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pWEB-TNC™ Cosmid Cloning Kit

Cat. No. WEBC931 and TNC9401

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Cat. #	Concentration	Quantity
pWEB-TNC™ Cosmid Cloning Kit Contents		
pWEB-TNC™ Cosmid Vector (0.5 µg/µl)		20 µl
(Cloning-Ready; linearized at the unique *Sma* I site and dephosphorylated)		
End-Repair 10X Buffer		100 µl
(330 mM Tris-acetate [pH 7.5], 660 mM potassium acetate, 100 mM magnesium acetate, 5 mM DTT)		
dNTP Mix (2.5 mM each)		100 µl
End-Repair Enzyme Mix		50 µl
(including T4 DNA Polymerase and T4 Polynucleotide Kinase)		
*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		200 µl
(2.0 M Bis-Tris [pH 6.0], 2.0 M NaCl)		
Ammonium Acetate Solution (5 M)		2 X 1.8 ml
EPI100™-T1^R Phage T1-Resistant *E. coli* Plating Strain, glycerol stock		250 µl
[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]		
†MaxPlax™ Lambda Packaging Extracts		10 Extracts
Ligated Lambda Control DNA λc1857 *Sam*7)	(1 µg @ 0.02 µg/µl)	50 µl
Control strain LE392MP, glycerol stock		250 µl
[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$^-$]		

***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-TNC/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.*

Overview of the pWEB-TNC™ Cosmid Cloning Kit

The pWEB-TNC™ 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), for subsequent use in making nested deletion sublibraries[1]. Genomic DNA is first sheared by passing it through a syringe needle (not supplied with the kit). 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-TNC 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.

pWEB-TNC (derived from pWE15[2]) is a 5,812-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 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) Chloramphenicol-resistance as an antibiotic selectable marker for transposition events.
5) Bacteriophage lambda *cos* site for lambda packaging or lambda-terminase cleavage.
6) M13 Forward Primer binding site flanking the cloning site.
7) Bacteriophage T7 RNA polymerase promoter flanking the cloning site.
8) pWEB-TNC Sequencing Primer binding site for sequencing transposition deletion clones.

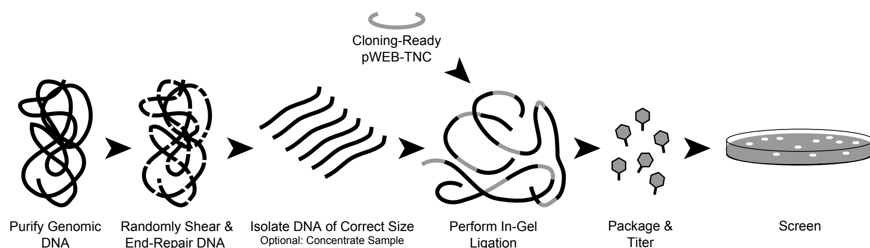

Figure 1. Production of a cosmid library using the pWEB-TNC™ Cosmid Cloning Kit.

Quality Control: The pWEB-TNC 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-TNC (*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.

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. After thawing, store the MaxPlax Control DNA at 4°C.

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

Related Products: The following products are also available:

– pWEB-TNC™ Sequencing Primer

– pWEB™ 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

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 (30-45 kb) for ligation into the pWEB-TNC vector. Occasionally however, the DNA will require shearing to create molecules of this optimal length.

   Users should avoid exposing genomic DNA to UV light. Even exposure for short periods of time 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.

2. **Ligation Reaction Conditions:** The protocol as outlined includes two ligation options. The in-gel ligation procedure (Part D) is a rapid and convenient means for users to obtain initial data on cloning efficiencies. For many smaller genomes in-gel ligation is sufficient to generate a library containing a representative number of clones. For large genomes, users may need to concentrate their insert DNA before ligation into pWEB-TNC vector (Part F). Appendix A includes a formula to determine

the number of cosmid clones required to reasonably ensure that any DNA sequence is contained within the library. The reaction conditions specified in either of the two ligation procedures utilizes a molar excess of vector-to-insert to minimize ligation of noncontiguous insert DNA molecules.[3] Users should aim for a 10:1 molar ratio of pWEB-TNC vector to insert DNA.

