GC Cloning & Amplification Kits with pSMART® GC VECTORS

IMPORTANT!

-80°C and -20°C Storage Required

Immediately Upon Receipt

Lucigen® Corporation

Advanced Products for Molecular Biology

2120 W. Greenview Drive
Middleton, WI 53562

Toll Free: (888) 575-9695
Tel: (608) 831-9011
FAX: (608) 831-9012
Email: lucigen@lucigen.com
Web: www.lucigen.com

MA034 v1.0

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.

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

Lucigen's products are sold for research use only and are not to be used in humans or for medical diagnostics. Lucigen's liability with respect to any CloneSmart product is limited to the replacement of the product. No other warranties of any kind, expressed or implied, including without limitation, any implied fitness for any particular use, are provided by Lucigen. Lucigen is not liable for any direct, indirect, incidental or consequential damages arising out of or in connection with the use or inability to use any of its CloneSmart products.

Limited Label License

This product is the subject of U.S. Patent #6,709,861 and pending patent applications owned by Lucigen Corporation. The consideration paid for this product grants a Limited License to use the product pursuant to the terms set forth in this Limited Label License. Academic, Not-for-Profit and For-Profit institutions acquire certain limited nontransferable rights with the purchase of this product (see below).

The purchase price of this product includes limited, nontransferable rights to use only the purchased amount of the product to perform CloneSmart Blunt Cloning Technology and only as described in CloneSmart Blunt Cloning Kit Instruction Manual. This limited license specifically excludes manufacture of the pSMART vector or derivatives thereof. Lucigen Corporation reserves all other rights; in particular, the purchaser of this product may not transfer or otherwise sell this product or its components or derivatives to a third party, and no rights are conveyed to the purchaser to use the product or its components or derivatives for commercial purposes. "Commercial purposes" includes any activity for which a party receives consideration and may include, but is not limited to, (1) use of the product or its components or derivatives in manufacturing, (2) use of the product or its components or derivatives for diagnostic purposes, (3) transfer or sale of vectors made with the product or components or derivatives of the product, (4) use of this product or materials made therefrom to provide a service, information, or data (e.g., DNA sequence) to a third party in return for a fee or other consideration, or (5) resale of the product or its components or derivatives, whether or not such product or its components or derivatives are resold for use in research. Academic, Not-for-Profit, and For-Profit institutions must obtain a separate license from Lucigen Corporation to use this product for any purpose other than those permitted above. It is the sole responsibility of the buyer to ensure that use of the product does not infringe that patent rights of third parties.

If the purchaser is not willing to accept these use limitations, Lucigen Corporation is willing to accept return of the product for a full refund. For information on obtaining a license, contact Lucigen Corporation, 2120 W. Greenview Dr., Middleton, WI 53562. Email: lucigen@lucigen.com. Phone: 608-831-9011. Fax 608-831-9012.

Table of Contents

GC Cloning Kit Designations

Several versions of the GC Cloning Kit are available. The kits differ in the number of reactions, version of GC 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 GC Vector and *E. cloni*® Cell Combinations

Vector	Reactions	10G ELITE Electrocompetent Cells (SOLOs)	10G SUPREME Electrocompetent Cells (SOLOs)	10G Chemically Competent Cells (SOLOs)
pSMARTGC HK (High Copy)	10	40731-1	40732-1	40733-1
	20	40731-2	40732-2	40733-2
pSMARTGC LK (Low Copy)	10	40736-1	40737-1	40738-1
	20	40736-2	40737-2	40738-2

Components & Storage Conditions

The GC Cloning Kit components are shipped in Container 1, which should be stored at **-20°C**. *E. cloni* 10G Cells are shipped in Container 2, which must be stored at **-80°C**.

Container 1: GC Cloning Kit Components — Store at -20°C

	10 Reactions	20 Reactions
4X GC Vector Premix Includes Buffer, ATP, and ***one*** type of ligation-ready Vector: pSMARTGC HK Premix *or* pSMARTGC LK Premix	25 µl	50 µl
CloneSmart® DNA Ligase (2 U/µl)	12 µl	24 µl
T4 Polynucleotide Kinase (10 U/µl) 10X Primer Kinase Buffer (containing ATP)	20 µl 20 µl	20 µl 20 µl
PCR Control *lac*Z template plus primers (5 ng/µl template, 25 pmol/µl each primer)	12 µl	12 µl
EconoTaq™ DNA Polymerase (5 U/µl) EconoTaq 10X Reaction Buffer 2.5 mM dNTPs	50 µl 1.5 ml 50 µl	50 µl 1.5 ml 50 µl
CloneSmart Sequencing Primers (200 reactions each) SL1 Primer (3.2 pmol/µl) SR2 Primer (3.2 pmol/µl)	 200 µl 200 µl	 200 µl 200 µl

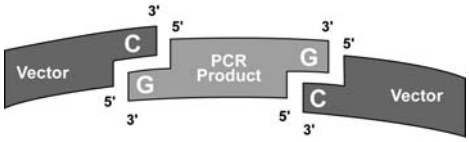
Container 2: *E. cloni* 10G Competent Cells — Store at -80°C

	Catalog #	Reactions
E. cloni 10G ELITE Electrocompetent Cells ***or***	60052-1 60052-2	12 (6 x 50 µl) 24 (12 x 50 µl)
E. cloni 10G SUPREME Electrocompetent Cells ***or***	60080-1 60080-2	12 (6 x 50 µl) 24 (12 x 50 µl)
E. cloni 10G Chemically Competent Cells	60107-1 60107-2	12 (6 x 50 µl) 24 (12 x 50 µl)
Control pUC19 DNA (1 ng/µl) Store at **-20°C** or **-86°C**	----	10 (1 x 10 µl)
Recovery Medium Store at **-20°C** or **-86°C**	---- ---- 80030-1	12 (1 x 12 ml) 24 (1 x 24 ml) 96 (8 x 24 ml)

GC Cloning Kit Description

The GC Cloning Kits contain everything needed to amplify and efficiently clone PCR products into an unbiased, high-fidelity cloning vector. The Kits are compatible with both NON-proofreading and proofreading PCR polymerases. They can also be used to clone any blunt DNA up to 10 kb.

