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Stratagene's pSCOS1 as a cloning vector in the construction of a genomic library

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STRATAGENE'S pSCOS1 AS A CLONING
VECTOR IN THE
CONSTRUCTION OF A GENOMIC LIBRARY

A Thesis

Presented to

The Faculty of the Department of

Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

Timothy S. Andriese

May 1998

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ABSTRACT

STRATAGENE'S pSCOS1 AS A CLONING VECTOR IN THE CONSTRUCTION OF A GENOMIC LIBRARY

by Timothy S. Andriese

An evaluation is made of Stratagene's SuperCos1 (pSCos) cosmid cloning system, a 7.9 kilobase pair cosmid designed to construct genomic, cDNA or other libraries for use in the analysis of complex genomes. Though the protocol appears to be reasonably simple, there are, in fact, numerous steps which have stringent requirements. The resources necessary to meet these requirements include the use of systems that can accurately quantify DNA, the use of a field shifting electrophoretic unit, and the development of a positive insert control. The first two are often unavailable to a laboratory with limited resources, while the third does not exist. With the first two resources available, this vector proved to be of no use in the attempt to construct a genomic library. Though well designed, the problems associated with the vector's successful use proved insurmountable. Improvements in the vector and/or methods associated with its use are necessary.

Acknowledgments

It is now appropriate to thank those involved in a project of this type. From the outset, I would like to extend a special thanks to my advising professor Dr. Steven White, the person primarily responsible for providing this opportunity to me. His dedication and insight into the challenges associated with this work were invaluable, his enthusiasm about the work contagious, his patience greatly appreciated.

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Introduction

The field of molecular biology, generally speaking, defines the study of the mechanism, structure, or function of any subcellular system. This can range from the mechanisms controlling gene expression, to the cell to cell interaction of lymphocytes, to subcellular trafficking systems, to biochemical kinetic studies. In characterizing any such system, it is often necessary to clone a given DNA sequence. The cloning system to be used must be specific in its capabilities and is often chosen based on the dictated needs of the given protocol.

The word "clone" has taken on multiple definitions over the years. Recent research efforts have allowed for the asexual production of genetic duplicates of mammals, clones. But to a molecular biologist, cloning refers to the production of identical copies of a given piece of DNA, regardless of size. The method by which one goes about DNA cloning depends on the size, type, and application of the cloning effort.

The overall goal of this paper is to critique the usefulness of a previously characterized cosmid vector (SuperCos1 from the Stratagene Corporation) as a cloning vector. For purposes of comparison, it would be useful at this point to discuss some common cloning techniques prior to discussing SuperCos1 (pSCos1) in detail.

The Plasmid as a Cloning Vector

A commonly used DNA cloning vector is the plasmid, defined as an extrachromosomal DNA molecule usually found in a circular state, potentially

existing in multiple copy numbers per cell. Naturally occurring plasmids exist but are of little use in the laboratory (Old and Primrose, 1994). Many engineered plasmids, however, have been produced to increase vector effectiveness and to improve the efficiency or ease of recombinant selection. To better understand the advantages of the vector, one must consider the use of the plasmid. Having obtained an appropriate amount of plasmid DNA (via any number of conventional methods), the DNA would then be linearized using a restriction enzyme (RE) that cuts the plasmid only once. Insert DNA would then be modified to give not only DNA of an appropriate size (when insert size is crucial), but also DNA with cohesive ends to the plasmid. The insert and plasmid DNA would then be ligated to give a circular piece of recombinant DNA. This DNA would then be introduced into a host cell via one of several methods (i.e., chemical or electrical transformation, transduction, etc.), where transformed or transduced cells would then be selected for using antibiotic resistance, some type of reporter enzyme (i.e., beta-galactosidase) activity, or other appropriate marker systems. One commonly used selection system involves plating transformed cells out on Luria Agar (LA) plates supplemented with an antibiotic. In this example, the plasmid contains a "marker" (a gene that confers antibiotic resistance) that allows for selection. Selection between transformed and nontransformed cells is based on their ability to survive in media containing the antibiotic. Transformed cells survive, divide and form colonies whereas nontransformed cells die or fail to divide. Using a single colony as an inoculum, a culture could then be grown in selective medium allowing for the amplification of the cloned DNA. Subsequent plasmid isolation and modification would then allow one to isolate and apply the clone to further studies.

There are notable advantages in using a plasmid as a cloning vector. First, most commonly used plasmids are of relatively low molecular weight, with a range from 2.8 to 14 kbp (Singer and Berg, 1991; Old and Primrose, 1994). This makes them easy to manipulate and resistant to mechanical damage. Second, many plasmids have a region known as a multiple cloning site, a short portion of DNA containing multiple unique restriction sites, any one of which may be used for DNA insertion. This site also allows one to enzymatically excise the fragment using an appropriate restriction enzyme. Third, most commercially available plasmids have a well characterized selection marker (ampicillin resistance, tetracycline resistance, beta-galactosidase activity, etc.) allowing for easily detectable transformants. Fourth, most commonly used plasmids have origins of replication that allow for many copies of the plasmids per cell (Singer and Berg, 1991), ultimately producing large amounts of recombinant DNA. Finally, cloning using a plasmid typically is inexpensive and simple.

DNA cloning using a plasmid vector, however, has some important limitations. First, the transformation efficiency in general is inversely proportional to the size of the insert DNA (Old and Primrose, 1994). In addition, the size of insert DNA is typically limited to small fragments (i.e., below 14 kbp). Using a vector whose insert is size limited might not allow for the isolation of an entire gene (sometimes a crucial requirement). For example, the chicken ovalbumin gene is 7.7 kbp in length (Singer and Berg, 1991). It would be impractical to attempt to capture the entire gene, including introns and flanking sequences, on a 10 kbp insert. During insert DNA modification using the appropriate RE, the insert DNA strand would most often be cut in the middle of the ovalbumin gene, ultimately producing a clonable fragment that

contains only a portion of the entire gene. And lastly, the types of hosts that can accommodate a plasmid vector are limited primarily to prokaryotes.

Phage as a Cloning Vector

The bacteriophage lambda has been studied extensively for more than 30 years, where its life cycles and genome sequence (Sanger et al., 1982) have been well characterized. With the advent of cloning systems whose insert DNA capacities are larger (cosmids, bacterial artificial chromosomes, or BACs, and yeast artificial chromosomes, or YACs), alternatives to the lambda cloning system have been produced. Yet, due to its relative simplicity, it is still widely used in the rapid construction of genomic libraries (Hohn and Murray, 1977).

Lambda phage is a double-stranded DNA virus whose linear genome is 48.5 kbp in length (Sanger et al., 1982). Flanking the genome are two identical, single-stranded DNA segments of 12 base pairs, called "cos" sites. These cos sites are complementary and thus cohesive, and allow the genome to circularize in vivo following infection of any host cell. Lambda's usefulness in cloning comes from the fact that a center portion, approximately 25% of its genome, is not essential for viral replication and propagation. This center section can be removed enzymatically and replaced with insert DNA from another source. Subsequent viral replication allows for replication of the foreign DNA insert (Thomas et al., 1974; Leder et al., 1977).

The protocol for using this system involves a few conceptually simple steps. Insert DNA must first be isolated from any chosen source. It must then be optimally digested with a RE to give segments of around 10-14 kbp. A "backfill" reaction then follows the optimal digestion of insert DNA. Using only two of the four possible dNTPs, this reaction converts the complementary

four base pair overhang regions on the ends of digested DNA fragments to a noncomplementary two base pair overhang. These modified insert DNA fragments are ligated to lambda DNA flanking sequences called "lambda arms". Both of these arms possess the lambda *cos* site sequence and allows this ligation product to be packaged *in vitro* using lambda coat protein extracts, where the resulting lambda viral particles (Hohn and Murray, 1977) are used to infect a host cell strain. (Note, these reagents—lambda arms and coat proteins—can be produced arduously in the lab, or purchased from one of many reputable vendors).

As previously mentioned, this cloning strategy is reasonably straightforward and comparatively inexpensive. It does, however, have inherent disadvantages. The insert DNA size is limited to no more than 23 kbp. As previously mentioned, this might be disadvantageous and/or prohibitive in any cloning effort. In addition, the host cell is limited to *Escherichia coli*.

The Cosmid as a Cloning Vector

A modern cloning vector that allows for the cloning of DNA ranging from 30-45 kbp is the cosmid. A number of different cosmids have been produced whose applications, screening methods, and host cells all differ. Applications of the cosmid include both cDNA and genomic library production and high-resolution restriction mapping. In addition, the capacity of the cosmid to accept larger foreign DNA inserts allows the cosmid to be used in chromosome mapping through a technique called "chromosome walking", which is used to map various genes of interest. By identifying a large series of

overlapping cosmid clones, a physical map is produced and can be used to identify the location of a given genetic sequence.

