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pWEB™ Cosmid Cloning Kit

Cat. No. PC8805

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1. Introduction

The pWEB™ Cosmid Cloning Kit provides all of the reagents needed to construct a complete and unbiased primary cosmid library in about two days using a novel cloning strategy (Fig. 1). Genomic DNA is first sheared by passing it through a standard pipette tip. 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 fragmenting the DNA by partial restriction endonuclease digestion. The sheared DNA is end-repaired, using reagents in the kit, to generate blunt ends and size selected on a low-melting-point agarose gel by comparison with a supplied 36-kb standard. Finally, the size-selected DNA is ligated into the supplied blunt-ended Cloning-Ready pWEB cosmid vector, packaged using ultra-high efficiency MaxPlax™ Lambda Packaging Extracts (>10^9 pfu/µg for phage lambda), and plated on the included EPI100™-T1^R Phage T1-Resistant *E. coli* Plating Strain.

The benefits of the pWEB strategy over BAC cloning are several-fold.[1] First, users do not have to isolate high molecular weight (HMW) DNA (>200 kb), which can be difficult from some organisms. HMW DNA is required in the standard approach because if the DNA is of insufficient length, few of the fragments generated by partial restriction digestion will have restriction sites at both ends, limiting the number of clones in the library.[2] Second, in the standard approach, generating optimal size fragments of genomic DNA for cloning using partial restriction digests often requires extensive optimization. The pWEB system generates DNA of the optimal size by random shearing, thus avoiding this difficulty. And third, the distribution of restriction sites throughout the genome is rarely random; therefore following the standard approach could result in the omission of certain sequences from the library.[2] Since genomic DNA is randomly sheared in the pWEB system, it is more likely that all sequences will be represented in the library.

pWEB (derived from pWE15[3]) is a 8179-bp cosmid cloning vector which is provided in a "ready-to-use" state for researcher convenience.

The vector has been linearized at the unique *Sma* I restriction enzyme recognition site, dephosphorylated and rigorously tested for purity and recombinant cloning efficiency (Cloning-Ready).

Features of the vector include:

1) Pairs of *Bam*H I, *Eco*R I, and *Not* I sites flank the *Sma* I site for excision and mapping of insert DNA.
2) ColE1 origin of replication for growth in *E. coli*.
3) Ampicillin-resistance as an antibiotic selectable marker for growth in *E. coli*.
4) SV40 origin of replication for growth in eukaryotic cells.
5) Neomycin-resistance as an antibiotic selectable marker for growth in eukaryotic cells.
6) Bacteriophage lambda *cos* site for lambda packaging or lambda-terminase cleavage.
7) M13 Forward Primer binding site flanking the cloning site.
8) Bacteriophage T7 RNA polymerase promoter flanking the cloning site.

2. Product Specifications

Storage: Store the EPI100-T1^{R} Phage T1-Resistant *E. coli* Plating Strain and the MaxPlax Lambda Packaging Extracts at –70°C. Exposure to higher temperatures will greatly compromise packaging extract efficiency. Store the remainder of the kit components at –20°C.

Size: Reagents included in the kit are sufficient to construct 10 cosmid libraries.

3. Kit Contents

Desc.	Concentration	Quantity
pWEB™ Cosmid Vector (Cloning-Ready; linearized at the unique *Sma* 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)	100 µl
End-Repair Enzyme Mix (including T4 DNA Polymerase and T4 Polynucleotide Kinase)		50 µl
*Cosmid Control DNA,	(100 ng/µl)	20 µl
ATP	(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
Ammonium Acetate Solution	(5 M)	2 x 1.8 ml
EPI100™-T1R Phage T1-Resistant *E. coli* Plating Strain, glycerol stock ([F$^-$ mcrA Δ(*mrr-hsd*RMS-*mcr*BC) φ80d*lac*ZΔM15 Δ*lac*X74 *rec*A1*end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U galK λ$^-$ *rps*L *nup*G *ton*A])		250 µl
†MaxPlax™ Lambda Packaging Extracts		10 Extracts
Ligated Lambda Control DNA (λc1857 *Sam7*)	(1 µg @ 0.02 µg/µl)	50 µl
Control strain LE392MP, glycerol stock ([F$^-$ e14$^-$(McrA$^-$) Δ(*mcr*C-mrr) (Tet^R) *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:* *The "T7 Control DNA" has been replaced with "Cosmid Control DNA". The Cosmid Control DNA is used both as a size marker and as a control insert for cosmid library production. Packaged "pWEB/Control DNA" results in colony formation as will cosmid cloning of genomic DNA.*

