重组 DNA 的分离、克隆与测序实验手册

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August 5, 1995

A printed version of this protocol book can be obtained from:

"DNA Isolation and Sequencing" (Essential Techniques Series)

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Published by John Wiley & Sons, List Price $49.95

ISBN 0-471-97324-0

QP625.N89R64 1996

John Wiley & Sons

Introduction

This manual is a compilation of many of the everyday methods used in the average molecular biology laboratory, with emphasis on the techniques for large scale DNA sequencing protocols and DNA sequencing automation techniques. The manual has been written in a protocol format, with little theoretical discussion. For theory and additional information, users of this manual are referred back to the original literature, or to other textual manuals such as those published by Maniatis (1) et al. and Glover (2).

The following persons are acknowledged for contributing methods and suggestions during the assembly of this manual: Stephanie Chissoe, Sandy Clifton, Dennis Burian, Rick Wilson, Din-Pow Ma, James Wong, Leslie Johnston-Dow, Elaine Mardis, Zhili Wang, Kala Iyer, Steve Toth, Goughay Zhang, Hua Qin Pan and other members of the Roe laboratory, both past and present.

1. Sambrook, J., Fritsch, E.F., and Maniatis, T., in Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3 (1989).

2. Glover, D.M. DNA Cloning Volume I: A Practical Approach. IRL Press, Oxford, 1985.

I. General methods

A. Phenol extraction of DNA samples

Phenol extraction is a common technique used to purify a DNA sample (1). Typically, an equal volume of TE-saturated phenol is added to an aqueous DNA sample in a microcentrifuge tube. The mixture is vigorously vortexed, and then centrifuged to enact phase separation. The upper, aqueous layer carefully is removed to a new tube, avoiding the phenol interface and then is subjected to two ether extractions to remove residual phenol. An equal volume of water-saturated ether is added to the tube, the mixture is vortexed, and the tube is centrifuged to allow phase separation. The upper, ether layer is removed and discarded, including phenol droplets at the interface. After this extraction is repeated, the DNA is concentrated by ethanol precipitation.

Protocol

1. Add an equal volume of TE-saturated phenol to the DNA sample contained in a 1.5 ml microcentrifuge tube and vortex for 15-30 seconds.

2. Centrifuge the sample for 5 minutes at room temperature to separate the phases.

3. Remove about 90% of the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous:phenol interface. At this stage the aqueous phase can be extracted a second time with an equal volume of 1:1 TE-saturated phenol:chloroform, centrifuged and removed to a clean tube as above but this additional extraction usually is not necessary if care is taken during the first phenol extraction.

4. Add an equal volume of water-saturated ether, vortex briefly, and centrifuge for 3 minutes at room temperature. Remove and discard the upper, ether layer, taking care to remove phenol droplets at the ether:aqueous interface. Repeat the ether extraction.

5. Ethanol precipitate the DNA by adding 2.5-3 volumes of ethanol-acetate, as discussed below.

B. Concentration of DNA by ethanol precipitation

Typically, 2.5 - 3 volumes of an ethanol/acetate solution is added to the DNA sample in a microcentrifuge tube, which is placed in an ice-water bath for at least 10 minutes. Frequently, this precipitation is performed by incubation at -20C overnight (1). To recover the precipitated DNA, the tube is centrifuged, the supernatant discarded, and the DNA pellet is rinsed with a more dilute ethanol solution. After a second centrifugation, the supernatant again is discarded, and the DNA pellet is dried in a Speedy-Vac.

Protocol

1. Add 2.5-3 volumes of 95% ethanol/0.12 M sodium acetate to the DNA sample contained in a 1.5 ml microcentrifuge tube, invert to mix, and incubate in an ice-water bath for at least 10 minutes. It is possible to place the sample at -20degC overnight at this stage.

2. Centrifuge at 12,000 rpm in a microcentrifuge (Fisher) for 15 minutes at 4 degC, decant the supernatant, and drain inverted on a paper towel.

3. Add 80% ethanol (corresponding to about two volume of the original sample), incubate at room temperature for 5-10 minutes and centrifuge again for 5 minutes, and decant and drain the tube, as above.

4. Place the tube in a Savant Speed-Vac and dry the DNA pellet for about 5-10 minutes, or until dry.

5. Always dissolve dried DNA in 10 mM Tris-HCl, pH 7.6-8.0, 0.1 mM EDTA (termed 10:0.1 TE buffer).

6. It is advisable to aliquot the DNA purified in large scale isolations (i.e. 100 ug or more) into several small (0.5 ml) microcentrifuge tubes for frozen storage because repeated freezing and thawing is not advisable.

Notes on precipitation of nucleic acids

A. General rules

Most nucleic acids may be precipitated by addition of monovalent cations and two to three volumes of cold 95% ethanol, followed by incubation at 0 to -70 degC. The DNA or RNA then may be pelleted by centrifugation at 10 to 13,000 x g. for 15 minutes at 4degC. A subsequent wash with 70% ethanol, followed by brief centrifugation, removes residual salt and moisture.

The general procedure for precipitating DNA and RNA is:

1. Add one-tenth volume of 3M NaOAc, pH 5.2* to the nucleic acid solution to be precipitated,

2. Add two volumes of cold 95% ethanol,

3. Place at -70degC for at least 30 minutes, or at -20degC overnight.

or alternatively

1. Combine 95 ml of 100% ethanol with 4 ml of 3 M NaOAc (pH 4.8) and 1ml of sterile water. Mix by inversion and store at -20degC.

2. Add 2.5 volumes of cold ethanol/acetate solution to the nucleic acid solution to be precipitated.

3. Place at at -70degC for at least 30 minutes or -20degC for two hours to overnight.

* 5M NH4OAc, pH 7.4, NaCl and LiCl may be used as alternatives to NaOAc. DNA also may be precipitated by addition of 0.6 volumes of isopropanol.

B. Oligonucleotides

Add one-tenth volume of 3M NaOAc, pH 6.5, and three volumes of cold 95% ethanol.

Place at -70degC for at least one hour.

C. RNA

Add one-tenth volume of 1M NaOAc, pH 4.5, and 2.5 volumes of cold 95% ethanol.

Precipitate large volumes at -20degC overnight.

Small volume samples may be precipitated by placing in powdered dry ice or dry ice-ethanol bath for five to 10 minutes.

D. Isobutanol concentration of DNA

DNA samples may be concentrated by extraction with isobutanol. Add slightly more than one volume of isobutanol, vortex vigorously and centrifuge to separate the phases. Discard the isobutanol (upper) phase, and extract once with water-saturated diethyl ether to remove residual isobutanol. The nucleic acid then may be ethanol precipitated as described above.

E. Notes on phenol extraction of nucleic acids

The standard and preferred way to remove proteins from nucleic acid solutions is by extraction with neutralized phenol or phenol/chloroform. Generally, samples are extracted by addition of one-half volume of neutralized (with TE buffer, pH 7.5) phenol to the sample, followed by vigorous mixing for a few seconds to form an emulsion. Following centrifugation for a few minutes, the aqueous (top) phase containing the nucleic acid is recovered and transferred to a clean tube. Residual phenol then is removed by extraction with an equal volume of water-saturated diethyl ether. Following centrifugation to separate the phases, the ether (upper) phase is discarded and the nucleic acid is ethanol precipitated as described above.

A 1:1 mixture of phenol and chloroform also is useful for the removal of protein from nucleic acid samples. Following extraction with phenol/chloroform, the sample should be extracted once with an equal volume of chloroform, and ethanol precipitated as described above.

C. Restriction digestion

Restriction enzyme digestions are performed by incubating double-stranded DNA molecules with an appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier, and at the optimal temperature for that specific enzyme. The optimal sodium chloride concentration in the reaction varies for different enzymes, and a set of three standard buffers containing three concentrations of sodium chloride are prepared and used when necessary. Typical digestions included a unit of enzyme per microgram of starting DNA, and one enzyme unit usually (depending on the supplier) is defined as the amount of enzyme needed to completely digest one microgram of double-stranded DNA in one hour at the appropriate temperature. These reactions usually are incubated for 1-3 hours, to insure complete

digestion, at the optimal temperature for enzyme activity, typically 37degC. See the Appendix for a listing of restriction sites present in the M13 (pUC) MCS and a listing of various restriction enzymes, incubation conditions and cut sites.

Protocol

1. Prepare the reaction for restriction digestion by adding the following reagents in the order listed to a microcentrifuge tube:

sterile ddH20	q.s (where "q.s." means quantity sufficient)
10X assay buffer	one-tenth volume
DNA	x ul
restriction enzyme*	y ul (1-10 units per ug DNA)
Total volume z ul	

*If desired, more than one enzyme can be included in the digest if both enzymes are active in the same buffer and the same incubation temperature.

Note: The volume of the reaction depends on the amount and size of the DNA being digested. Larger DNAs should be digested in larger total volumes (between 50-100 ul), as should greater amounts of DNA.

Refer to the vendor's catalog for the chart of enzyme activity in a range of salt concentrations to choose the appropriate assay buffer (10X High, 10X Medium, or 10X Low Salt Buffers, or 10X SmaI Buffer for SmaI digestions). Restriction enzymes are purchased from Bethesda Research Laboratories, New England Biolabs, or United States Biochemicals.

2. Gently mix by pipetting and incubate the reaction at the appropriate temperature (typically 37degC) for 1-3 hours.

3. Inactivate the enzyme(s) by heating at 70-100degC for 10 minutes or by phenol extraction (see the vendor's catalog to determine the degree of heat inactivation for a given enzyme). Prior to use in further protocols such as dephosphorylation or ligation, an aliquot of the digestion should be assayed by agarose gel electrophoresis versus non-digested DNA and a size marker, if necessary.

D. Agarose gel electrophoresis

Agarose gel electrophoresis (2) is employed to check the progression of a restriction

enzyme digestion, to quickly determine the yield and purity of a DNA isolation or PCR reaction, and to size fractionate DNA molecules, which then could be eluted from the gel. Prior to gel casting, dried agarose is dissolved in buffer by heating and the warm gel solution then is poured into a mold (made by wrapping clear tape around and extending above the edges of an 18 cm X 18 cm glass plate), which is fitted with a well-forming comb. The percentage of agarose in the gel varied. Although 0.7% agarose gels typically are used, in cases where the accurate size fractionation of DNA molecules smaller than 1 kb is required, a 1, 1.5, or 2% agarose gel is prepared, depending on the expected size(s) of the fragment(s). Ethidium bromide is included in the gel matrix to enable fluorescent visualization of the DNA fragments under UV light. Agarose gels are submerged in electrophoresis buffer in a horizontal electrophoresis apparatus. The DNA samples are mixed with gel tracking dye and loaded into the sample wells. Electrophoresis usually is at 150 - 200 mA for 0.5-1 hour at room temperature, depending on the desired separation. When low-melting agarose is used for preparative agarose gels, electrophoresis is at 100-120 mA for 0.5-1 hour, again depending on the desired separation, and a fan is positioned such that the heat generated is rapidly dissipated. Size markers are co-electrophoresed with DNA samples, when appropriate for fragment size determination. Two size markers are used, phi-X 174 cleaved with restriction endonuclease HaeIII to identify fragments between 0.3-2 kb and lambda phage cleaved with restriction endonuclease HindIII to identify fragments between 2-23 kb. After electrophoresis, the gel is placed on a UV light box and a picture of the fluorescent ethidium bromide-stained DNA separation pattern is taken with a Polaroid camera.

Protocol

1. Prepare an agarose gel, according to recipes listed below, by combining the agarose (low gel temperature agarose may also be used) and water in a 500 ml Ehrlenmeyer flask, and heating in a microwave for 2-4 minutes until the agarose is dissolved.

	0.7%	1.0%	2.0%
agarose	1.05 g	1.5 g	3.0 g
20X TAE	7.5 ml	7.5 ml	7.5 ml

ddH2O	142.5 ml	142.5 ml	142.5 ml
EtBr (5 mg/ml)	25 ul	25 ul	25 ul
total vol	150 ml	150 ml	150 ml

Genetic technology grade (800669) or low gel temperature (800259) agarose from Schwarz/Mann Biotech.

2. Add 20X TAE and ethidium bromide (EtBr), swirl to mix, and pour the gel onto a taped plate with casting combs in place. Allow 20-30 minutes for solidification.

3. Carefully remove the tape and the gel casting combs and place the gel in a horizontal electrophoresis apparatus. Add 1X TAE electrophoresis buffer to the reservoirs until the buffer just covers the agarose gel.

4. Add at least one-tenth volume of 10X agarose gel loading dye to each DNA sample, mix, and load into the wells. Electrophorese the gel at 150-200 mA until the required separation has been achieved, usually 0.5-1 hour (100-120 mA for low gel temperature agarose), and cool the gel during electrophoresis with a fan. Visualize the DNA fragments on a long wave UV light box and photograph with a Polaroid camera.

E. Elution of DNA fragments from agarose

DNA fragments are eluted from low-melting temperature agarose gels using an unpublished procedure first developed by Dr. Roe. Here, the band of interest is excised with a sterile razor blade, placed in a microcentrifuge tube, frozen at -70degC, and then melted. Then, TE-saturated phenol is added to the melted gel slice, and the mixture again is frozen and then thawed. After this second thawing, the tube is centrifuged and the aqueous layer removed to a new tube. Residual phenol is removed with two ether extractions, and the DNA is concentrated by ethanol precipitation.

Protocol

1. Place excised DNA-containing agarose gel slice in a 1.5 ml microcentrifuge tube and freeze at -70degC for at least 15 minutes, or until frozen. It is possible to pause at this stage in the elution procedure and leave the gel slice frozen at -70degC.

