SuperCos 1 Cosmid Vector Kit

Instruction Manual

Catalog #251301

Revision C.0

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SuperCos 1 Cosmid Vector Kit

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MATERIALS PROVIDED

Materials provided	Quantity
SuperCos 1 cosmid DNA[a] (1 μg/μl)	25 μg in 10 mM Tris-HCl and 1 mM EDTA
T3 polymerase	1000 U
T7 polymerase	1000 U
RNA polymerase dilution buffer	500 μl
5× transcription buffer	1 ml
XL1-Blue MR[b]	1 vial

[a] On arrival store at –20°C. After thawing, aliquot and store at –20°C. Do not pass through more than two freeze–thaw cycles. For short-term storage, store at 4°C for 1 month.

[b] Genotype of XL1-Blue MR is *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*

STORAGE CONDITIONS

Cosmid vector: –20°C
T7 and T3 polymerase: –20°C
RNA polymerase dilution buffer: –20°C
5× transcription buffer: –20°C
XL1-Blue MR Bacterial Glycerol Stock: –80°C (see *Preparation of Host Cells*)

Revision C.0

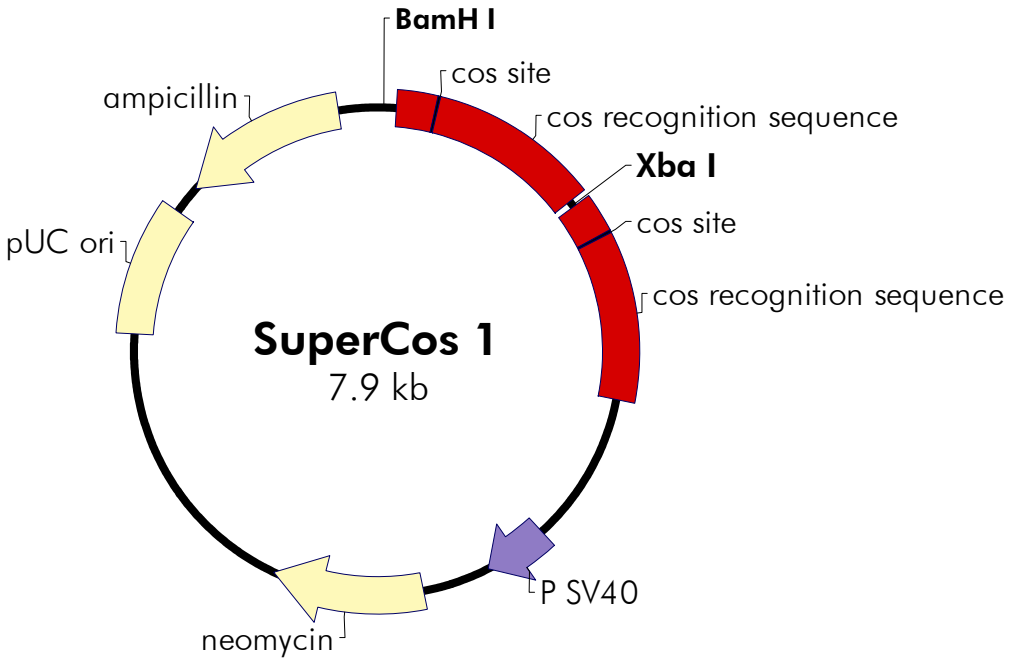
INTRODUCTION

The structural and functional analysis of complex genomes often requires the isolation and physical mapping of large regions of DNA. Cosmid vectors are valuable tools in this analysis, because they can accommodate genomic DNA fragments ranging in size from 30 to 42 kb. A procedure known as "walking" allows the isolation of sequentially overlapping cosmid clones as well as the physical linkage and extensive characterization of genes.[1,2,3,4]

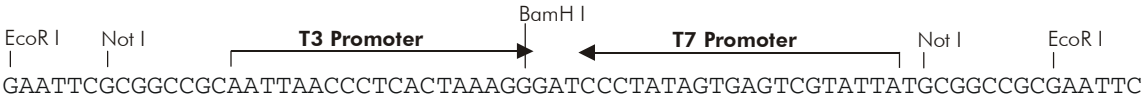
SuperCos 1 is a novel, 7.9-kb cosmid vector that contains bacteriophage promoter sequences flanking a unique cloning site (see Figure 1). This structure allows rapid synthesis of "walking" probes specific for the extreme ends of insert DNA. The SuperCos 1 vector is also engineered to contain genes for the amplification and expression of cosmid clones in eukaryotic cells. In addition, most genomic inserts can be excised as a single large restriction fragment using the *Not* I restriction site that flanks the SuperCos 1 polylinker.

