

# BIGEASY™ LINEAR CLONING KITS

Including  
DNATerminator® End Repair Kit

## **IMPORTANT!**

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

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# BigEasy™ Linear Cloning Kits

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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, especially the DNA targets to be cloned, are of the highest quality. Please follow the manual carefully and contact our technical service representatives if additional information is necessary. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

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# BigEasy™ Linear Cloning Kits

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# BigEasy™ Linear Cloning Kits

## BigEasy™ Linear Kit Designations

Several versions of the BigEasy Linear Cloning Kit are available. The kits differ in number of reactions, restriction digestion of the pJAZZ™-KA 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 Lucigen's cloning kits.

### Catalog numbers of vector and cell combinations

Vector	Reactions	Kit with BigEasy-pTEL Electrocompetent Cells
pJAZZ-KA-Blunt	5	43001-1
	10	43001-2
	20	43001-3
pJAZZ-KA-NotI	5	43012-1
	10	43012-2
	20	43012-3

## Components & Storage Conditions

The Ligation Components of the BigEasy Linear Cloning Kits are shipped in Container 1, which should be stored at **-20°C**. BigEasy-pTEL Competent Cells are shipped in Container 2, which must be stored at **-86°C**. Additional BigEasy-pTEL Competent Cells may be purchased separately.

### Container 1: BigEasy Ligation Components

Store at **-20°C**

	5 Reactions	10 Reactions	20 Reactions
pJAZZ™-KA Vector (50 ng/μl)	5 μl	10 μl	20 μl
CloneSmart™ DNA Ligase (2 U/μl)	10 μl	10 μl	20 μl
CloneDirect™ 10X Ligation Buffer (includes ATP)	100 μl	100 μl	100 μl
Positive Control Insert DNA (Blunt or NotI ends, 80 ng/μl)	5 μl	5 μl	5 μl
DNATerminator End Repair Enzyme	20 μl	20 μl	50 μl
DNATerminator 5X End Repair Buffer	100 μl	100 μl	500 μl
BigEasy Sequencing Primers (200 reactions each)			
NZ-F2 Primer (3.2 pmol/μl)	200 μl	200 μl	200 μl
NZ-R4 Primer (3.2 pmol/μl)	200 μl	200 μl	200 μl

### Container 2: BigEasy-pTEL™ Electrocompetent Cells

Store at **-86°C**

	Catalog #	Reactions
BigEasy-pTEL Electrocompetent Cells	60220-1	6 (6 x 25 μl)
	60220-2	12 (12 x 25 μl)
	60220-3	24 (24 x 25 μl)
Control pUC19 DNA (1 ng/μl)	Store at <b>-20°C</b> or <b>-86°C</b> .	10 (1 x 10 μl)
Induction Solution (1000 X)	Store at <b>-20°C</b> or <b>-86°C</b> .	(1 x 1 ml)
Recovery Medium	Store at <b>-20°C</b> or <b>-86°C</b> .	12 (1 x 12 ml)
		24 (1 x 24 ml)

# BigEasy™ Linear Cloning Kits

## BigEasy™ Blunt Cloning Kit Description

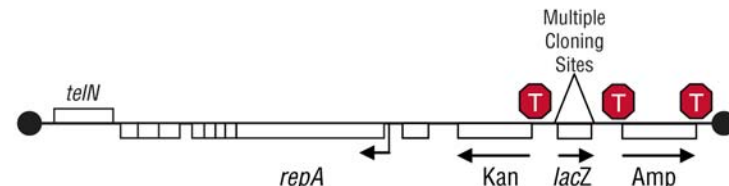
Lucigen's BigEasy Linear Cloning Kit (Patent Pending) is based on a novel linear cloning plasmid, pJAZZ™-KA (Figure 1) (1-3). Because of its lack of supercoiling, pJAZZ-KA has an unprecedented ability to maintain DNAs that are otherwise unclonable. Conventional circular plasmids are maintained in multiple states of supercoiling by the action of DNA topoisomerase and gyrase. Supercoiling induces torsional stress in the plasmid DNA, which is associated with structural instability of sequences that are AT-rich or contain inverted repeats (4). Because the pJAZZ-KA vector is linear, the ends of the plasmid can rotate freely as the molecule is replicated. Therefore, it is not under torsional stress, and numerous classes of structure-rich sequences are much more stable. In addition, the pJAZZ-KA vector incorporates Lucigen's patented CloneSmart® technology for transcription-free cloning (U.S. Pat. 6,709,861), which further reduces instability or loss of insert DNA. Large fragments or inserts with high AT content are cloned easily with this vector.