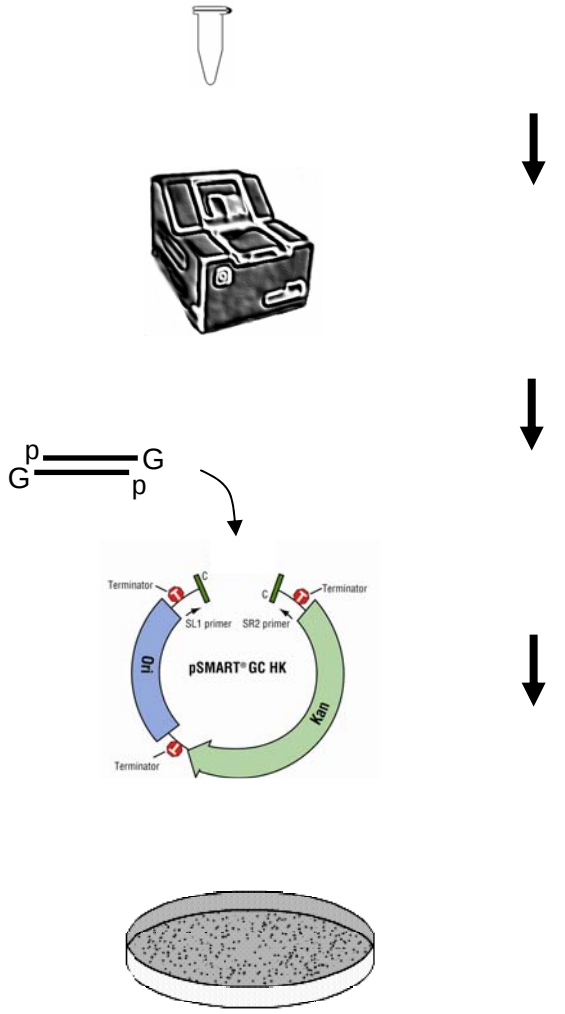
The GC Cloning Kits are based on a newly discovered attribute of non-proofreading DNA polymerases: these enzymes can add a single 3'-G residue to the ends of DNA molecules. The 3'G tailing occurs during PCR with a NON-proofreading, or it can be performed as a separate G-tailing reaction to the blunt products of proofreading polymerases (or any other type of blunt DNA fragments). The pSMARTGC vectors contain a single 3'-C overhang, which is compatible with the 3'-G overhang generated by the polymerase (see Figures 1 and 2). The unique combination of a G-tailed insert DNA and C-tailed vector is the basis for GC Cloning (patents pending).



GC Cloning technology is analogous to TA cloning® (Mead 1991), in which a non-proofreading polymerase, such as Taq, Tfl, Tth, or Tbr DNA polymerase, adds a single 3'-A residue to the insert DNA. A compatible T-tailed vector is used for the complementary cloning step. However, there are several notable differences between the two technologies: (a) the optimal time for the GC ligation reaction is minutes, compared to the hours required for standard TA cloning; GC Cloning technology rivals TOPO® TA cloning (Invitrogen Corp.) in the time required to complete the reaction; (b) the cloning efficiency and accuracy is higher with GC ends than with TA ends; and (c) the transcription-free pSMARTGC vectors demonstrate much greater stability for cloning large and otherwise unstable PCR products, as described below.

The pSMARTGC vectors incorporate Lucigen's CloneSmart® transcription-free cloning technology (U.S. Pat. 6, 709, 861) to reduce bias and maximize cloning efficiency. The pSMARTGC vectors (Figure 2) are supplied pre-digested, with single 3'-C tails and dephosphorylated 5' ends, and are qualified to produce >99% recombinant clones in typical experiments. The very low background of empty vector eliminates the need to screen for recombinants. It also enables PCR cloning and novel library construction methods from nanogram amounts of DNA. Because no screening is required, this technology eliminates the need for XGAL/IPTG and removes the uncertainty of false negatives (light blue pUC colonies) and false positives (white colonies that lack inserts). In contrast, conventional TA or TOPO TA 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 can be lost and consequently thought to be "unclonable". Further, the pZERO™ vectors typically have an empty vector background of 5% or more. In addition, the ampicillin-resistant pZERO™ transformants are often surrounded by non-transformed "satellite" colonies, which complicate colony picking and can contaminate cultures. The growth of satellite colonies is completely eliminated with the kanamycin-resistant pSMARTGC vectors.

The GC Cloning Kits are convenient to use, containing pre-cut, dephosphorylated pSMARTGC cloning vector premixed with buffer and ATP, as well as DNA ligase, Taq DNA polymerase, sequencing primers, and DNA controls. The Kits also contain high-efficiency *E. cloni*® 10G Electrocompetent or Chemically Competent Cells.



PHOSPHORYLATE PRIMERS
with T4 Polynucleotide Kinase
37°C, 10 min

AMPLIFY TARGET
with EconoTaq™ DNA Polymerase or other non-proofreading polymerase

LIGATE TO pSMARTGC
with CloneSmart® DNA Ligase
RT, 30 min
70ºC, 15 min.

TRANSFORM
***E. cloni*® 10G Cells**
15 min.

GROW and PLATE
30-60 min.

Figure 1. GC Cloning with EconoTaq DNA Polymerase. The simplest method of GC Cloning uses products from EconoTaq or other NON-proofreading polymerases. PCR primers are phosphorylated in a brief kinase reaction and are then added directly to the PCR reaction. After amplification, PCR fragments are directly ligated to the pSMARTGC vector and transformed into *E. cloni* 10G competent cells. Alternate protocols for Proofreading polymerases are described in the Manual.

pSMARTGC Cloning Vectors

The pSMARTGC vectors contain high- or low-copy replication origins and kanamycin resistance (Figure 2). 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 *lacZ*α 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 pSMARTGC vectors do not use a promoter or 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 pSMARTGC vectors, strong transcription terminators flank the cloning site to block this transcription (Figure 2), eliminating another source of cloning bias and sequencing gaps.

