BIGEASY® Long PCR Cloning Kit

For
Proofreading DNA Polymerases
(pJAZZ®-OK Blunt Vector)
Or
Non-Proofreading DNA Polymerases
(pJAZZ-OK GC Vector)

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

Lucigen® Corporation
Advanced Products for Molecular Biology

MA048 v1.4

BigEasy® Long PCR Cloning Kits

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® Long PCR Cloning Kits

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Kit Designations

The BigEasy Long PCR Cloning Kits are available in two different sizes and with a choice of two different vectors with prepared cloning sites. One vector is blunt (pJAZZ®-OK Blunt vector) for cloning DNA amplified using proofreading DNA polymerases, and one vector is C-tailed (pJAZZ-OK GC vector) for cloning DNA amplified using non-proofreading DNA polymerases. The catalog numbers are listed below. Please refer to Appendix B: Application Guide for more information and recommended uses of Lucigen's cloning kits.

Catalog numbers

Reactions	BigEasy Long PCR Cloning Kit	
	Proofreaders Kit pJAZZ-OK Blunt vector	Non-Proofreaders Kit pJAZZ-OK GC vector
5	43054-1	43066-1
10	43054-2	43066-2
20	43054-3	43066-3

Components & Storage Conditions

The Ligation Components of the BigEasy Long PCR Cloning Kits are shipped in Container 1, which should be stored at **-20°C**. BigEasy-TSA™ Competent Cells are shipped in Container 2, which must be stored at **-80°C**. Additional BigEasy-TSA Competent Cells may be purchased separately.

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

	5 Reactions	10 Reactions	20 Reactions
pJAZZ®-OK Blunt Vector (100 ng/µl) or pJAZZ-OK GC Vector (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
PCR Control Cm^R template plus primers (5 ng/µl template, 25 pmol/µl each primer)	12 µl	12 µl	2 X 12 µl
T4 Polynucleotide Kinase (10 U/µl) 10X Primer Kinase Buffer (containing ATP)	20 µl 100 µl	20 µl 100 µl	2 X 20 µl 2 X 100 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) Store at **-80°C.**	60224-1	6 (6 x 25 µl)
	60224-2	12 (12 x 25 µl)
	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 Store at **-20°C** or **-80°C.**	---- ---- 80026-1	12 (1 x 12 ml) 24 (2 x 12 ml) 96 (8 x 12 ml)

YT Agar (powder)	----	

Kit Description

The BigEasy Long PCR Cloning Kits contain everything needed to efficiently clone PCR products up to 30 kb into an unbiased, high-fidelity cloning vector. There are two different versions of the kits that are compatible with either proofreading or non-proofreading PCR polymerases. They can also be used to clone any blunt or G-tailed DNA up to 30 kb.

The BigEasy Long PCR Kit for Non-Proofreading DNA Polymerases is based on a newly discovered attribute of non-proofreading DNA polymerases: these enzymes can add a single 3'-G residue to the ends of DNA molecules. The 3'-G tailing occurs during PCR with a non-proofreading polymerase. The pJAZZ®-OK GC vector contains a single 3'-C overhang, which is compatible with the 3'-G overhang generated by the non-proofreading polymerase (see Figure 1). The unique combination of a G-tailed insert DNA and C-tailed vector is the basis for GC Cloning (patents pending). The pJAZZ®-OK Blunt vector contains a blunt end which is compatible with DNA amplified using proofreading DNA polymerases.

GC Cloning technology is analogous to TA cloning® (Mead 1991), in which a non-proofreading polymerase, such as Taq, Tfl, Tth, or Tbr DNA polymerase, adds a single 3'-A residue to the insert DNA. A compatible T-tailed vector is used for the complementary cloning step. However, there are several notable differences between the two technologies: (a) the optimal time for the GC ligation reaction is minutes, compared to the hours required for standard TA cloning; GC Cloning technology rivals TOPO® TA cloning (Invitrogen Corp.) in the time required to complete the reaction; (b) the cloning efficiency and accuracy is higher with GC ends than with TA ends; and (c) the transcription-free pJAZZ-OK vectors demonstrate much greater stability for cloning large and otherwise unstable PCR products, as described below.

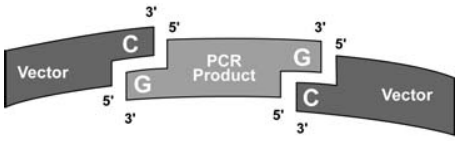


Figure 1. GC cloning schematic.

