pBC Phagemids

Instruction Manual

Catalog #212215, #212218

Revision C.0

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212215-12

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pBC Phagemids

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pBC Phagemids

MATERIALS PROVIDED

Materials Provided	Amount			
	Catalog # 212215	**Catalog # 212216**	**Catalog # 212217**	**Catalog # 212218**
pBC SK+ phagemid	20 µg			
pBC SK– phagemid		20 µg		
pBC KS+ phagemid			20 µg	
pBC KS– phagemid				20 µg
XL1-Blue MRF′ (1-ml glycerol stock)	1 tube	1 tube	1 tube	1 tube

STORAGE CONDITIONS

pBC Phagemids: –20°C
Bacterial Glycerol Stocks: –80°C

Revision C.0

INTRODUCTION

The pBC vectors were derived from the pBluescript II phagemid. The ampicillin-resistance gene has been replaced with the chloramphenicol-resistance gene. pBC 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 pBC phagemids have an extensive polylinker with 21 unique restriction enzyme recognition sites. Flanking the polylinkers are T7 and T3 RNA polymerase promotors that can be used to synthesize RNA *in vitro* (see Figures 1 and 2). The choice of promotor used to initiate transcription determines which strand of the insert cloned into the polylinker will be transcribed.

The polylinker and T7 and T3 RNA polymerase promotor sequences are present in the N-terminal portion of a *lac*Z gene fragment. There are 36 amino acids from the MET sequence to the *Eco*R I site in the pBC plasmid. There are a total of 131 amino acids, but this is interrupted by the large polylinker. pBC phagemids having no inserts in the polylinker will grow as blue colonies on the appropriate strains of bacteria (i.e., strains containing the *lacZΔM15* on an F´ episome, XL1-Blue MRF´, among others). pBC phagemids which have inserts will grow as white colonies on the same strain, because the inserts disrupt the coding region of the *lac*Z gene fragment.

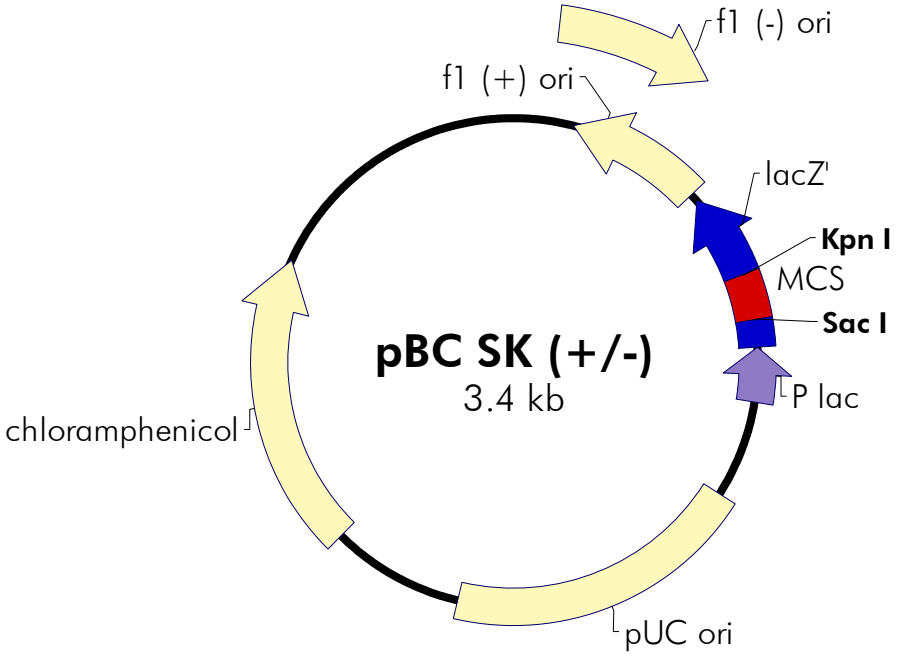
pBC (+) and (–) are available with two polylinker orientations designated as either KS or SK using the following convention: (1) in the KS orientation, the *Kpn* I restriction site is nearest the *lac*Z promotor and the *Sac* I restriction site is farthest from the *lac*Z promotor; and (2) in the SK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

