cSMART™ cDNA Cloning Kits

IMPORTANT!
-80°C and -20°C Storage Required
Immediately Upon Receipt

Lucigen® Corporation
Advanced Products for Molecular Biology

MA006 v 5.3

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.

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cSMART™ cDNA Cloning Kits

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cSMART™ Kit Designations

Several versions of the cSMART cDNA Cloning Kit are available. The kits differ in the included number of reactions, digest of pSMART®-cDNA vector, and *E. cloni*® cells. The catalog numbers are listed below. Please refer to Appendix B: Application Guide for more information.

Catalog numbers of vector and cell combinations

Vector	Reactions	10G ELITE Electrocompetent Cells (DUOs)	10G SUPREME Electrocompetent Cells(DUOs)	10G Chemically Competent Cells(DUOs)
pSMART-cDNA Blunt (blunt insertion sites)	10	41047-1	41049-1	41042-1
	20	41047-2	41049-2	41042-2
pSMART-cDNA BluntN (Blunt + NotI insertion sites)	10	41036-1	41038-1	41032-1
	20	41036-2	41038-2	41032-2
pSMART-cDNA RN (EcoRI + NotI insertion sites)	10	41015-1	41018-1	41009-1
	20	41015-2	41018-2	41009-2

Components & Storage Conditions

The Ligation Components of the cSMART Kits are shipped in Container 1, which should be stored at **–20°C**. *E. cloni* Cells are shipped in Container 2, which must be stored at **-80°C**. Additional *E. cloni* Competent Cells may be purchased separately. Please note that 20-reaction Kits are packaged as two10-reaction Kits.

Container 1: cSMART Ligation Components — Store at -20°C

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

Container 2: *E. cloni* Competent Cells — Store at -80°C

	Catalog #	Reactions
E. cloni 10G ELITE Electrocompetent Cells (DUOs)	60052-1	12 (6 x 50 µl)
	60052-2	24 (12 x 50 µl)
or		
E. cloni 10G SUPREME Electrocompetent Cells (DUOs)	60080-1	12 (6 x 50 µl)
	60080-2	24 (12 x 50 µl)
or		
E. cloni 10G Chemically Competent Cells (DUOs)	60107-1	12 (6 x 50 µl)
	60107-2	24 (12 x 50 µl)
Control pUC19 DNA (1 ng/µl) Store **-20°C** or **-80°C**	----	10 (1 x 10 µl)
Recovery Medium Store at **-20°C** or **-80°C**	----	12 (1 x 12 ml)
	----	24 (2 x 12 ml)
	80026-1	96 (8 x 12 ml)
YT Agar (powder)	----	

cSMART™ cDNA Cloning Kit Description

The cSMART family of cloning kits represents a new system 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 1,000,000 recombinant clones from 50 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®-cDNA cloning vector premixed with buffer and ATP, as well as DNA ligase, high-efficiency *E. cloni* Competent Cells, sequencing primers, and DNA controls.

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.

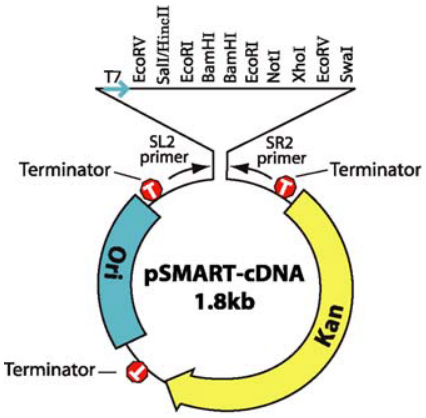


Figure 1. Schematic diagram of the pSMART-cDNA vectors. Ori, origin of replication; Kan, Kanamycin resistance gene; T7, phage T7 promoter. Approximate positions of sequencing primers and transcription terminators (T) are indicated.

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-cDNA 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. pSMART-cDNA vectors contain a T7 promoter, for in vitro transcription/translation or in vivo expression in Lucigen's EXPRESS BL21(DE3) or OverExpress™ Competent Cells. The vector sequence is shown in Appendix F.

E. cloni® 10G Competent Cells

Lucigen's cSMART cDNA Cloning Kits are available with the following *E. cloni* 10G Competent Cells in convenient DUO packaging (two transformations per tube):

***E. cloni* 10G SUPREME Electrocompetent Cells** deliver $\geq$ 4 x 10^{10} cfu/μg. SUPREME Cells are ideal for the most demanding applications that require the greatest number of transformants, such as construction of large, high complexity libraries or cloning difficult targets.

