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EpiFOS™ Fosmid Library Production Kit

Cat. No. FOS0901

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1. Overview of the EpiFOS Fosmid Library Production Process

Fosmid vectors[1-3] provide an improved method for cloning and the stable maintenance of cosmid-sized (35-45 kb) libraries in *E. coli*. The stability of such large constructs *in vivo* is facilitated by the pEpiFOS™-5 vector that maintains the clones at single copy in the cell.

The EpiFOS Fosmid Library Production Kit will produce a complete and unbiased primary fosmid library. The kit utilizes a novel strategy of cloning randomly sheared, end-repaired DNA. Shearing the DNA leads to the generation of highly random DNA fragments in contrast to more biased libraries that result from fragmenting the DNA by partial restriction digests.

The steps involved (protocols for steps 2-7 are included in this manual):

1. Purify DNA from the desired source (the kit does not supply materials for this step).
2. Shear the DNA to approximately 40-kb fragments.
3. End-repair the sheared DNA to blunt, 5'-phosphorylated ends.
4. Size-resolve the end-repaired DNA by Low Melting Point (LMP) agarose gel electrophoresis.
5. Purify the blunt-end DNA from the LMP agarose gel.
6. Ligate the blunt-end DNA to the Cloning-Ready pEpiFOS-5 vector.
7. Package the ligated DNA and plate on EPI100™-T1^R Plating Strain. Grow clones overnight.

pEpiFOS-5 is a 7518 bp. fosmid cloning vector which is provided Cloning-Ready - linearized at the unique *Eco*72 I (blunt) restriction enzyme recognition site, dephosphorylated and rigorously tested for purity and recombinant cloning efficiency. Features of the vector include:

1) Chloramphenicol-resistance as an antibiotic selectable marker.
2) *E. coli* F factor-based partitioning and copy number regulation system.
3) Bacteriophage lambda *cos* site for lambda packaging or lambda-terminase cleavage.
4) Bacteriophage P1 *lox*P site for Cre-recombinase cleavage.
5) Bacteriophage T7 RNA polymerase promoter flanking the cloning site.
6) Fosmid end-sequencing primers are available separately.

A pEpiFOS-5 Vector map and complete restriction table is provided in Appendix E.

2. Kit Contents

Desc.	Concentration	Quantity
Reagents included in the kit are sufficient to construct 10 fosmid libraries.		
pEpiFOS™-5 Fosmid Vector: (Cloning-Ready; linearized at the unique *Eco*72 I site and dephosphorylated)	(0.5 µg/µl)	20 µl
End-Repair 10X Buffer: (330 mM Tris-acetate [pH 7.5], 660 mM potassium acetate, 100 mM magnesium acetate, 5 mM DTT)		100 µl
dNTP Mix: (2.5 mM each of dATP, dCTP, dGTP, dTTP)		100 µl
End-Repair Enzyme Mix: (including T4 DNA Polymerase and T4 Polynucleotide Kinase)		50 µl
ATP Solution	(10 mM)	100 µl
Fast-Link™ DNA Ligase	(40 U @ 2 U/µl)	20 µl
Fast-Link™ 10X Ligation Buffer		100 µl
GELase™ Enzyme Preparation	(25 U @ 1 U/µl)	25 µl
GELase™ 50X Buffer: (2.0 M Bis-Tris [pH 6.0], 2.0 M NaCl)		200 µl
***Fosmid Control DNA**	(100 ng/µl)	50 µl
EPI100™-T1^R Phage T1-Resistant *E. coli* Plating strain: glycerol stock [F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ– *rps*L *nup*G *ton*A]		250 µl
MaxPlax™ Lambda Packaging Extracts		10 Extracts
Ligated Lambda Control DNA: (λcI857 *Sam*7)	(1 µg @ 0.02 µg/µl)	50 µl
LE392MP *E. coli* Control strain: glycerol stock [F– e14–(McrA–) Δ(*mcr*C-*mrr*) (TetR) *hsd*R514 *sup*E44 *sup*F58 *lac*Y1 or Δ(*lac*IZY)6 *gal*K2 *gal*T22 *met*B1 *trp*R55 λ–]		250 µl

Note: *MaxPlax Lambda Packaging Extracts are supplied as freeze-thaw/sonicate extracts in unlabeled single tubes. The extracts, Ligated Lambda Control DNA, and LE392MP Control Plating Strain are packaged together in a CO2-impermeable foil pouch.*

Storage: Store the EPI100-T1^R Plating Strain and the MaxPlax Lambda Packaging Extracts at –70°C. Exposure to higher temperatures will greatly compromise packaging extract efficiency. Once the MaxPlax Packaging Extracts are opened, do no expose them to dry ice. Store the remainder of the kit components at –20°C. After thawing, store the Ligated Lambda Control DNA at 4°C.

**The "T7 DNA Size Marker" and the "Fosmid Control Insert DNA" have been replaced with a single tube of "Fosmid Control DNA". The Fosmid Control DNA is used both as a size marker and as a control insert for fosmid library production. The amount of DNA used for sizing and library production remains the same.*

Additional Required Reagents

In addition to the component supplied, the following reagents are required:

LB broth + 10 mM $MgSO_4$
LB plates + 12.5 µg/ml chloramphenicol
Low melting point (LMP) agarose
Ethanol
3 M Sodium Acetate (pH 7.0)
Phage Dilution Buffer (10mM Tris-HCl [pH 8.3], 100mM NaCl, 10mM $MgCl_2$)
TE Buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA)

Quality Control

The EpiFOS Fosmid Library Production Kit is function-tested using the Fosmid Control DNA provided in the kit. Each kit must yield >10^7 cfu/µg of the Fosmid Control DNA. The MaxPlax Lambda Packaging Extracts have been tested to give >10^8 pfu/µg of Ligated Lambda Control DNA.