Note *The Agilent T7 22-mer primer (Catalog #300302) is not suitable for use with the SuperCos 1 vector. The primer is designed specifically for the T7 site present in the pBluescript vectors.*

The SuperCos 1 Vector



SuperCos 1 Cloning Site Region
(sequence shown 1–71)



Note The *neo*^{r} gene confers resistance to kanamycin in *E. coli* and to G418 in eukaryotic cells.

Feature	Nucleotide Position
T3 promoter	15–34
BamH I cloning site	33
T7 promoter	37–56
cos recognition sequence	101–1150
cos site	300–311
Xba I restriction site	1173
cos recognition sequence	1183–2237
cos site	1382–1393
SV40 promoter	3037–3375
neomycin resistance ORF	3732–4526
pUC origin of replication	6049–6727
ampicillin resistance (*bla*) ORF	6878–7735

Figure 1 Circular map and features of the SuperCos 1 cosmid vector. The complete sequence and list of restriction sites are available at *www.genomics.agilent.com*.

PREPARATION OF HOST CELLS

Note *The host strains may thaw during shipment. Store the vials immediately at –20° or –80°C (most strains remain viable longer if stored at –80°C). Avoid repeated freeze-thaw cycles of the host strains in order to maintain extended viability.*

The host strain is provided as a bacterial glycerol stock. Refer to the following table for the appropriate media and plates.

Bacterial strain	Plates for bacterial streak	Media for glycerol stock	Media for bacterial cultures for titering cosmid (final concentration)
XL1-Blue MR	LB plates	LB broth	LB with 0.2% (v/v) maltose and 10 mM $MgSO_4$

Reviving the Cells

1. Upon arrival, scrape several splinters of ice from the frozen bacterial stock with a sterile wire loop.

2. Streak the splinters onto an LB agar plate (see *Preparation of Media and Reagents*).

3. Restreak the cells each week.

Preparing –80°C Glycerol Stocks

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

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

3. Aliquot into sterile microcentrifuge tubes (1 ml/tube) and store at –20°C for 1–2 years or –80°C for more than 2 years.

PREPARATION OF VECTOR DNA

Digestion of Vector DNA with *Xba* I

1. Digest 25 µg of the SuperCos 1 cosmid vector (1.0 µg/µl) with 9 U/µg of *Xba* I enzyme in a total volume of 200 µl at standard buffer conditions for 1 hour at 37°C.

2. Check for complete digestion on a 0.8% (w/v) agarose gel. A single linear cosmid band should be seen at 7.9 kb.

3. Extract DNA once with phenol–chloroform–isoamyl alcohol (25:24:1) saturated with 50 mM Tris-HCl (pH 8.0) and once with chloroform. Adjust aqueous phase to 0.3 M sodium acetate (pH 5.5) and ethanol-precipitate. Wash the DNA pellet with 70% (v/v) ethanol.

4. Resuspend DNA in distilled, deionized water at a concentration of 1 µg/µl.

Dephosphorylation of *Xba* I-Digested Vector DNA

1. Dephosphorylate the DNA with calf intestinal alkaline phosphatase (CIAP) (Catalog #600015 (1000 U)). Use 0.1 U CIAP/1-20 pmol of DNA termini in 1× CIAP buffer for 30 minutes at 37°C.

2. Adjust the reaction to 15 mM EDTA and incubate at 68°C for 10 minutes to inactivate the CIAP.

3. Phenol-extract the DNA and re-extract the aqueous layer with chloroform.

4. Adjust the aqueous phase to 0.3 M sodium acetate (pH 5.5) and ethanol-precipitate. Wash the DNA pellet with 70% (v/v) ethanol.

5. Resuspend the vector DNA at 1 µg/µl in TE buffer (see *Preparation of Media and Reagents*). Store at –20°C.

Digestion of *Xba* I- and CIAP-Treated DNA with *Bam*H I

1. Digest vector DNA (~25 µg) with 5 U/µg of *Bam*H I enzyme in a total volume of 200 µl at standard buffer conditions for 1 hour at 37°C.