All GC Cloning vectors are supplied pre-cut with dephosphorylated 5' ends and single 3'-C overhangs. The copy number of pSMARTGC HK is similar to that of pUC plasmids (~300 copies/cell); the copy number of pSMARTGC LK is similar to that of pBR322 (~20/cell). The only difference between the high copy and low copy plasmids is the presence of the ROP gene in the low copy versions, which inhibits plasmid replication. The low copy number version of pSMARTGC further increases the ability to clone intact sequences that are otherwise difficult to maintain.

Insert DNA that contains 3'-G tails and 5'-phosphate groups is ligated to the GC Cloning vector, transformed into competent cells, and spread on plates containing kanamycin. pSMARTGC transformants do NOT require additional screening against colonies containing empty vector, as they typically are not present at detectable levels. The GenBank accession numbers for the pSMARTGC HK and LCKan vectors are not yet available; the vector sequences are supplied in the Appendix at the back of this manual.

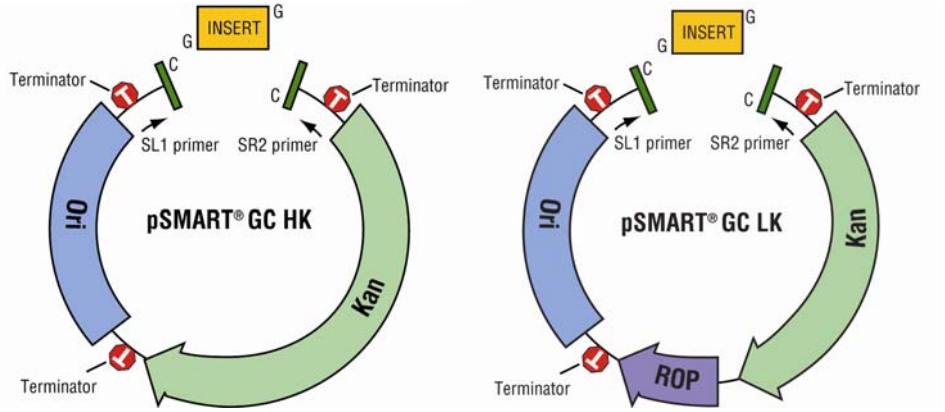


Figure 2. Schematic diagram of the transcription-free pSMARTGC vectors. Ori, origin of replication; Kan, Kanamycin resistance gene; ROP, Repressor of primer (lowers plasmid copy number). Approximate positions of sequencing primers and transcription terminators are indicated. After being linearized, the preparation of pSMARTGC HK is 1845 bp and pSMARTGC LK is 2037 bp.

Purification and Size Fractionation of DNA

A PCR reaction created with phosphorylated primers and a NON-proofreading polymerase can be used directly for cloning with the GC Cloning vectors. However, PCR products often contain spurious bands, primer dimers, and unused primers that can be cloned efficiently. Isolation of the desired DNA fragments by agarose gel electrophoresis is strongly recommended to avoid cloning irrelevant inserts.

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 3). 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.

Use a long wavelength (e.g., 360 nm) low intensity UV lamp and short exposure times when isolating DNA fragments from agarose gels.

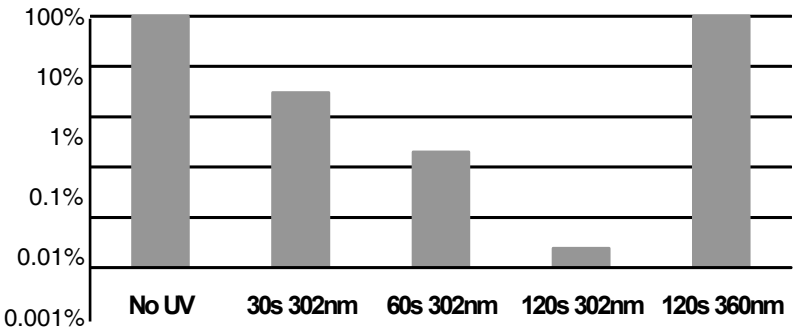


Figure 3. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 120 seconds ("30s 302nm, 60s 302nm,120s 302nm") or to 360 nm UV light for 120 seconds ("120s 360nm"). Cloning efficiencies were calculated relative to un-irradiated pUC19 DNA.

E. cloni® 10G Competent Cells

For maximum cloning efficiency, Lucigen's *E. cloni* 10G Competent Cells are supplied with all GC Cloning Kits. *E. cloni* 10G Competent Cells are *E. coli* strains optimized for high efficiency transformation. They are ideal for cloning and propagation of BAC, fosmid, or plasmid clones.

E. cloni 10G Cells give high yield and high quality plasmid DNA due to the *end*A1 and *rec*A1 mutations, and are phage resistant (*ton*A mutation). *E. cloni* 10G strains also contain the inactive *mcr* and *mrr* mutations, allowing methylated genomic DNA that has been isolated directly from mammalian or plant cells to be cloned without deletions or rearrangements.

Lucigen's GC Cloning Kits are available with the following *E. cloni* 10G Competent Cells in convenient SOLO packaging (one transformation 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*

- *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 electroporation. Use of TB or other media may result in lower transformation efficiencies.

Materials and Equipment Needed

The GC Cloning Kits supply most of the items needed to efficiently generate recombinant clones. While simple and convenient, successful use of the 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). Successful results are obtained with cuvettes from BTX (Model 610), BioRad (Cat. #165-2089), or Eppendorf (4307-000-569). Users have reported difficulties using *E. cloni* cells with Invitrogen cuvettes (Cat. # 65-0030).

  or

  Water bath at 42°C (for chemically competent cells).
- Thermocycler and gel electrophoresis equipment.
- Wet ice.
- Sterile 17 x 100 mm culture tubes.
- Terrific Broth.
- YT agar plates containing kanamycin (see Appendix for recipes).

