PCR-SMART™
CLONING KITS

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

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The Molecular Cloning Company™

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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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PCR-SMART™ Kit Designations

Several versions of the PCR-SMART Cloning Kits are available. The kits are specific for cloning PCR products generated from either proofreading or non-proofreading polymerases, and they can be ordered with or without competent cells. The catalog numbers are listed below.

Catalog numbers of kit and cell combinations

Kit	Reactions	10G Elite Electrocompetent Cells	No Cells
PCR-SMART Proofreaders Cloning Kit	10	41103-1	---
	20	41103-2	41101-2
	40	---	41101-4
PCR-SMART Non-Proofreaders Cloning Kit	10	41124-1	---
	20	41124-2	41120-2
	40	---	41120-4

Components & Storage Conditions

The Ligation Components of the PCR-SMART Kits 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 PCR-SMART Ligation Components and *E. cloni* Competent Cells may be purchased separately.

Container 1: PCR-SMART Ligation Components

(Proofreaders *or* Non-Proofreaders) **Store at -20°C**

	10 Reactions	20 Reactions	40 Reactions
4X pcrSMART™ Vector Premix Includes Buffer, ATP, and pcrSMART-HC Kan vector	25 µl	2 x 25 µl	4 x 25 µl
CloneSmart® DNA Ligase (2 U/µl)	10 µl	2 x 10 µl	4 x 10 µl
Control Insert PCR Product (50 ng/µl, 5'-phosphorylated) Proofreaders Ampicillin Resistant PCR fragment (blunt) *or* Non-proofreaders Ampicillin Resistant PCR fragment (3'-dA)	5 µl	5 µl	5 µl
PCR-SMART 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
T4 Polynucleotide Kinase (10 U/ul) 10X T4 Polynucleotide Kinase buffer (containing ATP) (Proofreaders Kit only)	20 µl 20 µl	20 µl 20 µl	20 µl 20 µl
PCR-Terminator® End Repair Enzyme Mix 5X PCR-Terminator End Repair Buffer (Non-Proofreaders Kit only)	25 µl 100 µl	2 x 25 µl 2 x100 µl	125 µl 500 µl

Container 2: *E. cloni*® Electrocompetent Cells **Store at -86°C**

	Catalog #	Reactions
E. cloni 10G Elite Electrocompetent Cells	60052-1	12 (6 x 50 µl)
	60052-2	24 (12 x 50 µl)
Control pUC19 DNA (10 pg/µl) store at **-20°C** or **-86°C**	---	10 (1 x 10 µl)
Recovery Medium store at **-20°C** or **-86°C**	---	12 (1 x 12 ml)
		24 (1 x 24 ml)

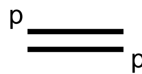
PCR-SMART Cloning Kit Description

The PCR-SMART Cloning Kits incorporate the pcrSMART™ transcription-free vector to eliminate bias and maximize efficiency in cloning PCR products. There are two versions of these kits, one for proofreading and one for non-proofreading polymerases, which generate different types of DNA ends. The pcrSMART Proofreaders Cloning Kit is designed for simplified cloning of PCR products generated by proofreading thermostable DNA polymerases, such as Vent or *Pfu* DNA polymerase, which leave blunt DNA ends. For optimum efficiency, we recommend the use of 5' phosphorylated primers in the PCR, to allow ligation of products into the dephosphorylated cloning vector pcrSMART. The pcrSMART Non-Proofreaders Cloning Kit is designed for cloning PCR products generated by non-proofreading thermostable DNA polymerases, such as *Taq* DNA polymerase, which leave single 3'-dA overhangs on their reaction products. The 3'-A overhangs are removed and 5' phosphates are added during an end repair step. The treated DNA is then purified and ligated to the vector. The figure below diagrams the basic steps for each type of cloning kit. See Appendix G for complete details and alternate strategies for cloning PCR products.