2. Check 0.5 µg for complete digestion on a 0.8% (w/v) agarose gel. Two cosmid bands should be seen at 1.1 and 6.5 kb.

3. Extract DNA once with phenol–chloroform–isoamyl alcohol (25:24:1) saturated with 50 mM Tris-HCl (pH 8.0) and once with chloroform. Adjust aqueous phase to 0.3 M sodium acetate (pH 5.5) and ethanol-precipitate. Wash the DNA pellet with 70% (v/v) ethanol.

4. Resuspend DNA in distilled, deionized water at a concentration of 1 µg/µl. Store at –20°C.

PREPARATION OF GENOMIC DNA

Isolation of Genomic DNA

Because large fragments are required for cloning in the cosmid system, the genomic DNA must be ~150 kb before digestion. This is the most critical step in the cosmid cloning protocol. It is important that any mixing or aliquoting of the DNA solution be done by gentle inversion or gentle pipetting.

1. Suspend cells or tissue in 15 ml of 10 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 µg/ml proteinase K and 0.5% (w/v) SDS.

2. Mix gently and incubate 6 hours or overnight at 50°C.

3. Extract the DNA by gentle inversion with an equal volume of phenol–chloroform for 10 minutes at room temperature.

4. Centrifuge the DNA at 4000 rpm at 10–12°C for 20 minutes and remove the upper aqueous layer with a wide-bore pipet, being careful not to collect any interphase.

5. Adjust the DNA sample to 0.3 M sodium acetate (pH 5.5) and gently layer 2 volumes of ethanol on top of the DNA solution. Spool out the DNA at the aqueous ethanol interphase using a sterile glass rod.

6. Wash the DNA with 70% (v/v) ethanol, touching the DNA to the side of a sterile tube to drain the ethanol and air dry.

7. Dissolve the DNA in 5 ml of TE buffer. Store at 4°C overnight without shaking. Allow up to 2 days to resuspend the sample.

8. Check the quality of the genomic DNA by electrophoresis through a 0.3% (w/v) agarose gel or field inversion gel using T4 DNA (160 kb) and lambda DNA (50 kb) as size standards. Good-quality DNA will comigrate with T4 DNA.

Partial Digestion of the Chromosomal DNA

In order to clone into the *Bam*H I site of the SuperCos 1 cosmid vector (see Figure 1), the chromosomal DNA should be partially digested with either *Mbo* I or *Sau* 3A I. A series of test partial digests may be done to determine the ideal conditions for obtaining the desired insert size range (30–42 kb).

1. To set up the test partial digest, add the following to a microcentrifuge tube:

   10 µg of genomic DNA
   10 µl of 10× restriction digest buffer
   Water to a final volume of 100 µl

2. Pre-equilibrate at 37°C for 5 minutes and add 0.5 U of *Mbo* I or *Sau*3A I.

3. Remove 15-µl aliquots at various time points from the digestion: 0-, 5-, 10-, 20-, 30- and 45-minute time points. Add ~10 µl of DNA gel loading buffer and check by electrophoresis on a 0.3% (w/v) agarose gel. Be sure to run the gel long enough to get good separation and accurate sizing.

4. After the optimal time interval has been determined, perform a partial *Mbo* I or *Sau*3A I digest of 100 µg of genomic DNA in a 1-ml total reaction volume. Scale up the reaction to best mimic the test partial digest including enzyme concentration, temperature and reaction time.

5. After the digest, add 15 µl of 0.5 M EDTA (pH 8.0) to the sample. Check a 10-µl aliquot on a 0.3% gel to ensure appropriate size distribution.

6. Extract the DNA once with phenol–chloroform saturated with 50 mM Tris-HCl (pH 8.0) and once with chloroform. Adjust aqueous phase to 0.3 M sodium acetate (pH 5.5) and ethanol-precipitate by adding 2.5 volumes of 100% (v/v) ethanol.

7. Resuspend the DNA in 50 µl of TE buffer.

Dephosphorylation of the Partially Digested Chromosomal DNA

1. Dephosphorylate the 50 µl of DNA from above by adding 2 µl of CIAP and diluting the mixture to a final volume of 100 µl in 1× CIAP (Final concentration of CIAP will be 0.1–0.5 U/µl).

