Lucigen®
Simplifying Genomics

pGC™ Blue Cloning & Amplification Kits

IMPORTANT!
-20 °C Storage Required
Immediately Upon Receipt

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE
MA036 Rev. 01JAN2016

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Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the reagents supplied by the user are of the highest quality. Please follow the instructions 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.

Lucigen Technical Support

Email: techserv@lucigen.com

Phone: (888) 575-9695

Product Guarantee: Lucigen guarantees that this product will perform as specified for one year from the date of shipment. Please avoid using reagents for greater than one year from receipt.

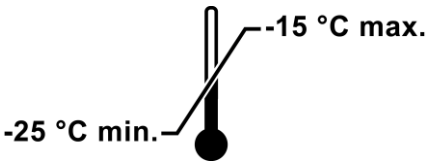
Product Designations

GC Cloning & Amplification Kits (pGC Blue Vector) are available with different numbers of reactions included (see table below). Please refer to Appendix B: Application Guide for more information and recommended uses of the kits.

Catalog Numbers

Vector	Reactions	Catalog #
GC Cloning and Amplification Kit (pGC Blue)	10	40744-1
	20	40744-2

Components & Storage Conditions



GC Cloning Kit Components

	Cap Color	10 Reactions	20 Reactions
4X pGC Blue Vector Premix Includes Buffer, ATP, and pGC Blue Vector	Blue	25 µL	50 µL
CloneSmart® DNA Ligase (2 U/ µL)	Green	12 µL	24 µL
T4 Polynucleotide Kinase (10 U/ µL) 10X Primer Kinase Buffer (containing ATP)	Green Brown	20 µL 100 µL	20 µL 100 µL
PCR Control Cm^R template plus primers (5 ng/ µL template, 25 pmol/ µL each primer)	Yellow	12 µL	12 µL
EconoTaq® DNA Polymerase (5U/ µL) EconoTaq 10X Reaction Buffer 2.5 mM dNTPs	Red Brown Clear	50 µL 1.5 mL 50 µL	50 µL 1.5 mL 50 µL
CloneSmart Sequencing Primers (200 reactions each) Z FOR Primer [M13 For (-41)] (3.2 pmol/ µL) Z REV Primer [M13 Rev (-48)] (3.2 pmol/ µL)	 Clear Clear	 200 µL 200 µL	 200 µL 200 µL

Competent cells are available separately from Lucigen (www. lucigen.com).

Abbreviated Protocol (with EconoTaq® PCR)

Please see page 9 for a Detailed Protocol.

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 incubation, add 2-5 µL directly to a 50-100 µL PCR reaction and amplify using standard protocols.

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

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. Ligation to pGC Blue 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 pGC Blue 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. Transformation. Competent cells are available separately from Lucigen (www. lucigen.com). Thaw *E. cloni*® Competent Cells on wet ice. Pipet cells into a pre-chilled tube on ice. Add 1-2 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 nutrient agar (e.g., LB)+KanXI agar plate. Incubate at 37 °C.