PCR-SMART Proofreaders Cloning Kit

Phosphorylate Primers
(or synthesize with 5' phosphates)

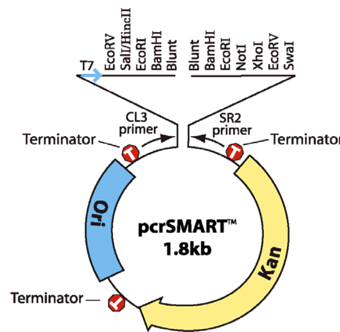
Amplify Target Using Proofreading Polymerase and Phosphorylated Primers

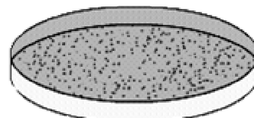


LIGATE TO pcrSMART
30' to 2 hr

TRANSFORM E. cloni 10G
15 min

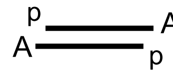
GROW and PLATE
1 hr





PCR-SMART Non-Proofreaders Cloning Kit

Amplify Target Using Non-Proofreading Polymerase and Phosphorylated Or Non-phosphorylated Primers



END REPAIR Rxn and DNA Purification
45 min

LIGATE TO pcrSMART
30' to 2 hr

TRANSFORM E. cloni 10G
15 min

GROW and PLATE
1 hr

pcrSMART™ Vector

The unique design of the pcrSMART vector (Figure 2) 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 of toxic coding sequences, strong secondary structure, or other deleterious features. The pcrSMART vectors do not use a promoter or an indicator gene for screening, 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 pcrSMART vectors, strong transcription terminators flank the cloning site to block this transcription (Figure 2), eliminating another source of cloning bias and sequencing gaps. The T7 promoter of the pcrSMART vector is inactive in most cell lines, including Lucigen's *E.cloni* 10G, but it can be activated in cells containing the gene for T7 RNA polymerase [e.g., BL21(DE3)].

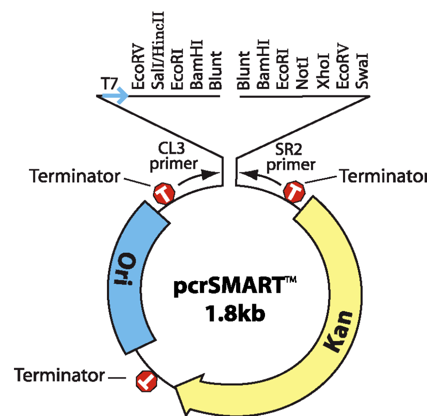


Figure 2. Schematic diagram of the pcrSMART vector. Ori, origin of replication; Kan, Kanamycin resistance gene; T7, phage T7 promoter. Approximate positions of sequencing primers and transcriptional terminators are indicated.

The ultra-low background of the PCR-SMART system is unparalleled. The pcrSMART vector undergoes a proprietary processing method to assure blunt, dephosphorylated ends. As a result, > 99% of clones will have an insert, so there is no need for blue/white screening or direct selection schemes...just pick colonies at random! 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.

The copy number of pcrSMART-HCKan is similar to that of pUC plasmids (~300 copies/cell). Blunt-ended, 5'-phosphorylated insert DNA is ligated to the pcrSMART vector, transformed into competent cells, and spread on plates containing kanamycin. pcrSMART transformants do NOT require additional screening against colonies containing empty vector, as they typically are not present at detectable levels. The sequence of the pcrSMART vector is identical to that of Lucigen's pSMART-cDNA-blunt vector.

Growth of satellite colonies is completely eliminated with the kanamycin resistant pcrSMART vector. In contrast, conventional ampicillin resistant plasmids are often surrounded by non-transformed "satellite" colonies, which complicate colony picking and contaminate cultures.

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 $\geq$ 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, and they are NOT capable of activating the T7 promoter of the pcrSMART vector.

E. cloni 10G Genotype: F^{-} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *end*A1 *rec*A1 φ80d*lac*ZΔM15 Δ*lac*X74 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K *rps*L *nup*Gλ *ton*A

The plasmid pUC19 (ampicillin resistant) is provided at a concentration of10 pg/µl, as a control for transformation of *E. cloni* cells. Recovery media is also included with all *E. cloni* cells.

Materials and Equipment Needed

The PCR-SMART Blunt Cloning Kits supply most of the items needed to efficiently generate recombinant clones. While simple and convenient, successful use of the PCR-SMART 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 and 0.1 cm cuvettes (for electrocompetent cells). With Lucigen's *E. cloni* cells, successful results are obtained with cuvettes from BTX (Model 610), BioRad (Cat. #165-2089), or Eppendorf (Cat. #940001005). Users have reported difficulties when using Invitrogen cuvettes (Cat. # 65-0030).

  or

  Water bath at 42 °C (for chemically competent cells). Note: most chemically competent cells will yield only 5-10% of the number of transformants expected from Lucigen's *E. cloni* Electrocompetent Cells.

