High-Efficiency Cosmid Library Construction Using the New pWEB™
Cosmid Cloning Kit

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Introduction
Cosmid vectors are high capacity genomic cloning vectors that contain an origin of replication, a selectable drug resistance marker, and the cos site of phage lambda. In a typical cosmid cloning experiment, a sized Sau3A I partial digest of genomic DNA (30-45 kb) is ligated to a cosmid vector that has been linearized with BamH I. A properly sized segment of genomic DNA flanked by cosmid molecules with cos sequences in the same orientation is then packaged using a lambda in vitro packaging system. Following infection of the appropriate host strain, the cosmid clones are selected on an antibiotic plate. There are several problems commonly encountered using this approach. First, the genomic DNA must be at least 200 kb in length before partial digestion or only a small fraction of the 30-45 kb molecules will contain a restriction site at both ends. Isolating quality DNA of this size from some organisms and tissues can be difficult. Secondly, obtaining satisfactory partial digests of high molecular weight eukaryotic DNA can be difficult and time consuming. Finally, a Sau3A I partial digest is not truly random, possibly leading to failure to yield a particular clone.

To circumvent these problems, we have developed the pWEB™ Cosmid Cloning Kit. The pWEB Kit utilizes a vector containing a blunt-ended cloning site (Sma I). Randomly sheared genomic DNA is end-repaired, sized on a low melting point agarose gel, and subsequently cloned into the pWEB Vector. In this report, we demonstrate the high efficiencies obtainable using this approach, and present results for the construction of both eukaryotic DNA (wheat and mouse) and bacterial DNA libraries. The pWEB Cosmid Cloning Kit allowed us to construct a high efficiency (4 x 10⁶ cfu/µg insert DNA) wheat DNA library in two days.

The pWEB System

The pWEB Vector
The pWEB Vector, a derivative of pWE15, is depicted in Figure 1. The vector has a number of features that make it well suited for use in cosmid cloning. pWEB contains a unique Sma I site for the cloning of blunt-ended genomic DNA. This site is flanked by rare cutting Not I sites for the subsequent excision of insert DNA. The vector contains a col E1 origin of replication and ampicillin resistance for growth in E. coli, and an SV40 origin of replication and neomycin resistance for selection in eukaryotic cells. The vector contains M13 forward and T7 promoter primer binding sites that can be used for sequencing insert ends and generating probes for chromosome walking. Restriction mapping of insert DNA can be performed using lambda terminase and labeled oligonucleotides complementary to the cos sites (ON-R and ON-L). The pWEB Vector is conveniently supplied as a Sma I-linearized, dephosphorylated vector.

Control insert
DNA from phage T7 is provided as a control for the pWEB system. At 40 kb in size, the T7 Control DNA cloned into the pWEB Vector results in a 48 kb molecule, the optimal size for lambda packaging. The blunt-ended T7 Control DNA is ligated to the pWEB Vector and packaged using MaxPlax™ Lambda Packaging Extract. When the packaged pWEB/T7 molecule is transfected into a cell, T7 phage development ensues, resulting in the formation of T7 plaques, instead of the colonies normally obtained in a cosmid cloning experiment.

General pWEB procedure
A general outline of the procedure is shown in Figure 2. Because genomic DNA should be in the 30-45 kb range for optimal cloning efficiency, DNA greater than 50 kb is first sheared to the correct size range by expelling the DNA solution from a syringe through a small bore needle. The sheared DNA is then end-repaired to generate blunt ends for cloning into the pWEB Vector. The end-repaired DNA is resolved on a low melting point agarose gel and the DNA in the correct size range is excised. Next, the insert DNA is purified and concentrated from the gel slice using GELase™ Preparation and then ligated to the pWEB Vector. Alternatively, the ligation can be carried out directly in the gel. This in-gel ligation procedure is faster and more convenient, and in...
many cases, yields sufficient clones for a complete library. The ligation products are packaged using MaxPlax Lambda Packaging Extract, a restriction-free, high-efficiency in vitro packaging system. EPI305™ E. coli cells (restriction-free and recA) are then infected with the phage particles and plated onto LB/ampicillin plates. Use of restriction-free MaxPlax Extracts and the EPI305 strain is especially important for packaging of DNA bearing mammalian methylation patterns.

