BIGEASY® V2.0
LINEAR CLONING KIT

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

Lucigen® Corporation
Advanced Products for Molecular Biology

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MA033 v2.2

Technical Support

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BigEasy® v2.0 Linear Cloning Kits

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BigEasy® v2.0 Linear Cloning Kits

Kit Designations

The BigEasy v2.0 Linear Cloning Kits are available with either a blunt or a NotI digestion of the pJAZZ®-OC or the pJAZZ®-OK vector. 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 Kits

Reactions	pJAZZ-OC Blunt	pJAZZ-OC NotI	pJAZZ-OK Blunt	pJAZZ-OK NotI
5	43018-1	43024-1	43036-1	43042-1
10	43018-2	43024-2	43036-2	43042-2
20	43018-3	43024-3	43036-3	43042-3

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 TSA™ Electrocompetent Cells are shipped in Container 2, which must be stored at **-80°C**. The DNATerminator Kit, provided with the Blunt digest of the vector, is shipped in Container 3, and should be stored at **-20°C**. BigEasy TSA Electrocompetent Cells and DNATerminator Kits may be purchased separately.

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

	5 Reactions	10 Reactions	20 Reactions
pJAZZ-OC or -OK Vector (NotI ends, 50 ng/µl) ***or*** pJAZZ-OC or -OK Vector (Blunt ends, 100 ng/µl)	5 µl	10 µl	2 x 10 µl
CloneSmart® DNA Ligase (2 U/µl)	12 µl	12 µl	2 x 12 µl
CloneDirect™ 10X Ligation Buffer (includes ATP)	100 µl	100 µl	2 x 100 µl
Positive Control Insert DNA Includes ***one*** type of insert control: lambda *PmeI, SmaI* (blunt, 500 ng/µl) ***or*** lambda *Psp*OMI, *EagI* (NotI compatible, 500 ng/µl)	5 µl	5 µl	2 x 5 µl
BigEasy Sequencing Primers (200 reactions each) SL1 Primer (3.2 pmol/µl) NZ-RevC Primer (3.2 pmol/µl)	 200 µl 200 µl	 200 µl 200 µl	 2 x 200 µl 2 x 200 µl

Container 2: BigEasy TSA Electrocompetent Cells — Store at -80°C

	Catalog #	Reactions
BigEasy TSA Electrocompetent Cells (SOLOs)	60224-1	6 (6 x 25 µl)
	60224-2	12 (12 x 25 µl)
Store at **-80°C.**	60224-3	24 (24 x 25 µl)
Transformation Control pKanR DNA (1 ng/µl) Store at **-20°C** or **-80°C.**	----	(1 x 5 µl)
Arabinose Induction Solution (1000 X) Store at **-20°C** or **-80°C.**	----	(1 x 1 ml)
Recovery Medium	---- ---- 80026-1	12 (1 x 12 ml) 24 (2 x 12 ml) 96 (8 x 12 ml)
Store at **-20°C** or **-80°C.**		
YT Agar (powder)	----	

Container 3: DNATerminator® End Repair Kit (provided with Blunt kits only) **Store at -20°C**

	5 Reactions	10 Reactions	20 Reactions
DNATerminator End Repair Enzyme	20 µl	20 µl	40 µl
DNATerminator 5X End Repair Buffer	100 µl	100 µl	200 µl

Kit Description

Lucigen's BigEasy v2.0 Linear Cloning Kits (patent pending) provide an unprecedented ability to maintain DNAs that are otherwise unclonable. 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 Kit is based on the novel linear cloning plasmids, pJAZZ® (Figure 1, Refs. 1-3), which are not subject to supercoiling in the cell. 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). The ends of the pJAZZ vectors can rotate freely as the molecule is replicated, so it is not under torsional stress. As a result, numerous classes of structure-rich sequences are much more stable. In addition, the pJAZZ vectors incorporate 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 Cloning Kits are convenient to use, containing pre-cut, dephosphorylated pJAZZ-OC or pJAZZ-OK cloning vector; DNATerminator End Repair enzymes and buffer (with the blunt kit only); ligase and ligation buffer containing ATP; sequencing primers; competent cells; and DNA controls.

Improvements over original BigEasy Kit

The BigEasy v2.0 Kit contains two major changes from the original Big Easy Kit (see Table below). The new pJAZZ-OC vector is resistant only to chloramphenicol, and the new pJAZZ-OK vector is resistant to only kanamycin. The original pJAZZ-KA vector was resistant to kanamycin plus ampicillin.