6. Colony Growth. Pick white colonies and grow in media (e.g., LB)+Kan.

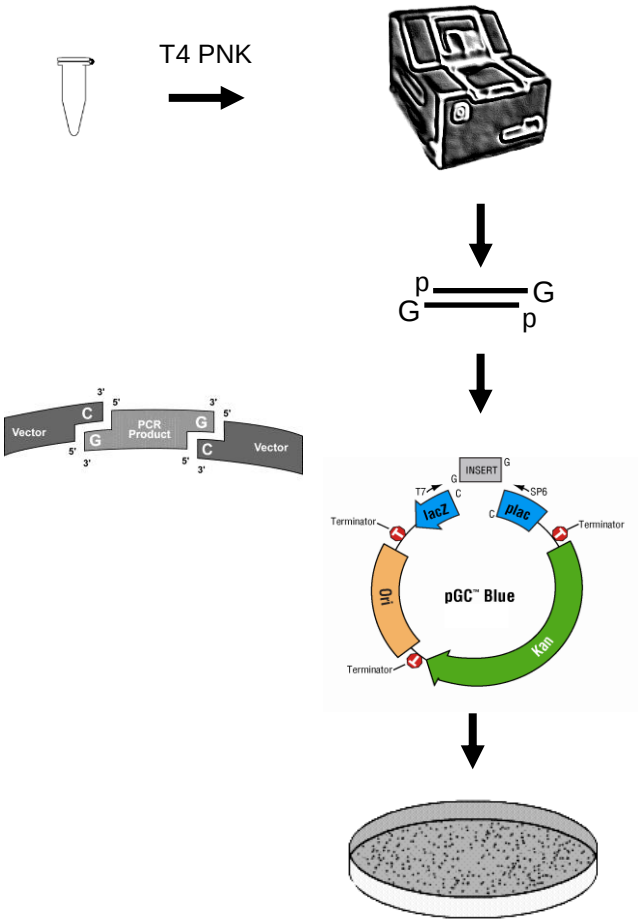
Product Description

GC Cloning & Amplification Kits (with pGC® Blue vectors)

The GC Cloning Kits provide everything needed to amplify and clone PCR products into an unbiased, high-fidelity vector. The GC Cloning Kits contain pre-cut, dephosphorylated pGC Blue cloning vector premixed with buffer and ATP, as well as CloneSmart DNA ligase, EconoTaq® DNA Polymerase, sequencing primers, and DNA controls. The Kits are compatible with both NON-proofreading and proofreading PCR polymerases and can be used to clone any blunt DNA up to ~10 kb.

Two major advantages of GC Cloning are: 1) the GC Cloning vectors 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 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 pGC Blue vector (see Figures 1 and 2) (patents pending).



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

AMPLIFY TARGET
with EconoTaq® DNA Polymerase or other NON-proofreading polymerase

LIGATE TO pGC Blue vector
with CloneSmart® DNA Ligase
RT, 30 min
70 ºC, 15 min.

TRANSFORM
E. cloni® 10G Cells
15 min.

RECOVER 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 pGC Blue vector and transformed into *E. cloni* 10G competent cells. An alternate protocol for Proofreading polymerases requires a short G-tailing step (see detailed Instructions).

pGC Blue Cloning Vector

The pGC Blue vector Genbank Accession # EU729726 (Figure 2) incorporates Lucigen's CloneSmart® transcription-free cloning technology to reduce bias and maximize cloning efficiency (U.S. Pat. 6, 709, 861). The pGC Blue vector is supplied pre-digested, with single 3'-C tails and dephosphorylated 5' ends, and is qualified to produce >99% recombinant clones in typical experiments. The very low background enables cloning and library construction from nanogram amounts of DNA. In contrast, TA or TOPO TA vectors typically have an empty vector background of 5% or more. In addition, the ampicillin-resistant transformants are often surrounded by NON-transformed "satellite" colonies, which complicate colony picking and can contaminate cultures. The growth of satellite colonies is eliminated with the kanamycin-resistant pGC Blue vector. The vector also includes opposed SP6 & T7 promoters for expression studies.

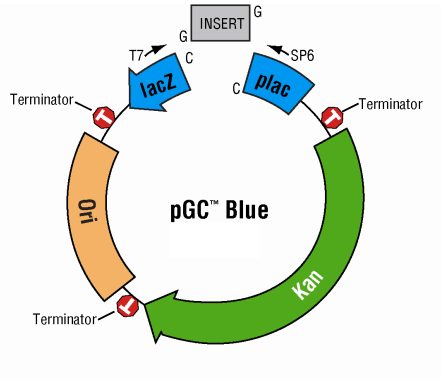


Figure 2. pGC Blue vector. Ori, origin of replication; Kan, Kanamycin resistance gene; plac, lac promter; lacZ, *lac*Zalpha ORF. Approximate positions of sequencing primers and transcriptional terminators (T) are indicated. See Appendix D.

