



IMPORTANT!
-86°C and -20°C Storage Required
Immediately Upon Receipt

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CopyRight™ Cloning Kits

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CopyRight™ Cloning Kits

Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to ensure that 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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CopyRight™ Cloning Kits

CopyRight™ Kit Designations

Lucigen offers several versions of the CopyRight™ Cloning Kits. The kits differ in number of reactions, vector, and cells that are included. The catalog numbers are listed below. Please refer to Appendix B: Application Guide for recommended uses of the kits.

Catalog numbers of CopyRight kits with and without cells

Vector	Reactions	Replicator™ Electrocompetent Cells	No Cells
pEZ™ BAC BamHI	10	42001-1	42012-1
	20	42001-2	42012-2
pEZ™ BAC Blunt	10	42005-1	42016-1
	20	42005-2	42016-2
pSMART® VC BamHI	10	42008-1	42019-1
	20	42008-2	42019-2
pSMART® VC Blunt	10	42011-1	42022-1
	20	42011-2	42022-2

Components & Storage Conditions

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

Container 1: CopyRight™ Cloning Components

Store at -20°C

	10 Reactions	20 Reactions
CopyRight Vector (25 ng/ul) Includes one type of ligation-ready Vector: pEZ BAC BamHI or pEZ BAC Blunt or pSMART VC BamHI or pSMART VC Blunt	10 µl	20 µl
CloneSmart™ DNA Ligase (2 U/µl)	20 µl	40 µl
CloneDirect™ 10X Ligation Buffer (includes ATP)	100 µl	200 µl
Positive Control Insert DNA Includes one type of insert control: lambda <i>BamHI</i> (80 ng/µl) or lambda <i>HpaI</i> (80 ng/µl)	5 µl	5 µl
Replicator Induction Solution (1000X)	1 ml	1 ml
Sequencing Primers (200 reactions each) BEZ-F1 Primer (3.2 pmol/µl) BEZ-R1 Primer (3.2 pmol/µl) or BSM-F1 Primer (3.2 pmol/µl) BSM-R1 Primer (3.2 pmol/µl)	200 µl 200 µl 200 µl 200 µl	200 µl 200 µl 200 µl 200 µl

Container 2: *E. cloni*® Replicator™ Electrocompetent Cells **Store at -86°C**

	Catalog #	Reactions
<i>E. cloni</i> ™ Replicator Electrocompetent Cells	60201-1	12 (6 x 50 µl)
	60201-2	24 (12 x 50 µl)
Positive Control Plasmid (pAKL 10 pg/µl) store at -20°C or -86°C	----	10 (1 x 10 µl)
Recovery Medium store at -20°C or -86°C	----	12 (1 x 12 ml)
		24 (2 x 12 ml)

CopyRight™ Cloning Kits

CopyRight™ Cloning Kit Description

Lucigen's CopyRight Cloning system introduces the ultimate in vector design. Most single-copy vectors yield small amounts of DNA and are prone to transcription error and instability. The unique design of Lucigen's CopyRight vectors reduces or eliminates transcription both into and out of the insert DNA, reducing the cloning bias commonly found with standard plasmids. CopyRight kits feature "on command" amplification of vector and copy number, increasing yield to as many as 50 copies per cell. The combination of Inducible copy amplification and Lucigen's patented transcription-free cloning technology provides maximum stability of large and small otherwise unclonable sequences. When used with Lucigen's *E. cloni* Replicator cells, thousands of recombinant clones can be routinely obtained.

CopyRight amplification permits easy isolation of plasmid DNA for sequencing, shotgun subcloning, or restriction mapping. CopyRight kits are ideal for cloning and library construction of BAC, fosmid, and other large insert DNAs, as well as general purpose cloning and cloning blunt PCR or restriction products, especially those containing unstable genetic elements. Lucigen's pSMART VC and pEZ BAC vectors are pre-cut and dephosphorylated, eliminating the need for vector preparation. CopyRight kits contain pEZ BAC or pSMART VC cloning vector, buffer, ligase, sequencing primers, induction solution, and control DNA.

Bacterial artificial chromosome (BAC) and fosmid vectors are important tools for positional cloning, physical mapping, and genomic sequencing of large DNAs. BAC vectors can maintain inserts of up to 350 kb, and fosmids can carry inserts of ~40 kb. Both types of vector utilize the single-copy origin of the *E. coli* F plasmid. In addition, these vectors are extremely useful for cloning smaller unstable and recalcitrant DNAs (10-20 kb), which appear to be more stable at lower copy numbers.

The CopyRight vectors, in conjunction with the *E. cloni* Replicator™ competent cells, incorporate controllable genetic elements that allow the single copy BAC or fosmid DNA to be amplified *in vivo*. The single-copy state is controlled by the *ori2* (*oriS*) origin of replication, *repE* gene, and *parABC* partition loci. Addition of L-arabinose induces expression of the TrfA replicator protein, which is under the control of the *araC*- P_{BAD} promoter system (Wild *et al.*, 2002). The TrfA replicator, incorporated into the genome of *E. cloni* Replicator cells, activates the medium-copy origin of replication (*oriV*), and plasmid accumulates up to 50 copies per cell (Figure 1).