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

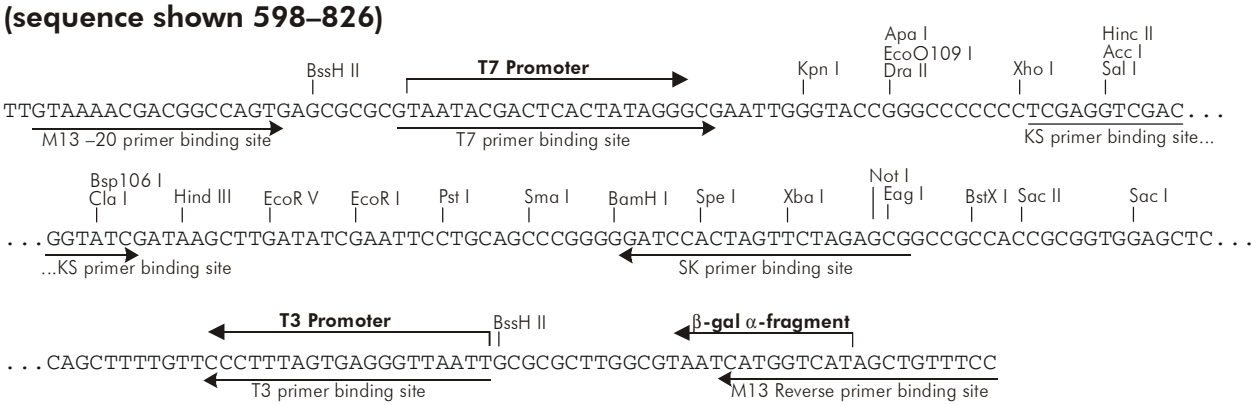
pBC phagemids can be rescued as single-stranded (ss) DNA. pBC phagemids contain a 454-nucleotide filamentous f1 phage intergenic region (M13 related). The (+) and (–) orientations of the f1 intergenic region allow the rescue of sense or antisense ssDNA by a helper phage. This ssDNA can be used for dideoxynucleotide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

Note *We have discovered that the use of excess amounts of EcoR I to digest pBC results in EcoR I prime activity. This appears to cut 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, try reducing the units of EcoR I and check whether a normal restriction pattern is restored.*

The pBC SK Vectors



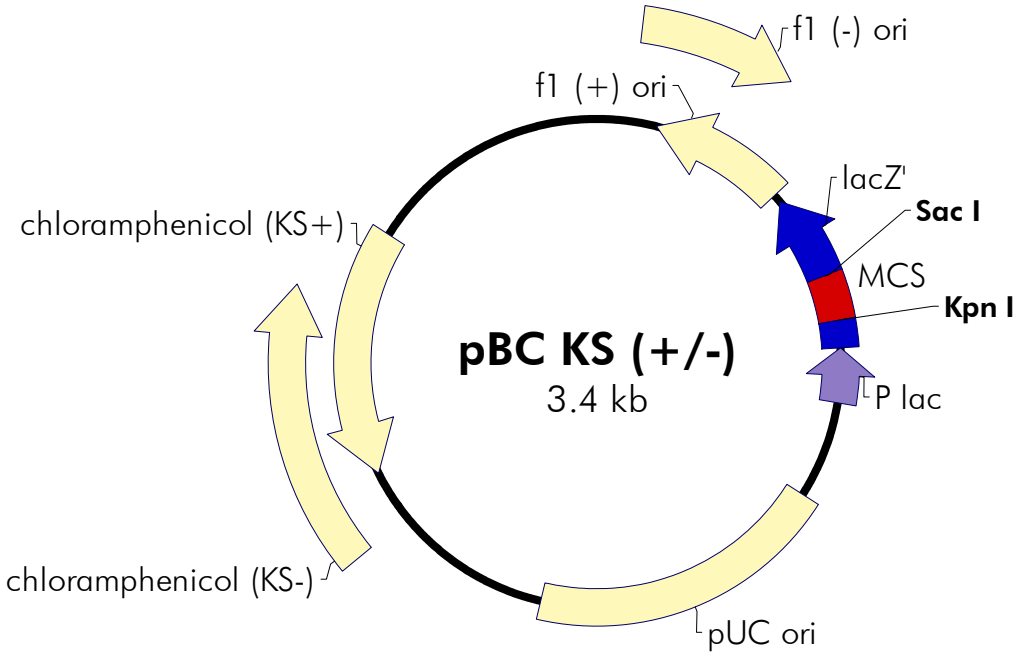
pBC SK (+/–) Multiple Cloning Site Region (sequence shown 598–826)