2. Incubate at 37°C for 1 hour.

3. Add 3 µl of 0.5 M EDTA and incubate at 68°C for 10 minutes.

4. Extract the DNA once with phenol–chloroform saturated with 50 mM Tris-HCl (pH 8.0) and once with chloroform. Adjust aqueous phase to 0.3 M sodium acetate (pH 5.5) and ethanol- precipitate by adding 2.5 volumes of 100% (v/v) ethanol.

5. Resuspend the DNA to 1 µg/µl in TE buffer.

6. Run a sample of DNA on a 0.3% (w/v) agarose gel.

Note *The additional cos site in the SuperCos 1 vector eliminates the need to dephosphorylate the vector DNA to prevent packaging of cosmid concatemers. The insert DNA can be dephosphorylated, which eliminates the need to size-fractionate the insert DNA in order to prevent multiple insert ligation. While the size-fractionation is not necessary to produce a library, you may observe that consistently higher titer libraries will be obtained using size-fractionated DNA. Cosmids with inserts smaller than 30 kb will not be packaged, so any DNA fragments smaller than 30 kb in the ligation reaction will effectively act as competitive inhibitors, binding up vector and removing it from the pool of clones that can be packaged.*

LIGATION AND PACKAGING OF DNA

Ligation

Note *In all ligations, the final glycerol content should be less than 5%.*

Use large-bore or cut-off pipet tips when pipetting to avoid shearing of chromosomal DNA.

1. Set up the ligation by adding the following components to a microcentrifuge tube:

   2.5 µg of partially digested CIAP genomic DNA
   1.0 µl of SuperCos 1 DNA [*Xba* I–CIAP and digested with *Bam*H I (1 µg/µl)]
   2.0 µl of 10× ligase buffer
   2.0 µl of 10 mM rATP
   Water to a final volume of 20 µl

2. Set up a negative control ligation to check for background by adding the following components to a microcentrifuge tube:

   1.0 µl of SuperCos 1 DNA [*Xba* I–CIAP and digested with *Bam*H I (1 µg/µl)]
   2.0 µl of 10× ligase buffer
   2.0 µl of 10 mM rATP
   Water to a final volume of 20 µl

3. Remove a 1-µl aliquot from each reaction and store at 4°C for later gel analysis.

4. Add 1 µl of T4 DNA ligase (1–2 Weiss U) to the remaining 19 µl of the reaction and incubate at 4°C overnight. **Do not exceed 5% glycerol!**

Packaging

For optimal packaging efficiency, use Gigapack III gold packaging extract [Catalog #200201 (Gold-4), #200202 (Gold-7) and #200203 (Gold-11)]. If selecting for large inserts, Agilent recommends using Gigapack III XL packaging extract [Catalog #200207 (XL-4), #200208 (XL-7) and #200209 (XL-11)].

TITERING THE COSMID PACKAGING REACTION

1. Streak the bacterial glycerol stock onto the appropriate agar plates (see *Preparation of Host Cells*). Incubate the plates overnight at 37°C.

   Note *Do not add antibiotic to the medium in the following step. The antibiotic will bind to the bacterial cell wall and will inhibit the ability of the phage to infect the cell.*

2. Inoculate an appropriate medium, supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose, with a single colony.

3. Grow at 37°C, shaking for 4–6 hours (do not grow past an OD_{600} of 1.0). Alternatively, grow overnight at 30°C, shaking at 200 rpm.

   Note *The lower temperature keeps the bacteria from overgrowing, thus reducing the number of nonviable cells. Phage can adhere to nonviable cells resulting in a decreased titer.*

4. Pellet the bacteria at 500 × *g* for 10 minutes.

5. Gently resuspend the cells in half the original volume with sterile 10 mM $MgSO_4$.

6. Dilute the cells to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$.

   Note *The bacteria should be used immediately following dilution.*

7. Prepare a 1:10 and a 1:50 dilution of the cosmid packaging reaction in SM buffer.

8. Mix 25 µl of each dilution with 25 µl of the appropriate bacterial cells at an OD_{600} of 0.5 in a microcentrifuge tube and incubate the tube at room temperature for 30 minutes.