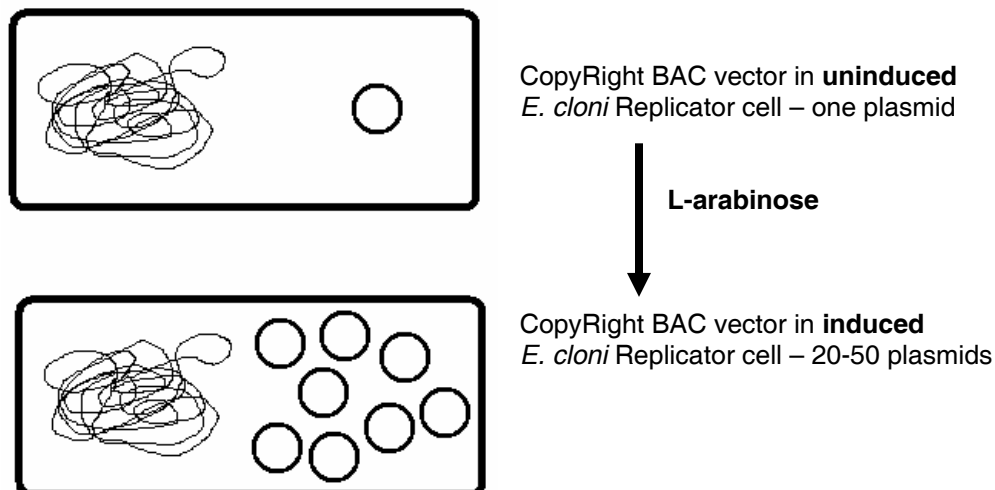


Figure 1. *E. cloni* Replicator cell with the CopyRight vector. Addition of L-arabinose results in amplification of the vector copy number.

CopyRight™ Cloning Kits

pEZ™ BAC and pSMART® VC Vectors

Two versions of the CopyRight cloning vectors are available (Figure 2). The pEZ™ BAC vector adds transcriptional terminators to the *lacZ*-based blue/white screening system to enhance insert stability. The pSMART VC vector eliminates the *lacZ* gene entirely, resulting in transcription-free cloning for the highest stability possible. All CopyRight vectors are supplied pre-cut at the BamHI or blunt (HpaI) sites with dephosphorylated ends. Insert DNAs with 5'-phosphorylated ends are ligated to the CopyRight vector, transformed into Lucigen's *E. coli*® Replicator Electrocompetent Cells, and spread on plates containing chloramphenicol.

In conventional BAC vectors, transcription from the chloramphenicol resistance gene promoter is oriented toward the insert cloning site. The CopyRight vectors have the chloramphenicol promoter facing away from the cloning sites, protecting inserts from the destabilizing influence of transcription.

Direct selection plasmids employ a toxic gene such as *sacB* to select against non-recombinant plasmids. Active transcription and translation coming from insert DNA is a possible source of clone gaps in such plasmids. A high level of instability can also be induced by the vector-driven transcription inherent to the blue/white screen, especially with inserts containing toxic coding sequences, strong secondary structure, or other deleterious features. The pSMART VC vector does not use a promoter or an indicator gene, avoiding transcription and translation across the insert (Figure 2).

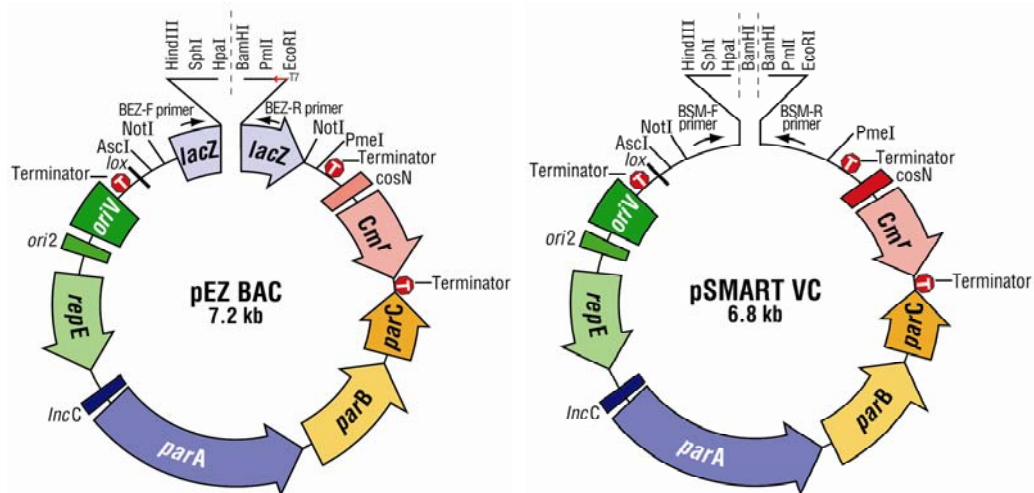


Figure 2. CopyRight vectors: pEZ BAC and pSMART VC. ori2, repE, IncC - origin of replication (single copy); oriV - inducible origin of replication; par A,B,C- partition genes; Cm^r- chloramphenicol resistance gene; cosN - lambda packaging signal; T - transcriptional terminators. Approximate positions of sequencing primers and transcriptional terminators are indicated.