There are many common characteristics between plasmid and cosmid cloning systems. The cosmid by itself acts like a plasmid *in vivo* by autonomously replicating in multiple copies, is circular, and usually has multiple selection markers. The cosmid does, however, possess at least one (sometimes two) sequence(s) not found in a typical plasmid, the lambda cos site. Figure 1 shows the steps followed in the creation of a genomic library using a cosmid vector. In general, this cosmid system requires that a lambda cos site be ligated to each end of an insert DNA segment. For any given insert DNA fragment, it must be of the appropriate size (which itself depends on the vector) along with cohesive ends to the vector, allowing for the ligation of vector to insert. The resulting recombinant DNA fragment, an insert flanked by cos sites, can then be packaged using lambda coat proteins where the resulting viral particles are then used to infect a host cell line. Any appropriately ligated DNA fragments between 46 and 52 kbp in length (following ligation) can successfully package using lambda coat proteins and can potentially infect a suitable host. Once infection has taken place, the linear recombinant DNA circularizes and begins to replicate as a plasmid *in vivo*. One can then select for transformants, grow up individual colonies for the use in plasmid isolation, and carry out further DNA manipulations as required.

Other Cloning Systems

When it is necessary to clone segments of DNA that are greater than 50 kbp, one must shift to another system. One system that allows for the cloning

Figure 1. A flow chart showing the series of steps in the creation of a genomic library using Stratagene's cosmid vector SuperCos1.

of segments up to 1000 kbp is the yeast artificial chromosome (YAC). The vectors are engineered DNA molecules designed to be maintained and/or amplified as plasmids in a bacterial host strain. Following isolation, the vectors are linearized and ligated to foreign DNA to form extremely long chromosome-like molecules. The resulting linear DNA molecule is then introduced into a yeast host, where transformants are then selected. For the resulting YAC to be stable in the yeast cell through multiple rounds of mitosis, it should be greater than 150 kbp (Murray and Szostak, 1983), and possess telomeric regions for stable DNA replication, a centromeric region (CEN), a replication region (ARS), and a selectable marker (LEU2, HIS3). Any YAC consisting of less than 20 kbp was found to be unstable (Singer and Berg, 1991; Old and Primrose, 1994).

The advantages of using YACs as cloning vectors have already been mentioned, yet the disadvantages and/or difficulties are great. First, YACs are difficult to isolate without shearing the large 50 kbp plasmid, which is true of any DNA molecule greater than approximately 50 kbp. In addition, transformation of yeast is neither easy nor efficient, making a YAC cloning vector difficult to use.

Library Types

As mentioned previously, present studies in molecular biology can vary greatly. Often times it is necessary to probe, or look into the genome of a given organism to study the structure or function of discrete areas of its genome. To do this it is necessary to have a complete and accurate representation of the genome itself. Unfortunately, a sample of isolated genomic DNA by itself is of little use to the researcher since any given region is found in reasonably low

numbers. It is often necessary to have many copies of a particular DNA sequence, necessitating its cloning. In order to probe an entire genome, it is therefore necessary to have a complete representation of the genome in a cloned form. A complete representation of the organism's genome cloned into a chosen vector is called a *library*, in this case, a genomic library.

In order to get a complete (or nearly complete) representation of the DNA sequences in the genome of interest, it is necessary to produce an appropriate *number* of recombinant DNA molecules, or clones. This number itself depends on the genome size, insert DNA size, and the ultimate desired probability that the full genome has been cloned. If one produces this number, a viable genomic library has been produced, one that may represent the entire sequence of the total genome.

The formula (Clark and Carbon, 1976) that describes the number of recombinants (N) needed to give the probability (P) that all sequences of the genome have been captured is:

$$N = \ln(1-P) / \ln[1-(1/n)]$$

where n represents the size of the genome relative to the average size of a cloned fragment (i.e., genome size divided by average insert size).

Another library to be considered is the complementary DNA (cDNA) library. Using reverse transcriptase, DNA is produced *in vitro* from isolated mRNA, ligated into a vector, and used to transform a host cell strain to produce recombinants. The major difference in this case is that the library itself does not represent the whole genome of the organism, but that of the genes being expressed at the time of mRNA isolation, whether they be involved in

embryonic development or simply cellular "house keeping." This is a major tool used by developmental biologists interested in gene expression at temporally discrete windows during embryonic development.

Stratagene's pSCos1 as a Cosmid Cloning Vector

One of the few commercially available cosmids is pSCos1 (pSCos) from the Stratagene Corporation (San Diego, CA). A map of this vector is seen in Figure 2. This vector is designed to be used in the creation of genomic and

Figure 2. A map of Stratagene's SuperCos1 cosmid vector.

cDNA libraries for further use in chromosome walking, restriction mapping studies, or as an expression vector (Stratagene Corp, 1997). This 7.9 kbp cosmid has two lambda phage cos sites that are reported to allow for highly efficient production of packagable recombinants from DNA that has not been sorted based on insert fragment size. An ampicillin resistance gene and prokaryotic origin of replication sequence allows for prokaryotic selection and amplification, respectively. SV40 and neomycin resistance (NEO) sequences allow for eukaryotic amplification and selection. In addition to dual EcoRI

restriction sites, dual NotI restriction sites, and a single BamHI restriction site, its multiple cloning site (MCS) has both T3 and T7 promoters for gene expression. Since the restriction enzyme NotI cuts DNA strands at a theoretical average of only one in 65,536 base pairs (4⁸), the dual NotI restriction sites allows for specific excision of cloned DNA.

A Practical Assessment of pSCos1 Use in the Small Lab

The original purpose of this project was to construct a chicken (*Gallus gallus*) genomic library, followed by a low stringency screening of the library using a probe derived from either a mouse or frog developmental "master control gene", the latter effort, of course, dependent upon the success of the former. There are, however, many subtle techniques associated with the DNA modifications and manipulations required in cosmid library construction that are not readily obvious nor fully documented. When it became clear that the protocol supplied by Stratagene, even when faithfully followed, was doomed to repeated failure, the nature of this project shifted. It was decided at that point that what was required was a careful and rigorously controlled investigation into the individual steps inherent in library construction using pSCos. Special emphasis was therefore to be placed on the design of controls that allowed the assessment of efficiencies and completeness of reaction at each step. This project thus became an effort to formally reveal the problems associated with each step in cosmid library construction, to convert art to science.

Materials and Methods

I. Genomic DNA Isolation and Modification

Genomic DNA Isolation

To isolate high molecular weight (HMW) DNA, a classical dodecyltrimethylammonium bromide:cetyltrimethylammonium bromide (DTAB:CTAB) DNA isolation protocol (Gustincich *et al*, 1991) was initially employed, then modified to the following protocol. A fertilized white leghorn chicken egg (*Galus galus*) was obtained and incubated until approximately 8 days post coitum. The embryo was then removed from its shell using a modified, hook-shaped Pasteur pipette under sterile conditions. In a sterile petri dish, the head, legs and wings were removed using a sterile razor blade. The weight of the torso was then determined to get a general idea of the volume of tissue. In 2 ml sterile water, the torso was minced using sterile razor blades and placed into a 50 ml conical plastic centrifuge tube. To this mixture, 4 ml of DTAB (8% dodecyltrimethylammonium bromide, 1.5 M NaCl, 100 mM Tris, 50 mM EDTA, pH 8.6) were added. This solution was mixed via multiple gentle inversions and placed into a 65° C water bath for 30 minutes. The mixture was then equilibrated to 37° C where lyophilized Proteinase K was added to give a final enzyme concentration of 0.43 mg/ml, and digested at 37° C overnight. Protein and DTAB were extracted by adding an equal volume of chloroform, whereupon the solution was gently mixed by multiple inversions. Centrifugation followed at 2,000 x g for 5 minutes, whereupon the aqueous phase was then transferred to a new 50 ml conical plastic centrifuge tube avoiding the white precipitate at the interphase. (Note: All pipetting of HMW

DNA was done using large bore pipette tips to minimize the amount of hydrodynamic shearing of DNA.) This extraction procedure was repeated two more times. The aqueous phase was then stored at 4 ° C. Eight ml of 1.2 M NaCl were added to 2 ml of this DNA:DTAB mixture. The resulting very viscous solution was placed in a 55° C water bath for 3 days to facilitate DNA solubilization.