2. Melt the slice by incubating the tube at 65degC.

3. Add one-volume of TE-saturated phenol, vortex for 30 seconds, and freeze the sample at -70degC for 15 minutes.

4. Thaw the sample, and centrifuge in a microcentrifuge at 12,000 rpm for 5 minutes at room temperature to separate the phases. The aqueous phase then is removed to a clean tube, extracted twice with equal volume ether, ethanol precipitated, and the DNA pellet is rinsed and dried.

F. Kinase end-labeling of DNA

Typical 5'-kinase labeling reactions included the DNA to be labeled, [[gamma]]-32-P-rATP, T4 polynucleotide kinase, and buffer (3). After incubation at 37degC, reactions are heat inactivated by incubation at 80degC. Portions of the reactions are mixed with gel loading dye and loaded into a well of a polyacrylamide gel and electrophoresed. The gel percentage and electrophoresis conditions varied depending on the sizes of the DNA molecules of interest. After electrophoresis, the gel is dried and exposed to x-ray film, as discussed below for radiolabeled DNA sequencing.

Protocol

1. Add the following reagents to a 0.5 ml microcentrifuge tube, in the order listed:

sterile ddH2O	q.s
10X kinase buffer	1 ul
DNA	x ul
[[gamma]]-[32-P]-rATP	10 uCi
T4 polynucleotide kinase	1 ul (3U/ul)
	10 ul

[[gamma]]-[32-P]-rATP (35020) ICN and T4 polynucleotide kinase (70031) from United States Biochemicals.

2. Incubate at 37degC for 30-60 minutes.

3. Heat the reaction at 65degC for 10 minutes to inactivate the kinase.

G. Bacterial cell maintenance

Four strains of E. coli are used in these studies: JM101 for M13 infection and

isolation (4), XL1BMRF' (Stratagene) for M13 or pUC-based DNA transformation (5), and ED8767 for cosmid DNA transformation (6-8). To maintain their respective F' episomes necessary for M13 viral infection (9), JM101 is streaked onto a M9 minimal media (modified from that given in reference (1) plate and XL1BMRF' is streaked onto an LB plate (1) containing tetracycline. ED8767 is streaked onto an LB plate. These plates are incubated at 37degC overnight. For each strain, 3 ml. of appropriate liquid media are inoculated with a smear of several colonies and incubated at 37degC for 8 hours, and those cultures then are transferred into 50 ml of respective liquid media and further incubated 12-16 hours. Glycerol is added to a final concentration of 20%, and the glycerol stock cultures are distributed in 1.3 ml aliquots and frozen at -70degC until use (1).

Protocol

1. Streak a culture of the bacterial cell strain onto an agar plate of the respective medium, listed below, and incubate at 37degC overnight.

E. coli strain	Agar Medium/Liquid Media
XL1BMRF' (Stratagene)	LB-Tet
JM101	M9
ED8767	LB

2. Pick several colonies into a 12 X 75 mm Falcon tube containing a 2 ml aliquot of the respective liquid media, and incubate for 8-10 hours at 37degC with shaking at 250 rpm.

3. Transfer the 2 ml culture into an Ehrlenmeyer flask containing 50 ml of the respective liquid media and further incubate overnight (12-16 hours) at 37degC with shaking at 250 rpm.

4. Add 12.5 ml of sterile glycerol for a final concentration of 20%, and distribute the culture in 1.3 ml aliquots into 12 X 75 mm Falcon tubes.

5. Store glycerol cell stocks frozen at -70degC until use.

Notes on Restriction/Modification Bacterial Strains:

1. EcoK (alternate=EcoB)-hsdRMS genes=attack DNA not protected by adenine methylation. (ED8767 is EcoK methylation -). (10)

2. mcrA (modified cytosine restriction), mcrBC, and mrr=methylation requiring systems that attack DNA only when it IS methylated (Ed8767 is mrr+, so methylated adenines will be restricted. Clone can carry methylation activity.) (10)

3. In general, it is best to use a strain lacking Mcr and Mrr systems when cloning genomic DNA from an organism with methylcytosine such as mammals, higher plants , and many prokaryotes.(11)

4. The use of D(mrr-hsd-mcrB) hosts=general methylation tolerance and suitability for clones with N6 methyladenine as well as 5mC (as with bacterial DNAs). (12)

5. XL1-Blue MRF'=D(mcrA)182, D(mcrCB-hsdSMR-mrr)172,endA1, supE44, thi-1, recA, gyrA96, relA1, lac, l-, [F' proAB, lacIqZDM15, Tn10(tetr)].

Host Mutation Descriptions:

ara Inability to utilize arabinose.

deoR Regulatory gene that allows for constitutive synthesis for genes involved in deoxyribose synthesis. Allows for the uptake of large plasmids.

endA DNA specific endonuclease I. Mutation shown to improve yield and quality of DNA

from plasmid minipreps.

F' F' episome, male E. coli host. Necessary for M13 infection.

galK Inability to utilize galactose.

galT Inability to utilize galactose.

gyrA Mutation in DNA gyrase. Confers resistance to nalidixic acid.

hfl High frequency of lysogeny. Mutation increases lambda lysogeny by inactivating specific

protease.

lacI Repressor protein of lac operon. LacIq is a mutant lacI that overproduces the

repressor protein.

lacY Lactose utilization; galactosidase permease (M protein).

lacZ b-D-galactosidase; lactose utilization. Cells with lacZ mutations produce white

colonies in the presence of X-gal; wild type produce blue colonies.

lacZdM15 A specific N-terminal deletion which permits the a-complementation segment present

on a phagemid or plasmid vector to make functional lacZ protein.

Dlon Deletion of the lon protease. Reduces degradation of b-galactosidase fusion proteins

to enhance antibody screening of l libraries.

malA Inability to utilize maltose.

proAB Mutants require proline for growth in minimal media.

recA Gene central to general recombination and DNA repair. Mutation eliminates general

recombination and renders bacteria sensitive to UV light.

rec BCD Exonuclease V. Mutation in recB or recC reduces general recombination to a hundredth

of its normal level and affects DNA repair.

relA Relaxed phenotype; permits RNA synthesis in the absence of protein synthesis.

rspL 30S ribosomal sub-unit protein S12. Mutation makes cells resistant to streptomycin.

Also written strA.

recJ Exonuclease involved in alternate recombination pathways of E. coli.

strASee rspL.

sbcBC Exonuclease I. Permits general recombination in recBC mutants.

supE Supressor of amber (UAG) mutations. Some phage require a mutation in this gene in order

to grow.

supF Supressor of amber (UAG) mutations. Some phage require a mutation in this gene in order

to grow.

thi-1 Mutants require vitamin B1(thiamine) for growth on minimal media.

traD36 mutation inactivates conjugal transfer of F' episome.

umuC Component of SOS repair pathway.

uvrC Component of UV excision pathway.

xylA Inability to utilize xylose.

damDNA adenine methylase/ Mutation blocks methylation of Adenine residues in the recognition

sequence 5'-G*ATC-3' (*=methylated)

dcmDNA cytosine methylase/Mutation blocks methylation of cytosine residues in the recognition

sequences 5'-C*CAGG-3' or 5'-C*CTGG-3' (*=methylated)

hsdM E. coli methylase/ Mutation blocks sequence specific methylation AN6*ACNNNNNNGTGC or

GCN6*ACNNNNNNGTT (*=methylated). DNA isloated from a HsdM- strain will be restricted by a HsdR+

host.

hsd R17 Restriction negative and modification positive.

(rK-, mK+) Allows cloning of DNA without cleavage by endogenous restriction endonucleases. DNA

prepared from hosts with this marker can efficiently transform rK+ E. coli hosts.

hsdS20 Restriction negative and modification negative.

(rB-, mB-) Allows cloning of DNA without cleavage by endogenous restriction endonucleases . DNA

prepared from hosts with this marker is unmethylated by the hsdS20 modificationsystem.

mcrA E. coli restriction system/ Mutation prevents McrA restriction of methylated DNA of

sequence 5'-C*CGG (*=methylated).

mcrCB E. coli restriction system/ Mutation prevents McrCB restriction of methylated DNA of

sequence 5'-G5*C, 5'-G5h*C, or 5'-GN4*C (*=methylated).

mrr E. coli restriction system/ Mutation prevents Mrr restriction of methylated DNA of sequence

5'-G*AC or 5'-C*AG (*=methylated). Mutation also prevents McrF restriction of methylated cytosine

sequences.

Other Descriptions:

cmr Chloramphenicol resistance

kanr Kanamycin resistance

tetr Tetracycline resistance

strr Streptomycin resistance

D Indicates a deletion of genes following it.

Tn10 A transposon that normally codes for tetr

Tn5 A transposon that normally codes for kanr

spi- Refers to red-gam- mutant derivatives of lambda defined by their ability to form plaques on E. coli P2 lysogens.

Commonly used bacterial strains

C600 - F-, e14, mcrA, thr-1 supE44, thi-1, leuB6, lacY1, tonA21, l-

-for plating lambda (gt10) libraries, grows well in L broth, 2x TY, plate on NZYDT+Mg.

-Huynh, Young, and Davis (1985) DNA Cloning, Vol. 1, 56-110.

DH1 - F-, recA1, endA1, gyrA96, thi-1, hsdR17 (rk-, mk+), supE44, relA1, l-

-for plasmid transformation, grows well on L broth and plates.

-Hanahan (1983) J. Mol. Biol. 166, 557-580.

XL1Blue-MRF' - D(mcrA)182, D(mcrCB-hsdSMR-mrr)172,endA1, supE44, thi-1, recA, gyrA96, relA1,

lac, l-, [F'proAB, lac IqZDM15, Tn10 (tetr)] -For plating or glycerol stocks, grow in LB

with 20 mg/ml of tetracycline. For transfection, grow in tryptone broth containing 10 mM

MgSO4 and 0.2% maltose. (No antibiotic--Mg++ interferes with tetracycline action.) For picking

plaques, grow glycerol stock in LB to an O.D. of 0.5 at 600 nm (2.5 hours?). When at 0.5, add

MgSO4 to a final concentration of 10 mM.

SURE Cells - Stratagene - e14(mcrA), D(mcrCB- hsdSMR-mrr)171, sbcC, recB, recJ, umuC::Tn5

(kanr), uvrC, supE44, lac, gyrA96, relA1, thi-1, end A1[F'proAB, lacIqDM15, Tn10(tetr)].

An uncharacterized mutation enhances the a - complementation to give a more intense blue color

on plates containing X-gal and IPTG.

GM272 - F-, hsdR544 (rk-, mk-), supE44, supF58, lacY1 or 茕 acIZY6, galK2, galT22, metB1m, trpR55,

l-

-for plasmid transformation, grows well in 2x TY, TYE, L broth and plates.

-Hanahan (1983) J. Mol. Biol. 166, 557-580.

HB101 - F-, hsdS20 (rb-, mb-), supE44, ara14, galK2, lacY1, proA2, rpsL20 (strR), xyl-5, mtl-1,

l-, recA13, mcrA(+), mcrB(-)

-for plasmid transformation, grows well in 2x TY, TYE, L broth and plates.

-Raleigh and Wilson (1986) Proc. Natl. Acad. Sci. USA 83, 9070-9074.

JM101 - supE, thi, ?lac-proAB), [F', traD36, proAB, lacIqZ 芃 15], restriction: (rk+, mk+), mcrA+

-for M13 transformation, grow on minimal medium to maintain F episome, grows well in 2x TY,

plate on TY or lambda agar.

-Yanisch-Perron et al. (1985) Gene 33, 103-119.

XL-1 blue recA1, endA1, gyrA96, thi, hsdR17 (rk+, mk+), supE44, relA1, l-, lac, [F', proAB,

lacIqZ 芃 15, Tn10 (tetR)]

-for M13 and plasmid transformation, grow in 2x TY + 10 礸/ml Tet, plate on TY agar + 10 礸/ml

Tet (Tet maintains F episome).

-Bullock, et al. (1987) BioTechniques 5, 376-379.

GM2929 - from B. Bachman, Yale E.coli Genetic Stock Center (CSGC#7080); M.Marinus strain; sex F-;

(ara-14, leuB6, fhuA13, lacY1, tsx-78, supE44, [glnV44], galK2, galT22, l-, mcrA, dcm-6, hisG4,[Oc],

rfbD1, rpsL136, dam-13::Tn9, xyl-5, mtl-1, recF143, thi-1, mcrB, hsdR2.)

MC1000 - (araD139, D[ara-leu]7679, galU, galK, D[lac]174, rpsL, thi-1). obtained from the McCarthy

lab at the University of Oklahoma.

ED8767 (F-,e14-[mcrA],supE44,supF58,hsdS3[rB-mB-], recA56, galK2,galT22,metB1, lac-3 or lac3Y1 -

obtained from Nora Heisterkamp and used as the host for abl and bcr cosmids.

H. Fragment purification on Sephacryl S-500 spin columns

DNA fragments larger than a few hundred base pairs can be separated from smaller fragments by chromatography on a size exclusion column such as Sephacryl S-500. To simplify this procedure, the following mini-spin column method has been developed.

1. Thoroughly mix a fresh, new bottle of Sephacryl S-500, distribute in 10 ml portions, and store in screw cap bottles or centrifuge tubes in the cold room.

2. Prior to use, briefly vortex the matrix and without allowing to settle, add 500 ul of this slurry to a mini-spin column (Millipore) which has been inserted into a 1.5 ml microcentrifuge tube.

3. Following centrifugation at 2K RPM in a table top centrifuge, carefully add 200 ul of 100 mM Tris-HCl (pH 8.0) to the top of the Sephacryl matrix and centrifuge for 2 min. at 2K RPM. Repeat this step twice more. Place the Sephacryl matrix-containing

spin column in a new microcentrifuge tube.