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-TNC 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.

Cosmid Library Construction Protocol

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

A. Insert DNA Preparation

1. Prepare high molecular weight genomic DNA by one of several standard methods[3] and resuspend the DNA in TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) at a concentration of about 0.5 µg/µl. Most of these methods will generate a random distribution of DNA molecules, a small proportion of which will be of the optimal size (30-45 kb) for ligation into the pWEB-TNC vector. Determine the size distribution of DNA molecules by examining an aliquot of the DNA on a 0.7-1% agarose gel. Include 100 ng of the T7 control DNA as a molecular weight marker. If the smear of DNA overlaps the T7 marker, proceed with the End-Repair reaction in Part B. If the DNA is larger than the T7 markers, shear the DNA as directed in Part A, Step 2.
2. Randomly shear the DNA to 30-45 kb molecules by expelling the DNA from a syringe through a small bore needle (e.g., Hamilton HPLC/GC syringe). To determine the appropriate number of passes, expel 25 µl containing 10 µg of DNA from the syringe. Following the first pass, remove 1-2 µl to a clean tube. Repeat the procedure with the remaining DNA; following the second pass, remove 1-2 µl to a clean tube. Expel the remaining DNA from the syringe a third time and remove another 1-2 µl aliquot. Examine the three samples of sheared DNA by using a 0.7-1% agarose gel; include 100 ng of T7 control DNA as a size marker. Shearing is sufficient when the DNA overlaps the T7 DNA marker. Generally, 1 or 2 passes is sufficient to generate DNA of optimal length.
3. Shear 25-50 µg of the high molecular weight DNA using the appropriate number of passes, as determined in Step 2.
4. Proceed with the End-Repair reaction in Part B.

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-TNC 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 (70°C for 10 minutes) 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, e.g., 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- to 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 with the in-gel ligation in Part D.

D. In-Gel Ligation

The ligation of vector to insert DNA can be performed directly without first purifying the insert DNA from the gel slice.

1. Melt the 2- to 4-mm LMP gel slice at 70°C for 10-15 minutes; transfer the tube to 45°C immediately and incubate for 5 minutes.
2. In a separate tube at room temperature, combine the following reagents in the order listed and mix thoroughly after each addition.

15.5	µl	sterile water
5	µl	10X Fast-Link™ Ligation Buffer
2.5	µl	10 mM ATP
1	µl	pWEB-TNC Vector
25	µl	molten agarose from step 1 (Store the remainder at 4°C.)
1	µl	Fast-Link DNA Ligase
50	µl	Total reaction volume

3. Mix thoroughly, (the mixture will reform a gel) then incubate at room temperature for 2 hours.
4. Transfer the reaction to 70°C for 10 minutes to melt the gel mixture and to inactivate the Fast-Link Ligase.
5. Following inactivation, transfer the reaction to 45°C.
6. Add 1 µl (1 U) of GELase™ Agarose Gel-Digesting Enzyme Preparation and mix thoroughly (leaving the tube at 45°C).

   Note: *Do not add 50X GELase Buffer to this reaction, as the buffer will inhibit the subsequent packaging reaction.*

7. Incubate at 45°C for 15 minutes to digest the agarose.
8. Transfer the reaction to 70°C for 10 minutes to inactivate the GELase enzyme.
9. Proceed with *In Vitro* Packaging in Part E.

E. *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 $MgSO_4$ 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 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 ml of prepared EPI100-T1^R host cells.
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/µl, (where cfu represents colony forming units) of this reaction would be:

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

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., 1.1×10^4 cfu/ml x 0.5 ml = 5.5×10^3 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, it is not necessary to perform the remainder of the protocol (omit Parts F and G). See the Appendix for instructions on Amplification and Storage of Cosmid Libraries.

If the number of clones obtained from the in-gel ligation is less than the number of clones determined to ensure that a given sequence is within a library, users will have to generate additional clones either by packaging more ligated DNA or by concentrating the insert DNA and performing another ligation reaction. If the number of additional clones required is only two- or three-fold higher, users may choose to package an additional 2 or 3, 10-µl aliquots of the ligated DNA generated by the in-gel ligation Part E. Alternatively, if the number of clones required is much greater than three-fold, users should continue with Part F.

