CopyRight® v2.0 Fosmid Cloning Kits

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE.

Note: Two different storage temperatures required

Containers 1 & 3

Container 2

Lucigen Corporation 2905 Parmenter St, Middleton, WI 53562 USA
Toll Free: (888) 575-9695 | (608) 831-9011 | FAX: (608) 831-9012
lucigen@lucigen.com www.lucigen.com

MA027 Rev. D

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Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the reagents supplied by the user are of the highest quality. Please follow the instructions 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.

Lucigen Technical Support

Email: techserv@lucigen.com

Phone: (888) 575-9695

Product Guarantee: Lucigen guarantees that this product will perform as specified for one year from the date of shipment. Please avoid using reagents for greater than one year from receipt.

Kit Designations

Lucigen offers the CopyRight v2.0 Fosmid Cloning Kit in several different sizes (see Table). Please note that the Kit does NOT contain phage Lambda packaging extract, which must be purchased from other sources. Refer to Appendix B: Application Guide for recommended uses of the kit.

Catalog numbers of CopyRight Fosmid kits

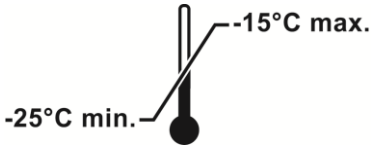
Vector	Reactions	Catalog Number
pSMART® FOS Blunt	5	42027-1
	10	42027-2
	20	42027-3

Note: YT Agar packets are no longer included in this kit

Components & Storage Conditions

The Cloning Components of the CopyRight v2.0 Fosmid Kits are shipped in Container 1, which should be stored at **-20°C**. The glycerol stock of Replicator FOS strain and related components are shipped in Container 2, which must be stored at **-80°C**. The DNATerminator® End Repair Kit is shipped in Container 3, which should be stored at **-20°C**.

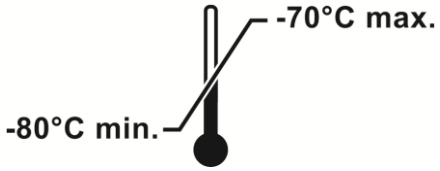
Container 1 must be stored at -20°C



Container 1: CopyRight v2.0 Fosmid Blunt Cloning Components

	5 Reactions	10 Reactions	20 Reactions
CopyRight pSMART FOS Blunt Vector (500 ng/µl)	5 µl	10 µl	2 x 10 µl
CloneDirect™ 10X Ligation Buffer (contains ATP)	100 µl	100 µl	2 x 100 µl
CloneSmart® DNA Ligase (2 U/µl)	12 µl	12 µl	2 x 12 µl
Sequencing Primers (200 reactions each)			
SL1 (3.2 pmol/µl)	200 µl	200 µl	2 x 200 µl
SR4 (3.2 pmol/µl)	200 µl	200 µl	2 x 200 µl

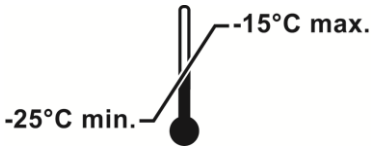
Container 2 must be stored at -80°C



Container 2: Replicator FOS Strain and Components

	5 Reactions	10 Reactions	20 Reactions
Glycerol stock of Replicator FOS strain	1 ml	1 ml	2 x 1 ml
20% (w/v) Maltose	12 ml	12 ml	2 x 12 ml
1M $MgSO_4$	12 ml	12 ml	2 x 12 ml
SM Buffer	12 ml	12 ml	2 x 12 ml
Arabinose Induction Solution (1000X)	1 ml	1 ml	2 x 1 ml

Container 3 must be stored at -20°C



Container 3: DNATerminator® End Repair Kit

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

Kit Description

Fosmid vectors are important tools for positional cloning, physical mapping, and genomic sequencing. In fosmid cloning, DNA fragments of ~35-45 kb are cloned into a fosmid vector and packaged into phage particles *in vitro* by bacteriophage Lambda packaging extract. The phage particles are then used to insert the fosmid DNA into bacteria, which typically maintain it as a single-copy plasmid.