4. Then, carefully add 40 ul of nebulized cosmid, plasmid or P1 DNA which has been end repaired to the Sephacryl matrix (saving 2 ul for later agarose gel analysis) and centrifuge at 2K RPM for 5 minutes. Remove the column, save the solution containing the eluted, large DNA fragments (fraction 1). Apply 40 ul of 1xTM buffer and recentrifuge for 2 minutes at 2K RPM to obtain fraction 2 and repeat this 1xTM rinse step twice more to obtain fractions 3 and 4.

5. To check the DNA fragment sizes, load 3-5 ul of each eluant fraction onto a 0.7% agarose gel that includes as controls, 1-2 ul of a PhiX174-HaeIII digest and 2 ul of unfractionated, nebulized DNA saved from step 4 above.

6. The fractions containing the nebulized DNA in the desired size ranges (typically fractions 1 and 2) are separately phenol extracted and concentrated by ethanol precipitation prior to the kinase reaction.

[NextPage]

II. Random subclone generation

A. Sonication

The generation of DNA fragments by sonication is performed by placing a microcentrifuge tube containing the buffered DNA sample into an ice-water bath in a cup-horn sonicator and sonicating for a varying number of 10 second bursts using maximum output and continuous power (10), essentially as described by Bankier and Barrell (11). During sonication, temperature increases result in uneven fragment distribution patterns, and for that reason, the temperature of the bath is monitored carefully during sonication, and fresh ice-water is added when necessary. The exact conditions for sonication are determined for a given DNA sample before a preparative sonication is performed. Approximately 100 ug of DNA sample, in 350 ul of buffer, is distributed into ten aliquots of 35 ul, five of which are subjected to sonication for increasing numbers of 10 second bursts. Aliquots from each time point are electrophoresed on an agarose gel versus the phi-X 174 size marker (12) to determine the approximate DNA fragment size range for each sonication time point. Once optimal sonication conditions are determined, the remaining five DNA aliquots

(approximately 50 ug) are sonicated according to those pre-determined conditions. After sonication, the five tubes are placed in an ice-water bath until fragment end-repair and size selection, discussed below.

Protocol

1. Prepare the following DNA dilution, and aliquot 35 ul into ten 1.5 ml microcentrifuge tubes:

DNA	100 ug
10X TM buffer	35 ul
sterile ddH2O	q.s.
Final Volume	350 ul

2. To determine the optimal sonication conditions, sonicate the DNA samples in five of the tubes in a Heat Systems Ultrasonics W-375 cup horn sonicator set on 'HOLD', 'CONTINUOUS', and maximum 'OUTPUT CONTROL' = 10 under the following conditions:

Tube	No. 10 second bursts
1	1
2	2
3	3
4	4
5	5

We have recently learned that the Genome Center at Washington University and the Sanger Center set the OUTPUT CONTROL to the lowest possible settings. Because at present we use the Nebulizer (see the next section below), we have not investigated this further.

2. Cool the DNA samples by placing the tubes in an ice-water bath for at least 1 minute between each 10 second burst. Replace the ice-water bath in the cup horn sonicator between each sample.

3. Centrifuge the samples to reclaim condensation and electrophorese a 10 ul aliquot from each sonicated DNA sample on a agarose gel versus the phi-X 174/HaeIII size marker (Pharmacia 15611-015).

4. Based on the fragment size ranges detected from agarose gel electrophoresis, sonicate the remaining 5 tubes according to the optimal conditions and then place the tubes in a ice-water bath.

B. Nebulization

You can purchase Nebulizer, Number 4101 or 4101UO, from a local supplier, whose name you can obtain by calling the manufacturer:

IPI Medical Products Inc.

3217 North Kilpatrick

Chicago, IL 60641

phone: (773) 777-0900

The president of IPI is Walter Levine so if you have any troubles ordering them be sure to ask for him and/or to tell them that these devices are:

"NOT INTENDED FOR PATIENT USE"

Basically we follow a protocol sent to us by Steve Surzycki at the Department of Biology, Indiana University.

There are two small problems that we solved as follows:

1. You have to cover the hole where normally the mouth piece gets attached to; cover that hole with a cap QS-T from ISOLAB Inc. (Drawer 4350 Akron, OH 44303, 100 caps for $ 9.50).

2. The other problem that may occur is that the nebulizer leaks where the hose for the nitrogen gets attached. It seems that Nalgene tubing (VI grade 3/16" ID) seals better that the tubing which comes with the nebulizer. The nebulizer might still leak somewhat at the top, you can't avoid that.

Nebulizer Summary:

A nebulizer containing 2 ml of a buffered DNA solution (approximately 50 ug) containing 25-50% glycerol is placed in an ice-water bath and subjected to nitrogen gas at a pressure of 8-10 psi for 2.5 minutes for nebulizing BACs (10,13). Nitrogen gas pressure is the primary determinant of DNA fragment size, and although pressure studies should be performed with each BAC, cosmid or plasmid, a pressure of 8-10 psi almost always resulted in the desired (1kb-4kbp) fragment size range. As

discussed above for sonication, the use of an ice-water bath for nebulization also is critical to the generation of evenly distributed DNA fragments. During the nebulization process, unavoidable leaks are minimized by securely tightening the lid for nebulizer chamber and sealing the larger hole in the

top piece with a plastic cap. To prepare for fragment end-repair, the nebulized DNA typically is divided into four tubes and concentrated by ethanol precipitation.

Protocol

1. Modify a nebulizer (IPI Medical Products, Inc. 4207) by removing the plastic cylinder drip ring, cutting off the outer rim of the cylinder, inverting it and placing it back into the nebulizer. Seal the large hole inthe top cover (where the mouth piece was attached) with a plastic stopper and connect a 1/4 inch id length of Tygon tubing (which eventually should beconnected to a compressed air source) to the smaller hole.

2. Prepare the following DNA sample and place in the nebulizer cup:

DNA	50 ug
10X TM buffer	200 ul
sterile glycerol	0.5-1 ml
sterile ddH2O	q.s.
	2 ml

3. Nebulize in an ice-water bath at 30 psi for 2.5 minutes for plasmid, or 8-10 psi for 2.5 minutes for BACs, PACs, fosmids or cosmids.

4. Briefly centrifuge at 2500 rpm to collect the sample by placing the entire unit in the rotor bucket of a table top centrifuge (Beckman GPR tabletop centrifuge) fitted with pieces of styrofoam to cushion the plastic nebulizer.

5. Distribute the sample into four 1.5 ml microcentrifuge tubes and ethanol precipitate. Resuspend the dried DNA pellet in 35 ul of 1X TM buffer prior to proceeding with fragment end-repair.

C. Random fragment end-repair, size selection, and phosphorylation

Since both sonicated and nebulized DNA fragments usually contain single-stranded ends, the samples are end-repaired prior to ligation into blunt-ended vectors (10,11). A combination of T4 DNA polymerase and Klenow DNA polymerase are used to

"fill-in" the DNA fragments by catalyzing the 3'-5' incorporation of complementary nucleotides into resultant double-stranded fragments with a 5' overhang. Additionally, the single-stranded 3'-5' exonuclease activity of T4 DNA polymerase is used to degrade 3' overhangs. The reactions included the two enzymes, buffer, and deoxynucleotides and are incubated at 37degC.

Following fragment end-repair, the DNA samples are electrophoresed on a preparative low-melting temperature agarose gel versus the phi-X 174 marker, and after appropriate separation, the fragments in the size range from 1-2Kbp and 2-4Kbp are excised and eluted separately from the gel, as discussed above. Alternatively, the fragments can be purified by fractionation on a Sephacryl S-500 spin column as also discussed above. In both instances, the purified fragments are concentrated by ethanol precipitation followed by resuspension in kinase buffer, and phosphorylation using T4 polynucleotide kinase and rATP. The polynucleotide kinase is removed by phenol extraction and the DNA fragments are concentrated by ethanol precipitation, dried, resuspended in buffer, and ligated into blunt-ended cloning vectors. It should be noted that because a significant portion of nebulized DNA fragments are easily cloned without end-repair or kinase treatment, these two steps can be combined without significantly affecting the overall number of resulting transformed clones (see section V.B. on purification of PCR fragments for cloning, which describes a method for simultaneous end-repair and kinase treatment).

Protocol

1. To each tube containing 35 ul of DNA fragments (five of sonicated DNA and four of nebulized DNA), add:

0.25 mM dNTPs	2 ul
T4 DNA polymerase	3 ul (3 U/ul)
Klenow DNA polymerase	2 ul (5 U/ul)
	42 ul

T4 (203L) and Klenow (210L) DNA polymerases from New England Biolabs.

2. Incubate at room temperature for 30 minutes.

3a. Add 5 ul of agarose gel loading dye and apply to separate well of a 1% low gel

temperature agarose gel and electrophorese for 30-60 minutes at 100-120 mA.

4a. Elute the DNA from each sample lane, ethanol precipitate, and resuspend the dried DNA in 36 ul of sterile ddH2O and add 4 ul of 10X denaturing buffer. There should be five tubes for sonicated fragments and four tubes for nebulized fragments.

5a. Incubate at 70degC for 10 minutes, and place the samples in an ice-water bath.

6a. Add the following reagents for the kinase reaction and incubate at 37 degC for 10-30 minutes:

10 mM rATP	1 ul
10 X kinase buffer	5 ul
T4 polynucleotide kinase	1 ul (30 U/ul)
Final Volume	47 ul

T4 polynucleotide kinase (70031) from United States Biochemicals.

7a. Pool the kinase reactions, phenol extract, ethanol precipitate, and resuspend the dried DNA fragments in 40 ul of 10:0.1 TE buffer. This yields a typical concentration of 500-1000 ng/ul.

Alternatively the end-repair and phosphorylation steps can be combined:

1b. Resuspend DNA in 27 ul of 1X TM buffer. Add the following:

10X kinase buffer	5 ul
10 mM rATP	5 ul
0.25 mM dNTPs	7 ul
T4 polynucleotide kinase	1 ul (3 U/ul)
Klenow DNA polymerase	2 ul (5 U/ul)
T4 DNA polymerase	3 ul (3 U/ul)
Final Volume	50 ul

note: if the DNA has been sheared by nebulizing, the T4 DNA polymerase addition here may not be necessary.

2b. Incubate at 37degC for 30 minutes

3b. Add 5 ul of agarose gel loading dye and apply to separate well of a 1% low melting temperature agarose gel and electrophorese for 30-60 minutes at 100-120 mA.

4b. Elute the DNA from each sample lane, ethanol precipitate, resuspend in 10 ul of 10:0.1 TE buffer.

D. DNA ligation

DNA ligations are performed by incubating DNA fragments with appropriately linearized cloning vector in the presence of buffer, rATP, and T4 DNA ligase (10,11). For random shotgun cloning, sonicated or nebulized fragments are ligated to either SmaI linearized, dephosphorylated double-stranded M13 replicative form or pUC vector by incubation at 4degC overnight. A practical range of concentrations is determined based on the amount of initial DNA, and several different ligations, each with an amount of insert DNA within that range, are used to determine the appropriate insert to vector ratio for the ligation reaction. In addition, several control ligations are performed to test the efficiency of the blunt-ending process, the ligation reaction, and the quality of the vector (10,11). These usually included parallel ligations in the absence of insert DNA to determine the background clones arising from self-ligation of inefficiently phosphatased vector. Parallel ligations also are performed with a known blunt-ended insert or insert library, typically an AluI digest of a cosmid, to insure that the blunt-ended ligation reaction would yield sufficient insert containing clones, independent of the repair process.

Protocol

1. Combine the following reagents in a microcentrifuge tube, and incubate overnight at 4degC:

DNA fragments	100-1000 ng
cloning vector	2 ul (10 ng/ul)
10X ligation buffer	1 ul
T4 DNA ligase (NEB 202L)	1 ul (400 U/ul)
sterile ddH2O	q.s.
	10 ul

The cloning vector typically is SmaI-linearized, CIAP-dephosphorylated pUC vector (Pharmacia 27-4860-01) as several years ago we switched from M13 to pUC-based shotgun cloning. The advantage of obtaining two sequence reads off one isolated shotgun sub-clone seems to outweigh the disadvantage of a few bases less in double-stranded vs single-stranded read lengths. In some instances, including 5% PEG in the ligation reactions also seems to slightly improve the ligation efficiency.

2. Include control ligation reactions with no insert DNA and with a known blunt-ended insert (such as AluI digested cosmid).

E. Competent cell preparation

There are two main methods for preparation of competent bacterial cells (14) for transformation, the calcium chloride and the electroporation method. For the calcium chloride method, a glycerol cell culture stock of the respective E. coli strain is thawed and added to 50 ml of liquid media. This culture then is preincubated at 37degC for 1 hour, transferred to an incubator-shaker, and is incubated further for 2-3 hours. The cells are pelleted by centrifugation, resuspended in calcium chloride solution, and incubated in an ice-water bath. After another centrifugation step, the resulting cell pellet again is resuspended in calcium chloride to yield the final competent cell suspension. Competent cells are stored at 4degC, for up to several days.

Calcium Chloride Protocol

1. Thaw a frozen glycerol stock of the appropriate strain of E. coli, add it to an Erlenmeyer flask containing 50 ml of pre-warmed 2xTY (1) media, and pre-incubate in a 37degC water bath for 1 hour with no shaking. Further incubate for 2-3 hours at 37degC with shaking at 250 rpm.

2. Transfer 40 ml of the cells to a sterile 50 ml polypropylene centrifuge tube, and collect the cells by centrifugation at 3000 rpm for 8 minutes at 4deg C in a GPR centrifuge (Beckman) or 6000 rpm for 8 minutes at 4degC in an RC5-B centrifuge (DuPont) equipped with an SS-34 rotor. For M13-based transformation, save the remaining 10 ml of culture in an ice-water bath for later use.

3. After centrifugation, decant the supernatant and resuspend the cell pellet in one-half volume (20 ml) of cold, sterile 50 mM calcium chloride, incubate in an ice-water bath

for 20 minutes, and centrifuge as before.