The new BigEasy TSA™ Electrocompetent Cells have an ampicillin resistance gene integrated into the chromosome and no exogenous plasmids. The original BigEasy pTel cells had an integrated chloramphenicol gene and an exogenous plasmid that encoded gentamycin resistance.

Kit Version	Vector	Cells
BigEasy v2.0	pJAZZ-OC (chloramphenicol^R) pJAZZ-OK (kanamycin^R)	BigEasy TSA: ampicillin^R No Endogenous plasmids
Original BigEasy	pJAZZ-KA kanamycin^R + ampicillin^R	BigEasy pTel: chloramphenicol^R gentamycin^R on endogenous plasmid

pJAZZ®-OC and pJAZZ-OK Vectors

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

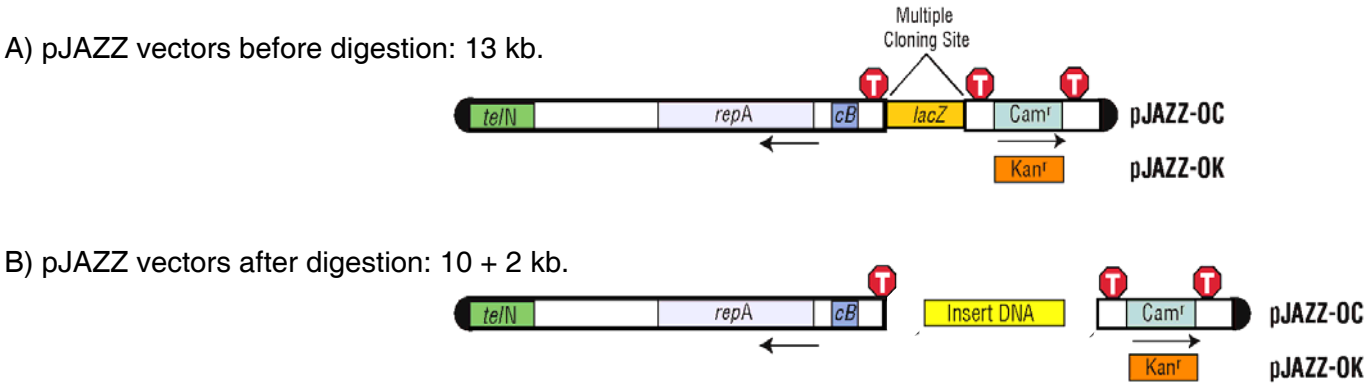


Figure 1. The pJAZZ vectors before digestion (A) and after digestion (B). During cloning, the *lac*Z fragment is replaced by the experimental insert DNA. *Tel*N, protelomerase gene; *rep*A, replication factor gene and origin of replication; *Cam*^{r}, chloramphenicol resistance gene; *Kan*^{r}, kanamycin resistance gene. Approximate positions of transcriptional terminators are indicated. After digestion, the *lac*Z fragment is dephosphorylated to prevent its ligation into the vector, but it is NOT removed from the preparation.

The pJAZZ-OC and -OK vectors also employ the CloneSmart transcription-free cloning technology (US Patent #6,709,861), which eliminates transcription both into and out of the insert DNA. During preparation of the vector, the *lac*Z promoter is excised along with the coding region. Thus, 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 *lac*Z or a negative selection gene, such as *ccd*B. Transcription from the promoter causes loss of plasmids containing toxic coding sequences, strong secondary structure, or other deleterious features. In the pJAZZ vectors (as in most Lucigen vectors), transcription across the insert is avoided, so this loss is minimized.

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 Lucigen's pJAZZ vectors, strong transcription terminators flank the cloning site to block this transcription, eliminating another source of cloning bias and sequencing gaps.

The left arm of the vector contains the origin of replication, and the right arm encodes resistance to chloramphenicol or kanamycin (Figure 1). Selection with the appropriate antibiotic results in recombinant clones containing both vector arms flanking the insert DNA.

The pJAZZ-OC and -OK vectors contain an inducible origin of replication. The copy number is ~2-4/cell prior to induction; it is increased by approximately 5-20 fold by induction in BigEasy TSA cells (see below).

The GenBank Accession number of the pJAZZ-OC vector is EF583812. The Accession number for the pJAZZ-OK vector will be available shortly. The DNA sequences are also provided in Appendices F and G.