Lucigen's CopyRight v2.0 Cloning system introduces the ultimate in fosmid vector design. Most fosmid vectors yield small amounts of DNA and are prone to instability due to transcriptional interference. The unique design of Lucigen's CopyRight vectors reduces or eliminates transcription both into and out of the insert DNA, reducing the cloning bias found with conventional plasmid and fosmid vectors. The CopyRight vectors also incorporate a *lac*Z marker and a direct selection gene to completely eliminate the background of uncut vector. However, unlike other vectors, the marker and direct selection genes, as well as their promoters, are completely removed from the final vector preparation, eliminating the risk of clone loss.

CopyRight kits further feature inducible amplification of copy number, increasing yield to as many as 50 copies per cell. CopyRight amplification permits easy isolation of plasmid DNA for sequencing, subcloning, or restriction mapping. The combination of inducible copy number amplification and

Lucigen's patented transcription-free cloning technology provides maximum stability of otherwise unclonable sequences.

The pSMART® FOS vector, in conjunction with the Replicator FOS strain, incorporates controllable genetic elements that allow the single copy fosmid to be amplified *in vivo*. The single-copy state is controlled by the *ori*2 (*ori*S) origin of replication, *rep*E gene, and *par*ABC partition loci. Induction of the gene for TrfA replicator protein, incorporated into the genome of Replicator FOS cells, activates the medium-copy origin of replication (*ori*V), and plasmid accumulates up to 50 copies per cell (Figure 1).

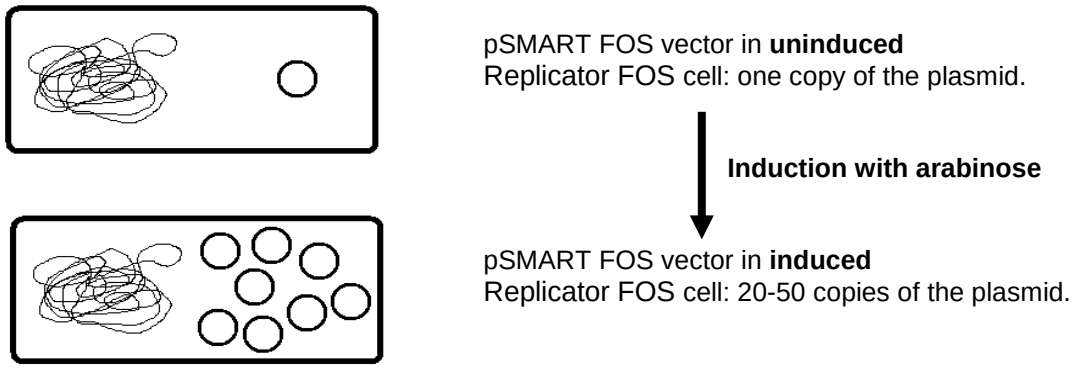


Figure 1. Replicator FOS cell with the pSMART FOS vector. Addition of Arabinose Induction Solution results in amplification of the vector copy number.

CopyRight v2.0 Fosmid Kits contain the pSMART FOS blunt cloning vector, buffer, DNA ligase, sequencing primers, and induction solution. The pSMART FOS vectors are pre-cut and dephosphorylated, eliminating the need for vector preparation. The kit does NOT provide Lambda packaging extract. Suitable Lambda packaging extract is available from Stratagene (see "Materials and Equipment Needed" section below).

pSMART® FOS Vectors

The CopyRight v2.0 pSMART FOS cloning vector incorporates transcription-free cloning for the highest stability possible. The pSMART FOS vector is supplied pre-cut and dephosphorylated at the blunt *Pml*I site. Insert DNAs with blunt 5'-phosphorylated ends are ligated to the pSMART FOS vector, packaged, and transfected into Lucigen's Replicator FOS strain.

The unprocessed pSMART® FOS vector has a *lac*Z/*sac*B stuffer region flanked by dual cloning sites (Figure 2). The *sac*B gene, which encodes the *Bacillus subtilis* levansucrase, is lethal to *E. coli* in the presence of 5% sucrose. Therefore, traces of uncut vector can be selected against by plating on sucrose. In the absence of sucrose, they can be detected by blue/white screening. Complete elimination of the empty-clone background is a great benefit for automated robotic colony picking and arraying. Moreover, unlike other cloning vectors, both the promoter and the coding sequence of the stuffer fragment are completely removed during processing. This design prevents active expression of the insert DNA by the *lac*Z or *sac*B promoter, contributing greatly to plasmid stability, especially for inserts containing toxic coding sequences, strong secondary structure, or other deleterious features. To further protect against unwanted transcription, the pSMART FOS vector has the chloramphenicol promoter facing away from the cloning site.

