

CopyRight[®] v2.0

BAC Cloning Kits

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

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Advanced Products for Molecular Biology

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Technical Support

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Table of Contents

Kit Designations.....	4
Components & Storage Conditions.....	4
Kit Description.....	5
pSMART® BAC and pEZ™ BAC Vectors	6
BAC-Optimized Replicator™ v2.0 Electrocompetent Cells.....	7
Construction of BAC Libraries.....	7
Materials and Equipment Needed.....	8
BAC Cloning Protocol	9
Transformation of BAC-Optimized Replicator v2.0 Electrocompetent Cells	12
DNA Isolation & Sequencing.....	14
References	14
Appendix A: Media Recipes.....	15
Appendix B: Application Guide	15
Appendix C: Abbreviated Protocol.....	16
Appendix D: Vector Maps and Sequencing Primers.....	17
Appendix E: Troubleshooting Guide	19
Appendix F: Vector Sequences	20

CopyRight® v2.0 BAC Cloning Kits

Kit Designations

Lucigen offers several versions of the CopyRight v2.0 BAC Cloning Kits. The kits differ in the number of reactions. The catalog numbers are listed below. Please refer to Appendix B: Application Guide for recommended uses of the Kits.

Catalog numbers of CopyRight v2.0 BAC Cloning kits with electrocompetent cells

Reactions	pSMART BAC BamHI	pSMART BAC EcoRI	pSMART BAC HindIII	pEZ BAC BamHI	pEZ BAC Blunt
10	42030-1	42031-1	42032-1	42007-1	42009-1
20	42030-2	42031-2	42032-2	42007-2	42009-2

Components & Storage Conditions

The Cloning Components of the CopyRight v2.0 BAC Kits are shipped in Container 1, which should be stored at **-20°C**. BAC-Optimized Replicator v2.0 Electrocompetent Cells are shipped in Container 2, which must be stored at **-80°C**. Additional Ligation components and Electrocompetent Cells may be purchased separately.

Container 1: CopyRight v2.0 BAC Ligation Components **Store at -20°C**

	10 Reactions	20 Reactions
CopyRight Vector (25 ng/μl, ligation-ready) pSMART BAC BamHI <i>or</i> pSMART BAC EcoRI <i>or</i> pSMART BAC HindIII <i>or</i> pEZ BAC BamHI <i>or</i> pEZ BAC Blunt	10 μl	2 X 10 μl
CopyRight 5X Ligation Buffer	100 μl	2 X 100 μl
CopyRight DNA Ligase (1 U/μl)	20 μl	2 X 20 μl
Positive Control Insert DNA lambda BamHI or EcoRI or HindIII or HpaI (80 ng/μl)	5 μl	2 X 5 μl
Sequencing Primers (200 reactions each)		
SL1 (3.2 pmol/μl) with pSMART kits	200 μl	2 X 200 μl
SR4 (3.2 pmol/μl) or	200 μl	2 X 200 μl
BEZ F1 (3.2 pmol/μl) with pEZ kits	200 μl	2 X 200 μl
BEZ R (3.2 pmol/μl)	200 μl	2 X 200 μl

Container 2: BAC-Optimized Replicator v2.0 Electrocompetent Cells **Store at -80°C**

	Catalog #	Reactions
BAC-Optimized Replicator v2.0 Electrocompetent Cells (DUOS) Store at -80°C	60210-1	12 (6 x 50 μl)
	60210-2	24 (12 x 50 μl)
Positive Control Plasmid (pKanR 1 ng/μl) Store at -20°C or -80°C	----	1 x 5 μl
Recovery Medium Store at -20°C or -80°C	----	12 (1 x 12 ml)
	----	24 (2 x 12 ml)
	80026-1	96 (8 x 12 ml)
Arabinose Induction Solution (1000X) Store at -20°C or -80°C	----	1 ml
	----	2 x 1 ml
YT Agar (powder)	----	

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Kit Description

Bacterial artificial chromosomes (BACs) are important tools for positional cloning, physical mapping, and genomic sequencing of large DNAs. BAC vectors can maintain inserts of up to 350 kb, utilizing 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 number.

Lucigen's CopyRight v2.0 BAC Cloning System introduces the ultimate in BAC 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. Lucigen's CopyRight vectors also completely eliminate uncut vector background without the risk of clone loss. CopyRight kits further feature "on command" amplification of vector and copy number, increasing yield to as many as 50 copies per cell. The combination of inducible copy number amplification and Lucigen's patented transcription-free cloning technology provides maximum stability of otherwise unclonable sequences. When used with Lucigen's BAC-Optimized Replicator v2.0 Electrocompetent Cells, hundreds or thousands of recombinant clones can be routinely obtained per transformation.