The pGC Blue vector contains a high-copy replication origin. Strong transcription terminators flank the *lac*Z gene to eliminate fortuitous transcription from cloned inserts (Figure 2). The copy number is similar to that of pUC plasmids (~300 copies/cell). The sequence of the pGC Blue vector (Genbank EU729726) is supplied at the back of this manual (Appendix B).

E. cloni® Competent Cells

Competent cells are available separately from Lucigen (www. lucigen.com).

For maximum cloning efficiency, we strongly recommend the use of Lucigen's *E. cloni*® 10G ELITE or 10G SUPREME Electrocompetent Cells. For less demanding applications, *E. cloni* Chemically Competent cells may be used. The number of clones will decrease in proportion to the competency of the cells.

E. cloni 10G Competent Cells are optimized for high efficiency transformation. They are ideal for cloning and propagation of BAC, cosmid, or plasmid clones. They give high yield and high quality plasmid DNA due to the *rec*A1 and endA1 mutations, and are phage T1-resistant (*ton*A mutation). *E. cloni* 10G strains 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. The *rps*L mutation confers resistance to streptomycin.

Cell Preparation	24 Reactions		Transformation Efficiency
	SOLO*	SOLO*	
***E. cloni* 10G SUPREME Electrocompetent Cells**	60081-2	60081-2	$\geq$ 4 x 10^{10} cfu/µg
E. cloni* 10G ELITE Electrocompetent Cells*	60051-2	60051-2	$\geq$ 2 x 10^{10} cfu/µg
***E. cloni* 10G Chemically Competent Cells**	60106-2	60106-2	$\geq$ 1 x 10^{9} cfu/µg

*SOLO and DUO formats contain either 1 or 2 transformations per vial.

**E. cloni 10G ELITES are also available in 150 µL aliquots (SixPacks), sufficient for six transformation reactions of 25 µL each.

Genotype of *E. cloni* 10G: F- mcrA Δ(mrr-hsdRMS-mcrBC) endA1 recA1 Φ80dlacZΔM15 ΔlacX74 araD139 Δ(ara,leu)7697galU galK rpsL nupG λ- tonA (StrR)

Considerations For Use

Please read the entire manual and prepare the necessary equipment and materials before starting.

Preparation of DNA Inserts

The GC Cloning Kit can be used with PCR products from Non-Proofreading and Proofreading DNA polymerases. A short G-tailing step is required for blunt products produced by Proofreading polymerases.

All DNA fragments used for GC Cloning MUST have three features:

1) 5' phosphate groups added by phosphorylating the PCR primers prior to performing PCR.

2) Single 3'G overhangs added during PCR. 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) also 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) Amplicon purity. 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.

Avoid exposing the DNA to short wave UV radiation

Exposure to short wavelength ultraviolet light (e.g., 254, 302, or 312 nm) can reduce cloning efficiencies by several orders of magnitude (Figure 3).

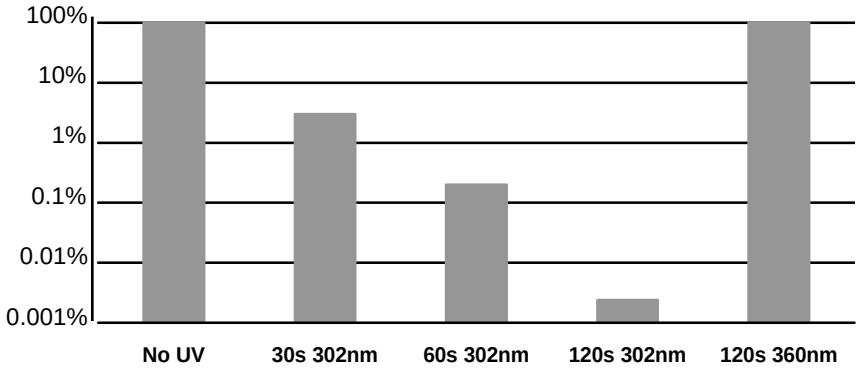


Figure 3. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 90 seconds ("30 s 302 nm, 60s 302 nm,120 s 302 nm") or to 360 nm UV light for 120 seconds ("120 s 360 nm"). Cloning efficiencies were calculated relative to un-irradiated pUC19 DNA.

