NEN-Phage™

Phage P1 DNA Packaging System

NEP-113

User Manual

CAUTION: This kit contains components that require different storage temperatures. If the kit cannot be opened immediately, it must be stored at -70°C.

This kit is designed to clone and propagate DNA in an *E. coli*-based system

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NEP-113A
March, 1992

TECHNICAL DATA SHEET

Product Name: pAd10SacBII Vector

Catalogue Number: NEP-113V

Lot Number: 0127WK2

Concentration: 500 µg/ml

Storage Buffer: 10mM Tris-HCl (pH 8.0), 1 mM EDTA

Recommended Storage Conditions: 2-8° C

Description:

The P1 cloning vector, pAd10SacBII, is a new improved vector for use with the Phage P1 DNA Packaging System (NEP-113) to generate genomic libraries. It differs from the Ad10 vector in that it allows positive selection of P1 clones containing DNA inserts due to the presence of the Sac BII gene: the gene which is regulated by an upstream synthetic *E. coli* promoter codes for an enzyme converting sucrose to levan; levan accumulates in the periplasmic of the *E. coli* cell causing cell death. Located between the gene and its promoter is a unique BamH1 cloning site. Insertion of DNA fragments at this site disrupts expression of the SacBII gene. Thus, P1 clones with inserts are able to grow on sucrose-containing medium whereas those without inserts are unable to grow.

Also engineered into the SacBII vector are sequences to facilitate the characterization of the cloned DNA: flanking the BamHI site are rare cutting restriction sites Not 1 and Sfi 1 for easier recovery of the insert DNA as well as T7 and Sp6 promoter sequences for RNA probe analysis of cloned DNA ends.

Details for the use of the vector to clone large DNA fragments (75-100 kb) are given in the manual which accompanies the Phage P1 DNA Packaging System (NEP-113)

Concentration:

The concentration was determined by ultraviolet spectrophotometry in 10mM Tris-HCl (pH 8.0), 1 mM EDTA.

Restriction Enzyme Digests:
The vector was incubated with restriction enzymes Sca 1, BamH1 and Spe 1 in separate reactions according to manufacturer's instructions. Reaction samples were subjected to 0.8% agarose gel electrophoresis. Sca 1 and BamH1 digestion results in linearization of the vector; Spe 1 digestion yields three fragments: one of about 22kb; one of about 6.5kb; and one of 1.7kb which is the SacBII gene.

References:

Sternberg, N.L. (1990) Proc. Natl. Acad Sci (USA) 87, 103.
Pierce J.C. and Sternberg, N.L. Methods in Enzymology, in press.

FIGURE 1. pAd10SacBII vector

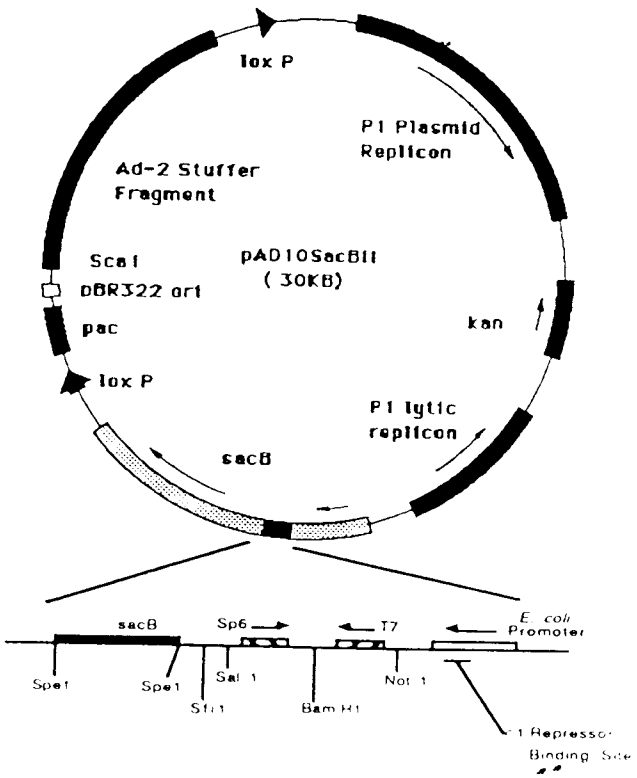


Table of Contents

P1 Packaging System Instructions

Introduction

The construction of detailed gene-linkage maps of complex genomes facilitates the localization of genes to specific regions on chromosomes. Further precise localization of specific genes requires cloning of high molecular weight DNA if it is to be done efficiently. Two methods commonly used are to either insert large genomic DNA fragments into cosmid vectors, which are then packaged into Phage λ heads and propagated in *Escherichia coli* [1] or into yeast artificial chromosome (YAC) vectors propagated in *Saccharomyces cerevisiae* [2]. The use of cosmids results in high transformation efficiencies, but insert size is limited by the size of the Phage λ head to 45-48 kilobase pairs (kbp). The advantage of using YAC vectors is their ability to accommodate DNA as large as 200-800 kbp. Disadvantages are the low transformation efficiency, which decreases with insert size, the need to process transformants individually prior to screening, and the difficulty in obtaining large amounts of recombinant DNA from transformed cells [3].

To overcome some of the problems associated with using cosmid or YAC systems, a novel method for cloning and packaging DNA fragments using a Bacteriophage P1 system has been developed [3] which offers the ability to clone large genomic DNA fragments of between 70-95 kb in size with efficiencies approaching those of cosmids. In addition, the P1 DNA Packaging System uses host *E. coli* strains and *in vitro* packaging extracts obtained from strains which are deficient in restriction and recombination abilities. These prevent the degradation and recombination of methylated genomic DNA [4].

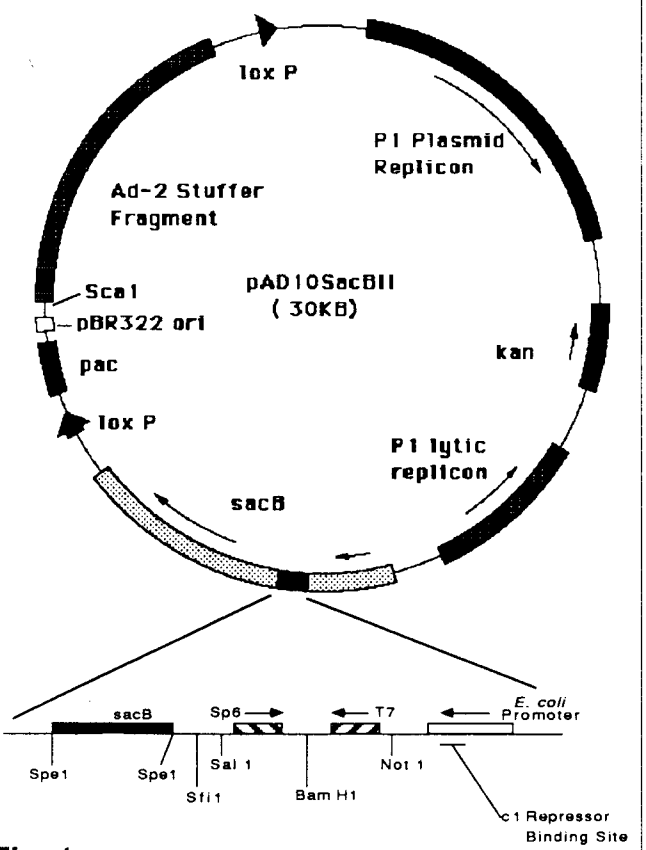


Fig. 1

Plasmid map of pAd10-SacB II and cloning region.

Principle of the Method

Using a strategy analogous to Phage λ packaging[1], partially digested and size selected genomic DNA between 70kb and 95kb is ligated onto linearized plasmid vector arms. The SacBII vector used contains a Phage P1 *pac*-cleavage site and two Phage P1 *lox*P recombination sites in addition to replication origins and antibiotic resistance gene (Fig.1). The recombinant vector is cleaved at the *pac*-cleavage site in a "*Pac*ase" extract and the resulting DNA is then inserted in an empty P1 phage head using a second extract containing phage packaging proteins. The attachment of P1 phage tails to the heads results in the formation of infectious recombinant phage particles which are then used to infect a restriction minus *Escherichia coli* host strain containing an expressed *cre* gene. After injection into the host strain, the recombinant DNA is circularized between the two Phage P1 *lox*P sites by Cre recombinase. DNA which does not circularize is degraded by host nucleases. The circular DNA molecule now replicates and is maintained stably at one copy per host cell by the P1 plasmid replicon.

Prior to alkaline lysis plasmid isolation [5], the recombinant plasmid copy number is increased more than 25-fold by isopropyl β-D-thiogalactopyranoside (IPTG) induction of the *lac* promoter controlled high-copy P1 lytic replicon. A schematic detailing the P1 DNA packaging strategy is shown in Figure 2.