Transcription from cloned promoters is another source of clone gaps in conventional plasmids. Cloned promoters can express the *lac*Z or *sac*B genes in the conventional vectors, leading to loss of

the recombinant plasmid. 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 terminators on either side of the cloning site to prevent this interference.

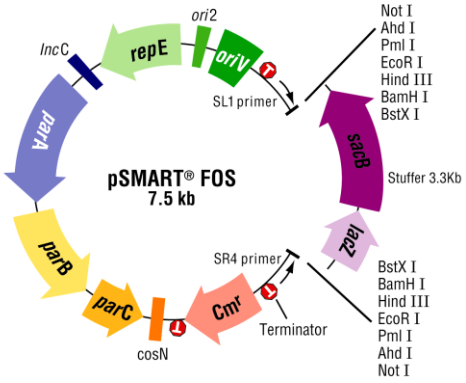


Figure 2. CopyRight v2.0 vector pSMART FOS. sacB - sucrase gene; lacZ - alpha peptide of beta galactosidase; Cm^r - chloramphenicol resistance gene; ori2 - single copy origin of replication; oriV - inducible origin of replication; par A,B,C- partition genes; cosN - lambda packaging signal; T - transcription terminators. Approximate positions of sequencing primers are indicated.

The CopyRight v2.0 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
- *lac*Z/*sac*B stuffer that is completely removed for minimal background and no bias
- Bacteriophage Lambda *cos* site for Lambda packaging or terminase cleavage
- *lox*P site for Cre-recombinase recognition
- Rare-cutting restriction sites on either side of insert
- Chloramphenicol resistance gene

Replicator™ FOS Strain

Lucigen's Replicator FOS strain contains an inducible *trf*A gene, which is required for amplification of the pSMART FOS clones to high copy number. Most lab strains of *E. coli* do not contain a *trf*A gene, and thus will not support copy number amplification of the CopyRight v2.0 vectors.

Replicator FOS cells are ideal for cloning and propagation of fosmid or plasmid clones. In addition to the *trf*A gene for induction of the *ori*V origin, they contain the *end*A1 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. The *rps*L mutation confers resistance to streptomycin.

Replicator FOS genotype: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *end*A1 *rec*A1 ϕ80d*lac*ZΔM15 Δ*lac*X74 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K *rps*L (Str^R) *nup*G (*att*L *ara*C-P_{BAD}-*trf*A250 *bla att*R) λ^-

Purification of Large DNA

Lucigen has optimized the preparation and design of the CopyRight v2.0 vectors. However, 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 into CopyRight v2.0 vectors.

1. Do not pipette HMW DNA with standard pipette tips. Fosmid libraries require an average insert size of 35-45 kb. High molecular weight DNA such as this is readily sheared by pipeting. The use of wide bore pipette tips will minimize unwanted shearing. Purification and fragmentation of high molecular weight DNA can be accomplished by any of several different methods (Refs 1, 5, 7, 8, 11, 12).

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

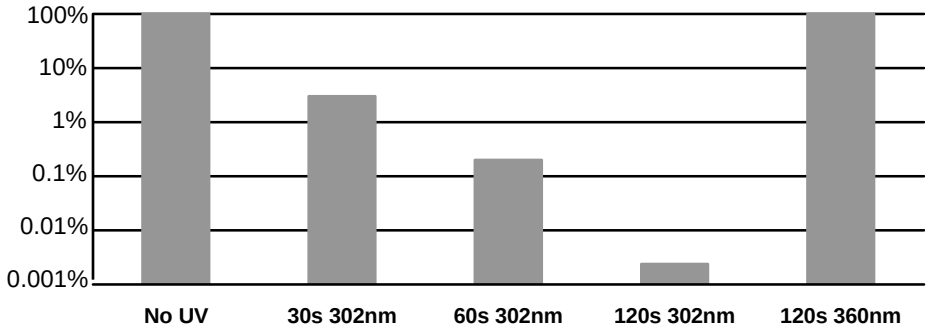


Figure 3. Relative cloning efficiency of pUC19 after exposure to short wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 90 seconds ("30s 302nm, 60s 302nm,120s 302nm"). Cloning efficiencies were calculated relative to un-irradiated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

The wavelength of most UV transilluminators, even those designated specifically for DNA visualization, is typically 302 nm or 312 nm. This light can cause significant damage to DNA. Visualize DNA only with a handheld UV lamp of 360 nm wavelength, or use other methods that do not rely on UV illumination (Reference 11).