†Note: *MaxPlax Lambda Packaging Extracts are supplied as freeze-thaw/sonicate extracts in unlabeled single tubes. The extracts, Ligated Lambda Control DNA, and Control Plating Strain LE392MP are packaged together in a CO_2-impermeable foil pouch.*

Quality Control: The pWEB Cosmid Cloning Kit is function-tested using the provided Cosmid Control DNA (efficiencies in colonies/µg of DNA). Each kit must yield at least 10^7 cfu/µg of Cosmid Control DNA. pWEB (*Sma* I-Cloning-Ready) vector preparations, in recircularization assays (+ligase, –insert) must minimally show a 10^5 reduction in background colony formation as compared to control cloning (+ligase, +insert) assays as described above.

4. Related Products

The following products are also available:

– pWEB-TNC™ Cosmid Cloning Kit
– MasterPure™ DNA Purification Kits
– Colony Fast-Screen™ Kits
– Lambda-Terminase
– EZ-Tn5™ Insertion Kits
– T7 RNA Polymerase
– Plasmid-Safe™ ATP-Dependent DNase

5. General Considerations

1. **Insert DNA Quality and Quantity:** The final number of unique clones in a cosmid library is in part determined by the average size of the insert DNA molecules and the concentration of insert DNA present in the ligation reaction. Most standard methods of isolating genomic DNA will generate a random distribution of DNA molecules, a proportion of which will be of the optimal size (40 kb) for ligation into the pWEB vector. Occasionally however, the DNA will require shearing to create molecules of this optimal length.

   Users should avoid exposing genomic DNA to UV light and ethidium bromide. Exposure for even short periods of time (as little as 30 seconds) can decrease the efficiency of cloning by two or more orders of magnitude. The best method for localizing DNA molecules in an agarose gel is to stain the DNA with SYBR® Gold followed by illumination with a Dark Reader™ Transilluminator. Using this method, the DNA can be visualized while the band is being excised, without loss of cloning efficiency.

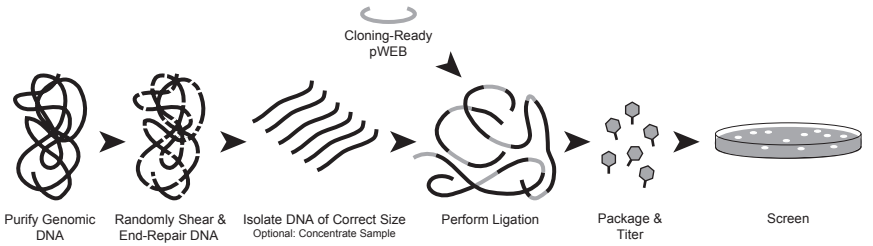

Figure 1. Production of a Cosmid Library using the pWEB™ Cosmid Cloning Kit.

2. **Ligation Reaction Conditions:** The in-gel ligation method outlined in previous versions of this protocol is no longer supported due to inconsistent performance. The new approach described in Part E of this protocol has consistently provided higher yields of clones, and is therefore strongly recommended.
3. **Plating of Packaging Extracts:** The efficiency of plating is dependent upon several factors including the growth state of host bacterial cells. We recommend a bacterial strain such as EPI100-T1^{R} *E. coli* for infection with the packaged pWEB cosmid DNA. This bacterial strain is genetically deficient in both recombination and restriction systems to minimize the rearrangement or loss of clones *in vivo*. To determine the packaging efficiency of the MaxPlax Lambda Packaging Extracts only, users can package the control lambda DNA supplied in the kit and infect the control bacterial strain LE392MP.