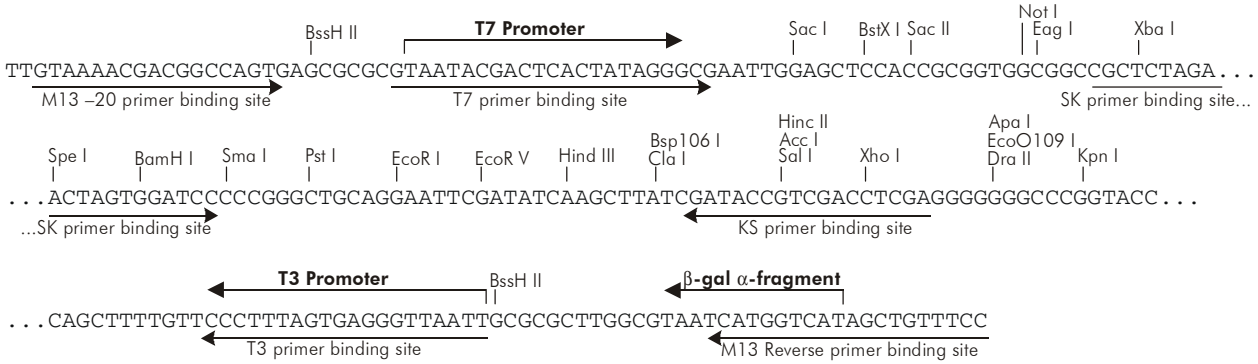
Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication [pBC SK (+) only]	135–441
f1 (–) origin of ss-DNA replication [pBC SK (–) only]	21–327
β-galactosidase α-fragment coding sequence (lacZ′)	460–816
T7 promoter transcription initiation site	643
multiple cloning site	653–760
T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
chloramphenicol resistance ORF	2125–2781

Figure 1 The pBC SK (+/–) phagemid vectors. The complete sequence and list of restriction sites are available at *www.genomics.agilent.com*.

The pBC KS Vectors



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



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T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
chloramphenicol resistance ORF [pBC KS (+) only]	2194–2850
chloramphenicol resistance ORF [pBC KS (–) only]	2125–2781

Figure 2 The pBC KS (+/–) phagemid vectors. The complete sequence and list of restriction sites are available at *www.genomics.agilent.com*.

LIGATION INTO pBC PHAGEMIDS

We suggest dephosphorylation of the digested pBC 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 purification and ethanol precipitation of the DNA, resuspend in a volume of TE buffer [5 mM Tris (pH 7.5) and 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:

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

We suggest the following protocol which includes three controls:

	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 Weiss 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 using blunt ends, ligate overnight at 12–14°C.

2. Transform 1–2 µl of the ligation mix into the appropriate competent bacteria. Plate on selective media.

Interpretation of test results:

Controls 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.

Expected test 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 pBC PHAGEMIDS

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

Suggested Host Strain and Genotype

The XL1-Blue MRF´ host strain[1] is recommended for propagation of pC 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.[2]

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 are available for purchase (Catalog #200301). Alternatively, high-efficiency XL1-Blue MRF´ frozen competent cells are also available (>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 tetracycline.