To remove the DTAB and NaCl, the solution was then dialyzed (from Spectropore Dialysis Tubing - 10,000 MW cutoff) in Tris/EDTA (TE--5 mM Tris, 1 mM EDTA, pH 7.4) supplemented with NaCl, where the concentration of NaCl progressively decreased. Approximately 10 cm of dialysis tubing was boiled for 15 min in a 50 mM EDTA solution. Two milliliters of the DNA:DTAB:NaCl solution were placed into approximately 5 cm of dialysis tubing allowing for an increase in volume due to osmotic movement of fluid into the bag. The tubing was then sealed using two plastic dialysis tubing clamps. The partially filled dialysis bag was then placed into 100 ml of TE at 1.2 M NaCl and allowed to dialyze overnight at 4° C. The dialysis bag was then transferred to 100 ml of TE at 1.0 M NaCl and allowed to dialyze for 3 hr at room temperature, then transferred and dialyzed through a series of TE buffers containing decreasing concentrations of NaCl (TE at 0.8 M NaCl, then 0.6 M NaCl, then 0.3 NaCl, then 0.0 M NaCl). Dialysis times varied alternately from 3 hr at room temperature to overnight at 4° C. Dialysis in simple TE buffer was repeated at the end to help ensure complete removal of detergents, salts, peptides, small oligonucleotides, etc. Three small aliquots of the genomic DNA were removed (10 μ l, 5 μ l, and 2.5 μ l) and analyzed on a pulse-field (see Appendix 1) agarose gel (1% agarose in Tris-Acetate-EDTA, or TAE, 14 cm X 14 cm X 0.5 cm, hexagonal electrode set-up, 8.9 V/cm, 15 sec pulse time- NS vs. EW, 14° C, 27.5 hr) along with DNA standards

consisting of 100 ng of lambda HindIII-digested DNA, 100 ng of lambda DNA, and approximately 650 ng Pulse-Field Midrange Markers from New England BioLabs (see Appendix 1). Following electrophoresis, the gel was stained in ethidium bromide (EtBr) at a concentration of 0.5 $\mu\text{g}/\text{ml}$ for 2 hr, and visualized on a BioRad Gel Doc 1000 gel imaging system using Molecular Analyst software. Here the extent of DNA digestion was assessed, whereupon DNA concentration was determined by measuring absorbance at OD₂₆₀ nm.

Partial Digestion of Genomic DNA

To define *Sau3A* partial digestion conditions able to generate 30-42 kbp fragments of DNA, an optimization experiment was performed. To seven microfuge tubes, approximately 1.0 μg dialyzed genomic DNA (DgDNA at 14.4 μl at 68.2 ng/ μl) and 1.6 μl of 10X restriction enzyme (RE) buffer were added and allowed to come to room temperature. Just prior to enzymatic digestion, 1 μl of *Sau3A* (4 U/ μl) was added to 159 μl of 1X RE solution. This solution (2.5×10^{-2} U/ μl) was then used to create a 2-fold dilution series of the RE. Ten microliters of each dilution was added to one of the previously prepared seven tubes to give a RE:DNA ratio of 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256 U/ μg DNA. Each tube was then immediately transferred to a 37° C water bath and incubated for exactly one hour. Following the one hour digestion, 1 μl of 0.5 M EDTA was added to each tube (in the same order that the RE was added). The tubes were then transferred to a 65° C water bath for 15 min. to heat inactivate the RE. The digestion products were then assessed via pulse field agarose gel electrophoresis as previously described, with the sole exception of duration which was 26.5 hr.

A preparative partial digestion of HMW dialysed, genomic DNA (DgDNA) was done as follows. Three separate 500 μ l aliquots of HMW DgDNA (24 ng/ μ l, 12 μ g total) were digested with *Sau*3A at 1/8, 1/12, and 1/16 U/ μ g DNA. To each 500 μ l volume, 55 μ l of restriction enzyme buffer at ten times normal reaction concentration (10X RE buffer) were added. In a different microfuge tube, 39 μ l of 1X RE buffer and 1 μ l of *Sau*3A (4 U/ μ l) were mixed via trituration to give a RE solution at 0.1 U/ μ l. To each of the three tubes containing the 500 μ l aliquots of DgDNA, 15 μ l, 10 μ l, and 7.5 μ l aliquots of RE (0.1 U/ μ l) were added to give the RE concentrations of 1/8, 1/12, and 1/16 U/ μ g DNA. The tubes were mixed via gentle inversions and immediately transferred into a 37° C water bath and incubated for exactly one hour. Following the digestion period, 12 μ l of 0.5 M EDTA were added to each tube and the tubes were then transferred to a 65° C water bath for 30 minutes to thermally inactivate the RE. Following this period, 10 μ l of each digestion product were removed for pulse field gel electrophoretic analysis as previously described. Each of the three tubes were then phenol:chloroform:isoamyl (25:24:1) alcohol extracted (P:C:IAA) as follows. Equal volumes of P:C:IAA were added to each tube, whereupon the tubes' contents were mixed by multiple inversion, spun at 12,000 x g for 1 min, then each of the three aqueous phases were transferred to a new microfuge tubes. These solutions were then extracted twice with an equal volume of chloroform in a similar fashion. The aqueous phases were then sodium acetate:ethanol (NaAc:EtOH) precipitated as follows. One ninth the total volume (56 μ l) of 3M NaAc (pH 5.5) and 2.5 volumes of ice-cold, 100% EtOH were added to each of the tubes. The solution was then mixed by multiple inversions, spun at 12,000 x g for 15 min, and the supernatant was then discarded. To each tube, 300 μ l of ice-cold 75% EtOH were added. Each tube was

inverted a number of times to dislodge the pellet from the bottom of the microfuge tube. At this point, the three pellets and the 300 μ l of EtOH suspending each of them were pooled in the same tube. This tube was then spun at 12,000 x g for 2 min, then the supernatant was gently pipetted off and discarded. The pellets were washed two more times with 300 μ l 75% EtOH with multiple inversions and subsequent centrifugation, where the supernatant was discarded. The resulting white pellet was then vacuum dried and resuspended in 150 μ l TE (10 mM Tris, 1 mM EDTA, pH 7.4) in a 55° C water bath overnight.

Dephosphorylation of Genomic DNA with Calf Intestinal Alkaline Phosphatase

The partially digested, dialyzed genomic DNA (approximately 36 μ g) was then digested with calf intestinal alkaline phosphatase (CIAP) as follows. To the 150 μ l of DgDNA, 15.5 μ l of CIAP 10X buffer and 75 units of CIAP (7.5 μ l at 10 U/ μ l) were added. The solution was then mixed via inversion and incubated at 37° C for 1 hr. The tube was then transferred to a 65° C water bath for 15 min, then P:C:IAA extracted, followed by two chloroform extractions as previously described. The solution was then NaAc:EtOH precipitated as previously described. The pellet was dried by vacuum dessication, resuspended in 100 μ l TE, and stored at 4° C.

Finally, a 1% agarose TAE gel was run to assess genomic DNA concentration. Serial dilutions of the modified gDNA were run through the gel for 1.5 hr at 5 V/cm at room temperature. Following electrophoresis, the gel was stained in EtBt for at least 2 hr, then genomic DNA masses were determined using the BioRad Digital Gel Imaging System (Fig. 4, see Appendix 2).

II. Cosmid Vector Isolation and Modification

Cosmid Vector Isolation

The *E. coli* strain XL1 Blue MR (Δ [mcrA]183, Δ [mcrCB-hsdSMR-mrr]173, *endA1*, *supE44*, *thi1*, *recA1*, *gryA96*, *relA1*, *lac*) containing the Stratagene product Super Cos1 (pSCos) was inoculated into 50 ml of Luria Broth (LB) (Ausubel, 1992) supplemented with ampicillin to a final concentration of 50 μ g/ml (LB/Amp). This culture was incubated overnight in a 37° C shaking hot air incubator at 200 rpm. As controls, an ampicillin sensitive strain of *E. coli* (LE392) was grown in both 20 ml of LB/Amp and 20 ml of LB to ensure that both the ampicillin stock solution and the sensitive cell strain were viable.

Using this overnight culture, cosmid DNA was isolated using Bio-Rad's Quantum Prep Plasmid Miniprep Kit. Two plasmid preps were run in parallel as dictated by the vendor with the following noted exceptions. Instead of using 2.0 ml of the overnight cell culture per preparation, approximately 7.5 ml of culture was used per preparation. Two 100 μ l elution volumes of TE were used per preparation instead of a single elution volume of 100 μ l. Following cosmid isolation, the final elution volumes were pooled in one microfuge tube totaling 400 μ l, where the overall DNA mass was later estimated to be around 50 μ g.

Digestion of pSCos with the Restriction Enzyme *Xba*I

In order to set a RE:DNA reaction ratio of 3 U *Xba*I/ μ g vector DNA, 44 μ l of 10X RE Buffer D (60 mM Tris-HCl, 60 mM MgCl₂, 1.5 M NaCl, 10 mM DTT, pH 7.9) were added to the above cosmid miniprep and mixed via trituration. One hundred and fifty units of *Xba*I RE (New England BioLabs, 15 μ l at 10 U/ μ l)

were added and mixed. The reaction tube was incubated in a 37° C water bath for 3 hr. A 3 μ l aliquot of the digestion product was removed for agarose gel electrophoretic analysis, where the tubes were then returned to the 37° C water bath.