The CopyRight vectors contain the following features:

- Single-copy replication origin and inducible medium copy replication origin
- Transcriptional terminators to stabilize recombinant clones
- Transcription/translation free cloning for unstable DNAs (pSMART VC only)
- Bacteriophage lambda cos site for lambda packaging or terminase cleavage
- Phage T7 polymerase promoter for *in vitro* transcription (pEZ BAC only)
- *loxP* sites for Cre-recombinase cleavage
- Rare-cutting restriction sites on either side of insert
- Chloramphenicol resistance gene
- Minimal vector size

CopyRight™ Cloning Kits

E. cloni® Replicator™ Electrocompetent Cells

Lucigen's *E. cloni* Replicator Electrocompetent Cells are an *E. coli* strain that contains an inducible *trfA* gene, which is required for amplification of the CopyRight clones to high copy number. Most lab strains of *E. coli* do not contain a *trfA* gene, and thus will not support copy number amplification of the CopyRight vectors.

E. cloni Replicator cells are optimized for high efficiency transformation by electroporation. *E. cloni* Replicator Elite cells yield $\geq 2 \times 10^{10}$ cfu/ μ g supercoiled pUC19 DNA. These cells are ideal for cloning and propagation of BAC, fosmid, or plasmid clones. In addition to the *trfA* gene for induction of the *oriV* origin, they contain the *endA1* mutation for high yield and high quality plasmid DNA. They also contain the inactive *mcr* and *mrr* mutations, allowing methylated genomic DNA isolated directly from mammalian or plant cells to be cloned without deletions or rearrangements. They do not contain the F plasmid.

E. cloni Replicator Genotype: F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) *endA1* *recA1* ϕ 80d*lacZ* Δ M15 Δ *lacX74* *araD139* Δ (*ara,leu*)7697 *galU* *galK* *rpsL* *nupG* *tonA* (*attL* *araC*-P_{BAD}-*trfA250* *bla* *attR*) λ ⁻

The plasmid pAKL19 (Ampicillin and Kanamycin resistant) is provided as a control for transformation of *E. cloni* Replicator cells. Note that Replicator cells are Amp^R (*bla* gene), so kanamycin is required for selection of pAKL transformants.

Construction of Plasmid, BAC, and Fosmid Libraries

Lucigen has optimized the preparation and design of the CopyRight vectors. The quality of results obtained is directly related to the quality of the input DNA preparation. The following points are critical for cloning all inserts, especially large inserts, into CopyRight vectors.

1. Do not pipette HMW DNA with standard pipette tips. High molecular weight DNA is readily sheared by pipetting. The use of wide bore pipette tips will minimize unwanted shearing. Purification and fragmentation of very high molecular weight DNA can be accomplished using a number of different methods. References 2-5 provide details for preparing DNA for BAC and fosmid cloning.

2. Do not expose DNA to 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 in a few seconds (Figure 3).

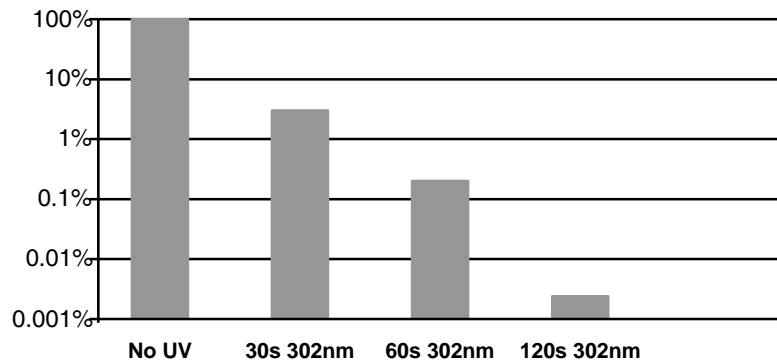


Figure 3. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 90 seconds ("30s 302nm, 60s 302nm, 120s 302nm"). Cloning efficiencies were calculated relative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

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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. Avoid exposing DNA to UV radiation. See reference 2 for methods to isolate DNA from gels without exposing DNA to UV.

3. *E. coli* Replicator cells are required for amplification. Induction of the CopyRight vectors from single copy to high copy number requires the use of *E. coli* Replicator cells, which carry the *trfA* gene. Amplification will not occur in standard laboratory strains of *E. coli*.