BigEasy TSA™ Electrocompetent Cells

The BigEasy TSA strain is derived from Lucigen's *E. cloni*® 10G cells. This strain contains several genes from phage N15, which are essential for high transformation efficiency and induction of copy number of the pJAZZ linear vectors. Transformation with other strains will be 20-200 X less efficient. The N15 genes are *tel*N, which encodes pro-telomerase for efficient replication of the linear plasmid; the *sop*AB genes for stable inheritance; and the *ant*A gene to regulate copy number.

These cells give high yield and high quality plasmid DNA due to the *end*A1 and *rec*A1 mutations. They contain the *mcr* and *mrr* mutations, which allow stable cloning of methylated genomic DNA that has been isolated directly from mammalian or plant cells.

BigEasy TSA cells contain an integrated copy of the *bla* gene; they are therefore resistant to ampicillin.

BigEasy TSA Genotype:

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*dlacZ*ΔM15 Δ*lac*X74 *end*A1 *rec*A1*ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K *rps*L *nup*G λ- *tonA bla (Amp*^{R}*) sopAB telN antA*

- BigEasy TSA Electrocompetent Cells are provided with supercoiled control pKanR plasmid DNA at a concentration of 1 ng/µl. The pKanR control plasmid is kanamycin resistant.
- For highest transformation efficiency, use the provided Recovery Medium to resuspend the cells after electroporation. Use of TB, SOC, or other media may result in lower transformation efficiencies.

Purification and Size Fractionation of DNA

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

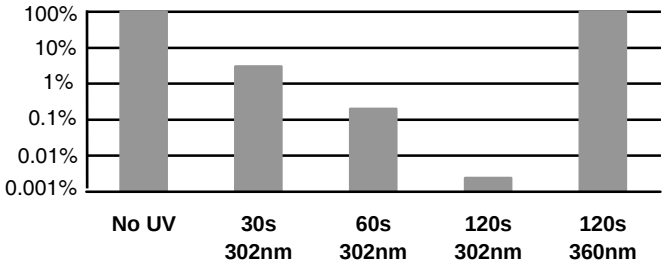


Figure 2. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 90 seconds ("30s 302nm, 60s 302nm,120s 302nm") or to 360 nm UV light for 120 seconds ("120s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

To safely gel-isolate large fragments, we recommend running a duplicate lane of the insert DNA. Stain, photograph, and physically mark the band in the duplicate lane, place it next to an unstained lane containing

the desired sample, and excise the region of the unmarked gel corresponding to the position of the desired band. This method completely avoids exposure of the band to ethidium bromide and UV light.

Materials and Equipment Needed

The BigEasy v2.0 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:

- 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.
- Prepare YT Agar from powder included in the kit.
- YT+CXI agar plates, containing chloramphenicol plus XGAL/IPTG (See Appendix A).
- YT+KXI agar plates, containing kanamycin plus XGAL/IPTG (See Appendix A).

Note: Colony growth may be slow or variable on LB agar plates.

Overview of the BigEasy v2.0 Cloning Process

1) Preparation and Purification of DNA Fragments for Cloning

A) NotI ends

B) Blunt ends

Restriction fragments

Blunt PCR products (from proofreading PCR enzymes)

Tailed PCR products (from non-proofreading PCR enzymes)

Mechanically sheared fragments

C) End-repair of fragments for blunt cloning

2) Ligation of DNA Fragments to pJAZZ® vector

3) Transformation of BigEasy TSA™ Electrocompetent Cells

Detailed Protocol

1) Preparation and Purification of DNA Fragments for Cloning

A) Generation of NotI Restriction Fragments

DNA fragments created by digestion with NotI can be cloned directly into the pJAZZ®-OC/NotI or pJAZZ-OK/NotI vector preparation. For optimum results, the DNA should be purified after digestion by standard methods, e.g., agarose gel electrophoresis or binding to a purification column. After purification, proceed with "Ligation to the pJAZZ Vector" (p.10).

B) Generation of Blunt Fragments

Restriction Fragments

DNA fragments created by digestion with blunt-cutting restriction enzymes (e.g., EcoRV or HincII) should be purified by standard methods, e.g., agarose gel electrophoresis or binding to a purification column. After purification, proceed with 'Ligation to the pJAZZ Vector' (p.10).