CopyRight amplification permits easy isolation of plasmid DNA for sequencing, shotgun subcloning, or restriction mapping. Lucigen's pSMART® or pEZ™ BAC vector is pre-cut and dephosphorylated, eliminating the need for vector preparation. CopyRight kits contain the pSMART or pEZ BAC cloning vector, buffer, ligase, sequencing primers, induction solution, and control DNA, as well as BAC-Optimized Replicator v2.0 Electrocompetent Cells.

The CopyRight vectors, together with the BAC-Optimized Replicator v2.0 Electrocompetent Cells, incorporate controllable genetic elements that allow the single copy BAC to be amplified *in vivo*. The single-copy state is controlled by the *ori2* (*oriS*) origin of replication, *repE* gene, and *parABC* partition loci, all of which are located on the vector backbone. The CopyRight vectors also carry the *oriV* medium-copy origin of replication, which is active only in the presence of the TrfA replication protein. The gene for TrfA is incorporated into the genome of BAC-Optimized Replicator v2.0 Cells under control of the *araC*- P_{BAD} promoter (Wild *et al.*, 2002). Addition of L-arabinose induces expression of TrfA, which activates *oriV*, causing the plasmid to replicate to up to 50 copies per cell (Figure 1).

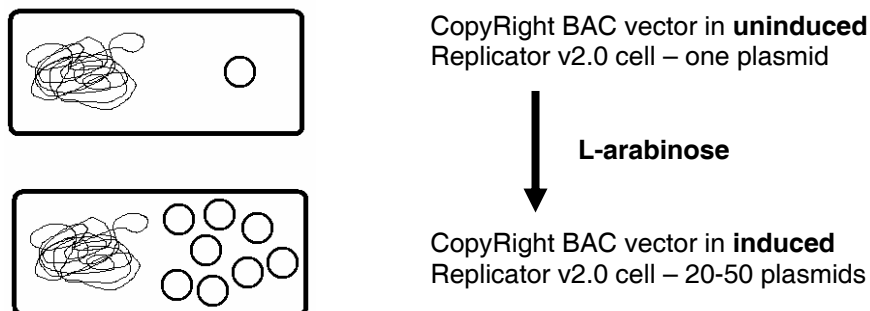


Figure 1. BAC-Optimized Replicator v2.0 Cell with the CopyRight vector. Addition of L-arabinose results in amplification of the vector copy number.

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pSMART and pEZ BAC Vectors

The CopyRight **pSMART** BAC cloning vector incorporates transcription-free cloning for the highest stability possible. The vector is supplied pre-cut at BamHI, EcoRI or HindIII sites, with dephosphorylated ends. The **pEZ** BAC vector is supplied pre-cut at BamHI or HpaI sites, with dephosphorylated ends. Insert DNAs with 5'-phosphorylated ends are ligated to the CopyRight vector and transformed into Lucigen's BAC-Optimized Replicator v2.0 Electrocompetent Cells.

The pSMART[®] BAC vector has a *lacZ/sacB* stuffer region, allowing uncut vector to be detected by blue/white screening and selected against by plating on sucrose (Figure 2). This feature completely eliminates the background of uncut vector, improving the efficiency of colony picking. However, unlike other cloning vectors, the *promoter* as well as the coding sequence of the stuffer fragment is completely removed during processing. This design prevents active expression of the insert DNA by the *lacZ* or *sacB* promoter, contributing greatly to plasmid stability, especially for inserts containing toxic coding sequences, strong secondary structure, or other deleterious features.

On the other hand, the **pEZ** BAC vector retains the *lacZ* indicator gene to allow blue-white screening of BAC colonies.

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

Transcription from cloned promoters is another source of clone gaps in libraries made in conventional plasmids. Cloned promoters can express the *lacZ* or *sacB* genes in the conventional vectors, leading to selection against true recombinant plasmids. Transcription from the insert into the vector can also interfere with plasmid replication or expression of drug resistance, contributing to difficulty in cloning and to library bias. The CopyRight vectors contain CloneSmart[®] terminators on either side of the cloning site to prevent this interference.