Inserts of <20 kb can be cloned following routine methods, outlined in the following section. For construction of a BAC library, there are several additional considerations, listed below:

1. Purify high molecular weight (HMW) genomic DNA.
2. Partially digest or randomly shear DNA to desired size.
3. Size select DNA by PFGE.
4. Test ligations of genomic and vector DNA.
5. Electroporate into *E. coli*.
6. Plate on selective media.
7. Pick transformants.
8. Assay for insert size and background.
9. Repeat until acceptable and scale up.

Protocols specific to the ligation and transformation of large insert BAC and fosmid libraries constructed in the CopyRight vectors are provided below. Detailed protocols for manipulating HMW DNA, BAC cloning, and handling BAC recombinant clones can be found in references 2-5.

Materials and Equipment Needed

The CopyRight™ Blunt Cloning Kits supply many of the items needed to efficiently generate recombinant clones. While simple and convenient, successful use of the CopyRight 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. Successful results are obtained with cuvettes from Eppendorf (4307-000-569), BTX (Model 610), or BioRad (Cat. #165-2089). Users have reported difficulties using *E. coli* cells with Invitrogen cuvettes (Cat. # 65-0030).
- Wet ice.
- Sterile 17 x 100 mm culture tubes.
- TY agar plates containing chloramphenicol, XGAL, and IPTG (see Appendix for recipes).

Plasmid and BAC Cloning Protocols

The CopyRight vectors are an efficient method for cloning extremely difficult DNA. These vectors can be used for cloning fragments of any size, from <1 kb up to 100's of kb (see BAC Cloning Protocol).

Approximately 50-100 ng of DNA insert is required per ligation. Calculate the concentration of the insert by comparison to mass standards on an agarose gel. Occasionally DNA may be more difficult to clone, so over 100 ng may be needed. Either the Bam HI or blunt CopyRight vectors can be used, depending on the ends of the insert. For highest insert stability, the pSMART VC vector is recommended.

The following protocols are designed for cloning fragments of various sizes.

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Cloning fragments up to 20 kb

Fragments up to 20 kb are ligated in a 10 µl reaction, containing up to 100 ng of insert.

1. Combine the following components in a 1.5-ml tube:

x µl Insert DNA (35-100 ng)
y µl H₂O
1.0 µl 10X CloneDirect Ligation buffer
1.0 µl pEZ BAC or pSMART VC Vector (25 ng/µl)
1.0 µl CloneSmart Ligase (2 U/µl)
10.0 µl total reaction volume

2. Incubate at room temperature for 2 hours.

3. **Critical: Heat denature at 70°C for 15 minutes.**

4. Cool to room temperature for 15 seconds followed by 0-4°C for 15 seconds to condense water vapor inside the tube. Spin briefly in a microcentrifuge.

5. The sample is ready for transformation; precipitating or desalting the DNA is not necessary and is not recommended.

Cloning BACs

A typical BAC library construction reaction is carried out in a volume of 100 µl. It contains 100 ng of insert DNA with an average size of 100-200 kb and 25-75 ng vector. The resulting molar ratio of vector to insert is approximately 10:1. For each new batch of insert DNA, set up trial ligations with varying amounts of vector to optimize the vector:insert ratio.

The ligation is performed as follows:

1. Combine the following components in a 1.5-ml tube, using a large bore pipette tip.

x µl Insert DNA (100 ng)
y µl H₂O
10.0 µl 10X CloneDirect Ligation buffer
1.0 - 3.0 µl pEZ BAC or pSMART VC Vector (25 ng/µl)
98.0 µl total reaction volume

Note: Cloning efficiency may be increased by using 2-3 µl of vector per reaction.

2. Add 2 µl CloneSmart Ligase, and mix by slowly stirring contents. Incubate at room temperature for 4 hours.

3. Heat denature the ligation reaction at 65°C for 15 minutes.

4. Cool to room temperature for 15 seconds followed by 0-4°C for 15 seconds to condense water vapor inside the tube. Spin briefly in a microcentrifuge.

5. The sample is ready for transformation; desalting the DNA is not necessary and is not recommended. The sample may be concentrated if desired.

CopyRight™ Cloning Kits

Transformation

BAC cloning applications demand the highest transformation efficiency possible. To ensure optimal cloning results, we strongly recommend the use of Lucigen's *E. cloni* Replicator Electrocompetent Cells. These cells yield the equivalent of $\geq 2 \times 10^{10}$ cfu/ug of pUC19, to maximize the number of transformants. Chemically competent cells are not recommended. The following protocols are specific for transformation of *E. cloni* Replicator Electrocompetent Cells.

ELECTROPORATION OF *E. CLONI* REPLICATOR ELECTROCOMPETENT CELLS

Lucigen's *E. cloni* Replicator Electrocompetent Cells are provided in 50- μ l aliquots, sufficient for two transformation reactions. Optimal settings for electroporation are listed in the table below. Typical time constants are 3.5 to 4.5 msec.