Use a long wavelength (e.g., 360 nm) low intensity UV lamp and short exposure times when isolating DNA fragments from agarose gels. Alternatively, fluorescent DNA stains with optimum illumination wavelengths outside the UV range can be used for DNA visualization.

Obtaining 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 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.

Materials and Equipment Needed

Please read the entire manual and prepare the necessary equipment and materials before starting. Following ligation, the following items are required for transformation:

- **Chemically or electrically competent *E. coli*** (see recommendations on page 6).
- 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).
- Water bath at 42 °C (for chemically competent cells).
- Thermocycler and gel electrophoresis equipment.
- Wet ice.
- Sterile 17 x 100 mm culture tubes.
- Make media (e.g., LB) + KanXI agar plates, containing kanamycin, XGAL (50 µg/ml), and IPTG (1 mM).

Detailed Protocol

An Abbreviated Protocol for experienced users can be found on page 4.

A control template and primers are supplied to produce a PCR product of 781 bp that encodes chloramphenicol acetyl transferase (CAT). The control PCR product must be created by amplification and must contain 5' phosphate groups for ligation into the GC Cloning vector.

Phosphorylation of Primers

T4 Polynucleotide Kinase (included in the GC Cloning Kits) adds 5' phosphates to PCR primers before PCR. In rare cases, the Primer Kinase Buffer may interfere with the PCR reaction resulting in reduced yield or smeared bands. Primers synthesized with 5´ phosphate groups should then be used.

1. Combine the following in a PCR tube. For the control reaction, use 4.0 µL PCR control CAT template plus primers (5 ng/µL template, 25 pmol/µL each primer).

   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 H_2O
   4.0 µL T4 PNK (10 U/µL)
   10.0 µL total

2. Incubate at 37°C, 10 minutes

3. Add 1-5 µL of this reaction directly to a 50-100 µL PCR mix and amplify by standard PCR.

Amplification by PCR

1. Amplify target using standard methods. EconoTaq DNA Polymerase, 10X Reaction Buffer, and dNTPs are included with the GC Cloning Kit for PCR amplification using standard methods. If desired, other polymerases can be used.
2. For the control reaction combine the following in a PCR tube:

   5.0 µL Phosphorylated PCR control plus primers, from step 1
   5.0 µL 10X EconoTaq Reaction Buffer
   4.0 µL 2.5 mM dNTP mix
   0.5 µL EconoTaq DNA Polymerase
   35.5 µL DIUF water
   50.0 µL Total

   Thermal cycler conditions:

Step	Temperature	Time	Cycles
1	94 °C	2 minute	1
2 3 4	94 °C 60 °C 72 °C	15 seconds 15 seconds 1 minute	2-4 x25
5	72 °C	10 minutes	1
6	4 °C	Indefinitely	1

3. For PCR products made with EconoTaq or other NON-proofreading polymerase (including the control), proceed directly to Step 4: Purification of Insert DNA.
4. For products made with Proofreading Polymerases (Vent, Pfu, etc.), proceed to G-Tailing of Blunt DNA.

G-Tailing of Blunt DNA (required only when using Proofreading polymerases)

1. EconoTaq DNA Polymerase is used to add single 3'G tails to blunt, 5' phosphorylated PCR products made with Proofreading polymerases. PCR reagents are required for G-tailing, so the PCR product should NOT be purified before G-tailing. 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).
2. Add 1 µL EconoTaq DNA Polymerase (5 unit/µL) to the completed PCR reaction (50-100 µL)
3. Incubate at 72 °C, 10 minutes. Do not cycle.
4. Proceed to Step 4: Purification of Insert DNA.

