAATACGACTCACTATAGGG*GAAGATATCGTCGACGAATTCGGATCCTGAATGTC/
CAACTAACGTCAGGTCAATGCGATTTAATTATGCTGAGTGATATCCCCTTCTATAGCAGCTGCTTAAGCCTAGGACTTACAG/

(blunt) BamHI EcoRI NotI HindIII XhoI EcoRV SwaI

/GTCATCAATGGATCCGAATTCGCGGCCGCAAGCTTCTCGAGGATATCACTGACCATTTAAATCATACCTGACCTCC
/CAGTAGTTACCTAGGCTTAAGCGCCGGCGTTCGAAGAGCTCCTATAG**TGACTGGTAAATTTAGTATGGACTGG**AGG

← **SR2 Primer**

Appendix E: Troubleshooting Guide

Problem	Probable Cause	Solution
Very few or no transformants	Inappropriate ends.	Check the insert DNA for self-ligation by gel electrophoresis. Repeat restriction digestion if necessary. Make sure ends of fragment are compatible with the ends of the vector.
	Contaminating enzymes in ligation reaction.	Heat-denature 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.	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 TB (Terrific Broth) for electrocompetent cells. Use SOC for chemically competent cells.
	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 Kanamycin to molten agar at 55°C before pouring plates (see Appendix). DO NOT spread antibiotic onto the surface of agar plates.
High background of transformants that do not contain inserts.	Contaminating enzymes in ligation reaction.	Purify DNA after restriction digestion. 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 to molten agar at 55°C before pouring plates (see Appendix A).
	Unstable DNA Inserts	Use a CloneSmart LCKan Blunt Cloning Kit for maximum clone stability.

Appendix F. Sequence of pSMART-cDNA vector

CCCGTGAAGGTGAGCCAGTGA**GTTGATTGCAGTCCAGTTACGCT**AAATTA 50

EcoRV EcoRI BamHI
ATACGACTCACTATAGGGGAAGATATCGTCGACGAATTCGGATCCTGAAT 100

↓ (blunt) BamHI EcoRI NotI XhoI EcoRV
GTCGTCATCAATGGATCCGAATTCGCGGCCGCAAGCTTCTCGAGGATATC 150

ACTGACCATTTAAATCATACCTGACCTCCATAGCAGAAAGTCAAAAGCCT 200

CCGACCGGAGGCTTTTGACTTGATCGGCACGTAAGAGGTTCCAACTTTCA 250
CCATAATGAAATAAGATCACTACCGGGCGTATTTTTTGAGTTATCGAGAT 300
TTTCAGGAGCTAAGGAAGCTAAAATGAGCCATATTCAACGGGAAACGTCT 350
TGTTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGTA 400
TAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGAT 450
TGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGT 500
AGCGTTGCCAATGATGTTACAGATGAGATGGTCAGGCTAAACTGGCTGAC 550
GGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATG 600
ATGCATGGTTACTCACCACTGCGATCCCAGGGAAAACAGCATTCCAGGTA 650
TTAGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGT 700
GTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAACG 750
GCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGT 800
TTGGTTGGTGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGA 850
ACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCATTCTCACCGGATTCAG 900
TCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGG 950
AAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATA 1000
CCAGGATCTTGCCATCCTATGGAACTGCCTCGGTGAGTTTTCTCCTTCAT 1050
TACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAAT 1100
AAATTGCAGTTTCACTTGATGCTCGATGAGTTTTTCTAATGAGGGCCCAA 1150
ATGTAATCACCTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTGCTGGCGT 1200
TTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGATGCTCA 1250
AGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCC 1300
CCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCG 1350
GATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGC 1400
TCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGG 1450
CTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTA 1500
ACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCA 1550
GCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTAC 1600
AGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTAT 1650
TTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTCGGAAAAAGAGTTGGTA 1700
GCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTT 1750
TGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTT 1800
GATTTTCTACCGAAGAAAGGCCCA 1824