Electroporation conditions for *E. cloni* Replicator Cells

Optimal Setting	Alternate Settings (~ 20-50% lower efficiencies)
1.0 mm cuvette 10 μ F 600 Ohms 1800 Volts	1.0 mm cuvette 25 μ F 200 Ohms 1400 – 1600 Volts
Bio-Rad: Micro Pulser #165-2100; E. coli Pulser #165-2102; Gene Pulser II #165-2105; BTX: ECM630 Electroporation System; Eppendorf: Model 2510	

Optional transformation control reactions include electroporation with 1 μ l (10 pg) of supercoiled pAKL DNA (kanamycin resistant).

1. Have room-temperature Recovery Medium (supplied with kit) and 17 x 100 mm sterile culture tubes readily available (one tube for each transformation reaction). Transformation efficiency may decrease substantially with SOC or other media.
2. Place electroporation cuvettes (0.1 cm gap, BTX or BioRad brand) and microcentrifuge tubes on wet ice (one cuvette and one tube for each transformation reaction).
3. Remove *E. cloni* Replicator cells from the -86°C freezer and thaw completely on wet ice (20-30 minutes).
4. Add 25 μ l of *E. cloni* cells to the chilled microcentrifuge tube.
5. Add 1 μ l of the CopyRight ligation reaction to the 25 μ l of cells on ice. Stir briefly with a pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells.
6. Carefully pipet 25 μ 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 on the bottom of the well. Electroporate according to the conditions described above.
7. Within 10 seconds of the pulse, add 975 μ 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 225-250 rpm for 1 hour at 37°C.
9. Spread the following amounts of experimental reactions on 8.5 cm chloramphenicol plates (+XGAL/IPTG if desired). Plate the pAKL control on a kanamycin plate. Incubate the plates overnight at 37°C.

Table 1. Plating Transformed Cells

Reaction Plate	μ l/Plate
Experimental Insert (100 ng per ligation)	100
Lambda <i>Bam</i> HI Insert (Positive Control)	100
No-Insert Control (Vector Background)	100
pAKL Supercoiled Plasmid Control (10 pg)	2

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Expected Results

The results presented below are expected when cloning 100 ng of intact, purified DNA fragments, with 5' phosphate groups and *Bam*HI or blunt ends, into Lucigen's *E. coli* Replicator Elite Electrocompetent Cells (transformation efficiency > 2 x 10¹⁰ cfu/ug pUC19 DNA).

The number of recombinant clones is typically 100-fold greater than the background of self-ligated CopyRight vector. The background number of empty CopyRight vectors is constant (<10 colonies per 100 µl of cells plated), unless kinase, nuclease, or other contaminants are introduced. However, use of too little insert DNA, insert DNA with improper ends, or modified DNA, yields significantly lower recombinant cloning efficiencies. With fewer recombinant clones, the fraction of empty vector colonies becomes relatively higher. For example, if the Experimental Insert ligation reaction produces only 25 colonies from 100 ul of cells plated, then the 10 colonies obtained from 100 ul of the No-Insert Control ligation will represent a background of 40%.

Use of competent cells with a transformation efficiency of less than 1 x 10¹⁰ will severely compromise the desired results, yielding less than 5% the number of CFUs listed below. The ratio of recombinants to empty vector will not be affected.

Table 2. Expected Transformation Results from Electroporation

Reaction	CFU/Plate	Efficiency
CopyRight plus 100 ng control Insert	> 200	> 95% inserts
No-Insert Control (Vector Background)	< 10	< 5.0% background
pAKL Supercoiled Plasmid Control (10 pg) (Kan ^R)	> 200	2 x 10 ¹⁰ cfu/ug

1. Using Lucigen's *E. coli* Replicator Electrocompetent Cells (transformation efficiency > 2 x 10¹⁰ cfu/ug pUC19 DNA), a CopyRight ligation reaction with 100 ng of positive control insert DNA is expected to yield >200 colonies from a 100 µl aliquot of transformed cells, with >95% inserts. Results with experimental DNA may vary significantly, particularly with larger insert sizes, skewed base composition, encoded peptides, etc.
2. A 100 µl aliquot of the empty vector control reaction should produce < 10 colonies, representing less than 1% background.
3. A 2 µl aliquot of transformed cells from the supercoiled pAKL reaction (diluted into 90 µl of TB) should yield > 200 colonies, or 2 x 10¹⁰ colonies per µg plasmid.

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Fosmid Cloning Protocol

A fosmid clone is identical to a cosmid clone, except that it carries the F factor origin of replication from *E. coli*. As a result, it exists only as a single copy per cell. The CopyRight vectors allow for subsequent amplification to 20-50 copies per cell for DNA isolation. Genomic DNA inserts for fosmid cloning are always size selected to 40 kb to allow packaging into phage heads. Following ligation of the 40 kb DNA fragments into pEZ BAC or pSMART VC, the DNA is packaged *in vitro* using lambda packaging extracts. (Packaging extract is NOT provided with the CopyRight Kits.) The packaged phage are then transfected into the Replicator cells. Colonies, rather than plaques, are selected using chloramphenicol plates.

