pBluescript® II Phagemid Vectors

INSTRUCTION MANUAL

Catalog #212205, #212206, #212207 and #212208
Revision #083001m

For In Vitro Use Only

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pBluescript® II Phagemid Vectors

MATERIALS PROVIDED

Material Provided	Catalog Number			
	#212205	#212206	#212207	#212208
pBluescript® II SK(+) phagemid, 1 µg/µl	20 µg	—	—	—
pBluescript® II SK(–) phagemid, 1 µg/µl	—	20 µg	—	—
pBluescript® II KS(+) phagemid, 1 µg/µl	—	—	20 µg	—
pBluescript® II KS(–) phagemid, 1 µg/µl	—	—	—	20 µg
XL1-Blue MRF´ host strain, glycerol stock, Catalog #200301	1 tube	1 tube	1 tube	1 tube

STORAGE CONDITIONS

Phagemids: –20°C
Bacterial Strains: –80°C

Revision #083001m

INTRODUCTION

The pBluescript® II phagemids (plasmids with a phage origin) are cloning vectors designed to simplify commonly used cloning and sequencing procedures, including the construction of nested deletions for DNA sequencing, generation of RNA transcripts *in vitro* and site-specific mutagenesis and gene mapping. The pBluescript II phagemids have an extensive polylinker with 21 unique restriction enzyme recognition sites. Flanking the polylinker are T7 and T3 RNA polymerase promoters that can be used to synthesize RNA *in vitro*.[1,2] The choice of promoter used to initiate transcription determines which strand of the insert cloned into the polylinker will be transcribed.

Circular maps and lists of features for the pBluescript II phagemids are shown in figures 1 and 2. The polylinker and T7 and T3 RNA polymerase promoter sequences are present in the N-terminal portion of a *lac*Z gene fragment. A total of 131 amino acids of β-galactosidase coding sequence is present in the pBluescript II phagemid, but the coding sequence is interrupted by the large polylinker. (There are 36 amino acids from the initiator Met sequence to the *Eco*R I site.) pBluescript II phagemids having no inserts in the polylinker will produce blue colonies in the appropriate strains of bacteria (i.e., strains containing *lac*Z*ΔM15* on an F′ episome, such as XL1-Blue MRF′, among others). pBluescript II phagemids that have inserts will produce white colonies using the same strain, because the inserts disrupt the coding region of the *lac*Z gene fragment.

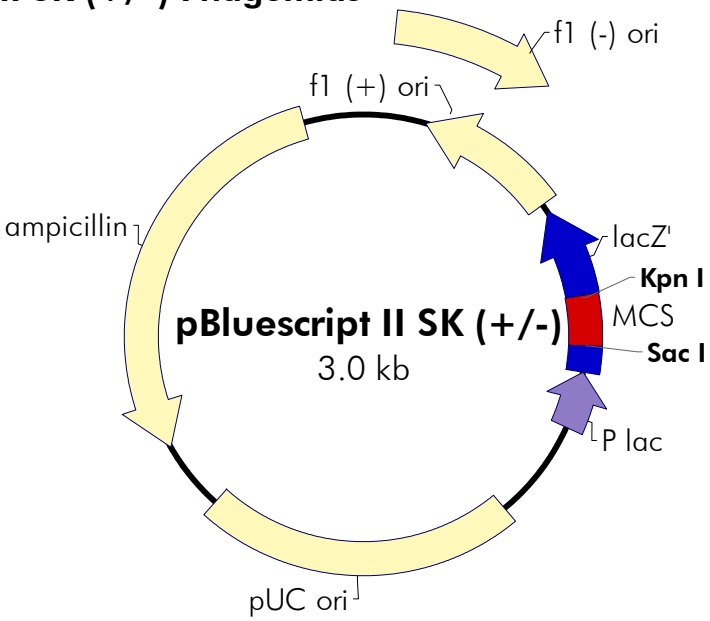
pBluescript II (+) and (–) are available with two polylinker orientations designated as either KS or SK using the following convention: (1) in the KS orientation, the *Kpn* I restriction site is nearest the *lac*Z promoter and the *Sac* I restriction site is farthest from the *lac*Z promoter; and (2) in the SK orientation, the *Sac* I site is the closest restriction site to the *lac*Z promoter and the *Kpn* I site is the farthest.

Flanking the T3 and T7 promoters are *Bss*H II sites. This rare six-base cutter will allow the insert plus the T phage RNA promoters to be excised and used for gene mapping.

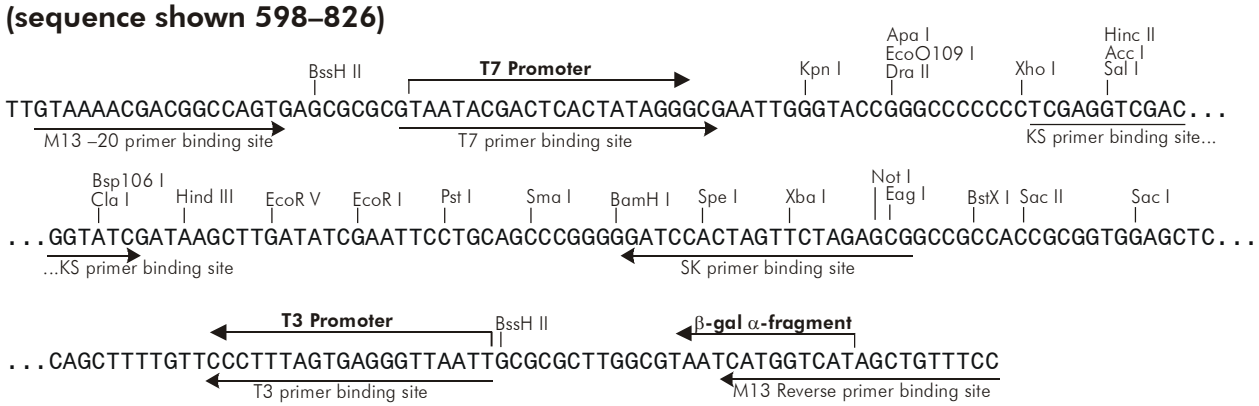
pBluescript II phagemids can be rescued as single-stranded (ss) DNA. pBluescript II phagemids contain a 454-bp filamentous f1 phage intergenic region (M13 related), which includes the 307-bp origin of replication. The (+) and (–) orientations of the f1 intergenic region allow the rescue of sense or antisense ssDNA by a helper phage. This ssDNA can be used for dideoxynucleotide sequencing (Sanger method) or site-specific mutagenesis.

Note *We have discovered that the use of excess amounts of* Eco*R I to digest pBluescript II results in* Eco*R I prime activity. This appears as cleavage at a non-EcoR I site at the 3′ end of the f1 intergenic region, causing confusion when interpreting results from an agarose gel. If a restriction pattern appears incorrect, check whether reducing the units of* Eco*R I restores a normal restriction pattern.*

pBluescript® II SK (+/–) Phagemids



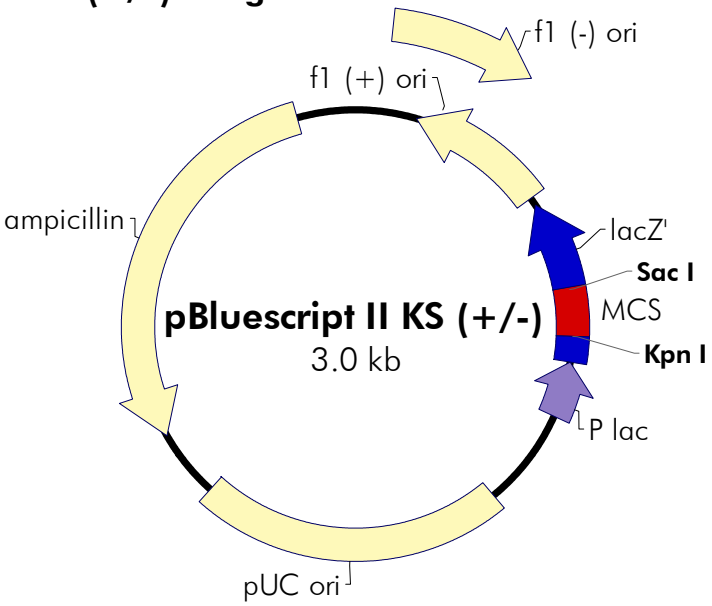
pBluescript II SK (+/–) Multiple Cloning Site Region (sequence shown 598–826)