To ensure that enzymatic digestion of pSCos was complete, the 3 μ l aliquot was used to create a 4-fold dilution series using TE as the diluent, and the series was run out on an 0.8% agarose TAE gel (14 cm x 10 cm x 0.5cm, at 2 V/cm for 4 hr at room temperature). Following electrophoresis, the gel was stained in EtBr (0.5 μ g/ml) for 2 hr. The DNA fragment sizes and masses were assessed using the aforementioned gel imaging system. Following RE digestion, the contents of the reaction tube were P:C:IAA extracted followed by two chloroform extractions, followed by an NaAc:EtOH precipitation as previously described. Following precipitation, the pellets were dried by vacuum dessication and then resuspended in 90 μ l sterile deionized (DI) water.

Since restriction enzyme digestion of pSCos was incomplete, 10 μ l of 10X RE Buffer D was added and mixed via trituration. To this solution, 50 units of *Xba*I (5 μ l at 10 U/ μ l) were added giving a RE:DNA ratio of 1.0 U/ μ g DNA. The solution was mixed and then transferred to a 37° C water bath overnight. A 0.5 μ l aliquot of the digestion product was used to create a 2-fold dilution series (similar to previous serial dilutions). This series was then run out on a 1% agarose TAE gel, 10 cm x 8 cm x 0.5 cm, at 5 V/cm for 2 hr at room temperature. Following electrophoresis, the gel was stained and analyzed as before.

At this point, another 5 μ l of *Xba*I (10 U/ μ l) was added to the tube giving a RE:DNA ratio of up to 2.0 U/ μ g DNA, and the mixture was allowed to digest overnight in a 37° C water bath. A 0.5 μ l aliquot of the digestion product was run out on a 1% agarose TAE gel and analyzed as described above. The digested

DNA was then P:C:IAA/chloroform extracted and NaAc:EtOH precipitated as previously described. Following precipitation, the pellet was then dried by vacuum dessication, and the pellet was resuspended in 42.5 μ l sterile DI water.

Dephosphorylation of pSCos with Calf Intestinal Alkaline Phosphatase

The digested pSCos (approximately 35.9 μ g in 42.5 μ l) was mixed with 5 μ l of calf intestinal alkaline phosphatase (CIAP) 10X reaction buffer (0.5 M Tris-HCl, 10 mM MgCl₂, 1 mM ZnCl₂, 10 mM spermidine, pH 9.3), followed by the addition of 2.5 μ l of CIAP (10 U/ μ l) to give a final CIAP concentration of 0.5 U/ μ l. This mixture was incubated overnight at 37° C, followed by heat inactivation at 65° C for 15 min to inactivate the CIAP. The solution was then P:C:IAA extracted once, chloroform extracted twice, then NaAc:EtOH precipitated as previously described. Following precipitation, the DNA was dried by vacuum dessication and resuspended in 100 μ l TE giving a DNA concentration of approximately 350 ng/ μ l, and then stored at -20° C.

Test Ligation of XbaI-digested, Dephosphorylated pSCos

To ensure that the phosphatase reaction was complete, XbaI-digested, dephosphorylated pSCos was test ligated to itself. The reaction mixture was prepared by mixing 2.0 μ l modified vector (300 ng/ μ l) with 3.0 μ l 10X rATP, 3.0 μ l 10X ligase buffer, 22.0 μ l sterile distilled water, and 0.25 μ l T₄ DNA ligase (Promega, 10 U/ μ l). Control ligations of XbaI-digested pSCos and lambda HindIII-digested DNA were also performed in a similar fashion. All solutions

were mixed and allowed to incubate overnight at 14° C. The incubation products were electrophoresed on a 1% agarose TAE gel and analyzed as before.

Digestion of *Xba*I-digested, Dephosphorylated pSCos with *Bam*HI

One hundred microliters of *Xba*I-digested, dephosphorylated pSCos (approximately 350 ng/ μ l) were mixed with 11 μ l of 10X RE reaction buffer E (60 mM Tris-HCl, 60 mM MgCl₂, 1.0 M NaCl, 10 mM DTT, pH 7.5), followed by the addition of 90 units of *Bam*HI (9 μ l at 10 U/ μ l). The solution was mixed via trituration, then incubated at 37° C overnight. A small aliquot of the digestion product was run out on a 1% agarose TAE gel and analyzed as before.

Determination of Modified Vector DNA Concentration

In order to accurately determine the concentration of *Xba*I-digested, dephosphorylated, *Bam*HI digested pSCos solutions, serial dilutions (using TE as the diluent) were run out on a 1% agarose TAE gel, 10 cm x 8 cm x 0.5 cm, at 5.0 V/cm for 1.5 hr at room temperature. Following electrophoresis, the gel was stained and analyzed, where DNA concentration was calculated using BioRad's digital gel imaging system.

III. Ligation of Modified pSCos and Modified Genomic DNA

As dictated by the vendor, the ratio of vector to insert DNA ligated was 1.0 μ g to 2.5 μ g, respectively. Approximately 25.0 μ l of insert DNA (200 ng/ μ l) were mixed with 3.0 μ l modified pSCos (800 ng/ μ l) along with 3.7 μ l of both 10X ATP and 10X ligation buffer. At this point, 2.5 μ l (533 ng DNA) were removed for later pulse-field gel electrophoresis. To the remaining solution, 2.0 μ l T₄ DNA ligase (10 U/ μ l, 20 U total) were added. To ensure efficient ligation, a

control ligation reaction was performed in parallel in a similar fashion using lambda HindIII-digested DNA. Both final reaction solutions were mixed via thorough stirring with the pipette tip and briefly centrifuged to pool all liquid at the bottom of the reaction vessel. Both mixtures were then incubated at 14° C overnight.

Following overnight ligation, a 1% agarose pulse-field gel was run as described previously (except duration of run was extended for 3.0 hr) to assess the ligation products. Following electrophoresis, the gel was stained in EtBr as before and both the DNA fragment sizes and masses were assessed using BioRad's gel imaging system.

IV. Packaging of DNA and Infection of Host Cells

Packaging of Experimental and Control DNA

The day prior to packaging the experimental and control DNA, an *E. coli* culture of XL1Blue MR was started. From a cryopreserved culture in 7% DMSO (Ausubel, 1992), 10 μ l of XL1Blue MR were inoculated into LB supplemented with maltose and MgSO₄ to give final concentrations of 0.2% and 10 mM, respectively (LB-maltose/MgSO₄). The culture was put into a 37°C shaking hot air bath at 200 rpm overnight.

Packaging of ligated insert and vector DNA with lambda phage coat proteins (Packagene from Promega) was done, along with vendor-supplied control lambda DNA. Both packaging reactions were done as dictated by the vendor. Both reaction mixtures were allowed to incubate at room temperature for exactly 3 hr, where 400 μ l phage buffer (PB: 20 mM Tris-HCl, 100 mM NaCl, 10 mM MgSO₄, pH 7.4) and 22.5 μ l of chloroform were added to the experimental packaging reaction, and 45 μ l PB and 2.5 μ l chloroform were added to the

control packaging reaction. Both reaction solutions were mixed via inversion and stored at 4° C.

Infection of the XL1Blue MR with Packaged Cosmid

The day of DNA packaging, a new XL1Blue MR cell culture was set up by inoculating 100 μ l of the overnight culture into 20 ml of LB-maltose/MgSO₄, and grown in similar conditions until OD₆₀₀=0.6.

A two-fold dilution series of packaged insert and vector DNA was created using a sterile phage suspension medium (SM: 0.01% gelatin, 50 mM Tris-Cl, 100 mM NaCl, 8 mM MgSO₄, pH 7.4) to give six dilutions of 30 μ l each whose dilution factors were 10, 20, 40, 80, 160, and 320. To each of these 30 μ l dilutions, 30 μ l of the above XL1Blue MR culture (OD₆₀₀ = 0.6) were added to each tube, and mixed via gentle finger flicks. Each tube was incubated at room temperature (22° C) for 30 min, after which 200 μ l of fresh sterile LB were added to each tube. All tubes were placed into a 37° C water bath and incubated for 1 hr, shaken gently every 15 min. At the end of this 1 hr incubation period, all tubes were centrifuged for 1 min at 12,000 x g and resuspended in 50 μ l fresh sterile LB. Each 50 μ l volume was then sterilely plated out onto a Luria Agar (LA) culture plate supplemented with ampicillin at 50 μ g/ml (LA/AMP). Each plate was then incubated in a 37° C warm air incubator overnight. The next morning, cell colonies were counted, and titer of the original packaged cosmid solution was calculated.

Infection of the XL1Blue MR with Packaged Control DNA

A ten-fold dilution series of packaged control DNA was created using SM to give eight dilutions whose dilution factors were 10¹ through 10⁸. To a 25 μ l

aliquot of each dilution, an equal volume of the aforementioned XL1Blue MR culture ($OD_{600}=0.6$) was added to each tube, and mixed via gentle finger flicks. Each tube was incubated at room temperature (22°C) for 30 min, and each dilution culture tube was then plated out as follows. The $50\ \mu\text{l}$ volume (10^1 phage and cells) was added to 5 ml of sterile, liquid lambda top agar (Ausubel, 1992) which had been pre-equilibrated at 55°C . The top agar was then quickly vortexed to thoroughly mix in the phage, and poured out onto an appropriately labeled LA plate. This procedure was repeated for each phage dilution. Approximately 15 min after pouring the agar (during which time the top agar had solidified), each plate was transferred to a 37°C warm air incubator and allowed to incubate overnight. The next morning, plaques were counted and the titer of the original packaging solution was calculated.