Important: Note that growth of the cells must be started *two days* before the packaging/transfection steps are performed (see below for more details).

Ligation to the CopyRight vector

Approximately 65 ng of DNA insert is required per ligation. Calculate the concentration of the insert by comparison to mass standards on an agarose gel. Occasionally DNA may be more difficult to clone, requiring up to 100 ng. Either the Bam HI or blunt CopyRight vectors can be used, depending on the ends of the insert.

1. Two days before packaging/transfection: Streak the *E. coli* Replicator cells onto an agar plate without antibiotic. Incubate the plate overnight at 37°C.
2. One day before packaging/transfection: Inoculate a single colony into LB media supplemented with 10 mM MgSO₄ and 0.2% (w/v) maltose. *Do not add antibiotic to the medium.* Grow overnight at 37°C with shaking. Antibiotic will bind to the bacterial cell wall and will inhibit the ability of the phage to infect the cell.
3. Ligation (One day before or same day as packaging/transfection):

Combine the following components in a 1.5-ml tube using a large bore pipette tip.

x	µl	Insert DNA (65 ng)
y	µl	H ₂ O
	1.0	µl 10X CloneDirect Ligation buffer
	5.0	µl pEZ BAC or pSMART VC Vector (125 ng total)
	1.0	µl CloneSmart Ligase (2 U/µl)
	10.0	µl total reaction volume

Incubate at room temperature for 4 hours.

Heat denature at 70°C for 15 minutes. The ligation reaction may be stored at -20°C.

4. Packaging: Package 1 µl of the ligation reaction with lambda packaging extract (e.g., Gigapack™ XL, Stratagene) according to the manufacturer's instructions.
5. Cell growth and transfection: Dilute 0.1 ml of overnight culture of cells into 5 ml of LB media supplemented with 10 mM MgSO₄ and 0.2% (w/v) maltose. Grow cells by shaking at 37°C for ~2 hours, to an OD₆₀₀ of 0.5 (do not grow past an OD₆₀₀ of 1.0). Pellet the bacteria at 10,000 × g for 30 seconds.
6. Gently resuspend the cells in 1 ml sterile 10 mM MgSO₄ to an OD₆₀₀ of ~0.5. The bacteria should be used immediately following dilution.
7. Combine 50 µl cells and 10 µl packaged DNA/extract. Incubate 30 minutes at room temperature. (Store the remaining DNA/extract at 4°C.)
8. Add 200 µl of LB broth, supplemented with 10 mM MgSO₄ and 0.2% (w/v) maltose, and incubate for 1 hour at 37°C, shaking the tube gently once every 15 minutes. Plate the cells on LB agar plates with 12.5 µg/ml chloramphenicol. Incubate the plates overnight at 37°C.

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DNA Isolation & Sequencing

Transformants are grown in TB medium plus 12.5 µg/ml chloramphenicol. Stable inserts of 10-40 kb can be grown overnight with shaking at 37°C in the presence of 1X Replicator Induction solution. DNA minipreps can be performed by standard methods.

For BACs and unstable smaller inserts, it is necessary to grow the cultures without induction to an OD₆₀₀ of 0.2-0.3. To reach this OD, it is convenient to grow the cultures overnight at 37°C without shaking. The following morning, dilute the cultures 2-10 fold, and grow at 37°C with shaking at 225 rpm for 30 minutes.

For each ml of culture, add 1 ul of 1000 X Replicator Induction solution. Continue growth for 2-3 hours at 37 °C with shaking at 225 rpm.

Prepare DNA minipreps according to standard protocols.

The *E. coli* Replicator Electrocompetent cells are *recA endA* deficient and will provide high quality plasmid DNA. The CopyRight Kits are provided with sequencing primers: BEZ-F1 and BEZ-R1 for pEZ BAC; for pSMART VC the primers are BSM-F1 and BSM-R1. The sequence and location of primers are shown in Appendix D.

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CopyRight™ Cloning Kits

Appendix A: Media Recipes

YT+Chl or YT+Chl+XGal+IPTG Agar Medium for Plating of Transformants. Per liter: 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, plus antibiotic. Mix all components except antibiotic; autoclave and cool to 55°C. Prepare YT+Chl agar medium by adding chloramphenicol to a final concentration of 12.5 µg/ml. Prepare YT+Chl+XGal+IPTG agar medium by adding chloramphenicol to a final concentration of 12.5 µg/ml, X-Gal to 40 µg/ml, and IPTG to 0.4 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 (K₂HPO₄; anhydrous), 2.2 g potassium dihydrogen phosphate (KH₂PO₄; anhydrous), 0.4% glycerol. Mix all components except glycerol; autoclave and cool to 55°C. Before use, add 8 ml filter-sterilized 50% glycerol and add chloramphenicol to 12.5 µg/ml. 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 at -20°C.