- Wet ice.
- Sterile 17 x 100 mm culture tubes.
- Terrific Broth (for electrocompetent cells) ***or*** SOC (for chemically competent cells).
- TY agar plates containing ampicillin or kanamycin (see Appendix for recipes).

Detailed Protocol

The most efficient way to clone PCR products is to perform the amplification with a proofreading polymerase (e.g., Vent™ or Pfu polymerase) and use primers synthesized with 5' phosphates. The resulting products can be cloned with the PCR-SMART Proofreaders Cloning Kit. Products made by other PCR protocols can also be cloned with the PCR-SMART kits. See Appendix G for a flowchart illustrating methods of cloning PCR products created with non-proofreading polymerases and/or non-phosphorylated primers.

Preparation and Purification of Insert DNA

Proofreaders Kit

Use of Phosphorylated PCR Primers

Reactions carried out with **5'-phosphorylated** primers and a **proofreading** polymerase are suitable for the PCR-SMART ligation reaction. However, size selection on an agarose gel is highly recommended to remove contaminating DNA, truncated or aberrant PCR products, and PCR primers. We recommend purifying the DNA bands from the gel by commercial purification kits (e.g., Zymoclean Gel DNA Recovery Kit, Zymo Research).

If the DNA is not gel fractionated, it can be purified using commercial purification kits (e.g., DNA Clean & Concentrate Kit, Zymo Research, cat. # D4003) or ethanol precipitation. The DNA should be eluted or dissolved in water.

Use of Non-Phosphorylated PCR Primers

PCR primers that do not contain 5' phosphates can be enzymatically phosphorylated by T4 polynucleotide kinase (T4 PNK) before performing the PCR. T4 PNK enzyme and T4 PNK buffer mix (containing ATP) are included in the PCR SMART Proofreaders Cloning Kit. Perform the primer kinase reaction as follows:

Primer kinase reaction

4 ul Forward primer @ 100 pmol/ul
4 ul Reverse primer @ 100 pmol/ul
1 ul 10 X T4 PNK buffer mix
1 ul T4 PNK

10 ul total

37°C, 15 minutes

After the incubation, add 5 ul of this reaction directly to a 100-ul PCR mix and amplify according to standard protocols.

Alternately, the PCR product may be amplified with non-phosphorylated primers and subsequently phosphorylated with T4 PNK. Use 1 ul of T4 PNK to phosphorylate up to 200 pmol of product.

T4 PNK is not completely inactivated by heat treatment or thermal cycling. Traces of T4 PNK can carry over into the ligation reaction, resulting in a high background of self-ligated empty vector clones. Therefore, T4 PNK must be removed from the PCR product before ligation to the pcrSMART vector.

Agarose gel electrophoresis can be used both to size-select the desired PCR product and to remove the T4 PNK. Other methods of DNA purification can be used if desired.

Non-Proofreaders Kit

PCR products created with a non-proofreading polymerase (e.g., Taq or Tfl polymerase) contain 3' single base overhangs, so an end repair step is required. The PCRTerminator End Repair reaction will remove 3' overhangs from PCR products and add 5' phosphates, so use of phosphorylated primers is not necessary. The reaction leaves blunt, phosphorylated ends suitable for ligation to the pcrSMART vector.

Buffers used for PCR must be removed before beginning the PCRTerminator reaction. We recommend electrophoresis on an agarose gel, followed by purification of the desired band using a commercial purification column (e.g., Zymoclean Gel DNA Recovery Kit, Zymo Research, cat. # D4001; www.zymoresearch.com). Gel fractionation assures cloning of the correct PCR product rather than truncated variants, aberrant products, or multimers of PCR primers.

If the DNA is not gel fractionated, it can be purified using commercial purification kits (e.g., DNA Clean & Concentrate Kit, Zymo Research, cat. # D4003) or ethanol precipitation. The DNA should be eluted or dissolved in water.