Time Course of Fosmid Cloning

Fosmid cloning requires several days of preparation. An overview of the protocol is listed below:

1. **Two days before** packaging: Streak out the Replicator FOS strain.
2. **One day before** packaging: Use a colony from the streak plate to start an overnight culture of the Replicator FOS cells.
3. On the **day of packaging**, prepare the cells for transfection.
4. Purify high molecular weight (HMW) genomic DNA.
5. Partially digest or randomly shear the HMW DNA to 35-75 kb.
6. End-repair sheared DNA using the provided DNATerminator® reagents.
7. Size select DNA by PFGE.
8. Test ligations of genomic and vector DNA.
9. Package fosmid ligation using Lambda packaging extract (NOT included).
10. Infect *E. coli* Replicator FOS Cells.
11. Plate on selective media.
12. Assay transformants for insert size and background.
13. Repeat until acceptable and scale up.

Protocols specific to the ligation of fosmid libraries into the pSMART FOS vector are provided below. Detailed protocols for manipulating HMW DNA, fosmid/BAC cloning, and handling fosmid/BAC recombinant clones can be found in references 5-12.

Materials and Equipment Needed

Successful use of the CopyRight Fosmid Kit requires proper planning for each step. Please read the entire manual and prepare the necessary equipment and materials before starting.

The CopyRight v2.0 Fosmid Cloning Kit supplies many of the items needed to ligate the DNA inserts into the pSMART FOS vector. However, it does NOT contain the lambda packaging extract. The following items are required for fosmid cloning:

- **Dialysis tubing** (MW cutoff 12~14 kD, Spectrum, catalog # 132676) and the tube closures.
- **Lambda packaging extract.** The following packaging kits from Stratagene are recommended:
  Catalog #200201, #200202, or #200203 (Gigapack® III Gold-4, -7, or -11);
  Catalog #200204, #200205, or #200206 (Gigapack III Plus-4, -7, or -11);
  Catalog #200207, #200208, or #200209 (Gigapack III XL-4, -7, or -11).
  Packaging extracts from other manufacturers may be used, but their efficiency may be lower.
- **Sterile 17 x 100 mm culture tubes.**
- **LB medium.**
- **YT agar plates** with no antibiotic**.**
- **YT+CXIS agar plates** containing Cam, XGAL, IPTG, and sucrose (Appendix A for recipes).

Preparing the Replicator FOS strain

1. Prepare YT Agar plates. (Colonies do not grow as well on LB.)
2. The Replicator FOS strain is supplied as a glycerol stock. At least two days before packaging, streak the Replicator FOS cells onto a YT plate with NO antibiotic.
3. On the day before packaging, pick a single colony of Replicator FOS Cells into 5 ml of LB + Maltose + $MgSO_4$ (see Appendix A for recipe). Shake overnight at 37°C.
4. On the day of packaging, dilute 0.5 ml of the overnight culture of Replicator FOS Cells into 50 ml of LB broth+maltose+$MgSO_4$. Shake at 37°C to OD_{600} = 0.8-1.0. Pellet the bacteria at 500 x g for 10 minutes, gently resuspend the cells in one third of the original volume with LB + Maltose + $MgSO_4$, and store the cells at 4°C until needed (up to 3 days).

Preparation of Target DNA

Random Shearing

Fosmid cloning requires insertion of DNA fragments of 35-45 kb. Random shearing is preferred over partial restriction digestion, because it is not biased by the frequency of restriction sites. Hydrodynamic shearing (e.g., HydroShear® device, Genomic Solutions) is the most reliable and efficient method of random shearing (Figure 4). Repeated pipeting of the DNA may also yield fragments in the desired size range. Typically, 10~30 μg of HMW genomic DNA is sheared.