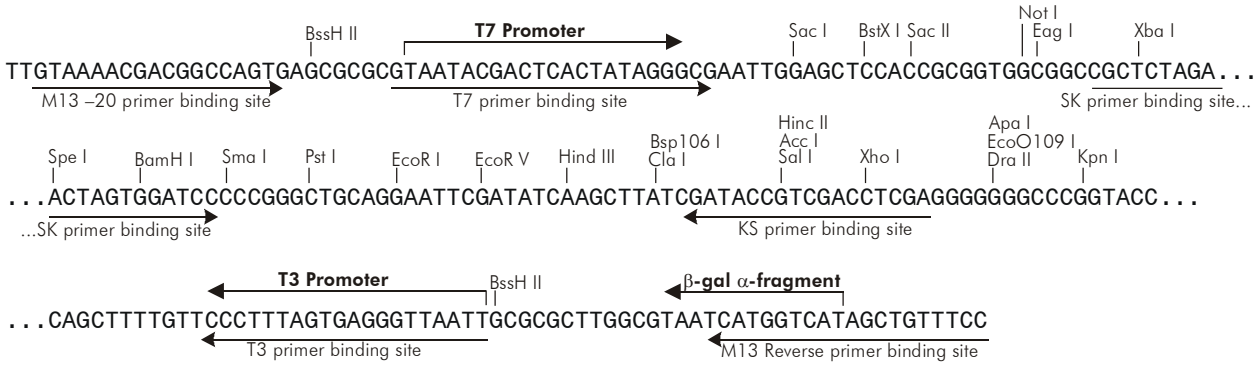
Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication [pBluescript SK (+) only]	135–441
f1 (–) origin of ss-DNA replication [pBluescript SK (–) only]	21–327
β-galactosidase α-fragment coding sequence (lacZ')	460–816
multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (*bla*) ORF	1976–2833

FIGURE 1 The pBluescript® II SK (+/–) phagemid vectors. The complete sequence and list of restriction sites are available at *www.stratagene.com*. Genbank® #X52328 [SK(+)] and #X52330 [SK(–)].

pBluescript® II KS (+/–) Phagemids



pBluescript II KS (+/–) Multiple Cloning Site Region (sequence shown 598–826)



Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication [pBluescript KS (+) only]	135–441
f1 (–) origin of ss-DNA replication [pBluescript KS (–) only]	21–327
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multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (*bla*) ORF	1976–2833

FIGURE 2 The pBluescript® II KS (+/–) phagemid vectors. The complete sequence and list of restriction sites are available at *www.stratagene.com*. Genbank® #X52327 [KS(+)] and #X52329 [KS(–)].

LIGATION INTO pBLUESCRIPT® II PHAGEMIDS

Stratagene suggests dephosphorylation of the digested pBluescript II phagemid with calf intestinal alkaline phosphatase (CIAP) prior to ligating to the insert DNA. If more than one restriction enzyme is used, the background can be reduced further by electrophoresing the DNA on an agarose gel and removing the desired vector band through electroelution, leaving behind the small fragment that appears between the two restriction enzyme sites.

After gel purification and ethanol precipitation of the DNA, resuspend in a volume of TE buffer [5 mM Tris (pH 7.5), 0.1 mM EDTA] that will allow the concentration of the vector DNA to be the same as the concentration of the insert DNA(~0.1 µg/µl).

For ligation, the ideal ratio of insert to vector DNA is variable; however, a reasonable starting point is 2:1 (insert:vector), measured in available picomole ends. This is calculated as:

picomole ends/micrograms of DNA = $(2 \times 10^6) \div$ (number of base pairs × 660)

We suggest the following protocol, which includes three controls:

Component	1	2	3	4	5
Prepared vector (0.1 µg/µl)	1 µl	1 µl	1 µl	1 µl	0 µl
Prepared insert (0.1 µg/µl)	*X* µl	*X* µl	0 µl	0 µl	1 µl
10 mM rATP (pH 7.0)	1 µl	1 µl	1 µl	1 µl	1 µl
10× Ligase buffer	1 µl	1 µl	1 µl	1 µl	1 µl
T4 DNA ligase (4 U/µl)	0.5 µl	0.5 µl	0.5 µl	0 µl	0.5 µl
ddH_2O (to 10 µl)	*X* µl	*X* µl	*X* µl	*X* µl	*X* µl

1. Ligate for 2 hours at room temperature (22°C) or overnight at 4°C. When ligating blunt ends, incubate the ligation overnight at 12–14°C.

2. Transform 1–2 µl of the ligation mix into the appropriate competent bacteria. (See *Transformation with pBluescript II Phagemids*.) Plate on selective media.

3. Interpretation of test results:

   Reactions 1 and 2 vary the insert:vector ratio.
   Control 3 tests for the effectiveness of the CIAP treatment.
   Control 4 indicates if the vector was cleaved completely or if residual uncut vector remains.
   Control 5 verifies that the insert alone is not contaminated with any vector DNA.

4. Expected plating results:

   Plates 1 and 2 should have mostly white colonies, representing recombinants.

   Plate 3 should have low numbers of blue colonies if the CIAP treatment was effective.

   Plate 4 should have no colonies if the digest was complete.

   Plate 5 should have no colonies if the insert was pure.

TRANSFORMATION WITH pBLUESCRIPT II® PHAGEMIDS

Note *pBluescript II phagemids will replicate autonomously as plasmids. Therefore, colonies—**not plaques**—are obtained following transformation.*

Suggested Host Strain and Genotype

Stratagene recommends the host strain XL1-Blue MRF´ for propagation of pBluescript II phagemids and for transformation of recombinant phagemids. XL1-Blue MRF´ allows blue-white color selection and single-stranded DNA rescue, and is restriction-deficient aiding in the construction of libraries made from methylated DNA.[3]

XL1-Blue MRF´ Genotype: Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F´ *pro*AB *lac*I^{q}ZΔM15 Tn10 (Tet^r)]

Note *The XL1-Blue MRF´ is provided as a glycerol stock. Additional tubes of glycerol stock can be purchased from Stratagene (Catalog #200301); alternatively, XL1-Blue MRF´ is available from Stratagene as high-efficiency frozen competent cells (>1 × 10^9 colonies/μg of pUC 18, Catalog #200230).*

For the appropriate media and plates for growth of XL1-Blue MRF´, please refer to the following table:

Bacterial strain	Plates for bacterial streak	Media for glycerol stock
XL1-Blue MRF´	LB–tetracycline agar[a]	LB–tetracycline[a]

[a]12.5 μg/ml.

Streaking Cells from a –80°C Bacterial Glycerol Stock

Prepare the following from a frozen glycerol stock:

Note *Do not allow the contents of the vial to thaw. The vials can be stored at –20° or –80°C, but most strains remain viable longer if stored at –80°C.*

1. Revive the stored cells by scraping off splinters of solid ice with a sterile wire loop.

2. Streak the splinters onto an LB plate containing the appropriate antibiotic.

   Restreak the cells fresh each week.

Preparation of a –80°C Bacterial Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of the appropriate liquid media with one or two colonies from a plate of freshly-streaked cells. Grow the cells to late log phase.