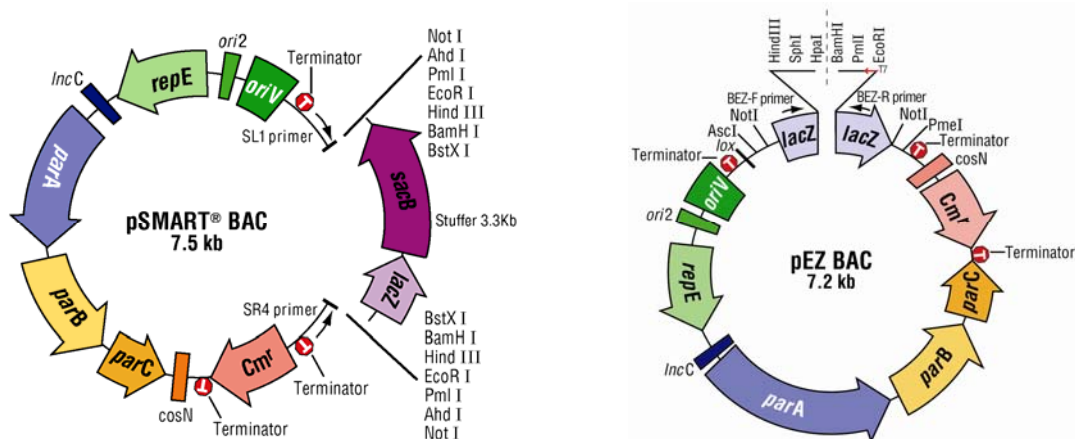


Figure 2. Vectors pSMART and pEZ BAC. *ori2*, *repE*, *IncC* - origin of replication (single copy); *oriV* - inducible origin of replication; *par A,B,C*- partition genes; *Cm^r*- chloramphenicol resistance gene; *cosN* - lambda packaging signal; T – CloneSmart transcription terminators; *sacB*, sucrase gene; *lacZ*, alpha peptide portion of the beta galactosidase gene. Approximate positions of sequencing primers and transcription terminators are indicated.

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The CopyRight vectors contain the following features:

- Single-copy replication origin and inducible medium-copy replication origin
- Transcription terminators to stabilize recombinant clones
- Transcription/translation-free cloning for unstable DNAs
- *lacZ/sacB* stuffer that is completely removed for minimal background and no bias (pSMART BAC vector only)
- Bacteriophage lambda cos site for lambda packaging or terminase cleavage
- *loxP* site for Cre-recombinase recognition
- Rare-cutting restriction sites on either side of insert
- Chloramphenicol resistance gene

BAC-Optimized Replicator v2.0 Electrocompetent Cells

Lucigen's BAC-Optimized Replicator v2.0 Electrocompetent Cells are genotypically similar to *E. coli* strain DH10B, but they contain an inducible *trfA* gene, which provides an optional amplification of the CopyRight clones to higher copy number (20-50 copies per cell). Common lab strains of *E. coli* do not contain a *trfA* gene and thus will not support copy number amplification of the CopyRight vectors.

BAC-Optimized Replicator v2.0 Electrocompetent Cells provide the highest transformation efficiency of BAC-sized DNA by electroporation. These cells yield $\geq 5 \times 10^7$ cfu/ μ g of 200-kb supercoiled BAC DNA. Thus they are ideal for cloning and propagation of BAC clones. They also contain the inactive *mcr* and *mrr* mutations, allowing methylated genomic DNA to be cloned without deletions or rearrangements. They do not contain the F plasmid. The BAC-Optimized Replicator v2.0 cells contain the *endA1* and *recA1* mutations for high yield and high quality plasmid DNA.

BAC-Optimized Replicator v2.0 Genotype: F^- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80d*lacZ* Δ M15 Δ *lacX74* *endA1* *recA1* *araD139* Δ (*ara,leu*)7697 *galU* *galK* *rpsL* *nupG* (*attL* *araC-P_{BAD}-trfA250* *bla* *attR*) λ^-

The plasmid pKanR (Kanamycin resistant) is provided as a control for transformation of BAC-Optimized Replicator v2.0 Cells.

Construction of BAC 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 pipeting. 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 efficiency by several orders of magnitude in a few seconds (Figure 3).

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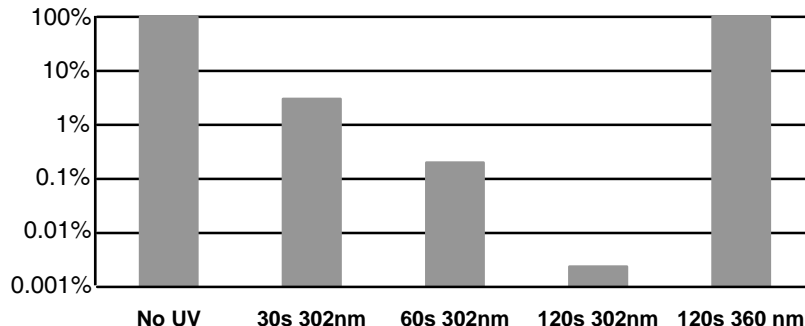


Figure 3. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure (“No UV”) or exposure to 302 nm UV light for 30, 60, or 90 seconds (“30s 302nm, 60s 302nm, 120s 302nm”). Cloning efficiencies were calculated relative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

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. See Reference 1 for methods to isolate DNA from gels without exposing DNA to UV.

