BLUE/WHITE CLONING KIT

Blue/White cloning without the blues™

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

Lucigen® Corporation
Advanced Products for Molecular Biology

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MA005 v10.4

Technical Support

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pEZSeq™ Blunt Cloning Kit

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pEZSeq Blunt Cloning Kit Designations

The pEZSeq Blunt Cloning Kits are supplied with pEZSeq High Copy Vector (either Kan^R or Amp^R) and *E. cloni*® Competent Cells. Kits are also available with no cells. Please refer to "Appendix B: Application Guide" for more information and recommended uses of the kits.

Catalog numbers of vector and cell combinations

Vector	Reactions	10G ELITE Electrocompetent Cells (DUOs) (≥ 2 x 10^{10} cfu/µg)	10G SUPREME Electrocompetent Cells(DUOs) (≥ 4 x 10^{10} cfu/µg)	10G Chemically Competent Cells(DUOs) (≥ 1 x 10^8 cfu/µg)	10GF' ELITE Electrocompetent Cells(DUOs) (≥ 2 x 10^{10} cfu/µg)	10GF' Chemically Competent Cells(DUOs) (≥ 5 x 10^8 cfu/µg)	No Cells
pEZSeq Amp (High Copy)	10	40475-1	40486-1	40488-1	40494-1	40496-1	---
	20	40475-2	40486-2	40488-2	40494-2	40496-2	40464-2
pEZSeq Kan (High Copy)	10	40501-1	40512-1	40514-1	40524-1	40526-1	---
	20	40501-2	40512-2	40514-2	40524-2	40526-2	40500-2

Components & Storage Conditions

The Ligation Components of the pEZSeq Kits are shipped in Container 1, which should be stored at **-20°C**. If *E. cloni* Cells are ordered with the Kit, they are shipped in Container 2, which must be stored at **-80°C**. Additional pEZSeq Ligation Components and *E. cloni* Competent Cells may be purchased separately.

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

	10 Reactions	20 Reactions
4X pEZSeq Vector Premix Includes Buffer, ATP, and either pEZSeq-HC Amp *or* pEZSeq-LC Amp	25 µl	2 x 25 µl
CloneSmart® DNA Ligase (2 U/µl)	12 µl	2 X 12 µl
Positive Control Insert DNA (500 ng/µl Lambda *Hinc*II)	5 µl	2 X 5 µl
Sequencing Primers (200 reactions each)		
Z-For Primer [M13 For (-41)] (3.2 pmol/µl)	200 µl	2 X 200 µl
Z-Rev Primer [M13 Rev (-48)] (3.2 pmol/µl)	200 µl	2 X 200 µl

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

	Catalog #	Reactions
E. cloni 10G ELITE Electrocompetent Cells	60052-1 60052-2	12 (6 x 50 µl) 24 (12 x 50 µl)
or *E. cloni* 10G SUPREME Electrocompetent Cells	60080-1 60080-2	12 (6 x 50 µl) 24 (12 x 50 µl)
or *E. cloni* 10GF' ELITE Electrocompetent Cells	60061-1 60061-2	12 (6 x 50 µl) 24 (12 x 50 µl)
or *E. cloni* 10G Chemically Competent Cells	60106-1 60106-2	12 (6 x 80 µl) 24 (12 x 80 µl)
or *E. cloni* 10GF' Chemically Competent Cells	60062-1 60062-2	12 (6 x 80 µl) 24 (12 x 80 µl)
Control pUC19 DNA (1 ng/µl) Store at **-20°C** or **-80°C**	----	10 (1 x 10 µl)
Recovery Medium Store at **-20°C** or **-80°C**	---- ---- 80026-1	12 (1 x 12 ml) 24 (2 x 12 ml) 96 (8 x 12 ml)
YT Agar (powder)	----	

pEZSeq Blunt Cloning Kit Description

The pEZSeq Blunt Cloning Kits provide a improved vector incorporating the traditional blue/white colony screen. Up to one million recombinant clones may be obtained routinely from less than 500 ng of insert DNA, with no vector preparation required. As little as 100 ng of insert DNA is sufficient to yield thousands of recombinant clones. The kit is ideal for constructing shotgun libraries or for general purpose cloning, especially when amounts of target DNA are limited. The pEZSeq Blunt Cloning Kit is convenient to use. It contains pre-digested, dephosphorylated pEZSeq cloning vector, ligase, buffer, sequencing primers, electrocompetent or chemically competent cells, and DNA controls. The Kit may also be ordered without cells.

