

Please note: The concentration of the included pUC19 has been changed from 1ng/μl to 10pg/μl. Do not dilute the plasmid before performing the transformation positive control. Please contact Lucigen if you have any questions.

GC Cloning & Amplification Kits with pSMART[®] GC VECTORS

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

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Advanced Products for Molecular Biology

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MA034 v3.2

GC Cloning & Amplification Kits (with pSMART® GC vectors)

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. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

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GC Cloning & Amplification Kits (with pSMART[®] GC vectors)

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GC Cloning & Amplification Kits (with pSMART[®] GC vectors)

GC Cloning Kit Designations

Several versions of the GC Cloning Kit with pSMARTGC vector 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 pSMARTGC Vector and *E. coli*[®] Cell Combinations

Vector	Reactions	10G ELITE Electrocompetent Cells (SOLOs)	10G SUPREME Electrocompetent Cells (SOLOs)	10G Chemically Competent Cells (SOLOs)
pSMARTGC HK (High Copy)	5	---	---	40733-0
	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

GC Cloning & Amplification Kits (with pSMART[®] GC vectors)

GC Cloning Kit Description

The GC Cloning Kits contain everything needed to amplify and clone PCR products into an unbiased, high-fidelity 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.

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 nucleotide to the 3' end of the PCR product. Although most PCR products from these enzymes have 3'A tails, a fraction of the PCR products have 3'G tails instead. In GC Cloning, these G-tailed products are ligated to 3'C overhangs on the pSMARTGC vectors (see Figures 1 and 2) (patents pending).