Phage P1 uses a headful packaging strategy. Once the phage head is filled with DNA (about 110-115kb), a "headful cut" occurs, cleaving any remaining DNA away from the head before it is packaged. This packaging mode suggests that if the insert DNA is too large (>95kb) it will be packaged but not recovered in bacteria because the "headful" packaging process will terminate before the distal *lox*P is incorporated into the phage head. It also suggests that DNA that is too small to generate a headful when inserted into the vector should not be packaged into phage particles. However, it has been observed that DNA that is less than a P1 "headful" is packaged *in vitro* in viable phage particles with an efficiency of about 10-15% that of "headful" DNA (Pierce & Sternberg, manuscript in preparation). While it is not completely clear why this should occur, it points out the need to size-select the insert DNA on sucrose gradient before attempting to ligate it to vector in order to maximize the recovery of large inserts. In particular, size fractions that maximize DNA fragments in the 70-95kb range, and minimize the presence of shorter fragments should be used. The presence of small fragments is undesirable also because it increases the possibility that they will be ligated together and then recovered in one clone. This would significantly complicate subsequent screening and gene localization processes.

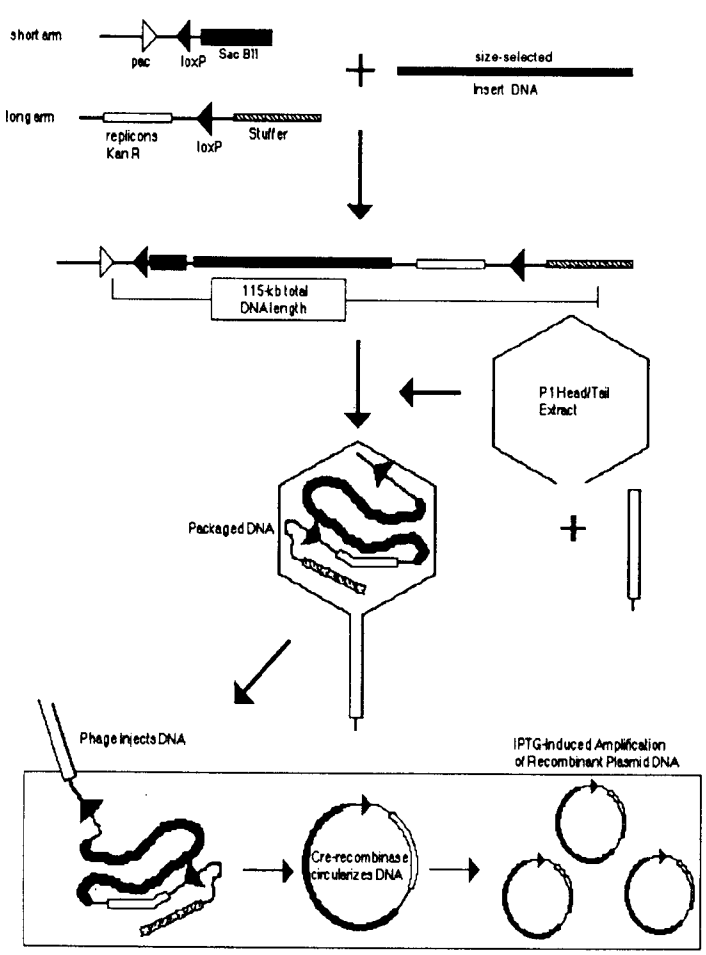


Figure 2. The P1 DNA Packaging Strategy. The SacBII vector is digested with restriction endonucleases *Sca*I and *Bam*HI and the ends dephosphorylated. This generates two vector "arms", one consisting of the "short" *Sca*I to *Bam*HI fragment, and the other the "long" *Sca*I to *Bam*HI fragment. Genomic DNA is partially digested with *Bam*HI or other compatible end restriction endonucleases and size-selected on a sucrose gradient. Fragments between 70kb and 95kb in length are isolated and ligated to the vector arms, generating a series of linear molecules. If ligation occurs between two "short" arms, the resulting molecule will neither contain the origins of replication nor the kan^r gene, and will be nonviable. If both arms are "long", there will be no *pac* site, and no packaging into the phage heads will occur. The only viable recombinant will be one consisting of the insert sequence flanked by both a short and long arm. Phage P1 uses a headful packaging strategy and can accomodate a total DNA length of approximately 110-115 kb. Any inserts longer than 95-100kb will result in truncation of the packaged DNA before the distal *loxP* site is inserted, and the molecule will be unable to circularize upon injection into the host. Once injected into the cre$^+$ host cell, the *cre* protein circularizes the injected DNA at the *loxP* sites, and the DNA now replicates using the plasmid origin of replication. Propagation of cells on sucrose containing media only permits growth of colonies with genomic DNA inserts (positive selection). Plasmid copy number is increased by induction with IPTG. The recombinant DNAs are then isolated as plasmids using traditional methods.

Finally, as the second stage packaging extract contains about 5-10% small heads (headful size 47kb), small DNA fragments can be recovered by a headful packaging process in phage particles containing these heads.

An 11kb "stuffer" region of Adenovirus type 2 DNA has been engineered into the pAd10-SacBII cloning vector. It is designed to provide a segment of DNA in which the "headful" cut can be made.

Using the P1 DNA Packaging System, genomic DNA from 70-95 kb can be readily cloned and manipulated. The major advantages of the P1 DNA packaging method over other genomic cloning methods are: 1) the ability to clone inserts two to three times the size of those used with cosmids and lambda vectors, 2) no rearrangement or deletion of methylated DNA occurs because of the use of restriction-minus host strains and 3) recombinant DNA is easily recovered as plasmids for further screening and manipulations.

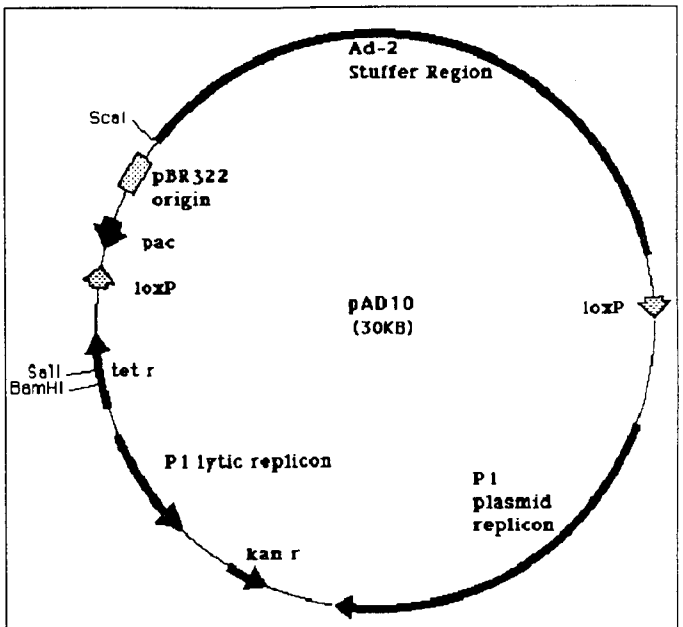


Fig. 3 Plasmid map of pAd10

P1 CLONING VECTORS

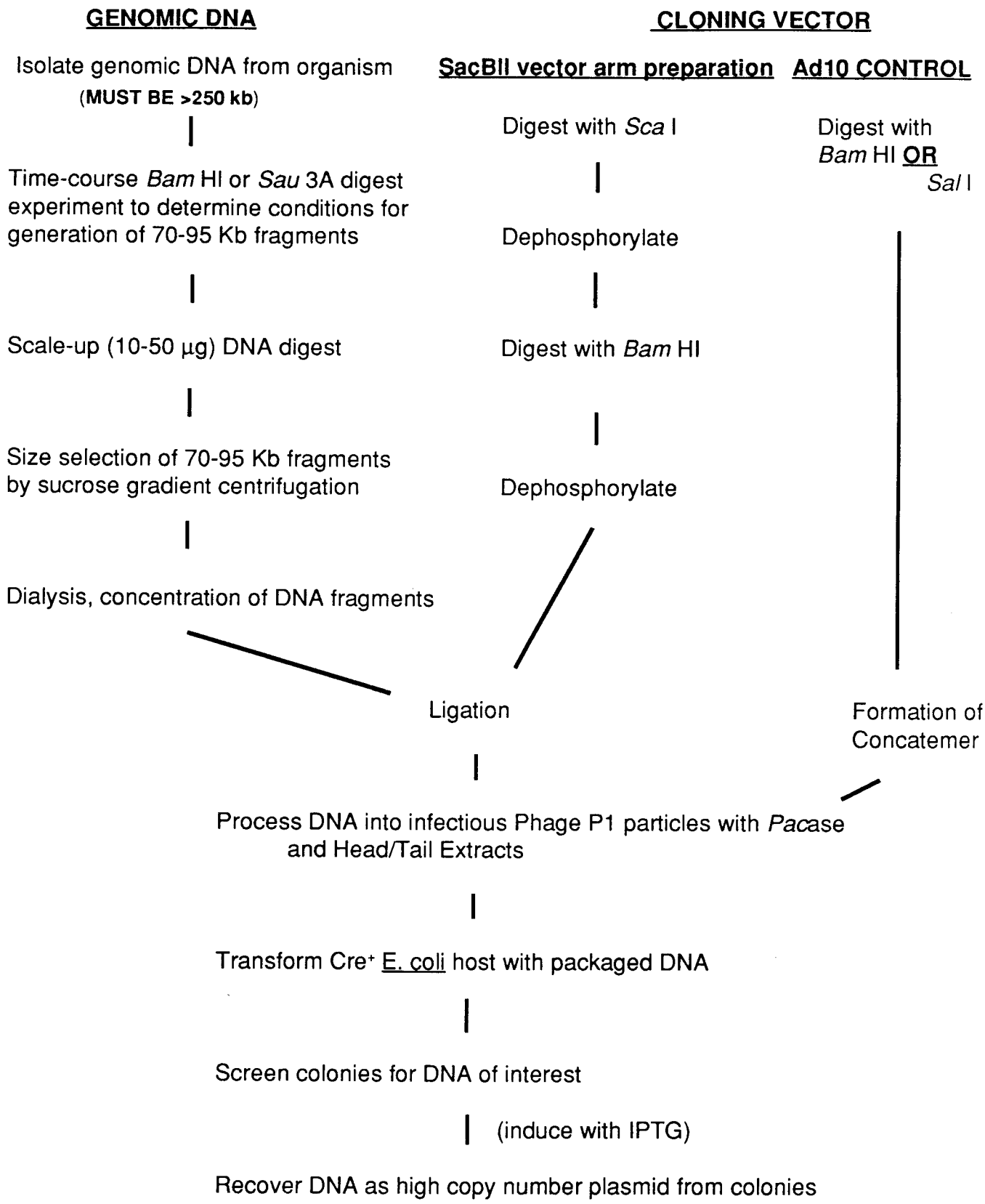
One of the key components in the construction of a P1 DNA library is the genetically engineered P1 plasmid vector. The original vector was pAd10 (see Fig.3). A new improved, more versatile version of the Ad10 vector has been constructed. This vector is designated pAd10-SacBII (see Fig. 1).