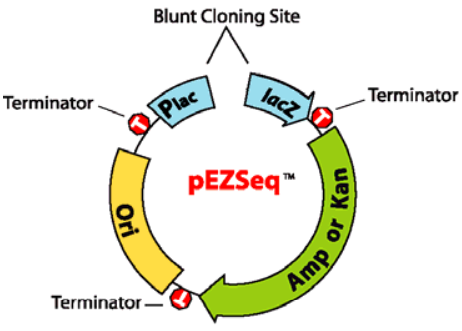


Figure 1. Schematic diagram of the CloneSmart® pEZSeq vector. Ori, origin of replication; Amp or Kan, ampicillin or kanamycin resistance gene. Positions of transcription terminators are indicated.

The pEZSeq vector incorporates the familiar blue/white screen for detecting recombinant clones. Ligation of an insert into the cloning site interrupts the coding sequence of the lacZα peptide; when

plated on XGAL/IPTG indicator plates, recombinant clones are white and non-recombinants are blue. The pEZSeq vectors have several major advantages over conventional cloning vectors.

The pEZSeq vectors are processed and assayed to assure blunt dephosphorylated ends. When transformed into *E. cloni*® Competent Cells, > 99% of the colonies recovered will have an insert. In contrast, conventional vectors often produce a dense background of blue colonies. The extremely low background of the pEZSeq vectors reduces colony picking errors and simplifies screening.

The pEZSeq vectors are designed to allow cloning of a wide range of inserts. Conventional plasmids can be destabilized by transcription initiated within the cloned insert. For example, fragments that contain *E. coli* promoters, as well as random AT-rich sequences that may act as promoters, can cause plasmid instability by transcribing into essential regions of the vector. In the pEZSeq vectors, strong transcription terminators flank the cloning site to block this transcription (Figure 1), eliminating a significant source of cloning bias and sequencing gaps.

The pEZSeq vectors contain a minimal amount of vector DNA between the sequencing primers and the cloning site (Figure 1 and Appendix D). As a result, less vector sequence must be trimmed from each trace, so sequence reads may contain up to 10% more insert sequence.

The pEZSeq system reduces the growth of “satellite” or “feeder” colonies, which often grow near colonies harboring conventional ampicillin resistant plasmids. Contamination of recombinant clones with non-transformed bacteria is greatly reduced with the pEZSeq Blunt Cloning Kit. Satellite colonies are completely eliminated with the use of kanamycin-resistant versions of the pEZSeq vectors.

pEZSeq Vectors

pEZSeq vectors are supplied with blunt, dephosphorylated ends (Figure 1). The copy number is similar to that of pUC plasmids (~300 copies/cell), yielding 20–100 µg of plasmid DNA per ml of culture. Blunt-ended, 5’-phosphorylated insert DNA is ligated to pEZSeq, transformed into competent cells, and spread on plates containing XGAL and either ampicillin or kanamycin. When using 10GF’ cells (or other cells containing the lacIq gene), IPTG must be added to induce expression of the blue/white screen.

E. cloni® Competent Cells

E. cloni 10G and 10GF’ Competent Cells are *E. coli* strains 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 *end*A1 mutation.

E. cloni 10G and 10GF’ 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. These strains are phage T1-resistant, due to the *ton*A mutation. The *rps*L mutation confers resistance to streptomycin.

E. cloni 10GF’ has the same chromosomal genotype as 10G, but it harbors the F’ plasmid. This plasmid confers tetracycline resistance and allows the cells to be infected with M13 for ssDNA production. The F' plasmid also carries the lacIq repressor allele; therefore, IPTG must be added to induce expression of the lacZα peptide for the blue/white screen. DNA inserted into the cloning site of pEZSeq will also be transcribed at high levels in the presence of IPTG.

E. cloni 10G and 10GF’ ELITE Electrocompetent Cells produce $\geq 2 \times 10^{10}$ cfu/µg supercoiled pUC19 DNA. The pEZSeq Kits are also available with *E. cloni* 10G SUPREME Electrocompetent Cells ($\geq 4 \times 10^{10}$ cfu/µg); or 10G ($\geq 1 \times 10^{8}$ cfu/µg) or 10GF’ Chemically Competent Cells.