Restriction fragments that have 3'- or 5'-overhangs must undergo an end-repair reaction to generate blunt ends prior to ligation. Lucigen's DNATerminator® Kit is supplied with the BigEasy Kit to repair these fragments (see below).

Blunt PCR products

PCR fragments created with proofreading polymerases, such as Vent™, Phusion™, or Pfu polymerase, have blunt ends. However, it is essential that the PCR products also have 5'-phosphate groups.

The simplest method to generate phosphorylated PCR products is to perform the reaction with phosphorylated primers. The primers can be synthesized directly with terminal 5'-phosphate groups, or they can be treated briefly with T4 PNK plus ATP prior to the PCR. The following is a protocol for phosphorylation of primers:

Primer kinase reaction

4 µl	Forward primer @ 100 pmol/ul
4 µl	Reverse primer @ 100 pmol/ul
1 µl	10 X T4 PNK buffer mix (containing ATP, final conc.= 1 mM)
1 µl	T4 PNK
10 µl	Total

Incubate at 37°C for 15 minutes.
Add 2-4 µl of this reaction directly to a 50-100 µl PCR mix and amplify.

Occasionally, the kinase buffer interferes with the PCR reaction. In these cases, we recommend either ordering primers synthesized with 5'-phosphates or kinasing the final PCR product (see below).

Alternately, blunt PCR products that lack 5'-phosphate groups can be treated with T4 PNK or with the DNATerminator Kit (included) to add the phosphates. The PCR products must first be purified to remove the PCR buffer, as ammonium ions in the buffer strongly inhibit the phosphorylation reaction. After phosphorylation, the products must be purified again to remove the kinase activity.

Tailed PCR products

PCR products created with non-proofreading enzymes, such as Taq or Tfl polymerases, have single 3'-single-base overhangs that must be removed before ligation. In addition, they require addition of 5'-phosphate groups. To clone these PCR products, we recommend using Lucigen's PCRTerminator® End Repair Kit (Cat. # 40037-1), which generates blunt, 5'-phosphorylated ends.

NOTE: *All* PCR products should be purified by gel fractionation before cloning to remove PCR primers and spurious PCR products. After gel isolation and purification of the desired fragment, proceed with "Ligation to the pJAZZ® Vector" (p.10).

Sheared DNA

Because the pJAZZ vectors have minimal cloning bias and can maintain large inserts, they are 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 cloning bias (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 amount of DNA available for cloning (5). The shearing results are also highly reproducible.

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. The DNATerminator End Repair Kit is included in the BigEasy Kit to ensure maximal efficiency of blunt cloning (see below).

C) DNATerminator Kit for End Repair of Fragments for Blunt Cloning

Lucigen's DNATerminator® End Repair Kit (Cat. # 40035-1 and 40035-2) provides an efficient and convenient method for end-repairing DNA fragments.

The insert DNA needs to be relatively free of RNA before end repairing. Even moderate amounts of contaminating RNA will severely impair the efficiency of the end repair reaction, resulting in poor cloning results. We recommend the use of RNase I, which breaks RNA down into nucleotides, to remove residual RNA associated with DNA purification. RNase I (DNase-free) is available from Lucigen (Cat. # 30104-1 and 30104-2).

We do NOT recommend use of RNase A, because it is a site specific endonuclease that will not degrade the RNA sufficiently.

DNATerminator End Repair Reaction

The DNATerminator End Repair Kit has been optimized for processing approximately 0.2-15 pmol of DNA fragments (equivalent to ~1-10 μg of DNA fragmented 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 DNA purification column or precipitation with ethanol and sodium acetate.

Mix the following components in a microfuge tube:

y μl	fragmented DNA in water
10 μl	5X DNATerminator End Repair Buffer
2 μl	DNATerminator End Repair Enzymes
x μl	H_2O
50 μl	final volume

Incubate 30 minutes at room temperature. Stop the reaction by incubation at 70ºC for 15 minutes.

IMPORTANT: Do not exceed the recommended enzymatic treatment of the fragment. Excessive enzyme treatment can lead to nucleolytic degradation of the fragments. For less than 0.2 pmol 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 reaction is stopped and the DNA is *immediately* purified by addition of a protein denaturing reagent (e.g., phenol or 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 at a concentration of at least 30 ng/µl in deionized water or column elution buffer (e.g., Qiagen Buffer EB or 10 mM Tris pH8.5).