F. Ligation of Concentrated Insert DNA

As the amount of insert DNA included in the ligation reaction is proportional to the eventual number of clones in the library, follow the protocol below to concentrate the insert DNA before ligation into the pWEB-TNC vector (see General Considerations).

Concentration of Insert DNA:

1. Incubate the remainder of the gel slice from Part D at 70°C for 10-15 minutes to melt the agarose.
2. Transfer the tube to 45°C immediately and equilibrate for 5 minutes.

   Note the volume of molten agarose and add 10 µl of 50X GELase Buffer (at 45°C) for every 500 µl of molten agarose (i.e., add 20 µl of 50X GELase Buffer to 1,000 µl of molten agarose).
3. While keeping the tube at 45°C, add 3 µl (3 U) of GELase enzyme to the tube, mix thoroughly and continue incubation at 45°C for another 30 minutes.
4. Transfer the reaction to 70°C for 10 minutes to inactivate the GELase enzyme.
5. Remove a 20 µl aliquot into a clean tube for measuring DNA concentration (below).
6. Add an equal volume of 5 M ammonium acetate and mix thoroughly.
7. Pellet the insoluble material by centrifugation in a microcentrifuge for 10 minutes.
8. Carefully transfer 95% of the supernatant to a clean tube and precipitate the DNA by adding two volumes of 100% ethanol. Recover the DNA by microcentrifugation for 10 minutes.
9. Resuspend the pellet(s) in a total volume of 25 µl of TE buffer.

Measuring Insert DNA Concentration:

To follow the recovery of insert DNA from the agarose plug and to determine the efficiency of cloning, users must measure the amount of DNA contained in the agarose plug and in the final concentrate. We recommend determining the DNA concentration by using a fluorometer (e.g., DyNA Quant™ 200) in conjunction with Hoechst dye #33258. Follow the instructions provided by the manufacturer to measure the DNA concentration of the sample from Step 7 above and a sample of the final concentrate. Alternatively, users can obtain an estimate of the concentration by comparing the signal intensity of the DNA samples with a known amount of Cosmid Control DNA using agarose gel electrophoresis.

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

Ligation of Insert DNA into the pWEB-TNC Vector:

1. Combine the following reagents in the order listed and mix thoroughly after each addition.
   Aim for a 10:1 molar ratio of pWEB-TNC vector to insert DNA.

x µl	sterile water
2 µl	10X Fast-Link Ligation Buffer
1 µl	10 mM ATP
1 µl	pWEB-TNC Vector (0.5 mg, ~6 kb)
x µl	concentrated insert DNA (~0.3 mg, ~40 kb)
1 µl	Fast-Link DNA Ligase
20 µl	Total reaction volume

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

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

Package the ligated insert DNA as directed in Part E.

Titering Phage Extracts:

1. Make a 10^{-1} dilution of the packaged cosmids in phage dilution buffer (10 mM Tris-HCl [pH 8.3], 100 mM NaCl, 10 mM $MgCl_2$). Vortex mix 10 µl of the cosmids with 90 µl of phage dilution buffer.
2. Add 10 µl of the 10^{-1} dilution, and 10 µl of the packaged cosmids to separate 100 µl aliquots of EPI100-T1^R host cells.
3. Adsorb at 37°C for 20 minutes.
4. Spread the infected bacteria on LB-ampicillin selection plates and incubate at 37°C overnight.
5. Count colonies and calculate the titer as directed in Part E.

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-TNC/Control DNA" results in colony formation as will cosmid cloning of genomic DNA.*

A. Control DNA Ligation into pWEB-TNC 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-TNC 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 E.

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

Appendix

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:

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

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 E.

Perform the packaging reaction as directed in Part E.

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/µl) and packaging efficiency as directed on the next page. MaxPlax Lambda Packaging Extracts are guaranteed to maintain a packaging efficiency of 1×10^9 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:

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

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

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

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.[4] 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[4] 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 $MgSO_4$ 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- to 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: 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.

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 (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.