OVERVIEW OF PROTOCOL

SECTION 1: PREPARATION OF PCR PRODUCTS FOR GC CLONING

PROTOCOL FOR ECONOTAQ (AND OTHER NON-PROOFREADING POLYMERASES)

Steps:
1. Phosphorylate primers
2. Amplify by PCR
3. Purify product

OR

Steps:
1. Amplify by PCR
2. Phosphorylate product
3. Purify product

PROTOCOL FOR PROOFREADING POLYMERASES

Steps:
1. Phosphorylate primers
2. Amplify by PCR
3. Add 3′-G tails
4. Purify product

OR

Steps:
1. Amplify by PCR
2. Add 3′-G tails
3. Phosphorylate product
4. Purify product

SECTION 2: LIGATION AND TRANSFORMATION

Detailed Protocol

The GC Cloning Kit can be used with PCR products from either Non-Proofreading or Proofreading DNA polymerases. However, all DNA fragments used for GC Cloning **MUST** have three features:

1) 5' phosphate groups: PCR products usually do NOT have 5' phosphate groups, regardless of the type of enzyme used for PCR amplification. The required 5' phosphate groups are added by phosphorylating either the PCR primers (Protocol A) or the PCR product (Protocol B). Protocols A and B are provided for both EconoTaq and for Proofreading polymerases.

2) Single 3'G overhangs: EconoTaq™ DNA Polymerase is a NON-proofreading enzyme supplied with the GC Cloning Kit. It adds 3'G tails during the PCR reaction. Therefore, only a subsequent phosphorylation step is needed before ligation to the GC vector. Other NON-proofreaders (e.g., Taq, Tfl, Tbr polymerases) similarly add 3'G tails.

   Proofreading polymerases (e.g., Vent®, Phusion®, Pfu) do NOT add 3'G tails, so both a G-tailing step and a 5'-phosphorylation step are needed. The EconoTaq enzyme is used after the initial PCR to add the 3'G tails.

3) Sufficient purity: In all cases, gel electrophoresis is ***highly recommended*** for purification of the insert DNA, to avoid cloning spurious bands or PCR primers.

Section 1 describes the preparation of 5-phosphorylated, G-tailed fragments. Separate protocols are provided for preparing fragments from NON-proofreading or proofreading polymerases. Section 2 details their ligation into the GC Cloning vector and transformation of *E. cloni* cells.

SECTION 1: PREPARATION OF PCR PRODUCTS FOR GC CLONING

Phosphate groups can be incorporated into the product by either of two methods: Protocol A describes phosphorylation of the primers before starting the PCR; Protocol B describes phosphorylation of the product after the PCR is finished. The following section presents Protocols A and B for EconoTaq and other NON-proofreading polymerases. The subsequent section presents Protocols A and B for Proofreading polymerases

GC Cloning with EconoTaq (and other NON-proofreading polymerases)

Protocol A. Use of Phosphorylated PCR Primers

Step 1: Phosphorylate Primers.

Option A) Synthesize PCR Primers with 5' Phosphates

The easiest way to generate a phosphorylated PCR product is to use PCR primers that have been chemically synthesized with 5 phosphates already attached. Nearly all manufactures of oligonucleotides provide this option, but the user must specifically request addition of 5 phosphates.

Option B) Add 5' phosphates to the primers by T4 PNK

T4 PNK can be used to add 5' phosphates to PCR primers before performing the PCR reaction. T4 PNK and Primer Kinase Buffer (containing ATP) are included in the GC Cloning Kits. Perform the primer kinase reaction as follows:

Primer kinase reaction

2.0 µl Forward primer @ 100 pmol/µl
2.0 µl Reverse primer @ 100 pmol/µl
1.0 µl 10 X Primer Kinase Buffer
1.0 µl T4 PNK (10 U/µl)
4.0 µl H_2O
10.0 µl total
Incubate at 37°C, 10 minutes

After the incubation, add 2-5 µl of this reaction directly to a 50-100 µl PCR mix and amplify by standard PCR (see Step 2).

In rare cases, the Primer Kinase Buffer interferes with the PCR reaction, resulting in reduced yields or smeared bands. In these cases, use chemically phosphorylated primers (Option A, above) or use non-phosphorylated primers followed by kinase treatment of the PCR product (Protocol B, below).

Step 2: Amplify by PCR.

EconoTaq DNA Polymerase, 10X Reaction Buffer, and dNTPs are included with the GC Cloning Kit for amplification. If desired, other NON-proofreading polymerases can be used in this protocol.

Step 3: Purify Insert DNA

DNA products can be cloned directly after the PCR reaction, if desired. However, any spurious PCR products, primers, or primer dimers may also be cloned efficiently. Size selection on an agarose gel therefore is ***highly recommended*** to remove contaminating DNA, aberrant PCR products, PCR primers, and primer-dimers. Purify the DNA bands from the gel by a commercial DNA purification kit.

If the DNA is not gel fractionated, it can be purified using a commercial purification kit or ethanol precipitation.

Proceed to Section 2: Ligation and Transformation.

Protocol B) Addition of 5′ phosphates to the PCR product

PCR products created with non-phosphorylated primers can also be used for GC Cloning. The PCR product MUST be purified away from the PCR buffer before the kinase reaction, because ammonium ions in the PCR buffer strongly inhibit T4 PNK. We recommend use of a purification column to remove the PCR buffer. The product is then treated with T4 PNK, followed by a second purification.

Step 1: Amplify by PCR.

EconoTaq DNA Polymerase, 10X Reaction Buffer, and dNTPs are included with the GC Cloning Kit for amplification. If desired, other NON-proofreading polymerases can be used for PCR in this protocol.

After the PCR is complete, purify the PCR product using a standard DNA purification column and elute in 44 µl of H_2O or the recommended elution buffer. Alternately, precipitate the PCR reaction using sodium acetate plus ethanol. Do not use ammonium acetate for precipitation, as this salt will severely inhibit the PNK reaction. Resuspend the DNA in 44 µl H_20.

Step 2: Phosphorylate PCR Product.

After removal of the PCR buffer from the PCR product, perform the following kinase reaction:

Post-PCR Kinase Reaction

44 µl purified PCR product (No PCR buffer present)
5 µl 10 X Primer Kinase Buffer (containing ATP)
1 µl T4 PNK (10 U/µl)
50 µl total

Incubate at 37°C, 10 minutes.

Do not use this material directly in a ligation reaction, as the PNK will phosphorylate the vector, resulting in a high background of non-recombinants. Purification is essential after this step.

Step 3: Purify Insert DNA

Size selection on an agarose gel is ***highly recommended*** to remove contaminating DNA, aberrant PCR products, PCR primers, and primer-dimers. Purify the DNA bands from the gel by a commercial purification kit.

If the DNA is not gel fractionated, it can be purified using a commercial DNA purification kit or ethanol precipitation.