3. BAC-Optimized Replicator v2.0 Cells are required for copy number amplification. Induction of the CopyRight vectors from single copy to high copy number requires the use of BAC-Optimized Replicator v2.0 Cells, which carry the *trfA* gene. Amplification will not occur in standard laboratory strains of *E. coli*.

For construction of a BAC library, there are several important steps, 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 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 1, 5-8, 11, and 12.

Materials and Equipment Needed

The CopyRight v2.0 BAC 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 cuvettes. For optimal BAC transformation, we recommend the Whatman Biometra Cell-Porator® electroporation system (Cat.#11609013) and Voltage booster (Cat.

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#11612017), and 0.15 cm cuvettes (Cat.#11608031). Other electroporation equipment can be used, but the transformation efficiency will be lower (See p. 10).

- Wet ice.
- Sterile 17 x 100 mm culture tubes.
- YT agar plates (make from YT Agar powder included with cells) containing chloramphenicol, XGAL, and IPTG, with or without sucrose (see Appendix for recipes). Alternatively, LB Lennox medium may be used, but DO NOT use standard LB agar.

BAC Cloning Protocol

Partial Digestion of High Molecular Weight (HMW) genomic DNA

A BAC library construction typically requires ~10-30 µg of HMW genomic DNA, embedded in five~ten plugs of Low Melting Temperature (LMT) agarose. The following protocol describes digestion of the embedded DNA with restriction enzymes. Each restriction enzyme must be titrated to determine the optimal amount for partial digestion. Typical digests use 0.2-10 units of enzyme per 1-10 µg of DNA. We strongly recommend using enzymes compatible with BamHI, EcoRI, or HindIII for digestion of insert DNA. Construction of BAC libraries with blunt inserts is not recommended.

1. Cut each 100-µl plug of HMW DNA into 9 slices with a glass cover slip. Use 3 slices for each digestion reaction.
2. Prepare a master mix of enzyme incubation buffer in a 15 ml Falcon tube.

Pre-Incubation Buffer (10 digestion reactions)

H ₂ O	8670 µl
10 x enzyme buffer	1000 µl
1 M spermidine	20 µl
1 M DTT	10 µl
Total	9700 µl

3. Transfer the desired number of gel slices into a plastic weigh boat. Add 970 µl of Pre-Incubation Buffer per 3 plug slices. Place on ice for 30 minutes. Discard the buffer and repeat once.

Transfer 3 plug slices into a 1.5 ml microfuge tube for each desired restriction digestion.

4. Prepare Digestion Buffer as follows:

1X Digestion Buffer (10 digestion reactions)

H ₂ O	1324.9 µl
10 x enzyme buffer*	170 µl
1 M spermidine	3.4 µl
1 M DTT	1.7 µl
10 mg/ml BSA	100 µl
Total	1600 µl

*Manufacture's recommended buffer

5. To each 1.5 ml tube containing 3 gel plug slices, add 160 µl of the prepared 1X Digestion Buffer. Then add 1-10 µl of restriction enzyme (0.2 to 20 Units total) and water to bring the final volume of digestion mixture

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to 170 µl. **Note:** If necessary, dilute the enzyme in 1x Digestion Buffer before adding to the reaction mixture.

6. Incubate the plug slices on ice for 60 minutes to allow the enzyme to diffuse into the plugs. Transfer the tubes to 37°C, and digest for 10 minutes.
7. Stop the reaction by immediately transferring the tubes on ice and adding 1/10 volume of 0.5 M EDTA, pH 8.0.
8. Analyze the partial digestion by pulsed-field gel electrophoresis under the following conditions:
1% agarose in 0.5 x TBE, 12.5°C (cooler settings), 80 (pump settings), 120° angle, 6 V/cm, initial pulse time of 50 seconds and final pulse time of 50 seconds for 18 - 24 hours.
9. Stain and photograph the gel. Determine the concentration of the enzyme that produces the highest proportion of fragments in the size range of 100 - 350 kb (Figure 4).
10. Use the selected concentration of enzyme for large-scale partial digestion for BAC cloning.