3. EpiFOS Fosmid Library Production Protocol

General Considerations

1. ***Important!*** *Users should avoid exposing DNA to UV light. Even exposure for short periods of time can decrease the efficiency of cloning by two orders of magnitude or more. Two methods for excising DNA from an LMP agarose gel without exposure to UV are presented in Part C.*
2. The 36-kb Fosmid Control DNA supplied in the kit is used as a size marker for agarose gel size-fractionation of the DNA to be cloned.
3. The Ligated Lambda Control DNA (λcI857 Sam7) and the Control Strain LE392MP are used to test the efficiency of the MaxPlax Lambda Packaging Extracts (see Appendix C).

Preparation

1. Prepare high molecular weight genomic DNA from the organism using standard methods.[4] Resuspend the DNA in TE buffer at a concentration of about 0.5 µg/µl. This DNA will be the source of the clones and will be referred to as the "insert DNA" throughout this manual.
2. The EPI100-T1^R Plating Strain is supplied as a glycerol stock. Prior to beginning the Fosmid Library Production procedure, streak out the EPI100™-T1^R cells on an LB plate. Do not include any antibiotic in the medium. Grow the cells at 37°C overnight and then seal and store the plate at 4°C. The day before the Lambda Packaging reaction (Part F), inoculate 50 ml of LB broth + 10 mM $MgSO_4$ with a single colony of EPI100™-T1^R cells and shake overnight at 37°C.

A. Shearing the Insert DNA

Kit component used in this step: **Fosmid Control DNA.**

Shearing the DNA into approximately 40-kb fragments leads to the highly random generation of DNA fragments in contrast to more biased libraries that result from partial restriction endonuclease digestion of the DNA. Frequently genomic DNA is sufficiently sheared, as a result of the purification process, that additional shearing is not necessary. Test the extent of shearing of the DNA by first running a small amount of it by Pulse Field Gel Electrophoresis (e.g., Field Inversion Gel Electrophoresis [FIGE], CHEF, etc.) with voltage and ramp times recommended by the manufacturer for separation of 10-100 kb DNA. If a PFGE apparatus is not available, run the sample on a 20-cm long, 1% agarose gel at 30-35 V overnight. Load 100 ng of the Fosmid Control DNA in an adjacent gel lane. Run the gel and stain with ethidium bromide.

If 10% or more of the genomic DNA migrates with the Fosmid Control DNA, then the user can proceed to Part B. If the genomic DNA migrates slower (higher MW) than the Fosmid Control DNA, then the DNA needs to be sheared further as described in Part A, Step 1 below. If the genomic DNA migrates faster than the Fosmid Control DNA (lower MW) then it has been sheared too much and should be reisolated.

1. We recommend that at least 2.5 µg of DNA be used. Randomly shear the DNA by passing it through a 200-µl small bore pipette tip. Aspirate and expel the DNA from the pipette tip 50-100 times. Examine 1-2 µl of the DNA on a 20-cm agarose gel using the Fosmid Control DNA. If 10% or more of the genomic DNA migrates with the Fosmid Control DNA, then the user can proceed to Part B. If the DNA is still too large, aspirate and expel the DNA from the pipette tip an additional 50 times. Examine 1-2 µl of this DNA by agarose gel as described previously.

B. End-Repair of the Insert DNA

Kit components used in this step: **End-Repair Enzyme Mix and 10X Buffer, dNTPs and ATP**.

This step generates blunt-ended, 5′-phosphorylated DNA. The end-repair reaction can be scaled up or scaled down as dictated by the amount of DNA available.

1. Thaw and thoroughly mix all of the reagents listed below before dispensing; place on ice. Combine the following on ice:

x	µl	sterile water
8	µl	End-Repair 10X Buffer
8	µl	2.5 mM dNTP Mix
8	µl	10 mM ATP
up to 20	µg	sheared insert DNA (approximately 0.5 µg/µl)
4	µl	End-Repair Enzyme Mix
80	µl	Total reaction volume

2. Incubate at room temperature for 45 minutes.
3. Add gel loading buffer and incubate at 70°C for 10 minutes to inactivate the End-Repair Enzyme Mix. Proceed with **Size Selection of the End-Repaired DNA** in Part C.

C. Size Selection of the End-Repaired DNA

Kit components used in this step: **Fosmid Control DNA.**

Size select the end-repaired DNA by low melting point **(LMP)** agarose gel electrophoresis. Ideally, use Pulse Field Gel Electrophoresis (e.g., FIGE, CHEF, etc.) with voltage and ramp times recommended by the manufacturer for separation of 10- to 100-kb DNA. If a PFGE apparatus is not available, run the sample on a 20 cm long, 1% agarose gel at 30-35 V overnight. **Minigels (e.g., 10 cm) do not provide sufficient resolution of DNA in the 20- to 60-kb size range.**

1. Prepare a 1% **LMP** agarose gel in 1X TAE or 1X TBE buffer.

   Note: Do not include *ethidium bromide in the gel solution.*

2. Load 100 ng of Fosmid Control DNA into each of the outside lanes of the gel and load the end-repaired insert DNA in the lane(s) between the Fosmid Control DNA marker lanes.
3. Resolve the samples by gel electrophoresis (for example at room temperature overnight) at a constant voltage of 30-35 V. Visualizing and excising the end-repaired DNA can be done by one of two methods described below.