The pJAZZ vectors incorporate Lucigen's CloneSmart® transcription-free cloning technology (U.S. Pat. 6, 709, 861) to reduce bias and maximize cloning efficiency. The pJAZZ vectors (Figure 2) are supplied pre-digested, with single 3'-C tails or blunt ends and dephosphorylated 5' ends, and are qualified to produce >99% recombinant clones in typical experiments. The very low background of empty vector eliminates the need to screen for recombinants. It also enables PCR cloning and novel library construction methods from nanogram amounts of DNA. Because no screening is required, this technology removes the uncertainty of false negatives (light blue pUC colonies) and false positives (white colonies that lack inserts). In contrast, conventional TA or TOPO TA vectors utilizing the blue/white screen can generate a dense background of blue colonies and many ambiguous "light blue" colonies, both of which may contain inserts but are often discarded. The DNA contained in such clones can be lost and consequently thought to be "unclonable". Further, the pZERO™ vectors typically have an empty vector background of 5% or more. In addition, the ampicillin-resistant pZERO transformants are often surrounded by non-transformed "satellite" colonies, which complicate colony picking and can contaminate cultures. The growth of satellite colonies is completely eliminated with the pJAZZ-OK kanamycin-resistant vectors.

pJAZZ-OK Vectors

The BigEasy Long PCR Cloning Kits (patent pending) are based on a novel linear cloning plasmid, the pJAZZ-OK vector (Figure 2) (1-3). The pJAZZ-OK vector is not supercoiled *in vivo*, giving it unprecedented ability to maintain DNA fragments that are otherwise unclonable. Conventional circular plasmids are maintained in multiple states of supercoiling by 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-OK 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. Large fragments or inserts with high AT content are cloned easily with this vector.

The BigEasy Long PCR Kit is ideal for cloning long PCR amplicons. The BigEasy Cloning Kit is convenient to use, containing pre-cut, dephosphorylated pJAZZ®-OK cloning vector; T4 polynucleotide kinase and buffer; ligase and ligation buffer containing ATP; sequencing primers; competent cells; and DNA controls.

Several versions of the pJAZZ vector are available. BigEasy Long PCR Cloning Kits contain either the pJAZZ-OK GC or pJAZZ-OK Blunt vector. These vectors differ only in the type of end they possess for cloning.

The pJAZZ-OK GC preparation is supplied with 3'-C overhang sites and has dephosphorylated ends for cloning amplicons made with non-proofreading DNA polymerases such as Taq. The pJAZZ-OK Blunt vector preparation is supplied with blunt dephosphorylated ends for cloning amplicons made with proofreading DNA polymerases such as Vent® or enzyme mixes containing a proofreading polymerase. The pJAZZ-OK vectors contain a pair of nearly identical Multiple Cloning Sites on either side of the *lac*Z reporter gene (Figure 2). During preparation of the vectors, 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 vector fragments are then dephosphorylated, preventing their re-ligation. Insert DNA is ligated between the two arms to re-create a viable linear plasmid. The left arm of the vector contains the origin of replication; the right arm encodes resistance to kanamycin (Figure 2). Selection with kanamycin results in recombinant clones containing an insert flanked by the two arms.