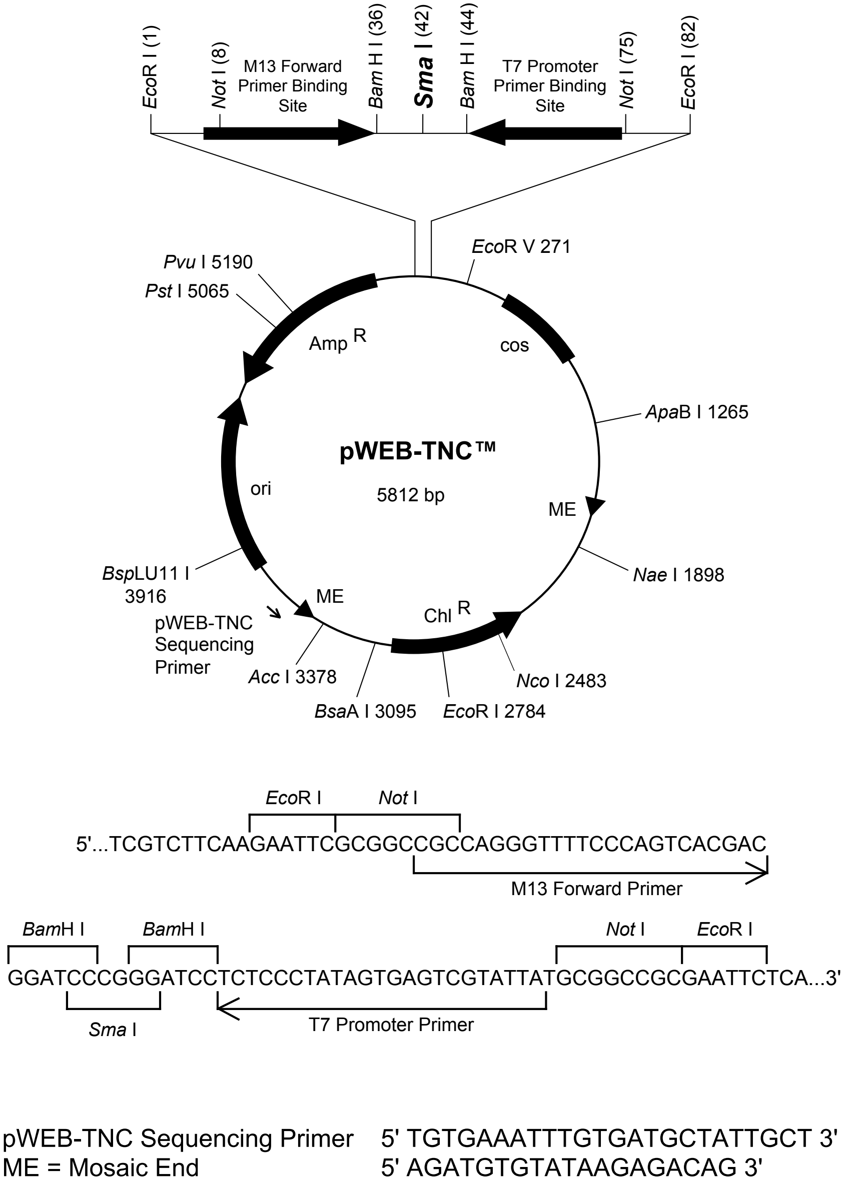

Figure 2. **pWEB-TNC™ Cosmid Vector.**

Figure 3. **Deletion and Inversion Transposition.**

Restriction Enzymes that cut pWEB-TNC one to three times:

Enzyme	Sites	Location	Enzyme	Sites	Location
Aat II	1	5742	Eag I	2	8, 75
Acc I	1	3378	Ear I	2	3800, 5615
Afl III	2	1428, 3916	EcoR I	3	1, 82, 2784
Age I	1	2180	EcoR V	1	271
Ahd I	1	4820	Fsp I	3	346, 3602, 5042
ApaB I	1	1265	Hinc II	2	679, 5361
ApaL I	2	4241, 5487	Hind III	1	113
Ase I	1	4992	Mfe I	1	926
Ava I	1	40	Mlu I	1	1428
BamH I	2	36, 44	Msc I	1	2521
Bbs I	3	1857, 3739, 5798	Nae I	1	1898
BbvC I	1	1227	Nco I	1	2483
Bcl I	1	2080	NgoM IV	1	1896
Bgl I	2	471, 4940	Nhe I	2	313, 3366
Bme1580 I	3	2321, 4245, 5491	Not I	2	8, 75
BmgB I	1	1683	Nsp I	1	3920
Bmr I	3	19, 383, 4860	Pci I	1	3916
Bsa I	1	4881	PflF I	2	1323, 2240
BsaA I	1	3095	PflM I	2	2559, 3126
BsaB I	1	1984	PpuM I	2	902, 3627
BsaH I	3	1276, 5357, 5739	PshA I	1	1843
BseY I	1	4231	Psi I	1	3477
BsmB I	3	1312, 2560, 3113	Pst I	1	5065
BspD I	1	108	Pvu I	1	5190
BspE I	2	1976, 2788	Pvu II	3	667, 2888, 3300
BspLU11 I	1	3916	Sap I	1	3800
BsrB I	3	1763, 3849, 5661	Sca I	2	2371, 5300
BssS I	3	4100, 5484, 5791	SexA I	2	1125, 1438
BstAP I	1	1264	Sma I	1	42
BstB I	2	1312, 2256	Ssp I	3	1411, 2476, 5624
BstE II	2	1158, 1218	Sty I	2	1195, 2483
BstX I	1	987	Tat I	2	2369, 5298
BstZ17 I	1	3379	Tfi I	3	2435, 3671, 3891
Bsu36 I	1	2131	Tth111 I	2	1323, 2240
Cla I	1	108	Xma I	1	40
Drd I	2	2240, 4035	Xmn I	3	840, 1308, 5419

Restriction Enzymes that cut pWEB-TNC four or more times:

Aci I	BsiHKA I	Btg I	Hae II	Mbo II	Sau96 I
Acl I	Bsl I	Bts I	Hae III	Mly I	ScrF I
Afe I	Bsm I	Cac8 I	Hha I	Mnl I	SfaN I
Alu I	BsmA I	CviJ I	Hinf I	Mse I	Sfc I
Alw I	Bsp1286 I	Dde I	HinP I	Msl I	Sim I
AlwN I	BspH I	Dpn I	Hpa II	Msp I	Sml I
Apo I	Bsr I	Dra I	Hph I	MspA1 I	Taq I
Ava II	BsrD I	Dsa I	Hpy188 I	Mwo I	Tse I
Ban I	BsrF I	Eae I	Hpy99 I	Nci I	Tsp45 I
BciV I	BssK I	Eco47 III	HpyCH4 III	Nla III	Tsp4C I
Bfa I	BstDS I	EcoO109 I	HpyCH4 IV	Nla IV	Tsp509 I
Bpu10 I	BstF5 I	Fau I	HpyCH4 V	Ple I	TspR I
BsaJ I	BstN I	Fnu4H I	Mae II	PspG I	
BsaW I	BstU I	Gdi II	Mae III	Rsa I	
BsiE I	BstY I	Hae I	Mbo I	Sau3A I	

Restriction Enzymes that do not cut pWEB-TNC:

Acc65 I	BfuA I	Fse I	Pme I	Sfi I	Swa I
Afl II	Bgl II	Hpa I	Pml I	Sfo I	Tli I
Ale I	Blp I	Kpn I	PspOM I	SgrA I	Xba I
Apa I	BsiW I	Nar I	Rsr II	SnaB I	Xcm I
Asc I	BspM I	Nde I	Sac I	Spe I	Xho I
AsiS I	BsrG I	Nru I	Sac II	Sph I	
Avr II	BssH II	Nsi I	Sal I	Srf I	
Ban II	Dra III	Pac I	SanD I	Sse8647 I	
BfrB I	EcoN I	PaeR7 I	Sbf I	Stu I	

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@epicentre.com) or by calling Technical Service.

MaxPlax™ Lambda Packaging Extracts Protocol

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

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.

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.

Related Products: The following products are also available:

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

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:

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

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