PCRTerminator End Repair Reaction

Mix the following components in a microfuge tube:

Volume	Component
37.5 µl	purified amplification product (1-5 µg)
10.0 µl	5X PCRTerminator End Repair Buffer
1.0 - 2.5 µl	PCRTerminator End Repair Enzyme*
50.0 µl	final volume

* Use 2.5 µl End Repair enzyme to treat > 2 µg amplification product.
Use 1 µl of End Repair enzyme to treat 1-2 µg amplification product.
For less than 1 µg of amplification product, reduce incubation time to 15 minutes.

Incubate 30 minutes at room temperature.
Stop the reaction by incubation at 70ºC for 15 minutes.
Purify the DNA as described above.

Note: the heat denaturation step may be omitted if the DNA is *immediately* purified using a protein denaturing reagent (e.g., phenol or column binding buffer that contains guanidinium HCl).

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*. Note that the wavelength of most UV transilluminators, even those designated specifically for DNA visualization, is typically 302 nm or 312 nm, which can cause significant damage to DNA. **When preparing samples for cloning, use a long UV wavelength (e.g., 360 nm) low intensity lamp and short exposure times.**

Ligation to the pcrSMART™ Vector

In the PCR-SMART ligation reaction, the pre-processed pcrSMART vector is ligated with blunt, phosphorylated insert in a total volume of 10 µl. We recommend using 100-200 ng of insert DNA in the size range of 300 - 4000 bp. 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. The ligation is performed as follows:

1. Briefly centrifuge the PCR-SMART 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 (100-200 ng, blunt-ended, 5'-phosphorylated)
y μl H_2O
2.5 μl 4X PCR-SMART Vector Premix (pcrSMART™, ATP, buffer)
1.0 μl CloneSmart DNA Ligase (2 U/μl)
10.0 μl total reaction volume

1. 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:

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.
Vector Background	To determine the background of empty vector, omit Insert DNA in the above reaction.

Preparation for Transformation

1. CRITICAL: 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 pcrSMART 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, chemically competent cells can be used. For expression from the T7 promoter of pcrSMART, recombinant plasmids must be transferred to a strain containing the T7 RNA polymerase gene, such as BL21(DE3). Cloning directly into BL21(DE3) may lead to instability of the insert caused by transcription from the T7 promoter. 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 Setting (~ 20-50% less efficient)
1.0 mm cuvette 10 μF 600 Ohms 1800 Volts		1.0 mm cuvette 25 μF 200 Ohms 1400 – 1600 Volts
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 1 µl (10 pg) of supercoiled pUC19 DNA.

1. Have room temperature Recovery Medium and 17 x 100 mm sterile culture tubes readily available (one tube for each transformation reaction). Transformation efficiency may decrease with SOC or other media.
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 (15-20 minutes).
4. Add 25 µl of *E. cloni* cells to the chilled microcentrifuge tube.
5. Add 1 µl of the PCR-SMART ligation reaction to the 25 µl of cells on ice. Stir briefly with a pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells. Note: adding the ligated DNA to the tube before adding the cells may decrease transformation efficiency by up to 50%.
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 on the bottom of the well. Electroporate according to the conditions described 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 the following amounts of experimental and control reactions on the appropriate ampicillin or kanamycin plates. Carbenicillin may be substituted for ampicillin if desired. Spread up to 100 µl of the experimental insert transformation per 100 mm petri plate.

Table 1. Plating Transformed Cells

Reaction Plate	Antibiotic	µl/Plate
Experimental Insert (50 ng per ligation)	Kanamycin	5, 20, & 100
Amp^R gene fragment (Positive Control)	Kan. and/or Amp.	5
No-Insert Control (Vector Background)	Kanamycin	50
pUC19 Supercoiled Plasmid Control (10 pg)	Ampicillin	2

10. 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 blunt 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 pcrSMART vector. The background number of empty pcrSMART 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 ul of cells plated, then the 25 colonies obtained from 50 ul of the No-Insert Control ligation will represent a background of 10%.