4. Decant the supernatant and gently resuspend the cell pellet in one-tenth volume (4 ml) of cold, sterile 50 mM calcium chloride to yield the final competent cell suspension.

Preparation of calcium chloride competent cells for frozen storage

1. Transfer 166 ul of the competent cell suspension to sterile Falcon culture tubes.

2. Add 34 ul of sterile 100% glycerol to the 166 ul aliquots of the final competent cell suspension prepared above, giving a final concentration of 17 % glycerol.

3. The competent cells then should be placed at -70degC and can be stored indefinately.

4. To use competent cells for transformation, remove from freezer and thaw for a few minutes at 37degC. Place on ice, add plasmid DNA and incubate for one hour as in the standard transformation procedure. Then heat shock at 42degC for 2 minutes, cool briefly, add 1 ml of 2xTY and incubate for 1 hour at 37degC before spreading on plates.

Electroporation Protocol

Preparation of Electro-competent Cells:

1. Grow XL1-Blue cells on a tetracycline plate (20 ug tet/ml of LB agar)

2. Inoculate 3 ml of YENB and grow overnight at 37 degrees C with shaking at 250 rpm in the New Brunswick incubator shaker.

3. Inoculate the 3 ml of overnight growth into 1 liter of YENB (7.5 grams of Bacto Yeast Extract and 8 grams of Bacto Nutrient Broth brought to 1 liter with distilled water and autoclaved) and grow to an A600 of 0.5 (typically requires 3-4 hours of shaking at 250 rpm in the New Brunswick incubator shaker at 37 degrees C.

4. Distribute the 1 liter of cells into four 500 ml Sorval (GS-3) centrifuge bottles and centrifuge at 5000 rpm at 4 degrees C for 10 minutes.

Note: Steps 5-9 should be performed in the cold room and typically ~600 ml of ice cold sterile water and 150 ml of ice cold sterile 10% glycerol are required for manipulating the cells from a 1 liter growth.

5. Resuspend each pellet in 100 ml of ice cold sterile double distilled water and

combine the resuspended pellets into two Sorval centrifuge bottles (i.e each bottle then will contain 200 ml of resuspended pellet).

6. Centrifuge at 5000 rpm at 4 degrees C for 10 minutes in the Sorval GS-3 Rotor.

7. Resuspend each of the two pellets in 100 ml of ice cold sterile double distilled water and combine the resuspended pellets into one Sorval centrifuge bottle and centrifuge at 5000 rpm at 4 degrees C for 10 minutes in the Sorval GS-3 Rotor once more. Note: The purpose of all these centrifugation/resuspension/centrifugation steps is to insure that the cells are essentially "salt-free" as salt causes arching during the electroporation step.

8. Resuspend the pellet in 100 ml of 10% ice cold sterile glycerol, centrifuge as above, and finally resuspend the pellet in 2 ml of 10% ice cold sterile glycerol to give salt-free, concentrated electrocompetent cells.

9. Aliquote 40 ul of these electrocompetent cells into small snap cap tubes and immediately freeze by placing in curshed dry ice and then store at -70 degrees C until needed.

Electroporation Protocol for transformations using double-stranded plasmids

1. Thaw the electro-competent cells on ice for about one minute.

2. Add 2-3 ul of the ligation mix to the cells.

3. transfer 40 ul of the cells into to BTX Electroporation cuvettes PLUS and MAKE SURE THAT THE CELLS COVER THE BOTTOM OF THE CUVETTE.

4. Turn on the Bio Rad E. coli Pulser and set the current to 2.5 KV by pushing the "Lower" and "Raise" bottoms simultaneously twice.

5. Place the cuvette in the holder and slide it into position.

6. Charge by pressing the "Charge" bottom until you hear the beep.

7. Immediately, suspend the cells in 1 ml of YENB and transfer into a Falcon tube.

8. Incubate the cells at 37 degrees C for 30 minutes at 250 rpm shaker.

9. Spin the cells in BECKMAN table-top centrifuge for 8 minutes at 2500 rpm

10. Resuspend the cells in 200 ul fresh YENB and add 30 ul of 20 mg/ml XGAL and 30 ul of 25 mg/ml IPTG

11. Plate ~130 ul of the cells on pre-warmed LB-amp plates.

Reference:

Rakesh C. Sharma and Robert T. Schimke, "Preparation of Electro-competent E. coli Using Salt-free Growth Medium", Biotechniques 20, 42-44 (1996).

F. Calcium Chloride treated bacterial cell transformation

A brief background discussion of transformation and transfection can be found in the Appendix.

For DNA transformation (14,15), the entire DNA ligation reaction is added to an aliquot of competent cells, which is mixed gently, and incubated in an ice-water bath. This mixture then is heat-shocked briefly in a 42degC water bath for 2-5 minutes. At this point in the transformation, the method varied slightly depending on whether the cloning vector is M13-based or pUC-based.

For M13-based transformation (14), an aliquot of non-competent cells is added to the heat-shocked mixture, as is the lac operon inducer homologue, IPTG, and the b-galactosidase chromogenic substrate, x-gal. Melted top agar is added, and the transformation mixture then is poured onto the surface of an agar plate. After the top agar solidified, the plates are inverted and incubated overnight at 37degC.

For pUC-based transformation (15), an aliquot of liquid media is added to the heat-shocked mixture, which then is incubated in a 37degC water bath for 15-20 minutes. After recovery, the cell suspension is concentrated by centrifugation and then gently resuspended in a smaller volume of fresh liquid media. IPTG and x-gal are added to the cell mixture, which is spread onto the surface of an ampicillin-containing agar plate. After the cell mixture had diffused into the agar medium, the plates are inverted and incubated overnight at 37degC.

Protocol

1. Add the entire ligation reaction to a 12 X 75 Falcon tube containing 0.2-0.3 ml of competent cells, mix gently, and incubate in an ice-water bath for 40-60 minutes. (For retransformation of recombinant DNA, add approximately 10-100 ng of DNA directly to competent cells).

2. Heat shock the cells by incubation at 42degC for 2-5 minutes.

For M13-based transformation:

3a. Add the following reagents to the heat shocked transformation mixture:

Non-competent cells	0.2 ml
IPTG (25 mg/ml H2O)	25 ul
x-gal (20 ml/ml DMF)	25 ul
lambda top agar	2.5 ml

4a. Mix by briefly vortexing, and then quickly pour onto the surface of a pre-warmed lambda agar plate.

5a. Allow 10-20 minutes for the agar to harden, and then invert and incubate overnight at 37degC.

For pUC-based transformation:

3b. Add the following reagents to the heat shocked transformation mixture, add 1 ml of fresh 2xTY and incubate in a 37degC water bath for 15-30 minutes.

4b. Collect the cells by centrifugation at 3000 rpm for 5 minutes, decant the supernatant, and gently resuspend in 0.2 ml of fresh 2xTY.

5b. Add 25 ul IPTG (25 mg/ml water) and 25 ul x-gal (20 mg/ml DMF), mix and pour onto the surface of a pre-warmed LB-Amp plate. Spread over the agar surface using a sterile bent glass rod or sterile inoculating loop.

6b. Allow 10-20 minutes for the liquid to diffuse into the agar, and then invert and incubate overnight at 37degC.

For pBR322, pAT153 or other non-lacZ containing vectors:

3b. Add 1 ml of fresh 2xTY to the cells and incubate for 15-30 minutes at 37 degC. Spread approximately 50 ul on L plates containing antibiotic using a sterile glass spreader. Incubate the plates overnight at 37degC.

G. Microcentrifuge Tube Transformation

Microcentrifuge transformations are recommended when a single plasmid is being retransformed or for qualitative transformation experiments. Shotgun cloning experiments should be transformed using the large scale transformation, since the objective is to efficiently obtain transformation of hundreds of distinct recombinant plasmids.

1. Inoculate 50 ml of fresh 2xTY media with 3 to 5 ml of a fresh overnight culture of

a suitable host strain (GM272) and incubate for 2 to 3 hours at 37deg C.

2. Transfer 1 ml of the culture into a 1.5 ml tube and centrifuge for 5 min at room temperature. Use 1 tube of culture per DNA sample to be transformed.

3. Decant supernatant, and resuspend the cell pellet in 500 ul (1/2 volume) of sterile, cold 50 mM calcium chloride. Gently vortex if necessary.

4. Incubate 5 min. on ice.

5. Centrifuge as before, decant and resuspend the competent cell pellet in 100 ul (1/10 volume) of calcium chloride.

6. Transfer each 100 ul sample of competent cells to chilled 12 x 75 mm Falcon tubes which contain 3 to 5 ul of DNA sample (about 2 ng/ul to 20 ng/ul).

7. Incubate on ice for 15 minutes.

8. Heat shock the sample at 42degC for 5 minutes.

9. Add 1 ml of fresh 2xTY to each sample and recover the cells by incubating at 37degC for 15 min.

10. For lacZ containing vectors add 25 ul of 20 mg/ml IPTG (in water) and 25 ul of 24 mg/ml X-Gal (in DMF).

11. Add 2.5 ml of soft top agar to each sample, vortex and quickly pour onto the surface of a TYE-AMP agar plate. Allow at least 15-30 min. for the agar to solidify.

12. Invert the plates and incubate overnight at 37degC.

[NextPage]

III. Methods for DNA isolation

A. Large scale double-stranded DNA isolation

The method used for the isolation of large scale cosmid and plasmid DNA is an unpublished modification (16) of an alkaline lysis procedure (17,18) followed by equilibrium ultracentrifugation in cesium chloride-ethidium bromide gradients (1). Briefly, cells containing the desired plasmid or cosmid are harvested by centrifugation, incubated in a lysozyme buffer, and treated with alkaline detergent. Detergent solubilized proteins and membranes are precipitated with sodium acetate, and the lysate is cleared first by filtration of precipitate through cheesecloth and then by centrifugation. The DNA-containing supernatant is transferred to a new tube, and the

plasmid or cosmid DNA is precipitated by the addition of polyethylene glycol and collected by centrifugation. The DNA pellet is resuspended in a buffer containing cesium chloride and ethidium bromide, which is loaded into polyallomer tubes and subjected to ultracentrifugation overnight. The ethidium bromide stained plasmid or cosmid DNA bands, equilibrated within the cesium chloride density gradient after ultracentrifugation, are visualized under long wave UV light and the lower band is removed with a 5 cc syringe. The intercalating ethidium bromide is separated from the DNA by loading the solution onto an equilibrated ion exchange column. The A260 containing fractions are pooled, diluted, and ethanol precipitated, and the final DNA pellet is resuspended in buffer and assayed by restriction digestion as detected on agarose gel electrophoresis.

During the course of this work several modifications to the above protocol were made. For example, initially cell growth times included three successive overnight incubations, beginning with the initial inoculation of 3 ml of antibiotic containing media with the plasmid or cosmid-containing bacterial colony, and then increasing the culture volume to 50 ml, and then to 4 l. However, it was observed that recombinant cosmid DNA isolated from cell cultures grown under these conditions, in contrast to recombinant plasmid DNA, was contaminated with deleted cosmid DNA molecules. However, these deletions are avoided by performing each of the three successive incubations for eight hours instead of overnight, although a slight yield loss accompanied the reduced growth times.

Recently, a diatomaceous earth-based (19-22) method was used to isolate the plasmid or cosmid DNA from a cell lysate. The cell growth, lysis, and cleared lysate steps are performed as described above, but following DNA precipitation by polyethylene glycol, the DNA pellet is resuspended in RNase buffer and treated with RNase A and T1. Nuclease treatment is necessary to remove the RNA by digestion since RNA competes with the DNA for binding to the diatomaceous earth. After RNase treatment, the DNA containing supernatant is bound to diatomaceous earth in a chaotropic buffer of guanidine hydrochloride by incubation at room temperature. The DNA-associated diatomaceous earth then is collected by centrifugation, washed several times with

ethanol buffer and acetone, dried, and then resuspended in buffer. The DNA is eluted during incubation at 65degC, and the DNA-containing supernatant is collected after centrifugation and separation of the diatomaceous earth particles. The DNA recovery is measured by taking absorbance readings at 260 nanometers. After concentration by ethanol precipitation, the DNA is assayed by restriction digestion.

Protocol

1. Pick a colony of bacteria harboring the plasmid or cosmid DNA of interest into a 12 X 75 mm Falcon tube containing 2 ml of LB media supplemented with the appropriate antibiotic (typically ampicillin at 100 ug/ml) and incubate at 37deg C 8-10 hours with shaking at 250 rpm. Transfer the culture to an Ehrlenmeyer flask containing 50 ml of similar media, and incubate further for 8-10 hours. Transfer 12.5 ml of the culture to each of 4 liters of similar media, and incubate for an additional 8-10 hours.

2. Harvest the cells by centrifugation at 7000 rpm for 20 minutes in 500 ml bottles in the RC5-B using the GS3 rotor. Resuspend the cell pellets in old media and transfer to two bottles, centrifuge as before, and decant the media. The cell pellets can be frozen at -70degC at this point.

3. Resuspend the cell pellets in a total of 70 ml of GET/Lysozyme solution (35 ml for each bottle) by gently teasing the pellet with a spatula and incubate for 10 minutes at room temperature. (Note: Do not vortex the lysate at any time because this may shear the chromosomal DNA).

4. Add a total of 140 ml of alkaline lysis solution (70 ml for each bottle), gently mix, and incubate for 5 minutes in an ice-water bath.

5. Add 105 ml of 3M NaOAc, pH 4.8 (52.5 ml for each bottle), cap tightly, gently mix by inverting the bottle a few times, and incubate in an ice-water bath for 30-60 minutes.

6. Clear the lysate of precipitated SDS, proteins, membranes, and chromosomal DNA by pouring through a double-layer of cheesecloth. Transfer the lysate into 250 ml centrifuge bottle, centrifuge at 10,000 rpm for 30 minutes at 4deg C in the RC5-B using the GSA rotor.