6. Cosmid Library Construction Protocol

EPI100-T1^{R} E. coli bacterial cells are required for the completion of this protocol.

See Part F for instructions.

A. Insert DNA Preparation

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.

1. Test the extent of shearing of the DNA by first running a small amount of it by Pulse Field Gel Electrophoresis (PFGE) (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 36-kb Cosmid Control DNA in an adjacent gel lane. Run the gel and stain with ethidium bromide.
2. If 10% or more of the genomic DNA migrates with the Cosmid Control DNA, then proceed to Part B. If the genomic DNA migrates slower (higher MW) than the Cosmid Control DNA, then the DNA needs to be sheared further as described in step 3 below. If the genomic DNA migrates faster than the Cosmid Control DNA (lower MW) then it has been sheared too much and should be reisolated.
3. We recommend that at least 2.5 µg and up to 20 µg of DNA be used in the shearing process. 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 Cosmid Control DNA. If 10% or more of the genomic DNA migrates with the Cosmid Control DNA, then 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. Insert DNA End-Repair Reaction

1. Treat the sheared DNA with the End-Repair Enzyme Mix to generate blunt ends for cloning into the prepared pWEB vector. The volumes listed below are for one end-repair reaction containing a total volume of 80 µl.
2. Thaw and thoroughly mix all of the reagents listed below before dispensing; place on ice.

   Combine the following on ice:

8	µl	10X End-Repair Buffer
8	µl	2.5 mM dNTP Mix
8	µl	10 mM ATP
x	µl	sterile water
20	µg	sheared insert DNA
4	µl	End-Repair Enzyme Mix
80	µl	Total reaction volume

3. Incubate at room temperature for 45 minutes.
4. Add gel loading buffer and incubate at 70°C for 10 min. to inactivate the End-Repair Enzyme Mix.
5. Proceed with Insert DNA Size Selection (Part C).

C. Insert DNA Size Selection

1. Prepare a 1% low melting point (LMP) agarose gel in 1X TAE buffer (40 mM Tris-acetate [pH 8], 1 mM EDTA).

   Note: *Do not include ethidium bromide in the gel solution.*
2. Load 100 ng of the Cosmid Control DNA as a size marker in each of the outside lanes and load the end-repaired insert DNA between the marker lanes.
3. Resolve the samples via gel electrophoresis at room temperature overnight at a constant voltage of 30-35 V. Alternatively, users may perform Pulse Field Gel Electrophoresis or Field Inversion Gel Electrophoresis.
4. Following completion of electrophoresis, users should stain the gel with SYBR Gold according to the manufacturer's instructions. Place the gel on a Dark Reader Transilluminator and visualize the DNA. Excise a 2-4 mm slice of gel containing insert DNA migrating between the size markers; transfer the slice to a clean tube.
5. If a Dark Reader Transilluminator is not available, cut off the outer lanes of the gel containing the T7 DNA marker. Stain the size marker lanes with ethidium bromide and visualize the DNA with UV light. Mark the position of the size markers using a pipet tip.

   Note: *Do not expose the insert DNA to UV irradiation, as even short exposures can decrease cloning efficiencies by 2-3 orders of magnitude. Reassemble the gel and excise a 2 to 4-mm wide gel slice containing insert DNA migrating between the size markers; transfer to a clean tube.*
6. Store the gel slice at 4°C to –20°C for up to one year.
7. Proceed to Part D when ready.

D. Recovery of the Size-Fractionated DNA

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 (in Step 2 below).
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 can also be performed without any ill effects to the DNA).
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 (~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 to 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. If the pellet is dislodged, centrifuge the tube at maximum rpm to reattach.
   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. Many of our customers have had success measuring the concentration using the Nanodrop® series of UV spectrophotometers.