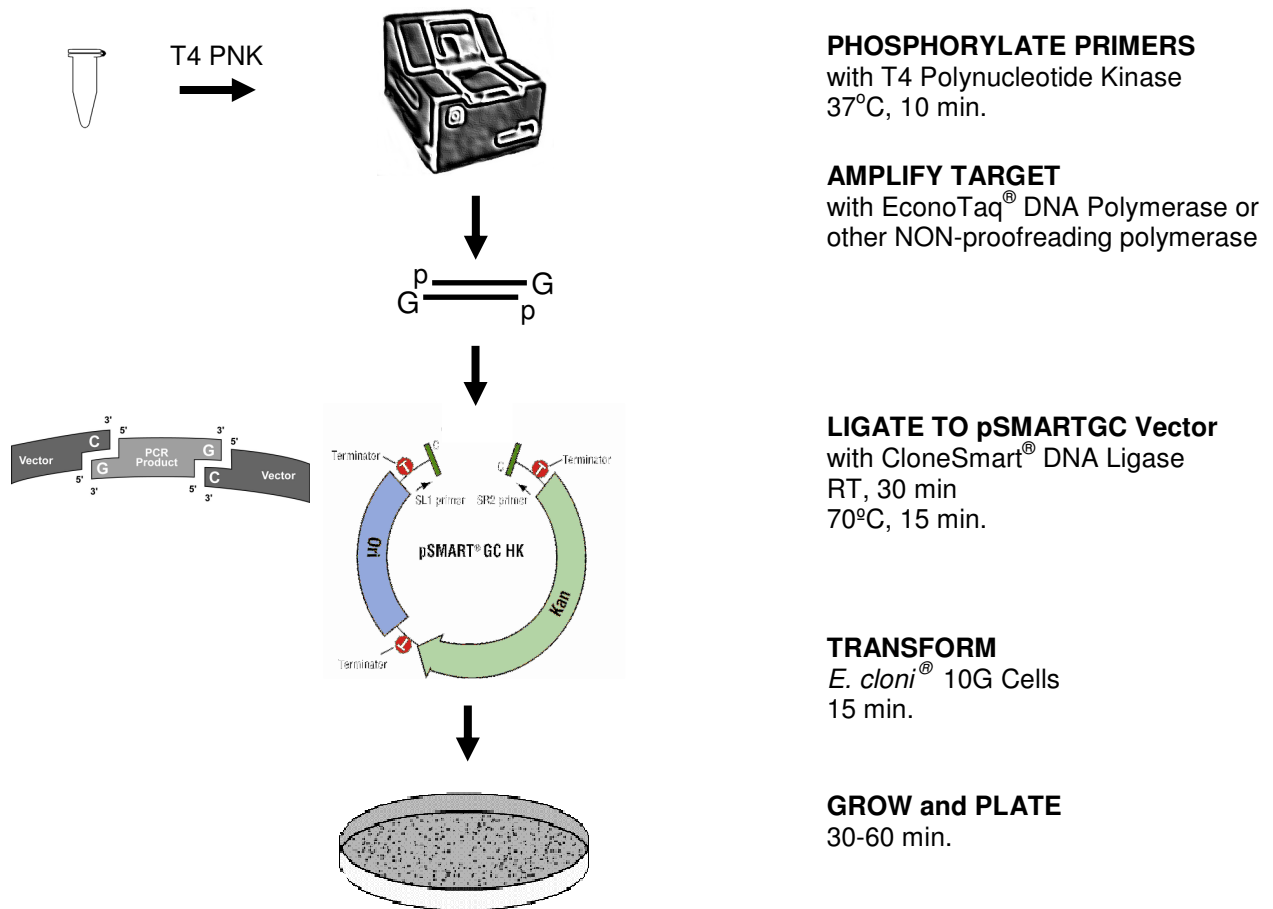


Figure 1. GC Cloning with EconoTaq DNA Polymerase. The simplest method of GC Cloning uses products from EconoTaq or other NON-proofreading polymerases. PCR primers are phosphorylated in a brief kinase reaction and are then added directly to the PCR reaction. After amplification, PCR fragments are directly ligated to the pSMARTGC vector and transformed into *E. coli* 10G competent cells. An alternate protocol for Proofreading polymerases requires a short G-tailing step (see detailed Instructions).

Two major advantages of GC Cloning are: 1) the transcription-free pSMARTGC vectors readily maintain large or otherwise unstable PCR products, as described below; and 2) the cloning efficiency and accuracy is higher with GC ends than with TA ends.

GC Cloning & Amplification Kits (with pSMART[®] GC vectors)

pSMARTGC Cloning Vectors

The pSMARTGC vectors (Figure 2) incorporate Lucigen's CloneSmart[®] transcription-free cloning technology to reduce bias and maximize cloning efficiency (U.S. Pat. 6,709,861). 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 *ccdB*. 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.

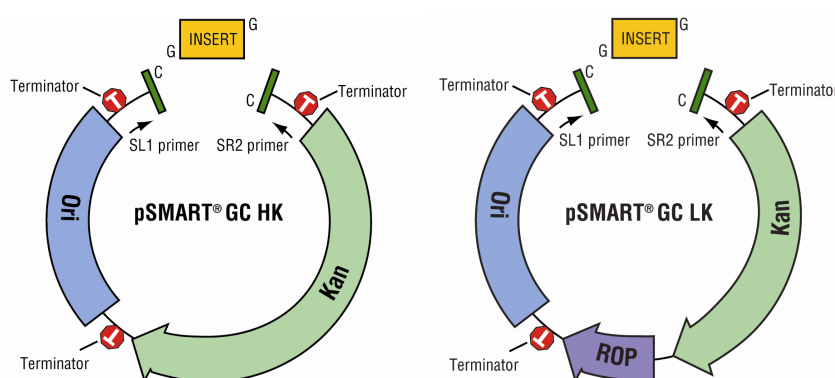


Figure 2. Transcription-free pSMARTGC vectors. Ori, origin of replication; Kan, Kanamycin resistance gene; ROP, Repressor of primer (reduces plasmid copy number). Approximate positions of sequencing primers and transcription terminators are indicated. After being linearized, the preparation of pSMARTGC HK is 1797 bp and pSMARTGC LK is 1993 bp.

The pSMARTGC vectors are supplied pre-digested, with 3'-C tails and dephosphorylated 5' ends. They 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, the blue/white screen of conventional TA or TOPO TA vectors 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 consequently thought to be "unclonable".

Further, the pZERO[™] vectors typically have an empty vector background of 5% or more. 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 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 difference in copy number is due to the presence of the ROP gene in the low copy vector, which inhibits plasmid replication. The low

GC Cloning & Amplification Kits (with pSMART[®] GC vectors)

copy number of the pSMARTGC LK vector further increases the ability to clone sequences that are otherwise difficult to maintain.

Insert DNA that contains 5'-phosphate groups and 3'-G tails 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 significant levels. The GenBank accession number for the pSMARTGC HK vector is EU729724 and for the LCKan vector is EU729725; the vector sequences are supplied in the Appendix at the back of this manual.