Method 1

Following electrophoresis, cut off the outer lanes of the gel containing the Fosmid Control DNA. Stain the Fosmid Control DNA lanes with ethidium bromide and visualize the DNA with UV light. Mark the position of the Fosmid Control DNA in the gel using a Pasteur pipet.

Note: *Do not expose the sample DNA to UV irradiation! Even short UV exposure can decrease cloning efficiencies by 100- to 1,000-fold. Reassemble the gel and excise a 2- to 4-mm wide gel slice containing sample DNA that migrated with and just slightly above (higher MW) the position of the 36-kb Fosmid Control DNA markers.*

Caution: *Be sure to cut the gel slice so that the DNA recovered is ≥25 kb. Cloning DNA smaller than about 25 kb may result in unwanted chimeric clones. Transfer the gel slice to a tared, sterile, 15-ml screw-cap tube.*

Method 2

Following electrophoresis, stain the gel with SYBR® Gold (Molecular Probes, Inc.) according to the manufacturer's instructions. Place the gel on a Dark Reader™ Transilluminator (Clare Chemical Research) and visualize the DNA. The Dark Reader Transilluminator allows the DNA to be visualized without UV light thus preventing damage to the DNA. Excise a 2- to 4-mm wide gel slice containing sample DNA that migrated with and slightly above (higher MW) the position of the 36-kb Fosmid Control DNA markers.

Caution: *Be sure to cut the gel slice so that the DNA recovered is greater than or equal to 25 kb. Cloning DNA smaller than about 25 kb may result in unwanted chimeric clones. Transfer the slice to a tared, sterile, 15-ml screw-cap tube.*

4. Proceed with **Recovery of the Size-Fractionated DNA** in Part D or store the gel slice at 4°C to –20°C for up to one year.

D. Recovery of the Size-Fractionated DNA

Kit components used in this step: **GELase 50X Buffer, GELase Enzyme Preparation**.

Before beginning this Step, prepare a 70°C and a 45°C water bath or other temperature regulated apparatus.

1. Weigh the tared tubes to determine the weight of the gel slice(s). Assume 1 mg of solidified agarose will yield 1 µl of molten agarose upon melting.
2. Warm the GELase 50X Buffer to 45°C. Melt the LMP agarose by incubating the tube at 70°C for 10-15 minutes. Quickly transfer the tube to 45°C.
3. Add the appropriate volume of warmed GELase 50X Buffer to 1X final concentration. Carefully add 1 U (1 µl) of GELase Enzyme Preparation to the tube for each 100 µl of melted agarose. Keep the melted agarose solution at 45°C and gently mix the solution. Incubate the solution at 45°C for at least one hour (overnight incubation is acceptable if desired).
4. Transfer the reaction to 70°C for 10 minutes to inactivate the GELase enzyme.
5. Remove 500 µl aliquots of the solution into sterile, 1.5-ml microfuge tube(s).
6. Chill the tube(s) in an ice bath for 5 minutes. Centrifuge the tubes in a microcentrifuge at maximum speed (approximately 10,000 rpm) for 20 minutes to pellet any insoluble oligosaccharides. Any "pellet" will be gelatinous, and translucent-to-opaque. Carefully remove the upper 90%-95% of the supernatant, **which contains the DNA**, to a sterile 1.5-ml tube. Be careful to avoid the gelatinous pellet.
7. Precipitate the DNA.
   a) Add 1/10 volume of 3 M Sodium Acetate (pH 7.0) and mix gently.
   b) Add 2.5 volumes of ethanol. Cap the tube and mix by gentle inversion.
   c) Allow precipitation for 10 minutes at room temperature.
   d) Centrifuge the precipitated DNA for 20 minutes in a microcentrifuge, at top speed (typically 10,000-16,000 rpm).
   e) Carefully aspirate the supernatant from the pelleted DNA.
   f) Wash the pellet 2X with cold, 70% ethanol, repeating steps d) and e) using care not to disrupt the DNA pellet.
   g) After the second 70% ethanol wash carefully invert the tube and allow the pellet to air-dry for 5-10 minutes (longer dry times will make resuspension of the DNA difficult).
   h) Gently resuspend the DNA pellet in TE Buffer.
8. Determine the DNA concentration by fluorimetry. Alternatively, estimate the concentration of the DNA by running an aliquot of the DNA on an agarose gel using dilutions of known amounts of the Fosmid Control DNA as standard.

   Note: *Measuring the DNA concentration by spectrophotometry (A_{260}) is not recommended because the DNA concentration will not be high enough to be measured accurately.*

E. Ligation Reaction

Kit components used in this step: **Fast-Link 10X Ligation Buffer, Fast-Link DNA Ligase, ATP, pEpiFOS-5 Cloning-Ready Vector.**

1. Please refer to Appendix A to determine the approximate number of fosmid clones that you will need for your library. A single ligation reaction will produce 10^3-10^6 clones depending on the quality of the insert DNA. Based on this information calculate the number of ligation reactions that you will need to perform. The ligation reaction can be scaled-up as needed.
2. Combine the following reagents in the order listed and mix thoroughly after each addition.

   A 10:1 molar ratio of pEpiFOS-5 Vector to insert DNA is optimal.

   0.5 µg pEpiFOS-5 (Cloning-Ready) Vector ≈ 0.1 pmol vector

   0.25 µg of ≈40 Kb insert DNA ≈ 0.009 pmol insert DNA

x	µl	sterile water
1	µl	10X Fast-Link Ligation Buffer
1	µl	10 mM ATP
1	µl	pEpiFOS-5 Vector (0.5 µg/µl)
x	µl	concentrated insert DNA (0.25 µg of ≈40 kb DNA)
1	µl	Fast-Link DNA Ligase
10	µl	Total reaction volume

3. Incubate at room temperature for 2 hours.
4. Transfer the reaction to 70°C for 10 minutes to inactivate the Fast-Link DNA Ligase.