Methods

Wheat germ DNA library construction using two different ligation procedures

Wheat germ DNA was isolated using standard methods. The DNA was sheared by expelling the DNA from a syringe through a 25-gauge needle. The DNA was then end-repaired in a reaction containing 1X End-Repair Buffer, 0.25 mM each dNTP, 1 mM ATP, 20 µg of sheared genomic DNA, and 4 µl of End-Repair Enzyme Mix in a total volume of 80 µl. After a 45-minute incubation at room temperature, 16 µl of gel loading dye were added and the reaction was incubated for 10 minutes at 70°C. The end-repaired DNA was loaded onto a 1% low melting point (LMP) agarose preparative gel with 100 ng of T7 DNA (40 kb) in the outside lanes as a marker. The gel was run overnight at 30 volts at room temperature. The outside lanes containing the T7 markers were cut from the gel, stained with ethidium bromide, and the position of the T7 bands marked by inserting a pipet tip. The gel was reassembled and the genomic DNA migrating between the marker bands was excised. Alternatively, pulsed field gel electrophoresis (PFGE) or field inversion gel electrophoresis (FIGE) can be used to allow for more precise size fractionation.

Ligation was then performed either directly in the gel, or following purification and concentration of the DNA. The 50 µl in-gel ligation reaction contained 25 µl of melted 1% LMP gel containing the size-selected insert DNA, 1X Fast-Link™ Buffer, 1 mM ATP, 2 units of Fast-Link DNA Ligase, and 0.5 µg of pWEB Vector. The mixture was allowed to solidify and the ligation was allowed to proceed for two hours at room temperature. The reaction was then heated at 70°C for 10 minutes to melt the gel and inactivate the ligase, cooled to ~40-45°C, and treated with 1 unit of GELase Preparation for 15 minutes. The DNA (10 µl) was directly packaged using MaxPlax Packaging Extract according to the protocol3 and dilutions of the extract were mixed with EPI305 host bacteria and plated onto LB/ampicillin plates. The plates were incubated overnight at 37°C.

For ligation using purified and concentrated DNA, the excised gel slice was first incubated for 10 minutes at 70°C to melt the agarose. The DNA was then treated with 3 units of GELase Preparation for 30 minutes at 42°C. The sample was incubated for 10 minutes at 70°C to inactivate the GELase enzyme and the DNA was precipitated with two volumes of 100% ethanol. Then, 1 µg of pWEB Vector was ligated with the concentrated wheat DNA for 2 hours in a 10 µl ligation reaction. After heating for 10 minutes at 70°C, the entire ligation reaction was packaged using MaxPlax Packaging Extract. Dilutions of the extract were mixed with EPI305 host bacteria, spread on LB/ampicillin plates, and the plates were incubated overnight at 37°C.

Control template and background reactions

A control experiment was performed by ligating 0.5 µg of T7 Control DNA with 1.0 µg of pWEB Vector (vector:insert ratio = 10:1) in a 10 µl overnight ligation reaction at room temperature. The ligase was inactivated by heating for 10 minutes at 70°C and the entire reaction was packaged using MaxPlax Packaging Extract. The packaging reaction was serially diluted, adsorbed to LE392MP indicator bacteria, and plated to determine T7 phage titer. To test for vector background, an in-gel ligation was carried out as described above, except no insert DNA was added to the ligation. The self-ligated vector DNA was packaged and plated on EPI305 host cells as described above.