2. Add 4.5 ml of a sterile glycerol–liquid media solution (prepared by combining 5 ml of glycerol + 5 ml of liquid media) to the bacterial culture from step 1. Mix well.

3. Aliquot into sterile centrifuge tubes (1 ml/ tube). This preparation may be stored at –20°C for 1–2 years or at –80°C for more than 2 years.

Blue-White Color Selection

The XL1-Blue MRF´ strain allows blue–white color selection for pBluescript II phagemids because of *lac*ZΔ*M15* complementation on the F´ episome. The color selection may be seen when plating on LB plates containing 100 µg/ml of ampicillin, 80 µg/ml of fresh X-gal, and 20 mM IPTG. Alternatively, plates for color selection can be prepared by spreading 100 µl of 40 mM IPTG and 100 µl of 2% X-gal on LB–ampicillin plates 30 minutes prior to plating your transformants. X-gal should be prepared in dimethyl formamide and IPTG in sterile, distilled H_2O (store stock solutions at –20°C until use). Colonies containing phagemids without inserts will be blue after incubation for 12–18 hours at 37°C. Colonies with phagemids containing inserts will remain white. Further enhancement of the blue color may be obtained by placing plates at 4°C for 2 hours following overnight growth at 37°C.

Occasionally, β-galactosidase fusion proteins are toxic to the host bacteria. If there is any suspicion that an insert might be toxic, the X-gal and IPTG may be left out of the ampicillin plates. Under these conditions there will be no color selection, but recombinants will express lower levels of the potentially toxic proteins.

Background White Colonies

Since the Δ*M15 lac* gene carried on the F´ episome is needed for the blue–white color assay, host bacteria that have lost the F´ episome will remain as white colonies on an X-gal/IPTG agar plate even if the pBluescript II phagemid does not contain an insert. XL1-Blue MRF´ is a *lac*⁻ AG1 derivative with Tn*10*, *lacI*^{q}, and *lacZ*Δ*M15* on the F´. Selection for bacteria containing the F´ in this strain is accomplished by plating on 12.5 µg/ml tetracycline instead of minimal media plates. XL1-Blue MRF´ transformants containing pBluescript II phagemids can be plated on tetracycline–ampicillin plates to select for colonies that contain both the F´ and the pBluescript II phagemid. This advantage further reduces the background of false positives.

For bacteria containing an F´ without a Tn*10* gene, growth on a minimal medium plate supplemented with 1 mM thiamine-HCl will maintain selection for the F´; however, colonies will grow more slowly. If there is any doubt about whether a white colony represents a pBluescript II recombinant or a colony lacking the F´, streak it onto a minimal medium plate.[4] A cell lacking an F´ will not grow; an F^{+} will grow slowly since it carries the *proAB* genes on the F´ episome.

SCREENING COLONIES

Colonies containing pBluescript II phagemids may be screened for recombinants by double-stranded DNA, RNA, or oligonucleotide hybridization.[5] Colonies may also be screened by restriction mapping or by sequencing miniprep plasmid DNA. Antibodies may be used to screen colonies[6] since cDNA cloned into the appropriate reading frame of the *lac*Z gene will be expressed as fusion proteins.

When screening with antibodies, the bacteria produce fusion proteins containing several amino acids from the amino-terminus of the β-galactosidase protein (3.5 kDa to the *Eco*R I site). Some fusion proteins are toxic to *E. coli*. Therefore, it is best to initially plate transformants on nitrocellulose filters on top of ampicillin plates lacking IPTG. After 8–10 hours (when the colonies are 1 mm in diameter), transfer the filters to plates containing 5 mM IPTG for several hours. This will induce synthesis of the fusion proteins. When screening with antibodies, Stratagene's *pico*Blue™ immunoscreening kit is recommended. To synthesize large amounts of the fusion proteins in liquid culture, grow the cells to an OD_{600} = 0.7 in the absence of IPTG. Add IPTG to 5 mM and grow for another 2–3 hours. The β-galactosidase portion of the fusion protein is ~3.5 kDa from the Met amino acid to the *Eco*R I site in the polylinker.

Identification of recombinant clones within pBluescript II can be performed by colony hybridization. The following protocol minimizes problems associated with colony screening procedures. For the following protocol to be effective, the screening should be performed on **duplicate sets of filters**.

Fixing Replica Sets of Colonies to Nitrocellulose Filters

Use the following protocol to make multiple replica plates of transformants. Keep the original or master filter to pick colonies identified by the screening of the replica filters.

1. Place 100–mm Duralon–UV™ or nitrocellulose filters on 150–mm LB–ampicillin plates.

2. Spread ~1.0×10^6 cfu on the filters.

3. Incubate the plates at 37°C overnight or until colonies are 1.0 mm in diameter (~7–10 hours).

4. Make a replica of the library growing on the nitrocellulose filter:

   a. Place a piece of sterile Whatman® 3MM paper on a glass surface.

   b. Remove the filter from the agar and place it colony side up on the Whatman 3MM paper.

   c. Align a fresh filter, prewetted on an LB plate, over the master filter and cover with another piece of Whatman 3MM paper. Press in place with a glass plate.

   d. Mark the filters with a small needle to aid in realignment after hybridization.

   e. Separate the master and replica filters and place face up on LB agar plates containing ampicillin.

   f. Incubate both the master and replica filters for at least 4 hours at 37°C.

   g. Seal the master plate with Parafilm® and store at 4°C.

5. The replica filter is then prepared for hybridization:

   a. Place the replica filter colony side up for 30 seconds on the surface of Whatman 3MM paper prewetted with 0.5 M NaOH.

   b. Remove filter and place on another sheet of Whatman 3MM paper prewetted with 1 M Tris-HCl (pH 7.6) for 30 seconds.

   c. Remove the filter and place on a third piece of Whatman 3MM paper prewetted with 1 M Tris-HCl (pH 7.6) and 1.5 M NaCl for 30 seconds.

   d. Immerse the filter in 1 M Tris-HCl (pH 7.6) and 1.5 M NaCl and remove bacterial debris by rubbing the filter gently with a gloved hand.

e. Rinse the filter in 1 M Tris-HCl (pH 7.6) and 1.5 M NaCl. Blot dry on paper towels.

f. Crosslink the DNA to the filters using the autocrosslink setting on the Stratalinker® UV crosslinker (120,000 μJ of UV energy). Alternatively, oven bake at 80°C for ~1.5–2 hours.

Prehybridization

Prehybridization Solution for Oligonucleotide Probe

6× SSC
20 mM NaH_2PO_4
0.4% sodium dodecyl sulfate* (SDS)
5× Denhardt's
Denatured, sonicated salmon sperm DNA (500 μg/ml)

OR

Prehybridization Solution for Double-Stranded Probe

2× Pipes buffer
50% Deionized formamide
0.5% SDS*
Denatured, sonicated salmon sperm DNA (100 μg/ml)

The amount of prehybridization solution to make is dependent on the number of filters used (generally 2–3 ml/membrane).

1. Preheat the prehybridization solution to ~50°C without the salmon sperm DNA. Preboil the salmon sperm DNA for ~10 minutes and add it to the warm prehybridization solution.

2. Wet each filter (quickly) in the prehybridization buffer in a tray, placing each filter on top of the next, until each is wet through. Add more prehybridization solution as necessary. (This helps wet the filters completely to allow more even hybridization later.)

3. Put the wet prehybridization filter "stack" in a heat-seal bag, add the remaining prehybridization buffer and heat seal.

4. Calculate the hybridization temperature (generally 42°C) and prehybridize for a minimum of 1 hour.

5. Prehybridize and hybridize a blank filter ("background") along with the rest and wash it to determine when and at what temperature the background counts disappear.

* For Stratagene's Duralon-UV™ membranes, increase the SDS concentration to 1% (w/v).