As controls, an ampicillin-resistant cell strain (XL1 Blue MR with pSCos) and an ampicillin-sensitive cell strain (XL1Blue MR) were grown on both LA and LA/AMP plates to ensure that both the LA/AMP plates and cell strains were viable.

Results

I. Genomic DNA Isolation and Modification

The torso of the chick embryo (approximately 8 days post coitum) was found to be approximately 2.5 grams. When digested by Proteinase K in 2:1 DTAB to water, the resulting solution was very viscous (presumably due to HMW DNA), which prohibited further manipulation. This necessitated dilution of the solution with 8.0 ml of 2:1 DTAB:water.

The success of HWM DNA isolation is shown by a pulse-field agarose gel (Figure 3). When compared to the standard DNA, it is evident that there is a smear of DNA between 48.5 and 290 kbp, with the majority greater than 150 kbp. Absorbance (OD_{260nm}) of the DNA in solution at 1/2 the original concentration was 0.682, giving a DgDNA concentration of approximately 68.2 ng/ μ l.

Another pulse-field agarose gel was run to assess the digestion products of the optimal digestion of HMW DNA with *Sau*3A, shown in Figure 4. Since the optimal DNA fragment size for the insert ranges from 30-42 kbp, the ratio of RE used per microgram of genomic DNA must be determined. When comparing the various digestion products to the DNA standards, Figure 4 shows that the optimal ratio for this DNA isolate ranges from 1/16 to 1/8 U/ μ g DNA. Table 1 shows the gel imaging system's analysis of DNA masses per lane. These data were used to calculate the average mass of DNA per microliter of DgDNA, which was 24.1 ng/ μ l.

Another pulse-field agarose gel was run to assess the digestion products of the preparative partial *Sau*3A digestion of high molecular weight, dialyzed

Figure 3. This pulse-field agarose gel shows the size of DNA fragments from a DTAB DNA isolation protocol. Lanes 1 and 2 contain 100 ng of lambda *Hind*III digested DNA (fragment lengths of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56, and 0.125 kbp) and 100 ng uncut lambda DNA (fragment length of 48.5 kbp), respectively. Lane 3 contains Pulse-field Midrange Markers (291.0, 276.0, 257.5, 242.5, 227.5, 209.0, 194.0, 179.0, 160.5, 145.5, 130.5, 112.0, 97.0, 82.0, 63.5, 48.5, 33.5, and 15.0 kbp). Lanes 4, 5, and 6 all contain dialyzed genomic DNA, 10, 5, and 2.5 μ l, respectively. Lane 7 contains lambda concatamer DNA.

Figure 4. A pulse-field agarose gel showing the products of a genomic DNA digestion protocol designed to maximize the production of DNA fragments whose sizes range from 30-42 kbp. Lanes 1 and 2 contain lambda *Hind*III digested and lambda DNA, respectively. Lane 3 contains Pulse-field Midrange Markers. Lanes 4 through 10 contain dialyzed genomic DNA digested with *Sau*3A with a DNA:restriction enzyme ratio of 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256 U *Sau*3A/ μ g genomic DNA, respectively. Lane 11 contains undigested dialyzed genomic DNA.

Lane:	Volume Name	Type	Mass (ng):
1	Lambda <i>Hind</i> III-digested DNA	Standard	200
2	Lambda	Standard	100
3	PFG Markers	Standard	650
4	1/4 U <i>Sau</i> 3A/ μ gDNA	Unknown	156
5	1/8 U <i>Sau</i> 3A/ μ gDNA	Unknown	361
6	1/16 U <i>Sau</i> 3A/ μ gDNA	Unknown	336
7	1/32 U <i>Sau</i> 3A/ μ gDNA	Unknown	331
8	1/64 U <i>Sau</i> 3A/ μ gDNA	Unknown	367
9	1/128 U <i>Sau</i> 3A/ μ gDNA	Unknown	370
10	1/256 U <i>Sau</i> 3A/ μ gDNA	Unknown	351
11	Uncut DgDNA	Unknown	295
12	Blank	Standard	0

Table 1. A listing of the gel imaging system's analysis of the gel in Figure 2, showing the lane number, its contents, type and DNA masses. The DNA masses in lanes 4 through 11 were averaged, where the calculated figure was used to estimate the concentration of the original genomic DNA isolation, approximately 24.1 ng/ μ l.

Figure 5. A pulse-field agarose electrophoretic gel intended to assess the extent of partial digestion of dialyzed genomic DNA with the restriction enzyme *Sau*3A. Lanes 1 and 2 contain lambda *Hind*III digested and lambda DNA, respectively. Lanes 3 through 5 contain partially digested dialyzed genomic DNA at DNA:restriction enzyme ratios of 1/8, 1/12, and 1/16 U *Sau*3A/ μ g DNA. Lane 6 contains Pulse-field Midrange Markers. Lane 7 is a reiterative lane of lambda *Hind*III digested DNA of a different mass.

genomic DNA (HMW DgDNA). With digestion done in a similar fashion as before, Figure 5 shows these digestion products obtained using three different RE:DNA ratios (1/16, 1/12, and 1/8 U *Sau3A*/μg DgDNA). For all three RE:DNA ratios, a large portion of the DNA falls between the target DNA fragment length of 30 to 42 kbp.

The final concentration of modified DgDNA was determined via gel electrophoretic analysis using the gel imaging system where the final concentration of modified DgDNA was determined to be approximately 200 ng/μl.

II. Vector Isolation and Modification

When growing up the XL1 Blue bacterial culture prior to isolating pSCos, control cultures were run in parallel. As expected, the LE392 strain inoculated into LB/Amp showed no growth, but did grow in LB.

The vector proved to be difficult to restrict to completion as determined by gel electrophoresis (see Figures 6a and 6b). Following a third *Xba*I digestion of pSCos, a small portion of the product was electrophoresed showing that digestion of the circular vector to a 7.9 kbp linear form was completed (Figure 7). A 0.5 μl aliquot of the RE digestion product was run out with both lambda *Hind*III-digested and lambda DNA standards. The mass of DNA loaded onto the gel was quantified using the same gel imaging system and was found to be approximately 171 ng, whereupon the concentration of *Xba*I-digested pSCos was estimated to be 342 ng/μl.

To ensure that the phosphatase reaction was complete, the *Xba*I-digested pSCos and the associated controls were test-ligated to themselves as described and run out on a 1% agarose TAE gel (Figure 8). The ligation product of

Figure 6. (a) A 0.8% agarose TAE gel designed to assess both the size and masses of the various dilutions of *Xba*I restriction enzyme digestion of pSCos. Lanes 1 through 3 are serial dilutions ($3/2 \mu\text{l}$, $3/8 \mu\text{l}$, and $3/32 \mu\text{l}$ per lane, respectively) of pSCos RE digest reaction product. Lane 4 is 135 ng of lambda *Hind*III-digested DNA. (b) A 1% agarose TAE gel showing a 2-fold serial dilution of the second *Xba*I restriction enzyme digestion of pSCos in lanes 2 through 7 ($1/4$, $1/8$, $1/16$, $1/32$, $1/64$, and $1/128 \mu\text{l}$, respectively). Lane 1 contains a total of 150 ng of lambda *Hind*III-digested DNA. Both figures show multiple vector bands as noted.

Figure 7. A 1.0% agarose TAE gel designed to assess both the size and mass of $0.5 \mu\text{l}$ of *Xba*I-digested pSCos. Lane 1 is 150 ng of lambda *Hind*III-digested DNA, where lane 2 is *Xba*I-digested pSCos. Using the digital gel imaging system, the mass of the single band in lane 2 was estimated to be 171 ng giving an estimated concentration of $3.42 \text{ ng}/\mu\text{l}$.

Figure 8. A 1% agarose TAE electrophoretic gel showing the products of the test-ligation of phosphatased *XbaI*-digested pSCos and associated ligation controls. Lanes 1 and 6 are duplicate lanes of lambda *HindIII*-digested control DNA of 150 and 100 ng per lane, respectively. Lane 2 contains 300 ng of ligated, phosphatased *XbaI*-digested pSCos, while lane 3 contains 300 ng of ligated *XbaI*-digested pSCos. Lane 4 contains the positive control of 200 ng ligated lambda *HindIII*-digested DNA. Lane 5 contains 100 ng unligated, *XbaI*-digested pSCos.