Genotypes

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

***E. cloni* 10GF' Genotype:**
[F′ *pro*A+B+ *lacI*^{q}ZΔM15::Tn10 (TetR)] / *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *end*A1 *rec*A1 ϕ80d*lac*ZΔM15 Δ*lac*X74 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K *rps*L *nup*Gλ *ton*A

As a control for transformation, *E. cloni* Competent Cells are provided with supercoiled pUC19 DNA at a concentration of 1 ng/µl. For transformation, dilute 1:100 in dH_2O to a final concentration of 10 pg/µl.

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.

End Repair of Sheared DNA

Because of their low background and precut, blunt ends, the pEZSeq vectors are ideal for random shotgun cloning. This process typically entails a fragmentation step to randomly shear the DNA, an end-repair step to generate blunt ends, and a fractionation step to size select the fragments. Mechanical methods of DNA fragmentation (e.g., nebulization, sonication, hydrodynamic shearing) are often preferred over enzymatic methods, as they are more random and reduce the bias of sequencing projects (1). However, mechanical fragmentation results in a heterogeneous mix of blunt and 3'- and 5'-overhanging ends that may not ligate efficiently. Successful library construction requires a robust repair method to convert these ragged ends to blunt ends.

Lucigen has developed the DNATerminator® End Repair Kit (Cat. # 40035-1, 40035-2, and 40035-3) and PCRTerminator® End Repair Kit (Cat. # 40037-1, 40037-2, and 40037-3) to provide an efficient and convenient method for repairing DNA fragments. Use of the DNATerminator End Repair Kit is recommended for generating pEZSeq libraries from sheared or restriction-digested DNA. Use of the PCRTerminator End Repair Kit is recommended for generating libraries from PCR products generated by non-proofreading DNA polymerases (e.g., Taq, Tfl, Tth).

The DNA to be cloned needs to be free of RNA before end repairing. Significant amounts of contaminating RNA will severely impair the efficiency of the end repair reaction, resulting in DNA with poor cloning capabilities. We recommend the use of Lucigen's RNase I (Cat. # 30104-1 and 30104-2), which is an exonuclease that breaks RNA down into nucleosides, to remove the residual RNA often associated with DNA purification protocols. RNase A, which is a site-specific endonuclease, will not degrade the RNA sufficiently and is not recommended.

For shotgun library construction, Lucigen recommends using the HydroShear™ instrument by GeneMachines® to randomly fragment DNA. Fragments generated by the HydroShear device are repaired more efficiently than those produced through sonication or nebulization. It also generates a tight distribution of fragments in any desired size range, increasing the proportion of DNA available for cloning. The shearing results are also highly reproducible.

Purification and Size Fractionation of DNA

DNA must be purified from restriction or repair enzymes before ligation to pEZSeq vectors. Agarose gel electrophoresis, which is commonly used to size fractionate DNA fragments, is sufficient for purification. If end-repaired DNA is *not* fractionated by electrophoresis after repair or digestion, it must be purified by phenol/chloroform extraction or binding to a DNA purification column to remove the repair enzymes.

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 2). Note that the wavelength of most UV transilluminators, even those designated specifically for DNA visualization, is typically 302 nm or 312 nm, and can cause significant damage to DNA.

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

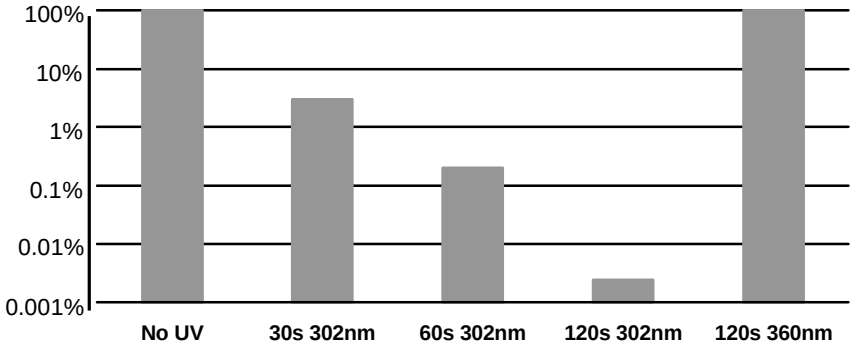


Figure 2. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 90 seconds ("30s 302nm, 60s 302nm,120s 302nm") or to 360 nm UV light for 120 seconds ("120s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

Materials and Equipment Needed

The pEZSeq Blunt Cloning Kit supplies many of the items needed to efficiently generate recombinant clones. While simple and convenient, successful use of the pEZSeq Kit requires proper planning for each step. Please read the entire manual and prepare the necessary equipment and materials before starting. It is assumed that common molecular biology equipment, supplies, and reagents are readily available. The following items are required for this protocol:

- 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 antibiotic (YT Agar powder included. Appendix has recipes).