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

E. Ligation Reaction

1. Please refer to Appendix A to determine the approximate number of pWEB cosmid 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 pWEB Cosmid Vector insert DNA is optimal.

0.5	µg	pWEB Vector ≈ 0.09 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	pWEB 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

   This reaction can be scaled up or down as necessary.
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 Step F or store at –20°C.

F. *In Vitro* Packaging (using MaxPlax Packaging Extracts)

Plating Bacteria Preparation:

1. The day before performing the packaging reactions, inoculate 50 ml of LB broth supplemented with 10 mM $MgSO_4$ and 0.2% maltose with a single colony of EPI100-T1^R cells and shake overnight at 37°C.
2. The day of the packaging reactions, inoculate 50 ml of supplemented 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 (50 µl) and 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 10 µl of the ligated cosmid 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.
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 and incubate the reaction(s) for an additional 90 minutes at 30°C.
7. Add 500 µl of phage dilution buffer (10 mM Tris-HCl [pH 8.3], 100 mM NaCl, 10 mM $MgCl_2$) and mix by gentle vortexing. Add 25 µl of chloroform and mix by gentle vortexing and store at 4°C.

Titering Phage Extracts:

1. To determine the titer of the packaged cosmids, add 10 µl of the packaged cosmids to 100 µl of prepared EPI100-T1^R host cells. Performing a dilution series of the phage may be helpful in determining phage titers.
2. Adsorb at 37°C for 20 minutes.
3. Spread the infected bacteria on LB-ampicillin selection plates and incubate at 37°C overnight.
4. Count colonies and calculate the titer as directed below.

   Sample Calculation:

   If there were 110 colonies on the plate, then the titer, cfu/ml, (where cfu represents colony forming units) of this reaction would be:

$$\frac{(\text{\# of colonies})\,(\text{dilution factor})\,(1000\ \mu l/ml)}{(\text{volume of phage plated [}\mu l\text{]})} \quad \textbf{OR} \quad \frac{(110\ \text{cfu})\,(1)\,(1000\ \mu l/ml)}{(10\ \mu l)} = 1.1 \times 10^4\ \text{cfu/ml}$$

5. Calculate the total number of clones contained within the cosmid library by multiplying the titer (cfu/ml) determined in Step 4 above by the total volume of the packaged phage (e.g., 1.1 x 10^4 cfu/ml x 0.5 ml = 5.5 x 10^3 clones).
6. The number of clones required to reasonably ensure that the cosmid library contains enough clones so that any given DNA sequence will be found in the library will vary with the size of the genome. For most prokaryotic genomes, a few hundred clones will be enough; for most eukaryotic genomes, several thousand clones are required. See Appendix A for a formula to estimate the number of clones required.

If the number of clones contained within the library is greater than the number of clones determined to ensure that a given sequence is within the library, the library is ready to use. See Appendices C and D for instructions regarding the Amplification and Storage of Cosmid Libraries.

If the number of clones obtained from the initial ligation and packaging reaction is lower than the desired number for the library, additional ligation and packaging reactions should be performed with the remaining size-fractionated DNA from Part C of this protocol.

7. Control DNA Reaction

Note: *The "T7 Control DNA" has been replaced with "Cosmid Control DNA". The Cosmid Control DNA is used both as a size marker and as a control insert for cosmid library production. Packaged "pWEB/Control DNA" results in colony formation as will cosmid cloning of genomic DNA.*

A. Control DNA Ligation into pWEB Vector

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

34.5	µl	sterile water
5	µl	10X Fast-Link Ligation Buffer
2.5	µl	10 mM ATP
2	µl	pWEB Vector
5	µl	Cosmid Control DNA
1	µl	Fast-Link DNA Ligase
50	µl	Total reaction volume

2. Incubate at room temperature overnight.
3. Transfer the reaction to 70°C for 10 minutes to inactivate the Fast-Link Ligase.
4. Proceed with in vitro packaging in Part B.