Table 1. Features of SacBII and Ad10 Plasmid Vectors

	SacBII	Ad10
Cloning	*Bam*HI*	*Bam*HI /*Sal* I
Screening Method	positive Kan/sucrose	antibiotic Kan/Tet
Rare 8-base R.E sites	*Sfi* I/*Not* I	none
RNA Analysis	Sp6/T7	none

**Sal* I can also be used to clone inserts, but this prevents use of Sp6 and T7 promoters to prepare RNA-probes from cloned insert.

P1 PACKAGING SYSTEM: PROCESS OVERVIEW



REFERENCES:

1. Murray, N.E. (1983) in *Lambda II*, eds.Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg, R.A. (Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.) pp.395-432.
2. Burke, D.T., Carle, G.F. and Olson, M.V.(1987) *Science* **236**: 806-812.
3. Sternberg, N.L. (1990). *Proc. Nat. Acad. Sci.* USA **87**:103-107.
4. Blumenthal, R. (1989).*Focus* **2**:41-46.
5. Birnboim, H.C. and Doly, J. (1979)*Nucleic Acids Res.* **7**:1513-1523.
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7. Sternberg, N., Reuther, J. and deRiel, K. (1990) *The New Biologist* **2**:151-162.
8. Pierce, J.C. and Sternberg, N.L. (In Press) *Methods in Enzymology.*
9. Pierce, J.C., Sauer, B.S. and Sternberg, N.L. (In Press) PNAS.

DuPont's P1 DNA Packaging System
ORDERING INFORMATION

Call 1-800-551-2121 1,1 at the prompt

NEP-113

NEP-113 contains all of the components found in both NEP-113C and NEP-113P, listed below. **(NOTE: NEP-113 does not include "SacBII" positive selection cloning vector).**

NEP-113C

The P1 DNA Cloning System contains the components needed for ligating size-fractionated genomic DNA into the P1 "SacBII" cloning vector (ordered as **NEP-113V**). The kit includes: P1 control "Ad-10" vector , T4 DNA ligase and ligation buffer, Calf Intestinal Alkaline Phosphatase and high molecular weight DNA markers (used for selecting DNA of optimal length for cloning and packaging).

NEP-113P

The P1 DNA Packaging System consists of all components needed for five packaging reactions of genomic DNA to P1 vector clones including the phage P1 Head/Tail extract, the *Pac*ase extract for cleaving the recombinant DNA and guiding it into phage heads, the recombination and restriction-minus bacterial plating host strains and all necessary buffers.

NEP-113V

New P1 cloning vector pNS582tet14-Ad10-SacBII, the positive selection cloning vector used for genomic DNA library generation. **Must be ordered separately; not included with NEP-113 or NEP-113C .**

Description of P1 DNA packaging extracts and cloning vector

*Pac*ase extract description:

The *Pac*ase extract is from P1 prophage lysogen strain NS3208. It contains all phage P1 proteins necessary for cleavage of *pac*-containing DNA and for attachment of necessary proteins to the termini of the cleaved DNA for insertion into P1 phage heads.

Genotype:

recD⁻ **hsd**R⁻ **hsd**M⁺ **mcr**(AB)⁻ P1**cm**-2 **cI**:100**rm**⁻ **am** 10.1

***E. coli* mutations**

recD⁻ reduces recombination.
hsdR⁻ prevents endonuclease B and K restriction of unmodified DNA.
hsdM⁺ methylates DNA to prevent restriction by host nucleases.
mcr(AB)⁻ prevents the restriction of MeCpG-containing DNA.

P1 prophage mutations

cm-2 is a chromosomal rearrangement where transposon Tn9, containing the chloramphenicol resistance gene, cm-R, is inserted into P1 map coordinate 24, deleting a 10 kb region of adjacent P1 DNA. This mutation renders the prophage partially lysis defective, allowing for later harvesting of bacteria than would otherwise be possible.
cI:100 renders the phage c1 repressor temperature sensitive. At temperatures above 33°C, the P1 prophage is induced to enter the lytic phase, and cell lysis occurs.
rm⁻ inactivates P1 restriction and modification systems.
am 10.1 is an amber mutation in P1 gene 10, rendering the prophage defective for all late gene products, including head and tail proteins.

Head/Tail extract description:

The Head/Tail extract is from P1 prophage lysogen strain NS3210. It contains all Phage P1 proteins necessary for production of empty phage heads and tails.

Genotype:

recD⁻ **hsd**R⁻ **hsd**M⁺ **mcr**(AB)⁻ P1**cm cI**:100**rm**⁻ **am** 131

***E. coli* mutations**

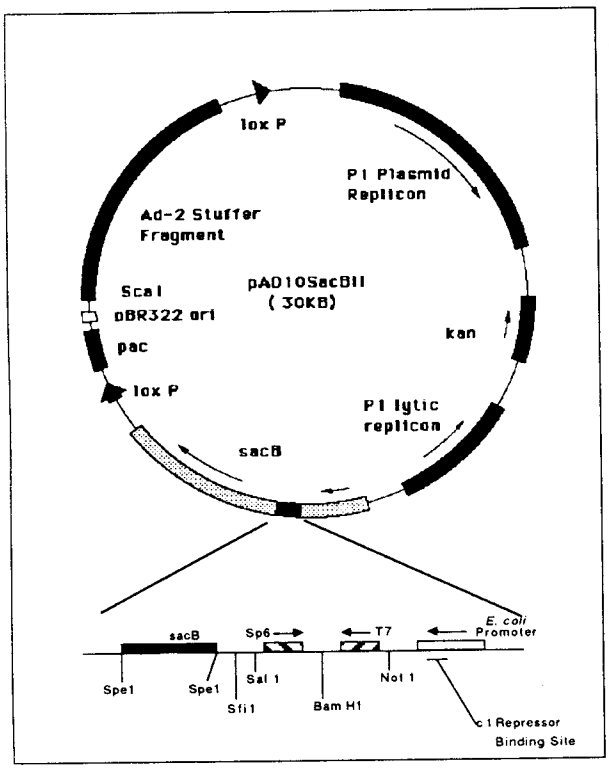
recD⁻ reduces recombination.
hsdR⁻ prevents endonuclease B and K restriction of unmodified DNA.
hsdM⁺ methylates DNA to prevent restriction by host nucleases.
mcr(AB)⁻ prevents the restriction of MeCpG-containing DNA.