9. Add 200 µl of LB broth to each sample and incubate for 1 hour at 37°C, shaking the tube gently once every 15 minutes. This incubation will allow time for expression of the antibiotic resistance.

10. Spin the microcentrifuge tube for 1 minute and resuspend the pellet in 50 µl of fresh LB broth.

11. Using a sterile spreader, plate the cells on LB agar plates with the required amount of the appropriate antibiotic. Incubate the plates overnight at 37°C.

AMPLIFYING COSMID LIBRARIES

1. In a series of 15-ml culture tubes, mix packaged recombinant cosmid DNA (50,000 cfu/tube) with an equal volume of XL1-Blue MR host cells (OD_{600} = 0.5). Incubate the tubes at room temperature for 30 minutes.

2. Add 4 volumes of LB broth to each tube and incubate at 37°C for 1 hour with shaking.

3. Harvest the cells by centrifugation for 10 minutes at room temperature.

4. Resuspend the cells in 500 µl of LB broth per plate and spread onto 150-ml LB-ampicillin or LB–kanamycin agar plates (see *Preparation of Media and Reagents*).

5. Incubate the plates at 37°C overnight.

6. Pour 3 ml of LB broth onto each plate and scrape the colonies into the liquid using a sterile spreader. Pipet the broth into a fresh tube and wash the plate with another 3 ml of LB broth.

7. Pool the LB broth from all the plates into a single tube.

8. Add sterile glycerol to a final concentration of 20% and add ampicillin to 100 µg/ml.

9. Shake well to mix and then aliquot into several samples. Allow one aliquot to remain unfrozen for immediate titering and store the remaining aliquots at –80°C.

TITERING AMPLIFIED COSMID LIBRARIES

1. Perform 1:10^2, 1:10^4, and 1:10^6 serial dilutions in LB–ampicillin or LB–kanamycin broth (see *Preparation of Media and Reagents*).

2. Using a sterile spreader, plate the cells on LB–ampicillin or LB–kanamycin agar plates.

3. Incubate the plates overnight at 37°C. Determine the number of cfu/ml.

4. The diluted aliquots may be stored at 4°C overnight and used to plate for screening.

SCREENING COSMID LIBRARIES

1. Place 100-mm Duralon-UV [Catalog #420100–#420105] or nitrocellulose filters on 150–mm LB–ampicillin agar plates.

2. Spread 50,000 cfu/filter. Screen ~1.0×10^6 cfu in total (e.g., 20 plates).

3. Incubate the plates at 37°C overnight or until colonies are ~1 mm in diameter.

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

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

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

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

8. Separate the master and replica filters and place face up on LB-ampicillin agar plates.

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

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

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

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

13. 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.

14. 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.

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

16. 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.

Prehybridizing

Prepare the appropriate prehybridization solution:

Prehybridization Solution for Oligo Probe

6× SSC
20 mM NaH_2PO_4
0.4% sodium dodecyl sulfate (SDS) (increase to 1% for Duralon-UV membranes)
5× Denhardt's reagent
Denatured, sonicated salmon sperm DNA (500 µg/ml) [Catalog #201190 (10 × 1 ml)]

Prehybridization Solution for Double-Stranded Probe

2× PIPES [1,4-Piperazine diethanesulfonic acid] buffer
50% Deionized formamide
0.5% SDS (increase to 1% for Duralon-UV membranes)
Denatured, sonicated salmon sperm DNA (100 µg/ml)

1. Preheat the prehybridization solution to ~50°C without the salmon sperm DNA. Boil 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.

Hybridizing

Labeling Oligonucleotide Probes

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

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

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

3. Run the solution over a G-50 column or a NucTrap® probe purification column [Catalog #400701 (25 columns)] to get rid of the unincorporated counts.

Labeling Double-Stranded Probes

Notes *Agilent offers the Prime-It II random primer kit (Catalog #300385) designed to produce high-specific-activity DNA probes in 2 minutes.*

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

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

Hybridizing Oligonucleotide Probes

1. Prepare the hybridization solution [6× SSC, 20 mM NaH_2PO_4, 0.4% SDS (increase to 1% for Duralon-UV membranes)] and heat to ~50°C.