Purification of Insert DNA

1. DNA products can be cloned directly after G-tailing. However, 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 using a commercial DNA purification kit. If few contaminating bands are present, the product may be purified using a commercial column purification kit.
2. NOTE: the control PCR product may be cloned directly after G-tailing, without further purification.

Ligation to pGC Blue Cloning Vector

The G-tailed, phosphorylated insert is ligated with pre-processed pGC Blue 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.

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

***ESSENTIAL:* The ligation reaction must be heat inactivated!**

4. Incubate ligation reaction at 70 °C for 15 minutes.
5. Cool to room temperature for 15 seconds, then cool to 4 °C on ice for 15 seconds.
6. Briefly to collect the mixture at the bottom of the tube and proceed to transformation.

Electroporation of *E. cloni* Electrocompetent Cells

Competent cells are available separately from Lucigen (www.lucigen.com).

Transformation is carried out in a 0.1 cm gap cuvette. Typical time constants are 3.5 to 4.5 msec. Optimal settings for electroporation are listed in the table below.

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; Eppendorf Model 2510.

Suggested Electroporation Cuvettes:

BTX (Model 610) or BioRad (Cat. #165-2089)

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

- ✓ ***ESSENTIAL:*** **the ligation reaction must be inactivated at 70 °C for 15 minutes!**
- ✓ Microcentrifuge tubes and electroporation cuvettes must be thoroughly pre-chilled on ice.
- ✓ The cells must be completely thawed **on ice** before use.
- ✓ Recovery Medium should be used after electroporation. Use of SOC or other media may decrease transformation efficiency.

Transformation Protocol (Electrocompetent cells)

1. Prepare at room temperature:
   - 17 mm x 100 mm sterile culture tubes (one tube for each transformation)
   - Recovery Medium
1. Prepare on wet ice:
   - Electroporation cuvettes with 0.1 cm gap (1 cuvette for each transformation)
   - Microcentrifuge tubes on ice (one tube for each transformation)
2. Remove *E. cloni* cells from the -80 °C freezer and place on wet ice until they thaw **completely** (10-20 minutes).
3. When cells are thawed, mix them by tapping gently. Add 25 µL of *E. cloni*® cells to the chilled microcentrifuge tube on ice.
4. Add 1 µL of the **heat-inactivated** CloneSmart Ligation reaction to the 25 µL of cells on ice. 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.

   For transformation control, use 1 µL (10 pg) of supercoiled pUC19 DNA.
2. 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.
3. 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.
4. Place the tube in a shaking incubator at 250 rpm for 1 hour at 37 °C.
5. Spread up to 100 µL of transformed cells on bacterial medium agar plates containing the appropriate antibiotic.
6. **Note: When transforming pUC19 control, plate cells on agar containing <u>ampicillin</u>.**
7. 10. Incubate the plates overnight at 37 °C.
8. Transformed clones can be further grown in any rich culture medium (e.g., LB).

Heat Shock Transformation of Chemically Competent cells

Competent cells are available separately from Lucigen (www.lucigen.com).

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

- ✓ ***ESSENTIAL:*** **the ligation reaction must be heat inactivated at 70 °C for 15 minutes!**
- ✓ Microcentrifuge tubes must be thoroughly pre-chilled on ice before use.
- ✓ The cells must be completely thawed **on ice** before use.

✓ Recovery Medium should be used after electroporation. Use of SOC or other media may decrease transformation efficiency.

Transformation Protocol (Chemically Competent cells)

1. Chill 17 mm x 100 mm sterile culture tubes on ice (one tube for each transformation reaction).
2. Remove *E. cloni* cells from the -80 °C freezer and place on wet ice until they thaw **completely** (10-20 minutes).
3. Add 40 µL of *E. cloni* cells to the chilled culture tube.
4. Add 1 µL of the heat-inactivated CloneSmart Ligation reaction to the 40 µL of cells on ice. Stir briefly with pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells.