Detailed Protocol

Preparation and Purification of Insert DNA

Generation of Blunt-Ended Fragments

DNA fragments created by digestion with blunt-cutting restriction enzymes (e.g., *Eco*RV or *Hin*cII) can be used with the pEZSeq Blunt Cloning Kits without further processing. However, an end-repair reaction is required for cloning fragments generated by physical shearing (e.g. sonication or hydrodynamic shearing), by PCR with polymerases having terminal transferase activity (e.g. Taq or Tfl), or by restriction enzymes that leave 3' or 5' overhangs. The end-repair reaction must generate blunt ends with 5' phosphate groups.

For cloning physically sheared DNA, we recommend using Lucigen's DNATerminator® End Repair Kit, which has been optimized for this purpose. The DNATerminator End Repair Kit also very efficiently removes 3' or 5' overhanging ends created by restriction digestion. Standard protocols for DNA end-repair, typically consisting of a series of steps incorporating a DNA polymerase or exonuclease, also can be used for repairing restriction fragments.

For cloning PCR products with 3' single base overhangs, we recommend using Lucigen's PCRTerminator® End Repair Kit to generate blunt phosphorylated ends. Alternately, PCR may be carried out with a proof-reading thermostable polymerase, such as Vent™ or Pfu DNA polymerase, which leaves blunt ends. After the reaction is complete, the PCR products must be phosphorylated with T4 polynucleotide kinase. Kinase treatment of the PCR product is unnecessary if the PCR primers were treated with kinase prior to the PCR or if they were synthesized with terminal 5' phosphate groups.

*Note: End-repaired or kinased fragments **must** be purified to remove the enzymes before ligation to the pEZSeq vectors.*

Purification of Repaired Fragments

If repaired or kinased fragments are subsequently fractionated by gel electrophoresis, no further purification is necessary to remove the repair enzymes. Use of short-wavelength UV light (e.g., 254, 302, or 312 nm) **must** be avoided. After electrophoresis, DNA may be isolated using your method of choice.

If the DNA is *not* fractionated by electrophoresis after end repair, it must be purified by extraction or binding to a purification column to remove the repair enzymes. Heat denaturation is NOT sufficient to inactivate the enzymes. Failure to completely remove residual enzymes may result in a large background of empty vector clones or a greatly decreased ligation efficiency.

Ligation to the pEZSeq Vector

In the pEZSeq ligation reaction, the pre-processed pEZSeq vector is ligated with blunt, phosphorylated insert in a total volume of 10 µl. For library construction, we recommend using 300-500 ng of insert DNA in the size range of 1,000 to 4,000 bp. For cloning a single DNA species, 100-200 ng of insert is recommended. Successful cloning can be achieved routinely with as little as 100 ng of insert, but use of low amounts of insert will result in significantly fewer transformants. The ligation is performed as follows:

1. Prepare YT Agar from powder included with the cells. (Colonies are small & grow slowly on LB.)
2. Briefly centrifuge the pEZSeq Vector Premix before use. Mix by gently pipeting up and down several times.

3. Combine the following components in a 1.5-ml tube, adding the ligase last:

x µl Insert DNA (300-500 ng blunt-ended, 5'-phosphorylated)
y µl H_2O
2.5 µl 4X pEZSeq Vector Premix (pEZSeq, ATP, buffer)
1.0 µl CloneSmart® DNA Ligase (2 U/µl)
10.0 µl total reaction volume

4. Mix by gently pipeting the reaction mixture up and down. Incubate at room temperature (21-25°C) for 30 minutes. If the maximum number of transformants is desired, ligation time can be extended to 2 hours.

Optional Control Reactions include the following:

Positive Control Insert DNA	To determine the ligation and transformation efficiency with a known insert, use 1 µl (500 ng) of λ/*Hinc*II DNA.
Vector Background	To determine the background of empty vector, use water instead of insert in the above reaction.

Preparation for Transformation

1. Heat denature the ligation reaction at 70°C for 15 minutes.
2. Cool to room temperature for 15 seconds followed by 0-4 °C for 15 seconds to condense water vapor inside the tube.
3. Spin 1 minute at 12000 rpm to collect condensation and pellet precipitated material.
4. The sample is ready for transformation; precipitating the DNA is not necessary.