Hybridization

Labeling Oligonucleotide Probes

Label oligonucleotides with fresh [γ-^{32}P]ATP. High-specific-activity γ-label yields the best results.

a. Perform a polynucleotide kinase (PNK) labeling in 1× ligase buffer for 30 minutes at 37°C.

b. Incubate for 15 minutes at 65°C to inactivate the kinase.

c. Run the solution over a G-50 column or a NucTrap® probe purification column to get rid of the unincorporated counts.

Labeling Double-Stranded Probes

When using double-stranded probes, nick translate with fresh [α-^{32}P]dATP.

Stratagene offers the Prime-It® II random primer kit designed to produce high-specific-activity DNA probes in 2 minutes.

It is best to use ~1 × 10^6–5 × 10^6 counts/ml of hybridization solution. Keep the concentration of counts high and use ~1 × 10^7 counts/filter.

Hybridization Solution

Hybridization Solution for Oligonucleotide Probes

6× SSC
20 mM NaH_2PO_4
0.4% SDS*
Denatured, sonicated salmon sperm DNA (500 µg/ml)

1. Make the hybridization solution.

2. Boil the salmon sperm DNA and then add it to the prewarmed hybridization solution.

3. Pour out the prehybridization buffer from the filter bag. Add the hybridization solution and then the appropriate amount of labeled oligonucleotide.

* For Stratagene's Duralon-UV™ membranes, increase the SDS concentration to 1% (w/v).

4. Heat seal and hybridize at 5–10°C below T_m. Calculate T_m using the following formula:

Note *The first method below overestimates the T_m of hybrids involving longer nucleotides.*

OLIGONUCLEOTIDES SHORTER THAN 18 BASES

$$T_m = 2°C(A + T) + 4°C(G + C)$$

OLIGONUCLEOTIDES 14 BASES AND LONGER (UP TO 60–70 NUCLEOTIDES)

$$T_m = 81.5 - 16.6\ (\log_{10}[Na^+]) + 0.41(\%G + C) - (600/N), \text{ where } N = \text{chain length}$$

Hybridization Solution for Double-Stranded Probes

2× Pipes buffer
50% Deionized formamide
0.5% SDS*
Denatured, sonicated salmon sperm DNA (100 µg/ml)

1. Prepare the hybridization solution.

2. Warm the solution, boil the appropriate amount of salmon sperm DNA with the probe for 4 minutes and then add it to the hybridization buffer.

3. Decant the prehybridization buffer and replace it with the hybridization solution and probe. Hybridize overnight at 42°C.

Washes

Oligonucleotide Probes

Use 6× SSC buffer and 0.1% (w/v) SDS. Wash the filters three times for 5 minutes each at room temperature. The final washing temperature depends on the GC ratio of the probe. It is best to stay several degrees below the melting temperature. A rough estimate of the melting temperature of an oligonucleotide probe can be determined by the following formula:

$$T_m = 4(G + C) + 2(T + A)$$

If the probe sequence is unknown, start with a room temperature wash and gradually increase the temperature until the background diminishes. DO NOT allow the membranes to completely dry out or the probe may be irreversibly bound.

Double-Stranded Probes

Use 0.1× SSC buffer and 0.1% (w/v) SDS. Wash the filters at 50–65°C with agitation.

* For Stratagene's Duralon-UV™ membranes, increase the SDS concentration to 1% (w/v).

Exposure to Film

After washing, remove the excess liquid by blotting on Whatman 3MM paper and place the filters between two sheets of plastic wrap in cassettes with intensifying screens. Leave overnight at –80°C. (By keeping the filters slightly moist between plastic wrap, you can wash again if the background is high.)

T3 AND T7 RNA TRANSCRIPTION

The RNA transcripts synthesized from inserts cloned into vectors containing either T3 or T7 polymerase promoters can be used for many purposes. Transcripts can be used for both Southern and Northern hybridization experiments and for either S1 or RNase A analysis. In addition, RNA transcripts can be used to produce protein by translation in vitro or translation in vivo after microinjection into *Xenopus* oocytes or tissue culture cells. Stratagene's mCAP™ RNA capping kit may be used with both T3 and T7 RNA polymerases to incorporate 5´-7MeGpppG-5´ cap analogs, increasing RNA stability by up to 95%.

The pBluescript II vectors have a *Bss*H II site outside each RNA promoter. This feature allows the excising of the insert with the promoters and subsequent mapping using phosphorylated T3 and/or T7 primers.

Handling RNA

Note *Wear gloves at all times to prevent RNase contamination.*

When working with RNA, caution must be used to eliminate RNase contamination from any source. The following general principles will help in the production of full-length transcripts:

1. Make all buffers, DTT, and rNTPs in highly pure water treated with diethylpyrocarbonate (DEPC) as follows:

   Add DEPC to water to a final concentration of 0.1%, heat to 37°C for 8 hours and autoclave. If DEPC scent remains after autoclaving, place the water in a 90°C water bath for at least 1 hour or until the scent is gone.

   Note *Do not treat Tris solutions with DEPC!! Instead, use water that has been treated with DEPC to make up all Tris solutions.*

   Stratagene's RNAMaxx™ high-yield transcription kit (Catalog #200339) may be used for transcription reactions performed with T7 RNA polymerase.

2. All tubes and pipet tips should be autoclaved and baked for several hours at 80°C. A common source of RNase contamination on gel electrophoresis equipment comes from DNA mini–preps which have been treated with RNase A. Thoroughly clean all gel tanks, gel combs, gel spacers and glassware, using soap and water. Followed with an ethanol rinse. Next, soak the equipment in 3% hydrogen peroxide for 10 minutes at room temperature and rinse with DEPC-treated water. Keep cleaned items covered and away from bare hands. Autoclave all glass plates and other appropriate materials on dry cycle prior to use.

3. Phagemid templates for transcription must be RNase-free. Cesium chloride preps are advisable, but minipreps may be used if care is taken to remove contaminating RNases. Generally the plasmid template is linearized with an enzyme that cleaves "downstream" of the RNA polymerase promoter and the insert in the multiple cloning site. It is strongly advised to purify the post-restriction digest DNA by adding 50 µg/ml proteinase K to the restriction buffer at 37°C for 30 minutes, followed by two phenol–chloroform [1:1 (v/v)] extractions and ethanol precipitation prior to the transcription reaction. Resuspend digested, proteinase K treated DNA at 1 mg/ml in a 10 mM Tris (pH 7.4) and 0.1 mM EDTA solution made with DEPC-treated water.

4. Working with RNA is simplified by using a ribonuclease inhibitor in transcription reactions. Stratagene's RNase Block Ribonuclease Inhibitor has been tested and adjusted to work optimally with Stratagene transcription kits.

Nonspecific Initiation with T7 and T3 RNA Polymerases

T7 and T3 RNA polymerases are highly specific for their respective promoters,[1] however, nonspecific initiation of RNA transcripts may occur at the ends of the DNA template. This is most prevalent with a 3′-protruding terminus. Nonspecific initiation may be reduced by increasing the NaCl concentration in the transcription buffers to 100 mM, although this will result in a decrease of the total transcription efficiency by ~50%. When possible, use restriction enzymes that leave blunt or 5′-protruding ends.

When the T7 or T3 polymerase enzymes are used in molar excess of the DNA template, there is a risk of polymerization from the wrong promoter. T7 polymerase can synthesize RNA inefficiently from a plasmid containing only a T3 promoter. Conversely, T3 polymerase can synthesize RNA inefficiently from a plasmid containing only a T7 promoter. Synthesis is extremely promoter specific when both promoters are present, provided that the enzyme is not in molar excess of the specific promoter. Do not use excessive amounts of the polymerases if promoter specificity is important to your experiment. Best results are obtained when the ratios stated in this manual are followed.