The BigEasy Kit is ideal for constructing shotgun libraries with large inserts or for cloning smaller products, particularly when the target DNA is especially difficult to clone in conventional vectors. The BigEasy Cloning Kits are convenient to use, containing pre-cut, dephosphorylated pJAZZ-KA cloning vector; DNATerminator End Repair enzymes and buffer; ligase and ligation buffer containing ATP; sequencing primers; competent cells; and DNA controls.

## pJAZZ™-KA Vector

The pJAZZ-KA vector is supplied pre-digested at NotI or SmaI (blunt) sites and has dephosphorylated ends. The pJAZZ-KA vector contains a pair of nearly identical Multiple Cloning Sites on either side of the *lacZ* reporter gene (Figure 1). During preparation of the vector, both Multiple Cloning Sites are cleaved by restriction digestion, which completely removes the *lacZ* marker gene and its promoter from the left and right vector "arms". The vector fragments are then dephosphorylated, preventing their re-ligation. Insert DNA is ligated between the two arms to re-create a viable linear plasmid.

A) pJAZZ-KA before digestion: 14.6 kb.



B) pJAZZ-KA after digestion: 11.8 + 2.2 kb.

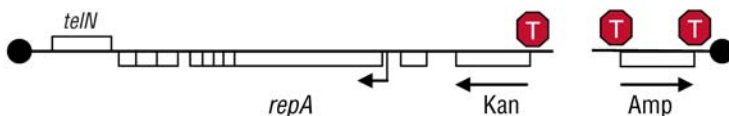


Figure 1. Schematic diagram of the pJAZZ-KA vector before digestion (A) and after digestion (B). During cloning, the *lacZ* fragment is replaced by the experimental insert DNA. *telN*, protelomerase gene; *repA*, replication factor and origin of replication; Kan, Kanamycin resistance gene; Amp, ampicillin resistance gene. Approximate positions of transcriptional terminators are indicated.

Because the *lacZ* promoter region is removed during preparation of the vector, cloned fragments are not subjected to vector-driven transcription. In conventional plasmids, inserts are cloned downstream of a strong promoter, within the coding sequence of *lacZ* or a negative selection gene, such as *ccdB*. Transcription from the promoter causes loss of plasmids containing toxic coding sequences, strong secondary structure, or other deleterious features. In the pJAZZ-KA vector (and all Lucigen pSMART® vectors), transcription across the insert is avoided, so this loss is minimized.

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The pJAZZ-KA vector also employs the CloneSmart transcription-free cloning technology, which eliminates transcription both into and out of the insert DNA. Inserts containing *E. coli*-like promoters are often difficult to clone in conventional plasmids, because transcription from these promoters can interfere with the plasmid's replication or expression of its drug resistance gene. In pJAZZ-KA vectors, strong transcription terminators flank the cloning site to block this transcription, eliminating another source of cloning bias and sequencing gaps.

The left and right arms of the vector encode resistance to kanamycin and ampicillin, respectively (Figure 1). Selection with both antibiotics results in recombinant clones containing an insert flanked by the two arms. After recombinant colonies are obtained, they can be grown in culture with only one drug for selection. We recommend using kanamycin for selection, as ampicillin is rapidly degraded by the beta-lactamase secreted by ampicillin resistant clones.

The pJAZZ-KA vector contains an inducible origin of replication. The copy number is ~4-10/cell prior to induction; it is increased by approximately 5-20 fold by induction in BigEasy-pTEL cells (see below).

## BigEasy pTEL Electrocompetent Cells

Although the pJAZZ-KA linear vector can be propagated in most laboratory strains of *E. coli*, only Lucigen's BigEasy-pTEL strain will provide high transformation efficiency and induction of copy number. The BigEasy-pTEL strain is derived from Lucigen's *E. cloni*® 10G strain. These cells give high yield and high quality plasmid DNA due to the *endA1* and *recA1* mutations. They contain the *mcr* and *mrr* mutations, allowing methylated genomic DNA that has been isolated directly from mammalian or plant cells to be cloned without deletions or rearrangements.