Do NOT attempt to clone the products directly after the phosphorylation reaction, without performing a purification step; doing so will produce a high background of empty vector clones.

Proceed to Section 2: Ligation and Transformation.

GC Cloning with PROOFREADING DNA polymerases

Proofreading DNA polymerases, such as Vent® or Pfu polymerases, result in blunt DNA fragments. A 5' phosphorylation reaction and a 3' G-tailing reaction are both necessary for ligation of these products into the GC Cloning vector.

Preparation of PCR fragments

The most efficient method of GC Cloning with proofreading polymerases is to use phosphorylated primers followed by G-tailing (Protocol A, below). Alternately, the PCR product can be G-tailed first and subsequently phosphorylated (Protocol B). For both methods, EconoTaq™ DNA Polymerase is provided in the GC Cloning Kit to add single 3' G tails to the PCR product.

Proofreaders Protocol A: Phosphorylation followed by G-tailing

Step 1 of this protocol describes two options for incorporating phosphate groups into the PCR primers. Step 2 describes G-tailing of the 5'-phosphorylated PCR products.

Step 1. Phosphorylate PCR Primers

Option A) Synthesize PCR Primers with 5' Phosphates

The easiest way to generate a phosphorylated PCR product to use PCR primers that have been synthesized with 5 phosphates chemically attached. Nearly all manufactures of oligonucleotides provide this option, but the user must specify that 5 phosphates are desired.

Option B) Add 5' phosphates to the primers by T4 PNK

Standard (non-phosphorylated) primers can be treated with T4 PNK to phosphorylate the primers before the PCR reaction. T4 PNK and Primer Kinase Buffer are provided with the GC Cloning Kit. Perform the primer kinase reaction as follows:

Primer kinase reaction

2.0 µl Forward primer @ 100 pmol/µl
2.0 µl Reverse primer @ 100 pmol/µl
1.0 µl 10 X Primer Kinase Buffer (containing ATP)
1.0 µl T4 PNK (10 U/µl)
4.0 µl H_2O
10.0 µl total

Incubate at 37°C, 10 minutes

After the incubation, add 2-5 ul of this reaction directly to a 50-100 µl PCR mix and amplify by standard protocols (see Step 2: PCR Amplification).

In rare cases, the Primer Kinase buffer interferes with the PCR reaction, resulting in reduced yields or smeared bands. In these cases, use chemically phosphorylated primers (Option A) or use non-phosphorylated primers followed by a kinase reaction (Protocol 2, below).

Step 2: PCR Amplification

Amplify the template by standard PCR methods using the phosphorylated PCR primers and a proofreading DNA polymerase (e.g., Vent, Pfu).

After the PCR is complete, proceed directly to Step 3: G-tailing. Do NOT purify the PCR reaction product, as the PCR buffer is required for the G-tailing reaction.

Step 3: G Tailing of Blunt DNA

After the PCR is complete, EconoTaq™ DNA Polymerase adds single 3'G tails to blunt PCR products that contain 5' phosphate groups. The G-tailing reaction is performed in the same tube and buffer as the PCR:

G-Tailing Reaction

50-100 µl completed PCR reaction (AFTER thermal cycling, but NOT purified)
1 µl EconoTaq DNA Polymerase (5 U/µl)
51-101 µl total

Incubate at 72°C, 10 minutes. Do not cycle.

After G-tailing, proceed to Step 4: Purification of Insert DNA.

Step 4: Purification of Insert DNA

DNA products can be cloned directly after the G-tailing reaction, if desired. However, if spurious products or primer dimers are present, they may also be cloned efficiently. Size selection on an agarose gel therefore is ***highly recommended*** to remove contaminating DNA, aberrant PCR products, PCR primers, and primer-dimers. Purify the DNA bands from the gel by a commercial DNA purification kit.

If the DNA is not gel fractionated, it can be purified using a commercial purification kit or ethanol precipitation.

Proceed to Section 2: Ligation and Transformation.

Proofreaders Protocol B: G Tailing followed by phosphorylation

In this protocol, a blunt PCR product is created with a proofreading DNA polymerase using non-phosphorylated primers. Subsequently, the product is G-tailed in Step 2 and phosphorylated with T4 PNK in Step 3.

Step 1: Amplify by PCR

Amplify a template using non-phosphorylated primers and a proofreading polymerase (e.g., Vent, Pfu). After the reaction is complete, proceed directly to Step 2: G-tailing reaction.

Do NOT purify the PCR reaction product, as the G-tailing reaction requires the PCR buffer.

Step 2: G-tailing reaction

EconoTaq™ DNA Polymerase is used to add single 3'G tails to blunt PCR products, as follows:

G-Tailing Reaction

50-100 µl *complete* PCR reaction mix (AFTER thermal cycling, but NOT purified)
1 µl EconoTaq DNA Polymerase (5 U/µl)
51-101 µl total

Incubate at 72°C, 10 minutes. Do not cycle.

Purify the G-tailed PCR reaction to remove the PCR buffer. We recommend use of a DNA purification column, with elution in 44 µl of H_2O or the recommended elution buffer.

Ethanol precipitation with sodium acetate can also be used. Do NOT use ammonium acetate for precipitation, because the ammonium ions strongly inhibit the T4 PNK reaction.

Step 3: Phosphorylation of G-tailed product

The G-tailed product MUST be purified before performing phosphorylation with T4 PNK. The reaction is carried out as follows:

Post-PCR Kinase Reaction
44 µl *purified* PCR product (No PCR buffer present)
5 µl 10 X Primer Kinase Buffer mix
1 µl T4 PNK (10 U/µl)
50 µl total

Incubate at 37°C, 10 minutes.

Proceed to Step 4: Purification of Insert DNA.

Do not use this material directly in a ligation reaction, as the PNK will phosphorylate the vector, resulting in a high background of non-recombinants.

Step 4: Purification of Insert DNA

Size selection on an agarose gel is ***highly recommended*** to remove contaminating DNA, aberrant PCR products, PCR primers, and primer-dimers. Purify the DNA bands from the gel by a commercial purification kit.

If the DNA is not gel fractionated, it can be purified using a commercial purification kit or ethanol precipitation.