Figure 9. A 1.0% agarose TAE gel was run showing the fragment sizes of *BamHI*-digested, dephosphorylated, *XbaI*-digested pSCos in lane 2 showing DNA bands at the predicted sizes of 6.8 and 1.1 kb. Lane 1 contains 150 ng lambda *HindIII*-digested DNA used as a mass standard, where lane 3 contains *XbaI*-digested pSCos.

previously *Xba*I-digested, phosphatased vector shows only a single band of DNA at approximately 8.0 kbp indicating that the 5' terminal phosphate group has effectively been removed. The ligation product of *Xba*I-digested vector shows a smear of DNA including multiple bands greater than 10 kbp. This shows that the vector itself is still capable of ligating to other DNA fragments with compatible ends. The positive control of ligated, *Hind*III-digested lambda DNA (lane 4) was performed to demonstrate the functionality of the ligase, showing multiple bands that range upwards of 23 kbp.

In order to show that pSCos was digested to completion with *Bam*HI, the reaction products were electrophoresed on a 1% agarose gel (Figure 9). A change in DNA mobility can be noted when comparing pre- and post-digestion samples. The digestion product shows two bands of DNA, one of approximately 6.7-6.8 kbp, the other of approximately 1.1 kbp, both of which are the expected lengths of the hemi-phosphorylated, ligation-ready vector DNA fragments.

Using the BioRad gel imager, the final concentration of modified vector DNA was estimated at 800 ng/ μ l.

III. Ligation of Insert and Vector DNA

To assess the products of the described ligation reaction, a 1% agarose TAE pulse-field gel was run as described, and can be seen in Figure 10. When comparing the pre- and post-ligation reaction mixtures of insert and vector DNA, there are notable differences. Most importantly, there is a disappearance of the vector band comparing pre- to post-ligation reaction mixtures suggesting that ligation was successful. In addition, an increase in fluorescence of DNA in the 23 to 48 kbp range in the post-ligation mixture adds

Figure 10. A 1.0% agarose TAE pulse-field gel was run to assess the products of the previously described ligation of insert and vector DNA. Lambda *Hind*III-digested DNA standards were run in lanes 1 and 7, 150 ng and 100 ng per lane, respectively. Lane 2 contains 100 ng of lambda DNA, where lane 3 is approximately 350 ng of pulse-field midrange markers. Lane 4 contains a mixture of insert and vector DNA (approximately 533 ng total) prior to ligation, where lane 5 contains an identical mass of ligated insert and vector. Lane 6 contains the product of a ligation control of *Hind*III-digested lambda DNA.

support to the hypothesis that vector had indeed ligated to insert DNA. The positive control ligation of *Hind*III-digest lambda DNA confirms ligase activity.

IV. Infection of XL1Blue MR Cells

In order to confirm successful packaging and packaging efficiency, the titer of the control DNA packaging reaction was assessed via plating out cells infected with a 10-fold dilution series of phage particles as described. This is shown in Table 2 below.

Table 2. The volumes and concentrations of phage solution used in a control phage plaque assay, with the corresponding number of plaques counted per plate. (The abbreviation "TMTC" indicates "too many to count".)

Plate #:	11	12	13	14	15	16	17	18
Volume Plated (μ l):	25	25	25	25	25	25	25	25
Dilution Factor:	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
Plaques:	TMTC	TMTC	TMTC	109	12	2	0	0

The plaque-forming unit (pfu) titer was calculated to be 4.36×10^7 pfu/ml using plate #14, giving 4.36×10^8 pfu/ μ g input DNA.

The titer of infection packaged cosmid and insert DNA was assessed via the plating out of infected cells on selective media (LA/Amp) in a 2-fold dilution series of phage particles as described, as shown in Table 3. The titer of packaged cosmid was determined to be 0 cfu/ml.

Table 3. The volumes and concentrations of recombinant cosmids plated out along with their respective colony count.

Plate #:	1	2	3	4	5	6
Volume Plated (μ l):	30	30	30	30	30	30
Dilution Factor:	10	20	40	80	160	320
Colonies:	0	0	0	0	0	0

Control Growth of Ampicillin Resistant and Sensitive Cell Strains

As expected, the ampicillin resistant cell strain (XL1Blue MR with pSCos) grew on both the LA and LA/Amp plates, while the ampicillin sensitive strain grew only on the LA plate.

Discussion

This research has revealed unexpected technical difficulties associated with library construction using Stratagene's pSCos1. Based on these findings, an initial step-by-step critique of the work done will be made, emphatically noting that the vendor's protocol was strictly followed, with few noted exceptions. A set of hypotheses as to *why* this system failed, and proposed experiments designed to test these hypotheses will be presented.

Genomic DNA Isolation and Modification

As dictated by the Stratagene protocol, it is necessary to begin with genomic DNA (gDNA) whose fragments are 150 kbp or greater. Figure 3 shows that the gDNA isolate ranges from ~48 kbp to ~280 kbp, with the majority greater than 150 kbp. The production of gDNA less than 150 kbp could be due to a number causes: hydrodynamic sheer during the mixing of liquid, molecular sheer during DNA precipitation, and/or enzymatic degradation. At this point, the majority of the gDNA appears to be greater than 150 kbp, and thus appears to be usable in this protocol.

An initial experiment was conducted to determine the optimal DNA to restriction enzyme (DNA:RE) ratio since insert DNA must be produced that ranges from 30 to 42 kbp. The optimization of enzymatic digestion would allow for the greatest production of DNA in this size range. Figure 4 shows the results of this initial experiment, where DNA:RE ratio of 1/8 to 1/32 U *Sau3A* per μ g gDNA produced a DNA "smear" in the 30 to 42 kbp range. In addition, the ability for *Sau3A* to restrict the genomic DNA strongly suggests that the

isolated DNA using this modified DTAB protocol was sufficiently purified, having removed any inhibitory moieties (i.e., detergents, salts, proteins, etc.).

The preceding DNA restriction protocol was then scaled up. Since protein nonspecifically binds to the walls of a plastic reaction vessel (in this case a microfuge tube), the amount of RE that was chosen for this next step was on the high side of the optimal range of the DNA:RE ratio. In order to ensure that the appropriate 30-42 kbp range of DNA fragments was produced, a range of 3 RE concentrations was used (1/8, 1/12, and 1/16 U *Sau3A* per μ g gDNA), where the digestion products were ultimately pooled. As shown in Figure 5, all three DNA:RE ratios produced a bright band, or "smear", of gDNA between 23 and 48.5 kbp with an obvious shift from the undigested gDNA size range. The *Sau3A* digested gDNA appears to be within the dictated size range.

Following optimal RE digestion, the gDNA was dephosphorylated using calf intestinal alkaline phosphatase (CIAP). This was done to prevent any two non-contiguous pieces of genomic DNA from ligating to each other, and ultimately producing a piece of DNA in the packagable range for this system.

Vector Isolation and Modification

pSCos was grown up in a suitable host (XL1 Blue MR) and isolated using the BioRad Quantum Miniprep system. The total mass of isolated vector DNA was not known until a gel could be run where the BioRad Gel Imaging System could be used to determine the approximate masses of DNA per lane. Due to its small size and high copy number per cell, the vector DNA could be isolated in reasonable quantities with relative ease. While it did prove difficult to restrict completely with *Xba1* (Figure 6a and 4b), Figure 7 clearly shows that pSCos

could be linearized where the linearized reaction product was of the expected size of approximately 7.9 kbp. Note that this value represents a rough estimate since the resolution of this gel is poor due to the gel parameters (high voltage with short run time). Subsequent run parameters were adjusted, allowing the size of DNA fragments to be estimated with much greater accuracy as seen in Figure 9. Under these modified run conditions, the size of the vector was indeed found to be approximately 7.9 kbp. At this point, the mass of DNA loaded per lane was determined (using the digital gel imaging system, see Appendix 2) and used to calculate the concentration of vector DNA for further modification as dictated by the vendor.

The vector was then dephosphorylated using CIAP to prevent the possibility that multiple vector molecules might ligate, producing unwanted packagable artifacts. To ensure that the phosphatase reaction was effective, linear dephosphorylated vector was test ligated to itself. The product of this reaction can be seen in Figure 8. The ligation of linearized, CIAP-digested vector (lane 2) shows the predicted single band of DNA around 7.9 kbp, as does lane 5 which contains linearized pSCos. Comparing these two, phosphatase treatment of linearized vector appears to be complete. As controls, *HindIII*-digested lambda DNA and linear pSCos were test ligated to themselves. Lanes 3 and 5 show that linearized vector and lambda *HindIII*-digested DNA still possessing their 5'-phosphate groups can ligate. Both lanes show many different concatameric combinations. These results thus support the conclusion that the phosphatase reaction was indeed successful.

This last step putatively demonstrated that the linearized, CIAP-treated vector could not self ligate. It is, however, not clear as to *why* the vector cannot self ligate. If the 4 base pair overhanging ends were themselves

modified or destroyed (i.e., by spurious exonuclease activity) during dephosphorylation, they would neither self ligate nor ligate to other "compatible ends". This would make the vector incompatible with the insert DNA in subsequent ligation reactions, rendering library construction impossible. The probability that this actually occurred is debatable, yet consideration of this possibility needs to be made.