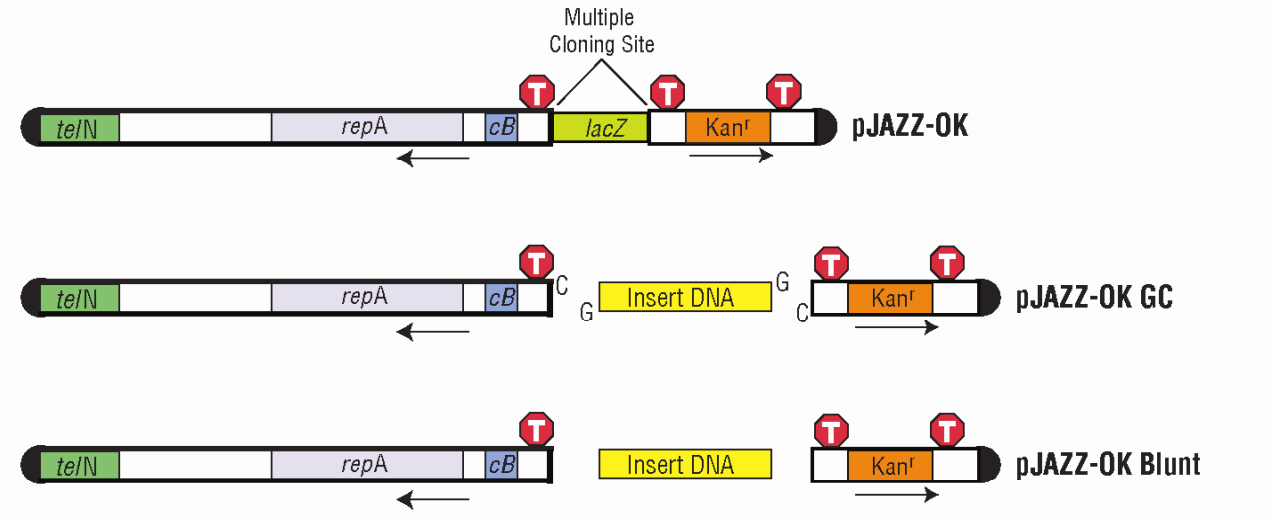


Figure 2. Processing and ligation of the pJAZZ-OK GC and Blunt vectors. Top Panel - Before processing, the pJAZZ-OK vector (12.9 kb) contains a *lac*Z stuffer fragment. Middle Panel - After processing, the vector has 3'-C tails and dephosphorylated ends. The left arm is 10 kb and the right arm is 2.2 kb. G-tailed insert DNA is ligated to vector arms. Bottom Panel - After processing, the vector has blunt dephosphorylated ends and blunt insert DNA is ligated to vector arms. *tel*N, protelomerase gene; *rep*A, replication factor and origin of replication; *c*B, regulator of copy number; *Kan*^{r}, kanamycin resistance gene. Approximate positions of transcription terminators (T) are indicated.

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-

OK vector (and all Lucigen pSMART® 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 pJAZZ-OK vectors, strong transcription terminators flank the cloning site to block this transcription, eliminating another source of cloning bias and sequencing gaps.

The pJAZZ® vectors contain an inducible origin of replication. The copy number is ~4-10/cell prior to induction; it is increased by approximately 10-20 fold by induction in BigEasy-TSA™ cells (see below).

BigEasy-TSA Electrocompetent Cells

Lucigen's BigEasy-TSA 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 20-200X less efficient. The BigEasy-TSA strain is derived from Lucigen's *E. cloni*® 10G cells. 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. These cells are also phage T1-resistant (*ton*A). The *rps*L mutation confers resistance to streptomycin.

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 Amp*^{R} *sopAB telN antA*

- BigEasy-TSA 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.

Producing PCR Products with 5' Phosphate Ends

The instructions in this kit describe how to clone PCR products amplified using either a proofreading or a non-proofreading DNA polymerase. There is an important difference in the type of DNA ends produced by these two classes of DNA polymerases. Non-proofreading polymerases, such as Taq, Tth, Tbr, or Tfl leave a single 3' extension, typically a G or an A residue, at both ends of the amplicon. The 3'-G extension is compatible with the C-tailed pJAZZ vector preparation termed pJAZZ-OK GC. Proofreading polymerases such as Vent and Pfu, or enzyme cocktails containing a mixture of non-proofreading and proofreading enzymes, will primarily produce a blunt end, which is compatible with the pJAZZ-OK Blunt vector. **Both vector preparations are supplied dephosphorylated, requiring that the PCR amplicons have 5' phosphate ends**. Reagents and protocols are provided to perform this required step.

Set up a 50 or 100 µl PCR reaction using the guidelines below:

Follow the manufacturer's instructions and recommendations for producing PCR products. Optimize conditions to produce your PCR product because small artifacts will preferentially clone over long products. Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product. Use a 7 to 30 minute final extension time to ensure that all PCR products are completely extended. After cycling, place the tube on ice or store at -20°C.