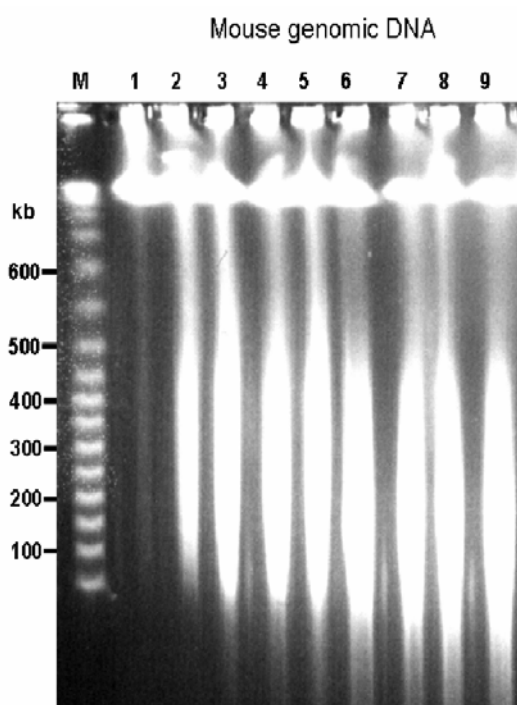


Figure 4. Partial digestion of HMW genomic DNA. Mouse genomic DNA was partially digested with EcoRI. Lanes 1-9 show results with various amounts of EcoRI (0, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2.0, 2.4 units). The amount of EcoRI that produced optimal fragmentation of 100-350 kb was 0.6~1.2 units. Lane M, DNA size marker (Lambda ladder).

Size Selection of the partially digested HMW DNA

Size select the partially digested DNA by agarose gel electrophoresis. Ideally, use Pulse Field Gel Electrophoresis (e.g., CHEF, FIGE, etc.) with voltage and ramp times recommended by the manufacturer for separation of 10-500 kb DNA. For example, 1% agarose CHEF gel, 0.5X TBE, 6V/cm, 50s, 50s, 18hrs, 120° angle, 12°C. If a PFGE apparatus is not available, run the sample overnight on a 20-cm, 1% agarose gel at 30-35 V. Minigels (e.g., 10 cm) do not provide sufficient resolution of DNA in the 10-500 kb size range.

1. Prepare a 1% agarose gel in 0.5X TBE buffer. **Note:** Do not include ethidium bromide in the gel or buffer.
2. Load DNA marker (e.g., MidRange I PFG Marker [New England BioLabs, catalog no. N3551S]) into each of the outside lanes of the gel. Load the digested DNA in a trough between the DNA markers.

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3. Resolve the samples by gel electrophoresis as described above. Following electrophoresis, cut off the outer lanes of the gel containing the DNA size marker. Stain the DNA marker lanes with ethidium bromide and visualize the DNA with UV light. Mark the 100-kb and 350-kb positions of the DNA marker in the gel using a razor blade. **Do not expose the bulk of sample DNA to UV irradiation!** Even short UV exposure can decrease cloning efficiencies by 100-1000 fold. Reassemble the gel and excise a 3-5 mm wide gel slice containing sample DNA that migrated between 100 and 350 kb. Transfer the gel slice to a sterile, 15-ml Falcon tube.
4. Proceed with Recovery of the Size-Fractionated DNA in the next step or store the gel slice at 4°C for up to one year.

Recovery of the Size-Fractionated DNA by Electroelution and Dialysis

Electroelute the DNA in each gel section into a dialysis tube (Spectrum, cutoff = 12~14 kD, catalog no. 132676) as follows:

1. Cut a piece of dialysis tube to 5 - 10 cm (~5 cm longer the gel section to be electroeluted). Rinse several times with ice-cold ddH₂O and then with ice-cold 0.5X TBE.

Place the gel section into the tube, close one end of the tube with a membrane tubing closure, fill the tube with ice-cold 0.5X TBE to completely submerge the gel slice in buffer (usually 100 - 300 µl). Remove all bubbles in the tube, and close the other end of the tube.

Submerge the dialysis tube in 0.5X TBE in the CHEF chamber and electroelute the DNA in the gel section by PFGE under the following conditions: 12°C (cooler settings), 80 (pump settings), 120° angle, 6 V/cm, initial pulse time 30 seconds and final pulse time 30 seconds for 4 hrs.

After four hours, turn the dialysis tube 180° and continue to run the tube for exactly one minute to run the eluted DNA off the dialysis tube wall.

If a PFGE apparatus is not available, run the electroelution sample at 30-35 V overnight.

2. Transfer the dialysis tube to ice-cold 0.5X TE for 1 hour. Repeat at least 2 more times. Carefully collect the DNA in the dialysis tube with a cut-off pipet tip into a 1.5 ml microfuge tube.
3. Determine the DNA concentration by fluorimetry. Alternately, estimate the concentration of the DNA by running an aliquot of the DNA on an agarose gel using dilutions of known amounts of the Lambda Control DNA as standard. Note: Measuring the DNA concentration by spectrophotometry (OD₂₆₀) is not recommended because the DNA concentration will not be high enough to be measured accurately. This DNA can be directly used for ligation.

Ligation of the Size-Fractionated DNA with the BAC Cloning Vector

The volume of a BAC library ligation reaction varies from 50 to 500 µl. For a 50 µl reaction, add 25 ng of vector and 100 ng of insert DNA with an average size of 100-200 kb. Set up trial ligations with varying amounts of vector to optimize the vector:insert ratio.