After shearing, run an aliquot of the DNA on a pulsed field gel electrophoresis apparatus (PFGE), along with size and mass standards, to measure size and concentration. The following PFGE conditions are suggested: 1% PFGE agarose or low melting temperature agarose (without ethidium bromide), 0.5X TBE, 6V/cm, 0.1 - 2s second ramp time, 11 hrs, 120° angle, and 12 °C. Stain the gel after electrophoresis to visualize the test sample. Consult the instructions of the PFGE apparatus for other specific recommendations. Fractionation on a standard 0.7% agarose gel (1 V/cm, 16 hours) can be used in place of a PFGE apparatus, but resolution of HMW DNA will be greatly reduced.

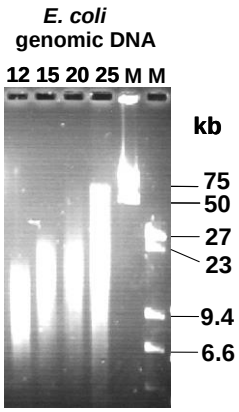


Figure 4. Hydrodynamic shearing of HMW genomic DNA. *E. coli* DNA was sheared using the HydroShear apparatus with the large pore orifice. Lanes 1-4 show results with various shear settings. The setting of 25 produced optimal fragmentation of 10-100 kb. Lanes 5 and 6 are DNA size markers ("M").

End-Repair of the Fragmented DNA

After shearing, the DNATerminator End Repair reaction is performed to create blunt ends with 5´ phosphate groups for ligation into the blunt, dephosphorylated vector. Perform the DNATerminator® End Repair reaction using the supplied reagents as follows:

1. Thaw and briefly vortex the DNATerminator Buffer. Set up the end-repair reaction:

y	µl sheared insert DNA (~10 µg)
10	µl 5X DNATerminator End Repair Buffer
x	µl sterile water
2	µl DNATerminator End Repair Enzyme
50	µl

2. Incubate at 37°C for 30 minutes.

Note: For less than 1 µg of DNA, use 1 µl of DNATerminator Enzyme and reduce incubation time to 15 minutes.

3. Incubate at 70°C for 15 minutes to inactivate the DNATerminator Enzyme.

Size Selection of the End-Repaired DNA

Size select the insert DNA by using the electrophoresis conditions determined above.

1. 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 end-repaired DNA in a trough between the DNA marker lanes.

2. After electrophoresis, excise the marker lanes along with 1-2 mm of the edge of the region of the gel containing the sample. Stain the marker lanes with ethidium bromide. View ONLY the marker lanes on a UV light box, and mark the positions of the 30-kb and 75-kb DNA bands. DO NOT expose the gel containing the bulk of the sample to ethidium bromide or UV light!

3. Re-position the marker lanes adjacent to the sample DNA, and excise the sample in the region that migrated between 30 and 75 kb.

Recovery of the Size-Fractionated DNA by Electroelution or β-Agarase treatment

Method 1: Electroelution

1. Electroelute the DNA in each gel slice into a dialysis tube as follows:

2. Cut the dialysis tubing to 5 - 10 cm, rinse with ice-cold ddH_2O four times, and then with ice-cold 0.5X TBE.

3. Place the gel section containing the size-selected DNA fragments 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 the buffer. Remove any bubbles and excess TBE from the tube (leaving 100 - 300 µl of TBE), and close the other end of the tube.

4. Submerge the dialysis tube in 0.5X TBE in the PFGE chamber and electroelute the DNA under the following conditions: 12°C, pump speed = 80, 120° angle, 6 V/cm, pulse time 30 seconds, 4 hours. If a PFGE apparatus is not available, run the electroelution sample at 30-35 V for 5 hours or overnight.

5. After 4 hours, rotate the dialysis tube 180°, and continue to run the electroelution for exactly one minute to elute the DNA off the inside of the dialysis tube wall.

6. The DNA eluted from the gel slice can be directly precipitated with 1 volume of isopropanol and 0.3 M NaOAc. Mix thoroughly and set at room temperature for 10 minutes. Centrifuge at 15,000 X g for 10 minutes.

7. Remove the supernatant, wash the pellet with cold 70% isopropanol, and dry the pellet at room temperature.

8. Gently resuspend the DNA pellet in TE.

9. Estimate the concentration of the DNA by running an aliquot of the DNA on an agarose gel using DNA mass standards. The OD_{260} will be too low to obtain an accurate measurement by spectrophotometry. This DNA can be directly used for ligation.