Purification and Size Fractionation of DNA

BigEasy® Long PCR Cloning Kits

A PCR reaction created with phosphorylated primers and a DNA polymerase can be used directly for cloning with the pJAZZ vectors. However, PCR products often contain spurious bands, primer dimers, and unused primers that can be cloned efficiently. **DNA must be purified from amplification and other enzymes before ligation to pJAZZ vectors.** Isolation of the desired DNA fragments by agarose gel electrophoresis is strongly recommended to avoid cloning irrelevant inserts. Agarose gel electrophoresis, which is commonly used to size fractionate DNA fragments, is sufficient for purification. If amplified DNA is *not* gel purified, it must be purified by binding to a DNA purification column or by phenol/chloroform extraction to remove enzyme contaminants. DNA should be eluted from the columns with deionized water or low-salt elution buffer (e.g., Qiagen Buffer "E.B.").

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 3). 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.

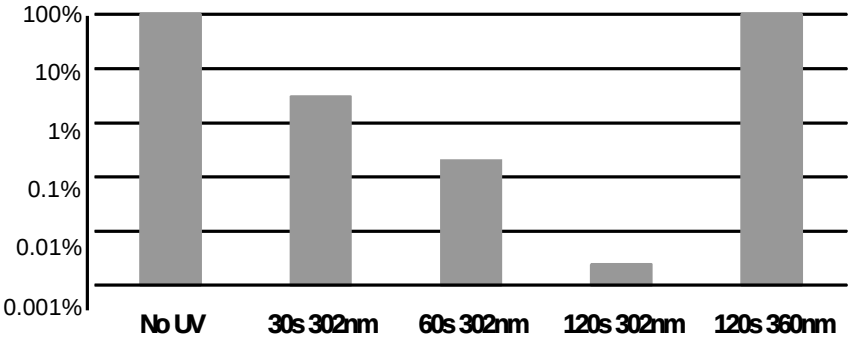


Figure 3. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 90 seconds ("30s 302nm, 60s 302nm,120s 302nm") or to 360 nm UV light for 120 seconds ("120s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

Materials and Equipment Needed

The BigEasy Long PCR Cloning Kit supplies 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.
- Terrific Broth (see Appendix A).
- YT agar plates containing kanamycin (see Appendix A).
- DNA Purification Columns
- Prepare YT Agar from powder included with cells

OVERVIEW OF PROTOCOL

SECTION 1: PREPARATION OF PCR PRODUCTS FOR GC OR BLUNT CLONING

PROTOCOL FOR NON-PROOFREADING OR PROOFREADING POLYMERASES

Protocol A	**OR**	Protocol B
Steps: 1. Phosphorylate primers 2. Amplify by PCR 3. Purify product		Steps: 1. Amplify by PCR 2. Phosphorylate product 3. Purify product

SECTION 2: LIGATION AND TRANSFORMATION

4. Proofreading Amplicon pJAZZ®-OK Blunt Ligation or Non-Proofreading Amplicon pJAZZ-OK GC Ligation
5. Electroporation

Detailed Protocol

The BigEasy Long PCR Cloning Kit can be used with PCR products from either non-proofreading or proofreading DNA polymerases. However, all DNA fragments used for GC Cloning **MUST** have three features:

1) **5' phosphate groups:** PCR products usually do NOT have 5' phosphate groups, regardless of the type of enzyme used for PCR amplification. The required 5' phosphate groups are added by phosphorylating either the PCR primers (**Protocol A**) or the PCR product (**Protocol B**). Protocols A and B are provided for both proofreading and non-proofreading polymerases.

2**) Single 3'-G overhangs:** Taq DNA Polymerase and similar enzymes are NON-proofreading enzymes that add 3'-G tails during the PCR reaction. Therefore, only a subsequent phosphorylation step is needed before ligation to the GC vector. Other NON-proofreaders (e.g. Tfl, Tth, Tbr polymerases) similarly add 3'-G tails.

3) **Sufficient purity:** In all cases, gel electrophoresis is highly ***recommended*** for purification of the insert DNA, to avoid cloning spurious bands or PCR primers.

DNA fragments used for blunt cloning must have features 1 and 3 above, but not feature 2 (G overhangs). Section 1 describes the preparation of 5'phosphorylated, DNA fragments. Section 2 details their ligation into the GC or blunt pJAZZ cloning vector preparations and transformation of BigEasy-TSA™ competent cells.