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

Purification and Size Fractionation of DNA

A PCR product created with 5'-phosphorylated primers and a NON-proofreading polymerase can be used directly for cloning with the GC Cloning vectors. Inserts created by Proofreading polymerases need a brief G-tailing treatment to facilitate cloning into the pSMARTGC vectors.

In either case, 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.

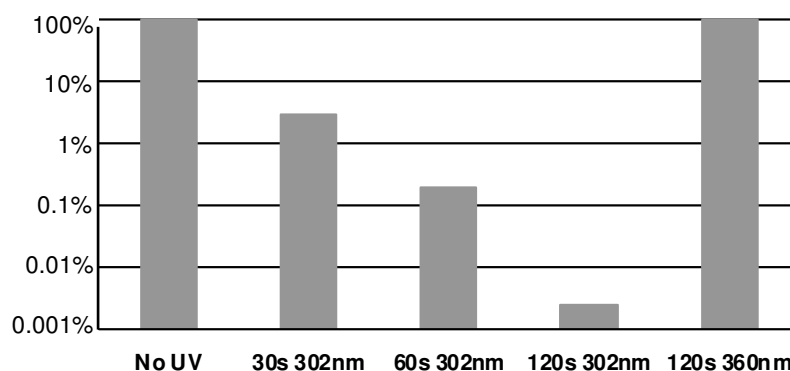


Figure 3. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure (“No UV”) or exposure to 302 nm UV light for 30, 60, or 120 seconds (“30s 302nm, 60s 302nm, 120s 302nm”) or to 360 nm UV light for 120 seconds (“120s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

GC Cloning & Amplification Kits (with pSMART® GC vectors)

***E. cloni*® 10G Competent Cells**

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 *endA1* and *recA1* mutations, and are phage resistant (*tonA* mutation). *E. cloni* 10G strains also contain the *mcr* and *mrr* mutations, allowing methylated genomic DNA that has been isolated directly from mammalian or plant cells to be cloned without deletions or rearrangements. The *rpsL* mutation confers resistance to streptomycin.

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 $\geq 4 \times 10^{10}$ cfu/ μ g. 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 $\geq 2 \times 10^{10}$ cfu/ μ g, providing a large number of transformants at a lower price than SUPREME Cells.

***E. cloni* 10G Chemically Competent Cells** deliver $\geq 1 \times 10^9$ cfu/ μ g and offer unbeatable performance and value for routine applications.

***E. cloni* 10G Genotype:**

F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*dlacZ* Δ M15 Δ *lacX74* *endA1* *recA1* *araD139* Δ (*ara, leu*)7697 *galJ* *galK* *rpsL* *nupG* λ - *tonA*

- *E. cloni* Competent Cells are provided with supercoiled pUC19 DNA at a concentration of 10 pg/ μ l. Use 1 μ l (10 pg) for transformation.
- **NOTE:** For optimal results, use the provided Recovery Medium to resuspend the cells after electroporation. Use of SOC, TB, or other media for recovery may result in substantially lower transformation efficiencies.

Materials and Equipment Needed

The GC Cloning Kits supply most of the items needed to efficiently generate recombinant clones. 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. The following items are required for transformation:

- Thermocycler and gel electrophoresis equipment.
 - 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).
 - Wet ice.
 - Sterile 17 x 100 mm culture tubes.
 - Make nutrient agar plates (e.g., LB). Add kanamycin to 30 μ g/ml before pouring the plates.

GC Cloning & Amplification Kits (with pSMART[®] GC vectors)

OVERVIEW OF PROTOCOL

SECTION 1: PREPARATION OF PCR PRODUCTS FOR GC CLONING

- Steps:
1. Phosphorylate primers
 2. Amplify by PCR
 3. Add 3'-G tails (*required for Proofreading polymerases only*)
 4. Purify product (recommended)

SECTION 2: LIGATION AND TRANSFORMATION

- Steps:
1. Ligate to pSMARTGC vector
 2. Heat inactivate the ligation reaction
 3. Transform *E. coli*[®] cells

Detailed Protocol

The GC Cloning Kit can be used with PCR products from either Non-Proofreading or Proofreading DNA polymerases. However, a short G-tailing step is required to clone blunt products produced by Proofreading polymerases. 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 enzyme used for amplification. The required 5' phosphate groups are added by phosphorylating the PCR primers.
- 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. 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 a G-tailing step is performed with EconoTaq enzyme after the initial PCR.