Also, perform a ligation control using 80 ng of the Positive Control Insert DNA in a parallel reaction.

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 (HpaI [blunt], BamHI, EcoRI, or HindIII compatible ends, 100 ng)
y µl H₂O
10.0 µl CopyRight® 5X Ligation Buffer
1.0 µl pSMART BAC Vector (25 ng/µl)
48.0 µl

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2. Add 2 µl CopyRight DNA Ligase, and mix by slowly stirring contents. Incubate at 16 °C overnight for BACs or for 4 hours for smaller DNA fragments.

3. The sample is ready for transformation; heat-denaturation and desalting the DNA are not necessary and are not recommended. The sample may be concentrated if desired.

Transformation of BAC-Optimized Replicator™ v2.0 Electrocompetent Cells

Electroporation conditions and protocols

BAC cloning applications demand the highest transformation efficiency and recovery of the largest clones possible. To ensure optimal cloning results, we strongly recommend the use of Lucigen's BAC-Optimized Replicator v2.0 Electrocompetent Cells. These cells are provided in 50-µl aliquots, sufficient for two transformation reactions. We also recommend use of the Cell Porator and Voltage Booster System by Whatman Biometra. Optimal settings for electroporation are listed below:

Electroporation conditions for BAC-Optimized Replicator v2.0 Cells

Optimal Setting	Alternate Settings (~ 20-50% lower efficiencies)
Cuvette gap: 1.5 mm Voltage: 358 V Capacitance: 330 µF Impedance: Low ohms Charge rate: Fast Voltage Booster Resistance: 4000 ohms	Cuvette gap: 1.0 mm Voltage: 1800 V Capacitance: 25 µF Impedance: 200 ohms
Whatman Biometra: Cell Porator and Voltage Booster System	Eppendorf: Model 2510; Bio-Rad: Gene Pulser II #165-2105; BTX: ECM630
1.5 mm Cuvettes: Whatman Biometra Cat.#11608031	1 mm Cuvettes: Eppendorf Model 4307-000-569, BTX Model 610, or BioRad Cat. #165-2089. Users have reported difficulties using Lucigen cells with Invitrogen cuvettes Cat. # 65-0030.

The following protocols are specific for transformation of BAC-Optimized Replicator v2.0 Electrocompetent Cells. These cells with the above conditions yield $\geq 5 \times 10^7$ cfu/µg of 200 kb BAC DNA.

1. Make YT Agar from powder supplied with the cells in the kit.
2. Have room-temperature Recovery Medium (supplied with the 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.**
3. Place electroporation cuvettes and microcentrifuge tubes on wet ice (one cuvette and one tube for each transformation reaction).
4. Remove BAC-Optimized Replicator v2.0 Cells from the -80°C freezer and thaw on wet ice (10-20 minutes).
5. Add 20 µl of cells to the chilled microcentrifuge tube.

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- Add 1 μl of the CopyRight ligation reaction to the 20 μl of cells on ice. Stir briefly with a pipet tip; **do not** pipette up and down, which can shear the DNA, introduce air bubbles, and warm the cells.
- Carefully pipette 20 μl of the cell/DNA mixture into a chilled electroporation cuvette without introducing bubbles. Electroporate according to the conditions described above.
- Transfer the cells immediately into a culture tube containing 1000 μl of Recovery Medium, and shake gently to resuspend the cells.
- Place the tube in a shaking incubator at 225-250 rpm for 1 hour at 37°C.
- Spread the following amounts of experimental reactions on YT+chl+5% sucrose plates (+XGAL/IPTG if desired). Plate the pAKL control on a YT+kanamycin plate. Incubate the plates overnight at 37°C.

Table 1. Plating Transformed Cells

Reaction Plate	$\mu\text{l}/\text{Plate}$
Experimental Insert (100 ng per ligation)	100
Lambda <i>Bam</i> HI, <i>Eco</i> RI, or <i>Hind</i> III Insert (Positive Control)	100
No-Insert Control (Vector Background)	100
pAKL Supercoiled Plasmid Control (10 pg)	2

Expected Results

The results presented below are expected when cloning 100 ng of intact, purified DNA fragments, with 5' phosphate groups and the appropriate cohesive ends, into Lucigen's BAC-Optimized Replicator™ v2.0 Electrocompetent Cells.

The number of recombinant clones is typically 100-fold greater than the background of self-ligated CopyRight vector. The background number of empty CopyRight vector 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 μl of cells plated, then the 10 colonies obtained from 100 μl of the No-Insert Control ligation will represent a background of 40%.

Use of competent cells with a transformation efficiency of less than 1×10^{10} cfu/ μg will severely compromise the desired results, yielding less than 5% the number of CFUs listed below.