B. *In Vitro* Packaging (using MaxPlax Packaging Extracts)

Package and titer the ligated insert DNA as directed in Part F.

Note: *After spreading infected bacteria on LB-ampicillin plates, incubate the plates for a minimum of 24 hours at 37°C.*

8. Appendices

Appendix A: Determining the Optimal Number of Clones in a Library

Using the following formula,[2] determine the number of cosmid clones required to reasonably ensure that any given DNA sequence is contained within the library.

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

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 cosmid clones.

For example, the number of clones required to ensure a 99% probability of a given DNA sequence of *E. coli* being contained within a cosmid library composed of 40 kb inserts is:

$N = \ln (1 - 0.99) / \ln (1 - [4 \times 10^4 \text{ bases} / 4.7 \times 10^6 \text{ bases}]) = -4.61 / -0.01 = 461$ clones

Appendix B: Testing the Efficiency of the MaxPlax Packaging Extracts

This protocol can be used to test the packaging efficiency of the MaxPlax Extracts only. The ligated lambda control DNA must be adsorbed to the control packaging strain LE392MP.

Prepare plating bacteria (using strain LE392MP) as directed in Part F.

Perform the packaging reaction as directed in Part F.

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. Melt 0.7% top agarose and cool to 45°C. (top agarose: LB broth containing 0.7% [w/v] agarose supplemented with 10 mM $MgSO_4$.)
4. Add 3 ml of top agarose (45°C) to the adsorbed phage, swirl, and immediately pour onto the surface of a prewarmed (37°C) LB plate.
5. Incubate the plates overnight at 37°C.
6. Count the plaques and determine the titer (pfu/ml) and packaging efficiency as directed on the next page. MaxPlax Lambda Packaging Extracts are guaranteed to maintain a packaging efficiency of $1x10^9$ pfu/µg of control lambda DNA for up to one year when stored as directed.

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:

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

Appendix C: Amplification of Cosmid Libraries

Cosmid libraries are generally stable at 4°C for up to several weeks when stored as packaged phage suspended in phage dilution buffer. For longer term storage or to generate additional materials for screening, users may wish to amplify the cosmid library. Several methods are available.[2] Users should note however, that amplification of the library may result in a change in the composition of clones contained within the library. This results because of the different growth rates of bacteria containing different cosmids. A simple method for amplifying cosmid libraries in liquid culture[2] is described below.

1. Inoculate 100 µl of a fresh overnight culture of EPI100-T1^R cells grown in LB broth supplemented with 10 mM $MgSO_4$ and 0.2% maltose with 10 µl of packaged phage containing 10^4 cosmid clones. If the library contains more than 10^4 clones, set up additional tubes. The addition of larger amounts of phage extract can inhibit adsorption by the bacteria. If the titer of the library is low, increase the amount of bacterial culture proportionately. Recover the infected bacteria following Step 3 below by centrifugation and suspend in 100 µl of LB broth.
2. Incubate at 37°C for 20 minutes.
3. Add 0.5 ml of LB broth to the infected culture and incubate at 37°C for 45 minutes.
4. Transfer 0.5 ml of the infected culture to the center of an LB-ampicillin plate (150 mm). Spread the culture leaving a 2-3 mm wide strip around the perimeter of the plate. Incubate at 37°C until colonies (0.2-0.3 mm in diameter) appear (12-14 hours).
5. Estimate the number of colonies. Add 10 ml of LB broth to the plate and scrape the colonies off of the plate. Transfer the bacteria to a sterile tube. Rinse the plate with an additional 5 ml of LB broth and transfer to the sterile tube. Transfer bacteria from additional plates to this same tube.
6. Vortex mix the tube of bacteria to disrupt clumps of bacterial cells.
7. Add sterile glycerol to a final concentration of 15% and mix well. Dispense the bacteria into 100 to 500-µl aliquots and store at –70°C.