P1 prophage mutations

cm is an insertion of the Tn9 transposon conferring chloramphenicol resistance. Unlike the cm-2 mutation, there is no deletion of P1 DNA. This mutation changes the ratio of large to small heads to 10:1.
cI:100 renders the phage c1 repressor temperature sensitive. At temperatures above 33°C, the P1 prophage is induced to enter the lytic phase, and cell lysis occurs.
rm⁻ inactivates P1 restriction and modification systems.
am 131 is an amber mutation in P1 gene 9, rendering the prophage defective for the production of *pac* cleavage proteins.

pAd10-SacBII Cloning vector:

The P1 DNA Packaging cloning vector is designated pAd10-SacBII. For simplicity, it will be referred to as "SacBII" in this manual. It is an approximately 30,000-bp double-stranded plasmid vector which contains the plasmid pBR322 origin of replication as its major replicon. It also contains the Phage P1 plasmid replicon which maintains the copy number of the packaged recombinant plasmid in the host cell to one copy. In addition, the vector has the Phage P1 lytic replicon, which is under *lac* repressor control. This replicon is used as an inducible means of amplifying plasmid copy number after DNA packaging. SacBII contains the Phage P1 *pac* recognition sequence and two Phage P1 *lox*P sequences. During DNA packaging, pacase extract proteins recognize and cleave the DNA at the *pac* site, and attach other proteins to allow for packaging into empty P1 phage heads. Once the packaged DNA is injected into a Cre$^+$ host, the Cre recombinase protein initiates circularization of the injected DNA at the *lox*P sites. In addition, the SacBII vector contains a 10.7-kb region of Adenovirus-2 as a "stuffer" region inserted between the distal *lox*P site and the unique *Sca*I restriction site. This allows for an approximately 10 kb range in size of clonable fragments. A key property of the SacBII vector is the presence of a sacB gene. This gene encodes an enzyme that converts sucrose to levan which is lethal to the cell, thus cells containing an active *Sac*B gene will not survive when grown on media containing greater than 2% sucrose. To take advantage of this property in a positive selection scheme for cloning, an *E. coli* promoter was placed upstream of sacB and a *Bam*HI cloning site was placed between these two elements. This site was, in turn, flanked by T7 and Sp6 promoters for RNA probe analysis of the ends of any cloned DNA, and by rare-cutting *Sfi*I, and *Not*I restriction sites to easily recover cloned DNA. Finally, a P1 c1 repressor binding site was positioned to overlap the *E. coli* promoter[8].



NS3529 Plating Host Description:

The plating host NS3529 is a derivative of *E.coli* DH5 that contains the *lac*Iq and *cre* genes on separate integrated λ prophages, λLP133 and λ **imm**434**nin**5X1-cre40, respectively. This Cre$^+$ host does not carry an F' plasmid and therefore has the advantage over NS3145 in allowing the isolation of plasmid vector DNA free of any F' plasmid DNA.

Genotype

recA$^-$, **mcr**A$^-$, Δ(**hsd**R, **hsd**M, **mcr**B, mrr) (λ**imm**λLP1) (λ**imm**434-P1: Cre$^+$)

recA$^-$ reduces recombination
mcrA$^-$ prevents the restriction of MeCpG-containing DNA
Δ (**hsd**R, **hsd**M, **mcr**B, **mrr**) indicates a deletion of these genes
(λ**imm**λIp1) location of *lac*Iq gene
Cre$^+$ signifies the production of the Cre recombinase protein

Section I.
P1 Packaging Buffers and Reagents

1. Components included in the NEN-Phage™ kit

NOTE: Components stored at recommended temperatures are usable for at least 3 months. The P1 Head/Tail Extract vials are single use only and must be kept at -70°C until needed. All other vials are stable when subjected to several freeze/thaw cycles.

Components that require -70°C storage

Frozen glycerol suspension of *E. coli* plating host strain NS3529 (0.5 ml)

P1 *Pac*ase extract (20 µl)

P1 Head/Tail extract (5 vials)

Components that require -20°C storage

10X dNTP mix (20 µl)
- 1 mM dATP
- 1 mM TTP
- 1 mM dCTP
- 1 mM dGTP

50 mM ATP (20 µl)

120 µg/ml deoxyribonuclease I (DNase I) (85 µl)

5X Ligation buffer (1 ml)

T4 DNA Ligase (60 µl, 5 U/µl)

25 mM DTT (20 µl)

Components that require 4°C storage

Calf Intestinal Alkaline Phosphatase 20µl,~15U/µl

10X *Pac*ase buffer (30 µl)
- 100 mM Tris-HCl, pH 8.0
- 500 mM NaCl
- 100 mM $MgCl_2$

TMG buffer (1 ml)
- 10 mM Tris-HCl, pH 8.0
- 10 mM $MgCl_2$
- 0.1% gelatin

P1 Head/Tail Extract buffer (50 µl)
- 6 mM Tris-HCl, pH 7.5
- 15 mM ATP
- 16 mM $MgCl_2$
- 60 mM Spermidine
- 30 mM β-mercaptoethanol
- 60 mM Putrescine

Purified Ad10 control plasmid (80µl, 0.5 µg/µl)

Phage T5 DNA Molecular Weight Markers (25 µl, 0.2 µg/µl)

Reagents sold separately

Ad10-SacBII CloningVector (20µg/40µl)
Cat. No. NEP-113V

2. Reagents needed by the researcher (not included in the NEN-Phage™ kit)

Restriction digests

*Bam*HI, *Sal* I and *Sca* I enzymes

10X *Sal* I buffer	10X *Sca* I buffer	10X *Bam*HI buffer
60 mM Tris-HCl, pH 8.0	60 mM Tris-HCl, pH 7.5	60 mM Tris-HCl, pH 8.0
(60 mM $MgCl_2$)**	60 mM $MgCl_2$	(60 mM $MgCl_2$)**
60 mM β-mercaptoethanol (BME)	10 mM dithiothreitol (DTT)	1 mg/ml nuclease-free BSA
1 mg/ml nuclease-free bovine serum albumin (BSA)	1 mg/ml nuclease-free BSA	1.5 M NaCl
1.5 M NaCl	1.0 M NaCl	

Note: When using *Sal* I or *Bam*HI for **genomic DNA partial digests, omit $MgCl_2$ from the buffer.

0.1M $MgCl_2$	0.2M EDTA, pH 8.0	2.5 mg/ml Nuclease-free Bovine serum albumin

Dephosphorylation
Calf Intestinal Alkaline Phosphatase buffer
50 mM Tris-HCl, 0.1 mM EDTA pH 8.0

Protein Extraction, Ethanol Precipitation, Resuspension *(NEW FORMULATION)*
Phenol (Molecular Biology Grade) equilibrated with 500 mM Tris-HCl pH 8.0.
Chloroform/isoamyl alcohol (24:1)
3 M Sodium Acetate (NaOAc)
100% ethanol and 70% ethanol
TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

Field Inversion Gel Electrophoresis
6x Loading Buffer *(NEW FORMULATION)*
- 40% sucrose (or glycerol)
- 0.1% SDS
- 20 mM EDTA, pH 8.0
- 0.2% bromophenol blue
- 0.2% xylene cyanol FF

5X TBE Buffer (1 liter)
- 54 g Tris base
- 27.5 g boric acid
- 20 ml 0.5 M EDTA, pH 8.0

1% SeaKem™ GTG-grade agarose in 0.5 X TBE buffer
20 mg/ml ethidium bromide (in H_2O)

Size-selection of genomic DNA fragments
10% and 40% sucrose (in 0.8 M NaCl, 10 mM EDTA , 20 mM Tris-HCl pH 8.0)
n-butanol
Millipore type VS 0.025 µm pore filters (cat. # VSWP04700)
Parafilm™

DNA Packaging and Plating of Recombinants
Chloroform
Bacterial growth media
- Luria-Bertaini (LB) broth (1 liter)
  - 10 g Bacto tryptone
  - 5 g yeast extract
  - 5 g NaCl
- Adjust to pH 7.0 with NaOH. Autoclave at 121°C, 15 PSI for 20 minutes.

LB-agar plates
- Add 15 g of Bacto agar per liter of LB before autoclaving.

Antibiotic Resistance
- Kanamycin plates:
  - Use a 25 mg/ml kanamycin stock solution (in sterile H_2O, store at -20°C).
  - Add kanamycin to a final concentration of 25 µg/ml after autoclaved LB-agar cools to 45-55°C.
- Kanamycin Plus 5% Sucrose: *(NEW FORMULATION)*
  - Add 1/10 volume sterile 50% sucrose to molten agar precooled to 75°C.
  - Add kanamycin as above

Section II.
DNA Preparation

1. Preparation of genomic DNA for ligation

(A procedure for isolation is included on page 23, which can also be found in reference 8.)

Genomic DNA isolation techniques vary depending on the source of the DNA. Follow the appropriate protocol for isolation. Use methods which yield intact genomic DNA greater than 300-400 kb in length; shorter pieces will likely result in non-ligatable ends. It is important to realize that DNA larger than 75 kb is highly susceptible to shearing through handling. One safeguard that should be exercised is the use of wide-bore pipet tips.

Partial digestion of genomic DNA

Because of the qualitative variability of genomic DNA, a time-course restriction digest should be performed for each type of DNA preparation. This involves mixing a sample of the genomic preparation with the appropriate restriction endonuclease, adding Mg^{2+} and incubating for various lengths of time. The aim is to determine what period of digestion is necessary so that the majority of fragments are in the 70-100 kb range.

*Bam*HI and *Sal* I are the two restriction sites used when cloning into the Ad10-SacBII vector. **(NOTE: only *Bam*HI compatible inserts are usable by the Sp6/T7 RNA Polymerase promoters, see Fig. 1)** Any restriction enzyme that generates compatible ends may be used to digest genomic DNA (i.e. *Sau*3A, *Bgl* II, *Mbo* I or *Bcl* I for *Bam*HI, and *Xho* I for *Sal* I). Restriction enzymes that recognize 4-base sites (*Sau*3A and *Mbo* I) usually generate a more representative library. The following protocol is written for the use of either *Bam*HI or *Sal* I. If another enzyme is to be used, substitute with the appropriate magnesium-free buffer for that enzyme.

