

pEZSeq™

BLUE/WHITE CLONING KIT

IMPORTANT!

**-86°C and -20°C Storage Required
Immediately Upon Receipt**

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The Molecular Cloning Company™

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pEZSeq™ Blunt Cloning Kit

Technical Support

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pEZSeq™ Blunt Cloning Kit

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pEZSeq Blunt Cloning Kit Designations

Several versions of the pEZSeq Blunt Cloning Kit are available. The kits differ in number of reactions, version of pEZSeq® 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 vector and cell combinations

Vector	Reactions	10G Elite Electrocompetent Cells	10G Supreme Electrocompetent Cells	10GF' Elite Electrocompetent Cells	No Cells
pEZSeq Amp (High Copy)	10	40475-1	40486-1	40494-1	---
	20	40475-2	40486-2	40494-2	40464-2
	40	---	---	---	40464-4
pEZSeq Kan (High Copy)	10	40501-1	40512-1	40524-1	---
	20	40501-2	40512-2	40524-2	40500-2
	40	---	---	---	40500-4

Components & Storage Conditions

The Ligation Components of the pEZSeq Kits are shipped in Container 1, which should be stored at **-20°C**. If *E. coli*® Cells are ordered with the Kit, they are shipped in Container 2, which must be stored at **-86°C**. Additional pEZSeq Ligation Components and *E. coli* Competent Cells may be purchased separately.

Container 1: pEZSeq Ligation Components

Store at -20°C

	10 Reactions	20 Reactions	40 Reactions
4X pEZSeq Vector Premix Includes Buffer, ATP, and either pEZSeq-HC Amp or pEZSeq-LC Amp	25 µl	50 µl	2 x 50 µl
CloneSmart DNA Ligase (2 U/µl)	10 µl	1 x 20 µl	1 x 40 µl
Positive Control Insert DNA (500 ng/µl lambda <i>HincII</i>)	5 µl	5 µl	5 µl
Sequencing Primers (200 reactions each) Z-For Primer (3.2 pmol/µl)	200 µl	200 µl	200 µl
Z-Rev Primer (3.2 pmol/µl)	200 µl	200 µl	200 µl

Container 2: *E. coli*® Competent Cells

Store at -86°C

	Catalog #	Reactions
<i>E. coli</i> 10G Elite Electrocompetent Cells or	60052-1	12 (6 x 50 µl)
	60052-2	24 (12 x 50 µl)
<i>E. coli</i> 10G Supreme Electrocompetent Cells or	60080-1	12 (6 x 50 µl)
	60080-2	24 (12 x 50 µl)
<i>E. coli</i> 10GF' Elite Electrocompetent Cells	60061-1	12 (6 x 50 µl)
	60061-2	24 (12 x 50 µl)
Control pUC19 DNA (1ng/µl) store at -20°C or -86°C	----	(1 x 5 µl)
Recovery Medium store at -20°C or -86°C	----	12 (1 x 12 ml) 24 (1 x 24 ml)

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pEZSeq Blunt Cloning Kit Description

The pEZSeq Blunt Cloning Kits are designed to provide blue/white screening and protein expression capability in a vector that has high cloning efficiency and minimal cloning bias. Up to a million recombinant clones may be obtained routinely from less than 500 ng of insert DNA, with no vector preparation required. 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, and DNA controls. The Kit may also be ordered with *E. coli* Electrocompetent Cells.

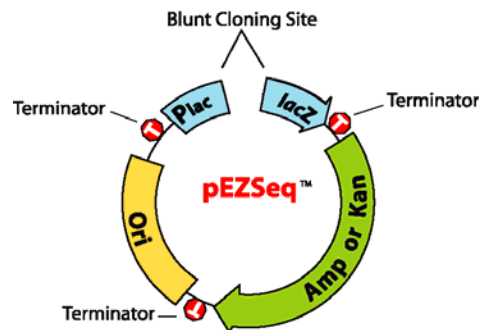


Figure 1. Schematic diagram of the CloneSmart® pEZSeq™ vector. Ori, origin of replication; Amp or Kan, ampicillin or kanamycin resistance gene. Positions of transcriptional terminators are indicated.

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 lacZ α 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. coli*® Electrocompetent 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 ug 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

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containing XGAL and either ampicillin or kanamycin. When using *E. cloni* 10GF' cells (or other cells containing the *lacIq* gene), IPTG must be added to induce expression of the blue/white screen.