   Proceed to Part F or store at –20°C.

F. Packaging the Fosmid Clones

Kit components used in this step: **MaxPlax Lambda Packaging Extracts, EPI100-T1^R Plating Strain.**

1. The day of the packaging reactions, inoculate 50 ml of LB broth + 10 mM $MgSO_4$ with 5 ml of the EPI100-T1^R overnight culture from the **Preparation** step on page 5. Shake at 37°C to an OD_{600} = 0.8-1.0. Store the cells at 4°C until needed (Part G). The cells may be stored for up to 72 hours at 4°C if necessary.
2. Thaw, on ice, 1 tube of the MaxPlax Lambda Packaging Extracts for every ligation reaction performed in Part E. For example thaw 1 tube of the MaxPlax Lambda Packaging Extracts if the standard 10 µl ligation reaction was done. Thaw 2 tubes if the ligation reaction was scaled up to 20 µl, etc.
3. When thawed, **immediately** transfer 25 µl (one-half) of each packaging extract to a second 1.5-ml microfuge tube and place on ice. Return the remaining 25 µl of the MaxPlax Packaging Extract on to –70°C freezer for use in Part F, Step 7.

   Note: *Do not expose the MaxPlax Packaging Extracts to dry ice or other CO_2 source.*

4. Add 10 µl of the ligation reaction from Part E to each 25 µl of the thawed, extracts being held on ice.
5. Mix by pipetting the solutions several times. Avoid the introduction of air bubbles. Briefly centrifuge the tubes to get all liquid to the bottom.
6. Incubate the packaging reactions at 30°C for 90 minutes.
7. After the 90 minute packaging reaction is complete, add the remaining 25 µl of MaxPlax Lambda Packaging Extract from Part F, Step 3 to each tube.
8. Incubate the reactions for an additional 90 minutes at 30°C.
9. At the end of the second 90 minute incubation, add Phage Dilution Buffer to **1 ml final volume** in each tube and mix gently. Add 25 µl of chloroform to each. Mix gently and store at 4°C. A viscous precipitate may form after addition of the chloroform. This precipitate will not interfere with library production. Determine the titer of the phage particles (packaged fosmid clones) in Part G, and then plate the fosmid library in Part H. Alternatively, store the phage particles for up to several weeks at 4°C.

G. Titering the Packaged Fosmid Clones

Kit components used in this step: **EPI100-T1^R Plating Strain from Part F, Step 1.**

Before plating the library we recommend that the titer of the phage particles (packaged fosmid clones) be determined. This will aid in determining the number of plates and dilutions to make to obtain a library that meets the needs of the user.

1. Make serial dilutions of the 1 ml of packaged phage particles from Part F, Step 9 into Phage Dilution Buffer (PDB) in sterile microfuge tubes.
   A) 1:10^2 Dilute 10 µl of packaged phage into 990 µl of PDB.
   B) 1:10^4 Dilute 10 µl of the 1:10^2 dilution into 990 µl of PDB.
   C) 1:10^5 Dilute 100 µl of the 1:10^4 dilution into 900 µl of PDB.
   D) 1:10^6 Dilute 100 µl of the 1:10^5 dilution into 900 µl of PDB.
2. Add 10 µl of each above dilution, individually, to 100 µl of the prepared EPI100-T1^R host cells from Step F1 above. Incubate each for 20 minutes at 37°C.
3. Spread the infected EPI100-T1^R cells on an LB plate + 12.5 µg/ml chloramphenicol and incubate at 37°C overnight to select for the fosmid clones.
4. Count colonies and calculate the titer of the packaged phage particles from Part F, Step 9.

Sample Calculation:

If there were 200 colonies on the plate streaked with the 1:10^4 dilution, then the titer in cfu/ml, (where cfu represents colony forming units) of this reaction would be:

$$\frac{(\#\text{ of colonies})\,(\text{dilution factor})\,(1{,}000\ \mu\text{l/ml})}{(\text{volume of phage plated [µl]})} \quad \textbf{OR} \quad \frac{(200\ \text{cfu})\,(10^4)\,(1{,}000\ \mu\text{l/ml})}{(10\ \mu\text{l})} = 2 \times 10^8\ \text{cfu/ml}$$

H. Plating and Selecting the Fosmid Library

Based on the titer of the packaged fosmid clones and the estimated number of clones required (see Appendix A), calculate the volume of the packaged fosmid clones that will be needed to prepare the fosmid library.

1. Based on the titer of the phage particles determined in Part G, dilute the phage particles from Part F, Step 9 with Phage Dilution Buffer to obtain the desired number of clones and clone density on the plate. Proceed to the next step or store the diluted phage particles at 4°C for up to several weeks.
2. Mix the diluted phage particles from Part H, Step 1 with EPI100-T1^{R} cells prepared in Part F, Step 1 in the ratio of 100 µl of cells for every 10 µl of diluted phage particles.
3. Adsorb at 37°C for 20 minutes.
4. Spread the infected bacteria on an LB plate + 12.5 µg/ml chloramphenicol and incubate at 37°C overnight to select for the fosmid clones.

4. Appendix

Appendix A

Determining the Approximate Number of Clones for a Complete Fosmid Library

Using the following formula[5], determine the number of fosmid clones required to reasonably ensure that any given DNA sequence is contained within the library.

$$N = \ln (1\text{-}P) / \ln (1\text{-}f)$$

Where P is the desired probability (expressed as a fraction); f is the proportion of the genome contained in a single clone; and N is the required number of fosmid clones.