Table 2. Expected Transformation Results from Electroporation

Reaction	CFU/Plate	Efficiency
pcrSMART plus 50 ng AmpR Insert	> 200	> 99.9% inserts
No-Insert Control (Vector Background)	< 25	< 0.1% background

pUC19 Supercoiled Plasmid Control (10 pg)	> 200	> 1 x 10^{10} cfu/ug plasmid

1. Using Lucigen's *E. cloni* Electrocompetent Cells, a pcrSMART 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 5, 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 2 µl aliquot of transformed cells from the supercoiled pUC19 reaction (diluted into 90 µl of TB) 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 PCR-SMART 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 (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 TB medium plus 50 µg/ml kanamycin. Use standard methods to isolate plasmid DNA suitable for sequencing. The pcrSMART 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 PCR-SMART Kit is provided with the sequencing primers CL3 and SR2. The sequence of the primers and their orientation relative to the pcrSMART 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+amp or TY+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. After cooling, add kanamycin to a final concentration of 30 mg/l (equal to 30 μg/ml). Pour into petri plates.

TB+kan 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. Just before use, add 8 ml filter-sterilized 50% glycerol per liter. Add kanamycin to a final concentration of 30 mg/l (equal to 30 μg/ml).

SOC Medium
Per liter: 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.186 g KCl. Adjust to pH 7.0 with NaOH. Autoclave, cool to 60 °C, add 20 ml of filter-sterilized 1 M glucose. Just before use add 5 ml of filter-sterilized 2 M $MgCl_2$.

Growing Transformed Cultures
Colonies obtained from a pcrSMART 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: PCR-SMART™ Application Guide

The pcrSMART vector is one member of the family of pSMART cloning vectors. A variety of vectors is available to accommodate nearly any cloning situation. For routine applications, we recommend using the pcrSMART or the pSMART-HCKan or -HCAmp vector. The copy number of these vectors is similar to that of pUC based vectors, yielding 20-80 ug of plasmid DNA per ml of culture. For cloning toxic genes or particularly difficult DNA sequences, we recommend using the low copy vector pSMART-LCKan. The plasmid yield is ~0.5-1 μg per ml culture.

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 10-G 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.
pcrSMART-HC Kan	High	+	+	++	+++	+
pSMART-HC Kan	High	+	+	++	+++	+
pSMART-LC Kan	Low	+	+	+++	+	+
pSMART-HC Amp	High	+	+	+	+++	+
pSMART-LC Amp	Low	+	+	++	+	+

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

Insert DNA Preparation

1. Generate target DNA fragments by PCR.
2. If necessary, repair the DNA ends to make them blunt, with 5’ phosphate groups.
3. Heat denature the repair reaction 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 4X PCR-SMART Vector Premix.
2. Combine the following components in a 1.5-ml tube. Add ligase last.

x µl Insert DNA (100-500 ng, blunt-ended, 5’-phosphorylated)
y µl H_2O
2.5 µl 4X PCR-SMART Vector Premix (pcrSMART 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

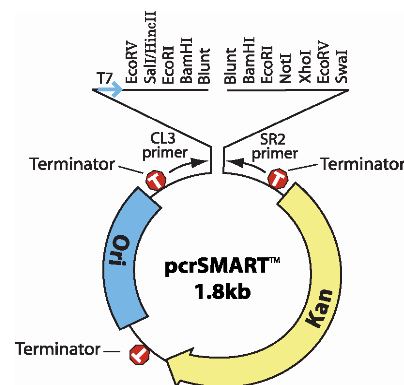
The pcrSMART™ vector is supplied predigested, with blunt, dephosphorylated ends. Transcriptional terminators border the cloning site to prevent transcription from the insert into the vector. Another terminator at the 3' end of the kanamycin resistance gene prevents this transcript from reading into the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