- 3) Sufficient purity: Successful results can be obtained without purification, if the PCR amplification produces predominantly the desired product. However, gel electrophoresis is highly recommended for purification of the insert DNA, to avoid cloning spurious bands or PCR primers. Column purification can be substituted, if few contaminating bands are present.

Section 1 (page 11) describes the preparation of 5-phosphorylated, G-tailed fragments.

Section 2 (page 13) details ligation into the GC vector and transformation of *E. coli* cells.

GC Cloning & Amplification Kits (with pSMART® GC vectors)

SECTION 1: PREPARATION OF PCR PRODUCTS FOR GC CLONING

Step 1: Phosphorylate Primers.

T4 Polynucleotide Kinase (PNK) is 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 H₂O

10.0 µl total

Incubate at 37°C, 10 minutes

After the incubation, add 1-5 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 may interfere with the PCR reaction, resulting in reduced yield or smeared bands. In these cases, primers with synthesized 5' phosphate groups should be used (these can be ordered from your oligonucleotide supplier).

Alternately, PCR can be performed with non-phosphorylated primers. The PCR products are purified to remove the PCR buffer and then phosphorylated with 10X Primer Kinase Buffer and T4 PNK.

Step 2: Amplify by PCR.

EconoTaq® DNA Polymerase, 10X Reaction Buffer, and dNTPs are included with the GC Cloning Kit for PCR amplification. If desired, other polymerases can be used. Regardless of the polymerase chosen, the target sequence can be amplified using standard methods.

Proofreading DNA polymerases, such as Vent® or Pfu polymerases, produce blunt DNA fragments. A 3' G-tailing reaction is necessary after the PCR for ligation of these products into the GC Cloning vector.

For products made with NON-proofreading polymerases (EconoTaq, Taq, Tfl, etc.), proceed directly to Step 4: Purification of Insert DNA.

For products made with Proofreading Polymerases (Vent, Pfu, etc.), proceed to Step 3: G-Tailing of Blunt DNA.

Step 3: G-Tailing of Blunt DNA (*required for Proofreading polymerases only*)

Products made with phosphorylated primers and Proofreading polymerases are blunt and 5'-phosphorylated. EconoTaq® DNA Polymerase is subsequently used to add single 3'G tails. The G-tailing reaction can also be used with blunt, 5'-phosphorylated fragments generated by any other method (e.g., end-repaired fragments or blunt restriction digests).

PCR buffer and reagents are required for the G-tailing reaction, so the product of the Proofreading polymerase should NOT be purified before the G-tailing reaction. 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, still containing PCR reagents)

1 µl EconoTaq DNA Polymerase (5 unit/µl)

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51-101 μ l 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 may be purified using a commercial purification kit.

Proceed to [Section 2: Ligation and Transformation](#).

Control Reaction

The control PCR product must be created by 5' phosphorylation of the primers and PCR amplification for ligation into the GC Cloning vector! A control template and primers are supplied to produce a diagnostic PCR product of 497 bp that encodes the *lacZ* gene. Successful cloning of the resulting PCR product will produce blue colonies on XGAL/IPTG plates. A few white colonies may also arise from cloning of aberrant, non-functional *lacZ* 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 *lacZ* template, which does not affect the results.

1. Primer Kinase reaction:

4.0 μ l PCR Control *lacZ* template plus primers (5 ng/ μ l template, 25 pmol/ μ l each primer)
1.0 μ l 10X Primer Kinase buffer
4.0 μ l H₂O
1.0 μ l T4 Polynucleotide kinase (10 U/ μ l)
10.0 μ l total

Incubate at 37°C for 10 minutes.

Use 5.0 μ l of the reaction directly for PCR amplification. Proceed with ligation and cloning as described above.