Do NOT attempt to clone the products directly after the phosphorylation reaction, without performing a purification step; doing so will produce a high background of empty vector clones.

Proceed to Section 2: Ligation and Transformation.

Control Reaction

The control PCR product must be created by amplification *and* must contain 5' phosphate groups for ligation into the GC Cloning vector! Performing the control reaction is recommended to help evaluate the results obtained with experimental samples. A control template and primers are supplied to produce a diagnostic PCR product of 497 bp that encodes the *lac*Z gene. Successful cloning of the resulting PCR product will produce blue colonies on XGAL/IPTG plates. A few white colonies may also arise from insertion of aberrant, non-functional *lac*Z mutants created by PCR.

For convenience, the control primers and template are supplied in a single tube. The Primer Kinase reaction will also phosphorylate the Control PCR template, which does not affect the results.

1. Primer Kinase reaction:

4.0 µl PCR control *lac*Z template plus primers (5 ng/µl template, 25 pmol/µl each primer)
1.0 µl 10X Primer Kinase buffer
4.0 µl H_2O
1.0 µl T4 Polynucleotide kinase (10 U/µl)
10.0 µl total

Incubate at 37°C for 10 minutes. Use 5.0 µl of the reaction directly for PCR amplification (below).

2. PCR reaction

5.0 µl Primer Kinase reaction (PCR control *lac*Z template plus primers)
5.0 µl 10X EconoTaq™ Reaction Buffer
2.5 µl dNTP mix (2.5 mM each)
37.0 µl H_2O
0.5 µl EconoTaq DNA Polymerase (5 unit/µl)
50.0 µl total

3. Amplify using the following cycling parameters:

STEP	TIME	TEMPERATURE	CYCLES
Denaturation	15 seconds	94ºC	25X
Annealing	15 seconds	60ºC	
Extension	60 seconds	72ºC	
Final Extension	10 minutes	72ºC	1X

4. Analyze 5 µl of the reaction by agarose gel electrophoresis. A distinct band at 497 bp should be visible. Use this product in the GC Cloning reaction described below.

Size Fractionation and Purification of Control PCR Fragments

Size selection on an agarose gel is highly recommended to remove aberrant PCR products, PCR primers, and primer-dimers. Purify the 497-bp DNA band from the gel by a commercial purification kit.

If the DNA is not gel fractionated, it can be purified using a commercial purification kit or ethanol precipitation.

DNA product can be cloned directly after the PCR reaction, if desired. However, if spurious products and primer dimers are present, they may also be cloned efficiently, resulting in numerous white colonies.

SECTION 2: LIGATION AND TRANSFORMATION

Ligation to the pSMARTGC Cloning Vectors

In the ligation reaction, the G-tailed, phosphorylated insert is ligated with pre-processed pSMARTGC Cloning vector. Successful cloning can be achieved routinely with as little as 10 ng of insert, but using low amounts of insert will result in significantly fewer transformants. The ligation is performed as follows:

1. Briefly centrifuge the GC Cloning 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 (10-400 ng, with 3′G tails and 5' phosphates)
2.5 μl 4X pSMARTGC Vector Premix
1.0 μl CloneSmart® DNA Ligase (2 U/μl)
y μl H_2O
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:

Positive Control Insert DNA	To determine the ligation and transformation efficiency with a known insert, use 100 ng (~1.0 μl) of the PCR amplified control *lac*Z template.
Vector Background	To determine the background of empty vector, omit Insert DNA in the above reaction.

4. Heat denature the ligation reaction at 70°C for 15 minutes.

Preparation for Transformation

1. Heat denature the ligation reaction at 70°C for 15 minutes if you have not done so already.
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 12,000 rpm to collect condensation and pellet precipitated material.
4. The sample is ready for transformation; precipitating the DNA is not necessary.

To ensure optimal cloning results, we strongly recommend the use of Lucigen's *E. cloni*® 10G ELITE or SUPREME Electrocompetent Cells. These cells yield $\geq 2 \times 10^{10}$ or $\geq 4 \times 10^{10}$ cfu/μg of pUC19, respectively, to maximize the number of transformants. For less demanding applications, *E. cloni* 10G Chemically Competent Cells may be used.

Most laboratory strains of competent *E. coli* can be effectively transformed with GC Cloning ligation reactions. The number of clones will be proportionate to the competency of the cells.

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 40 µl aliquots (SOLOs), sufficient for one transformation reaction. 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-killed 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 GC Cloning 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
*lac*Z PCR Amplified Insert (Positive Control)	50	> 200	> 99% inserts
No-Insert Control (Vector Background)	250	< 2	<1% background
Supercoiled pUC19 Transformation Control Plasmid (10 pg, Amp^R)	2	> 200	$\geq$ 1 x 10^8 cfu/µg plasmid

The results presented above are expected when cloning 100 ng of intact, PCR amplified *lac*Z DNA, with G-tailed ends and 5' phosphate groups, into Lucigen's pSMARTGC Cloning vectors. When transforming *E. cloni* 10G Chemically Competent Cells (transformation efficiency $\geq$1 x 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 GC Cloning 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 phosphorylated or G-tailed, 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 25-µl aliquots (SOLOs), sufficient for one transformation 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 GC Cloning 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
*lac*Z PCR Amplified 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 100 ng of intact, PCR amplified *lac*Z DNA, with G-tailed ends and 5' phosphate groups, into Lucigen's pSMARTGC 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 5′-phosphorylated or G-tailed, 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.

Getting More Recombinants

Increasing the ligation reaction time to 2 hours can increase the yield of recombinants by 4-5 fold. Ligation times beyond 2 hours will not improve the results further. Use of more efficiency competent cells will also increase recombinant yields. Use of more PCR amplicon in the ligation reaction can dramatically improve the number of recombinants.

Certain PCR products can prove recalcitrant to cloning due to a large size, the presence of toxic gene products or secondary structures, extremely biased base composition, or other unknown reasons. For these very challenging templates, we strongly recommend the use of the BigEasy™ v2.0 Linear Cloning Kit. The lack of supercoiling in the pJAZZ™-OC linear vector contained in the BigEasy v2.0 Cloning Kit alleviates many problems caused by secondary structure of the insert.