SECTION 1: PREPARATION OF PCR PRODUCTS FOR CLONING

Phosphate groups can be incorporated into the product by either of two methods: Protocol A describes phosphorylation of the primers before starting the PCR; Protocol B describes phosphorylation of the product

after the PCR is finished. The following section presents Protocols A and B for EconoTaq® DNA Polymerase and other non-proofreading polymerases. The subsequent section presents Protocols A and B for proofreading polymerases

Protocol A. Use of Phosphorylated PCR Primers

Step 1: Phosphorylate Primers.

Option A) Synthesize PCR Primers with 5' Phosphates

The easiest way to generate a phosphorylated PCR product is to use PCR primers that have been chemically synthesized with 5 phosphates already attached. Nearly all manufacturers of oligonucleotides provide this option, but the user must specifically request addition of 5 phosphates.

Option B) Add 5' phosphates to the primers by T4 PNK

T4 Polynucleotide Kinase can be used to add 5' phosphates to PCR primers before performing the PCR reaction. T4 PNK and Primer Kinase Buffer (containing ATP) are included in the Kits. Perform the primer kinase reaction as follows:

Primer kinase reaction

2.0 µl Forward primer @ 100 pmol/µl
2.0 µl Reverse primer @ 100 pmol/µl
1.0 µl 10 X Primer Kinase Buffer
1.0 µl T4 PNK (10 U/µl)
4.0 µl H_2O
10.0 µl total
Incubate at 37°C, 10 minutes

After the incubation, add 2-5 µl of this reaction directly to a 50-100 µl PCR mix and amplify by standard PCR (see Step 2 below).

In rare cases, the Primer Kinase Buffer interferes with the PCR reaction, resulting in reduced yields or smeared bands. In these cases, use chemically phosphorylated primers (Option A, above) or use non-phosphorylated primers followed by kinase treatment of the PCR product (Protocol B, below).

Step 2: Amplify by PCR.

Follow the manufacturer's instructions for DNA amplification.

Step 3: Purify Insert DNA

DNA products can be cloned directly after the PCR reaction, if desired. However, any spurious PCR products, primers, or primer dimers may also be cloned efficiently. Size selection on an agarose gel therefore is ***highly recommended*** to remove contaminating DNA, aberrant PCR products, PCR primers, and primer-dimers. Purify the DNA bands from the gel by a commercial DNA purification kit.

If the DNA is not gel fractionated, it can be purified using a commercial purification kit or ethanol precipitation.

Proceed to Section 2: Ligation and Transformation.

Protocol B. Addition of 5′ Phosphates to the PCR Product

PCR products created with non-phosphorylated primers can also be used for cloning. The PCR product MUST be purified away from the PCR buffer before the kinase reaction, because ammonium ions in the PCR buffer strongly inhibit T4 PNK. We recommend use of a purification column to remove the PCR buffer. The product is then treated with T4 PNK, followed by a second purification.

Step 1: Amplify by PCR.

Follow the manufacturer's instructions for DNA amplification.

After the PCR is complete, purify the PCR product using a standard DNA purification column and elute in 44 µl of H_2O or the recommended elution buffer. Alternately, precipitate the PCR reaction using sodium acetate plus ethanol. Do not use ammonium acetate for precipitation, as this salt will severely inhibit the PNK reaction. Resuspend the DNA with 44 µl H_20.

Step 2: Phosphorylate PCR Product.

After removal of the PCR buffer from the PCR product, perform the following kinase reaction:

Post-PCR Kinase Reaction

44 µl purified PCR product (No PCR buffer present)
5 µl 10 X Primer Kinase Buffer (containing ATP)
1 µl T4 PNK (10 U/µl)
50 µl total

Incubate at 37°C, 10 minutes.

Do not use this material directly in a ligation reaction, as the PNK will phosphorylate the vector, resulting in a high background of non-recombinants. **Purification is essential after this step.**

Step 3: Purify Insert DNA

Size selection on an agarose gel is highly ***recommended*** to remove contaminating DNA, aberrant PCR products, PCR primers, and primer-dimers. Purify the DNA bands from the gel by a commercial purification kit.

If the DNA is not gel fractionated, it can be purified using a commercial DNA purification kit or ethanol precipitation.

Do NOT attempt to clone the products directly after the phosphorylation reaction, without performing a purification step; doing so will produce a high background of empty vector clones.

Proceed to Section 2: Ligation and Transformation.