BigEasy-pTEL cells contain an additional plasmid that is compatible with the pJAZZ-KA vector. This plasmid is 5 kb, circular, low-copy, and contains the *colE1* (pUC) origin of replication. Unlike the parental *E. cloni*® 10G strain, BigEasy-pTEL cells are resistant to gentamycin and chloramphenicol.

### BigEasy-pTEL Genotype:

F- *mcrA*  $\Delta$ (*mrr-hsdRMS-mcrBC*)  $\phi$ 80*dlacZ* $\Delta$ M15  $\Delta$ *lacX74* *endA1* *recA1* *araD139*  $\Delta$ (*ara, leu*)7697 *galU galK rpsL nupG*  $\lambda$ - *tonA* *CatR*: *pTEL* (*Gen<sup>R</sup>*)

- BigEasy-pTEL Cells are provided with supercoiled pUC19 DNA at a concentration of 1 ng/μl as a transformation control.
- For highest transformation efficiency, use the provided Recovery Medium to resuspend the cells after electroporation. Use of TB or other media may result in lower transformation efficiencies.

## Shearing and End Repair of DNA

Because of its lack of cloning bias and its ability to maintain large inserts, the pJAZZ-KA vector is ideal for random shotgun cloning of fragments up to 30 kb. 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 (5). For random shearing, Lucigen recommends using the HydroShear™ instrument by Genomic Solutions® (formerly GeneMachines®). 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 a desired size range, increasing the proportion of DNA available for cloning (5). The shearing results are also highly reproducible.

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Importantly, 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's DNATerminator® End Repair Kit (Cat. # 40035-1 and 40035-2) provides an efficient and convenient method for repairing DNA fragments. The DNATerminator End Repair enzymes and buffer are included in the BigEasy Kit to ensure maximal efficiency of blunt cloning.

The insert 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 residual RNA often associated with DNA purification. RNase A, which is a site specific endonuclease, will not degrade the RNA sufficiently and is not recommended. Purified RNase I (DNase-free) is available from Lucigen (Cat. # 30104-1 and 30104-2).

## Purification and Size Fractionation of DNA

DNA must be purified from restriction or repair enzymes before ligation to pJAZZ-KA 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.

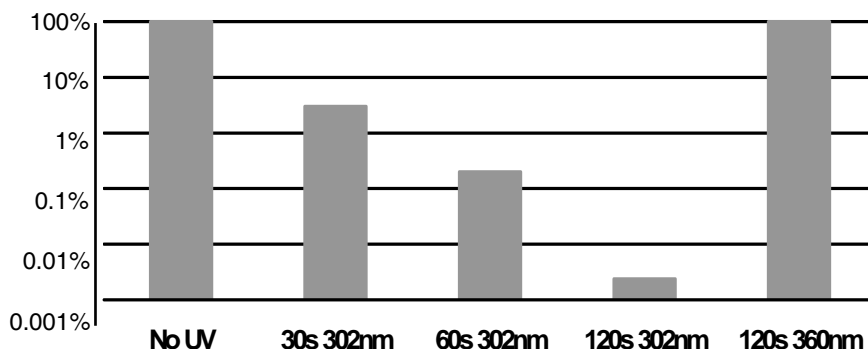


Figure 2. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 90 seconds ("30s 302nm, 60s 302nm, 120s 302nm") or to 360 nm UV light for 120 seconds ("120s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

## Materials and Equipment Needed

The BigEasy Linear Cloning Kits supply most of the items needed to efficiently generate recombinant clones. While simple and convenient, successful use of the BigEasy Kit requires proper planning for each step. Please read the entire manual and prepare the necessary equipment and materials before starting. Following ligation, the following items are required for transformation:

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- Electroporation apparatus and 0.1 cm cuvettes (for electrocompetent cells). Successful results are obtained with cuvettes from Eppendorf (Cat. #4307-000-569), BTX (Model 610), or BioRad (Cat. #165-2089). Users have reported difficulties using Lucigen's electrocompetent cells with Invitrogen cuvettes (Cat. # 65-0030).
- Sterile 17 x 100 mm culture tubes.
- TY agar plates containing ampicillin plus kanamycin (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* or *HincII*) can be used with the BigEasy Linear Cloning Kits following removal of the restriction enzymes and buffer. An end-repair reaction is required for cloning fragments generated by mechanical shearing (e.g. sonication or hydrodynamic shearing), by PCR with non-proofreading 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. The DNATerminator Kit is supplied with the BigEasy Kit for repairing mechanically sheared or restriction-digested DNA (see below).