Table 2. Expected Transformation Results from Electroporation

Reaction	CFU/Plate	Efficiency
CopyRight plus 80 ng control Insert	> 200	> 99% inserts
No-Insert Control (Vector Background)	< 2	< 1.0% background
pAKL Supercoiled Plasmid Control (10 pg) (Kan ^R)	> 200	2×10^{10} cfu/ μg

- Using Lucigen's BAC-Optimized Replicator v2.0 Electrocompetent Cells, a CopyRight ligation reaction with 80 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.
- A 100- μl aliquot of the empty vector control reaction should produce <10 colonies, representing less than 1% background.
- A 2- μl aliquot of transformed cells from the supercoiled pAKL reaction (diluted into 90 μl of TB) should yield >200 colonies, or 2×10^{10} colonies per μg plasmid.

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DNA Isolation & Sequencing

Transformants are grown in LB or TB medium plus 12.5 µg/ml chloramphenicol overnight with shaking at 37°C. DNA minipreps can be performed by standard methods.

Copy number amplification of BAC clones requires BAC-Optimized Replicator[™] v2.0 Electrocompetent Cells and Arabinose Induction Solution. Transformants are grown in LB or 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 Arabinose Induction Solution (provided in the Kit). DNA minipreps can be performed by standard methods.

For unstable BACs, first grow the cultures without induction to an OD₆₀₀ of 0.2-0.3. To reach this OD, 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 µl of 1000X Arabinose Induction Solution. Continue growth for 2-3 hours at 37°C with shaking at 225 rpm. Prepare DNA minipreps according to standard protocols.

BAC-Optimized Replicator v2.0 Electrocompetent cells are *recA endA* deficient and will provide high quality plasmid DNA. The CopyRight Kits are provided with the sequencing primers shown in Appendix D.

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Appendix A: Media Recipes

YT + CXI + Sucrose Agar Medium.

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 (6.25 mg of chloramphenicol per 500 ml or 12.5 µg/ml). Also add to a final concentration: X-Gal to 40 µg/ml, IPTG to 0.4 mM. To screen against uncut pSMART BAC vector, add sucrose to 5% (w/v). The pEZ BAC vector does not allow for sucrose selection.

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 is per liter: 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar. More YT Agar may be purchased separately as a 5-pack with catalog number 60025-1.

Note: Use of LB agar medium will result in small, slow-growing colonies.

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 primarily for BAC library construction and for cloning very unstable fragments. Alternately, for cloning fragments of 30 kb or less, Lucigen's BigEasy[®] v2.0 Linear Cloning Kit provides the highest level of plasmid stability.

The BAC-Optimized Replicator[™] v2.0 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.

Kit & Vector Name	BAC	Fosmid	AT-Rich, Unstable (<30 kb)
CopyRight v2.0 BAC Cloning Kit (pSMART [®] BAC BamHI, EcoRI or HindIII)	++	+	+
CopyRight v2.0 BAC Cloning Kit (pEZ BAC BamHI, or blunt)	++	+	+
CopyRight v2.0 Fosmid Cloning Kit (pSMART FOS Blunt)	-	++	+
BigEasy v2.0 Linear Cloning Kit (pJAZZ [®] -OC; pJAZZ-OK Blunt/NotI)	-	-	++

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Appendix C: Abbreviated BAC Cloning Protocol

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

BAC Ligation Reaction

- x μ l Insert DNA (*Bam*HI, *Eco*RI or *Hind*III compatible ends, 100 ng)
- y μ l H₂O
- 10.0 μ l CopyRight 5X Ligation buffer
- 1.0 μ l pSMART[®]-BAC or pEZ BAC vector (*Bam*HI, *Eco*RI or *Hind*III digested, 25 ng/ μ l)
- 48.0 μ l total reaction volume

Add 2 μ l CopyRight DNA Ligase (1U/ μ l), and mix by slowly stirring contents.

3. Incubate overnight at 16 °C and use DIRECTLY for transformation (no purification or heat denaturation).
4. Cool the reactions and spin 1 minute at 12,000 rpm.

BAC Transformation

1. Thaw Recovery Medium at room temperature. Aliquot 500~1000 μ l Recovery Medium to 15ml Falcon tubes.
2. Chill electroporation cuvettes and 1.5 ml microfuge tubes on ice.
3. Thaw the BAC-Optimized Replicator v2.0 Electrocompetent Cells on wet ice. Pipet 20 μ l of cells into a pre-chilled 1.5 ml tube on ice. Add 1 μ l ligation mixture.
4. Pipet the cell/DNA mixture to a chilled electroporation cuvette.
5. Electroporate. Immediately transfer the cell/DNA mixture to 500-1000 μ l of Recovery Medium at room temperature.
6. Shake at 225 rpm for 60 minutes at 37°C.
7. Spread 100 μ l per plate on YT agar plates containing chloramphenicol/ X-Gal/ IPTG/sucrose. Incubate overnight at 37°C.