For example, the number of clones required to ensure a 99% probability of a given DNA sequence of *E. coli* (genome = 4.7 Mb) being contained within a fosmid library composed of 40 kb inserts is:

$$N = \ln (1 - 0.99 / \ln (1 - [4 \times 10^4 \text{ bases} / 4.7 \times 10^6 \text{ bases}]) = -4.6 / -0.01 = 461 \text{ clones}$$

Appendix B

Control Fosmid Library Production

The Fosmid Control DNA provided in the kit can be used to test and familiarize the user with all the steps involved in producing a fosmid library.

The Fosmid Control DNA, as provided in the kit, is purified and blunt-ended and ready for ligation to the Cloning-Ready pEpiFOS-5 vector. If desired, the Control can be put through the end-repair and gel purification steps (Parts B, C, D) of the Fosmid Library Production procedure.

1. Prepare EPI100-T1^{R} host cells as described in Part F, Step 1.
2. Ligate the Fosmid Control DNA to the pEpiFOS-5 vector.

Combine the following reagents in the order listed and mix after each addition.

3.5	µl	sterile water
1	µl	10X Fast-Link Ligation Buffer
1	µl	10 mM ATP
1	µl	pEpiFOS-5 Vector (0.5 µg/µl)
2.5	µl	Fosmid Control DNA (100 ng/µl)
1	µl	Fast-Link DNA Ligase
10	µl	Total reaction volume

3. Incubate at room temperature for 2 hours.
4. Transfer the reaction to 70°C for 10 minutes to inactivate the Fast-Link DNA Ligase.
5. Package the entire 10 µl ligation reaction as directed in Part F, Steps 2-9.
6. Titer the packaged control clones by making a 1:1000 dilution of the packaged phage extract in Phage Dilution Buffer. Add 10 µl of the diluted packaged phage to 100 µl of EPI100-T1^R host cells. Adsorb at room temperature for 20 minutes.
7. Spread the infected EPI100-T1^R cells on LB medium + 12.5 µg/ml chloramphenicol. Incubate the plate overnight at 37°C to select for the control fosmid clones.
8. Count the colonies and determine the titer, cfu/ml of the reaction (refer to Part G, Step 4). Users should expect a packaging efficiency of >1 x 10^7 cfu/ml.

Appendix C

Testing the Efficiency of the MaxPlax Packaging Extracts

Kit components used in this step: **Ligated Lambda Control DNA, MaxPlax Lambda Packaging Extracts, LE392MP Plating Strain.**

Additionally required:

LB Plates without antibiotic

LB Top Agar (LB broth containing 0.7% [w/v] Bacto-agar supplemented with 10 mM $MgSO_4$)

Phage Dilution Buffer (10 mM Tris-HCl [pH 8.3], 100 mM NaCl, and 10 mM $MgCl_2$)

Please see the product literature for the MaxPlax Lambda Packaging Extracts, that is included on page 17, for details on how to test the efficiency of the extracts.

Appendix D

Amplification and Storage of the Fosmid Library

Short Term Storage: After dilution of the packaging reaction and addition of chloroform, the packaged fosmid library can be stored at 4°C for several days. For longer term storage, see recommendations below.

Long Term Storage: For longer term storage we recommend storage of the packaged DNA as a primary library or storage of the library in the EPI300-T1^R Phage T1-resistant *E. coli* plating strain using one of the methods described below.

Method A - Storage of Packaged DNA.

1. To the packaged fosmid library, add sterile glycerol to a final concentration of 20%, mix and store at –70°C.

Method B - Storage of Infected Cells.

1. Adsorb the packaged phage to bacterial cells (see Part H).
2. Based on the expected titer, resuspend the cells in an appropriate volume of liquid media.
3. Transfer the final resuspension to a sterile tube and add sterile glycerol to a final concentration of 20%. Mix the solution and store aliquots (which would each constitute a library of the desired coverage) at –70°C.

Method C - Storage of Amplified Library.

1. Adsorb the packaged phage to bacterial cells (see Part H).
2. Spread an appropriate volume of infected bacteria onto a plate(s) with the appropriate antibiotic and incubate at 37°C overnight.
3. Add ~2 ml of liquid media (i.e., LB) to a plate and resuspend all of the bacterial cells.
4. Transfer the resuspended cells and media to the next plate (if more than one overnight plate was used) and repeat resuspension process. Do this for as many plates as desired.
5. Transfer the final resuspension to a sterile tube and add sterile glycerol to a final concentration of 20%. Mix the solution and store aliquots (which would each constitute a library of the desired coverage) at –70°C.

Appendix E

pEpiFOS-5 Sequencing Primers and Vector Data

pCC1/pEpiFOS-5 Sequencing Primers

Primers are available separately:

pCC1™/pEpiFOS™ Forward Sequencing Primer Cat. No. F5FP010

5′ GGATGTGCTGCAAGGCGATTAAGTTGG 3′..... 1 nmol supplied in TE Buffer at 50 µM

pCC1™/pEpiFOS™ Reverse Sequencing Primer Cat. No. F5RP011

5′ CTCGTATGTTGTGTGGAATTGTGAGC 3′.....1 nmol supplied in TE Buffer at 50 µM

Note: *The sequence of the pCC1/pEpiFOS Forward and Reverse Primers do not function well as IRD800-labeled sequencing primers. We recommend using the T7 and pCC1/pEpiFOS RP-2 Primers instead of the pCC1/pEpiFOS Forward and Reverse Primers respectively, for this purpose.*