(NEW PROTOCOL)

1. Add the following to a microcentrifuge tube:
   - 0.5ml dialyzed genomic DNA (10-50 µg, should be very viscous)
   - 60 µl of 10 X *Bam*HI (or *Sal* I) buffer (without Mg^{2+})
   - 20 µl of 2.5 mg/ml nuclease-free BSA
   - 1-10 units of *Bam*HI (or *Sal* I)
2. Using a Labquake Shaker or equivalent, gently mix tube overnight at 4°C.
3. Dispense a 10 µl sample into a microcentrifuge tube for use as minus Mg^{2+} control.
4. Warm remainder at 30°C for 1 min, then add 60 µl 0.1M $MgCl_2$, gently stir.
5. Aliquots of 50 µl, are removed at various times, usually at 2 min. intervals between 2-20 min.
6. Aliquots are added to tubes containing 15 µl of 0.2 M EDTA, pH 8.0. Each tube is incubated 15 min. at 70°C, and then put on ice to stop the reaction.
7. Drop dialyze 10 µl of each aliquot plus control(s) in the following manner:
   a. Float a Millipore Type VSWP, 0.025 µm pore filter, shiny side up, on a pool of 1X TE buffer (50 ml).
   b. Gently place the sample on the filter (Note: It is possible to place several 10 µl samples on the same filter).
   c. Slowly mix the buffer with a stir bar for 30 minutes at ambient temperature.
   d. Mix each aliquot with 2 µl 6X Loading Buffer, and heat 5 min. at 70°C.
8. Analyze each sample against the T5 marker set (provided in the kit) by field inversion gel electrophoresis (FIGE; see Section II, part 5) to determine extent of digestion. The sample(s) with the highest concentration of 70-100 kb fragments reflect the optimum digestion period for the genomic DNA.
9. A linear scale-up of the above process for the remaining DNA preparation should generate the same fragment sizes (this reaction should also be checked by FIGE before ligation).

Size selection of partially digested genomic DNA

Once the genomic DNA is partially digested and FIGE demonstrates substantial 70-100 kb fragments, isolation of clonable fragments from the mix is necessary. Sucrose gradient centrifugation has proven to be the most successful method.

1. Make a linear gradient using 10% and 40% sucrose solutions, in 20mM Tris-HCl (pH 8.0), 0.8M NaCl, 10 mM EDTA for Sorvall TH-641 rotor tubes or equivalent.
2. Layer (pour not pipetted) the partially digested genomic DNA onto the sucrose gradient and centrifuge at 18-20,000 RPM, 4°C for 18 hours.
3. Collect fractions of 0.5-1 ml from the bottom of the centrifuge tube.
4. Dialyze 50 µl aliquots from each fraction for 1 hour at 25°C by drop dialysis on Millipore VSWP filters using 50 ml of 1X TE.
5. Analyze the fragments against T5 markers by FIGE.
6. Butanol concentrate the material from fractions containing 70-100 kb DNA in the following manner:
   a. Add an equal volume of n-butanol to DNA.
   b. Gently agitate for 1 hour. Aspirate butanol (top layer) from DNA
   c. Repeat a-b four times or until the volume is reduced to 35-50 µl.
   d. Transfer the DNA to Parafilm and evaporate residual butanol by air drying on bench.
   e. Drop dialyze the material to equilibrate salt concentration with 1X TE buffer (200-300ml). The DNA is ready to be used for cloning.

2. Preparation of a packaging control DNA

Generation of concatemerized Ad10 DNA

These procedures are used to generate a packaging control DNA. The pAd10 control plasmid DNA is linearized and then self-ligated to form concatemerized molecules, consisting of 2 to 10 (or more) multiples of the linear vector. The molecules comprised of **3 repeats** are primarily used as packaging substrates. **(NOTE: DO NOT USE SacBII VECTOR TO FORM CONCATEMERIZED MOLECULES; multiple copies of the SacB gene are detrimental even in the absence of sucrose.)**

A. Linearization *(NEW PROTOCOL)*

1. Add the following to a microcentrifuge tube:
   20 µl (10 µg) Ad10 DNA
   2.5 µl 10X *Bam*HI (or *Sal* I) buffer
   20 units *Bam*HI (or *Sal* I) restriction endonuclease
   H_2O to 25 µl
2. Incubate at 37°C for 1 hour.
3. Dilute the reaction to 400 µl with H_2O.
4. Extract protein and ethanol precipitate as follows:
   a. Add 200 µl of equilibrated phenol.
   b. Add 200 µl of chloroform/isoamyl alcohol and mix thoroughly.
   c. Centrifuge briefly at 12,000 RPM to separate phases.
   d. Transfer aqueous phase (upper layer) to new tube.
   e. Add 200 µl of chloroform/isoamyl alcohol and mix thoroughly.
   f. Centrifuge briefly at 12,000 RPM.
   g. Transfer aqueous phase to new tube.
   h. Add 1/10 volume 3M NaOAc and 800 µl chilled EtOH. Mix thoroughly.
   i. Precipitate DNA in ethanol/dry ice bath for 10 minutes.
   j. Centrifuge minimally for l0 minutes at 12,000 RPM (4°C).
   k. Aspirate supernatant and wash pellet with 70% EtOH.
   l. Air dry pellet.
   m. Resuspend pellet in 10 µl of TE.
5. Determine DNA concentration by gel analysis (ethidium bromide).

B. Ligation *(NEW PROTOCOL)*

1. Add the following to the microcentrifuge tube containing linearized Ad10 DNA (approx. 10 µg):
   10 µl 5x Ligation buffer
   27 µl H_2O (final volume should equal 50 µl)
   4 µl T4 DNA Ligase (~20 U)
2. Incubate for 3 hours at room temperature.
3. Heat inactivate the enzyme at 70°C for 15 minutes.
4. Adjust the DNA concentration to 100 ng/µl (based on analysis from step A.5) with 1X TE buffer.
5. Store at 4°C.

3. Generation of SacBII vector arms for ligation with genomic DNA *(NEW PROTOCOL)*

In this procedure, the vector is processed into short and long arms. Each digestion is followed by a dephosphorylation step so that, in the subsequent ligation, the vector cannot ligate to itself. The *Sca* I digestion generates blunt ends and dephosphorylation can be carried out with excess enzyme. The *Bam*HI (or *Sal* I) digestion generates cohesive ends so the quantity of enzyme used to dephosphorylate is critical. Thus, a double digest followed by dephosphorylation is not practical.

1. Add the following to a microcentrifuge tube:
   20 µl (10 µg) Ad10-SacBII DNA
   10 µl 10X *Sca* I buffer
   20 units *Sca* I
   H_2O to 100 µl
2. Incubate at 37° C for 1-2 hours.
3. Dilute reaction to 400 µl.
4. Extract protein and ethanol precipitate as above (Part 2. A. 4a - m)
5. Resuspend pellet in 50 µl of Calf Intestinal Alkaline Phosphatase buffer.
6. Add 200 units of Calf Intestinal Alkaline Phosphatase (13 - 15 µl).
7. Incubate at 37°C for 30 minutes.
8. Repeat step 4.
9. Resuspend pellet in 25 µl of TE buffer.
10. Determine DNA concentration by gel analysis.
11. Add the following to the microcentrifuge tube containing the DNA:
    5 µl 10X *Bam* HI buffer.
    2 units/µg DNA of *Bam*HI
    H_2O to 50 µl
12. Incubate *Bam*HI digest 15 minutes at 37°C (vector is highly susceptible to star activity by *Bam*HI).
13. Repeat step 4.
14. Resuspend pellet in 50 µl of Calf Intestinal Alkaline Phosphatase buffer.
15. Add Calf Intestinal Alkaline Phosphatase to a concentration of 0.005 units/µg DNA.
16. Incubate at 37°C for 30 minutes.
17. Repeat step 4.
18. Resuspend pellet in 10 µl of TE buffer.
19. Determine DNA concentration by gel analysis. Final vector arm concentration should be 100-200 ng/µl .
20. Store vector arms at 4°C.

4. Ligation of vector arms with genomic DNA

In the following procedure, vector arms are mixed with genomic DNA in approximately a 2:1 molar ratio. Because the vector arms are dephosphorylated, minimal vector-vector ligation is possible. Any insert-insert ligation will result in non-packageable DNA.

1. Add the following in a microcentrifuge tube: ***(NEW PROTOCOL)***
   2 µl (200-400 ng) vector arms
   500 ng insert DNA
   4 µl 5X Ligation buffer
   H_2O to 20 µl total volume
2. Incubate at 30°C for 1 hour.
3. Add 2 µl (10 U) DNA Ligase (supplied with kit) and incubate overnight at 16°C.
4. Heat inactivate enzyme at 70°C for 10 minutes.
5. The ligated DNA may be stored at 4°C until needed (stable at least 4 weeks).