Transformation

Most laboratory strains of *E. coli* (e.g., DH10B™, DH5α™, etc.) can be effectively transformed with pEZSeq ligation reactions. The number of clones will be proportionate to the competency of the cells. However, to ensure optimal cloning results, we strongly recommend the use of Lucigen's *E. cloni*® 10G or 10GF' ELITE or 10G SUPREME Electrocompetent Cells. These cells yield $\geq 2 \times 10^{10}$ or $\geq 4 \times 10^{10}$ cfu/ug of pUC19, respectively, to maximize the number of transformants. For less demanding applications, *E. cloni* 10G or 10GF' Chemically Competent Cells may be used.

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

Electroporation of *E. cloni* Electrocompetent Cells

E. cloni 10G Elite and Supreme Electrocompetent Cells are provided in 50 µl aliquots (DUOs), sufficient for two transformation reactions of 25 µl each. *E. cloni* 10G Elites are also available in 150 µl aliquots (SixPacks), sufficient for six transformation reactions of 25 µl 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:

- For best results, Lucigen CloneSmart® ligation reactions must be heat killed at 70ºC for 15 minutes before transformation. Alternately, the reactions may be purified, if desired. For other ligation reactions, follow the manufacturer's recommendations.
- Prepare YT Agar plus antibiotic from powder provided (see Media Recipes below).

  NOTE: Transformants grow slowly on LB agar.
- Microcentrifuge tubes and electroporation cuvettes must be thoroughly pre-chilled on ice before use. Successful results are obtained with cuvettes from BTX (Model 610) or BioRad (Cat.#165-2089). Users have reported difficulties using *E. cloni* cells with Invitrogen cuvettes (Cat.# 65-0030).
- The cells must be completely thawed **on ice** before use.
- For highest transformation efficiency, use the provided Recovery Medium to resuspend the cells after transformation.

Transformation Protocol

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) and microcentrifuge tubes on ice (one cuvette and one tube for each transformation reaction).
3. Remove *E. cloni*® Electrocompetent 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. Add 25 µl of *E. cloni* cells to the chilled microcentrifuge tube on ice.
5. Add 1 µl of the heat-denatured CloneSmart 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 the following amounts of experimental and control reactions on plates containing selective medium (plus IPTG and XGAL if desired). Spread up to 100 µl of the experimental insert transformation per 100 mm petri plate.

Reaction Plate	µl/Plate
Experimental Insert (500 ng per ligation)	5, 20, & 100
Lambda *Hinc*II Insert (Positive Control)	5
No-Insert Control (Vector Background)	50
Supercoiled pUC19 Control (10 pg; Amp^R)	2

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

The results presented below are expected when cloning 500 ng of intact, purified DNA fragments with blunt ends and 5' phosphate groups, into Lucigen's *E. cloni* 10G ELITE Electrocompetent Cells (transformation efficiency $\geq 2 \times 10^{10}$ cfu/ug pUC19 DNA).The number of recombinant (white) clones is typically 100-fold greater than the background of blue colonies from self-ligated pEZSeq vector and 1000-fold greater than the background of white colonies from self-ligated pEZSeq vector. The background number of empty pEZSeq vector is constant (< 25 total colonies per 50 µl of cells plated), unless kinase or nuclease is introduced as a contaminant. However, use of too little insert DNA, or insert DNA that is improperly end-repaired, or modified DNA that is not repairable yields significantly lower recombinant cloning efficiencies. Cloning AT-rich DNA and other recalcitrant sequences may also lead to fewer colonies. With relatively few recombinant clones, the number of empty vector colonies becomes noticeable. For example, if the Experimental Insert ligation reaction produces only 250 colonies from 50 ul of cells plated, then the 25 colonies obtained from 50 ul of the No-Insert Control ligation will represent a background of 10%.

Reaction	CFU/Plate	Efficiency
Experimental Insert (300-500 ng per ligation)	50 & 250	variable
pEZSeq plus 500 ng Lambda *Hinc*II Insert	> 200	> 99.9% inserts
No-Insert Control (Vector Background)	< 25	< 0.1% background
Supercoiled pUC19 Control (10 pg)	> 200	> 1 x 10^{10} cfu/ug plasmid

1. A pEZSeq ligation reaction, containing 500 ng of positive control lambda *Hinc*II DNA, is expected to yield > 200 white colonies from a 5 µl aliquot of transformed cells, with <1% non-recombinant blue colonies. Please note that up to 20% of the true recombinant colonies may be blue due to lambda DNA inserts that fail to completely disrupt the *lacZ*α peptide. To compensate for uncertainty in the nature or quantitation of the experimental DNA, we recommend plating 5, 20, and 100 µl of transformed cells to obtain a suitable number of clones.