2. Boil denatured, sonicated salmon sperm DNA (500 µg/ml) and then add it to the warm hybridization solution.

3. Pour out the prehybridization buffer from the filter 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 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)

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

Hybridizing Double-Stranded Probes

1. Prepare the hybridization solution [2× PIPES buffer, 50% Deionized formamide, 0.5% SDS (increase to 1% for Duralon-UV membranes)] and heat to ~50°C.

2. Boil denatured, sonicated salmon sperm DNA (100 µg/ml) with the probe for 4 minutes and then add it to the warm hybridization buffer.

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

Washing

Oligo Probes

Use 6× SSC, 0.1% SDS. Wash filters three times for 5 minutes each at room temperature. The final washing temperature depends upon 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 and 0.1% SDS. Wash the filters at 50–65°C with agitation.

Exposing 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.)

After isolation of the positive clones, these filters may be reused for hybridization in subsequent steps without further washing or removing of the hybridized probe. Repetitive hybridizations with sequential "walking" probes will show new hybridizing colonies at each step (see *Genomic "Walking"*).

PURIFYING POSITIVE CLONES

Because of the high density of colonies on the master filter, it is usually not possible to pick a purified colony directly. To isolate and purify cosmids overlapping with the reference clone:

1. Align the autoradiogram and master filter and remove a 1–5 mm region surrounding the hybridization signal by scraping the master filter with a sterile toothpick or wire loop. Inoculate into 100 µl of LB/ampicillin broth.

2. Dilute the bacteria and replate on 150–mm LB/ampicillin plates to give 100 to 1000 colonies per plate. This will depend on the viability of the particular cells and age of the master filter but usually plating 5–10 ml of a 10^{-3} dilution will suffice.

3. Repeat hybridization steps (see *Screening Cosmid Libraries*) to identify the positive clones. This colony can be used to inoculate a 3.0 ml culture and the resultant DNA (see *Preparation of Cosmid DNA from Mini-Lysates*) used for synthesis of a "walking" probe or for restriction mapping.

4. For long–term storage, add 160 µl of sterile glycerol/ml of bacterial culture and store at –80°C.

PREPARING COSMID DNA FROM MINI-LYSATES

Cosmid clones isolated from genomic libraries usually give a high yield of DNA when grown in the bacterial strain XL1-Blue MR. For most purposes, including genomic walking, preparation of hybridization probes for determination of overlaps, and traditional analysis of restriction fragments on agarose gels, the quantity of DNA prepared from 5.0 ml mini-cultures is sufficient. Though cosmid DNA can be prepared from large cultures and purified by CsCl banding techniques, this is usually not necessary for molecular mapping and expression studies.

1. Inoculate a 5.0–ml culture of LB broth containing 100 µg/ml ampicillin with a single colony from a fresh agar plate. For optimal yields, incubate the culture at 37°C with vigorous shaking for no longer than 12 hours. Longer incubation periods give consistently lower yields of cosmid DNA.

2. Collect the bacterial cells from the 5.0–ml culture by centrifugation for 2 minutes. Remove the supernatant by aspiration and resuspend the pellet by vortexing in 200 µl of an ice cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0).

3. Add 400 µl of a freshly prepared solution of 0.2 N NaOH, 1% SDS. Mix contents by inversion, do not vortex. Incubate on ice for 5 minutes.

4. Add 300 µl of an ice cold solution of potassium acetate (~pH 4.8). **Gently** invert twice and incubate on ice for 5 minutes.

5. Centrifuge for 5 minutes in a centrifuge at 4°C.

6. Transfer 500 µl of the supernatant to a fresh tube. Phenol–chloroform extract once.

7. Add 1 ml ethanol to precipitate and spin in a microfuge for 30 minutes.

8. Wash with 70% ethanol, dry and resuspend in 50 µl of TE buffer. 10 µl should be used for each restriction digest performed.

   Note *If using the boil prep procedure, boil for only 1 minute and phenol–chloroform extract twice.*

For a large scale preparation, inoculate a single colony into 500 ml prewarmed (37°C) LB/ampicillin. Grow for only 8 to 10 hours, shaking at 300 rpm for good aeration. Slower shaking speeds and increased growth periods severely drop yields. Do not grow overnight.