   For transformation control, use 1 µL (10 pg) of supercoiled pUC19 DNA.
5. Incubate on ice for 30 minutes.
6. Heat shock cells by placing them in a 42 ^{o}C water bath for 45 seconds.
7. Return the cells to ice for 2 minutes.
8. Add 960 µL of room temperature Recovery Medium to the cells in the culture tube.
9. Place the tubes in a shaking incubator at 250 rpm for 1 hour at 37 °C.
10. Plate up to 100 µL of transformed cells on bacterial medium agar plates containing the appropriate antibiotic.

    Note: When transforming pUC19 control, plate cells on agar containing <u>ampicillin</u>.
11. Incubate the plates overnight at 37 °C.
12. Transformed clones can be further grown in any rich culture medium (e.g., LB).

Expected Results

Expected Results Using *E. cloni*® 10G CHEMICALLY COMPETENT CELLS

Plating chemically transformed cells and expected results.

Reaction Plate	µL/Plate	CFU/Plate	Efficiency
Experimental Insert (100 ng per ligation)	50 & 250	variable	NA
CAT PCR Amplified Insert (Positive Control)	100	> 50	> 99% inserts
No-Insert Control (Vector Background)	250	< 2	<1% background
Supercoiled pUC19 Transformation Control Plasmid (10 pg, Amp^R)	50	>100	> 1 x 10^9 cfu/µg plasmid

The results presented above are expected when cloning 100 ng of intact, PCR amplified *CAT* DNA, with G-tailed ends and 5' phosphate groups, into Lucigen's pGC Blue Cloning vector. When transforming *E. cloni* 10G Chemically Competent Cells (transformation efficiency $\geq$1 x 10^9 cfu/µg 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.

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%.

EXPECTED RESULTS USING *E. cloni* 10G 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
CAT PCR Amplified Insert (Positive Control)	5	> 400	> 99% inserts
No-Insert Control (Vector Background)	100	< 25	<1% background
pUC19 Transformation Control Plasmid 1:100 Dilution (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 pGC Blue 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 5 µL 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.

Colony Screening

The pGC Blue vector uses the standard blue/white colony screen based on *lac*Zalpha complementation. Transformants are grown on plates containing kanamycin, XGAL, and IPTG. Recombinant colonies will be white, and non-recombinant colonies will be blue. However, some insert DNAs produce blue colonies in this screen (e.g., small open reading frames). Therefore, if the proportion of blue colonies is unexpectedly high, it may be useful to analyze blue colonies for the presence of inserts.

Inserts that are large or have unusual base composition may produce very few colonies. Lucigen's cloning kits containing transcription-free vectors are recommended for such inserts (see Appendix A and Lucigen's website: www.lucigen.com).

DNA Isolation and Sequencing

Grow transformants in nutrient agar medium plus 30 µg/mL kanamycin. Use standard methods to isolate plasmid DNA suitable for sequencing. The pGC Blue plasmid contains the high copy number pUC origin of replication, yielding 20-80 µg of plasmid DNA per mL of culture. The *E. cloni*® 10G Competent Cells are *recA* and *endA* deficient and will provide high quality plasmid DNA. GC Cloning Kits are provided with the sequencing primers Z FOR and Z REV. The sequence of the primers and their orientation relative to the pGC Blue vector 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: GC Cloning Application Guide

GC Cloning Kits accommodate any cloning situation. For routine applications, we recommend using the pSMART® GC HCKan vector or pGC Blue vector. For cloning toxic genes or particularly difficult DNA sequences, we recommend using the pSMARTGC LCKan vector or the pJAZZ® GC linear vector.

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.