5. Field Inversion Gel Electrophoresis (FIGE)

Field inversion gel electrophoresis utilizes a pulsed field electrical gradient to achieve a separation of high molecular weight DNA fragments that is not possible with standard techniques. Electrophoresis conditions may have to be adjusted to accomodate materials other than those which are described.

Switching unit:	DNASTAR PULSE™ (DNASTAR Inc., Madison, WI)
Gel apparatus:	LKB Miniphor™ unit
Agarose:	1% SeaKem™ GTG-grade agarose in 0.5X TBE
Electrophoresis buffer:	0.5X TBE
Gradient conditions:	120 Volts (constant voltage; 12 V/cm) 0:30 min. pre-run, 5:00 hour run Forward pulse: 0.15 sec. to 3.0 sec. Reverse pulse: 0.05 sec. to 1.0 sec.

1. Dilute 6X Loading buffer into DNA sample using a wide-bore pipet tip.
2. Heat sample at 65°C for 5 minutes to dissolve any DNA aggregates.
3. Load sample onto gel.
4. Use ~0.5 µg (2.5 µl) of the T5 DNA marker set (provided with kit) to compare sample sizes. The markers include 122, 102, 78 and 25 kb fragments.
5. After electrophoresis, soak the gel in 0.5X TBE with 20 µg/ml ethidium bromide for 15 minutes.
6. View the DNA bands on a UV transilluminator, using proper eye protection.

Section III.
Packaging and Propagation

1. Packaging ligated DNAs

In the following procedures, the vector-genomic recombinant DNA or the concatemer control DNA is modified (*Pacase* cleavage) and packaged into phage heads. Infectious particles result only if the necessary parameters described in the introduction section are fulfilled.

Pac-cleavage reaction

1. Add the following components to a microcentrifuge tube:
   - 1.5 µl *Pac*ase buffer
   - 1.5 µl 10X dNTP mix
   - 1.0 µl 25 mM DTT
   - 1.0 µl 50 mM ATP
   - 0.1-0.5 µg ligated DNA (with respect to vector concentration), **by gentle pipeting**
   - H_2O to 14 µl
   - 1.0 µl *Pac*ase extract (add last and gently mix reaction with pipet tip).

   Note: *Pac*ase extract can be subjected to several freeze-thaw cycles without compromising biological activity.
2. Incubate at 30°C for 15 minutes.
   Note: It is important to limit this step to 15 minutes. Longer incubation times will eventually degenerate the DNA into fragments that are too small for packaging.
3. Add: 3.0 µl Head/Tail buffer.
   1.0 µl 50 mM ATP
   Immediately go to next step.

Insertion of DNA in P1 phage heads

1. Using a wide-bore pipet tip, add the entire contents of the *Pac*-cleavage reaction to a vial of Head/Tail extract (partially thawed immediately before use). Mix by stirring with the pipet tip. **Note: Head/Tail extract must be kept at -70°C until use. Otherwise, severe loss of biological activity will result.**
2. Incubate at 30°C for 5 minutes.
3. Centrifuge at 12,000 RPM for 10 seconds to eliminate any bubbles in the mixture.
4. Continue incubation at 30°C for an additional 15 minutes.
5. Add 120 µl of TMG-buffer containing 10 µg/ml DNase I solution (dilute DNase I stock 1:12, 10 µl DNase I added to 110 µl TMG-Buffer). Vortex briefly.
6. Incubate at 37° C for 15 minutes.
7. Centrifuge at 12,000 RPM for 1 minute to remove cell debris from the reaction.
8. Transfer the supernatant to a new tube.
9. Add 10 µl of chloroform (without isoamyl alcohol) to prevent bacterial growth.
10. The phage-packaged DNA can be stored at 4°C for several weeks.

2. Plating of Packaged DNA using NS3529 Host Cells

Preparation of $MgSO_4$ stock of NS3529

1. Inoculate 30 ml LB broth with a loop of frozen NS3529 stock.
2. Incubate culture overnight at 37°C, 250 RPM.

3. Centrifuge saturated culture for 5 minutes at 5000 RPM (4°C).
4. Discard supernatant and resuspend pellet in 15 ml sterile 10mM $MgSO_4$/10mM Tris-HCl, pH 7.5.
5. The cell suspension can be stored at 4°C for several months.

Preparation of plating host

1. Mix the $MgSO_4$ cell stock so that it is a homogeneous suspension.
2. Make a 1:100 dilution of the stock into an appropriate volume of fresh LB (20 ml of culture will yield enough host for 10 platings).
3. Incubate at 37°C, 250 RPM until OD_{630}=0.3.
4. Centrifuge cells for 5 minutes at 5000 RPM (4°C).
5. Discard supernatant and resuspend pellet in 1/10 original volume with cold LB/5mM $CaCl_2$.
6. Store on ice. The suspension is stable for several hours.

Plating of Packaged DNA *(NEW PROTOCOL)*

In this procedure, phage particles adhere to host cells and inject them with packaged DNA. The DNA is recombined into a plasmid through the interaction of its *lox* P sites and host-produced Cre recombinase protein. Transformed cells with insert DNA will survive on LB-Kanamycin/sucrose agar plates

1. Add 10-20 µl of packaged DNA to a clean glass tube.
2. Evaporate any residual chloroform by incubating open tube at 37°C for 10 minutes.
3. Add 200 µl of plating host suspension to the tube.
4. Incubate at 37°C, without shaking, for 5-10 minutes to allow for phage adsorption.
5. Add 1 ml of LB to the tube and cap to avoid evaporation.
6. Incubate at 37°C, 250 RPM for 45 minutes.
7. Transfer the culture to a 1.5 ml microcentrifuge tube.
8. Centrifuge for 1-2 minutes at 12,000 RPM.
9. Discard supernatant.
10. Resuspend cells in 100 µl LB broth.
11. Spread 50 µl onto each (100 mm diameter): LB-kanamycin +5% sucrose and LB-kanamycin plates.
12. Incubate the plates overnight at 37°C.

Note: Generally the number of colonies containing insert genomic DNA on the sucrose plates is 100:1 over colonies without genomic DNA. Significant numbers of colonies may be found on Kan plates with and without sucrose.

3. Plasmid Isolation

Plasmids can be grown in high copy number (approximately 25/cell after several generations) by inducing the P1 lytic operon with IPTG. The DNA can be recovered using standard plasmid isolation techniques, such as in Birnboim, H.C. and Doly, J. (1979) *Nuc. Acids Res.* 7:1513-1523.

1. Inoculate LB-kanamycin (25 µg/ml) with a single colony.
2. Incubate culture at 37°C, 250 RPM until barely turbid.
3. Add IPTG to a final concentration of 1mM.
4. Continue incubation until culture reaches saturation.
5. Isolate plasmid from culture.

Note: A linear restriction map of the pAd10-SacBII (and pAd10) vector is available upon request as an aid for restriction analysis of DNA. Call DuPont/NEN Technical Support at 1-800-551-2121, option 1-2.

ADDENDUM:
GENERATION OF DNA LIBRARIES IN THE AD10 VECTOR

1. Preparation of genomic DNA for ligation

(A procedure for isolation is included on page 23, which can also be found in reference 8)

Genomic DNA isolation techniques vary depending on the source of the DNA. Follow the appropriate protocol for isolation. Use methods which yield intact genomic DNA greater than 300-400 kb in length; shorter pieces will likely result in non-ligatable ends. It is important to realize that DNA larger than 75 kb is highly susceptible to shearing through handling. One safeguard that should be exercised is the use of wide-bore pipet tips.

Partial digestion of genomic DNA

Because of the qualitative variability of genomic DNA, a time-course restriction digest should be performed for each type of DNA preparation. This involves mixing a sample of the genomic preparation with the appropriate restriction endonuclease, adding Mg^{2+} and incubating for various lengths of time. The aim is to determine what period of digestion is necessary so that the majority of fragments are in the 70-100 kb range.

*Bam*HI and *Sal* I are the two restriction sites used when cloning into the Ad10 vector. Any restriction enzyme that generates compatible ends may be used to digest genomic DNA (i.e. *Sau*3A, *Bgl* II, *Mbo* I or *Bcl* I for *Bam*HI, and *Xho* I for *Sal* I). Restriction enzymes that recognize 4-base sites (*Sau*3A and *Mbo* I) usually generate a more representative library. The following protocol is written for the use of either *Bam*HI or *Sal* I. If another enzyme is to be used, substitute with the appropriate magnesium-free buffer for that enzyme.