2. A 50 µl aliquot of the empty vector control reaction should produce < 25 blue colonies. White colonies in the empty vector control should represent less than 0.1% of the white colonies in the Lambda HincII control.

3. A 2 µl aliquot of transformed cells from the pUC19 reaction (diluted into 90 µl of TB) should yield > 200 colonies, or > 1 x 10^{10} colonies per µg plasmid.

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

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

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

- For best results, Lucigen CloneSmart® ligation reactions must be heat killed at 70ºC for 15 minutes before transformation. Alternately, the reactions may be purified, if desired. For other ligation reactions, follow the manufacturer's recommendations.
- Prepare YT Agar plus antibiotic from powder provided (see Media Recipes below).
  NOTE: Transformants grow slowly on LB agar.
- All microcentrifuge tubes must be thoroughly pre-chilled on ice before use.
- The cells must be completely thawed **on ice** before use.
- For highest transformation efficiency, use the provided Recovery Medium to resuspend the cells after transformation.

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 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

The results presented below are expected when cloning 500 ng of intact, purified DNA fragments with blunt ends and 5' phosphate groups, into Lucigen's *E. cloni* 10G Chemically Competent Cells (transformation efficiency $\geq$ 1 x 10^8 cfu/µg pUC19 DNA). The number of recombinant (white) clones is typically 100-fold greater than the background of blue colonies from self-ligated pEZSeq vector and 1000-fold greater than the background of white colonies from self-ligated pEZSeq vector. The background number of empty pEZSeq vector is constant (< 25 total colonies per 50 µl of cells plated), unless kinase or nuclease is introduced as a contaminant. However, use of too little insert DNA, or insert DNA that is improperly end-repaired, or modified DNA that is not repairable yields

significantly lower recombinant cloning efficiencies. Cloning AT-rich DNA and other recalcitrant sequences may also lead to fewer colonies. With relatively few recombinant clones, the number of empty vector colonies becomes noticeable. For example, if the Experimental Insert ligation reaction produces only 50 colonies from 250 ul of cells plated, then the then the 2 colonies obtained from 250 µl of the No-Insert Control ligation will represent a background of 4%.

Reaction Plate	µl/Plate	CFU/Plate	Efficiency
Experimental Insert (300-500 ng per ligation)	50 & 250	variable	NA
pEZSeq plus 500 ng Lambda *Hinc*II Insert (Positive Control)	50	> 20	> 99% inserts
No-Insert Control (Vector Background)	250	< 2	<1% background
Supercoiled pUC19 Transformation Control Plasmid	50	> 50	$\geq 1 \times 10^8$ cfu/µg plasmid

1. A pEZSeq ligation reaction, containing 500 ng of positive control lambda *Hinc*II DNA, is expected to yield > 20 white colonies from a 50 µl aliquot of transformed cells, with <1% non-recombinant blue colonies. Please note that up to 20% of the true recombinant colonies may be blue due to lambda DNA inserts that fail to completely disrupt the *lac*Zα peptide. To compensate for uncertainty in the experimental DNA, we recommend plating 50, 200, and 1000 µl of transformed cells to obtain a suitable number of clones.
2. A 50 µl aliquot of the empty vector control reaction should produce < 25 blue colonies. White colonies in the empty vector control should represent less than 0.1% of the white colonies in the Lambda HincII control.
3. A 50 µl aliquot of transformed cells from the pUC19 reaction (diluted into 90 µl of TB) should yield > 50 colonies, or > 1×10^8 colonies per µg plasmid.

Screening for Recombinants

Transformation by the empty pEZSeq cloning vector will result in a blue colored colony on plates containing XGAL/IPTG; recombinant inserts will result in white colonies. For most applications, no screening for recombinant colonies is required, as the design of the pEZSeq Kits ensure that > 99% of the colonies obtained from a typical transformation contain recombinant plasmid. Because the background of empty vector transformants is very low, colonies can be picked at random for growth and plasmid purification without using IPTG to induce the *lac*Z promoter for the blue/white screen.