Colony Screening

When using pSMARTGC vectors, no additional screening for recombinant colonies is required, as the CloneSmart® system typically delivers >99% 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. The low-copypSMART GC vector is recommended for such inserts. For the most difficult inserts, Lucigen's BigEasy Linear Cloning kit is the best option (see Appendix B and Lucigen's website: .www.lucigen.com).

DNA Isolation and Sequencing

Grow transformants in TB medium plus 30 μg/ml kanamycin. Use standard methods to isolate plasmid DNA suitable for sequencing. The pSMARTGC HK plasmid contains the high copy number pUC origin of replication, yielding 20-80 μg of plasmid DNA per ml of culture. The pSMARTGC LK plasmid contains the low copy number pBR322 origin of replication, reducing plasmid yields to 0.5-1.0 μg per ml of cells. The *E. cloni* 10G Competent Cells are *recA endA* deficient and will provide high quality plasmid DNA. Sequencing primers SL1 and SR2 are provided with the GC Cloning Kits. The sequence of the primers and their orientation relative to the GC Cloning plasmids are shown in Appendix D.

References

1. Mead DA, Pey NK, Herrnstadt C, Marcil RA, Smith LM. A universal method for the direct cloning of PCR amplified nucleic acid.Biotechnology (N Y). 1991 Jul;9(7):657-63.

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

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+kan 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 pSMARTGC 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 indefinitely at –20 °C.

Appendix B: CloneSmart® Application Guide

GC Cloning Kits accommodate any cloning situation. For routine applications, we recommend using the pSMARTGC HK or pGC™ Blue vectors. For cloning toxic genes or particularly difficult DNA sequences, we recommend using the pSMARTGC LK vector or the pJAZZ™-OC linear vector (GC version coming soon).

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

Vector		Insert DNA Source			Intended Use
Vector Name	Copy #	PCR (NON-proofreader)	PCR (Proofreaders)	AT-Rich, "Difficult"	Digestion, Subcloning, Sequencing
pSMARTGC LK	Low	++	+	++	+
pSMARTGC HK	High	++	+	+	++
pGC Blue	High	++	+	-	++
pSMART LCKan	Low	+	++	++	+
pSMART HCKan	High	+	++	+	++
pJAZZ-OC	Low-Mid	+	+	+++	+

Appendix C: Abbreviated Protocol (with EconoTaq™ PCR)

(Please see Manual for detailed instructions.)

1. Phosphorylate the primers.

Primer kinase reaction

2.0 µl Forward primer @ 100 pmol/µl
2.0 µl Reverse primer @ 100 pmol/µl
1.0 µl 10 X Primer Kinase Buffer
1.0 µl T4 PNK (10 U/µl)
4.0 µl H_2O
10.0 µl total

Incubate at 37°C, 10 minutes

After the incubation, add 2-5 ul of this reaction directly to a 50-100 µl PCR mix and amplify according to standard protocols

2. PCR amplify DNA using the provided EconoTaq DNA Polymerase (or other non-proofreading DNA polymerase)**.**

3. Purify DNA by affinity matrix or gel electrophoresis. Do NOT use short wave UV light.

4. Ligate to pSMARTGC Cloning Vector. Mix the following in a 1.5-ml tube. Add ligase last.

x µl Insert DNA (10-400 ng, 5'-phosphorylated, G tailed)
y µl H_2O
2.5 µl 4X pSMARTGC Vector Premix
1.0 µl CloneSmart® DNA Ligase (2 U/µl)
10.0 µl total reaction volume

Incubate 30 minutes at room temperature (incubate 2 hours for maximum number of clones). Heat denature the ligation reaction 15 minutes at 70°C.

5. Transform *E. cloni* Competent Cells. *Important*: Use only Electrocompetent cells for Electroporation and Chemically Competent cells for Heat Shock Transformation!

Thaw *E. cloni®* Competent Cells on wet ice. Pipet cells into a pre-chilled tube on ice. Add 1-4 µl of heat-treated ligation reaction to an aliquot of chilled cells on ice.

Electroporation	Heat Shock Transformation
A) Pipet 25 µl of the cell/DNA mixture to a chilled electroporation cuvette. B) Electroporate. Immediately add 975 µl of room temperature Recovery Medium. C) Place in culture tube.	A) Incubate 30 minutes on ice. B) Incubate 45 seconds at 42 °C; then 2 minutes on ice. Add 260 µl of room temperature Recovery Medium to the culture tube.

Shake at 250 rpm for 1 hour at 37°C. Spread up to 100 µl on YT+kan agar plate.

Incubate overnight at 37°C.

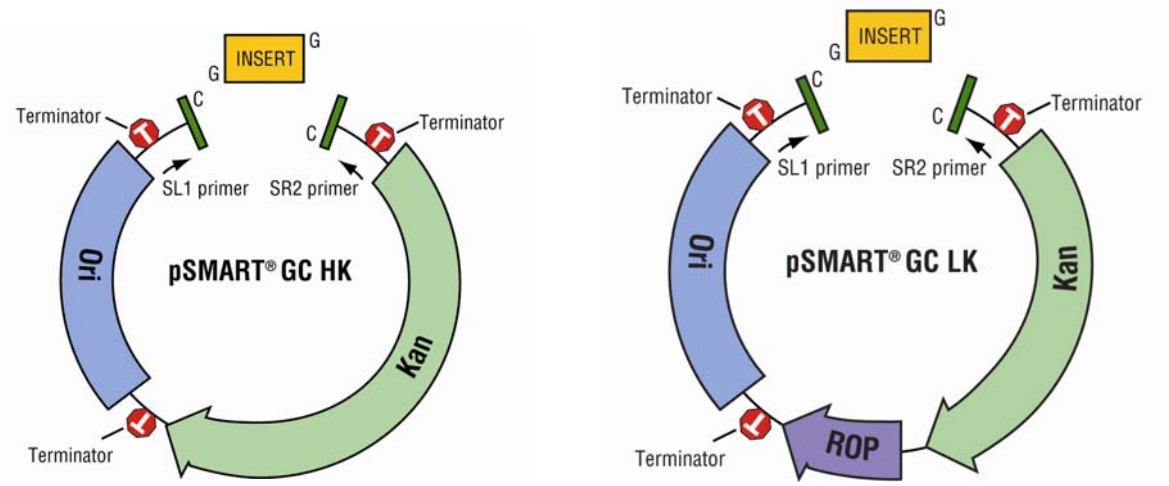
6. Colony Growth. Pick colonies at random and grow in TB+kan.