SECTION 2: LIGATION OF PCR AMPLICONS TO pJAZZ® VECTOR

Ligation of Non-Proofreader PCR Amplicons to the pJAZZ-OK GC Vector

In the BigEasy ligation reaction, the pre-processed pJAZZ-OK GC vector is ligated with phosphorylated G-tailed 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 1-30 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-OK GC 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, G-tailed, 5'-phosphorylated)
y µl H_2O
1.0 µl pJAZZ-OK GC vector (100 ng)

1.0 µl 10X CloneDirect Ligation buffer (contains ATP)
1.0 µl CloneSmart DNA Ligase (2 U/µl)
10.0 µl total reaction volume

3. Mix by gently pipeting the reaction mixture up and down. Incubate at room temp. (21-25°C) for 2 hours.

4. **Prepare YT Agar from powder included with cells**. Transformants do not grow well on LB agar.

Ligation of Proofreader PCR Amplicons to the pJAZZ®-OK Blunt Vector

In the BigEasy ligation reaction, the pre-processed pJAZZ-OK Blunt 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 1-30 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-OK Blunt 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, blunt, 5'-phosphorylated)
y µl H_2O
1.0 µl pJAZZ-OK Blunt vector (100 ng)
1.0 µl 10X CloneDirect Ligation buffer (contains ATP)
1.0 µl CloneSmart DNA Ligase (2 U/µl)
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.

Control Reactions

The control PCR product must be created by amplification *and* must contain 5' phosphate groups for ligation into the vector! This control PCR product will work in both proofreader and non-proofreader vector kits. Performing the control reaction is recommended to help evaluate the results obtained with experimental samples. A control template and primers are supplied to produce a diagnostic PCR product of 781 bp that encodes chloramphenicol acetyl transferase (CAT). Successful cloning of the resulting PCR product will produce chloramphenicol-resistant colonies. A few white colonies containing small, non-functional CAT inserts may also be observed if the transformants are grown on non-selective plates.

For convenience, the control primers and template are supplied in a single tube. The Primer Kinase reaction will also phosphorylate the Control PCR template, which does not affect the results.

1. Primer Kinase reaction:

4.0 µl PCR control Cm^R template plus primers (5 ng/µl template, 25 pmol/µl each primer)
1.0 µl 10X Primer Kinase buffer
4.0 µl H_2O
1.0 µl T4 Polynucleotide kinase (10 U/µl)
10.0 µl total

Incubate at 37°C for 10 minutes. Use 5.0 µl of the reaction directly for PCR amplification using the manufacturer's instructions for DNA amplification.

2. Analyze 5 µl of the reaction by agarose gel electrophoresis. A distinct band at 781 bp should be visible.

Size Fractionation and Purification of Control PCR Fragments

Size selection on an agarose gel is highly recommended to remove aberrant PCR products, PCR primers, and primer-dimers. Purify the 781-bp DNA band from the gel by a commercial purification kit.

If the DNA is not gel fractionated, it should be purified using a commercial purification kit or ethanol precipitation.

DNA product can be cloned directly after the PCR reaction, if desired. However, if spurious products and primer dimers are present, they may also be cloned efficiently, resulting in numerous white colonies.

Amplification with a proofreading enzyme will generate a blunt fragment and allow the PCR product to be ligated into the pJAZZ®-OK Blunt vector, and amplification with a non-proofreader will generate a fragment with G overhangs and will allow this PCR product to be ligated into the pJAZZ-OK GC vector.

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

Transformation

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

Electroporation of BigEasy-TSA Electrocompetent Cells

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 0.1-cm gap. Optimal settings for electroporation are listed in the table below. Typical time constants are 4.0 to 5.0 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		1400 – 2000 Volts

Suggested Electroporation Systems:

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

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).

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-TSA™ cells from the -80°C freezer and place on wet ice until they thaw **completely** (10-15 minutes).
4. When cells are thawed, mix them by tapping gently. Add 25 μl of BigEasy-TSA cells to the chilled microcentrifuge tube on ice.
5. Add 1 μl of the heat-denatured BigEasy Ligation reaction to the 25 μl of cells on ice. **(Failure to heat-inactivate the ligation reaction will prevent transformation.)** Stir briefly with pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells. Use of more than 2 μl of ligation mix may cause electrical arcing during electroporation.
6. Carefully pipet 25 μl of the cell/DNA mixture into a chilled electroporation cuvette without introducing bubbles. Quickly flick the cuvette downward with your wrist to deposit the cells across the bottom of the well. Electroporate according to the conditions recommended above.
7. Within 10 seconds of the pulse, add 975 μl of Recovery Medium to the cuvette and pipet up and down three times to resuspend the cells. Transfer the cells and Recovery Medium to a culture tube.
8. Place the tube in a shaking incubator at 250 rpm for 1 hour at 37°C.
9. Spread up to 100 μl of transformed cells on YT + KXI agar plates.
10. Incubate the plates overnight at 37°C.
11. Transformed clones can be further grown in TB or in any other rich culture medium with 30 μg/ml kanamycin. If higher copy number is desired, add Arabinose Induction Solution (provided in the Kit) to the cultures (1 μl per ml of culture; final concentration 0.01%). (See table below for recommended plating volumes).