Blunt fragments are also created by PCR carried out with a proof-reading polymerase, such as Vent™ or Pfu polymerase. 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. PCR products should be purified by gel fractionation before cloning. Using only column purification may lead to clones containing only PCR primers.

For cloning PCR products with 3' single base overhangs, we recommend using Lucigen's PCR Terminator® End Repair Kit to generate blunt phosphorylated ends.

#### DNATerminator End Repair Reaction

The DNATerminator End Repair Kit has been optimized for processing 1 to 10 µg of fragmented DNA (approximately 1 to 5 kb). Buffers used for fragmentation of the DNA must be removed before beginning the DNATerminator reaction. **Ammonium ions strongly interfere with the end repair reaction, so they must be removed prior to the reaction.** The most common sources of ammonium ions are PCR buffer and ammonium acetate used for ethanol precipitation. Fragments should be purified by binding to a silica matrix column or precipitation with ethanol and sodium acetate.

Mix the following components in a microfuge tube:

y µl fragmented DNA in water (1-10 µg)
x µl H <sub>2</sub> O
10 µl 5X DNATerminator® End Repair Buffer
2 µl DNATerminator® End Repair Enzymes
<hr/>
50 µl final volume

Incubate 30 minutes at room temperature.

Stop the reaction by incubation at 70°C for 15 minutes.

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**Do not exceed the recommended enzymatic treatment of the fragment.** Excessive enzyme or incubation time can lead to nucleolytic degradation of the fragments. For less than 1 µg of DNA, the amount of enzyme may be scaled down and the time decreased to 15 minutes. The heat denaturation step may be omitted if the DNA is *immediately* purified using a protein denaturing reagent (e.g., phenol or column binding buffer that contains guanidinium HCl).

## 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 end repair enzymes. Failure to completely remove residual enzymes may result in a large background of empty vector clones or greatly decreased ligation efficiency.

Elute or resuspend the DNA in deionized water for the following ligation steps.

## Ligation to the pJAZZ™-KA Vector

In the BigEasy ligation reaction, the pre-processed pJAZZ-KA vector is ligated with phosphorylated insert fragments in a total volume of 10 µl. For library construction, we recommend using 200-500 ng of insert DNA in the size range of 1-40 kb. For cloning a single DNA species, 100 ng of insert is usually sufficient. Successful cloning can be achieved routinely with less than 100 ng of insert, but use of low amounts of insert will result in significantly fewer transformants. The ligation is performed as follows:

1. Before use, briefly centrifuge the tube containing the pJAZZ-KA vector. Mix by gently pipeting up and down several times. Likewise, centrifuge and mix the CloneDirect™ Buffer.

2. Combine the following components in a 1.5-ml tube, adding the CloneSmart® DNA Ligase last:

x µl Insert DNA (100-500 ng, 5'-phosphorylated, blunt or NotI ends)

y µl H<sub>2</sub>O

1.0 µl pJAZZ-KA Vector (50 ng)

1.0 µl 10X CloneDirect Ligation buffer (contains ATP)

1.0 µl CloneSmart DNA Ligase (2 U/µl)

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10.0 µl total reaction volume

3. Mix by gently pipeting the reaction mixture up and down. Incubate at room temperature (21-25°C) for 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 (80 ng) of the supplied <i>control</i> DNA.
Vector Background	To determine the background of empty vector, omit Insert DNA in the above reaction.

## Preparation for Transformation

**1. Essential: 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.

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3. Spin 1 minute at 12,000 rpm to collect condensation and pellet precipitated material.
4. The sample is ready for transformation; precipitating the DNA is not necessary.

## Transformation

Lucigen's BigEasy-pTEL Electrocompetent Cells must be used for high efficiency transformation with pJAZZ-KA ligation reactions. These cells yield  $\geq 4 \times 10^{10}$  cfu/ug of pUC19.