***E. cloni*® Electrocompetent Cells**

E. cloni 10G and 10GF' Electrocompetent 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 *endA1* 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.

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/ μ g supercoiled pUC19 DNA. The pEZSeq Kits are also available with *E. cloni*® 10G Supreme Electrocompetent Cells which produce $\geq 4 \times 10^{10}$ cfu/ μ g.

Genotypes

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

F' *mcrA* Δ (*mrr-hsdRMS-mcrBC*) *endA1 recA1* ϕ 80d*lacZ* Δ M15 Δ *lacX74 araD139* Δ (*ara,leu*)7697 *galU galK rpsL nupGλ tonA*

***E. cloni* 10GF' Genotype:**

[F' *proA+B+ lacI^qZ* Δ M15::Tn10 (TetR)] / *mcrA* Δ (*mrr-hsdRMS-mcrBC*) *endA1 recA1* ϕ 80d*lacZ* Δ M15 Δ *lacX74 araD139* Δ (*ara,leu*)7697 *galU galK rpsL nupGλ tonA*

As a control for transformation, *E. cloni* Electrocompetent Cells are provided with supercoiled pUC19 DNA at a concentration of 1ng/ μ l.

End Repair of Sheared DNA

Because of their low backgrounds and pre-cut, 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 and 40035-2) and PCR Terminator® End Repair Kit (Cat. # 40037-1 and 40037-2) 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 PCR Terminator End Repair Kit is recommended for generating libraries from PCR products generated by non-proofreading DNA polymerases (e.g., Taq, Tfl, Tth).

The DNA needs to be relatively free of RNA before end repairing. Large 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 RNase I, which is an exonuclease that breaks RNA down into nucleosides, to remove

pEZSeq™ Blunt Cloning Kit

most of 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 (Genomic Solutions, Inc.) 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.

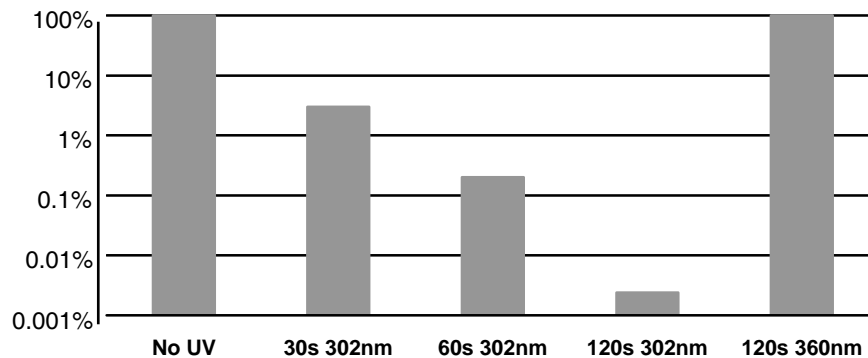


Figure 2. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure (“No UV”) or exposure to 302 nm UV light for 30, 60, or 90 seconds (“30s 302nm, 60s 302nm, 120s 302nm”) or to 360 nm UV light for 120 seconds (“120s 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:

- Microcentrifuge and tubes.

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- Electroporation apparatus and 0.1 cm cuvettes. Successful results are obtained with cuvettes from Eppendorf (Model 940001005), BTX (Model 610), or BioRad (Cat. #165-2089). Users have reported difficulties using *E. coli* cells with Invitrogen cuvettes (Cat. # 65-0030).
- Sterile 17 x 100 mm culture tubes.
- Terrific Broth.
- TY agar plates containing ampicillin and XGAL/IPTG (see Appendix for 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., *EcoRV*, *HincII*, etc.) 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 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. Briefly centrifuge the pEZSeq Vector Premix before use. Mix by gently pipeting up and down.

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

- x μ l Insert DNA (300-500 ng blunt-ended, 5'-phosphorylated)
- y μ l H₂O
- 2.5 μ l 4X pEZSeq Vector Premix (pEZSeq, ATP, buffer)
- 1.0 μ l CloneSmart DNA Ligase (2 U/ μ l)
- 10.0 μ l total reaction volume

3. 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 μ l (500 ng) of λ /HindII 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, DH5 α , etc.) can be effectively transformed with pEZSeq ligation reactions. 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/ μ g of pUC19 to maximize the number of transformants.