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 pBC phagemids because of *lacZΔM15* complementation on the F´ episome. The color selection may be seen when plating on LB plates containing 30 µg/ml of chloramphenicol, 80 µg/ml of fresh X-gal, and 20 mM IPTG. Alternatively, plates for color selection can be made by spreading 100 µl of 40 mM IPTG and 100 µl of 2% X-gal on LB–chloramphenicol plates 30 minutes prior to plating your transformants. Prepare X-gal in dimethyl formamide and prepare 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 chloramphenicol 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 *ΔM15 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 pBC phagemid does not contain an insert. XL1-Blue MRF´ is a *lac*$^-$ AG1 derivative with Tn*10*, *lacI*^{q}, and *lacZΔM15* 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 pBC phagemids can be plated on tetracycline–chloramphenicol plates to select for colonies that contain both the F´ and the pBC 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 pBC recombinant or a colony lacking the F´, streak it onto a minimal medium plate.[3] 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 pBC phagemids may be screened for recombinants by double-stranded DNA, RNA, or oligonucleotide hybridization.[4] Colonies may also be screened by restriction mapping or by sequencing mini-prep plasmid DNA. Antibodies may be used to screen colonies since cDNA cloned into the appropriate reading frame of the *lac*Z gene will express proteins by the *lac*Z promotor.[5]

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 *EcoR* I site). Some fusion proteins are toxic to *E. coli*. Therefore, it is best to initially plate transformants on nitrocellulose filters on top of chloramphenicol 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.[5] When screening with antibodies, the Agilent *pico*Blue immunoscreening kit (Catalog #200371, 200372) 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 pBC 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 nitrocellulose membranes on 150-mm LB–chloramphenicol (30 µg/ml) agar plates (see *Preparation of Media and Reagents*).

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-chloramphenicol agar plates.

    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 Whatmann 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 Whatmann 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 buffer[§]
20 mM NaH_2PO_4
0.4% (w/v) sodium dodecyl sulfate[‡] (SDS)
5× Denhardt's reagent[§]
Denatured, sonicated salmon sperm DNA (500 µg/ml) (Catalog #201190, 10 mg/ml)

or

Prehybridization Solution for Double-Stranded Probe

2× Pipes buffer[§]
50% Deionized formamide
0.5% (w/v) sodium dodecyl sulfate[‡] (SDS)
Denatured, sonicated salmon sperm DNA (100 µg/ml) (Catalog #201190, 10 mg/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-sealable 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.

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

Hybridization

Labeling Oligonucleotide Probes

Label oligonucleotides with fresh [γ-^{32}P]ATP. High-specific-activity γ 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 to remove the unincorporated [γ-^{32}P]ATP.

Labeling Double-Stranded Probes

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

Alternatively, we offer the Prime-It II random primer kit (Catalog #300385, 300392, 600069,) 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 buffer
20 mM NaH_2PO_4
0.4% (w/v) SDS‡
Denatured, sonicated salmon sperm DNA (500 µg/ml) (Catalog #201190, 10 mg/ml)

1. Prepare 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 hybridization bag. Add the hybridization solution and then the appropriate amount of labeled oligonucleotide.

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

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

The second formula works only for Na^+ concentrations of ≤1 M.

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)

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

Hybridization Solution for Double-Stranded Probes

2× PIPES buffer
50% Deionized formamide
0.5% (w/v) SDS
Denatured, sonicated salmon sperm DNA (100 µg/ml) (Catalog #201190, 10 mg/ml)

1. Prepare the hybridization solution.

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

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

Washes

Oligonucleotide Probes

Use a 6× SSC buffer and 0.1% (w/v) SDS wash solution. Wash the membranes 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 using the T_m equations in *Hybridization Solution*.

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 a 0.1× SSC buffer and 0.1% (w/v) SDS wash solution. Wash the membranes at 50–65°C with agitation.

Exposure to Film

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

TRANSCRIPTION BY T3 AND T7 RNA POLYMERASE

The RNA transcripts synthesized from inserts cloned into the pBC vectors and transcribed from 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.

The pBC 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 kinased 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 triphosphates 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.*

   The Agilent 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 RNaseA. 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 mini-preps 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 promotor 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 (Catalog #300140, 300141) to the restriction buffer at 37°C for 30 minutes, followed by two phenol:chloroform (1:1) extractions and ethanol precipitation prior to the transcription reaction. Resuspend digested, proteinase K treated DNA at 1 mg/ml in 10 mM Tris (pH 7.4) and 0.1 mM EDTA made with DEPC-treated water.