Appendix D: Amplification and Storage of Cosmid Libraries

Short Term Storage: After dilution of the packaging reaction and addition of chloroform, the packaged cosmid 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 cosmid 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.
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.

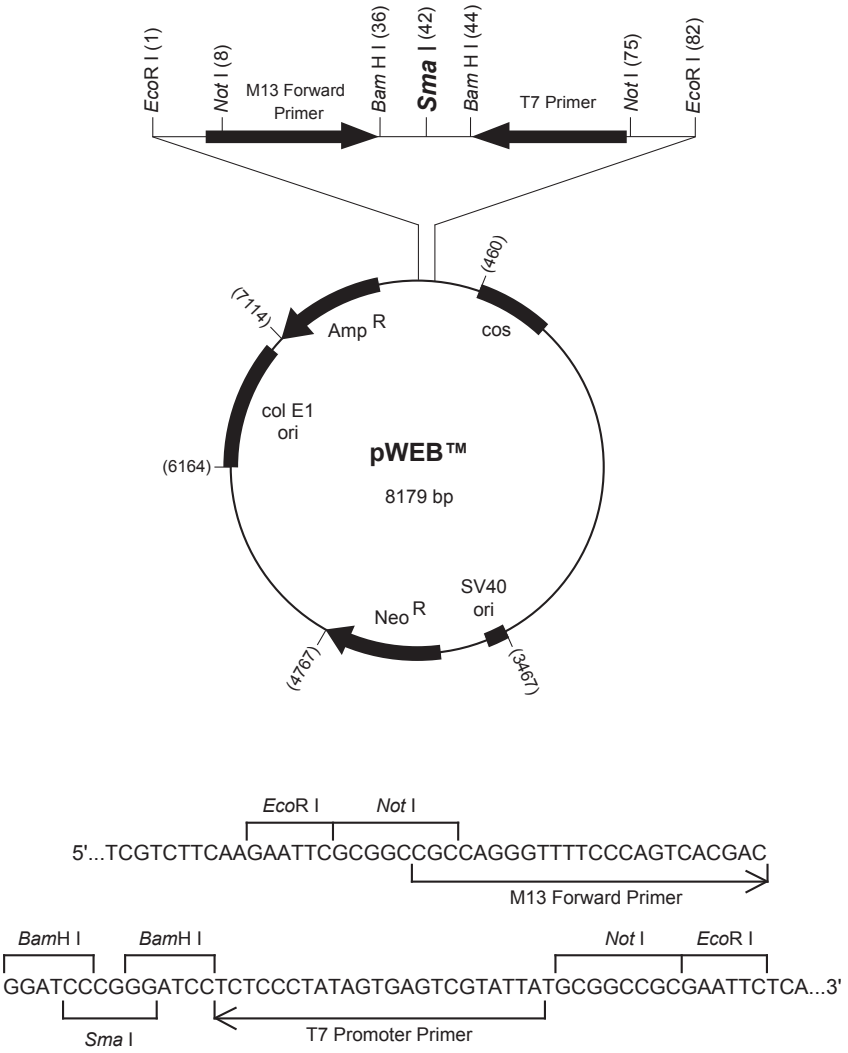

Figure 2. pWEB™ Cosmid Vector

Method C - Storage of Amplified Library.

1. Adsorb the packaged phage to bacterial cells.
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 (e.g., 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.