Table 1. Plating Transformed Cells

Reaction Plate		Drug Resistance	μl/Plate
Experimental Insert	(500 ng per ligation)	Kanamycin	20, & 100
Control Insert	(Positive Control, 500 ng/μl)	Kanamycin	50
No-Insert Control	(Vector Background)	Kanamycin	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 G-tailed ends and 5' phosphate groups, into Lucigen's BigEasy-TSA Electrocompetent Cells. The background number of empty pJAZZ-OK vector is constant (< 25 colonies per 50 μl 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 present in the pJAZZ®-OK 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-OK 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 μl of cells plated, then the 25 colonies obtained from 50 μl of the No-Insert Control ligation will represent a background of 10%.

Table 2. Expected Transformation Results from Electroporation

Reaction	CFU/Ligation	Efficiency
pJAZZ-OK vector plus Control Insert	> 100,000	> 95% inserts
No-Insert Control (Vector Background)	< 5,000	< 5% background
pKanR Transformation Plasmid Control (10 pg)	NA	$\geq 4 \times 10^{10}$ cfu/μg 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 control insert reaction should produce > 500 recombinant colonies and 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 > 4×10^{10} colonies per μg plasmid.

Screening

The BigEasy Long PCR 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 with NotI will release the insert DNA from the vector arms (See Figure 2 and Appendix D). 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 30 μg/ml kanamycin. 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-OK 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-OK 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-OK vector. The BigEasy Long PCR Kit is provided with the sequencing primers SL1 and NZ-RevC. The sequence of the primers and their orientation relative to the pJAZZ-OK 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+kan+XGAL+IPTG (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. After agar cools add antibiotic, etc. Prepare KXI agar medium by adding kanamycin to a final concentration of 15 mg/ 500 ml (30 μg/ml); XGAL to a final concentration of 10 mg/500 ml (20 μg/ml), and IPTG to 1 mM. Pour into petri plates. Per liter YT Agar is: 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar.

NOTE: BigEasy-TSA™ colonies will be small and slow-growing on standard LB plates.

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®-OK transformation can be further grown in TB or LB culture medium, containing the 30 μg/ml kanamycin. Add 1/1000th 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^{o}C. Unused portions of the ligation reactions may be stored indefinitely at –20 ^{o}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, BigEasy 2.0 Linear Cloning Kit or the CopyRight® BAC Cloning Kit is recommended. Regions containing long stretches of di-, tri-, or tetra-nucleotide repeats may be stable only in the BigEasy pJAZZ vector.

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	+	+	++	+	+
pSMART-HC Amp	High	+	+	+	++	+
pSMART-LC Amp	Low	+	+	+	+	+
pSMART VC	Single-Mid	+	+	++	+	+
pJAZZ-OC	Low-Mid	+	+	+++	+	+

BigEasy® Long PCR Cloning Kits

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

Insert DNA Preparation

1. Generate target DNA fragments by PCR.
2. Generate blunt of 3'-G ends with 5' phosphate groups.
3. Purify DNA. Elute with deionized water or low-salt buffer.

Ligation

1. Briefly centrifuge and gently mix the pJAZZ®-OK 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_2O
1.0 µl pJAZZ-OK GC or Blunt Vector (100 ng)
1.0 µl 10X CloneDirect Ligation buffer (contains ATP)
1.0 µl CloneSmart® DNA Ligase (2 U/µl)
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

1. Have Recovery Medium at room temperature for transformations.
2. Chill electroporation cuvettes and 1.5 ml microfuge tubes on ice.
3. Thaw BigEasy-TSA™ 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+KXI agar plates. Incubate overnight at 37°C.