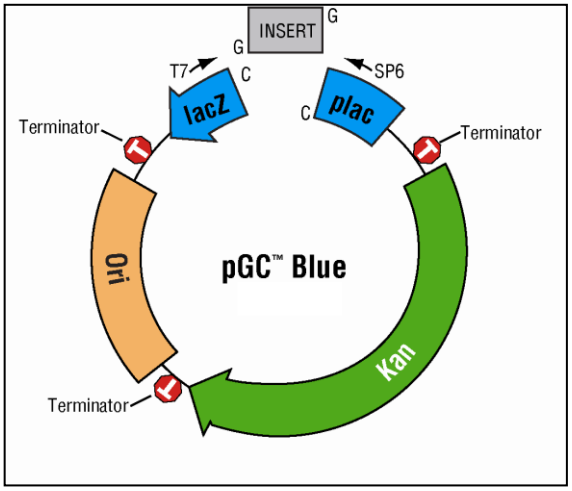
Vector		Insert DNA Source			Intended Use
Vector Name	Copy #	PCR (NON-proofreader)	PCR (Proofreaders)	AT-Rich, "Difficult"	Digestion, Subcloning, Sequencing
pSMARTGC LK	Low	++	+	++	+
pSMARTGC HK	High	++	+	+	++
pGC Blue	High	++	+	-	++
pSMART LCKan	Low	+	++	++	+
pSMART HCKan	High	+	++	+	++
pJAZZ-OC	Low-Mid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

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

The pGC Blue vector is supplied predigested, with dephosphorylated 5' ends and a single 3'-C overhang. Transcriptional terminators border the *lac*Z gene 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. Genbank Accession # EU729726.

The sequences of the Z FOR and Z REV primers are as follows:

Z FOR (M13 For (-41): 5'–CGCCAGGGTTTTCCCAGTCACGAC–3'
Z REV (M13 Rev (-48): 5'–AGCGGATAACAATTTCACACAGGA–3'



Z Rev Primer — *lac*Zα start — SP6

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AGCGGATAACAATTTCACACAGGAAACAGCT ATG ACC ATG ATT ACG CCA AGC TAT TTA GGT
TCGCCTATTGTTAAAGTGTGTCCTTTGTCGA TAC TGG TAC TAA TGC GGT TCG ATA AAT CCA
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Promoter

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                                          HindIII KpnI    SacI    BamHI
GAC ACT ATA GAA TAC TCA AGC TAT GCA TCA AGC TTG GTA CCG AGC TCG GAT CCA
CTG TGA TAT CTT ATG AGT TCG ATA CGT AGT TCG AAC CAT GGC TCG AGC CTA GGT

  SpeI          BstXI          EcoRI                              EcoRI
CTA GTA ACG GCC GCC AGT GTG CTG GAA TTC GAC CTC [PCR Product] GGG GTC GAA
GAT CAT TGC CGG CGG TCA CAC GAC CTT AAG CTG GAG [PCR Product] CCC CAG CTT

         EcoRV  BstXI         NotI      XhoI            XbaI    ApaI
TTC TGC AGA TAT CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC
AAG ACG TCT ATA GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG

AAT TCG CCC TAT AGT GAG TCG TAT TAC AAT TCA CTG GCC GTC GTT TTA CAA
TTA AGC GGG ATA TCA CTC AGC ATA ATG TTA AGT GAC CGG CAG CAA AAT GTT
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T7 Promoter

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CGT CGT GAC TGG GAA AAC CCT GGC GTT
GCA GCA CTG ACC CTT TTG GGA CCG CAA
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Z For Primer

Appendix C: Troubleshooting Guide

Problem	Probable Cause	Solution
Very few or no transformants	PCR amplicon is not 5'-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.
	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 LCKan or pJAZZ® GC linear vector for maximum clone stability.
High number of blue transformants	Some inserts may not disrupt lacZ expression.	Screen blue or light blue colonies for the presence of desired insert.

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