4. Use a ribonuclease inhibitor in transcription reactions. The Agilent RNase Block Ribonuclease Inhibitor has been tested and adjusted to work optimally with Agilent's transcription kits.

Nonspecific Initiation with T7 and T3 RNA Polymerases

T7 and T3 RNA polymerases are highly specific for their respective promotors, 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 which leave blunt or 5´ protruding ends.

The T7 or T3 polymerase enzymes are used in molar excess of the DNA template, there is a risk of polymerization from the wrong promotor. T7 polymerase can synthesize RNA inefficiently from a plasmid containing only a T3 promotor. Conversely, T3 polymerase can synthesize RNA inefficiently from a plasmid containing only a T7 promotor. Synthesis is extremely promotor specific when both promotors are present, provided that the enzyme is not in molar excess of the specific promotor. Do not use excessive amounts of the polymerases if promotor 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 (Catalog #600031). 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), add 1/10th of volume 3 M sodium acetate at pH 5.2 and precipitate RNA with 2.5 volumes of 100% ethanol.

High-Specific-Activity RNA Probes

The pBC phagemid vectors can be used to synthesize high-specific-activity, strand-specific RNA probes. The choice between T3 and T7 RNA polymerase promoters will determine which strand will be used as the template.

Note *When choosing between T3 and T7 polymerase, it is important to remember that probes used for Northern or S1 analysis must complement the RNA targeted for detection.*

To generate high-specific-activity probes, we suggest using radioactive rATP, rCTP or rUTP as the labeled nucleotide. rGTP should not typically be selected for labeling, since the initiation of RNA transcription requires rGTP, with a K_m of ~180 µM. Thus the concentration of rGTP in the labeling reaction should exceed 180 µM, requiring supplementation of the radioactive rGTP with a high concentration of nonradioactive rGTP, reducing the specific activity of the probe.

Transcription reaction conditions for probe synthesis are provided below. Ribonucleotide composition in this reaction may be adjusted according to the relative importance of achieving full-length and high-quantity of transcript versus high-specific-activity when producing probes. The elongation reaction has a K_m of 40 µM for each ribonucleotide. Adding 50 µCi of 500 Ci/mmol [^{32}P]rXTP to a 25-µl reaction produces an rXTP concentration of 4 µM. 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, supplement the reactions with nonradioactive rXTP.

Transcription Reaction

1. In this order add

   5 µl of 5× transcription buffer (see *Preparation of Media and Reagents*)
   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 I (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 an RNase-free G-50 column.Take care that there are no ribonucleases present in the column which could degrade the probe.

Note *Do not use a large excess of T3 polymerase (10 U per 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. (T3 polymerase in the recommended concentrations will not make T7 transcripts in the presence of a T3 promoter.) If T7 transcripts result from a T3 transcription, decrease the amount of T3 polymerase by 5–10 fold.*

† Use RNA polymerase dilution buffer supplied by the manufacturer to dilute enzyme 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 buffer
5× Denhardt's
20 mM NaH_2PO_4
500 µg/ml of denatured, sonicated salmon sperm DNA (Catalog #201190, 10 mg/ml)

Hybridization

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

6× SSC buffer
20 mM NaH_2PO_4
0.4% (w/v) SDS‡
500 µg/ml denatured sonicated salmon sperm DNA (Catalog #201190, 10 mg/ml)

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% (w/v) SDS‡
1 M NaCl
100 µg/ml of denatured sonicated salmon sperm DNA (Catalog #201190, 10 mg/ml)

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.

RECOVERY OF SINGLE-STRANDED DNA FROM CELLS CONTAINING pBC PHAGEMIDS

pBC is a phagemid[6] which 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), pBC 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.**

pBC phagemids are offered with the IG region in either of two orientations: pBC (+) is replicated so the coding strand of the β–galactosidase gene (the top strand in the enclosed map, the same strand as in the mp vectors) is secreted within the phage particles; pBC (–) is replicated so the noncoding strand of the β-galactosidase gene (the bottom strand in the enclosed map) is secreted in the phage particles.