For cesium chloride-gradient purification:

7a. Pool the cleared supernatants into to a clean beaker, add one-fourth volume of 50% PEG/0.5 M NaCl, swirl to mix, and incubate in an ice-water bath for 1-2 hours.

8a. Collect the PEG-precipitated DNA by centrifugation in 250 ml bottles at 7000 rpm for 20 minutes at 4degC in the RC5-B using the GSA rotor.

9a. Dissolve the pellets in a combined total of 32 ml of 100:10 TE buffer, 5 ml of 5 mg/ml ethidium bromide, and 37 g cesium chloride (Var Lac Oid Chemical Co., Inc.) (final concentration of cesium chloride should be 1 g/ml).

10a. Transfer the sample into 35 ml polyallomer centrifuge tubes, remove air bubbles, seal with rubber stoppers, and crimp properly.

11a. Centrifuge at 60,000 rpm to 16-20 hours at 15-20degC in the Sorvall OTD-75B ultracentrifuge (DuPont) using the T-865 rotor.

12a. Visualize the ethidium bromide stained DNA under long-wave UV light, and remove the lower DNA band using a 5 cc syringe and a 25 gauge needle. (It may be helpful first to remove and discard the upper band).

13a. To remove the ethidium bromide, load the DNA sample onto an equilibrated 1.5 ml Dowex column, and collect 0.5 ml fractions. Equilibrate the Dowex AG resin (BioRad) by successive centrifugation, resuspension, and decanting with 1M NaOH, water, and then 1M Tris-HCl, pH 7.6 until the Dowex solution has a pH of 7.6.

14a. Pool fractions with an A260 of 1.00 or greater into 35 ml Corex glass tubes, add one volume of ddH2O, and ethanol precipitate by adding 2.5 volumes of cold 95% ethanol. Incubate at least 2 hours at -20degC, centrifuge at 10,000 rpm for 45 minutes in the RC5-B using the SS-34 rotor. Gently decant the supernatant, add 80% ethanol, centrifuge as before, decant, and dry the DNA pellet in a vacuum oven.

15a. Resuspend the DNA in 10:0.1 TE buffer.

For diatomaceous earth-based purification:

7b. Pool the supernatants from step 6 into 500 ml bottles and add DNase-free RNase A and RNase T1 such that the final concentration of RNase A is 40 ug/ml and RNase T1 is 40 U/ml. Incubate in a 37degC water bath for 30 minutes.

8b. Add an equal volume of isopropanol and precipitate at room temperature for 5

minutes. Centrifuge at 9,000 rpm for 30 minutes in the RC5-B using the GS3 rotor. Decant the supernatant and drain the DNA pellet.

9b. Resuspend each DNA pellet in 20 ml 10:1 TE buffer, and add 40 ml of de-fined diatomaceous earth in guanidine-HCl (100 mg/ml) to each bottle. Allow the DNA to bind at room temperature for 5 minutes with occasional mixing. Centrifuge at 9,000 for 10 minutes in the RC5-B using the GS3 rotor.

10b. Decant the supernatant, resuspend each pellet in 40 ml of diatomaceous earth-wash buffer, and centrifuge as above.

11b. Decant the supernatant, resuspend each pellet in 40 ml of acetone, and centrifuge as above.

12b. Decant the supernatant and dry the pellet in a vacuum oven.

13b. Resuspend the pellet in 20 ml of 10:1 TE buffer, and elute the bound DNA by incubation at 65degC for 10 minutes with intermittent mixing.

14b. Remove the diatomaceous earth by centrifugation at 9,000 rpm for 10 minutes in the RC5-B using the GS3 rotor. Repeat if necessary.

15b. Combine the DNA-containing supernatants and precipitate the DNA in 35 ml Corex glass tubes adding 2.5 volumes of cold 95% ethanol/acetate.

16b. Resuspend the dried DNA pellet in 2 ml of 10:0.1 TE buffer and assay for concentration by absorbance readings at 260 nm or by agarose gel electrophoresis.

B. Midiprep double-stranded DNA isolation

A midi-prep double-stranded DNA isolation has been developed to generate a sufficient amount of template DNA for several Sequenase[TM] catalyzed fluorescent terminator reactions. Here, one bacterial colony which harbored the plasmid of interest is picked into 3 ml of liquid media containing ampicillin and incubated in a 37degC shaker for 8-10 hours. At this time, the culture is transferred into 50 ml of ampicillin-containing media and incubated further for 10-12 hours. After harvesting the cells by centrifugation, a diatomaceous earth-based alkaline-lysis purification method (19-22) is performed, similar to that discussed above for large scale DNA isolation. The purified DNA is crudely assayed for concentration and purity by agarose gel electrophoresis against known standards. The approximate yield of

double-stranded DNA using this method is 1 ug of DNA per ml of cell culture. For a 50 ml cell culture, about 50 ug of DNA are recovered, and 5 ug are used typically in a Sequenase[TM] terminator reaction.

Note: This procedure is the method of choice for isolating double stranded plasmid-based templates for the Sequenase Dye-Labeled Terminator Sequencing Reactions.

Protocol

1. Pick a colony of bacteria harboring the plasmid DNA of interest into a 12 X 75 mm Falcon tube containing 3 ml of 2xTY media supplemented with the appropriate antibiotic (typically ampicillin at 100 ug/ml) and incubate at 37deg C 8-10 hours with shaking at 250 rpm. Transfer the culture to an Ehrlenmeyer flask containing 50 ml of similar media, and incubate further for 11-14 hours.

2. Harvest the cells by centrifugation at 3000 rpm for 5 minutes in 50 ml conical tubes in the Beckman GPR tabletop centrifuge and decant the supernatant. The cell pellets can be frozen at -70degC at this point.

3. Resuspend the cell pellets in 2 ml of GET/Lysozyme solution, add 4 ml of alkaline lysis solution, gently mix, and incubate for 5 minutes in an ice-water bath.

4. Add 4 ml of 3M NaOAc, pH 4.8, gently mix by swirling, and incubate in an ice-water bath for 30-60 minutes.

5. Clear the lysate of precipitated SDS, proteins, membranes, and chromosomal DNA by pouring through a double-layer of cheesecloth into a new 50 ml conical tube. Centrifuge at 3,000 rpm for 20 minutes at 4degC in the Beckman GPR tabletop centrifuge.

6. Decant the supernatant to a 50 ml polypropylene centrifuge tube, add 20 ul of a 20 mg/ml DNase-free RNase A and incubate in a 37degC water bath for 30 minutes.

7. Add 7 ml (equal volume) of de-fined diatomaceous earth in guanidine-HCl (20 mg/ml) and allow the DNA to bind at room temperature for 5 minutes with occasional mixing. Centrifuge at 3,000 for 5 minutes in the Beckman GPR tabletop centrifuge.

8. Decant the supernatant, resuspend in 7 ml of diatomaceous earth-wash buffer, and centrifuge as above.

9. Decant the supernatant, resuspend in 7 ml of acetone, and centrifuge as above.

10. Decant the supernatant and dry in a vacuum oven.

11. Resuspend the pellet in 0.6 ml of 10:1 TE buffer, and elute the bound DNA by incubation at 65degC for 10 minutes with intermittent mixing.

12. Remove the diatomaceous earth by centrifugation at 3,000 rpm for 5 minutes in the in the Beckman GPR tabletop centrifuge.

13. Transfer the supernatant to a 1.5 ml microcentrifuge tube and centrifuge at 12,000 rpm for 5 minutes in a microcentrifuge at room temperature. Transfer the supernatant to a new 1.5 ml microcentrifuge tube and ethanol precipitate.

14. Resuspend the dried DNA pellet in 40 ul of 10:0.1 TE buffer and assay for concentration by agarose gel electrophoresis.

C. Miniprep double-stranded DNA isolation

The standard method for the miniprep isolation of plasmid DNA includes the same general strategy as the large scale isolation. However, smaller aliquots of antibiotic containing liquid media inoculated with plasmid-containing cell colonies are incubated in a 37degC shaker for 12-16 hours. After collecting the plasmid containing cells by centrifugation, the cell pellet is resuspended in a hypotonic sucrose buffer. The cells are successively incubated with an RNase-lysis buffer, alkaline detergent, and sodium acetate. The lysate is cleared of precipitated proteins and membranes by centrifugation, and the plasmid DNA is recovered from the supernatant by isopropanol precipitation. The DNA is crudely checked for concentration and purity using agarose gel electrophoresis against known standards. A typical yield for this method of DNA isolation is 10-15 ug of plasmid DNA from a 6 ml starting culture.

Since highly supercoiled DNA is desired for double-stranded DNA sequencing, a modification of this method employing diatomaceous earth (19-22) sometimes is used for isolation of double-stranded templates for DNA sequencing with fluorescent primers. After removal of the precipitated proteins and membranes, the plasmid containing supernatant is incubated with diatomaceous earth and guanidine hydrochloride and this mixture is added into one of the twenty-four wells in the BioRad Gene Prep Manifold. The supernatant is removed by vacuum filtration over a

nitrocellulose filter. The DNA-associated diatomaceous earth is washed to remove the guanidine hydrochloride with an ethanol buffer, and then dried by filtration. Elution buffer is added to the wells, and the DNA-containing solution then is separated from the diatomaceous earth particle by filtration into a collection tube. The collected DNA is concentrated by ethanol precipitation and crudely assayed for concentration and purity by agarose gel electrophoresis against known standards. The approximate yield of double-stranded DNA is 3-5 ug of DNA from 6 ml of starting culture.

Note: This is a typical mini-prep until step 7, where in step 7a you would precipitate the template and use it for Taq Cycle Sequencing with the Dye-Labeled Primers, or in step 7b proceed with the diatomaceous earth purification for Taq Dye-Labeled Terminator Cycle Sequencing Reactions. For Sequenase Dye-Labeled Terminator Sequencing Reactions use the Midi-prep procedure detailed above.

Protocol

1. Pick a colony of bacteria harboring the plasmid DNA of interest into a 17 X 100 mm Falcon tube containing 6 ml of TB media supplemented with the appropriate antibiotic (typically ampicillin at 100 ug/ml) and incubate at 37deg C 16-18 hours with shaking at 250 rpm.

2. Harvest the cells by centrifugation at 3000 rpm for 5 minutes in the Beckman GPR tabletop centrifuge and decant the supernatant. The cell pellets can be frozen at -70degC at this point.

3. Resuspend the cell pellets in 0.2 ml of TE-RNase solution (50:10 TE buffer containing 40 ug/ml RNase A; some also add RNase T1 to a final concentration of 10 U/ul) by gentle vortexing, add 0.2 ml of alkaline lysis solution, gently mix, and incubate for 15 minutes at room temperature.

4. Add 0.2 ml of 3M NaOAc, pH 4.8, gently mix by swirling, transfer to 1.5 ml microcentrifuge tubes, and incubate in an ice-water bath for 15 minutes.

5. Clear the lysate of precipitated SDS, proteins, membranes, and chromosomal DNA by centrifugation at 12,000 rpm for 15 minutes in a microcentrifuge at 4deg C.

6. Transfer the supernatant to a fresh 1.5 ml microcentrifuge tube, incubate in an ice-water bath for 15 minutes, centrifuge as above for an additional 15 minutes and

transfer the supernatant to a clean 1.5 ml tube.

For standard alkaline lysis purification:

7a. Precipitate the DNA by adding 1 ml of 95% ethanol, and resuspend the dried DNA pellet in 100-200 ul 10:0.1 TE buffer. Electrophorese an aliquot of the DNA sample on a 0.7% agarose gel to crudely determine the concentration and purity.

For diatomaceous earth-base purification:

7b. Add 1 ml of de-fined diatomaceous earth in guanidine-HCl (20 mg/ml) and allow the DNA to bind at room temperature for 5 minutes with occasional mixing. Meanwhile soak the Prep-A-Gene nitrocellulose membrane in isopropanol for at least 3 minutes, and assemble the Prep-A-Gene manifold as described in the manual.

8b. Turn on the vacuum pump and adjust the vacuum level to 8 in. Hg, let the membrane dry for 1 minute, and then release the vacuum.

9b. Pour the well mixed samples into the wells of the Prep-A-Gene manifold and filter through at 8 in. Hg until all the liquid is filtered through.

10b. Wash the samples four times with 250 ul of diatomaceous earth-wash buffer, using a repeat pipette, allowing all of the liquid to filter through between washes.

11b. Reduce the vacuum to 5 in. Hg before turning the vacuum off at the stopcock. Without unscrewing the black clamps, release the white clamps and place the collection rack with clean 1.5 ml screw-capped tubes into the manifold. Clamp the manifold with the white clamps, and apply 300 ul of 10:1 TE buffer heated to 65degC and pull the eluted DNA through at 5 in. Hg. After the liquid has filtered through, raise the vacuum to 10-12 in. Hg, and let the membrane dry for 1 minute.

12b. Turn off the vacuum at the stopcock and remove the collection rack containing the tubes. Ethanol precipitate the DNA and resuspend the dried DNA pellet in 30 ul of 10:0.1 TE buffer.

D. Large scale M13RF isolation (9)

Double-stranded M13RF is isolated for use in M13 SmaI cut, dephosphorylated vector preparation, described below. The growth conditions of M13-infected bacterial cells (see Figure 1) appears convoluted, but result in a maximal amount of M13 RF molecules per cell. After the M13RF containing bacterial cells are harvested by

centrifugation, the double-stranded molecules are isolated using the cesium chloride method for large scale plasmid isolation, as described above. This briefly entailed alkaline cell lysis, sodium acetate precipitation of detergent solubilized proteins and membranes, polyethylene glycol DNA precipitation, and extraction of ethidium bromide stained DNA from a cesium chloride gradient after ultracentrifugation. After removal of the ethidium bromide on an ion-exchange column, the DNA containing fractions are detected by A260 measurement and pooled, and the DNA is concentrated by ethanol precipitation and assayed by restriction enzyme digestion and agarose gel electrophoresis.