## Electroporation of BigEasy-pTEL Electrocompetent Cells

BigEasy-pTEL Electrocompetent Cells are provided in 25  $\mu$ l aliquots (SOLOs), sufficient for one transformation reaction 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 $\mu$ F 600 Ohms 1800 Volts	1.0 mm cuvette 25 $\mu$ F 200 Ohms 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 Model 2510.

Optional transformation control reactions include electroporation with 1  $\mu$ l of a 1:100 dilution of the supplied supercoiled pUC19 DNA (10 pg/  $\mu$ l final concentration).

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

- **ESSENTIAL: After ligation, the reaction must be heat denatured at 70°C for 15 minutes!**
- Successful results are obtained with cuvettes from Eppendorf (Cat. #4307-000-569), BTX (Model 610), or BioRad (Cat. #165-2089). Users have reported difficulties using Lucigen's electrocompetent cells with Invitrogen cuvettes (Cat. # 65-0030).
- The cells must be completely thawed **on ice** before use. Microcentrifuge tubes and electroporation cuvettes must be thoroughly pre-chilled 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 BigEasy-pTEL cells from the -86°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  $\mu$ l of BigEasy-pTEL cells to the chilled microcentrifuge tube on ice.

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5. Add 1  $\mu$ l of the heat-denatured BigEasy Ligation reaction to the 25  $\mu$ l of cells on ice. (Failure to heat-inactivate the ligation reaction will prevent transformation.) Stir briefly with pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells. Use of more than 2  $\mu$ l of ligation mix may cause electrical arcing during electroporation.
6. Carefully pipet 25  $\mu$ l of the cell/DNA mixture into a chilled electroporation cuvette without introducing bubbles. Quickly flick the cuvette downward with your wrist to deposit the cells across the bottom of the well. Electroporate according to the conditions recommended above.
7. Within 10 seconds of the pulse, add 975  $\mu$ l of Recovery Medium to the cuvette and pipet up and down three times to resuspend the cells. Transfer the cells and Recovery Medium to a culture tube.
8. Place the tube in a shaking incubator at 250 rpm for 1 hour at 37°C.
9. Spread up to 100  $\mu$ l of transformed cells on YT agar plates containing ampicillin plus kanamycin.
10. Incubate the plates overnight at 37°C.
11. Transformed clones can be further grown in TB or in any other rich culture medium. Selection requires only one antibiotic; we recommend using kanamycin, because ampicillin is rapidly degraded by resistant cultures.

Table 1. Plating Transformed Cells

Reaction Plate		$\mu$ l/Plate
Experimental Insert	(500 ng per ligation)	20, & 100
Control Insert	(Positive Control, 80 ng/ $\mu$ l)	50
No-Insert Control	(Vector Background)	50
Supercoiled pUC19 Transformation Control Plasmid (1 $\mu$ l diluted to 10 pg/ $\mu$ l, Amp <sup>R</sup> )		2

## Expected Results

The results presented below are expected when cloning 80 ng of intact, purified DNA fragments, with Blunt or NotI ends and 5' phosphate groups, into Lucigen's BigEasy-pTEL Electrocompetent Cells (transformation efficiency  $\geq 4 \times 10^{10}$  cfu/ $\mu$ g pUC19 DNA). The background number of empty pJAZZ-KA vectors is constant ( $< 25$  colonies per 50  $\mu$ l of cells plated), unless kinase is introduced as a contaminant. Note that two types of background colonies are possible: 1) Blue colonies are produced from the trace amounts of undigested vector supplied in the pJAZZ-KA preparation; 2) White colonies with no inserts may arise from ligation of the vector arms. The total number of recombinant clones is typically 100-fold greater than the background of white colonies from self-ligated pJAZZ-KA vector.

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 in these cases. For example, if the Experimental Insert ligation reaction produces only 250 colonies from 50  $\mu$ l of cells plated, then the 25 colonies obtained from 50  $\mu$ l of the No-Insert Control ligation will represent a background of 10%.