***E. cloni* 10G ELITE Electrocompetent Cells** deliver $\geq$ 2 x 10^{10} cfu/μg, providing large numbers of transformants from hard-to-clone fragments or limited DNA at a lower price than SUPREME Cells.

***E. cloni* 10G Chemically Competent Cells** deliver $\geq$ 1 x 10^{8} cfu/μg and offer unbeatable performance and value for routine applications.

***E. cloni* 10G Genotype:**
F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*dlacZ*ΔM15 Δ*lac*X74 *end*A1 *rec*A1*ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K *rps*L *nup*G λ- *tonA* (streptomycin^R)

- *E. cloni* Competent Cells are provided with supercoiled pUC19 DNA at a concentration of 1 ng/μl as a transformation control. Dilute the pUC plasmid 1:100 in dH_2O for transformation.
- **NOTE:** For optimal results, use the provided Recovery Medium to resuspend the cells after transformation. Use of TB, SOC or other media may result in lower transformation efficiencies.

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:

- **(Electrocompetent cells)** Electroporation apparatus with 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)**;**

  OR

  (Chemically competent cells) Water bath at 42 °C.
- Wet ice.
- Sterile 17 x 100 mm culture tubes.
- Recovery Medium (provided with the cells)
- YT 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-cDNA ligation reaction will lead to a very high background of empty vector clones.

Sensitivity of DNA to Short Wavelength UV Light

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.

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.

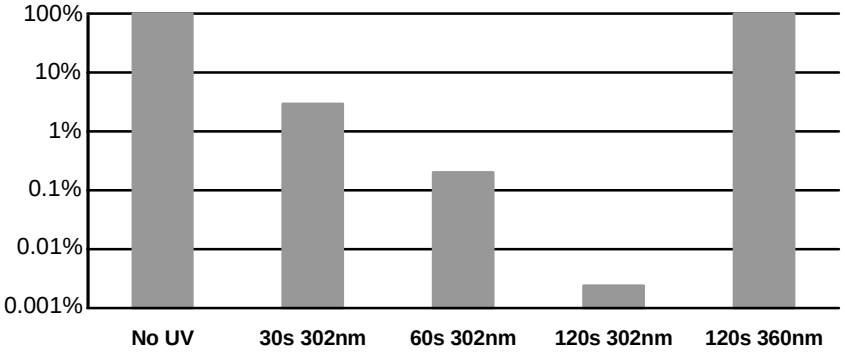


Figure 2. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 90 seconds ("30s 302nm, 60s 302nm,120s 302nm") or to 360 nm UV light for 120 seconds ("120s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

Detailed Protocol

Ligation to the pSMART®-cDNA 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 several times.

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. Prepare YT Agar from powder included with the cells. (Colonies do not grow well on LB.)

2. **IMPORTANT! Heat denature the ligation reaction at 70°C for 15 minutes. Failure to perform this step will prevent transformation.**

3. Cool to room temperature for 15 seconds followed by 0-4 ^{o}C for 15 seconds to condense water vapor inside the tube.

4. Spin 1 minute at 12000 rpm to collect condensation and pellet precipitated material.

5. 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 $\geq$ 2 X 10^{10} or $\geq$ 4 X 10^{10} cfu/ug of pUC19, respectively, to maximize the number of transformants. For less demanding applications,
E. cloni 10G Chemically Competent Cells can be used.

The following protocols are provided for transformation of *E. cloni* 10G Competent Cells.

Heat Shock Transformation of *E. cloni*® Chemically Competent Cells

E. cloni 10G Chemically Competent Cells are provided in 80 µl aliquots (DUOs), sufficient for two transformation reactions each. Transformation is performed by heat shock at 42ºC, followed by incubation on ice. To ensure successful transformation results, the following precautions must be taken:

***ESSENTIAL:* After ligation, the reaction must be heat killed at 70°C for 15 minutes!**

- Heat-inactiated ligation reactions can be used directly, without purification of the ligation products.
- All microcentrifuge tubes must be thoroughly pre-chilled on ice before use.
- The cells must be completely thawed **on ice** before use.