We offer helper phages that *preferentially* package pBC phagemids. Typically, 30–50 pBC molecules are packaged/helper phage DNA molecule. VCSM13 and R408 (Catalog #200251 and #200252, repectively) helper phage produce the largest amount of single-strand pBC. R408 (single-strand size ~4 kb) is more stable and can be grown more easily. VCSM13 (single-strand size ~6 kb), being more efficient, 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.

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 6 kb for VCSM13. This DNA mixture can be sequenced with primers that are specific for pBC and do not hybridize to the helper phage genome.

Site-specific mutagenesis is also possible using standard techniques. The advantages of using pBC phagemids for either purpose are as follows: (1) pBC 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 pBC phagemids containing inserts is efficient since the pBC vector is 3.5 kb (smaller than wild-type M13). (3) Oligonucleotide mutagenesis in pBC vectors is advantageous because the mutagenized insert is located between the T3 and T7 promotors. The resultant mutant transcripts can be synthesized *in vitro* without further subcloning.

Single-Stranded Rescue Protocol

1. Inoculate a single colony into 5 ml of 2× YT broth containing 30 µg/ml chloramphenicol 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 1–2 hours.

3. Add kanamycin to 70 µg/ml (selection for infected cells) only if using VCSM13.

4. Continue to grow at 37°C for 16–24 hours, or until growth has reached saturation.

5. Centrifuge 1.5 ml of cells for 5 minutes in a microfuge.

6. Remove 1 ml of supernatant and add 150 µl of 20% PEG and 2.5 M NaCl. Allow the phage particles to precipitate on ice for 15 minutes.

7. Centrifuge for 5 minutes in a microfuge (a pellet should be obvious).

8. Remove supernatant. Centrifuge the PEG pellets a few seconds more to collect residual liquid and remove.

9. Resuspend the pellet in 400 µl of 0.3 M NaOAc at pH 6.0/1 mM EDTA by vortexing vigorously.

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

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

12. Remove ethanol and dry the DNA pellet.

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

14. Analyze 1–2 µl on an agarose gel.

SITE-DIRECTED MUTAGENESIS

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

1. Kinase oligonucleotide—100 ng of oligonucleotide in a 40-µl reaction

   100 ng of oligonucleotide
   4 µl of 10× ligase buffer (see *Preparation of Media and Reagents*)
   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) (Catalog #201190, 10 mg/ml)

      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 µl of 10× ligase buffer
      2 µl of 2.5 mM dNTPs (N = A, C, G and T in equal concentration)
      4 µl of 10 mM rATP
      1 µg of single-stranded DNA binding protein (Catalog #600201)
      1.5 U of Klenow
      4 Weiss U of T4 DNA ligase (Catalog #600011)
      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 of mutants should be obtained.

APPENDIX: PLASMID BOILING MINIPREP PROTOCOL

The following protocol[7] 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.

1. Grow a 3-ml culture overnight in LB-chloramphenicol broth (30 µg/ml) (see *Preparation of Media and Reagents*) 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 microcentrifuge at 4°C 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.

Note *To purify the sample, StrataClean resin (Catalog #400714) may be used in place of the phenol–chloroform extraction.*

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

10. Add an equal volume of 7.5 M ammonium acetate and precipitate with 2.5 volumes of ethanol. Incubate on ice for 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.

PREPARATION OF MEDIA AND REAGENTS

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

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

20× SSC Buffer (per Liter)

175.3 g of NaCl
88.2 g of sodium citrate
800.0 ml of deionized H_2O
Adjust to pH 7.0 with a few drops of 10 N NaOH
Add deionized H_2O to a final volume of 1 liter

10× PIPES Buffer

4.0 M NaCl
0.1 M PIPES buffer (pH 6.5)

5× Transcription Buffer

200 mM Tris-HCl (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.*

LB–Chloramphenicol Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 3 ml of 10-mg/ml filter-sterilized chloramphenicol
Pour into petri dishes (~25 ml/100-mm plate)

LB–Chloramphenicol Broth (per Liter)

Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 3 ml of 10-mg/ml filter-sterilized chloramphenicol

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

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.

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ENDNOTES

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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.