Colony Growth

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

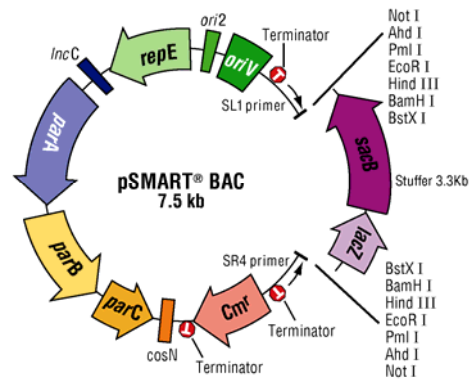
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Appendix D: Vector Maps and Sequencing Primers

The CopyRight vectors are supplied predigested with dephosphorylated ends. Transcription 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 pSMART BAC primers are as follows:

SL1: 5'–CAG TCC AGT TAC GCT GGA GTC–3'
SR4: 5'–TTG ACC ATG TTG GTA TGA TTT–3'

The GenBank accession number for the pSMART BAC v2.0 vector is EU101022.

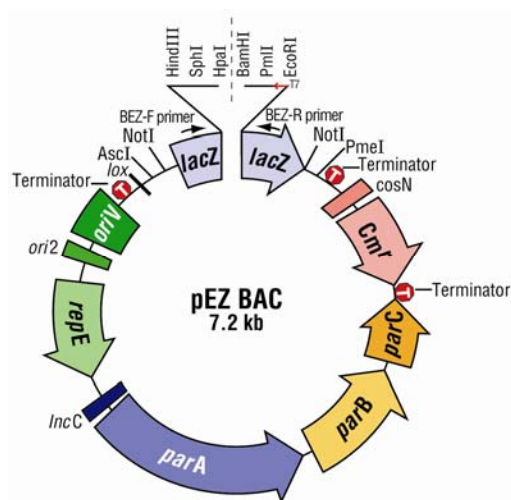


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The sequences of the pSMART BAC primers are as follows:

BEZ F1: 5'-CACTTTATGCTTCCGGCTCGTATG-3'

BEZ-R1: 5'-GGGATGTGCTGCAAGGCGATTAAG -3'



pEZ™ BAC Cloning site

BEZ-F1

CATTAGGCACCCAGGCTTTA**CACTTTATGCTTCCGGCTCGTATG**TTGTGTGGAATTGTGAGCG
GTAATCCGTGGGGTCCGAAATGTGAAATACGAAGGCCGAGCATAACAACACACCTTAACACTCGC

lacZα start

GATAACAATTTACACAGGAAACAGCT**ATG**ACCATGATTACGCCAAGCTATTTAGGTGAGACTA
CTATTGTTAAAGTGTGTCTTTGTCGATACTGGTACTAATGCGTTTCGATAAATCCACTCTGAT

HindIII SphI BsaBI HpaI BamHI PmlI EcoRI

TAGAATACTCAAGCTTGCATGCGATACGTATCGTTAACGATGGATCCGACGCACGTGCGAATTC
ATCTTATGAGTTCGAACGTACGCTATGCATAGCAATTGCTACCTAGGCTGCGTGCACGCTTAAG

GCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTTACAACGTCGTGACTGGGAAAAC
CGGGATATCACTCAGCATAATGTTAAGTGACCGGCAGCAAAATGTTGCAGCACTGACCCTTTTG

T7 Promoter

CCTGGCGTTACCCAACCTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCG
GGACCGCAATGGGTT**GAATTAGCGGAACGTCGTGTAGGG**GGAAAGCGGTTCGACCGCATTATCGC

BEZ-R1

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Appendix E: 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.
	Heat denaturation of ligation reaction.	DO NOT heat BAC ligation mixture. Unlike other Lucigen ligation reactions, heat treatment is NOT recommended for BAC cloning.
	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. 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.
	Unstable DNA Inserts	Use pSMART VC Transcription-Free Blunt Kit for maximum clone stability.
High background of transformants that do not contain inserts.	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.

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Appendix F: Vector Sequences

pSMART® BAC sequence, without stuffer fragment (7648 bp)

*Bam*HI, *Hind*III, and *Eco*RI sites are underlined

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CCACCACAGTGGGATCCGAAGCTTGGAAATTCACGTGACTTGAAGTCGCGGCCGCACTGACCCATAGTGAGTCGTATTAATTTAAATCAT
CCCAACATGGTCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCCTTTCGTTTTAATCTGATCGGCACGTAAGAGGTTCCAACCTTCA
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pEZ BAC Vector Sequence (7256 BP)

HpaI (blunt) and *Bam*HI sites are underlined

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