Nonradioactive Transcripts

Nonradioactive transcripts can be used for nucleotide sequencing, *in vitro* translation and injection into cells for *in vivo* translation. Set up the transcription reaction as described, but add 1 µl of 10 mM rUTP instead of radioactive rUTP. For larger amounts of RNA, scale up the reaction appropriately. Each molecule of DNA template yields 10–20 nonradioactive RNA molecules if the ribonucleotides are not a limiting factor.

DNase Treatment after Transcription

The DNA template will be present after the transcription reaction and can be removed with RNase-free DNase. After the transcription reaction, add 10 U of RNase-free DNase/µg of DNA template and incubate at 37°C for 15 minutes. Extract with phenol–chloroform [1:1 (v/v)], add 1/10 volume of 3 M sodium acetate at pH 5.2 and precipitate RNA with 2.5 volumes of 100% (v/v) ethanol.

High-Specific-Activity RNA Probes

Any vector containing T3 and T7 RNA promoters can be used to synthesize high specific activity, strand-specific RNA probes. The choice between T3 and T7 RNA polymerase will determine which strand will be used as the template. This is important because probes used for Northern or S1 analysis must complement the RNA targeted for detection.

The initiation of RNA transcription requires rGTP; the reaction has a K_m of ~180 µM. The elongation reaction has a K_m of 40 µM for each ribonucleotide. Therefore, radioactive rGTP should not be used to generate high specific-activity probes unless the concentration of rGTP exceeds 180 µM. This usually means supplementing the radioactive rGTP with cold rGTP. Adding 50 µCi of 500 Ci/mmol [^{32}P]rXTP to a 25-µl reaction only produces a rXTP concentration of 4 µM. To generate high specific-activity probes, we suggest using radioactive rATP, rCTP, or rUTP as the labeled nucleotide. However, any triphosphate present at just 4 µM will not produce many transcripts per template molecule because the reaction simply runs out of radioactive rXTP. To make large amounts of long, radioactive transcripts, the reactions must be supplemented with cold rXTP. It is therefore necessary to choose between full length, quantity and high-specific-activity when producing probes.

Transcription Reaction

Note *Stratagene's RNAMaxx high-yield transcription kit (Catalog #200339) may be used for transcription reactions performed with T7 RNA polymerase.*

1. In the order given, add
   5 µl of 5× transcription buffer§
   1 µg of restricted, proteinase K-treated DNA template
   1 µl of 10 mM rATP
   1 µl of 10 mM rCTP
   1 µl of 10 mM rGTP
   [1 µl of 1 mM rUTP is optional (see above)]
   1 µl of 0.75 M dithiothreitol (DTT)
   1 µl of RNase Block Ribonuclease Inhibitor (optional)
   5 µl of 400–800 Ci/mmol, 10 µCi/µl [α-^{32}P]rUTP
   10 U of T3 or T7 RNA polymerase*
   DEPC-treated water to a final volume of 25 µl

2. Incubate at 37°C for 30 minutes.

3. RNA transcripts may be purified away from the unincorporated nucleotides using Stratagene's NucTrap® probe purification column with a push-column beta-shield device.

Alternatively, an RNase-free G-50 column can be used. However, care must be taken that there are no ribonucleases present in the column that could degrade the probe.

Note *Do not use large excesses of T3 polymerase (10 U of polymerase/pmol of promoter is sufficient). T3 RNA polymerase may utilize the T7 promoter 1 in 20 times when the T3 enzyme concentration exceeds the T3 promoter concentration by 10-fold. However, T3 polymerase in the recommended concentrations will not make T7 transcripts in the presence of a T3 promoter. If any T7 hybridization should result from a T3 transcription, decrease the amount of T3 polymerase by a factor of 5 or 10.*

§ See *Preparation of Media and Reagents*.

* Use supplied RNA polymerase dilution buffer to dilute enzymes just before use.

HYBRIDIZATION CONDITIONS FOR RNA PROBES IN SOUTHERN BLOTS

Prehybridization

Prehybridize the membrane with 0.1–0.5 ml/cm^2 of the following solution for 2 hours at 42°C with constant agitation in a heat-sealable bag:

6× SSC
5× Denhardt's (see *Preparation of Media and Reagents*)
20 mM NaH_2PO_4
500 µg/ml of denatured, sonicated salmon sperm DNA

Hybridization

Pour off the prehybridization solution and add the probe to the bag with the minimum volume of the following hybridization solution:

6× SSC
20 mM NaH_2PO_4
0.4% SDS*
500 µg/ml denatured sonicated salmon sperm DNA

Incubate overnight at 42°C with constant agitation.

Washes

Wash in 2× SSC buffer and 0.1% (w/v) SDS twice for 15 minutes each at 55°C and twice in 0.1× SSC buffer and 0.1% (w/v) SDS for 15 minutes each at 55°C.

HYBRIDIZATION CONDITIONS FOR RNA PROBES IN NORTHERN BLOTS

Prehybridization

Prehybridize the membrane with 0.1–0.5 ml/cm^2 of the following solution for ~1 hour at 42°C with constant agitation in a heat-sealable bag:

50% deionized formamide
10% dextran sulfate
1% SDS*
1 M NaCl
100 µg/ml of denatured sonicated salmon sperm DNA

Hybridization

Hybridize overnight with the riboprobe at the same temperature and in the prehybridization solution.

Washes

Wash in 2× SSC buffer and 0.1% (w/v) SDS twice for 15 minutes each at 42°C and twice in 0.1× SSC buffer and 0.1% (w/v) SDS for 15 minutes each at 42°C. If a high background is observed, the temperature may be increased or the NaCl concentration may be decreased for greater stringency.

* For Stratagene's Duralon-UV™ and Illuminator™ membranes, increase the SDS concentration to 1% (w/v).

RECOVERY OF SINGLE-STRANDED DNA FROM CELLS CONTAINING pBLUESCRIPT II® PHAGEMIDS

pBluescript II is a phagemid that can be secreted as single-stranded DNA in the presence of M13 helper phage. These phagemids contain the intergenic (IG) region of a filamentous f1 phage. This region encodes all of the *cis*-acting functions of the phage required for packaging and replication. In *E. coli* with the F^+ phenotype (containing an F´ episome), pBluescript II phagemids will be secreted as single-stranded f1 "packaged" phage when the bacteria has been infected by a helper phage. Since these filamentous helper phages (M13, fI) will not infect *E. coli* without an F´ episome coding for pili, **it is essential to use XL1-Blue MRF´ or a similar strain containing the F´ episome.**[7,8]

Stratagene offers helper phages that *preferentially* package pBluescript II phagemids. Typically, 30–50 pBluescript II molecules are packaged/helper phage DNA molecule. pBluescript II phagemids are offered with the IG region in either of two orientations: pBluescript II (+) is replicated such that the sense strand of the β-galactosidase gene is secreted within the phage particles; pBluescript II (–) is replicated such that the antisense strand of the β-galactosidase gene is secreted in the phage particles.

Yields of single-stranded (ss)DNA depend on the specific insert sequence. For most inserts, over 1 µg of ssDNA can be obtained from a 1.5-ml miniprep if grown in XL1-Blue MRF´. A faint single-strand helper phage band may appear on a gel at ~4 kb for R408 or at 6 kb for VCSM13. This DNA mixture can be sequenced with primers that are specific for pBluescript II and do not hybridize to the helper phage genome.

Site-specific mutagenesis is also possible using standard techniques. The advantages of using pBluescript II phagemids for either purpose are as follows: (1) pBluescript II phagemids do not replicate via the M13 cycle, lessening the tendency to delete DNA inserts, therefore it is unlikely that even 10-kb inserts will be deleted. (2) "Packaging" of pBluescript II phagemids containing inserts is efficient since the pBluescript II vector is significantly smaller than wild-type M13. (3) Oligonucleotide mutagenesis in pBluescript II vectors is advantageous because the mutagenized insert is located between the T3 and T7 promoters. The resultant mutant transcripts can be synthesized *in vitro* without further subcloning.