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

The pSMARTGC vectors are supplied predigested, with dephosphorylated 5' ends and a single 3'-C overhang. 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 cloning sites and sequencing primers are identical for both pSMARTGC vectors. The sequences of the SL1 and SR2 primers are as follows:

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



SL1 Primer **EcoRV Hind III* EcoRI** **Insert DNA**

CAGTCCAGTTACGCTGGAGTCTGAGGCTCGTCCTGAATGATATCAAGCTTGAATTCGTCGACGAC...
GTCAGGTCAATGCGACCTCAGACTCCGAGCAGGACTTACTATAGTTCGAACTTAAGCAGCTGCT...

EcoRI XbaI EcoRV **SwaI**

...TCGTCGACGAATTCTCTAGATATCGCTCAATACTGACCATTTAAATCATACCTGACCTCCATAGCAGAAAG
...CAGCAGCTGCTTAAGAGATCTATAGCGAGTTA**TGACTGGTAAATTTAGTATGGACTGG**AGGTATCGTCTTTC

SR2 Primer

***The Hind III site is NOT unique in the pSMARTGC-HC and -LC vectors. Another Hind III site is present in the kanamycin resistance gene.**

Appendix E: Troubleshooting Guide

Problem	Probable Cause	Solution
Very few or no transformants	PCR amplicon is not phosphorylated.	The GC Cloning vectors are dephosphorylated, requiring insert DNA to have 5' phosphates.
	Contaminating enzymes in ligation reaction.	Heat-denature enzymes used to prepare DNA 15 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 G-tailing reaction if necessary. Be sure insert DNA is phosphorylated. Use the supplied control insert to test ligation reaction.
	Inadequate heat denaturation of ligation reaction.	Be certain to heat denature for 15 min at 70°C. Skipping this step may lower the number of transformants by 2-3 orders of magnitude.
	Loss of DNA during precipitation.	DO NOT precipitate DNA after ligation reaction. It is not necessary with this protocol and these cells.
	Incorrect recovery media.	Use Recovery Medium following transformation.
	Improper electroporation conditions.	Use Eppendorf, 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 A). DO NOT spread antibiotic onto the surface of agar plates.
High background of transformants that do not contain detectable inserts.	Small inserts from primer dimer amplification are preferentially cloned.	Gel purify PCR amplicons away from primer-dimers.
	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 pSMARTGC LK or pJAZZ™GC linear vector (when available) for maximum clone stability.

Appendix F: Sequence of pSMARTGC HK and LK vectors

Note: The vectors sequences shown below are linearized at the cloning site.

pSMARTGC HK

CTCGTCGACGAATTCTCTAGATATCGCTCAATACTGACCATTTAAATCATACCTGACCTCC
ATAGCAGAAAGTCAAAAGCCTCCGACCGGAGGCTTTTGACTTGATCGGCACGTAAGAGGTT
CCAACTTTCACCATAATGAAATAAGATCACTACCGGGCGTATTTTTTGAGTTATCGAGATT
TTCAGGAGCTAAGGAAGCTAAAATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTT
TTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATG
CTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGAT
CCTTGAGAGTTTACGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTA
TGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACT
ATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTCACGGATGGCAT
GACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTA
CTTCTGGCAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATC
ATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCG
TGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTA
CTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGAT
CACTTCTGCGCTCGGCCCTCCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGA
GCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGCATCGTA
GTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGA
TAGGTGCCTCACTGATTAAGCATTGGTAATGAGGGCCCAAATGTAATCACCTGGCTCACCT
TCGGGTGGGCCTTTCTGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCAT
CACAAAAATCGATGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGG
CGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATA
CCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTAT
CTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGC
CCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTT
ATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCT
ACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCT
GCGCTCTGCTGAAGCCAGTTACCTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAA
ACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAG
GATCTCAAGAAGATCCTTTGATTTTCTACCGAAGAAAGGCCCACCCGTGAAGGTGAGCCAG
TGAGTTGATTGCAGTCCAGTTACGCTGGAGTCTGAGGCTCGTCCTGAATGATATCAAGCTT
GAATTCGTTGACGAC

pSMARTGC LK

CTCGTCGACGAATTCTCTAGATATCGCTCAATACTGACCATTTAAATCATACCTGACCTCC
ATAGCAGAAAGTCAAAAGCCTCCGACCGGAGGCTTTTGACTTGATCGGCACGTAAGAGGTT
CCAACTTTCACCATAATGAAATAAGATCACTACCGGGCGTATTTTTTGAGTTATCGAGATT
TTCAGGAGCTAAGGAAGCTAAAATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTT
TTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATG
CTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGAT
CCTTGAGAGTTTACGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTA
TGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACT
ATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTCACGGATGGCAT
GACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTA
CTTCTGGCAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATC
ATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCG
TGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTA
CTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGAT
CACTTCTGCGCTCGGCCCTCCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGA
GCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGCATCGTA
GTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGA
TAGGTGCCTCACTGATTAAGCATTGGTAAGTGACCAAACAGGAAAAAACCGCCCTTAACAT
GGCCCGCTTTATCAGAAGCCAGACATTAACGCTTCTGGAGAAACTCAACGAGCTGGACGCG
GATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACCGCAGCT
GCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGATGAGGGCCCAAATGTAATCACCT
GGCTCACCTTCGGGTGGGCCTTTCTGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCT
GACGAGCATCACAAAAATCGATGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAA
GATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCT
TACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGC
TGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCC
CCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAG
ACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTA
GGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTAT
TTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTCGGAAAAAGAGTTGGTAGCTCTTGATCC
GGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCA
GAAAAAAAGGATCTCAAGAAGATCCTTTGATTTTCTACCGAAGAAAGGCCCACCCGTGAAG
GTGAGCCAGTGAGTTGATTGCAGTCCAGTTACGCTGGAGTCTGAGGCTCGTCCTGAATGAT
ATCAAGCTTGAATTCGTTGACGAC