DNA Isolation & Sequencing

Grow transformants in TB medium plus 100 µg/ml ampicillin or 30 µg/ml kanamycin. Use standard methods to isolate plasmid DNA. The pEZSeq plasmid contains the high copy number pUC origin of replication and produces DNA yields similar to that of pUC-based plasmids. *E. cloni* 10G and10GF' Competent Cells are *recA endA* deficient to provide high quality plasmid DNA. pEZSeq Z-For and Z-Rev Sequencing Primers are provided with the Kit. Their sequence and their orientation are shown in Appendix D.

References

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

2. Thorstenson YR, Hunicke-Smith SP, Oefner PJ, Davis RW. 1998. An automated hydrodynamic process for controlled, unbiased DNA shearing. Genome Res 8: 848-55.

Appendix A: Media Recipes

YT + (amp or kan) + XGAL + IPTG Agar Medium for Plating of Transformants
Add the YT Agar powder provided with the kit to 500 ml of deionized water. Autoclave and cool to 55°C. Add the appropriate filter-sterilized antibiotic to the cooled medium (e.g., 15 mg kanamycin for kanamycin-resistant transformants; 50 mg ampicillin or carbenicillin for ampicillin-resistant transformants).

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

YT Agar is per liter: 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar. Add XGAL to a final concentration of 50 μg/ml. For pEZSeq-HCAmp transformants, add ampicillin or carbenicillin to a final concentration of 100 μg/ml. For pEZSeq-HCKan transformants, add kanamycin to a final concentration of 30 μg/ml. If blue/white screening and expression of inserts is desired, add IPTG to 1mM final concentration.

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

TB Culture Medium
Per liter: 11.8 g Bacto-tryptone, 23.6 g yeast extract, 9.4 g dipotassium hydrogen phosphate (anhydrous), 2.2 g potassium dihydrogen phosphate (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 pEZSeq transformation can be further grown in rich medium, such as TB culture medium, containing a final concentration of 100 μg/ml ampicillin or carbenicillin.

Appendix B: pEZSeq Application Guide

The pEZSeq Blunt Cloning Kit is available with either ampicillin- or kanamycin-resistant vector, and with 10G or 10GF' cells. For most cloning applications, we recommend using the pEZSeq-HCKan 10G Blunt Cloning Kit, containing the high copy number, kanamycin-resistant pEZSeq-HCKan vector and *E. cloni* 10G Electrocompetent or Chemically Competent Cells.

The F' plasmid in strain 10GF' encodes the *lac*Iq repressor, which strongly inhibits transcription from the lac promoter. Therefore, in the absence of IPTG the blue/white screen will be inactive in 10GF' transformants. Expression of the insert DNA will also be at a low level, increasing the ability to clone several types of DNA sequences that are traditionally difficult to clone. Because the background of empty vector clones is very low, blue/white screening is NOT required. If high expression is desired, the clones can be grown in the presence of IPTG, which also allows the blue/white screen to be active.

The strain 10G lacks the F' plasmid, so the blue/white screen will be active regardless of whether IPTG is added. Likewise, inserts in pEZSeq will be transcribed, which may cause instability of the insert. A further complication of relying on the blue/white screen is that clones with small inserts or active promoters may appear blue or light blue.

For cloning any type of insert without the uncertainties of blue/white screening, we strongly recommend use of Lucigen's pSMART® plasmids (CloneSmart® Kits).

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

Insert DNA Preparation

1. Generate target DNA fragments by shearing, restriction digestion, or PCR.
2. If necessary, repair the DNA ends to make them blunt, with 5' phosphate groups.
3. Heat denature the repair reaction 10 minutes at 70°C.
4. Purify DNA by extraction, chromatography, or gel electrophoresis. **Do NOT use 256, 302, or 312 nm UV light to visualize the DNA.**

Ligation

1. Briefly centrifuge and gently mix the pEZSeq 4X Vector Premix.
2. Combine the following components in a 1.5-ml tube. Add ligase last.

x µl Insert DNA (300-500 ng, 1 – 4 kb, blunt-ended, 5'-phosphorylated)
y µl H_2O
2.5 µl 4X pEZSeq 4X Vector Premix (pEZSeq, ligation buffer, ATP)
1.0 µl CloneSmart® DNA Ligase (2 U/µl)
10.0 µl total reaction volume

3. Incubate 30 minutes at room temperature. (Incubate 2 hours for maximum number of clones.)
4. Heat denature the ligation reaction 15 minutes at 70°C.
5. Cool 15 seconds at room temperature and 15 seconds on ice.
6. Spin 1 minute at 12,000 rpm.