VCSM13 and R408 helper phage produce the largest amount of single-strand pBluescript II. R408 (single-strand size ~4 kb) is more stable and can be grown more easily. VCSM13 (single-strand size ~6 kb), is more efficient at single-stranded DNA rescue and yields more single-stranded phagemid; however it is more unstable and reverts to wild-type more frequently. This difficulty can be addressed by periodically propagating VCSM13 in the presence of kanamycin. VCSM13 (a derivative of M13KO7) has a kanamycin gene inserted into the intergenic region, while R408 has a deletion in that region. We suggest R408 for excision of pBluescript II from the Lambda ZAP vector and VCSM13 for single-stranded rescue.

Single-Stranded Rescue Protocol

1. Inoculate a single colony into 5 ml of 2× YT containing 100 µg/ml ampicillin and VCM13 or R408 helper phage at 10^7–10^8 pfu/ml (MOI ~10).

2. Grow the culture at 37°C with vigorous aeration for 16–24 hours, or until growth has reached saturation.

   Note *If using VCSM13, after 1–2 hours, add kanamycin to 70 µg/ml to select for infected cells.*

3. Centrifuge 1.5 ml of the cell culture for 5 minutes in a microcentrifuge.

4. Remove 1 ml of the supernatant to a fresh tube, then add 150 µl of a solution containing 20% PEG8000 and 2.5 M NaCl. Allow phage particles to precipitate on ice for 15 minutes.

   Note *For increased yield, perform the PEG precipitation overnight at 4°C.*

5. Centrifuge for 5 minutes in a microcentrifuge. (A pellet should be obvious.)

6. Remove supernatant. Centrifuge the PEG pellets a few seconds more to collect residual liquid, then remove and discard the residual liquid.

7. Resuspend the pellet in 400 µl of 0.3 M NaOAc (pH 6.0) and 1 mM EDTA by vortexing vigorously.

8. Extract with 1 volume phenol–chloroform and centrifuge for 1–2 minutes to separate phases.

9. Transfer the aqueous phase to a fresh tube and add 1 ml of ethanol. Centrifuge for 5 minutes.

10. Remove ethanol and dry the DNA pellet.

11. Dissolve the pellet in 25 µl of TE buffer.

12. Analyze 1–2 µl on an agarose gel.

SITE-DIRECTED MUTAGENESIS

Isolated single-stranded DNA (see *Recovery of Single–Stranded DNA from Cells Containing pBluescript II Phagemids*) can be used for site-directed oligonucleotide mutagenesis.[9] The following protocol is recommended:

1. Phosphorylation of the oligonucleotide with polynucleotide kinase:
   100 ng of oligonucleotide
   4 µl of 10× ligase buffer[§]
   4 µl of 10 mM rATP
   2 µl of polynucleotide kinase (10 U)
   Water to 40 µl final volume
   Incubate at 37°C for 30 minutes.

2. Synthesis of mutant DNA strand

   a. Anneal Oligonucleotide

      20 µl of oligonucleotide from the kinase reaction (50 ng)
      5 µl of salmon sperm DNA (1 µg template)
      Incubate at 65°C for 10 minutes, then at room temperature for 5 minutes.

   b. Primer Extension Reaction

      Add the following to the annealing reaction:
      4.0 µl of 10× ligase buffer[§]
      2.0 µl of 2.5 mM dNTPs (N = A, C, G and T in equal concentration)
      4.0 µl of 10 mM rATP
      1.0 µg of single-stranded DNA binding protein
      1.5 U of Klenow
      0.5 µl of T4 DNA ligase (2 U)
      Water to 40 µl final volume
      Incubate at room temperature for 3–4 hours.

3. Transform XL1-Blue MRF´ *E. coli* with 10 µl of synthesis reaction and plate onto nitrocellulose filters across three plates.

4. Screen as described in *Screening Colonies.* One percent mutants should be obtained.

[§] See *Preparation of Media and Reagents*.

EXONUCLEASE III/MUNG BEAN NUCLEASE DELETIONS

Stratagene's Exo III/Mung Bean Nuclease Deletion Kit (Catalog #200330) has been optimized to produce unidirectional deletions of predictable sizes. The technique takes advantage of the properties of exonuclease III and pBluescript II phagemids. Exonuclease III will not digest 3´-single-stranded overhangs ≥4 bases, but will digest 3´ ends from blunt ends or 5´ overhangs.[4] The polylinker in the pBluescript II phagemids has unique restriction sites on the outside edges of the polylinker with 3´ overhangs and internal sites with 5´-overhangs or blunt ended cleavage products. To create deletions in the insert but not in the vector DNA, simply double-digest the clone with a 3´-overhang-producing restriction enzyme and 5´-overhang or blunt end-producing restriction enzyme, creating a substrate for unidirectional exonuclease digestion by exonuclease III. Afterward, mung bean nuclease is used to digest the single-stranded DNA ends to allow blunt-end ligation of the deletion products. Taking advantage of the convenient restriction sites and the predictable progression of exonuclease III, nested deletion construction can be accomplished very quickly.

Stratagene's Exo III/Mung Bean Nuclease Deletion Kit provides the following buffers:

2× Exo III buffer[§]
10× mung bean buffer[§]
1× mung bean dilution buffer[§]

The 2× Exo III buffer and 10× mung bean buffer are used for the exonuclease III and mung bean nuclease digestions, respectively. The 10× mung bean buffer is also used to terminate the exonuclease III digestion. The 1× mung bean dilution buffer is used to dilute the mung bean nuclease to the appropriate concentration for the reaction. A fresh dilution of mung bean nuclease is necessary, because dilute concentrations of mung bean nuclease are not stable.

It is necessary to start the digestions with highly supercoiled DNA (>85%). Exonuclease III can initiate digestion from nicks in the DNA, producing high background and making it more difficult to interpret results. Restriction enzymes with any nicking activity will contribute to these problems. Therefore, use the highest quality restriction enzymes available.

Protocol Outline

1. Clone the inserts into internal restriction sites of pBluescript II phagemid (*Eco*R I or *Pst* I are best).

2. Perform cesium chloride banding and purification of the dsDNA.

[§] See *Preparation of Media and Reagents*.

3. Double-digest the clones to **COMPLETION** at a unique restriction site producing 3´-overhangs and a unique restriction site producing 5´ overhangs or blunt ends that lies between the insert and the 3´-overhang site chosen. **Check for completion of the first digest on an agarose gel.** Make sure that the 5´-overhang or blunt end-producing restriction site, where deletions will be initiated by Exo III, is between the 3´-overhang-producing restriction site and the insert. Ensure that the 3´-overhang is ≥4 nucleotides in length; shorter 3´-overhangs are susceptible to cleavage by Exo III. The 3´-overhang-producing digest can be replaced with a 5´-overhang-producing digest if the overhang is filled in with deoxythio-derivatives by Klenow fragment to block the end from Exo III digestion.[10] When protecting with deoxythioderivatives, Stratagene recommends the following protocol. If a 3'-overhang was produced in a double-digest, proceed directly to step 4.

Thioderivative Fill-In (for Dual 5′-Overhangs)

a. Select two unique, 5'-overhang-producing restriction sites on the same side of the insert within the polylinker. Digest ~20–30 µg of DNA in a 500-µl reaction with the restriction enzyme whose site is to be protected (i.e. the site farthest from the insert). Do not digest the DNA at the second site, to be used for unidirectional deletions, until step h.

b. Heat the restriction digest to 75°C for 15 minutes.

c. Add 2 µl of a 1 mM stock of thio-dNTP mix and 5 U of Klenow fragment.

d. Incubate at room temperature for 10 minutes.

e. Extract with phenol–chloroform [1:1 (v/v)].

f. Ethanol precipitate the DNA.

g. Verify the success of the fill-in reaction by incubating 1 µg of filled-in DNA with 20 U of exonuclease III for 15 minutes at 37°C. Run the products on an agarose gel to check for protection against deletion.

h. Proceed with the second 5´-overhang-producing restriction digestion.

i. Extract with phenol–chloroform [1:1 (v/v)].

j. Ethanol precipitate the DNA.