Transformation Protocol for Chemically Competent cells

1. Remove *E. cloni* cells from the -80°C freezer and thaw completely on wet ice (10-15 minutes).
2. Add 2-4 µl of the heat-denatured cSMART cDNA 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.
3. Incubate cells/ligation mixture on ice for 30 minutes.
4. Heat shock cells by placing them in a 42°C water bath for 45 seconds.
5. Return the cells to ice for 2 minutes.
6. Add 260 µl of room temperature Recovery Medium to the cells in the culture tube.
7. Place the tubes in a shaking incubator at 250 rpm for 1 hour at 37°C.
8. Plate 50-250 µl of transformed cells on YT agar plates containing 30 µg/ml kanamycin. Incubate the plates overnight at 37°C.
9. Transformed clones can be further grown in TB or any other rich culture medium

EXPECTED RESULTS USING *E. cloni* 10G CHEMICALLY COMPETENT CELLS

Expected results from plating chemically transformed cells.

Reaction Plate	µl/Plate	CFU/Plate	Efficiency
Experimental Insert (100 ng per ligation)	50 & 250	variable	NA
AmpR fragment Insert (Positive Control)	50	> 200	> 99% inserts
No-Insert Control (Vector Background)	250	< 2	<1% background
Supercoiled pUC19 Transformation Control (10 pg, Amp^R)	2	> 200	$\geq 1 \times 10^8$ cfu/µg plasmid

The results presented above are expected when cloning 50 ng of Positive Control Insert DNA into Lucigen's pSMART-cDNA Cloning vectors. When transforming *E. cloni* 10G Chemically Competent Cells (transformation efficiency $\geq 1 \times 10^8$ cfu/ug pUC19 DNA) the number of recombinant clones is typically 100-fold greater than the background of self-ligated vector. The background of empty pSMART-cDNA vector is constant (< 2 colonies per 250 µl of cells plated), unless contaminants are introduced. However, use of too little insert DNA, or insert DNA that is improperly prepared, can yield significantly fewer recombinant clones. 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 reaction produces 50 colonies

from 250 µl of cells, then the 2 colonies obtained from 250 µl of the No-Insert Control ligation will represent a background of 4%.

Electroporation of *E. cloni* 10G Electrocompetent Cells

E. cloni 10G SUPREME and ELITE Electrocompetent Cells are provided in 50-µl aliquots (DUOs), sufficient for two transformations each.

Transformation is carried out in a 0.1 cm gap cuvette. 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 – 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.

Optional transformation control reactions include electroporation with 10 pg of supercoiled pUC19 DNA (1 µl of a 1:100 dilution of the provided stock solution of pUC19).

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

***ESSENTIAL:* After ligation, the reaction must be heat killed at 70°C for 15 minutes!**

- Microcentrifuge tubes and electroporation cuvettes must be thoroughly pre-chilled on ice before use. Successful results are obtained with cuvettes from Eppendorf (Model 4307-000-569), 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.

Transformation Protocol for Electrocompetent cells

1. Have Recovery Medium and 17 mm x 100 mm sterile culture tubes readily available at room temperature (one tube for each transformation reaction). Transformation efficiency may decrease with the use SOC or other media.
2. Place electroporation cuvettes (0.1 cm gap) on ice.
3. Remove *E. cloni* cells from the -80°C freezer and place on wet ice until they thaw **completely** (10-15 minutes).
4. When cells are thawed, mix them by tapping gently.
5. Add 1 µl of the heat-denatured cSMART ligation reaction to the 25 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. Use of more than 2 µl of ligation mix may cause electrical arcing during electroporation.
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 YT agar plates containing 30 µg/ml kanamycin.
10. Incubate the plates overnight at 37°C.
11. Transformed clones can be further grown in TB or in any other rich culture medium.

EXPECTED RESULTS USING *E. cloni*® 10G ELITE ELECTROCOMPETENT CELLS

Plating electrocompetent transformed cells and expected results.

Reaction Plate	µl/Plate	CFU/Plate	Efficiency
Experimental Insert (100 ng per ligation)	5 & 50	variable	NA
AmpR fragment Insert (Positive Control)	5	> 400	> 99% inserts
No-Insert Control (Vector Background)	100	< 25	<1% background
Supercoiled pUC19 Transformation Control Plasmid (10 pg, Amp^R)	2	> 200	$\geq 2 \times 10^{10}$ cfu/µg plasmid

The results presented above are expected when cloning 50 ng of Positive Control Insert DNA into Lucigen's pSMART-cDNA Cloning vectors. When transforming *E. cloni* 10G ELITE Electrocompetent Cells (transformation efficiency $\geq 2 \times 10^{10}$ cfu/µg pUC19 DNA) the number of recombinant clones is typically 100-fold greater than the background of self-ligated vector (>400 colonies per 5µl plated). The background number of empty GC Cloning vectors is constant (< 25 colonies per 100 µl of cells plated), unless contaminants are introduced.