2) Ligation to the pJAZZ® Vector

In the BigEasy ligation reaction, the pre-processed pJAZZ 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 ligation, run a sample of the insert DNA on an agarose gel to verify the quantity and integrity of the fragment.

2. Briefly centrifuge the tube containing the pJAZZ vector. Mix by gently pipeting up and down several times. Likewise, centrifuge and mix the CloneDirect™ Buffer.

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

	NotI Ligation	Blunt Ligation
Insert DNA (100-500 ng, 5'-phosphorylated, NotI or Blunt ends)	x µl	x µl
pJAZZ-OC or -OK Vector	1.0 µl	1.0 µl *
10X CloneDirect Ligation buffer (contains ATP)	1.0 µl	1.0 µl
CloneSmart DNA Ligase (2 U/µl)	1.0 µl	1.0 µl
H_2O	y µl	y µl
Total reaction volume	10.0 µl	10.0 µl

* For Blunt ligation, up to 2.0 µl of vector may be used. The number of clones will increase proportionately with the amount of vector. The volume of the ligation reaction may be scaled up if necessary.

4. 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 (500 ng) of the supplied *control* DNA.
Vector Background	To determine the background of empty vector, omit Insert DNA in the above reaction.

Preparation for Transformation

1. Prepare YT Agar from powder included with cells. Add appropriate antibiotic as per Appendix A.
2. **Essential: Heat denature the ligation reaction at 70°C for 15 minutes.**
3. Cool to room temperature for 15 seconds followed by 0-4°C for 15 seconds to condense water vapor inside the tube.
4. Spin 1 minute at 12,000 rpm to collect condensation and pellet precipitated material.
5. The soluble sample is ready for transformation; precipitating the DNA is not necessary.

3) Transformation of BigEasy TSA™ Electrocompetent Cells

Lucigen's BigEasy TSA Electrocompetent Cells must be used for high efficiency transformation with pJAZZ® ligation reactions. These cells yield $\geq$ 4 X 10^{10} cfu/μg of supercoiled control plasmid.

Electroporation

BigEasy TSA Electrocompetent Cells are provided in 25-μl aliquots (SOLOs), sufficient for one transformation reaction each. Transformation is carried out in a cuvette with a gap of 0.1 cm. 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 μF	25 μF
600 Ohms	200 Ohms
1800 Volts	1600 – 2000 Volts

Suggested Electroporation Systems:
Bio-Rad Micro Pulser #165-2100; Bio-Rad E. coli Pulser #165-2102; Bio-Rad Gene Pulser II #165-2105; BTX ECM630 Electroporation System; Eppendorf Model 2510.

Optional transformation control reactions include electroporation with 1 μl of a 1:100 dilution of the supplied supercoiled pKanR plasmid DNA (10 pg/μl final concentration).

Transformation Protocol

ESSENTIAL: **Ligation reactions must be heat denatured at 70°C for 15 minutes before transformation!**

- 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. Electroporation cuvettes must be thoroughly pre-chilled on ice before use.

1. Have Recovery Medium and 17 mm x 100 mm sterile culture tubes readily available at room temperature (one tube for each transformation reaction). Transformation efficiency may decrease with the use SOC or other media.
2. Place electroporation cuvettes (0.1 cm gap) on ice.
3. Remove BigEasy TSA cells from the -80°C freezer and thaw **completely** on wet ice (10-15 minutes).

4. Add 1 µl of the heat-treated BigEasy v2.0 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.
5. 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.
6. 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.
7. Place the tube in a shaking incubator at 250 rpm for 1 hour at 37°C.
8. Spread up to 100 µl of transformed cells on YT + CXI agar plates.
9. Incubate the plates overnight at 37°C.
10. Transformed clones can be further grown in TB or in any other rich culture medium with 12.5 µg/ml chloramphenicol. If higher copy number is desired, add Arabinose Induction Solution to the cultures (1 µl per ml of culture; final concentration 0.01% arabinose).