GENOMIC "WALKING"

End-specific RNA probes may be synthesized from cosmid DNA isolated from 3.0 ml of bacterial culture. For the synthesis of "walking" probes, the DNA should first be digested with a restriction endonuclease with a frequently represented recognition sequence, such as *Pal* I or *Rsa* I. RNA probes prepared from *Pal* I restricted templates generally range in size from 50 to 100 nucleotides and are less likely to contain high repetitive sequences than longer probes.

Digesting Cosmid DNA

1. Digest the cosmid DNA with *Pal* I or *Rsa* I according to the specifications recommended by the manufacturer.

2. Check for complete digestion by electrophoresis on a 0.8% agarose "mini-gel." A smear of DNA up to 500 bp should be seen.

3. Extract the DNA once with phenol–chloroform–isoamyl alcohol and once with an equal volume of chloroform. Adjust the aqueous phase to 0.3 M sodium acetate (pH 5.5) and ethanol precipitate with 2.5 volumes of cold 100% ethanol. Five micrograms of carrier yeast tRNA or glycogen may be added to aid in quantitative recovery of the digested DNA. Collect the DNA pellet by spinning for 30 minutes at 4°C and wash in 70% ethanol. Air dry and resuspend at 1 µg/µl in DEPC-treated sterile water.

Synthesizing End-Specific RNA Probes

The following is a typical reaction for generating high-specific-activity probes.

1. Add the solutions in the following order:

   5 µl of 5× transcription buffer
   1 µg of restricted DNA template
   1 µl of 10 mM rATP
   1 µl of 10 mM rGTP
   1 µl of 10 mM rCTP
   1 µl of 0.75M dithiothreitol (DTT)
   5 µl of 400–800 Ci/mmol, 10 µCi/µl [α^{32}P]rUTP
   (**Optional:** add 40 U of RNase Block [Catalog #300151 (4000 U)] and 1 µl of 10 mM rUTP to inhibit RNase activity and enhance polymerase activity)
   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. Add 10 U of RNase-free DNase and incubate for 15 minutes at 37°C to remove the DNA template.

4. Terminate the reaction by extracting with an equal volume of phenol–chloroform, and an equal volume of chloroform.

5. Add 2.5 volumes of ice cold 100% ethanol to precipitate the RNA.

6. Store the radiolabeled probe at –20°C in 50% ethanol.

Determine the efficiency of synthesis by precipitating an aliquot of the RNA probe with trichloroacetic acid and calculate the incorporation. Typically, 1–2 µg of template cosmid DNA yields 1×10^7 cpm.

Examine a small aliquot of the probe on a 5% acrylamide–6 M urea gel with single-stranded radioactive size markers to determine the size of the probe. Probes less than 50 nucleotides in length will not be useful because they do not hybridize well. Probes greater than 1000 nucleotides in length run a higher risk of containing a highly repeated sequence. Because not all enzymes produce ideal probes, it is best to digest the DNA with several four-base restriction enzymes and test the probes in parallel. *Alu* I can be used if *Pal* I and *Rsa* I are not suitable. The probe is now ready to use for hybridization. These probes can be used to rescreen the library to isolate overlapping clones.

RESTRICTION MAPPING WITH OLIGONUCLEOTIDE PROBES

The restriction map of the cosmid clone is determined by digesting the cosmid recombinant with *Not* I to excise the insert, and then partially digesting the subsequent fragment. The series of digestion products is run on an agarose gel, transferred to nitrocellulose, and probed with an oligonucleotide probe. The oligonucleotide probe can either be labeled with radioactivity or conjugated to biotin or alkaline phosphatase and labeled with a color reaction.

Transferring DNA to Nitrocellulose

1. Soak the gel in 0.15 M HCl for 10 minutes to fragment the DNA. This aids in the transfer of DNA on the nitrocellulose. Agitate gently to ensure even exposure of the gel to the HCl. Alternatively, expose to UV light for 1 minute. Caution must be used with the UV light approach since the efficiency of nicking can decrease over time due to solarization of the filter. Always monitor transfer efficiency by staining the gel following transfer.

2. If HCl was used, rinse gel with dH_2O to remove excess acid.

3. Denature DNA by soaking the gel in 1.5 M NaCl, 0.5 M NaOH for 30 minutes.

4. Neutralize by soaking in 1.5 M NaCl, 1 M Tris–HCl (pH 8.0) for 30 minutes.

5. Place the gel on 3–4 sheets of Whatman 3MM filter paper 2–3 inches larger than the gel in all directions and saturate in 6× SSC or 6× SSPE.