Protocol

1. Prepare an early log phase culture of JM101 by inoculating an Ehrlenmeyer flask containing 50 ml of 2xTY with a glycerol stock of JM101 and pre-incubating for 1 hour in a 37degC water bath, with no shaking. Pick a plaque representing the desired M13 clone into four 1.5 ml aliquot of early log phase JM101, and incubate according to the procedure displayed in Figure 1 to result in 4 liters of M13-infected bacteria.

2. Harvest the cells by centrifugation at 7000 rpm for 20 minutes in 500 ml bottles in the RC5-B using the GS3 rotor. Resuspend the cell pellets in fresh 2xTY media to remove contaminating extracellular phage and transfer to two bottles, centrifuge as before, and decant the media. The cell pellets can be frozen at -70degC at this point.

3. Resuspend the cell pellets in a total of 120 ml (30 ml for each bottle) of 1X STB buffer by gently teasing the pellet with a spatula. Add a total 24 ml of lysozyme solution (6 ml for each bottle), gently mix, and incubate for 5 minutes in an ice-water bath.

4. Add 48 ml of 50:2:10 TTE buffer (12 ml for each bottle) and 2 ml of RNase A (10 mg/ml) (0.5 ml for each bottle), gently mix, and incubate in an ice-water bath for 5 minutes.

5. Clear the lysate of precipitated SDS, proteins, membranes, and chromosomal DNA by pouring through a double-layer of cheesecloth. Transfer the lysate into 250 ml centrifuge bottle, centrifuge at 10,000 rpm for 30 minutes at 4deg C in the RC5-B using the GSA rotor.

6. Add 6 ml of 5 mg/ml ethidium bromide, and cesium chloride such that the final concentration of cesium chloride is 1 g/ml.

7. Transfer the sample into 35 ml polyallomer centrifuge tubes and top off with a 1:1 solution of 100:10 TE buffer and cesium chloride, remove air bubbles, seal with rubber stoppers, and crimp properly.

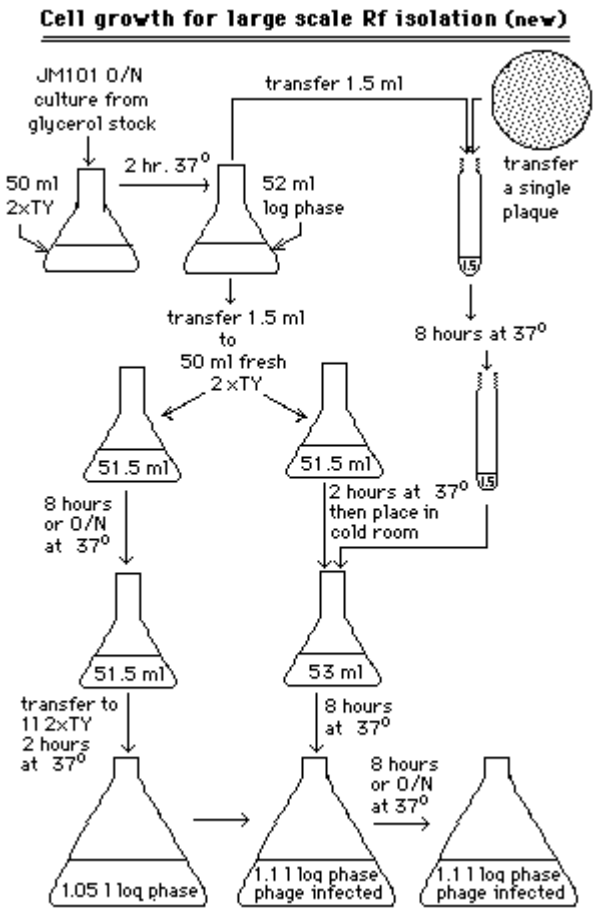
8. Centrifuge at 60,000 rpm to 16-20 hours at 15-20degC in the Sorvall OTD-75B ultracentrifuge using the T-865 rotor.

9. Visualize the ethidium bromide stained DNA under long-wave UV light, and remove the lower DNA band using a 5 cc syringe and a 25 gauge needle. (It may be helpful to remove and discard the upper band first).

10. To remove the ethidium bromide, load the DNA sample onto an 1.5 ml Dowex AG (BioRad) column, equilibrated as before, and collect 0.5 ml fractions.

11. Pool fractions with an A260 of 1.00 or greater into 35 ml Corex glass tubes, add one volume of ddH2O, and ethanol precipitate by adding 2.5 volumes of cold 95% ethanol. Incubate at least 2 hours at -20degC, centrifuge at 10,000 rpm for 45 minutes in the RC5-B using the SS-34 rotor. Gently decant the supernatant, add 80% ethanol, centrifuge as before, decant, and dry the DNA pellet in a vacuum oven.

12. Resuspend the DNA in 10:0.1 TE buffer.



E. Single-stranded M13 DNA isolation using phenol

This isolation procedure (23) is the method of choice for preparation of M13-based templates to be used in Sequenase[TM] catalyzed dye-terminator reactions. A pre-incubated early log phase JM101 culture is prepared by transferring a thawed glycerol stock into 50 ml of liquid media and incubating for 1 hour at 37degC with no shaking. M13 plaques are picked with a sterile toothpick and placed into 1.5 ml aliquots of the early log phase JM101 culture, which are incubated in a 37deg C shaker for 4-6 hours. After incubation, the bacterial cells are pelleted by centrifugation and the viral containing supernatant is transferred to a clean tube. The phage particle are precipitated with PEG, collected by centrifugation, and the pellet is resuspended in buffer. The phage protein coat is denatured and removed by one phenol and two ether extractions. After ethanol precipitation, the dried DNA pellet is resuspended in buffer, and the concentration and purity crudely are assessed by agarose gel electrophoresis against known standards.

Protocol

1. Prepare an early log phase culture of JM101, as above, and pick M13-based plaques with sterile toothpicks into 12 X 75 mm Falcon tubes containing 1.5 ml aliquots of the cells. Incubate for 4-6 hours at 37degC with shaking at 250 rpm.

2. Transfer the culture to 1.5 ml microcentrifuge tubes and centrifuge for 15 minutes at 12,000 rpm at 4degC.

3. Pipette the top 1 ml of supernatant to a fresh 1.5 ml microcentrifuge tube containing 0.2 ml 20% PEG/2.5 M NaCl to precipitate the phage particles. Mix by inverting several times and incubate for 15-30 minutes at room temperature.

4. Centrifuge for 15 minutes at 12,000 rpm at 4degC to collect the precipitated phage. Decant the supernatant and remove residual PEG supernatant by suctioning twice.

5. Resuspend the pellet in 100 ul of 10 mM Tris-HCl, pH 7.6 by vortexing, and add 50 ul of TE-saturated phenol.

6. Extract the DNA with phenol and twice with ether, as discussed above, and then ethanol precipitate.

7. Resuspend the dried DNA in 6 ul of 10:0.1 TE for use in single-stranded Sequenase[TM] catalyzed dye-terminator sequencing reactions.

F. Biomek-automated modified-Eperon isolation procedure for single-stranded M13 DNA

This semi-automated method is a modification of a previously reported procedure (24,25), and allowed the simultaneous isolation of 48 single-stranded DNAs per Biomek 1000 robotic workstation within 3 hours (26). Basically, M13 plaques are picked with sterile toothpicks into aliquots of early log phase JM101, prepared as discussed above. The phage infected cultures are incubated in a 37degC shaker for 4-6 hours, transferred into microcentrifuge tubes, centrifuged to separate bacterial cells from the viral supernatant, and then carefully placed on the Biomek tablet. For each sample, two 250 ul aliquots are robotically distributed into two wells of a 96-well microtiter plate, and this process is repeated for each of the 48 samples until the entire 96 wells are filled. A solution of polyethylene glycol (PEG) then is added robotically to each well and mixed. The microtiter plate is covered with an acetate plate sealer,

incubated at room temperature to precipitated the phage particles, and then centrifuged. The supernatant then is removed by inverting the plate and gently draining on a paper towel, without dislodging the pellet. After placing the microtiter plate back on the Biomek, a more dilute PEG solution is robotically added to each well. The plate then is covered with another sealer and centrifuged again. This rinse step aided in the removal of contaminating proteins and RNA. After removing the supernatant, as before, and placing the microtiter plate back on the Biomek, a Triton X-100 detergent solution is robotically added to each well. The plate is agitated gently and the sample from each pair of wells is robotically transferred to microcentrifuge tubes, which then are capped and placed in an 80deg C water bath for 10 minutes to aid in the detergent solubilization of phage coat proteins. After a brief centrifugation to collect condensation, the single-stranded DNA is ethanol precipitated, dried, and resuspended. An aliquot from each DNA sample is subjected to agarose gel electrophoresis to crudely assay concentration and purity. The yield of single-stranded template is approximately 2-3 ug per sample.

Protocol

The entire procedure will require 9 rows of P250 tips (counting from the center of the Biomek tablet towards the left) for the isolation of 48 templates (48ISOL). The reagent module should contain PEG-2000, Triton-Tris-EDTA, and ethanol-acetate, respectively.

1. Prepare an early log phase JM101 culture in 50 ml of 2xTY, as above.

2. Using sterile toothpicks, transfer individual M13 plaques into 12 X 75 mm Falcon tubes containing 1 ml early log phase cell cultures, and incubate for 4-6 hours at 37degC with shaking at 250 rpm. (Growth for longer than 6 hours results in cell lysis and contamination of the phage DNA by cellular proteins and nucleic acids).

3. Separate the bacterial cells from the viral-containing supernatant by centrifugation at 12,000 rpm for 15 minutes at 4degC. Carefully open the tubes and place on the Biomek tablet..

4. The Biomek will distribute two 250 ul aliquots of viral supernatant per sample into the wells of a 96-well flat-bottomed microtiter plate (Dynatech). The Biomek then

will add 50 ul of 20% PEG/2.5 M NaCl solution to each well, and mix by pipetting up and down.

5. Cover the plate with an acetate plate sealer and incubate at room temperature for 15 minutes.

6. Pellet the precipitated phage by centrifuging the plate at 2400 rpm for 20 minutes in a Beckman GPR tabletop centrifuge. Remove the plate sealer and drain the PEG from the plate by gently draining upside down on a Kimwipe.

7. Return the plate to the tablet, and the Biomek will robotically add 200 ul of PEG:TE rinse solution to each well. Cover the plate with a plate sealer, centrifuge, and drain, as above.

8. Return the plate to the tablet, and the Biomek will add 70 ul of TTE solution to each well. Remove and gently agitate to resuspend.

9. The Biomek then will robotically pool the contents from each pair of wells into 1.5 ml microcentrifuge tubes.

10. Incubate the tubes at 80degC for 10 minutes to denature the viral protein coat and then centrifuge briefly to reclaim condensation.

11. Ethanol precipitate the DNA by adding 500 ul ethanol/acetate to each tube, as described above.

12. Resuspend the DNA templates in 20 ul of 10:0.1 TE buffer.

G. 96 well double-stranded template isolation

A manual as well as an automated procedure is given below. The automated method is a modification of a previously reported procedure (4) which allows simultaneous isolation of 96 double stranded DNAs per Biomek 1000 Automated Laboratory Workstation within two hours. Basically colonies containing double-stranded plasmids are picked with sterile toothpicks into media and incubated at 37degC for 24 hours with shaking at 350 rpm. These cells are harvested by centrifugation and the pellets are either manually or robotically resuspended by the addition of TE-RNase solution. An alkaline lysis solution is used to lyse the cells and the lysate is precipitated with KOAc. The lysate is cleared by filtration and further concentrated by ethanol precipitation. An aliquot from each DNA sample is subjected to agarose gel

eletrophoresis to crudely assay concentration and purity. The yield of double stranded template is approximately 3 mg per sample.

Protocol

Manual Double stranded isolation method

The following is a manual, 96 well, double stranded sequencing template isolation procedure that has been developed in our laboratory. A similar procedure that has been automated on the Biomek is presented elsewhere herein.

1. Pick individual shotgun clones off a plate with a steril tooth pick and deposit each separately into 96 well block containing 1.75 ml of TB media per well. Keep toothpick in media for about 5 minutes to allow the cells to defuse into the media, remove the toothpicks, cover the 96 well block with the loose fitting lid, and allow the cells to grow for 24 hours in the 37degC shaker/incubator at 350 rpm.

2. Remove block from the shaker/incubator and collect the cells by centrifugation at 2500 rpm for 7 minutes. The cells can be stored frozen at -20degC in the block at this stage.

3. After thawing the cells, add 100ul TE-RNAse-A solution containing RNAse T1, mix by pipetting up and down 4-5 times to resuspend the cell pellet and then incubate in the 37degC incubator/shaker for 5 minutes at 350 rpm to mix more thoroughly.

4. Remove the block from the incubator/shaker and then add 100ul of alkaline lysis solution. Shake the block by hand to mix the reagents and then incubate at room temperature for 1 hour with intermittent swirling. 5. Then add 100ul of either 3M potassium or sodium acetate, pH 5, and place the block in the 37degC shaker/incubator for 5 minutes at 350 rpm to thoroughly mix and shear genomic DNA to reduce the viscosity of the solution. Place the block at -20degC for 30 minutes.

6. Centrifuge the block in the GPR centrifuge at 3000 rpm at 4degC for 30 minutes.

7. Carefully remove 200 ul of the supernatant from each well in the 96 well block with the 12 channel pipetter and transfer them to a v-bottom microtiter plate, being careful not to transfer any cell debris.

8. Transfer 10 ul of supernatant into the respective cycle sequencing reaction tubes, and precipitate with 150 ul of 95% ethanol (without added acetate). After storage at

-20degC for 30 minutes, the pellet is collected by centrifugation, washed three times with 70% ethanol, and dried directly in the cycle sequencing reaction tubes.