Table 1. Plating Transformed Cells

Reaction Plate	Drug Resistance	µl/Plate
Experimental Insert (500 ng per ligation)	Cam or Kan	20, & 100
Control Insert (Positive Control, 500 ng/µl)	Cam or Kan	50
No-Insert Control (Vector Background)	Cam or Kan	50
pKanR Transformation Control Plasmid (1 µl diluted 1:100 to 10 pg/µl)	Kanamycin	2

Expected Results

The results presented below are expected when cloning 500 ng of intact, purified DNA fragments, with Blunt or NotI ends and 5'-phosphate groups, into Lucigen's BigEasy TSA™ Electrocompetent Cells. The background number of empty pJAZZ® vectors is constant (< 25 colonies per 50 µl of cells plated), unless kinase or repair enzymes are introduced as contaminants. Two types of background colonies are possible: 1) Blue colonies are produced from the trace amounts of undigested vector supplied in the pJAZZ preparation; and 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 vector.

Table 2. Expected Transformation Results from Electroporation

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

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 control reaction (diluted into 90 μl of TB) should yield > 800 colonies, or > 4 x 10^{10} colonies per μg plasmid.

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

See Appendix E for additional troubleshooting advice if necessary.

Screening

The BigEasy system typically delivers >95% recombinant clones. 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 of mini-preps with NotI will release the insert DNA from the vector arms (See Figure 1B). The NotI fragment from the left arm is 10 kb and from the right arm is 2.2 kb.

DNA Isolation & Sequencing

Grow transformants in TB medium plus 12.5 μg/ml chloramphenicol. The BigEasy TSA™ 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® 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 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 vector. The BigEasy Kit is provided with the sequencing primers SL1 and NZ-RevC. The sequence of the primers and their orientation relative to the pJAZZ 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.

Appendix A: Media Recipes

YT-CXI, and YT-KXI 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/500 ml (30 μg/ml final) for KXI or 6.25 mg of chloramphenicol per 500ml (12.5 μg/ml final) for CXI). Add XGAL to a final concentration of 20 mg/L (20 μg/ml), and IPTG to 1 mM. YT Agar is available to purchase separately as 5 packets with catalog number 60025-1.

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 per liter: Mix 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar; autoclave and cool to 55°C.

TB Culture Medium. Per liter: 11.8 g Bacto-tryptone, 23.6 g yeast extract, 9.4 g dipotassium hydrogen phosphate (K_2HPO_4; anhydrous), 2.2 g potassium dihydrogen phosphate (KH_2PO_4; 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.

Arabinose 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® transformation can be further grown in TB or LB culture medium, containing the 12.5 μg/ml chloramphenicol. Add 1/1000 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. For cloning toxic genes or more difficult DNA sequences, we recommend using the low copy vector in the CloneSmart LCKan Blunt Cloning Kit. The pSMARTGC vectors contain similar features, but are optimized for cloning PCR products.

For cloning large inserts or very difficult DNAs, including regions containing long stretches of di-, tri-, or tetra-nucleotide repeats, the BigEasy v2.0 Linear Cloning Kits are recommended. The pSMART BAC vector in the CopyRight Cloning kits is useful for inserts up to 200 kb.

Use of the *E. cloni®* 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 [*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)].

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	+	+	++	+	+
pSMARTGC-HCK	High	+	+	+	++	++
pSMARTGC-LCK	Low	+	+	++	+	++
pSMART BAC	Single-Mid	+	+	++	+	+
pJAZZ-OC or -OK	Low-Mid	+	+	+++	+	+

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.**

Ligation

1. Briefly centrifuge and gently mix the BigEasy pJAZZ 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)
1.0 – 2.0 µl pJAZZ-OC or -OK Vector
1.0 µl 10X CloneDirect Ligation buffer (contains ATP)
1.0 µl CloneSmart® DNA Ligase (2 U/µl)
y µl H_2O
10.0 µl total reaction volume

4. Incubate 2 hours at room temperature.
5. **ESSENTIAL:** 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

1. Have Recovery Medium at room temperature for transformations.
2. Chill electroporation cuvettes on ice.
3. Thaw BigEasy TSA™ Electrocompetent Cells on wet ice.
4. Add 1 µl of heat-treated ligation reaction to the 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 the appropriate antibiotic. For the pKanR control, use YT+kanamycin (30 µg/ml). Incubate overnight at 37°C.

Colony Growth

1. Pick white colonies at random and grow in TB medium containing the appropriate antibiotic plus 1X Arabinose Induction Solution (if desired).

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

The pJAZZ-OC or -OK vector is supplied predigested at either SmaI (blunt) or NotI sites, with dephosphorylated ends.