SECTION 2: LIGATION AND TRANSFORMATION

Ligation to the pSMARTGC Cloning Vectors

In the ligation reaction, the G-tailed, 5'-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 μ l Insert DNA (10-400 ng, with 3'G tails and 5' phosphates)
2.5 μ l 4X pSMARTGC Vector Premix
1.0 μ l CloneSmart[®] DNA Ligase (2 U/ μ l)
y μ l H₂O

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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 product amplified from the control <i>lacZ</i> 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. Make nutrient agar plates (e.g., LB). Add kanamycin to 30 µg/ml final concentration before pouring the plates.
2. Heat denature the ligation reaction at 70 °C for 15 minutes if you have not done so already.
3. Cool to room temperature for 15 seconds followed by 0-4°C for 15 seconds to condense water vapor inside the tube.
4. Spin 1 minute at 12,000 rpm to collect condensation and to pellet unwanted particulate material.
5. 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. coli* 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. coli* 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. coli*® 10G Competent Cells.

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

E. cloni 10G Chemically Competent Cells are provided in 40- μ l aliquots (SOLOs), sufficient for one transformation reaction. Transformation is performed by heat shock at 42°C, 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 μ l of the heat-denatured GC Cloning ligation reaction to the 40 μ l 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 μ l 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 μ l of transformed cells on nutrient agar plates (e.g., LB) containing 30 μ g/ml kanamycin. Incubate the plates overnight at 37°C.
9. Transformed clones can be further grown in any rich culture medium

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

Expected results from plating chemically transformed cells.

Reaction Plate	μ l/Plate	CFU/Plate	Efficiency
Experimental Insert (100 ng per ligation)	50 & 250	variable	NA
<i>lacZ</i> PCR Amplified Insert (Positive Control)	100	> 50	> 99% inserts
No-Insert Control (Vector Background)	250	< 2	<1% background
Supercoiled pUC19 Transformation Control Plasmid (10 μ g, Amp ^R)	50	>100	$\geq 1 \times 10^9$ cfu/ μ g plasmid

The results presented above are expected when cloning 100 ng of intact, PCR amplified *lacZ* 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 $\geq 1 \times 10^9$ cfu/ μ g 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 μ l of cells plated), unless contaminants are introduced.

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

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Electroporation of *E. cloni*® 10G Electrocompetent Cells

E. cloni 10G SUPREME and ELITE Electrocompetent Cells are provided in 25- μ l 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 μ F 600 Ohms 1800 Volts	1.0 mm cuvette 25 μ F 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 1 μ l (10 pg) of supercoiled pUC19 DNA.

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 μ l of the heat-denatured GC Cloning ligation reaction to the 25 μ l 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 μ l of ligation mix may cause electrical arcing during electroporation.
6. Carefully pipet 25 μ l 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 μ l 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.

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9. Spread up to 100 µl of transformed cells on nutrient agar plates (e.g., LB) containing 30 µg/ml kanamycin.
10. Incubate the plates overnight at 37°C.
11. Transformed clones can be further grown in in any 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
<i>lacZ</i> 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 *lacZ* 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 efficient 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 be difficult to clone due to large size, toxic gene products, secondary structure, 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 transcription-free, linear pJAZZ®-OC linear vector in the BigEasy Kit is not supercoiled, alleviating many problems caused by secondary structure of the insert.

GC Cloning & Amplification Kits (with pSMART[®] GC vectors)

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-copy pSMARTGC vector is recommended for such inserts. For the most difficult inserts, Lucigen's BigEasy[®] Linear Cloning kit is the best option (see Appendix A and Lucigen's website: www.lucigen.com).

DNA Isolation and Sequencing

Grow transformants in nutrient 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. coli*[®]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 C.

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.
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GC Cloning & Amplification Kits (with pSMART[®] GC vectors)

Appendix A: 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[®]-GC linear vector.

Use of the *E. coli*[®] 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 [*mcrA* Δ (*mrr-hsdRMS-mcrBC*)]. 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	+	+	+++	+

GC Cloning & Amplification Kits (with pSMART[®] GC vectors)

Appendix B: 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 H₂O

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 according to standard protocols

2. PCR amplify DNA using the provided EconoTaq DNA Polymerase (or other NON-proofreading DNA polymerase, such as Taq, Tfl, Tth, or Tbr DNA polymerase).

2b. G-tailing (required for Proofreading polymerases only):

Add 1 µl EconoTaq, incubate 10 minutes at 70°C.

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, 3'G tails)
y µl H₂O
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. coli*[®] Competent Cells. Important: Use only Electrocompetent cells for Electroporation and Chemically Competent cells for Heat Shock Transformation!

Thaw *E. coli* 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.