Method 2: β-Agarase treatment

Kit needed: β-Agarase I and β-Agarase 10X Buffer (NEB catalog no. M0392S).

Preparation: before beginning this step, prepare a 70°C and a 42 °C water bath.

1. Weigh the microfuge tubes to determine the weight of the gel slice(s).

2. Add 1/10 volume of 10X β-Agarase I Reaction Buffer and melt together with the agarose by incubating at 65°C for 10 minutes. Cool to 42°C, add 1 unit of β-Agarase I, and digest at 42°C for 60 minutes. This procedure will digest up to 200 µl of 1% low melting point agarose. For larger volumes, adjust enzyme accordingly.

3. Heat inactivate the β-Agarase reaction at 70°C for 10 minutes.

4. Adjust the salt concentration of the β-Agarase I-treated solution to 0.3 M NaOAc. Chill on ice for 15 minutes.

5. Centrifuge at 15,000 X g for 15 minutes to pellet any remaining undigested carbohydrates.

6. Remove the supernatant containing the DNA. Add 1 volume of isopropanol. Mix thoroughly and set at room temperature for 10 minutes. Centrifuge at 15,000 X g for 10 minutes to precipitate the DNA.

7. Remove the supernatant, wash the pellet with cold 70% isopropanol, and dry the pellet at room temperature.

8. Gently resuspend the DNA pellet in TE buffer.

9. Estimate the concentration of the DNA by running an aliquot on an agarose gel using DNA mass standards. The OD_{260} will be too low to obtain an accurate measurement by spectrophotometry. The DNA can be directly used for ligation.

Ligation to the pSMART FOS Cloning Vector

A typical fosmid blunt ligation reaction is carried out in a volume of 10 µl. It contains 500 ng of vector and 250 ng of insert DNA with an average size of 30-75 kb. 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.

Blunt-end ligation reaction:

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

x µl Blunt insert DNA (250 ng)
1.0 µl CloneDirect™ 10X Ligation buffer
1.0 µl pSMART® FOS blunt vector (500 ng/µl)
y µl H_2O
1.0 µl CloneSmart® DNA Ligase
10.0 µl

2. Mix contents by stirring slowly. Incubate ~2-3 hours at room temperature.
3. **Heat denature the reaction 15 minutes at 70°C.**
4. Cool the reaction and spin 30 seconds at 13,000 rpm to collect the DNA sample.

Fosmid Packaging and Library Production

Packaging the pSMART FOS Fosmid Clones
Package the fosmid ligation reaction according to the packaging extract instructions. Use 4 - 10 µl of ligation + packaging extracts.

After incubation of the packaging reaction, add Phage Dilution Buffer (SM Buffer) to 0.5 ml final volume and mix gently. Add 20 µl of chloroform to each tube, mix gently, and store at 4°C. Components of the packaging extract may precipitate upon addition of the chloroform. Allow the precipitate to settle. The phage particles will remain suspended in the solution. They are stable at 4°C for several months.

Preparing the Replicator™ FOS strain
Refer to p. 8 for preparation of the Replicator FOS Cells for transfection with the packaged phage.

Titering the Packaged CopyRight Fosmid Clones
Before performing a large-scale transfection into Replicator FOS Cells, determine the titer of the packaged fosmid phage particles.

1. Dilute the phage particles 1:10 and 1:100 into SM Buffer (supplied in the Kit) in sterile microfuge tubes.
2. In a 15-ml culture tube, add 10 µl of each phage particle without and with dilution to 100 µl of the prepared Replicator FOS Cells separately. Shake at 225 rpm for 20 minutes at 37°C.
3. Plate the transfected Replicator FOS Cells on YT+CXIS plates. Incubate overnight at 37°C.
4. Calculate the packaged phage titer.

Plating and Selecting the CopyRight Fosmid Library
After performing the titering reaction, dilute the packaged phage if necessary to provide the desired number of colonies. Transfect as above, using 10 µl of packaged phage with or without dilution per 110 µl of infection reaction. And plate the transfected cells as above.