pCC1™/pEpiFOS™ RP-2 Reverse Sequencing Primer

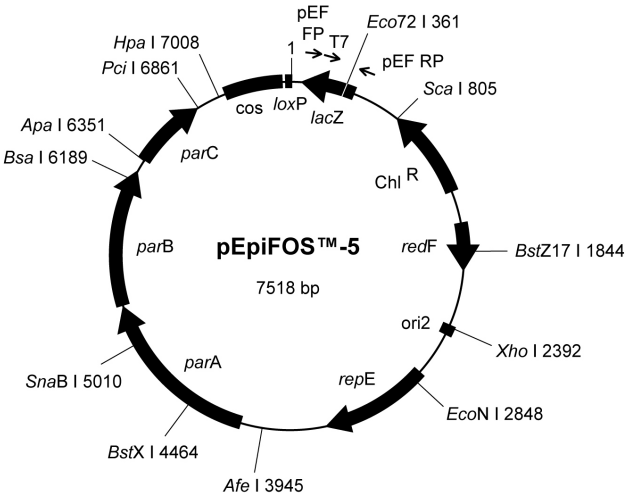
5′ TACGCCAAGCTATTTAGGTGAGA 3′

Orientation for Fosmid End-Sequencing

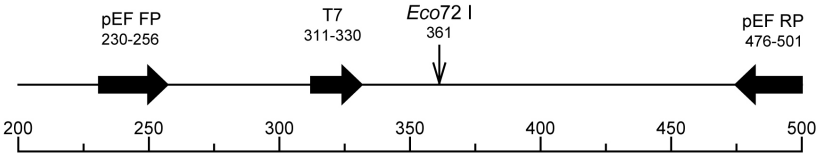
The following is the nucleotide sequence of pEpiFOS-5 (bases 230-501) from the pCC1/pEpiFOS Forward Sequencing Primer (230-256) to the pCC1/pEpiFOS Reverse Sequencing Primer (501-476) encompassing the T7 RNA polymerase promoter (311-330) and the *Eco*72 I site (359-364).

```
230 GGATGTGCTG CAAGGCGATT AAGTTGGGTA ACGCCAGGGT TTTCCCAGTC
280 ACGACGTTGT AAAACGACGG CCAGTGAATT GTAATACGAC TCACTATAGG
330 GCGAATTCGA GCTCGGTACC CGGGGATCCC AC - - Cloned Insert
    - - - Cloned Insert - - - - - -   GTGGGATC    CTCTAGAGTC
380 GACCTGCAGG CATGCAAGCT TGAGTATTCT ATAGTCTCAC CTAAATAGCT
430 TGGCGTAATC ATGGTCATAG CTGTTTCCTG TGTGAAATTG TTATCCGCTC
480 ACAATTCCAC ACAACATACG AG
```

An electronic copy of the pEpiFOS-5 sequence is available for downloading at our Web site (www.epibio.com/sequences) or can be requested via e-mail (techhelp@epicentre.com) or by calling Technical Service.



Note: Not all restriction enzymes that cut only once are indicated above.
See the following page for further information.
Primers are not drawn to scale.



pEF FP = pCC1™ / pEpiFOS™-5 Foward Sequencing Primer
5' GGATGTGCTGCAAGGCGATTAAGTTGG 3'

pEF RP = pCC1™ / pEpiFOS™-5 Reverse Sequencing Primer
5' CTCGTATGTTGTGTGGAATTGTGAGC 3'

T7 = T7 Promoter Primer
5' TAATACGACTCACTATAGGG 3'

Figure 1. pEpiFOS™-5 Fosmid Vector

Restriction Analysis of the pEpiFOS-5 Vector

Restriction Enzymes that cut pEpiFOS-5 1 to 3 times:

Enzyme	Sites	Location	Enzyme	Sites	Location
Acc65 I	2	344, 4586	EcoR V	2	3507, 3736
Acl I	2	1133, 4978	Fsp I	3	167, 3131, 6957
Afe I	1	3945	Hind III	1	395
Afl II	2	5987, 6227	Hpa I	1	7008
Afl III	3	4352, 4526, 6861	Kpn I	2	348, 4590
Age I	3	3206, 4436, 5329	Mfe I	1	4366
Ahd I	1	6865	Msc I	2	955, 4797
Ale I	1	5922	Nae I	2	3084, 7000
Apa I	1	6351	Nar I	1	146
ApaB I	3	96, 1946, 7025	Nco I	2	917, 6566
ApaL I	1	87	Nde I	2	94, 4384
BamH I	2	353, 365	NgoM IV	2	3082, 6998
Bbs I	3	4429, 4618, 5495	Not I	2	2, 643
Bcl I	1	5177	Nru I	2	1644, 7053
BfuA I	3	390, 3393, 7160	Nsp I	3	393, 1831, 6865
Bgl I	3	651, 2550, 6999	PaeR7 I	1	2392
Bgl II	2	2525, 4592	Pci I	1	6861
Blp I	1	3858	PflF I	1	4650
Bme1580 I	3	91, 755, 6351	Pml I	1	361
BmgB I	2	4416, 7176	PpuM I	2	1728, 7237
Bmr I	3	268, 6397, 6526	Psi I	1	2501
Bpu10 I	3	1446, 3306, 4501	PspOM I	1	6347
Bsa I	1	6189	Pst I	3	387, 3404, 4945
BsaB I	2	7133, 7217	Pvu I	2	188, 5252
BsaH I	1	146	Sal I	3	377, 657, 7041
BseY I	2	5269, 6026	Sap I	2	3982, 5192
Bsm I	2	824, 1231	Sbf I	2	387, 3404
BsmB I	3	994, 1547, 3321	Sca I	1	805
BspE I	2	1222, 5146	SexA I	1	6979
BspLU11 I	1	6861	Sfi I	1	651
BspM I	3	390, 3393, 7160	Sfo I	1	147
BsrB I	3	476, 1660, 2282	SgrA I	2	4436, 5593
BsrG I	1	3159	Sim I	2	4550, 7237
BssH II	2	4843, 5387	Sma I	3	350, 651, 2872
BssS I	3	4536, 6186, 6749	SnaB I	1	5010
BstAP I	3	95, 1945, 7024	Spe I	1	6101
BstE II	1	6983	Sph I	1	393
BstX I	1	4464	Srf I	1	651
BstZ17 I	1	1844	Sse8647 I	1	1728
Bts I	2	570, 4938	Stu I	1	2553
Dra III	2	1945, 7202	Tat I	3	77, 803, 3159
Eag I	2	2, 643	Tli I	1	2392
Eco47 III	1	3945	Tth111 I	1	4650
Eco72 I	**1**	**361**	Xba I	2	371, 2571
EcoN I	1	2848	Xho I	1	2392
EcoO109 I	2	1728, 7237	Xma I	3	348, 649, 2870
EcoR I	1	332			