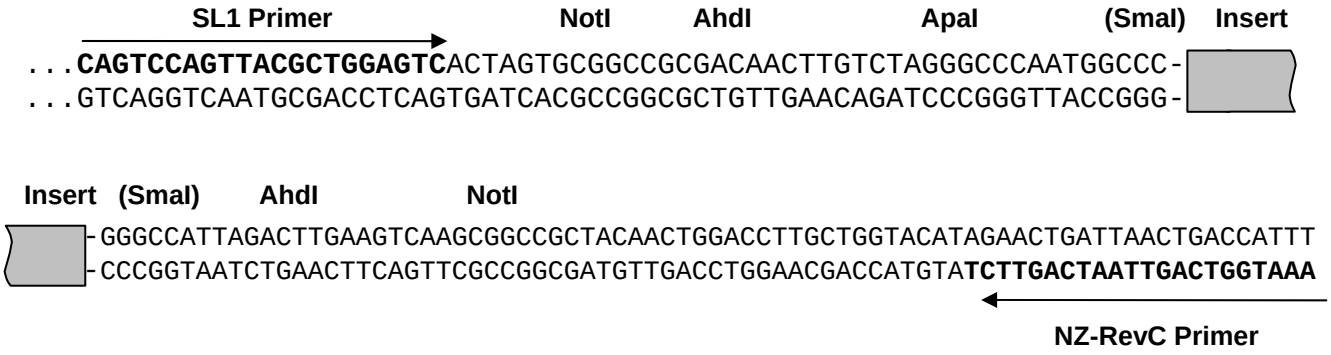
The sequences of the SL1 and NZ-RevC primers are as follows:

SL1: 5'-CAGTCCAGTTACGCTGGAGTC

NZ RevC: 5'- AAATGGTCAGTTAATCAGTTCT

The GenBank accession number for the pJAZZ-OC vector is EF583812.

The GenBank accession number for the pJAZZ-OK vector is not yet available.



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 the Recovery Medium provided in the Kit for electrocompetent and 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.
	Wrong antibiotic used.	Use chloramphenicol for pJAZZ-OC vector; use kanamycin for the pJAZZ-OK vector.
	Incorrect amounts of antibiotic in agar plates.	Add the correct amount of chloramphenicol or 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 Polynucleotide 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 chloramphenicol or kanamycin to molten agar at 55°C before pouring plates (see Appendix A).

Appendix F: Sequence of pJAZZ®-OC vector (12887 bp)

(*lac*Z stuffer fragment is underlined)

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CTAACTGTGGCCAGTCCAGTTACGCTGGAGTCACTAGTGCGGCCGCGACAACTTGTCTAGGGCCCAATGGCCCGGGAGGCCTACTTAAGT
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ACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGACAACAATTTCACACAGGAAACAGCTATGACCA
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GACCATTTAAATCATACCAACATGGTCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTAATCTGATCGGCACGTAAG
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AATTGCCCATTATACGC

Appendix G: Sequence of pJAZZ®-OK vector (13042 bp)

(*lac*Z stuffer fragment is underlined)

GCGTATAATGGACTATTGTGTGCTGATAAGGAGAACATAAGCGCAGAACAATATGTATCTATTCCGGTGTTGTGTTCCTTTGTTATTCTG
CTATTATGTTCTCTTATAGTGTGACGAAAGCAGCATAATTAATCGTCACTTGTTCTTTGATTGTGTTACGATATCCAGAGACTTAGAAAC
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Appendix H: Recommended conditions for High throughput sequencing of pJAZZ clones:

Use the Millipore Montage 96 well kit to prep the DNA.
Use Phenix 384-well FrameStar PCR plates to set up the reactions, do the ethanol ppt, and run the sequencing samples.

For sequencing reactions, use 3ul of template (60ng-180ng/rxn) + 3ul of the following master mix :
Master mix for 100rxns:

16.5 ul Big Dye V3.1
0.78 ul primer @ 320 pmol/ul (2.5 pmol/rxn)
118.0 ul 5X ABI sequencing buffer
164.72 ul DIUF water

300.0 ul total

Cycle : 95ºC for 4 min , then 25 cycles of:

95ºC for 15 sec
55ºC for 5 sec
60ºC for 2 min

Hold at 4C until ready for use.

Cleanup with ethanol precipitation or Sephadex (G-50).

Protocol courtesy of Laboratory for Genomics and Bioinformatics, The University of Oklahoma Health Sciences Center, OKC www.microgen.ouhsc.edu