Table 2. Expected Transformation Results from Electroporation

Reaction	CFU/Ligation	Efficiency
pJAZZ-KA Blunt plus Blunt Control Insert	$> 100,000$	$> 95\%$ inserts
pJAZZ-KA NotI vector plus NotI Control Insert	$> 500,000$	$> 95\%$ inserts
No-Insert Control (Vector Background)	$< 5,000$	$< 5\%$ background
pUC19 Supercoiled Plasmid Control (10 pg)	NA	$\geq 4 \times 10^{10}$ cfu/ $\mu$ g plasmid

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1. Results with experimental DNA may vary significantly, particularly with larger insert sizes, skewed base composition, encoded peptides, etc.
2. A 50 µl aliquot of the empty vector control reaction should produce < 25 colonies, representing less than 5% background.
3. A 2 µl aliquot of transformed cells from the supercoiled pUC19 reaction (diluted into 90 µl of TB) should yield > 800 colonies, or > 4 x 10<sup>10</sup> colonies per µg plasmid.

## Screening

The BigEasy system typically delivers >95% recombinant clones. The pJAZZ-KA vector is thoroughly digested and dephosphorylated; nonetheless, a low level of background is sometimes detectable. There are two classes of background clones: 1) Transformants from uncut, empty vector retain the lacZ stuffer fragment and therefore form blue colonies, 2) Clones from self-ligated vector arms, without the lacZ stuffer, form white colonies.

Insert DNAs that are large or have unusual base composition may produce very few colonies, in which case screening by insert size may be necessary to detect the recombinant plasmids. Digestion with NotI will release the insert DNA from the vector arms (See Figure 1B). The NotI fragment from the left arm is 11.8 kb and from the right arm is 2.2 kb.

## DNA Isolation & Sequencing

Grow transformants in TB medium plus 30 µg/ml kanamycin. The BigEasy-pTEL Electrocompetent Cells are *recA endA* deficient and will provide high quality plasmid DNA. Standard alkaline lysis methods of plasmid preparation are effective for isolation of linear pJAZZ-KA clones. For most clones, Induction Solution can be added to the culture medium before use. Overnight induction will yield approximately 5-20 µg of linear plasmid DNA per 1-ml culture. Without induction, the pJAZZ-KA vector yields 0.5-2 µg per ml of culture. In either case, yields generally decrease with larger inserts.

Approximately 150-400 ng of recombinant plasmid is sufficient for sequencing, with the higher range of template required for larger inserts. Standard protocols for cycle sequencing work well for the pJAZZ-KA vector. The BigEasy Kit is provided with the sequencing primers NZ-F2 and NZ-R4. The sequence of the primers and their orientation relative to the pJAZZ-KA plasmid is shown in Appendix D.

## References

1. Ravin NV, Ravin VK. (1999) Use of a linear multicopy vector based on the mini-replicon of temperate coliphage N15 for cloning DNA with abnormal secondary structures. *Nucleic Acids Res.* 27:e13.
2. Ravin NV, Ravin VK. (1998) Cloning of large imperfect palindromes in circular and linear vectors. *Genetika.* 34:38-44.
3. Godiska et al. Submitted.
4. Godiska R, Patterson M, Schoenfeld T, Mead DA. (2005) "Beyond pUC: Vectors for Cloning Unstable DNA." *In* DNA Sequencing: Optimizing the Process and Analysis. (J. Kieleczawa, ed.), Jones and Bartlett Publishers, Sudbury, MA.
5. Thorstenson YR, Hunicke-Smith SP, Oefner PJ, Davis RW. 1998. An automated hydrodynamic process for controlled, unbiased DNA shearing. *Genome Res* 8: 848-55.

# BigEasy™ Linear Cloning Kits

## Appendix A: Media Recipes

**YT+amp+low kan+XGAL+IPTG (ALKXI) Agar Medium for Plating of Transformants.** Per liter: Mix 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar; autoclave and cool to 55°C. Prepare ALKXI agar medium by adding ampicillin to a final concentration of 100 mg/l (100 µg/ml); kanamycin to a final concentration of 30 mg/l (30 µg/ml); XGAL to a final concentration of 20 mg/l (20 µg/ml), and IPTG to 1 mM. Pour into petri plates.

**TB Culture Medium.** Per liter: 11.8 g Bacto-tryptone, 23.6 g yeast extract, 9.4 g dipotassium hydrogen phosphate (K<sub>2</sub>HPO<sub>4</sub>; anhydrous), 2.2 g potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>; 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.