6. Briefly wet the nitrocellulose, cut to the size of gel, then presoak in 6× SSC or 6× SSPE.

7. Place nitrocellulose and 1–2 sheets of saturated filter paper carefully on top of the gel followed by a 1" stack of paper towels and a light weight to keep all layers compressed. Surround the gel with Parafilm wrap to prevent wicking of transfer buffer on to paper towels.

8. Transfer should be complete after 8 hours. The efficiency can be checked by restaining gel after transfer in 100 ml H_2O, 1 µg/ml ethidium bromide.

9. After transfer, crosslink the DNA to the filters using the autocrosslink setting on the Stratalinker UV crosslinker [Catalog #400071 (1800) and #400075 (2400)] (120,000 µJ of UV energy). Alternatively, oven bake at 80°C for ~1.5–2 hours.

Blots can be stored dry in sealed plastic bags at room temperature.

Prehybridizing

1. Prepare the prehybridization solution [6× SSC, 20 mM NaH_2PO_4, 0.4% SDS (increase to 1% for Duralon-UV membranes), 5× Denhardt's reagent] and heat to ~50°C.

2. Boil denatured, sonicated salmon sperm DNA (500 µg/ml) for ~10 minutes and add it to the warm prehybridization solution.

3. 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.)

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

5. Prehybridize for 1–2 hours at 42°C.

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

Hybridizing

Labeling Oligonucleotide Probes

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

1. Perform a PNK labeling reaction in 1× ligase buffer for 30 minutes at 37°C.

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

3. Run the solution over a G-50 column or a NucTrap® probe purification column to remove the unincorporated counts.

Hybridizing Oligonucleotide Probes

1. Prepare the hybridization solution [6× SSC, 20 mM NaH_2PO_4, 0.4% SDS (increase to 1% for Duralon-UV membranes)] and heat to ~50°C.

2. Boil denatured, sonicated salmon sperm DNA (500 µg/ml) and then add it to the warm hybridization solution.

3. Pour out the prehybridization buffer from the filter 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 T_m using the following formula:

   Note *The first method 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)

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

Washing

Use 6× SSC, 0.1% SDS at T_m – 10°C. 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.

Exposing 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 a cassette 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.)

PREPARATION OF MEDIA AND REAGENTS

All media must be autoclaved before use.

LB Broth (per Liter)

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

NZY Broth (per Liter)

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
Adjust the pH to 7.5 with NaOH

LB Agar (per Liter)

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

NZY Top Agar (per Liter)

1 liter of NZY broth
Add 0.7% (w/v) agarose

TE Buffer

10.0 mM Tris-HCl (pH 8.0)
0.1 mM EDTA

LB–Ampicillin Agar (per Liter)

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

LB–Ampicillin Broth (per Liter)

1 liter of LB broth, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin

LB–Kanamycin Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)

LB–Kanamycin Broth (per Liter)

Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml filter-sterilized kanamycin

20× SSC Buffer (per Liter)

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 a 10.0 N NaOH
Adjust volume to 1 liter with water

NZY Agar (per Liter)

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
15 g of agar
Adjust the pH to 7.5 with NaOH
Autoclave
Pour into petri dishes (~80 ml/150-mm plate)

10× STE

1 M NaCl
200 mM Tris-HCl (pH 7.5)
100 mM EDTA

20× SSPE

174.0 g of NaCl
27.6 g of NaH_2PO_4
7.4 g of EDTA, disodium salt (pH 8.0)
13.0 ml 5M NaOH
Adjust pH to 7.4 with NaOH
Add water to 1 liter
Mix well

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol

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

10× PIPES Buffer

4.0 M NaCl
0.1 M PIPES (pH 6.5)

SM Buffer (per Liter)

5.8 g of NaCl
2.0 g of $MgSO_4 \cdot 7H_2O$
50.0 ml of 1M Tris-HCl (pH 7.5)
5.0 ml of 2% (w/v) gelatin

Potassium Acetate (per 10 ml)

6.0 ml of 5 M potassium acetate
1.15 ml of glacial acetic acid
2.85 ml of water

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

REFERENCES

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ENDNOTES

MSDS INFORMATION

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