4. Treat the double-digested DNA with exonuclease III (as described in *Exo III Deletion Series and Mung Bean Nuclease Digestion*) so that a portion of the insert is made single stranded.

5. Digest the ssDNA with mung bean nuclease to create blunt ends.

6. Ligate the ends to recircularize.

7. Transform the DNA into competent *E. coli* cells.

To obtain unidirectional deletions, it is important that the DNA is completely digested, phenol–chloroform extracted and ethanol precipitated (as described in *Exo III Deletion Series and Mung Bean Nuclease Digestion*, below). When selecting sites to use for digestion prior to exonuclease treatment, select restriction sites as far apart as possible to increase the likelihood of obtaining a complete double-digestion. Stratagene has observed that the **overhang from *Sac* II digestion does not protect against exonuclease III digestion.**

Keep mung bean nuclease concentrated until just before use; store the mung bean nuclease on ice for only short periods of time. Check restriction enzymes for nicking activity before use (see *Troubleshooting*).

Exo III Deletion Series and Mung Bean Nuclease Digestion

The length of DNA converted from double stranded to single stranded by exonuclease III can be controlled by the reaction temperature and time of incubation:

At 37°C, ~400 bp are converted per minute
At 34°C, ~375 bp are converted per minute
At 30°C, ~230 bp are converted per minute
At 23°C, ~125 bp are converted per minute

When using the exonuclease III/mung bean nuclease system, it is possible to produce nested deletions of varying lengths simultaneously by setting up a single reaction for exonuclease III and removing aliquots at varying time points. Each aliquot is then treated with mung bean nuclease and is ligated separately. The following protocol has been optimized to obtain multiple nested deletions:

1. Prepare a stop solution for each exonuclease III time point. Dilute 20 µl of 10× mung bean buffer into 155 µl of water in a microcentrifuge tube for each time interval desired. Use this diluted mung bean buffer to terminate the exonuclease III deletions at the desired time points.

2. Start the reaction by adding 20 U of exonuclease III for each picomole of susceptible 3´ ends of DNA. Incubate reaction at the desired temperature (see the guidelines for conversion at different temperatures above) and remove 25-µl aliquots from the reaction mixture at the appropriate time intervals. Add the 25-µl aliquot directly to the tubes containing the 175-µl aliquots of diluted mung bean nuclease buffer prepared in step 1 above and place the tubes on dry ice.

The exonuclease reactions for all time points are started in a single tube, and aliquots are removed at each time point. For **each** time point, the reaction contains the following components:

5.0 µg of double-digested DNA (1 µg/µl)
12.5 µl of 2× Exo III buffer
2.5 µl of fresh 100 mM β-mercaptoethanol
X µl of exonuclease III (20 U/pmol end)
Water to 25 µl (total reaction volume per time point)

Multiply each component by the total number of time points to be taken. An example for an exonuclease III/mung bean nuclease deletion with five time points is as follows:

5.0 µl × 5 = 25 µl of double-digested DNA (1 µg/µl)
12.5 µl × 5 = 62.5 µl of 2× Exo III buffer
2.5 µl × 5 = 12.5 µl of fresh 100 mM β-mercaptoethanol
100.0 U × 5 = 500.0 U of exonuclease III
Water to 125 µl (total reaction volume for 5 time points)

3. When all aliquots have been removed, heat the tubes at 68°C for 15 minutes and then place the tubes on ice.

4. Dilute mung bean nuclease to 15 U/µl in 1× mung bean dilution buffer. Add 1 µl to each time point tube and incubate for 30 minutes at 30°C.

Optional *Steps 5–11 below are optional and are performed to completely remove residual mung bean nuclease from the DNA.*

5. Add the following components:
   10 µl of 1 M Tris-HCl (pH 9.5)
   20 µl of 8 M LiCl
   4 µl of 20% (w/v) SDS
   250 µl of buffer-equilibrated phenol–chloroform

6. Vortex and then spin for 1 minute in a microcentrifuge. Transfer the upper aqueous layer to a fresh tube and extract the upper layer with chloroform.

7. Add 25 µl of 3 M sodium acetate at pH 7.0 to the aqueous phase. Transfer RNA (tRNA) may be added to a final concentration of 10 ng/µl as a carrier for the precipitation.

8. Add 650 µl of cold ethanol. Chill on dry ice for 10 minutes and spin in a microcentrifuge for 20 minutes.

9. Drain off the supernatants and wash the pellets with 80% (v/v) ethanol.

10. Dry the pellet.

11. Redissolve the DNA pellet in 15 µl of TE buffer.

Ligation

12. Ligate the DNA deletions by adding the following:

1.0 µl (~3 µg) of exonuclease III/mung bean nuclease-treated DNA
2.0 µl of 10× ligase buffer
1.0 µl of 10 mM rATP (pH 7.0–7.5)
0.5 µl of T4 DNA ligase (2 U)
15.5 µl of water
20.0 µl total volume

Incubate at room temperature for 4 hours or at 4°C overnight.

13. Use 7 of the remaining 14 µl (20 of 200 µl if steps 5–11 were omitted) of the exonuclease/mung bean nuclease-treated DNA for gel electrophoresis analysis. The deletions can only be visualized after treatment with mung bean nuclease. Before treatment, there will be only a slight difference in mobility between the exonuclease-digested DNA and the full-length, linearized DNA.

14. Use 1 µl of the ligation reaction to transform 100 µl of *E. coli* competent cells (such as Stratagene's XL1-Blue MRF´ competent cells) and plate the cells on LB–ampicillin plates (100 µg/ml ampicillin).

Low-Melting-Temperature Agarose Enrichment Technique

To minimize screening of the deletions, run a portion of the deletion in low-melting-temperature (LMT) agarose, excise the band of interest and proceed with the ligation. Stratagene recommends keeping the agarose level below 0.5% in the ligation reaction.

1. Perform steps 1–4 from *Deletions*.

2. Add 10 µl of 3 M sodium acetate at pH 5.2 and 0.5 ml of cold ethanol. Chill on ice for 10 minutes and spin in a microcentrifuge for 20 minutes.

3. Dry the pellet.

4. Redissolve the DNA pellet in 15 µl of TE buffer.

5. Load 7 µl in a 1% low-melting-point agarose gel and separate by gel electrophoresis.

6. Excise deletion band. Heat agarose to 68°C for 30 minutes, then use 10 ng for ligation.

7. Ligate DNA deletions using the following conditions:
   1.0 µl of Exo/Mung-treated DNA
   2.0 µl of 10× ligase buffer[§]
   1.0 µl of 10 mM rATP (pH 7.0–7.5)
   0.5 µl of T4 DNA ligase (4 U/µl)
   15.5 µl of water
   (20.0 µl total reaction volume)
   Incubate at room temperature for 4 hours or 4°C overnight.

8. Use 1 µl of the ligation reaction to transform 100 µl of competent *E. coli* (e.g. Stratagene's XL1-Blue MRF´ competent cells) and plate on LB-ampicillin plates.

Quick Screen of the Transformants

1. Isolate three to four colonies from each time interval with sterile toothpicks and streak each as a single line onto LB–ampicillin plates (~12 streaks/plate).