(NEW PROTOCOL)

1. Add the following to a microcentrifuge tube:
   - 0.5ml dialyzed genomic DNA (10-50 µg, should be very viscous)
   - 60 µl of 10 X *Bam*HI (or *Sal* I) buffer (<u>without Mg^{2+}</u>)
   - 20 µl of 2.5 mg/ml nuclease-free BSA
   - 1-10 units of *Bam*HI (or *Sal* I)
2. Using a Labquake Shaker or equivalent, gently mix tube <u>overnight</u> at 4°C.
3. Dispense a 10 µl sample into a microcentrifuge tube, for use as minus Mg^{2+} control.
4. Warm remainder at 30°C for 1 min, then add 60 µl 0.1M $MgCl_2$, gently stir.
5. Aliquots of 50 µl, are removed at various times, usually at 2 min. intervals between 2-20 min.
6. Aliquots are added to tubes containing 15 µl of 0.2 M EDTA, pH 8.0. Each tube is incubated 15 min. at 70°C, and then put on ice to stop the reaction.
7. Drop dialyze 10 µl of each aliquot plus control(s) in the following manner:
   a. Float a Millipore Type VSWP, 0.025 µm pore filter, shiny side up, on a pool of TE buffer (50 ml).
   b. Gently place the sample on the filter (<u>Note</u>: It is possible to place several 10 µl samples on the same filter).
   c. Slowly mix the buffer with a stir bar for 30 minutes at ambient temperature.
   d. Mix each aliquot with 2 µl 6X Loading Buffer, and heat 15 min. at 70°C.
8. Analyze each sample against the T5 marker set (provided in the kit) by field inversion gel electrophoresis (FIGE; see Section II, part 5) to determine extent of digestion. The sample(s) with the highest concentration of 70-100 kb fragments reflect the optimum digestion period for the genomic DNA.
9. A linear scale-up of the above process for the remaining DNA preparation should generate the same fragment sizes (this reaction should also be checked by FIGE before ligation).

Size selection of partially digested genomic DNA

Once the genomic DNA is partially digested and FIGE demonstrates substantial 70-100 kb fragments, isolation of clonable fragments from the mix is necessary. Sucrose gradient centrifugation has proven to be the most successful method.

1. Make a linear gradient using 10% and 40% sucrose solutions, in 20mM Tris-HCl (pH 8.0), 0.8M NaCl, 10 mM EDTA for Sorvall TH-641 rotor tubes or equivalent.
2. Layer (poured not pipetted) the partially digested genomic DNA onto the sucrose gradient and centrifuge at 18-20,000 RPM, 4°C for 18 hours.
3. Collect fractions of 0.5-1 ml from the bottom of the centrifuge tube.
4. Dialyze 50 µl aliquots from each fraction for 1 hour at 25°C by drop dialysis on Millipore VSWP filters using 50 ml of 1X TE.
5. Analyze the fragments against T5 markers by FIGE.
6. Butanol concentrate the material from fractions containing 70-100 kb DNA in the following manner:
   a. Add an equal volume of n-butanol to DNA.
   b. Gently agitate for 1 hour. Aspirate butanol (top layer) from DNA
   c. Repeat a-b four times or until the volume is reduced to 35-50 µl.
   d. Transfer the DNA to Parafilm and evaporate residual butanol by air drying on bench.
   e. Drop dialyze the material to equilibrate salt concentration with 1X TE buffer (200-300 ml). The DNA is ready to be used for cloning.

2. Preparation of a packaging control DNA

Generation of concatemerized Ad10 DNA

These procedures are used to generate a packaging control DNA. The pAd10 control DNA is linearized and then self-ligated to form concatemerized molecules, consisting of 2 to 10 (or more) multiples of the linear vector. The molecules comprised of **3 repeats** are primarily used as packaging substrates. **(NOTE: DO NOT USE SacBII VECTOR TO FORM CONCATEMERIZED MOLECULES, multiple copies of the SacB gene are detrimental even in the absence of sucrose.)**

A. Linearization *(NEW PROTOCOL)*

1. Add the following to a microcentrifuge tube:
   20 µl (10 µg) pAd10 DNA
   2.5 µl 10X *Bam*HI (or *Sal* I) buffer
   20 units *Bam*HI (or *Sal* I) restriction endonuclease
   H_2O to 25 µl
2. Incubate at 37°C for 1 hour.
3. Dilute the reaction to 400 µl with H_2O.
4. Extract protein and ethanol precipitate as follows:
   a. Add 200 µl of equilibrated phenol.
   b. Add 200 µl of chloroform/isoamyl alcohol and mix thoroughly.
   c. Centrifuge briefly at 12,000 RPM to separate phases.
   d. Transfer aqueous phase (upper layer) to new tube.
   e. Add 200 µl of chloroform/isoamyl alcohol and mix thoroughly.
   f. Centrifuge briefly at 12,000 RPM.
   g. Transfer aqueous phase to new tube.
   h. Add 1/10 volume 3M NaOAc and 800 µl chilled EtOH. Mix thoroughly.
   i. Precipitate DNA in ethanol/dry ice bath for 10 minutes.
   j. Centrifuge minimally for l0 minutes at 12,000 RPM (4°C).
   k. Aspirate supernatant and wash pellet with 70% EtOH.
   l. Air dry pellet.
   m. Resuspend pellet in 10 µl of TE.
5. Determine DNA concentration by gel analysis (ethidium bromide).

B. Ligation *(NEW PROTOCOL)*

1. Add the following to the microcentrifuge tube containing the linearized Ad10 DNA (approx. 10 µg):
   10 µl 5x Ligation buffer
   27 µl H_2O (final volume should equal 50 µl)
   2 µl T4 DNA Ligase (~20 U)
2. Incubate for 3 hours at room temperature.
3. Heat inactivate the enzyme at 70°C for 15 minutes.
4. Adjust the DNA concentration to 100 ng/µl (based on analysis from step A.5) with TE buffer.
5. Store at 4°C.

3. Generation of Ad10 vector arms for ligation with genomic DNA *(NEW PROTOCOL)*

In this procedure, the vector is processed into short and long arms. Each digestion is followed by a dephosphorylation step so that, in the subsequent ligation, the vector cannot ligate to itself. The *Sca* I digestion generates blunt ends and dephosphorylation can be carried out with excess enzyme. The *Bam*HI (or *Sal* I) digestion generates cohesive ends so the quantity of enzyme used to dephosphorylate is critical. Thus, a double digest followed by dephosphorylation is not practical.

1. Add the following to a microcentrifuge tube:
   20 µl (10 µg) Ad10 DNA
   10 µl 10X *Sca* I buffer
   20 units *Sca* I
   H_2O to 100 µl
2. Incubate at 37° C for 1-2 hours.
3. Dilute reaction to 400 µl.
4. Extract protein and ethanol precipitate as above (Part 2. A. 4a - m)
5. Resuspend pellet in 50 µl of Calf Intestinal Alkaline Phosphatase buffer.
6. Add 200 units of Calf Intestinal Alkaline Phosphatase (13 - 15 µl).
7. Incubate at 37°C for 30 minutes.
8. Repeat step 4.
9. Resuspend pellet in 25 µl of TE buffer.
10. Determine DNA concentration by gel analysis.
11. Add the following to the microcentrifuge tube containing the DNA:
    5 µl 10X *Bam* HI buffer
    2 units/µg DNA of *Bam*HI
    H_2O to 50 µl
12. Incubate *Bam*HI digest 15 minutes at 37°C (vector is highly susceptible to star activity by *Bam*HI).
13. Repeat step 4.
14. Resuspend pellet in 50 µl of Calf Intestinal Alkaline Phosphatase buffer.
15. Add Calf Intestinal Alkaline Phosphatase to a concentration of 0.005 units/µg DNA.
16. Incubate at 37°C for 30 minutes.
17. Repeat step 4.
18. Resuspend pellet in 10 µl of TE buffer.
19. Determine DNA concentration by gel analysis. Final vector arm concentration should be 100-200 ng/µl .
20. Store vector arms at 4°C.

4. Ligation of vector arms with genomic DNA

In the following procedure, vector arms are mixed with genomic DNA in approximately a 2:1 molar ratio. Because the vector arms are dephosphorylated, minimal vector-vector ligation is possible. Any insert-insert ligation will result in non-packageable DNA.

1. Add the following in a microcentrifuge tube: ***(NEW PROTOCOL)***
   2 µl (200-400 ng) vector arms
   500 ng insert DNA
   4 µl 5X Ligation buffer
   H_2O to 20 µl total volume
2. Incubate at room temperature for 1 hour.
3. Add 2 µl DNA Ligase (supplied with kit) and incubate overnight at 16°C.
4. Heat inactivate enzyme at 70°C for 10 minutes.
5. The ligated DNA may be stored at 4°C until needed (stable at least 4 weeks).

5. Field Inversion Gel Electrophoresis (FIGE)

Field inversion gel electrophoresis utilizes a pulsed field electrical gradient to achieve a separation of high molecular weight DNA fragments that is not possible with standard techniques. Electrophoresis conditions may have to be adjusted to accomodate materials other than those which are described.

Switching unit:	DNASTAR PULSE™ (DNASTAR Inc., Madison, WI)
Gel apparatus:	LKB Miniphor™ unit
Agarose:	1% SeaKem™ GTG-grade agarose in 0.5X TBE
Electrophoresis buffer:	0.5X TBE
Gradient conditions:	120 Volts (constant voltage; 12 V/cm) 0:30 min. pre-run, 5:00 hour run Forward pulse: 0.15 sec. to 3.0 sec. Reverse pulse: 0.05 sec. to 1.0 sec.