DNA Isolation & Sequencing

Fosmid clones are grown in TB medium plus 12.5 µg/ml chloramphenicol. Stable inserts can be grown overnight with shaking at 37°C in the presence of 1X Arabinose Induction Solution (supplied in the Kit). DNA minipreps can be performed by standard methods.

For unstable inserts, it may be necessary to grow the cultures without induction to an OD_{600} 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 at 225 rpm for 30 minutes. For each ml of culture, add 1 µl of 1000 X Arabinose Induction Solution. Continue growth for 2-3 hours at 37 °C with shaking at 225 rpm. Prepare DNA minipreps according to standard protocols.

The Replicator FOS Cells are *recA endA* deficient and will provide high quality plasmid DNA. The CopyRight v2.0 Kits are provided with sequencing primers SL1 and SR4 for the pSMART® FOS vector. The sequence and location of primers are shown in Appendix D.

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Appendix A: Media Recipes

YT Agar Medium for streaking stock culture. Prepare YT agar, autoclave and cool to 55°C. Add the appropriate filter-sterilized antibiotic to the cooled medium.

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.

YT+CXIS for Selecting Transformants. Prepare YT Agar medium as described above. After cooling to 55°C, add chloramphenicol to a final concentration of 12.5 μg/ml, X-Gal to 40 μg/ml, IPTG to 0.4 mM, and sucrose to 5% (w/v). Pour into petri plates.

LB broth for growth of stock culture. Per liter: 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl.

LB + Maltose + $MgSO_4$. Per 50 mls: Add 0.5 mls 20% (w/v) maltose (supplied) to a final concentration of 0.2%. Add 0.5 mls of 1M $MgSO_4$ (supplied) to a final concentration of 10 mM.

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. 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 CopyRight v2.0 Fosmid Cloning Kit is designed for fosmid cloning of 35-45 kb inserts. The CopyRight BAC Cloning Kit is designed for BAC cloning of inserts up to 400 kb. For cloning very unstable fragments of 30 kb or less, Lucigen's BigEasy™ v2.0 Linear Cloning Kit provides the highest level of plasmid stability. Lucigen's CloneSmart series of cloning kits offers the best options for cloning inserts of 10 kb or less. They are particularly useful for shotgun library construction or for sub-cloning from fosmid or BAC vectors.

Appendix C: Abbreviated Fosmid Cloning Protocol

Preparation of Cells

1. Two days before transfection: Streak Replicator FOS Cells on YT plates (without antibiotic).
2. One day before transfection: Start an overnight culture in LB+0.2% Maltose+10 mM $MgSO_4$.

Fosmid Ligation Reaction

1. Combine the following components in a 1.5-ml tube. Add ligase last.

x μl Blunt Insert DNA (250 ng)
y μl H_2O
1.0 μl CloneDirect™ 10X Ligation buffer
1.0 μl pSMART® FOS Blunt Vector (500 ng/μl)
1.0 μl CloneSmart® DNA Ligase
10.0 μl total volume

2. Incubate 2~3 hours at room temperature.
3. **Heat denature the reaction 15 minutes at 70°C.**
4. Cool the reactions and spin briefly to collect the DNA sample at tube bottom.

Packaging the pSMART FOS Fosmid Clones

1. Package reaction according the Lambda packaging extract manual.

2. To each packaging reaction (4 - 10 µl ligation + packaging extracts), add SM Buffer to 0.5 ml final volume and mix gently. Add 20 µl of chloroform to each. Mix gently and store at 4°C.

Titering the Packaged pSMART FOS Fosmid Clones

1. Dilute packaged phage 1:10 and 1:100 in SM Buffer.
2. Add 10 µl of each phage particle without and with dilution to 100 µl of the prepared Replicator FOS Cells separately. Shake at 225 rpm for 20 minutes at 37°C.
3. Plate on YT+CXIS.
4. Calculate the titer.

Plating and Selecting the CopyRight Fosmid Library

Transfect additional phage without or with dilutions to yield desired number of clones.