9. Prior to adding the fluorscent terminator cycle sequencing reaction mix, the dried templates should be stored at -20degC. An additional 75 ul of the supernatant is transfered to a Robbins PCR reaction tube (in 96 well tube format) and precipitated with 200 ul of 95% ethanol, washed three times with 70% ethanol, and stored dry at -20degC for future use.

The following is an automated, 96 well, double stranded sequencing template isolation procedure that has been developed in our laboratory.

1. Pick colonies using a toothpick into 1.8 ml TB with TB salts containing appropriate antibiotic and shake for 22-24 hours at 350 rpm in a 96 well block with cover.

2. Harvest cells by centrifugation at 1800 rpm for 7 min. Pour off supernatant and allow pellets to drain inverted. Cell pellets may be frozen at this point if necessary.

3. Turn on Biomek, begin the program DSISOL2 and set up the Biomek as indicated in the configuration function on the screen. Specifically, you should put TE-RNase solution in the first module, alkaline lysis solution in the second reagent module and 3 M KOAc, pH 4.8 in the third module.

4. Place the 96 well block containing cells onto the Biomek tablet at the position labeled "1.0 ml Minitubes". Place a Millipore filter plate in the position labeled "96well flat bottomed microtitre plate".

5. Press ENTER to continue with the program.

6. First the Biomek will add 100 ml TE-RNase solution to the cell pellets and mix to partially resuspend.

7. Next, the biomek will add 100 ml alkaline lysis solution to the wells of the filter plate.

8. The biomek then will mix the cell suspension again, transfer the entire volume to the filter plate containing alkaline lysis solution, and mix again. Set up the filtration apparatus with a clean 96 well block to collect the filtrate (wash and reuse the block used for growth).

9. The biomek will add 100 ml 3M KOAc, pH 4.8 to the wells of the filter plate and

mix at the sides of the wells. Some choose to place the filter plate at -20degC for 5 minutes at this point. Transfer the filter plate to the QiaVac Vacuum Manifold 96 and filter using water vacuum only (do not do a harsh filtration as the plates are fragile and will loose their seal). This will typically take less than 20 minutes.

10. The supernatant collected in the 96 well block is the crude DNA and must be ethanol precipitated before use by the addition of 1 ml 100% ethanol and incubation at -20degC for at least 30 minutes.

11. Centrifuge for 25 minutes at 3000 rpm in a cooled Beckman GPR centrifuge.

12. Decant and wash with 500 ml 80% ethanol and centrifuge for an additional 5 minutes at 3000 rpm.

13. Decant the supernatant, drain inverted on a paper towel. Dry under vacuum.

14. Resuspend in 50 ml 10:0.1 TE for use in dye primer or dye terminator sequencing chemistry.

H. Genomic DNA isolation from blood

Genomic DNA isolation is performed according to the FBI protocol (27). After the blood samples (stored at -70degC in EDTA vacutainer tubes) are thawed, standard citrate buffer is added, mixed, and the tubes are centrifuged. The top portion of the supernatant is discarded and additional buffer is added, mixed, and again the tube is centrifuged. After the supernatant is discarded, the pellet is resuspended in a solution of SDS detergent and proteinase K, and the mixture is incubated at 55deg C for one hour. The sample then is phenol extracted once with a phenol/chloroform/isoamyl alcohol solution, and after centrifugation the aqueous layer is removed to a fresh microcentrifuge tube. The DNA is ethanol precipitated, resuspended in buffer, and then ethanol precipitated a second time. Once the pellet is dried, buffer is added and the DNA is resuspended by incubation at 55degC overnight, the genomic DNA solution is assayed by the polymerase chain reaction.

Protocol (updated 10/30/98)

1. Blood samples typically were obtained as 1 ml of whole blood stored in EDTA vacutainer tubes frozen at -70deg C.

2. Thaw the frozen samples, and to each 1 ml sample, add 0.8 ml 1X SSC buffer, and

mix. Centrifuge for 1 minute at 12,000 rpm in a microcentrifuge.

3. Remove 1 ml of the supernatant and discard into disinfectant.

4. Add 1 ml of 1X SSC buffer, vortex, centrifuge as above for 1 minute, and remove all of the supernatant.

5. Add 375 ul of 0.2M NaOAc to each pellet and vortex briefly. Then add 25 ul of 10% SDS and 5 ul of proteinase K (20 mg/ml H2O) (Sigma P-0390), vortex briefly and incubate for 1 hour at 55degC.

6. Add 120 ul phenol/chloroform/isoamyl alcohol and vortex for 30 seconds. Centrifuge the sample for 2 minutes at 12,000 rpm in a microcentrifuge tube.

7. Carefully remove the aqueous layer to a new 1.5 ml microcentrifuge tube, add 1 ml of cold 100% ethanol, mix, and incubate for 15 minutes at -20deg C.

8. Centrifuge for 2 minutes at 12,000 rpm in a microcentrifuge. Decant the supernatant and drain.

9. Add 180 ul 10:1 TE buffer, vortex, and incubate at 55degC for 10 minutes.

10. Add 20 ul 2 M sodium acetate and mix. Add 500 ul of cold 100% ethanol, mix, and centrifuge for 1 minute at 12,000 rpm in a microcentrifuge.

11. Decant the supernatant and rinse the pellet with 1 ml of 80% ethanol. Centrifuge for 1 minute at 12,000 rpm in a microcentrifuge.

12. Decant the supernatant, and dry the pellet in a Speedy-Vac for 10 minutes (or until dry).

13. Resuspend the pellet by adding 200 ul of 10:1 TE buffer. Incubate overnight at 55degC, vortexing periodically to dissolve the genomic DNA. Store the samples at -20degC

[NextPage]

IV. Methods for DNA sequencing

A. Bst-catalyzed radiolabeled DNA sequencing

Bst DNA polymerase-catalyzed radiolabeled two-step sequencing reactions (26) are modified from those presented earlier (25) by altering the absolute amounts and the relative deoxy/dideoxynucleotide ratios in the termination mixes. Two separate termination mixes provided optimal overlap for sequence data starting in the

polylinker and extending to approximately 600 bases from the priming site. This two-step format eliminated the need for the chase required in the Bst one-step reaction (25).

Each extension reaction contained 500-750 ng of Biomek isolated single-stranded DNA, reaction buffer, nucleotide extension mix, oligonucleotide primer (typically M13 (-40) universal sequencing primer, see Appendix D), either [a-32-P]dATP or [a-35-S]dATP and Bst polymerase. After the reactions are extended for 2 minutes at 67deg C and briefly centrifuged, four aliquots are removed and added to the appropriate base-specific termination mix. All nucleotide mixes contained the guanosine nucleotide analog, 7-deaza-dGTP, but differed in their deoxy/dideoxynucleotide ratios to yield fragments ranging in size from the beginning of the polylinker to greater than 300 bases from the primer, or fragments from about 150 to greater than 600 bases from the primer for "short" or "long" mixes, respectively. Following an incubation at 67degC for 10 min and a brief centrifugation, the reactions are stopped by the addition of dye/formamide/EDTA, and incubated at 100degC. When desired, sequencing reactions are stored at -70degC prior to the addition of loading dye.

When double-stranded pUC-based subclones are used as templates, the amount of primer is doubled and a denaturing/annealing step is added. Here, 3 ug of plasmid DNA, isolated by either the mini- or midi-prep diatomaceous earth method, is mixed with primer, placed in a boiling-water bath, and rapidly cooled by plunging into an ethanol/dry-ice bath (28). Following an incubation on ice, the remaining sequencing extension reagents (reaction buffer, nucleotide extension mix, either [a-32-P]dATP or [a-35-S]dATP, and Bst polymerase) are added. Reactions are performed as described above for single-stranded sequencing.

Protocol

For single-stranded DNA sequencing:

1. Prepare the following extension reaction in a microcentrifuge tube:

    750 ng M13 template DNA

    2 ul Bst reaction buffer

2 ul Bst nucleotide extension mix

1 ul oligonucleotide primer (2.5 ng/ul)

0.5-1 ul [alpha]-32-P-dATP or [alpha]-35-S-dATP

1 ul diluted Bst polymerase (0.1 U/ul)

q.s. sterile ddH2O

12 ul

[alpha]-32-P-dATP (PB 10384) and [alpha]-35-S-dATP (SJ 1304) from Amersham.

Dilute the Bst polymerase (BioRad 170-3406) in Bst dilution buffer.

2. Incubate the reactions for 2 minutes at 67degC, and briefly centrifuge to reclaim condensation.

3. Remove 2.5 ul aliquots for each reaction into the four base-specific termination mixes (either short or long), already pipetted into a V-bottomed microtiter plate (Dynatech).

4. Incubate the reactions for 10 minutes at 67degC, and briefly centrifuge to reclaim condensation. It is possible to store the reactions at -70degC at this stage.

5. Stop the reactions by the addition of 4 ul of agarose gel loading dye and incubate for 5-7 minutes at 100degC.

For double-stranded DNA sequencing:

1. To denature the DNA and anneal the primer, incubate the following reagents in a boiling water bath for 4-5 minutes and rapidly cool the reaction by plunging into an ethanol/dry ice bath.

3 ug plasmid DNA

5 ng oligonucleotide primer

q.s. sterile ddH2O

9 ul

2. Incubate the reaction in an ice-water bath for 5 minutes, and then add the following reagents:

2 ul Bst reaction buffer

2 ul Bst nucleotide extension mix

0.5-1 ul [alpha]-32-P-dATP or [alpha]-35-S-dATP

1 ul diluted Bst polymerase (0.1 U/ul)

15 ul

[alpha]-32-P-dATP (PB 10384) and [alpha]-35-S-dATP (SJ 1304) from Amersham.

Dilute the Bst polymerase (BioRad 170-3406) in Bst dilution buffer.

3. Proceed with the sequencing reaction as described above in steps 2-5 for single-stranded templates

B. Radiolabeled sequencing gel preparation, loading, and electrophoresis (26,29)

To prepare polyacrylamide gels for DNA sequencing, the appropriate amount of urea is dissolved by heating in water and electrophoresis buffer, the respective amount of deionized acrylamide-bisacrylamide solution is added, and ammonium persulfate and TEMED are added to initiate polymerization. Immediately after the addition of the polymerizing agents, the gel solution is poured between two glass plates, taped together and separated by thin spacers corresponding to the desired thickness of the gel, taking care to avoid and eliminate air bubbles. Prior to taping, these glass plates are cleaned with Alconox detergent and hot water, are rinsed with double distilled water, and dried with a Kimwipe. Typically, the notched glass plate is treated with a silanizing reagent and then rinsed with double distilled water. After pouring, the gel immediately is laid horizontally and a well forming comb is inserted into the gel and held in place by metal clamps. The polyacrylamide gels are allowed to polymerize for at least 30 minutes prior to use. After polymerization, the comb and the tape at the bottom of the gel are removed. The vertical electrophoresis apparatus is assembled by clamping the top and bottom buffer wells onto the gel, and adding running buffer to the buffer chambers. The wells are cleaned by circulating buffer into the wells with a syringe and, immediately prior to the loading of each sample, the urea in each well is suctioned out with a mouth pipette.

Each base-specific sequencing reaction terminated with the short termination mix is loaded using a mouth pipette onto a 0.15 mm X 50 cm X 20 cm, denaturing 5% polyacrylamide gel and electrophoresed for 2.25 hours at 22 mA. The reactions terminated with the long termination mix typically are divided in half and loaded onto two 0.15 mm X 70 cm X 20 cm denaturing 4% polyacrylamide gels. One gel is

electrophoresed at 15 mA for 8-9 hours and the other is electrophoresed for 20-24 hours at 15 mA. After electrophoresis, the glass plates are separated and the gel is blotted to Whatman paper, covered with plastic wrap, dried by heating on a Hoefer vacuum gel drier, and exposed to X-ray film. Depending on the intensity of the signal and whether the radiolabel is 32-P or 35-S, exposure times varied from 4 hours to several days. After exposure, the films are developed by processing in developer and fixer solutions, rinsed with water, and air dried. The autoradiogram then is placed on a light-box and the sequence is manually read and the data typed into a computer.

Protocol

1. Prepare 8 M urea, polyacrylamide gels according to the following recipe (100 ml), depending in the desired percentage:

	4%	5%	6%
urea	48 g	48 g	48 g
40% A & B	10 ml	12.5 ml	15 ml
10X MTBE	10 ml	10 ml	10 ml
ddH2O	42 ml	39.5 ml	37 ml
15% APS	500 ul	500 ul	500 ul
TEMED	50 ul	50 ul	50 ul

Urea (5505UA) is from Gibco/BRL.

2. Combine the urea, MTBE buffer, and water and incubate for 5 minutes at 55deg C and then stir to dissolve the urea.

3. Cool briefly, add the A & B, mix, and degas under vacuum for 5 minutes.

4. While stirring, add the APS and TEMED polymerization agents and then immediately pour in between two taped glass plates with 0.15 mm spacers. (Prior to taping, the notched, front glass plate should be treated with a small amount of silanizing reagent and then rinsed with ddH2O).

5. Insert the well forming comb, clamp, and allow the gel to polymerize for at least 30 minutes.

6. Prior to loading, remove the tape around the bottom of the gel and the well-forming comb. Assemble the vertical electrophoresis apparatus by clamping the upper and

lower buffer chambers to the gel plates, and add 1X MTBE electrophoresis buffer to the chambers.

7. Flush the sample wells with a syringe containing running buffer, and immediately prior to loading each sample, flush the well with running buffer using gel loading tips.