Restriction Enzymes that cut pEpiFOS-5 4 or more times:

Acc I	Bsp1286 I	Eae I	Mae II	Rsa I
Aci I	BspH I	Ear I	Mae III	Sac I
Alu I	Bsr I	Fau I	Mbo I	Sau3A I
Alw I	BsrD I	Fnu4H I	Mbo II	Sau96 I
AlwN I	BsrF I	Gdi II	Mly I	ScrF I
Apo I	BssK I	Hae I	Mnl I	SfaN I
Ase I	BstDS I	Hae II	Mse I	Sfc I
Ava I	BstF5 I	Hae III	Msl I	Sml I
Ava II	BstN I	Hha I	Msp I	Ssp I
Ban I	BstU I	Hinc II	MspA1 I	Sty I
Ban II	BstY I	Hinf I	Mwo I	Taq I
Bfa I	Btg I	HinP I	Nci I	Tfi I
BsaA I	Cac8 I	Hpa II	Nla III	Tse I
BsaJ I	CviJ I	Hph I	Nla IV	Tsp45 I
BsaW I	Dde I	Hpy188 I	PflM I	Tsp4C I
BsiE I	Dpn I	Hpy99 I	Ple I	Tsp509 I
BsiHKA I	Dra I	HpyCH4 III	PshA I	TspR I
Bsl I	Drd I	HpyCH4 IV	PspG I	Xmn I
BsmA I	Dsa I	HpyCH4 V	Pvu II	

Restriction Enzymes that do not cut pEpiFOS-5:

Aat II	BciV I	Bsu36 I	Nsi I	SanD I
Asc I	BfrB I	Cla I	Pac I	Swa I
AsiS I	BsiW I	Fse I	Pme I	Xcm I
Avr II	BspD I	Mlu I	Rsr II	Tsp509 I
BbvC I	BstB I	Nhe I	Sac II	

MaxPlax™ Lambda Packaging Extracts Protocol

(This protocol is also available as product literature #65 and is provided with Cat. Nos. MP5105, MP5110, and MP5120)

1. Introduction

MaxPlax™ Lambda Packaging Extracts are a convenient, high-efficiency system designed for *in vitro* lambda packaging reactions. MaxPlax Lambda Packaging Extracts are supplied as predispensed single-tube reactions that have been optimized for packaging of methylated and unmethylated DNA. The packaging extracts routinely yield packaging efficiencies of >1 x 10^9 pfu/µg of Control λ DNA. The extracts can be used in the construction of representative cDNA libraries and genomic cloning of highly modified (methylated) DNA into λ-phage or cosmid vectors.

Traditional packaging extracts are derived from two complementary lysogenic *E. coli* strains, BHB2690 and BHB2688, as described by Hohn (1979).[5] The MaxPlax extracts utilize a new packaging strain, NM759*, reported by Gunther, Murray and Glazer (1993).[6] This strain, which replaces strain BHB2690 in the preparation of the sonication extract, is a restriction-free K12-derived strain deficient in the production of λ-phage capsid protein D. When combined with the complementary freeze-thaw extract from strain BHB2688**,[5] deficient in the production of λ-phage capsid protein E, an extremely high-efficiency of packaging for λ DNA is obtained. Moreover, the ability to package λ DNA bearing the mammalian methylation pattern is greatly enhanced, as evidenced by the high efficiency of λ-vector rescue from transgenic mouse DNA.[6] The lack of restriction activity has been shown to be crucial for the high efficiency rescue of lambda shuttle vectors from transgenic mouse DNA.[6,7]

**NM759: [W3110 recA56, Δ(mcrA) e14, Δ(mrr-hsd-mcr), (λimm434, clts, b2, red3, Dam15, Sam7)/λ]*

***BHB2688: [N205 recA$^-$, (λimm434 clts, b2, red3, Eam4, Sam7)/λ]*

Store the MaxPlax Lambda Packaging Extracts at –70°C or below. Exposure to higher temperature will decrease packaging efficiencies.

2. Product Specifications

Storage: Store the control host bacteria and the MaxPlax Lambda Packaging Extracts at –70°C. Exposure to higher temperatures will greatly compromise packaging extract efficiency. Avoid long term storage of product in the presence of dry ice. Once removed from the foil package, avoid any exposure to dry ice. Store the remainder of the kit components at –20°C. After thawing, store the Control DNA at 4°C.

Storage Buffers: MaxPlax Lambda Packaging Extracts are supplied as unlabeled single tubes of freeze-thaw/sonicate extracts. Control plating bacteria are supplied as a glycerol stock. Control ligated λ DNA is supplied in 1X Ligation Buffer.