Appendix B: CopyRight™ Application Guide

The various CopyRight Cloning Kits are designed for three primary uses: 1) For BAC library construction, use the pEZ BAC BamHI Cloning Kit, which gives the highest cloning efficiency for HMW DNA; 2) For fosmid cloning, the pEZ BAC BamHI Cloning Kit is recommended; 3) For cloning very unstable fragments of 40 kb or less, use the pSMART VC Blunt Cloning Kit. Alternatively, Lucigen's BigEasy Linear Cloning Kit provides the highest level of plasmid stability.

The *E. coli* Replicator strain contains the inactive *mcr* and *mrr* alleles, which allows cloning of inserts that may be methylated, such as genomic DNA isolated directly from plant or mammalian cells.

Vector Name	BAC	Fosmid	AT-Rich, Unstable
CopyRight pEZ BAC BamHI	+	++	-
CopyRight pEZ BAC Blunt	-	+	-
CopyRight pSMART VC BamHI	-	+	+
CopyRight pSMART VC Blunt	-	+	+

CopyRight™ Cloning Kits

Appendix C: Abbreviated Plasmid and BAC Protocol

(Plasmid and BAC cloning only. Please see Manual for Fosmid Cloning and for detailed instructions.)

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

20 kb Insert Ligation Reaction	BAC Ligation Reaction
x μ l Insert DNA (100 ng)	x μ l Insert DNA (100 ng)
y μ l H ₂ O	y μ l H ₂ O
1.0 μ l 10X CloneDirect ligation buffer	10.0 μ l 10X CloneDirect ligation buffer
1.0 μ l CopyRight Vector (25 ng/ μ l)	1.0 μ l CopyRight Vector (25 ng/ μ l)
1.0 μ l CloneSmart Ligase	
<hr/>	<hr/>
10.0 μ l total volume	98.0 μ l total volume
	Add:
	2.0 μ l CloneSmart Ligase
	<hr/>
	100.0 μ l total volume

3. Incubate 2-4 hours at RT.
4. **Heat denature the reaction 15 minutes at 65°C.**
5. Cool the reactions and spin 1 minute at 12,000 rpm.

Transformation

1. Thaw Recovery Medium at room temperature.
2. Chill electroporation cuvettes and 1.5 ml microfuge tubes on ice.
3. Thaw *E. coli* Electrocompetent Cells on wet ice. Pipet 25 μ l of cells into a pre-chilled 1.5 ml tube on ice.
4. Add 2 μ l of heat-treated ligation reaction to an aliquot of chilled cells on ice.
5. Pipet the cell/DNA mixture to a chilled electroporation cuvette.
6. Electroporate. Immediately add 1 ml of room temperature Recovery Medium.
7. Shake at 225 rpm for 60 minutes at 37°C.
8. Spread 100 μ l per plate on TY agar plates containing the chloramphenicol. Incubate overnight at 37°C.

Colony Growth

1. Pick colonies and grow in TB medium containing 12.5 ug/ml chloramphenicol.
2. Add Induction solution to cultures when OD₆₀₀ reaches 0.2-0.3.
3. Grow 3 hours at 37°C with vigorous shaking.

CopyRight™ Cloning Kits

Appendix D: Vector Map and Sequencing Primers

The CopyRight™ vectors are supplied predigested with dephosphorylated ends. Transcriptional terminators border the cloning site to prevent transcription from the insert into the vector. Another terminator at the 3' end of the ampicillin or kanamycin resistance gene prevents this transcript from reading into the insert DNA. The sequences of the pEZ BAC and pSMART VC primers are as follows:

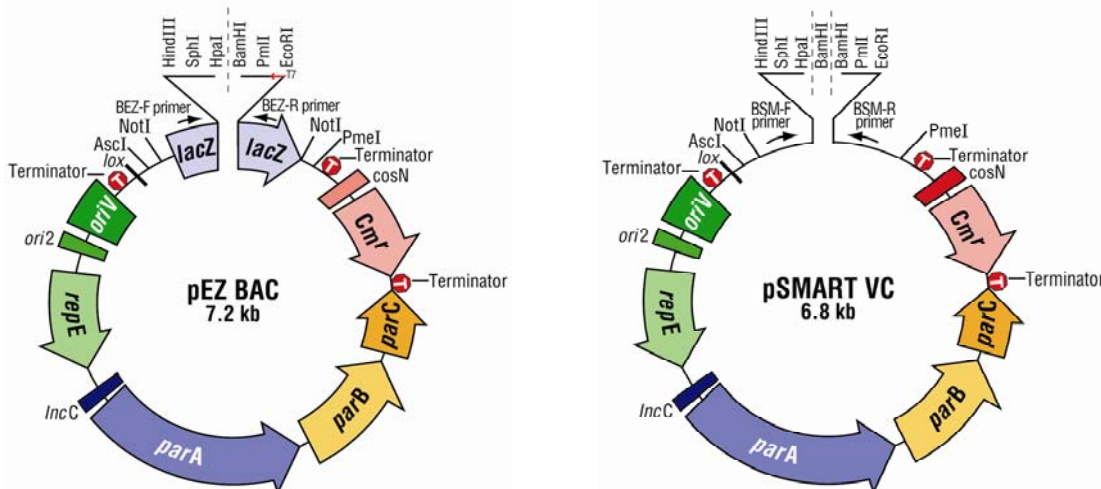
BEZ-F1: 5'CAC TTT ATG CTT CCG GCT CGT ATG 3'