**Induction Solution (1000X stock).** Dissolve L-(+)-arabinose in water to 10% w/v to make a 1000X stock. Filter sterilize.

**Growing Transformed Cultures.** Colonies obtained from a pJAZZ-KA transformation can be further grown in TB or LB culture medium, containing the 30 µg/ml kanamycin. Use of ampicillin in addition to kanamycin is not necessary. Add 1/1000<sup>th</sup> volume of Induction Solution to the medium for increased copy number. Transformed cultures can be stored by adding sterile glycerol to 20% (final concentration) and freezing at -70°C. Unused portions of the ligation reactions may be stored indefinitely at -20 °C.

## Appendix B: pSMART Application Guide

Numerous cloning kits are available from Lucigen to accommodate any cloning situation. For routine applications, we recommend using the CloneSmart® HCKan Blunt Cloning Kit, containing the high copy number pSMART®-HCKan vector. The copy number of this vector is similar to that of pUC based vectors, yielding 20-80 µg of plasmid DNA per ml of culture. For cloning toxic genes or more difficult DNA sequences, we recommend using the low copy vector in the CloneSmart® LCKan Blunt Cloning Kit. The plasmid yield is ~0.5-1 µg per ml culture.

For cloning large inserts or very difficult DNAs, Big Easy Linear Cloning Kit or the CopyRight Kit is recommended. Regions containing long stretches of di-, tri-, or tetra-nucleotide repeats may be stable only in the BigEasy Linear vector.

Use of the *E. coli*® 10G or BigEasy strains is essential for cloning inserts that may be methylated, such as genomic DNA isolated directly from plant or mammalian cells, as these strains contain the inactive *mcr* and *mrr* alleles [*mcrA* Δ(*mrr-hsdRMS-mcrBC*)].

Vector		Insert DNA Source			Desired Use	
Vector Name	Copy #	Cosmid, Plasmid, BAC, etc.	Genomic or cDNA	AT-Rich, Large, "Difficult"	Digestion, Subcloning, Sequencing	PCR, etc.
pSMART-HC Kan	High	+	+	+	++	+
pSMART-LC Kan	Low	+	+	++	+	+
pSMART-HC Amp	High	+	+	+	++	+
pSMART-LC Amp	Low	+	+	+	+	+
pSMART VC	Single-Mid	+	+	++	+	+
pJAZZ-KA	Low-Mid	+	+	+++	+	+

# BigEasy™ Linear Cloning Kits

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## 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 to generate blunt ends with 5' phosphate groups.
3. Heat denature the repair reaction 10 minutes at 70°C.
4. Purify DNA by binding to matrix, phenol/chloroform extraction, or gel electrophoresis. Elute in deionized water.

**Do NOT use 256, 302, or 312 nm UV light to visualize the DNA.**

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### Ligation

1. Briefly centrifuge and gently mix the BigEasy Vector.
2. Briefly centrifuge and gently mix the CloneDirect Buffer.
3. Combine the following components in a 1.5-ml tube. Add ligase last.

x	μl	Insert DNA (100-500 ng, 5'-phosphorylated, proper termini)
y	μl	H <sub>2</sub> O
1.0	μl	pJAZZ-KA Vector (50 ng)
1.0	μl	10X CloneDirect Ligation buffer (contains ATP)
1.0	μl	CloneSmart DNA Ligase (2 U/μl)
<hr/>		
10.0	μl	total reaction volume

4. Incubate 2 hours at room temperature.
5. Heat denature the ligation reaction 15 minutes at 70°C.
6. Cool 15 seconds at room temperature and 15 seconds on ice. Spin 1 minute at 12,000 rpm.

The ligation reaction can be used directly for electroporation, without further purification.