Results

The results of each experiment are shown in Table 1. The wheat germ DNA packaging reaction containing DNA that was concentrated prior to ligation yielded 2.8 x 10⁵ total clones, which corresponds to 2.6 x 10⁶ clones/µg of insert DNA. These values are much higher than the values typically reported for eukaryotic cosmids libraries, which range from 5 x 10⁴ clones/µg DNA to 1 x 10⁶ clones/µg DNA. These high ef-
ficiencies demonstrate that in some cases, it may be technically and economically feasible to construct and screen cosmid libraries without amplification using the pWEB system. This would be of great benefit since amplification of cosmid libraries often results in the selective loss of clones or allows for rearrangements of the genomic DNA.

When the in-gel ligation procedure was used, the packaging reaction yielded 5 x 10^6 clones. Using fluorometrically determined DNA concentrations in the gel slice, the efficiency was calculated to be 4.5 x 10^6 cfu/µg of insert DNA. Thus, the efficiency of cloning was approximately equal for the in-gel ligation procedure and the procedure using concentrated DNA; however, use of the concentrated DNA procedure produced more clones per packaging reaction.

DNA minipreps were prepared from several wheat germ clones and analyzed by restriction digestion with NotI, EcoR I, and BamHI (Figure 3). The 8.2 kb vector band is clearly visible in all three digests, and the insert DNA produced the expected variable patterns.

A mouse DNA library was produced from DNA that was purified from the gel and concentrated. The yield was 1.3 x 10^5 cfu/packaging extract and the efficiency was calculated to be 5.8 x 10^5 cfu/µg insert DNA. Whether the difference in cloning efficiency between wheat and mouse was due to an intrinsic difference in the DNA or just a reflection of the particular DNA preparation was not determined.

The yield of cosmid clones from the O. xanthineolytica packaging reaction was 5 x 10^4 clones per extract. From fluorometric measurements of insert DNA concentration in the gel slice, it was calculated that the yield of cosmid clones was 3.3 x 10^7 cfu/µg of insert DNA. Of 100 cosmid clones screened, 3 positives were found for the gene of interest.

The control experiment yielded 3-6 x 10^7 pfu/µg T7 DNA (Table 1). These efficiencies demonstrate that blunt-end ligation are not a limiting factor in this cosmid cloning system. Control experiments in which the pWEB Vector was omitted resulted in the formation of zero plaques. The vector background ligation resulted in the formation of 40 cfu per packaging extract. This background was at least several orders of magnitude lower than the number of clones obtained in the wheat germ DNA cloning experiment.

### Table 1. Cosmid Cloning Results.

<table>
<thead>
<tr>
<th>Sample</th>
<th>cfu (pfu) per extract</th>
<th>cfu (pfu) per µg insert DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat germ DNA (concentrated from gel)</td>
<td>2.8 x 10^5 cfu</td>
<td>2.6 x 10^6 cfu/µg</td>
</tr>
<tr>
<td>Wheat germ DNA (in-gel ligation)</td>
<td>5.0 x 10^4 cfu</td>
<td>4.5 x 10^5 cfu/µg</td>
</tr>
<tr>
<td>Mouse DNA</td>
<td>1.3 x 10^5 cfu</td>
<td>5.8 x 10^5 cfu/µg</td>
</tr>
<tr>
<td>Bacterial DNA</td>
<td>5.0 x 10^4 cfu</td>
<td>3.3 x 10^6 cfu/µg</td>
</tr>
<tr>
<td>T7 Control DNA</td>
<td>3.8 x 10^6 pfu</td>
<td>3.8 x 10^7 pfu/µg</td>
</tr>
</tbody>
</table>

### Summary

We have developed a high-efficiency, low-background cosmid cloning system for library construction. Randomly sheared genomic DNA is end-repaired to generate blunt ends for ligation into the SmaI pre-digested, dephosphorylated pWEB Vector. This approach eliminates the difficulties of isolating very high molecular weight genomic DNA and generating partial digests, and results in a truly random library. Sized, end-repaired genomic DNA can be ligated to the pWEB Vector directly in the gel. Following treatment with Gelase Preparation, the ligation products can be directly packaged using MaxPlax Lambda Packaging Extract without further purification. Using this approach, we have prepared bacterial and eukaryotic libraries that were ready for screening in 48 hours.

### References