BEZ-R1: 5'GGG ATG TGC TGC AAG GCG ATT AAG 3'

BSM-F1: 5'AAA GAA GGA AAG CGG CCG CCA GG 3'

BSM-R1: 5'CTA TAC GAA GTT ATG TCA AGC GG 3'

The sequences of the CopyRight™ vectors are available from Lucigen. GenBank accession number of pSMART VC is AY643800.



CopyRight™ Cloning Kits

Appendix E: Cloning Sites of CopyRight vectors

pEZ BAC Cloning site

BEZ-F1

CATTAGGCACCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCG
GTAATCCGTGGGGTCCGAAATGTGAAATACGAAGGCCGAGCATAACAACACACCTTAACACTCGC

lacZα start

GATAACAATTTACACAGGAAACAGCTATGACCATGATTACGCCAAGCTATTTAGGTGAGACTA
CTATTGTTAAAGTGTGTCCTTTGTTCGATACTGGTACTAATGCGGTTTCGATAAATCCACTCTGAT

HindIII SphI BsaBI HpaI BamHI PmlI EcoRI

TAGAATACTCAAGCTTGCATGCGATACGTATCGTTAACGATGGATCCGACGCACGTGCGAATTC
ATCTTATGAGTTCGAACGTACGCTATGCATAGCAATTGCTACCTAGGCTGCGTGCACGCTTAAG

GCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAAC
CGGGATATCACTCAGCATAATGTTAAGTGACCGGCAGCAAATGTTGCAGCACTGACCCTTTTG

← **T7 Promoter**

CCTGGCGTTACCCAACCTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCG
GGACCGCAATGGGTTGAATTAGCGGAACGTCGTGTAGGGGGAAAGCGGTCGACCGCATTATCGC

← **BEZ-R1**

pSMART VC Cloning site

AscI **BSM-F1** NotI

ATGGACTAGGCGCGCCAGAAGAGAGAAAGAAGGAAAGCGGCCCGCCAGGGTTTTCCAGTCACGA
TACCTGATCCGCGCGGTCTTCTCTCTTTCTTCTTTCGCGCGGTCGCCAAAAGGGTCAGTGCT

HindIII SphI BsaBI HpaI BamHI PmlI EcoRI XbaI*

CAAGCTTGCATGCGATACGTATCGTTAACGATGGATCCGACGCACGTGCGAATTCCTCTAGAGG
GTTTCGAACGTACGCTATGCATAGCAATTGCTACCTAGGCTGCGTGCACGCTTAAGGAGATCTCC

TTCCTAGTTGTAAGTCCAGGGCGGCCACTTGACATAACTTCGTATAGCATAATTATACGAAG
AAGGATCAACATTGACGGTCCC GCCGGTGAAGTGAAGCATATCGTATGTAATATGCTTC

← **BSM-R1**

Digestion at the HpaI site is used for preparation of blunt CopyRight vectors.

*XbaI site is not unique

CopyRight™ Cloning Kits

Appendix F: Troubleshooting Guide

Problem	Probable Cause	Solution
Very few or no transformants	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. Use the supplied control insert to test ligation reaction.
	Inadequate heat denaturation of ligation reaction.	Be certain to heat denature for 15 min at 65°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 outgrowth.
	Improper electroporation conditions.	Use BTX or BioRad electroporation cuvettes with a gap of 0.1 cm. Pre-chill cuvettes on ice. Add the DNA to 25 µl of pre-aliquotted cells on wet ice; DO NOT add the cells to the DNA.
	Addition of XGAL/DMSO to competent cells.	DO NOT add additional compounds to competent cells, as they are fragile.
	Incorrect amounts of antibiotic in agar plates. Wrong antibiotic used.	Add the correct amount of chloramphenicol to molten agar at 55°C before pouring plates (see Appendix A). DO NOT spread antibiotic onto the surface of agar plates.
	Contaminating enzymes in ligation reaction.	Purify DNA after partial digestion or end repair.
High background of transformants that do not contain inserts.	Unstable DNA Inserts	Use pSMART VC Transcription-Free Blunt Kit for maximum clone stability.
	Contaminating enzymes in ligation reaction.	Purify DNA after partial digestion or end repair.
	Contaminating DNA, oligos, or linkers in ligation reaction.	Purify DNA after partial digestion or end repair.
	Only large colonies were analyzed.	Colonies containing larger BAC inserts often grow somewhat slowly. Picking the smaller colonies on a plate may increase the likelihood of recovering recombinants.