Guaranteed Stability: MaxPlax Lambda Packaging Extracts are guaranteed to maintain a packaging efficiency of >1.0 x 10^9 pfu/µg of control λ DNA, when stored as directed for one year from the date of purchase.

3. Related Products

The following products are also available:

- pWEB-TNC™ Cosmid Cloning Kit
- pWEB™ Cosmid Cloning Kit
- EpiFOS™ Fosmid Library Production Kit
- Lambda Terminase

4. Protocol for Packaging Lambda DNA

This protocol can be used for the positive control reaction as well as for experimental reactions. The positive control reactions must be plated on the control host bacterial strain (LE392MP) included with the MaxPlax Extracts. The proper bacterial plating strain for the experimental reactions will vary depending on the cloning vector used. See the vector manufacturer's recommendations for the proper strain and plating media requirements. Ligation reactions may be added directly to the packaging extracts. When doing so, it is important to: a) add a volume of 10 µl or less to the packaging reaction, and b) heat inactivate the ligase (i.e., treatment at 65°C for 15 minutes) as active DNA ligase will decrease packaging efficiencies.

Solutions:

Phage Dilution Buffer
10 mM Tris-HCl (pH 8.3)
100 mM NaCl
10 mM $MgCl_2$

LB Broth (1 Liter)
10 g Bacto-tryptone
5 g Bacto-yeast extract
10 g NaCl
Adjust pH to 7.0 with NaOH

LB Plates
LB Broth with 1.5% (w/v) Bacto-agar

LB Top Agar
LB Broth with 0.7% (w/v) Bacto-agar

Plating Bacteria Preparation:

1. The day before performing the packaging reactions, inoculate 50 ml of supplemented (10 mM $MgSO_4$) LB broth with a single colony of the plating bacterial strain and shake overnight at 37°C.
2. The day of the packaging reactions, inoculate 50 ml of supplemented (10 mM $MgSO_4$ + 0.2% maltose) LB broth with 5 ml of the overnight culture and shake at 37°C to an OD_{600} = 0.8-1.0. Store the cells at 4°C until needed; cells may be stored for up to 72 hours.

Packaging Reactions:

1. Thaw the appropriate number of packaging extracts at room temperature. For every two packaging reactions, thaw one extract then place on ice.
2. When thawed, immediately transfer half (25 µl) of each packaging extract to a

second 1.5-ml tube and place on ice.

3. Add the substrate DNA (10 µl [0.2 µg] of the control DNA) to a tube containing 25 µl of extract. If performing an odd number of packaging reactions, the remaining 25 µl of extract can be refrozen at –70°C.
4. Mix by pipetting several times; avoid the introduction of air bubbles. Return all of the contents to the bottom of the tube by brief centrifugation if necessary.
5. Incubate the reaction(s) at 30°C for 90 minutes.
6. At the end of this incubation, add the additional 25 µl of thawed extract to each reaction tube at 30°C (If performing two packaging reactions, thaw another tube of extract and add 25 µl to each tube.) and incubate the reaction(s) for an additional 90 minutes at 30°C.
7. Add 500 µl of phage dilution buffer and mix by gentle vortexing.
8. Add 25 µl of chloroform and mix by gentle vortexing (store at 4°C).
9. Assay the packaged phage by titering on the appropriate bacterial strain (LE392MP for the control).

Titering Phage Extracts:

1. Make serial dilutions of the packaged phage in phage dilution buffer. Use 10^{-5} and 10^{-6} dilutions for the control reactions.

   10^{-2} dilution is 10 µl of packaged phage particles into 990 µl of phage dilution buffer; vortex mix.

   10^{-4} dilution is 10 µl of 10^{-2} dilution into 990 µl phage dilution buffer; vortex mix.

   10^{-5} dilution is 100 µl of 10^{-4} dilution into 900 µl phage dilution buffer; vortex mix.

   10^{-6} dilution is 10 µl of 10^{-4} dilution into 990 µl phage dilution buffer; vortex mix.
2. Add 100 µl of the appropriate serial dilutions to 100 µl of prepared plating bacteria (use LE392MP for the control reactions) and incubate for 15 minutes at 37°C.
3. Add 3.0 ml of melted supplemented (10 mM $MgSO_4$) LB top agar (cooled to ~48°C). Vortex gently and pour onto pre-warmed (37°C) LB plates. Allow the top agar to solidify and then incubate overnight at 37°C.
4. Count the plaques and determine the titer (pfu/ml) and packaging efficiency (See sample calculations).

Sample Calculations:

If there were 110 plaques on a 10^{-6} dilution plate, then the titer, pfu/ml, (where pfu represents plaque forming units) of this reaction would be:

$$\frac{(\text{\# of plaques})\ (\text{dilution factor})\ (1000\ \mu\text{l/ml})}{(\text{volume of phage plated [µl]})} \ \textbf{OR} \ \frac{(110\ \text{pfu})\ (10^6)\ (1000\ \mu\text{l/ml})}{(100\ \mu\text{l})} = 1.1 \times 10^9\ \text{pfu/ml}$$

The packaging efficiency (pfu/µg DNA) of this reaction would be:

$$\frac{\text{(\# of plaques) (dilution factor) (total reaction vol.)}}{\text{(vol. of dilution plated) (amount of DNA packaged)}} \textbf{ OR } \frac{\text{(110 pfu) (}10^6\text{) (550 µl)}}{\text{(100 µl) (0.2 µg)}} = 3 \times 10^9 \text{ pfu/µg}$$

5. References

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