Restriction Enzymes that cut pWEB one to three times:

Enzyme	Sites	Location	Enzyme	Sites	Location
Aat II	1	8109	Hind III	2	113, 3617
Acc I	1	2475	Hpa I	2	1717, 5824
Afl III	3	1428, 3671, 6283	Mfe I	2	926, 5811
Ahd I	1	7187	Mlu I	1	1428
ApaL I	2	6608, 7854	Msc I	1	4183
Ase I	2	5627, 7359	Nco I	2	3508, 4533
Ava I	2	40, 3248	Nhe I	1	313
Avr II	1	3601	Not I	2	8, 75
BamH I	2	36, 44	Nru I	3	2094, 2797, 4954
BbvC I	1	1227	Nsi I	2	3351, 3423
Bcl I	1	3942	Pci I	1	6283
BfrB I	2	3349, 3421	PflF I	2	1323, 4219
Bgl II	1	3937	PflM I	3	3144, 3193, 5260
BmgB I	1	1683	PpuM I	3	902, 3262, 5994
Bpu10 I	2	1227, 6094	PshA I	1	2539
Bsa I	1	7248	Psi I	3	5554, 5573, 5844
BsaA I	2	3674, 4405	Pvu I	1	7557
BsmB I	1	1312	Rsr II	1	4617
BspD I	1	108	Sal I	1	2474
BspE I	1	5105	Sap I	3	4445, 4655, 6167
BspLU11 I	1	6283	Sca I	1	7667
BssH II	1	4498	SexA I	3	1125, 1438, 3368
BstB I	2	1312, 4783	Sfi I	1	3554
BstE II	2	1158, 1218	SgrA I	1	2233
BstX I	1	987	Sma I	1	42
Bsu36 I	1	4975	Stu I	1	3600
Cla I	1	108	Tat I	1	7665
Drd I	2	4128, 6402	Tth111 I	2	1323, 4219
EcoN I	1	2449	Xcm I	1	3917
EcoR I	2	1, 82	Xma I	1	40
EcoR V	1	271	Xmn I	3	840, 1308, 7786

Restriction Enzymes that cut pWEB four or more times:

Aci I	BsiE I	CviJ I	Hpy188 I	Ple I
Acl I	BsiHKA I	Dde I	Hpy99 I	PspG I
Afe I	Bsl I	Dpn I	HpyCH4 III	Pst I
Alu I	Bsm I	Dra I	HpyCH4 IV	Pvu II
Alw I	BSma I	Dsa I	HpyCH4 V	Rsa I
AlwN I	Bsp1286 I	Eae I	Mae II	Sau3A I
ApaB I	BspH I	Eag I	Mae III	Sau96 I
Apo I	BspM I	Ear I	Mbo I	ScrF I
Ava II	Bsr I	Eco47 III	Mbo II	SfaN I
Ban I	BsrB I	EcoO109 I	Mly I	Sfc I
Ban II	BsrD I	Fau I	Mnl I	Sfo I
Bbs I	BsrF I	Fnu4H I	Mse I	Sim I
BciV I	BssK I	Fsp I	Msl I	Sml I
Bfa I	BssS I	Gdi II	Msp I	Sph I
BfuA I	BstAP I	Hae I	MspA1 I	Ssp I
Bgl I	BstDS I	Hae II	Mwo I	Sty I
Bme1580 I	BstF5 I	Hae III	Nae I	Taq I
Bmr I	BstN I	Hha I	Nar I	Tfi I
BsaB I	BstU I	Hinc II	Nci I	Tse I
BsaH I	BstY I	Hinf I	NgoM IV	Tsp45 I
BsaJ I	Btg I	HinP I	Nla III	Tsp4C I
BsaW I	Bts I	Hpa II	Nla IV	Tsp509 I
BseY I	Cac8 I	Hph I	Nsp I	TspR I

Restriction Enzymes that do not cut pWEB:

Acc65 I	AsiS I	Fse I	Pme I	SanD I	Sse8647 I
Afl II	Blp I	Kpn I	Pml I	Sbf I	Swa I
Age I	BsiW I	Nde I	PspOM I	SnaB I	Tli I
Ale I	BsrG I	Pac I	Sac I	Spe I	Xba I
Apa I	BstZ17 I	PaeR7 I	Sac II	Srf I	Xho I
Asc I	Dra III				

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

pWEB Cosmid Vector: Genbank Accession No. AF075573.

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:

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

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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