Transformation

***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.

Colony Growth

Pick colonies at random and grow in TB+amp or TB+kan medium.

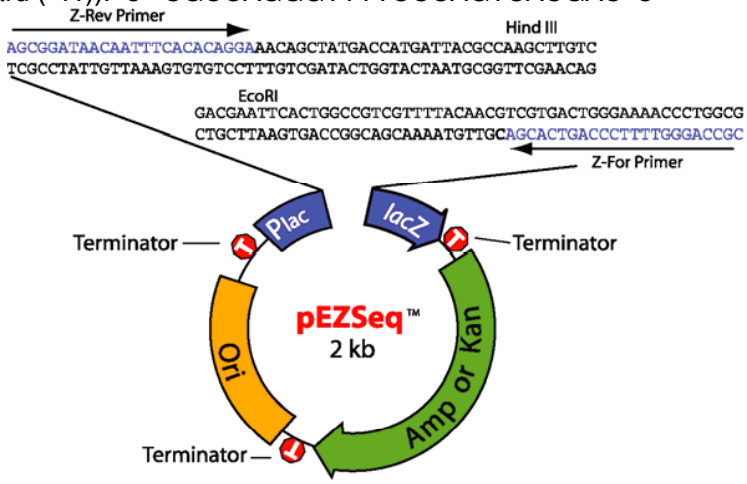
Appendix D: Vector Map and Sequencing Primers

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

The GenBank accession number of pEZSeq-Kan is AF532108 and of pEZSeq-Amp is AF532109.

The sequences of the Z-Rev and Z-For primers are the same as the M13 Reverse and Forward primers of pUC19:

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



Z-Rev Primer — **lacZα** → **Hind III*** **Insert DNA**

AGCGGATAACAATTTCACACAGGAAACAGCT **ATG** ACC ATG ATT ACG CCA AGC TTG TC.
TCGCCTATTGTTAAAGTGTGTCCTTTGTCGA TAC TGG TAC TAA TGC GGT TCG AAC AG.
Met Thr Met Ile Thr Pro Ser Leu Ser...

Insert DNA **EcoRI**

...GACGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCG
...CTGCTTAAGTGACCGGCAGCAAAATGTTG**CAGCACTGACCCTTTTGGGACCGC**

Z-For Primer

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

Appendix E: Troubleshooting Guide

Problem	Probable Cause	Solution
Very few or no transformants	Inefficient end repair.	Check the insert DNA for self-ligation by gel electrophoresis. Repeat end repair using Lucigen's DNATerminator.
	Contaminating enzymes in ligation reaction.	Heat-denature end repair reaction or restriction digest 10 minutes at 70°C. Purify DNA after end repair or restriction digestion reaction.
	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. Be sure insert DNA is phosphorylated. Repeat end repair with DNATerminator. Use the supplied control insert to test ligation reaction.
	Inadequate heat denaturation after ligation reaction.	DO 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.	DO use the provided Recovery Medium for optimal results. DO NOT use SOC or other recovery media.
	Improper electroporation conditions.	Use pre-chilled electroporation cuvettes. Use cuvettes with a gap of 0.1 cm (BTX or BioRad brand). Add the 1 µl of DNA to 25 µl of pre-aliquotted cells on wet ice; DO NOT add the cells to the DNA.
	Incorrect amounts of antibiotic in agar plates. Wrong antibiotic used.	Add the correct amount of Ampicillin or Kanamycin to molten agar at 55°C before pouring plates. DO NOT spread antibiotic onto the surface of agar plates.
High background of blue colonies or of transformants that do not contain inserts.	Contaminating enzymes in ligation reaction.	Purify DNA after DNA end repair reaction. DO NOT add T4 polynucleotide kinase to the ligation reaction.
	Inserts are small or contain active promoters.	Analyze blue colonies by PCR or restriction digestion to confirm the presence of inserts.
	Inserts are unstable.	Use 10GF' cells and plate without IPTG (blue/white screen will NOT be active). Clone into the pSMART-LCKan vector.
	Incorrect amount of antibiotic in agar plates.	Add the correct amount of Ampicillin or kanamycin to molten agar at 55°C before pouring plates. DO NOT spread antibiotic onto the surface of agar plates.