### ELECTROPORATION

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1. Have Recovery Medium at room temperature for transformations.
  2. Chill electroporation cuvettes, 1.5 ml microfuge tubes, and sterile culture tubes on ice.
  3. Thaw BigEasy-pTEL Electrocompetent Cells on wet ice. Pipet 25 μl of Electrocompetent cells into a pre-chilled 1.5 ml tube on ice.
  4. Add 1 μl of heat-treated ligation reaction to an aliquot of chilled cells on ice.
  5. Pipet 25 μl of the cell/DNA mixture to a chilled electroporation cuvette.
  6. Electroporate. Immediately add 975 μl of room temperature Recovery Medium. Place in culture tube.
  7. Shake at 250 rpm for 1 hour at 37°C.
  8. Spread up to 100 μl per plate on YT agar plates containing kanamycin plus ampicillin. Incubate overnight at 37°C.
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### Colony Growth

1. Pick white colonies at random and grow in TB medium containing 30 ug/ml kanamycin.

# BigEasy™ Linear Cloning Kits

## Appendix D: Vector Map, Cloning Site, and Sequencing Primers

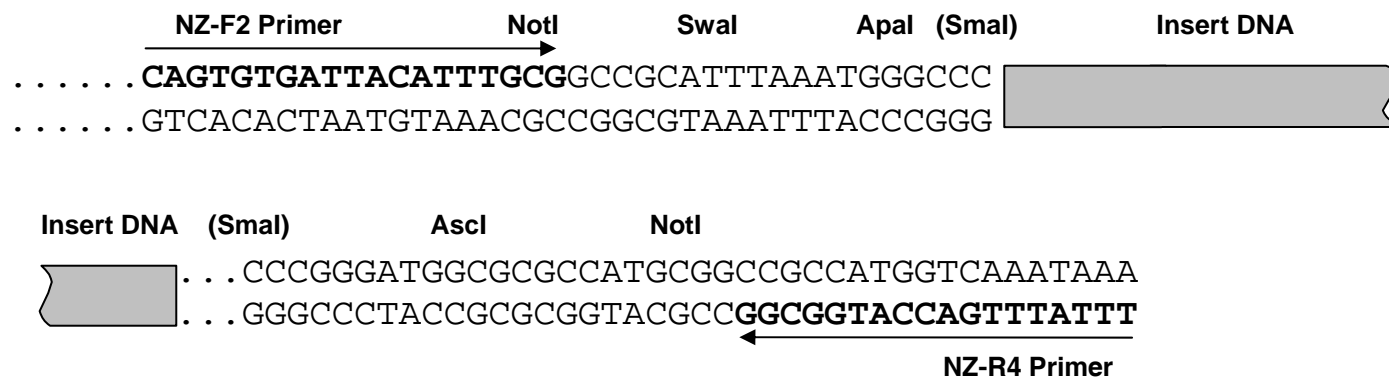
The pJAZZ-KA vector is supplied predigested, at either NotI or SmaI, with dephosphorylated ends.

The sequences of the NZ-F2 and NZ-R4 primers are as follows:

NZ-F2: 5'-CAGTGTGATTACATTTGCG

NZ-R4: 5'-TTTTATTTGACCATGGCGG

The GenBank accession number for the pJAZZ-KA vector is: DQ391279.



# BigEasy™ Linear Cloning Kits

## 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 if necessary.
	Contaminating enzymes in ligation reaction.	Heat-denature end repair reaction or restriction digest 10 minutes at 70°C. Purify DNA by extraction or adsorption to matrix.
	No DNA, degraded DNA, or insufficient amount of DNA.	Check insert DNA by gel electrophoresis. Determine concentration of insert and add the correct amount. Use the supplied control insert to test the system.
	Ligation reaction failed.	Check the insert DNA for self-ligation by gel electrophoresis. Repeat end repair 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 TB (Terrific Broth) for electrocompetent cells. Use SOC for chemically competent cells.
	Improper electroporation conditions.	Use 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.
	Incorrect amounts of antibiotic in agar plates. Wrong antibiotic used.	Add the correct amount of Ampicillin and 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 inserts.	Contaminating enzymes in ligation reaction.	Purify DNA after DNA End Repair reaction. DO NOT add T4 DNA Kinase to the ligation reaction.
	Contaminating oligo-nucleotides in ligation reaction.	Use multiple methods of size selection (e.g., column plus agarose gel). For purification of fragments from agarose gels, run gels without Ethidium Bromide, followed by post-staining.
	Incorrect amount of antibiotic in agar plates.	DO NOT spread antibiotic onto the surface of agar plates. Add the correct amount of Ampicillin and Kanamycin to molten agar at 55°C before pouring plates (see Appendix A).