Electroporation of *E. cloni* Electrocompetent Cells

E. cloni 10G Elite and Supreme Electrocompetent Cells are provided in 50- μ l aliquots (DUOs), sufficient for two transformation reactions of 25 μ l each. *E. cloni* 10G Elites are also available in 150- μ l aliquots (SixPacks), sufficient for six transformation reactions of 25 μ l 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	1.0 mm cuvette
10 μ F	25 μ F
600 Ohms	200 Ohms
1800 Volts	1400 – 1600 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 Electroporator 2510.

Optional transformation control reactions include electroporation with 1 μ l (10 pg) of supercoiled pUC19 DNA (1 μ l of a 1:100 dilution of the 1 ng/ μ l stock provided).

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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 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

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*® cells from the -86°C freezer and place on wet ice until they thaw **completely** (10-20 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. As a positive control for transformation, dilute the supplied pUC19 by 1:100 to a final concentration of 10 pg/µl using sterile water or TE. Use 1 µl of the diluted control for transformation.
7. 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.
8. 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.
9. Place the tube in a shaking incubator at 250 rpm for 1 hour at 37°C.
10. 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. For the pUC19 positive control transformation, dilute 10 µl of the transformed cells into 990 µl Recovery Medium and spread 100 µl on YT agar plate.

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

11. Incubate the plates overnight at 37°C.
12. Transformed clones can be further grown in TB or in any other rich culture medium.

EXPECTED RESULTS

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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. coli* 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 μ l 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 μ l of cells plated, then the 25 colonies obtained from 50 μ l of the No-Insert Control ligation will represent a background of 10%.

Reaction	CFU/Plate	Efficiency
pEZSeq plus 500 ng Lambda <i>HincII</i> Insert	> 200	> 99.9% inserts
No-Insert Control (Vector Background)	< 25	< 0.1% background
Supercoiled pUC19 Control (10 μ g)	> 200	> 1×10^{10} cfu/ug plasmid

1. A pEZSeq ligation reaction, containing 500 ng of positive control lambda *HincII* DNA, is expected to yield > 200 white colonies from a 5 μ l 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 μ l of transformed cells to obtain a suitable number of clones.
2. A 50 μ l 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 100 μ l aliquot of transformed cells from the pUC19 reaction (10 μ l diluted into 990 μ l of Recovery Medium) should yield > 200 colonies, or > 1×10^{10} colonies per μ g 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. However, 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 *lacZ* promoter for the blue/white screen.

DNA Isolation & Sequencing

Grow transformants in TB medium plus 100 μ g/ml ampicillin or 30 μ g/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. coli* 10G and 10GF' Electrocompetent 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, Hunnicke-Smith SP, Oefner PJ, Davis RW. 1998. An automated hydrodynamic process for controlled, unbiased DNA shearing. *Genome Res* 8: 848-55.

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Appendix A: Media Recipes

YT + (amp or kan) + XGAL + IPTG Agar Medium for Plating of Transformants

Per liter: 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar. Mix components, autoclave and cool to 55°C. 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. Pour into petri plates.

TB Culture Medium

Per liter: 11.8 g Bacto-tryptone, 23.6 g yeast extract, 9.4 g dipotassium hydrogen phosphate (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. coli* 10G electrocompetent cells.

The F' plasmid in strain 10GF' encodes the *lacIq* 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.

pEZSeq™ Blunt Cloning Kit

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 ₂ O
2.5	µl	4X pEZSeq 4X Vector Premix (pEZSeq, ligation buffer, ATP)
1.0	µl	CloneSmart DNA Ligase (2 U/µl)
<hr/>		
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

1. Have Recovery Medium and 17 mm x 100 mm sterile culture tubes readily available at room temperature (one tube for each transformation reaction).
 2. Chill electroporation cuvettes (0.1 cm gap) and microcentrifuge tubes on ice (one cuvette and one microcentrifuge tube per transformation).
 3. Thaw *E. coli* Electrocompetent Cells on wet ice. Pipet 25 µl of cells into a pre-chilled microfuge tube on ice.
 4. Add 1 µl of heat-treated ligation reaction to an aliquot of chilled cells on ice.
 5. Carefully pipet 25 µl of the cell/DNA mixture to a chilled electroporation cuvette so as not to introduce bubbles.
 6. Electroporate and immediately add 975 µl of room temperature Recovery Medium to the cells. Transfer the cells and Recovery Medium to a culture tube. Shake at 250 rpm for 1 hour at 37°C.
 7. Spread up to 100 µl per plate on selective plates containing XGAL (plus IPTG if required). Incubate overnight at 37°C.
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Colony Growth