Subsequent digestion of the vector with *Bam*H1 allowed for a single cut in the vector, producing two fragments of known sizes (6.8 and 1.1 kbp). As shown in Figure 9, two distinct DNA bands can be seen in lane 2, as compared to linear vector prior to *Bam*H1 digestion shown in lane 3. It again appears as though the vector has been sufficiently restricted to continue with the protocol.

Ligation of Insert and Vector DNA

Ligation of insert and vector DNA was carried out as dictated by the vendor. Figure 10 shows the product of this reaction along with the noted controls. When comparing lanes 4 and 5 (which contain identical masses of an insert and vector DNA mixture both prior to and after ligation), a change in staining intensity in the 23-40 kbp region of the gel can be seen. This suggests that larger DNA fragments (i.e., between 20 and 40 kbp) have been generated, presumably as a result of vector to insert ligation events. In addition, the disappearance of the vector band (seen in lane 4) suggests that a great percentage (most if not all) of the vector molecules present have been ligated. A control ligation of *Hind*III-digested lambda DNA was run to confirm ligase activity (lane 6).

This ligation reaction of insert and vector DNA can potentially produce a family of different ligation products. These reaction products might include DNA without any ligation modification, three different vector to vector ligation types, ligation of insert DNA with only one modified vector fragment (either the 1.1 or 6.8 kbp fragment), or the ligation of insert DNA with two vector fragments. Only the latter produces a packagable DNA molecule. Of all the ligation products of insert DNA with two modified vector fragments, only three possibilities exist: the ligation of insert DNA to two 1.1 kbp fragments, the ligation of insert DNA to one 1.1 kbp fragment and one 6.8 kbp fragment, and the ligation of insert DNA to two 6.8 kbp fragments. Having two *cos* sites (one on each end of the insert DNA), all of these are potentially packagable, and can ultimately infect the host cell. Note that the packaging reaction is only successful if the ligation product is between 46 and 52 kbp in length. This further limits the products of ligation. Following packaging and infection, the recombinant DNA will then circularize *in vivo*, and theoretically replicate as a plasmid. This is only possible when the recombinant DNA molecules contain a single origin of replication. The ligation product of two 1.1 kbp vector fragments with insert DNA contains no origin of replication, whereas the ligation product of two 6.8 kbp vector fragments with insert DNA contains two origins of replication that oppose each other (rendering it nonfunctional). This last requirement finally limits the ligation products down to a single possibility. Only a ligation product of two differently sized vector fragments with an appropriately size insert fragment will produce a packagable, functionally replicating clone.

It might be worthwhile to consider the following situation. Of the 533 ng of pre-ligated vector and insert DNA run out in lane 4 of Figure 10, approximately

131 ng should be found in the 6.8 kbp band. Comparatively, of the 100 ng of lambda *Hind*III DNA run in lane 7, approximately 47.7 ng should be found in the 23.1 kbp band. Based on the fluorescence of the noted control DNA in lane 7, there is far less than 131 ng of DNA in the 6.8 kbp band in lane 4, probably somewhere in the range of approximately 25 ng. This potentially (but not necessarily) presents a problem. Obviously, the overall mass of vector DNA was potentially less than that of what was estimated. This could have affected the insert to vector ratio to the point where no packagable ligation products were formed. However, it is possible that the insert to vector ratio was indeed accurate, but unfortunately, there is currently no way to test either of these two hypotheses.

Interestingly, even after repeated requests, Stratagene (the vendor of this cosmid vector) could not provide a positive insert control DNA, and evidently have yet to develop it. Note that the insert control must be free of *Bam*HI restriction sites in order to excise the insert from the vector during preparative isolation. One method to do this is presented here in an outline form. First, isolate and fully modify vector DNA as previously outlined by the vendor (an *Xba*I digestion, followed by a CIAP digestion, followed by a *Bam*HI digestion). Second, isolate genomic DNA from any suitable source (i.e., chicken, mouse, human, etc.). To produce this insert, first digest genomic DNA to completion with *Bam*HI. In order to destroy this *Bam*HI restriction site, follow this reaction by removing the 4 base pair, 5' overhanging ends using a 5'-3' exonuclease. The modified genomic DNA should then be an average of approximately 4096 (4^6) base pairs in length. Continue by blunt-end ligating these DNA fragments in an optimized reaction to produce the greatest amount of DNA fragments in the 30-42 kbp range. Purify this fragment range (30-42

kbp) by running the ligation products out on a low melting point agarose gel and digesting the harvested gel plug with agarase (Epicenter Technologies). The purified DNA fragments should then be blunt-end ligated to oligonucleotide adapters possessing *Bam*H1 overhanging ends. This ligation product should then be ligated to modified vector DNA, and ultimately packaged using lambda packaging proteins (Stratagene), where the subsequent packaging products would then be used to infect a suitable host and plated out appropriately on selective media plates. Healthy colonies could then be picked and grown up for cosmid isolation. In order to confirm that these control insert DNAs possess no internal *Bam*H1 restriction sites, an appropriate number of clones (perhaps 10 to 20) should be individually pick, grown, isolated, and separately digested with *Bam*H1. The digestion products should then be characterized via pulse-field electrophoresis to ensure that the digestion products are of the intended sizes (one fragment of 7.9 kbp, and a second ranging from 30-42 kbp). Any identified cosmid clones containing an insert with no internal *Bam*H1 restriction sites could then be isolated *en masse*, *Bam*H1 digested, where the control insert fragment could be purified from a low melting point gel as mentioned before. The developed control insert DNA could then be quantified, and then used in subsequent cloning efforts. Having this control insert would have been invaluable, serving as a trouble-shooting tool in the ligation, packaging, and infection steps of this protocol.

Packaging and Infection of Host Cell Line

The packaging and cell infection protocols were followed exactly as dictated by the vendor. The use of maltose in the growth medium induces greater expression of the maltose receptor, to which the viral particles bind.

Empirical evidence shows that the use of Mg^{+2} also increases infection efficiency, but there is currently no universally agreed upon explanation for this. Having run a control using vendor supplied lambda DNA, the lambda packaging extracts and cell culture used clearly appear to be functional and viable (respectively) giving an excellent transformation efficiency for the control of 4.36×10^8 pfu/ μ g input DNA.

The packaging of recombinant DNA and infection of host cells did not produce a single colony. Having evaluated each step of this protocol, considering each reaction against its respective control (from isolation of both vector and gDNA to the ligation of these two), the packaging and infection steps must now be considered as potential problem areas. Perhaps some uncharacterized problem exists (i.e. a packaging poison) that prevents the successful packaging of recombinant DNA, ultimately preventing any infectious particle from being formed. Another possibility might perhaps be that a DNA restriction system *in vivo* is still capable of recognizing and digesting foreign DNA. This too is purely speculation. Having sought out the advice of numerous people, including multiple calls to the vendor's technical support teams, cosmid cloning experts, and my advisors, no answer was found as to where the problem lies. Unfortunately, this is where a dead end was reached in this cloning effort.

Overall Critique of the Vector System

Having already discussed the advantages of any cosmid vector system, and this vector system, it would be useful to discuss the difficulties associated with the use of this system (assuming that one could successfully produce a

library). There are a number of practical tasks that one must accomplished to successfully use this vector, the first being able to quantify with accuracy the amount of DNA in solution. Many DNA digestion and or modification steps require that the concentration of DNA in solution be known. This includes restriction enzyme digestion, dephosphorylation, ligation and packaging reactions. The amount of enzyme used per reaction is ultimately based on the total mass sequence-dependent topology of DNA to be modified. Without the use of a digital gel imaging system (or something similar) which allows for a reasonably accurate estimate of DNA masses, most labs would run a small aliquot of the DNA through a gel, stain the gel, then photograph the gel using a Polaroid camera, and subjectively estimate the amount of DNA in a given lane based on comparative fluorescence with a known set of standards. This method of DNA quantification can be suprisingly accurate for an experienced research scientist. For the inexperienced, however, significant errors can be made very easily when quantifying DNA. Since the ratios of materials used during some steps (i.e., ligation of vector to insert, or the digestion of DNA with a specific amount of restriction enzyme) are crucial without much margin for error, the ability to accurately quantify DNA concentration is necessary.

Another critical step as dictated by the vendor is the isolation of high molecular weight DNA. In this case the DNA fragments need to be greater that 150 kbp. There are a number of difficulties associated with this step. DNA that is greater than approximately 100 kbp is extremely fragile, subject to hydrodynamic molecular shearing. Great care must be taken to prevent such breakage including extremely gentle pipetting and mixing of solutions. High molecular DNA can also be sheared via rapid precipitation (i.e., ethanol:sodium acetate precipitation) as DNA becomes more fragile as salt concentrations

increase (Maniatis *et al*, 1982). In addition, multiple freeze-thaw rounds of HMW gDNA can sufficiently shear the DNA during solute crystallization to where it is not usable in this effort. Again, the production of large DNA fragments, as dictated, is crucial in this cosmid cloning protocol.