2. Grow overnight at 37°C.

3. Scrape bacteria with sterile toothpick and resuspend in 40 µl of 1× STE buffer.[§]

4. Add 40 µl of phenol–chloroform and vortex.

5. Microcentrifuge for 1 minute.

6. Transfer supernatant to a microcentrifuge tube and add 1 µl of RNase A (1 mg/ml).

7. Incubate at room temperature for 2 minutes. Add loading buffer. Load 20 µl onto a 1% agarose gel and separate by gel electrophoresis to compare supercoiled Exo/Mung-deleted plasmids. *

[§] See *Preparation of Media and Reagents*.

* **WARNING: Samples cannot be restriction digested with this technique. Care must be taken when loading the gel since high sample viscosity may make it difficult to keep the sample in the wells. Make sure the wells are deeply immersed in running buffer while loading. If restriction digestion is desired, see *Plasmid Boiling Miniprep Protocol*.**

PLASMID BOILING MINIPREP PROTOCOL

The following protocol yields high-quality dsDNA template simply and rapidly. (Caution: *Escherichia coli* strain HB101 and derivatives give low yields using this protocol.) This DNA is suitable for restriction enzyme digestion or for enzyme sequencing.[11]

1. Grow a 3-ml culture overnight in LB broth plus ampicillin (100 µg/ml) from a single colony.

2. Pellet 1.5 ml of the culture in a microcentrifuge at 4°C for 2 minutes. Remove the supernatant by aspiration.

3. Resuspend the pellet in 110 µl of STETL buffer (see *Preparation of Media and Reagents*).

4. Place the tube in a boiling water bath for 30 seconds.

5. Immediately spin the tube in a microcentrifug for 15 minutes at room temperature.

6. Remove and discard the pellet with a sterile toothpick. Save the supernatant. [RNase treatment (20 µg/ml) is optional at this stage.)]

7. Add 110 µl of isopropanol to the supernatant and immediately spin the tube in a microcentrifuge for 15 minutes.

8. Resuspend the pellet in 100 µl of TE buffer.

9. Extract twice with an equal volume of phenol–chloroform [1:1 (v/v)] and once with chloroform.

   Note *To purify the sample, StrataClean™ resin may be used in place of the phenol–chloroform extraction.*

10. Add an equal volume of 7.5 M ammonium acetate and precipitate with 2.5 volumes of ethanol. Incubate on ice 15 minutes and spin at 4°C for 20 minutes.

11. Rinse with 1 ml of 80% (v/v) ethanol and spin in a microcentrifuge for 1 minute.

12. Vacuum dry the pellets.

13. Resuspend the pellets in 15 µl of TE buffer.

14. Use 5 µl of this DNA (about 2.0 µg) for sequencing.

IMPROVED SEQUENCING PRIMERS AND PCR PRIMER SETS

The traditional primers designed for the pBluescript phagemid vector and its derivatives were used primarily for primer extension reactions at 37°C or less. The advent of PCR and cycle sequencing requires that these primers bind efficiently at higher temperatures. Stratagene has redesigned these primers for exceptional performance in high-temperature primer extension reactions. The new primers maintain nearly the same template positions, but now have higher melting temperatures.

Improved Sequencing Primers

Primer	Sequence	Catalog #
T3	5′ AATTAACCCTCACTAAAGGG 3′	300301
T7	5′ GTAATACGACTCACTATAGGGC 3′	300302
M13 (–20)	5′ GTAAAACGACGGCCAGT 3′	300303
M13 reverse	5′ GGAAACAGCTATGACCATG 3′	300304
SK	5′ CGCTCTAGAACTAGTGGATC 3′	300305
KS	5′ TCGAGGTCGACGGTATC 3′	300306

Improved PCR Primer Sets

Primer set	Catalog #
T3/T7	302001
M13 (–20)/Reverse	302003
SK/KS	302005

TROUBLESHOOTING

Restriction Digests of pBluescript II Phagemids

Observation	Suggestion
Digestion with *Eco*R I produces multiple bands	Using excess amounts of *Eco*R I to digest pBluescript II vectors results in *Eco*R I prime activity. This appears as cleavage at a non-*Eco*R I site at the 3´ end of the f1 intergenic region, causing confusion when interpreting results from an agarose gel. Test whether reducing the units of *Eco*R I restores a normal restriction pattern
Nae I fails to cleave the pBluescript II vector	Stratagene has observed that the *Nae* I site in the pBluescript II phagemids presents a more challenging substrate for digestion than the sites in pBR322. Use 16U *Nae* I enzyme per μg DNA and increase the digestion period (overight digestion may be necessary). Even under these more stringent conditions, *Nae* I may not produce complete cleavage.

Exo/Mung Deletion Protocol

Observation	Suggestion
Gel electrophoresis analysis reveals the same size band before and after deletion. The band is the same molecular weight as the linearized starting plasmid vector. A large number of colonies are obtained after ligation and transformation	Incomplete digestion with restriction endonuclease that leaves a 5´ overhang. Increase the units of the appropriate restriction enzyme.
Transformants are obtained only for short fragments	Incomplete digestion with restriction endonuclease that leaves a 3´ overhang. Increase the units of the appropriate enzyme.
Deletions are observed on gel electrophoresis, but they are less extensive than expected. Few transformants are obtained after ligation of deleted DNA	Mung bean nuclease digestion did not go to completion. Increase the units of mung bean nuclease to remove all ssDNA.
Gel electrophoresis analysis reveals a smear on the gel instead of discrete deletion bands	Exonuclease III can delete from a 5´ overhang, a blunt end, or any nick in the plasmid. a. The initial plasmid should be greater than 85% supercoiled. b. The restriction enzymes should be checked for nonspecific nicking activity by incubating a supercoiled plasmid that does not contain the restriction site with the restriction enzyme and checking for change in mobility on a 1% agarose gel. Change in mobility indicates nicking activity.

PREPARATION OF MEDIA AND REAGENTS

5× Transcription Buffer
200 mM Tris, pH 8.0
40 mM $MgCl_2$
10 mM spermidine
250 mM NaCl

10× Ligase Buffer
500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

Note *rATP is added separately in the ligation reaction.*

2× Exo III Buffer
100 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$

10× Mung Bean Buffer
300 mM NaOAc (pH 5.0)
500 mM NaCl
10 mM $ZnCl_2$
50% (v/v) glycerol

1× Mung Bean Dilution Buffer
10 mM NaOAc (pH 5.0)
0.1 mM ZnOAc
1 mM cysteine
0.01% (v/v) Triton® X-100
50% (v/v) glycerol

20× SSC
175.3 g of NaCl
88.2 g of sodium citrate
800.0 ml of water
10.0 N NaOH
Adjust to pH 7.0 with a few drops of 10.0 N NaOH
Adjust volume to 1 liter with water

M9 Minimal Medium (per Liter)
750 ml of sterile deionized water (cooled to 50°C)
200 ml of 5× M9 salts
Sterile deionized water to 1 liter
20 ml of a 20% solution of the appropriate carbon source (e.g., 20% glucose)

50× Denhardt's Reagent (per 500 ml)
5 g of Ficoll
5 g of polyvinylpyrrolidone
5 g of BSA (Fraction V)
Add deionized H_2O to a final volume of 500 ml
Filter through a disposable filter
Dispense into aliquots and store at –20°C

LB Broth (per Liter)
10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.0 with 5 N NaOH
Autoclave

STETL Buffer
8.0% sucrose
0.5% Triton X-100
50.0 mM Tris (pH 8.0)
50.0 mM EDTA
0.5 mg/ml lysozyme
All components except lysozyme can be prepared and stored indefinitely at 4°C. The lysozyme is made as a 5 mg/ml stock and stored in small aliquots at –20°C. Do not reuse the lysozyme stock after thawing.

1× STE Buffer
100 mM NaCl
20 mM Tris-HCl (pH 7.5)
10 mM EDTA

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ENDNOTES

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on Stratagene's website at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.