1. Pick colonies at random and grow in TB medium containing the appropriate antibiotic.
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EZSeq™ Blunt Cloning Kit

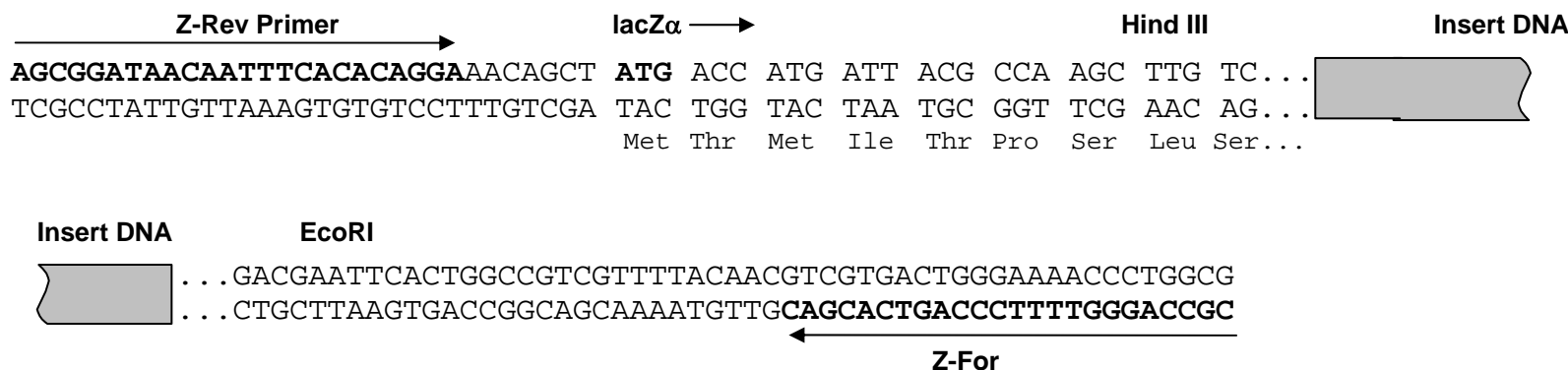
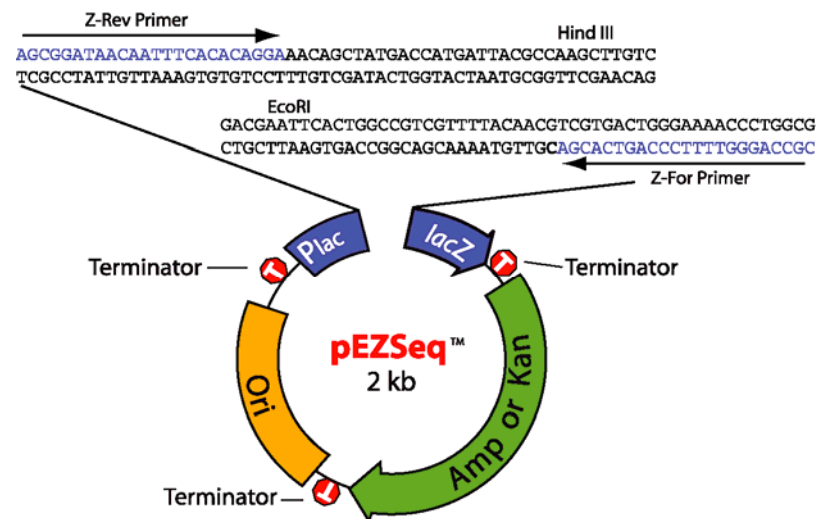
Appendix D: Vector Map and Sequencing Primers

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): 5'–AGCGGATAACAATTTACACACAGGA–3'

Z-For (M13 Forward): 5'–CGCCAGGGTTTTCCAGTCACGAC–3'



pEZSeq™ Blunt Cloning Kit

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 TB (Terrific Broth) 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 DNA 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.