Colony Growth

1. Pick white colonies at random and grow in TB medium containing 1X Arabinose Induction Solution (if desired) and 30 µg/ml kanamycin.

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

The pJAZZ®-OK GC vectors are supplied predigested with 3'-C extensions and dephosphorylated ends.

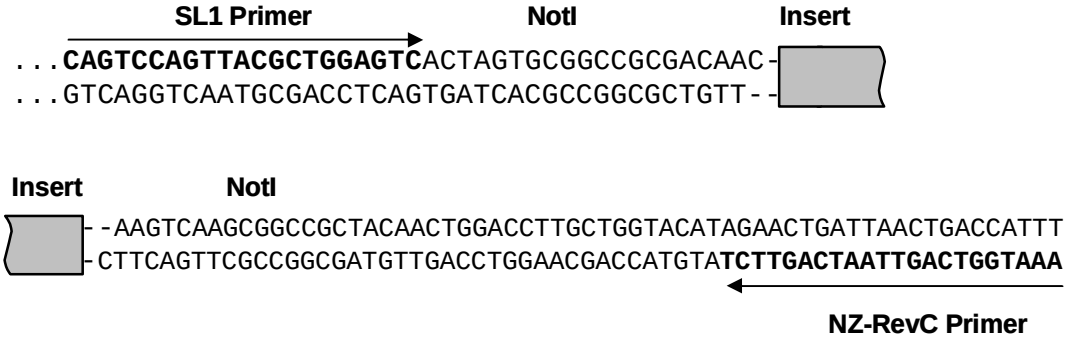
The sequences of the SL1 and NZ-RevC primers are as follows:

SL1: 5'-CAGTCCAGTTACGCTGGAGTC

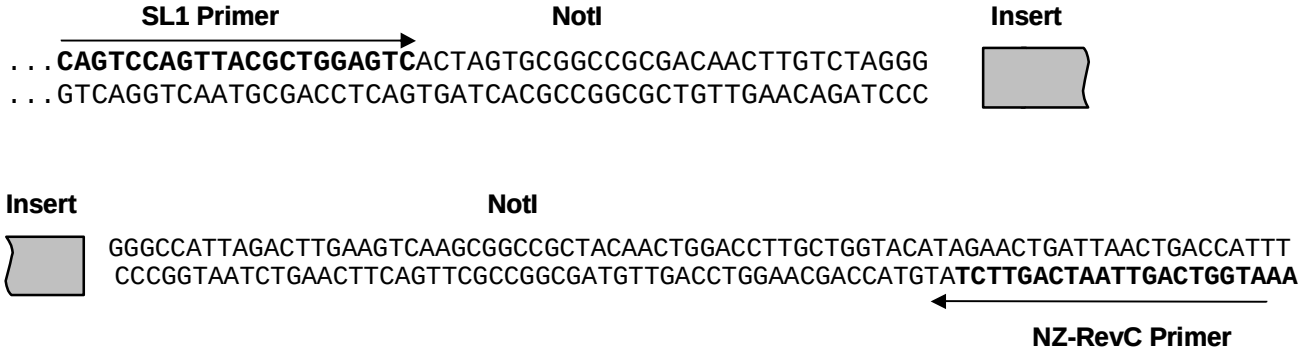
NZ RevC: 5'- AAATGGTCAGTTAATCAGTTCT

The GenBank accession number for the pJAZZ-OK vector will be released shortly.

Sequence of pJAZZ-OK processed for GC cloning:



Sequence of pJAZZ-OK processed for blunt cloning:



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 provided Recovery Medium.
	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 kanamycin to 30 µg/ml 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 kanamycin to 30 µg/ml to molten agar at 55°C before pouring plates (see Appendix A).

Appendix F: Sequence of pJAZZ-OK GC (13042 bp)

(*lac*Z stuffer fragment is underlined)

GCGTATAATGGACTATTGTGTGCTGATAAGGAGAACATAAGCGCAGAACAATATGTATCTATTCCGGTGTTGTGTTCCTTT
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AATGTATCAATAATTCGGAACATATGCACTATATCATATCTCAATTACGGAACATATCAGCACACAATTGCCCATTATACG
C

Appendix G: Recommended conditions for High throughput sequencing of pJAZZ clones:

BigEasy® Long PCR Cloning Kits

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