1. Dilute 6X Loading buffer into DNA sample using a wide-bore pipet tip.
2. Heat sample at 65°C for 5 minutes to dissolve any DNA aggregates.
3. Load sample onto gel using a wide-bore pipet tip.
4. Use ~0.5 µg (2.5 µl) of the T5 DNA marker set (provided with kit) to compare sample sizes. The markers include 122, 102, 78 and 25 kb fragments.
5. After electrophoresis, soak the gel in 0.5X TBE with 20 µg/ml ethidium bromide for 15 minutes.
6. View the DNA bands on a UV transilluminator, using proper eye protection.

Section II.
Packaging and Propagation

1. Packaging ligated DNAs

In the following procedures, the vector-genomic recombinant DNA or the concatemer control DNA is modified (*Pac*ase cleavage) and packaged into phage heads. Infectious particles result only if the necessary parameters described in the introduction section are fulfilled.

Pac-cleavage reaction

1. Add the following components to a microcentrifuge tube:
   1.5 µl *Pac*ase buffer
   1.5 µl 10X dNTP mix
   1.0 µl 25 mM DTT
   1.0 µl 50 mM ATP
   0.1-0.5 µg ligated DNA (with respect to vector concentration), **by gentle pipeting**
   H_2O to 14 µl
   1.0 µl *Pac*ase extract (add last and gently mix reaction with pipet tip).

   Note: *Pac*ase extract can be subjected to several freeze-thaw cycles without compromising biological activity.
2. Incubate at 30°C for 15 minutes.
   Note: It is important to limit this step to 15 minutes when using the concatemer DNA. Longer incubation times will eventually degenerate the DNA into fragments that are too small for packaging.
3. Add: 3.0 µl Head/Tail buffer.
   1.0 µl 50 mM ATP
   Immediately go to next step.

Insertion of DNA in P1 phage heads

1. Using a wide-bore pipet tip, add the entire contents of the *Pac*-cleavage reaction to a vial of Head/Tail extract (partially thawed immediately before use). Mix by stirring with the pipet tip. **Note: Head/Tail extract must be kept at -70°C until use. Otherwise, severe loss of biological activity will result.**
2. Incubate at 30°C for 5 minutes.
3. Centrifuge at 12,000 RPM for 10 seconds to eliminate any bubbles in the mixture.
4. Continue incubation at 30°C for an additional 15 minutes.
5. Add 120 µl of TMG-buffer containing 10 µg/ml DNase I solution (dilute DNase I stock 1:12, 10 µl DNase I added to 110 µl TMG-Buffer). Vortex briefly.
6. Incubate at 37° C for 15 minutes.
7. Centrifuge at 12,000 RPM for 1 minute to remove cell debris from the reaction.
8. Transfer the supernatant to a new tube.
9. Add 10 µl of chloroform (without isoamyl alcohol) to prevent bacterial growth.
10. The phage-packaged DNA can be stored at 4°C for several weeks.

2. Plating of Packaged DNA using NS3529 Host Cells

Preparation of $MgSO_4$ stock of NS3529

1. Inoculate 30 ml LB broth with a loop of frozen NS3529 stock.
2. Incubate culture overnight at 37°C, 250 RPM.

3. Centrifuge saturated culture for 5 minutes at 5000 RPM (4°C).
4. Discard supernatant and resuspend pellet in 15 ml sterile 10mM $MgSO_4$/10mM Tris-HCl, pH 7.5.
5. The cell suspension can be stored at 4°C for several months.

Preparation of plating host

1. Mix the $MgSO_4$ cell stock so that it is a homogeneous suspension.
2. Make a 1:100 dilution of the stock into an appropriate volume of fresh LB (20 ml of culture will yield enough host for 10 platings).
3. Incubate at 37°C, 250 RPM until OD_{630}=0.3.
4. Centrifuge cells for 5 minutes at 5000 RPM (4°C).
5. Discard supernatant and resuspend pellet in 1/10 original volume with cold LB/5mM $CaCl_2$.
6. Store on ice. The suspension is stable for several hours.

Plating of Packaged DNA

In this procedure, phage particles adhere to host cells and inject them with packaged DNA. The DNA is recombined into a plasmid through the interaction of its *lox* sites and host-produced *Cre* recombinase protein. Transformed cells will survive on LB-Kanamycin agar plates.

1. Add 10-20 µl of packaged DNA to a clean glass tube.
2. Evaporate any residual chloroform by incubating open tube at 37°C for 10 minutes.
3. Add 200 µl of plating host suspension to the tube.
4. Incubate at 37°C, without shaking, for 5-10 minutes to allow for phage adsorption.
5. Add 1 ml of LB to the tube and cap to avoid evaporation.
6. Incubate at 37°C, 250 RPM for 45 minutes.
7. Transfer the culture to a 1.5 ml microcentrifuge tube.
8. Centrifuge for 1 minute at 12,000 RPM.
9. Discard supernatant.
10. Resuspend cells in 100 µl LB broth.
11. Spread entire solution onto a 100 mm diameter LB-kanamycin plate.
12. Incubate the plate overnight at 37°C.

Note: Colonies which contain plasmid DNA with genomic inserts should be kan^r and tet^s. Checking a representative amount of kan^r colonies on LB-tetracycline media is encouraged to evaluate the success of each ligation reaction. If a plasmid does not contain an insert, the most likely cause is inadequate dephosphorylation of the vector arms resulting in vector-vector ligation.

3. Plasmid Isolation

Plasmids can be grown in high copy number (approximately 25/cell after several generations) by inducing the P1 lytic operon with IPTG. The DNA can be recovered using standard plasmid isolation techniques, such as in Birnboim, H.C. and Doly, J. (1979) *Nuc. Acids Res.* **7**:1513-1523.

1. Inoculate LB-kanamycin (25 µg/ml) with a single colony.
2. Incubate culture at 37°C, 250 RPM until barely turbid.
3. Add IPTG to a final concentration of 1mM.
4. Continue incubation until culture reaches saturation.
5. Isolate plasmid from culture.

Note: A linear restriction map of the pNS582tet14Ad10 vector is available upon request as an aid for restriction analysis of DNA. Call DuPont/NEN Technical Support at 1-800-551-2121, option 1-2.

ISOLATION OF GENOMIC DNA

The following procedure is included only as a suggested method for isolation of genomic DNA that has been used successfully to prepare DNA for P1 packaging. The procedure is from Pierce, J.C., and Sternberg, N.L., Methods in Enzymology (In Press). The procedure has been used to prepare DNA from blood, sperm, cultured lymphoblast and cultured fibroblast cells.

1. Pellet cells by centrifugation (Sorvall RT6000) at 1500 rpm for 10 min. at 4°C, 50 ml Oak-Ridge type tube.
2. Wash pellet with PBS (2.7 mM KCl, 1 mM KH_2PO_4 , 140 mM NaCl).
3. Resuspend cells in 50 mM Tris-HCl (pH 7.5), 0.1M NaCl, 0.15M EDTA at a concentration of about 10^7 - 10^8 cells/ml.
4. Add SDS (sodium dodecyl sulfate) to 1% final concentration.
5. Add proteinase K to a concentration of 1 mg/ml.
6. Incubate suspension for 4 hours at 60°C. (Cells are lysed and solution becomes very viscous.)
   (NOTE: DNA should never be vortexed, vigorously pipetted or centrifuged into a pellet from this stage on, as these procedures will shear it.)
7. The lysate is gently poured onto a 10 to 40% sucrose gradient prepared in 20 mM Tris-HCl (pH 8.0), 0.8M NaCl, and 10 mM EDTA. Generally, 16 or 36 ml gradient tubes are adequate depending upon volume of the lysate.
8. Centrifuge at 18,000 rpm for 16 hours at 4°C. The viscous DNA mass is usually located about half-way down the tube.
9. Aspirate off the overlying volume and pour the viscous DNA into a sterile dialysis bag. Care should be taken to place the bag in a sterile vessel before DNA is poured into it, as the viscous DNA flows as a mass. DNA failing to enter the bag may be recovered from the sterile vessel.
10. Dialyze DNA against four changes of 1L TE buffer at 4°C.
    (Quantitation of DNA at this stage is difficult because the solution is not homogeneous and much sampling bias occurs.)
11. Evaluate the size of DNA by field inversion gel electrophoresis (FIGE). If the majority of DNA is larger than 300-400 kb, it is suitable for subsequent restriction enzyme digestion. The following conditions are recommended:
    6 in. X 6 in. 1% agarose gel (GTG agarose, FMC) prepared in 0.5X TBE
    FIGE Conditions: -100V for 60 min.
    - initiate switching protocol of 0.3 sec. forward, 0.1 sec. backwards with a ramping factor of 15 to 240V for 6 hours at 4°C.
    Stain gel with ethidium bromide and visualize DNA by UV fluorescence.
12. Continue with the procedure outlined in the manual, page 11, for partial digestion of genomic DNA.

Important Product News

NEN PHAGE™ P1 DNA CLONING & PACKAGING SYSTEM

WARNING!

THERE IS A MISTAKE IN THE MANUAL ON PAGES 13 AND 19, GENERATION OF VECTOR ARMS, STEP 6.

THE CORRECT AMOUNT OF CALF INTESTINAL ALKALINE PHOSPHATASE TO USE FOR DEPHOSPHORYLATION OF THE Sca 1 DIGEST IS 5 UNITS PER UG.