Colony Growth

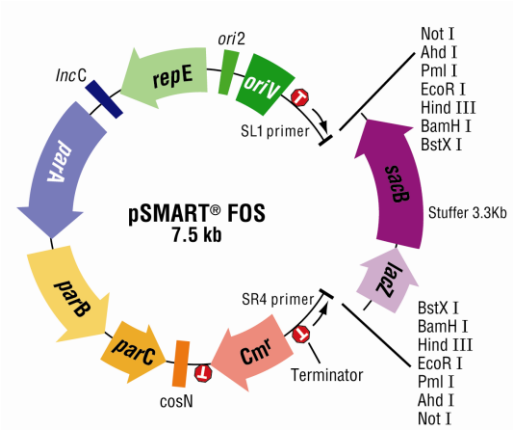
1. Pick colonies and grow in TB + chloramphenicol.
2. Add Induction solution to cultures when OD_{600} reaches 0.2-0.3.
3. Grow 3 hours at 37°C with vigorous shaking.

Appendix D: Vector Map and Sequencing Primers

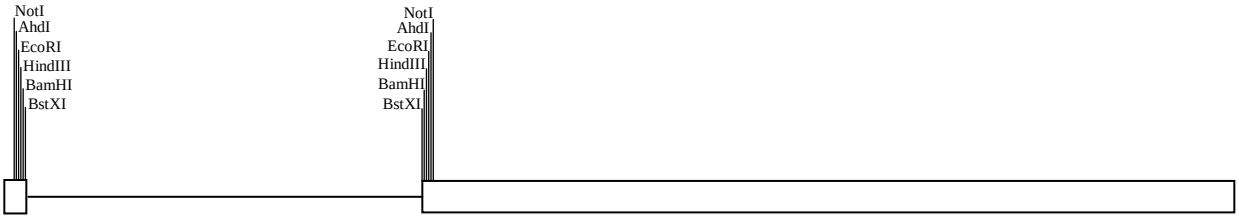
The pSMART® FOS vector is supplied predigested with dephosphorylated ends. Transcription terminators border the cloning site to prevent transcription from the insert into the vector. The sequences of the pSMART FOS primers are as follows:

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

The sequences of the CopyRight v2.0 vectors are available from Lucigen. GenBank accession number will be released shortly.



Appendix E: Cloning Sites of pSMART FOS vector



SL1 Primer → SP6 Promoter

GGCCTAACTGTGGC**CAGTCCAGTTACGCTGGAGTC**ACTAG*TATTTAGGTGACACTATAGA*
CCGGATTGACACCGGTCAGGTCAATGCGACCTCAGTGATCATAAATCCACTGTGATATCT

NotI AhdI PmlI EcoRI HindIII BamHI BstXI

AGCGGCCGCGACAACTTGTCCACGTGGAATTCTAAGCTTAGGATCCCAATGTGATGGCTA...
TCGCCGGCGCTGTTGAACAGGTGCACCTTAAGATTCGAATCCTAGGGTTACACTACCGAT...

.....*sac*B - *lac*Z Stuffer (3.3 kb).....

BstXI BamHI HindIII EcoRI PmlI AhdI NotI

ATCCACCACAGTGGGATCCGAAGCTTGGAATTCACGTGACTTGAAGTCGCGGCCGCACTG
TAGGTGGTGTCACCCTAGGCTTCGAACCTTAAGTGCACTGAACTTCAGCGCCGGCGTGAC

ACCCTATAGTGAGTCGTATTAATTTAAATCATACCAACATGGTCAAATAAAACGAAAGGC
T*GGGATATCACTCAGCATAAT*TAAA**TTTAGTATGGTTGTACC**

T7 Promoter ← SR4 Primer ←

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.
	No heat denaturation of ligation reaction.	Heat treatment of the ligation reaction at 70°C for 15 minutes is necessary for fosmid cloning.
	Loss of DNA during precipitation.	DO NOT precipitate DNA after ligation reaction. It is not necessary with this protocol and these cells.
	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	Clone smaller inserts (<30 kb) using Lucigen's pJAZZ®-OC linear vector (BigEasy™ v2.0 Kit).
	Contaminating DNA, oligos, or linkers in ligation reaction.	Purify DNA after partial digestion or end repair.
	Loss of efficiency of the packaging kit	The packaging kit is very sensitive to temperature and repeat thaw-frozen cycles. ALWAYS keep the kit at -80C. DO NOT thaw the package extract more than twice. Use a positive control to confirm the efficiency of an old packaging extract.
	Poor infection of Replicator FOS strain	Grow the Replicator FOS strain properly and to the correct OD. Use an isolated colony to start the overnight culture.

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