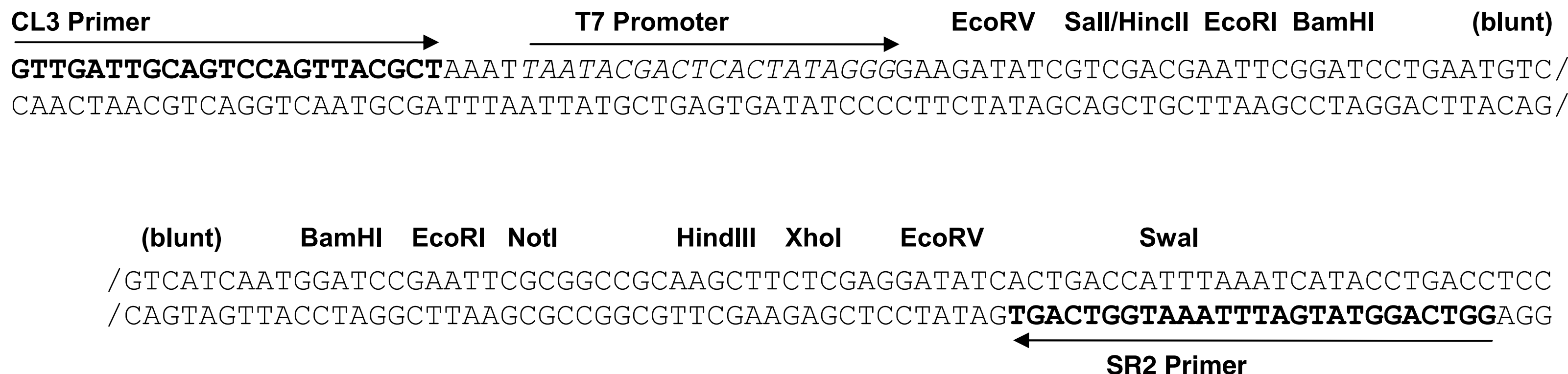
The sequences of the CL3 and SR2 primers are as follows:

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

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

The GenBank accession number of the pcrSMART™ vector is not available yet. Please see Appendix F for the sequence of the plasmid.





Appendix E: 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 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. Use recommended electroporation settings. Pre-chill cuvettes on ice. Add the ligated 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 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 to molten agar at 55°C before pouring plates (see Appendix A).
	Unstable DNA Inserts	Use pSMART-LCKan for maximum clone stability.

Appendix F. Sequence of the pcrSMART vector

CCCGTGAAGGTGAGCCAGTGA**GTTGATTGCAGTCCAGTTACGCT**AAATTA 50

EcoRV EcoRI BamHI
ATACGACTCACTATAGGGGAAGATATCGTCGACGAATTCGGATCCTGAAT 100

(blunt cut)
BamHI EcoRI NotI XhoI EcoRV
GTCGTCATCAATGGATCCGAATTCGCGGCCGCAAGCTTCTCGAGGATATC 150
SwaI
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
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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
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GCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTAC 1600
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TTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTCGGAAAAAGAGTTGGTA 1700
GCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTT 1750
TGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTT 1800
GATTTTCTACCGAAGAAAGGCCCA 1824

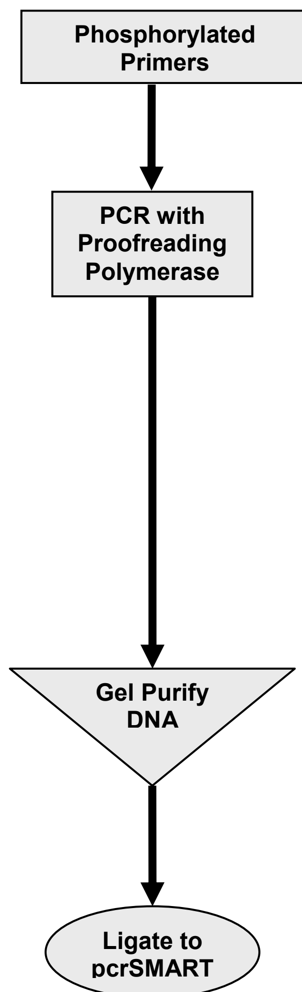
Appendix G: Flowchart for Cloning with the PCR-SMART Kit

A) Proofreaders. The most efficient way to clone with the PCR-SMART kit is to use primers chemically synthesized with 5'-phosphate groups and to perform PCR with a proofreading polymerase (shaded pathway). Primers synthesized without 5'-phosphate groups can be treated briefly with T4 polynucleotide kinase (included in the Kit) and used directly in PCR.

B) Non-Proofreaders. Products made with phosphorylated OR non-phosphorylated primers and a non-proofreading polymerase require an end-repair step to create blunt phosphorylated ends. The PCR product must be purified before end-repair, because the repair enzymes are not active in PCR buffer. The End Repair enzymes and buffer are included in the Kit.

In all cases, we recommend gel purification as one of the purification steps to remove primers and enzymes and to select for the correct-sized PCR product.

A) Proofreaders



B) Non-Proofreaders