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

Shake at 250 rpm for 1 hour at 37°C. Spread up to 100 µl on nutrient agar (e.g., LB)+kan plate.

Incubate overnight at 37°C.

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

GC Cloning & Amplification Kits (with pSMART[®] GC vectors)

Appendix C: 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.

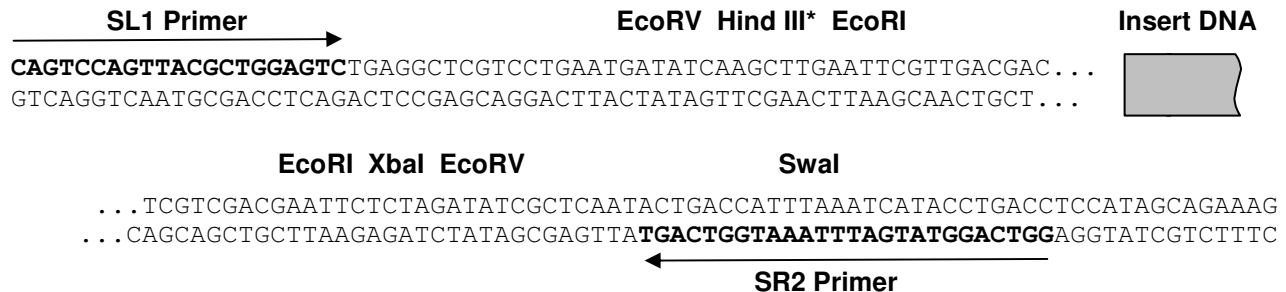
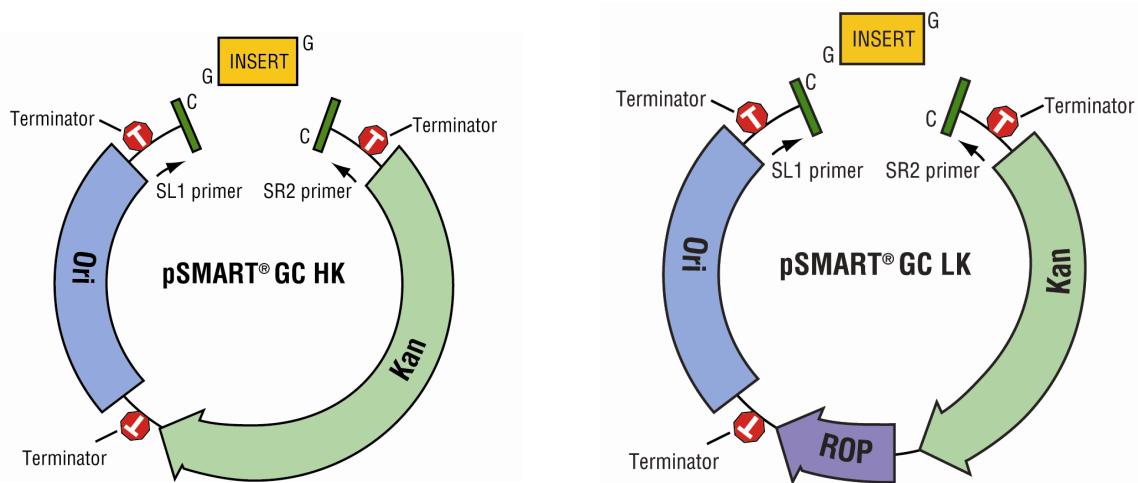
pSMARTGC HK (1797 bp). GenBank Accession # EU729724.

pSMARTGC LK (1993 bp). GenBank Accession #EU729725.

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'



*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.

GC Cloning & Amplification Kits (with pSMART[®] GC vectors)

Appendix D: 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. Purify DNA by extraction or adsorption to matrix.
	No DNA, degraded DNA, or insufficient amount of DNA.	Check concentration and integrity of insert DNA by gel electrophoresis. Use the supplied control insert to test the system.
	DNA damaged by UV exposure during gel purification.	Limit exposure to shortwave UV. Use 360-nm UV lamp for visualization, or use crystal violet staining for detection of DNA.
	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.
High background of transformants that do not contain detectable inserts.	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. DO NOT spread antibiotic onto the surface of agar plates.
	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.
	Unstable DNA Inserts	Use pSMARTGC LK or pJAZZ [®] GC linear vector (when available) for maximum clone stability.