Use of too little insert DNA, or insert DNA that is improperly prepared, can yield significantly fewer recombinant clones. 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 5 colonies from 5 µl of cells plated, then the 25 colonies obtained from 100 µl of the No-Insert Control ligation will represent a background of 2.5%.

Use of *E. cloni* SUPREME Electrocompetent cells (transformation efficiency $\geq 4 \times 10^{10}$ cfu/µg pUC19 DNA) will result in proportionately more colonies. Use of competent cells with a transformation efficiency of less than 2×10^{10} cfu/µg will result in proportionately fewer colonies. Most chemically competent cells will yield ~1% of the number of colonies shown above.

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-cDNA 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 Competent 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
Add the YT Agar powder provided with the kit to 500 ml of deionized water. Autoclave and cool to 55°C. Add the appropriate filter-sterilized antibiotic to the cooled medium (e.g., 15 mg kanamycin for kanamycin-resistant transformants).

Temperatures of >55°C may destroy the antibiotics. Do NOT add antibiotics to hot media! Pour approximately 20-25 ml per petri plate.

YT Agar is per liter: 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, plus antibiotic. Prepare YT+kan30 agar medium by adding kanamycin to a final concentration of 30 mg/l (equal to 30 μg/ml).

YT Agar is available to purchase separately as 5 packets with catalog number 60025-1.

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-LCKan vector. For cloning extremely difficult DNAs (e.g., very AT-rich; highly repetitive sequences) up to 30 kb, use Lucigen's BigEasy™ v2.0 Linear Cloning System. See our web site (www.lucigen.com) 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 cSMART 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 cSMART Vector Premix (pSMART-cDNA 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+kan agar plates. 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

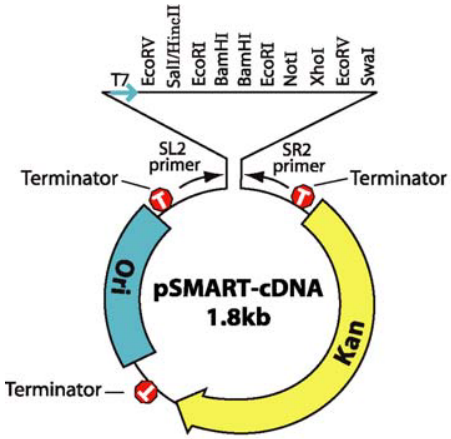
The pSMART®-cDNA vector is supplied predigested at various restriction sites, with dephosphorylated ends. Transcriptional terminators border the cloning site to prevent transcription from the insert into the vector. Another terminator at the 3' end of the ampicillin or kanamycin resistance gene prevents 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 →

					SalI/					
				EcoRV	HincII	EcoRI	BamHI		(blunt)	

GTTGATTGCAGTCCAGTTACGCTAAAT*TAATACGACTCACTATAGGG*GAAGATATCGTCGACGAATTCGGATCCTGAATGTC/
CAACTAACGTCAGGTCAATGCGATTTAATTATGCTGAGTGATATCCCCTTCTATAGCAGCTGCTTAAGCCTAGGACTTACAG/

(blunt)		BamHI	EcoRI	NotI		HindIII*	XhoI	EcoRV		SwaI

/GTCATCAATGGATCCGAATTCGCGGCCGCAAGCTTCTCGAGGATATCACTGACCATTTAAATCATACCTGACCTCC
/CAGTAGTTACCTAGGCTTAAGCGCCGGCGTTCGAAGAGCTCCTATAG**TGACTGGTAAATTTAGTATGGACTGG**AGG

← **SR2 Primer**

***The Hind III site is NOT unique in the pSMART-cDNA vector. Another Hind III site is present in the kanamycin resistance gene.**

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 Recovery Medium provided in the cSMART Kit.
	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 or BigEasy v2.0 Linear Cloning System for maximum clone stability.

Appendix F. Sequence of pSMART®-cDNA vector

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CCCGTGAAGGTGAGCCAGTGAGTTGATTGCAGTCCAGTTACGCTAAATTA   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
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