A second difficulty when isolating HMW DNA is the fact that this DNA does not readily go into solution following precipitation. Anywhere from a few days to two weeks might be necessary for resuspension of any appreciable amount of HMW DNA. One week of resuspension time at elevated temperature is not uncommon. To help facilitate DNA resuspension, the solution can be heated to 55° C with gentle mixing, but both increase the risk of shearing the DNA, and/or expression of residual DNase activity.

Following the actual isolation of high molecular weight DNA, it is necessary to determine with accuracy the size of genomic DNA isolated. The vendor dictates that the quality of the DNA be checked by electrophoresis through a 0.3% agarose gel or field inversion gel. The resolution produced even by the best 0.2% agarose gel is insufficient to allow for proper DNA quality assessment. It is absolutely necessary to run a gel with some kind of electric field shift (field inversion or pulse-field) in order to do this. It is also worth considering the time requirement of up to 32 hrs per electrophoretic run. Without such an electrophoretic set up, one can not expect to successfully use pSCos1.

A few other things are noteworthy in this discussion. First, there is some discrepancy in the actual size of the vector. The vector is actually 7.9 kbp in length as determined by DNA sequencing, where in many places both in the literature and the protocol it is quoted as being 7.6 kbp in length (Stratagene). Following the second RE digestion, two fragments were seen around 6.8 and 1.1

kbp which further supports the 7.9 kbp estimate. A second noteworthy point is that the vendor could not help determine the problem in this cloning effort. This in itself was troublesome. A vendor supplied product should work (and is expected to work) when the protocol is followed closely. In this case, it did not. Perhaps there are unknown factors in our lab leading to the unsuccessful cloning attempt (reagent contamination, incorrect reagent production, genetic rearrangements of the vector, etc.). Even after laborious examination of these possibilities, however, the exact causes for the failure of this system remain unclear to us.

This evaluation of Stratagene's pSCos1 use as a cosmid cloning vector in the small lab has been unfavorable. Multiple steps which necessitate the accurate quantification of DNA concentrations, the need to isolate high molecular weight DNA (and difficulties therein), and the resources required to successfully use this vector all act against this product. By all published accounts, the vector itself is a well designed cosmid with many useful characteristics. Unfortunately, it is my opinion that the problems associated with this cosmid's successful use are too many for a short-term project in a small lab. Another vector system, one with fewer technical requirements (i.e., expensive gel imaging systems, pulse-field electrophoretic systems, etc.), would probably be in order.

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Appendix 1: Use of the Pulse-field Agarose Gel Electrophoretic System

In the lab, an LKB Pulse-field Gel Electrophoresis system (LKB 2015 Pulsaphor Electrophoresis Unit along with the 2015 Pulsaphor Control Unit, and the 2301 Macrodrive Power Supply) was used to better resolve DNA fragments whose size ranged from 30 kbp to 300 kbp. Unlike standard, one-dimensional electrophoretic systems, the pulse-field system employs electronic hardware that can shift the electric field in 2 dimensions, from one vector axis to another. This system also employs both the use of an external refrigeration system to ensure that the gel does not overheat (and thus melt) during electrophoresis in addition to a buffer pump to ensure adequate buffer circulation. A typical gel set-up was as follows:

A square, rubber gasket was firmly affixed onto a plexiglass support plate where no gaps between the plate and gasket were allowed. This was to prevent liquid agarose from leaking out of the gel area, ultimately leading to a change in gel thickness. An appropriate well-molding "comb" was appropriately placed inside the gasket, where liquid agarose of approximately 55-60° C was poured into the mold allowing for a gel thickness of about 5 mm. Once the gel had solidified, the comb and gasket were removed making sure not to damage the gel. Unlike other electrophoretic systems, the DNA is loaded into the well "dry", which indicates that the gel has not yet been placed into the electrophoretic set-up and covered with buffer. The DNA samples to be run out on the gel were then loaded along with DNA standards of known sizes and masses and/or concentrations. During a typical gel run, the standard DNA used were 100 ng *HindIII*-digested lambda DNA, 100 ng uncut lambda DNA, and

350 ng of Pulse-Field Midrange Markers provided by New England BioLabs. Note that the *Hind*III-digested lambda DNA was always heated to 65° for 2 minutes prior to loading in order to dissociate the *cos* sites on the 4.4 and 23.1 kbp fragments. These standards allow one to quantify the amount DNA loaded per well for unknowns (see Appendix 2). Residual molten agarose was carefully pipetted on top of each DNA sample in order to seal each well. This is necessary since the buffer is vigorously circulated throughout the electrophoresis chamber, potentially creating eddies in unsealed wells, where the DNA samples would ultimately be washed away. (Note: a regularly used alternative does not seal the wells with molten agarose, but rather leaves the buffer pump off for the first 10 to 15 minutes of electrophoresis, allowing the DNA to move into the gel slightly, preventing the moving liquid from whisking it away.) The gel/plate assembly was then placed into the electrophoresis chamber, where a volume of 4° C 1X TAE was added in order to cover the gel with approximately 5 mm of buffer. (Note: the buffer can already be in the chamber so long as it has been equilibrated to 14° C.) A hexagonal electrode was then placed into the buffer, where the necessary electrical connections were made. This hexagonal electrode allows for a 120° difference in the vector axes. The cover was then affixed onto the gel box, where the power was turned on. The overall parameters of the run were as follows:

Agarose Concentration: 1% agarose
Buffer Type: TAE
Gel Dimensions: 14 cm X 14 cm X 0.5 cm
Electrode set-up: hexagonal
Voltage Gradient: 8.9 V/cm
Pulse Time Per Axis: 15 sec
Running Temperature: 14° C
Duration of Run: 27.5 hr.

Following the run, the gel was stained in a standard ethidium bromide solution (0.5 $\mu\text{g/ml}$) for approximately two hours, and then analyzed using the BioRad Gel Imaging System and software.

Appendix 2: Quantification of DNA Using the BioRad Gel ImagingSystem

The quantification of DNA concentrations in solution is an integral part of many DNA manipulation protocols. The amount of any modification enzyme to be used often depends on the overall mass of DNA. A typical method of estimating the concentration of DNA in solution is to run a small portion of the unknown out on a gel in parallel with DNA standard of known concentration. Following staining with ethidium bromide (EtBr at 0.5 $\mu\text{g}/\text{ml}$), DNA will fluoresce orange under the influence of UV light. The amount of fluorescence is proportional to the mass of DNA. Historically, the comparative fluorescence between known standards and unknowns has been made by the laboratory researcher in a nonquantitative, subjective method, or by "eyeballing it".

Presently, there are computer hardware and software packages available that allow the researcher to digitally image a gel using a video camera and digital acquisition software (BioRad Corporation). This unit allows one to quantify (comparatively) the amount of fluorescent light being emitted from various DNA samples. The following is a brief explanation as to the methods used to quantify DNA masses in agarose gels.

It is first necessary to run the DNA of unknown concentration out on a gel along with any appropriate mass and sized DNA standard, making sure to note their respective volumes and concentrations (if known). Figure 11 shows a serial dilution of the unknown, in

Figure 11. A thermal print of an electrophoretic gel

this case linearized pSCos, in lanes 2 through 7. Lambda *HindIII* standards were run in lane 1. (Note that this is a negative image of the original, done so to show the boxed areas.) From the initial mass of DNA loaded into the first lane, a total of 150 ng, one can calculate the approximate mass of DNA found in each of the lambda *HindIII* DNA fragments. Since the 23.1 kbp fragment represents approximately 47.7% of the lambda genome, approximately 72 ng of DNA should be found in that particular band. Similar calculations can be made to estimate the masses of DNA in the other bands. Using BioRad Gel Imaging System's software, one can "box in" any particular band. This box has an inherent surface area, measured in square millimeters. One then creates identically sized boxes for the other bands, including the unknown. The boxes must be of an identical size due to the fact that background fluorescence will also be detected by the imaging system. Using the software, one then distinguishes between figure types: either standard, blank, or unknown samples. (Note that blank samples are identical areas without any DNA bands.) When compared the relative fluorescence of the standards (Table 4), an

Table 4. Lambda *HindIII*-digested DNA standards and their respective masses along with the noted DNA unknown.

<u>Lane:</u>	<u>Fragment Size (kb):</u>	<u>Type:</u>	<u>Mass (ng):</u>
1	23.1	standard	72
1	9.4	standard	29
1	6.6	standard	21
1	4.4	standard	14
1	2.3	standard	7
1	2.0	standard	6
2	7.9 (pSCos)	unknown	171 (given by imager)

estimate of the unknown is made. Here the unknown DNA mass is estimated at 171 (ng). The concentration is then calculated knowing the overall volume of DNA sample loaded. In this case, the unknown is estimated to be at 342 ng/ μ l.

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