9. Load 1-2 ul of sample into each well using a Pipetteman with gel-loading tips, and then electrophorese according the following guidelines (during electrophoresis, cool the gel with a fan):

termination reaction	polyacrylamide gel	electrophoresis conditions
short	5%, 0.15 mm X 50 cm X 20 cm	2.25 hours at 22 mA
long	4%, 0.15 mm X 70 cm X 20 cm	8-9 hours at 15 mA
long	4%, 0.15 mm X 70 cm X 20 cm	20-24 hours at15 mA

10. After electrophoresis, remove the buffer wells, the tape, and pry the gel plates apart. The gel should adhere to back plate. Blot the gel to a 40 cm X 20 cm sheet of 3MM Whatman paper, cover with plastic wrap, and dry on a Hoefer gel dryer for 25 minutes at 80degC

11. Place the dried gel in a cassette and expose to Kodak XRP-1 film.

12. Develop the film for 1-5 minutes in Kodak GBX developer, rinse in distilled water for 30 seconds, fix in Kodak GBX fixer for 5 minutes, and then rinse again in distilled water for 30 seconds. Allow the film to air dry.

C. Taq-polymerase catalyzed cycle sequencing using fluorescent-labeled dye primers (10,26)

Each base-specific fluorescent-labeled cycle sequencing reaction routinely included approximately 100 or 200 ng Biomek isolated single-stranded DNA for A and C or G and T reactions, respectively. Double-stranded cycle sequencing reactions similarly contained approximately 200 or 400 ng of plasmid DNA, isolated using either the standard alkaline lysis or the diatomaceous earth modified alkaline lysis procedures. All reagents except template DNA are added in one pipetting step from a premix of previously aliquotted stock solutions stored at -20degC (see Appendix B). To prepare the reaction premixes, reaction buffer is combined with the base-specific nucleotide

mixes. Prior to use, the base-specific reaction premixes are thawed and combined with diluted Taq DNA polymerase and the individual fluorescent end-labeled universal primers (see Appendix C) to yield the final reaction mixes, that are sufficient for 24 template samples.

Once the above mixes are prepared, four aliquots of single or double-stranded DNA are pipetted into the bottom of each 0.2 ml thin-walled reaction tube, corresponding to the A, C, G, and T reactions, and then an aliquot of the respective reaction mixes is added to the side of each tube. These tubes are part of a 96-tube/retainer set tray in a microtiter plate format, which fits into a Perkin Elmer Cetus Cycler 9600. Strip caps are sealed onto the tube/retainer set and the plate is centrifuged briefly. The plate then is placed in the cycler whose heat block had been preheated to 95deg C, and the cycling program immediately started. The cycling protocol consisted of 15-30 cycles of seven-temperatures:

95degC denaturation

55degC annealing

72degC extension

95degC denaturation

72degC extension

95degC denaturation, and

72degC extension, linked to a 4deg C final soak file.

At this stage, the reactions frequently are frozen and stored at -20degC for up to several days. Prior to pooling and precipitation, the plate is centrifuged briefly to reclaim condensation. The primer and base-specific reactions are pooled into ethanol, and the DNA is precipitated and dried. These sequencing reactions could be stored for several days at -20degC.

Protocol

1. Pipette 1 or 2 ul of each DNA sample (100 ng/ul for M13 templates and 200 ng/ul for pUC templates) into the bottom of the 0.2 ml thin-walled reaction tubes (Robbins Scientific). Use the 1 ul sample for A and C reactions, and the 2 ul sample for G and T reactions. Meanwhile, preheat the PE Cetus Thermocycler 9600 to 95degC (Program

#2).

2. Prepare the Taq polymerase dilution. AmpliTaq polymerase (N801-0060) is from Perkin-Elmer Cetus.

30 ul	AmpliTaq (5U/ul)
30 ul	5X Taq reaction buffer
130 ul	ddH20
190 ul	diluted Taq for 24 clones

3. Prepare the A, C, G, and T base specific mixes by adding base-specific primer and diluted Taq to each of the base specific nucleotide/buffer premixes:

A,C/G,T	
60/120 ul	5X Taq cycle sequencing mix
30/60 ul	diluted Taq polymerase
30/60 ul	respective fluorescent end-labeled primer
120/240 ul	

4. Seal the reaction tubes carefully with the strip caps, and centrifuge briefly at 2500 rpm. Place the tube/retainer set in the 9600 Cycler, abort the soak file program, and run program #11. This program will cycle the sequencing reactions for 30 cycles of seven temperatures (30 cycles of 95degC denaturation for 4 seconds; 55degC annealing for 10 seconds; 72degC extension for 1 minute; 95degC denaturation for 4 seconds; 72degC extension for 1 minute; 95degC denaturation for 4 seconds; and 72degC extension for 1 minute), and then will link to a 4degC soak file until that program is aborted. (It is possible to freeze the reactions at -20degC after cycling, prior to the pooling step).

5. Briefly centrifuge the plate to reclaim condensation. Pool the four base specific reactions into 250 ul of 95% ethanol.

6. Precipitate the sequencing reactions, and store the dried samples at -20deg C.

D. Taq-polymerase catalyzed cycle sequencing using fluorescent-labeled dye terminator reactions

One of the major problems in DNA cycle sequencing is that when fluorescent primers (1) are used the reaction conditions are such that the nested fragment set distribution

is highly dependent upon the template concentration in the reaction mix. We have recently observed that the nested fragment set distribution for the DNA cycle sequencing reactions using the fluorescent labelled terminators (8) is much less sensitive to DNA concentration than that obtained with the fluorescent labelled primer reactions as described above. In addition, the fluorescent terminator reactions require only one reaction tube per template while the fluorescent labelled primer reactions require one reaction tube for each of the four terminators. This latter point allows the fluorescent labelled terminator reactions to be pipetted easily in a 96 well format. The protocol used, as described below, is easily interfaced with the 96 well template isolation and 96 well reaction clean-up procedures also described herein. By performing all three of these steps in a 96 well format, the overall procedure is highly reproducable and therefore less error prone.

Protocol

1. Place 0.5 ug of single-stranded or 1 ug of double-stranded DNA in 0.2 ml Robbins PCR tubes.

2. Add 1 ul (for single stranded templates) or 4 ul (for double-stranded templates) of 0.8 uM primer and 9.5 ul of ABI supplied premix to each tube, and bring the final volume to 20 ul with ddH2O.

3. Centrifuge briefly and cycle as usual using the terminator program as described by the manufacturer (i.e. preheat at 96oC followed by 25 cycles of 96oC for 15 seconds, 50oC for 1 second, 60oC for 4 minutes, and then link to a 4oC hold).

4. Proceed with the spin column purification using either the Centri-Sep columns or G-50 microtiter plate procedures given below.

Terminator Reaction Clean-Up via Centri-Sep Columns

1. Gently tap the column to cause the gel material to settle to the bottom of the column.

2. Remove the column stopper and add 0.75 ml dH2O.

3. Stopper the column and invert it several times to mix. Allow the gel to hydrate for at least 30 minutes at room temperature. Columns can be stored for a few days at 4 鶟; longer storage in water is not recommended. Allow columns that have been stored at 4

鹪 to warm to room temperature before use. Remove any air bubbles by inverting the column and allowing the gel to settle. Remove the upper-end cap first and then remove the lower-end cap. Allow the column to drain completely, by gravity. (Note: If flow does not begin immediately apply gentle pressure to the column with a pipet bulb.)

4. Insert the column into the wash tube provided.

5. Spin in a variable-speed microcentrifuge at 1300 g for 2 minutes to remove the fluid.

6. Remove the column from the wash tube and insert it into a Sample Collection Tube.

7. Carefully remove the reaction mixture (20 ml) and load it on top of the gel material. If the samples were incubated in a cycling instrument that required overlaying with oil, carefully remove the reaction from beneath the oil. Avoid picking up oil with the sample, although small amounts of oil (<1 ml) in the sample will not affect results. Oil at the end of the pipet tip containing the sample can be removed by touching the tip carefully on a clean surface (e.g., the reaction tube). Use each column only once.

8. Spin in a variable-speed microcentrifuge with a fixed angle rotor, place the column in the same orientation as it was in for the first spin--this is important because the surface of the gel will be at an angle in the column after the spin.

9. Dry the sample in a vacuum centrifuge. Do not apply heat. Do not overdry. If desired, reactions can be ethanol precipitated.

Terminator Reaction Clean-Up via Sephadex G-50 Filled Microtiter Format Filter Plates

The following protocol was developed at the C. Elegans Genome Sequencing Center at Washington University, St. Louis, Missouri, was conveyed to us by Dr. Richard Wilson, and now has been modified to take advantage of the Millipore 45 ul Column Loader (cat. # MACL 096 45). Additional information about this procedure also is available at the Millipore web site.

Preparation of Sephadex G-50 containing Microtiter Filter Plates:

1. Add dry Sephadex G-50 to the Millipore (cat.# MACL 096 45) 45 ul Column

Loader.

2. Remove the excess of resin from the top of the Column Loader with the scraper supplied.

3. Place MultiScreen HV Plate (Millipore MAHVN4550) upside-down on top of the Column Loader.

4. Invert both MultiScreen HV Plate and Column Loader.

5. Tap on top of the Millipore Column Loader to release the resin.

6. Using a multi-channel pipettor, add 300 ul of ddH2O to each well to swell the resin. and let stand at room temperature for 3 hours.

7. Once the minicolumns are swollen in MultiScreen plates, they can be sealed with saran wrap and stored in the refrigerator at 4 deg C for several weeks. A batch of plates also can be stored in the refrigerator at 4 deg C for several weeks in a sealed plastic container with a damp towel to assure the plates are kept moist.

8. When needed, the matrix containing filter plate is taped over a microtiter plate and centrifuged for 2 minutes at 1500 RPM in a Beckman GS-6R to pack the columns and to remove any access buffer.

** Older procedure no longer used, replaced by steps 1-8 above **

1. Weigh out 8 - 10 g of Sephadex G-50 Bead diameter: 50 - 150 micron (medium) and transfer to a sterile bottle.

2. Add 100 ml of sterile water.

3. Let it stand at room temperature for 3 hours or place at 4o C overnight.

Packing and using the columns:

1. Sephadex settles out; therefore, you must resuspend before adding to the plate and also after filling every 8 to 10 wells.

2. Add 400 ul of mixed Sephadex G-50 to each well of microtiter filter plate (Millipore MAHVN4550).

3. Place microtiter filter plate on top of another microtiter plate to collect water and tape sides so they do not fly apart during centrifugation.

4. Spin at 1500 rpm for 2 minutes.

5. Discard water that has been collected in the microtiter plate.

6. Again place the microtiter filter plate on top of the microtiter plate to collect water and tape sides so they do not fly apart during centrifugation.

7. Add an additional 100-200 ul of Sephadex G-50 to fill the microtiter filter plate wells.

8. Spin at 1500 rpm for 2 minutes.

** end of older procedure no longer used **

Using the 96 well format microtiter plate filters for dye-terminator clean-up

9. Discard water that has been collected in the lower microtiter plate.

10. Place the collection microtiter filter plate on top of a microtiter plate to collect sample and tape sides so they do not fly apart during centrifugation.

11. Add 20 ul terminator reaction to each Sephadex G-50 containing wells. If a smaller reaction volume was used (typically 5-7 ul), then add 10 ul of water to dilute to the larger volume and improve recovery.

12. Spin at 1500 rpm for 2 minutes.

13. Dry the collected effluent in a Speed-Vac for approximately 1-2 hours.

E. Sequenase[TM] catalyzed sequencing with dye-labeled terminators (29-32)

Single-stranded dye-terminator reactions required approximately 2 ug of phenol extracted M13-based template DNA. The DNA is denatured and the primer annealed by incubating DNA, primer, and buffer at 65degC. After the reaction cooled to room temperature, alpha-thio-deoxynucleotides, fluorescent-labeled dye-terminators, and diluted Sequenase[TM] DNA polymerase are added and the mixture is incubated at 37degC. The reaction is stopped by adding ammonium acetate and ethanol, and the DNA fragments are precipitated and dried. To aid in the removal of unincorporated dye-terminators, the DNA pellet is rinsed twice with ethanol. The dried sequencing reactions could be stored up to several days at -20degC.

Double-stranded dye-terminator reactions required approximately 5 ug of diatomaceous earth modified-alkaline lysis midi-prep purified plasmid DNA. The double-stranded DNA is denatured by incubating the DNA in sodium hydroxide at 65degC, and after incubation, primer is added and the reaction is neutralized by adding an acid-buffer. Reaction buffer, alpha-thio-deoxynucleotides,

fluorescent-labeled dye-terminators, and diluted Sequenase[TM] DNA polymerase then are added and the reaction is incubated at 37degC. Ammonium acetate is added to stop the reaction and the DNA fragments similarly are precipitated, rinsed, dried, and stored.

Protocol

For Single-stranded reactions:

1. Add the following to a 1.5 ml microcentrifuge tube:

4 ul ss DNA (2 ug)

4 ul 0.8 uM primer

2 ul 10x MOPS buffer

2 ul 10x Mn[2+]/isocitrate buffer

12 ul

2. To denature the DNA and anneal the primer, incubate the reaction at 65-70deg C for 5 minutes. Allow the reaction to cool at room temperature for 15 minutes, and then briefly centrifuge to reclaim condensation.

3. To each reaction, add the following reagents and incubate for 10 minutes at 37degC. (For more than one reaction, a pot of the reagents should be made).

7 ul ABI terminator mix (401489)

2 ul diluted Sequenase[TM] (3.25 U/ul)

1 ul 2 mM a-S dNTPs

22 ul

The undiluted Sequenase[TM] (70775) from United States Biochemicals is 13 U/ul and should be diluted 1:4 with USB dilution buffer prior to use resulting in a working dilution of 3.25 U/ul.

4. Add 20 ul 9.5 M ammonium acetate and 100 ul 95% ethanol to stop the reaction and vortex.

5. Precipitate the DNA in an ice-water bath for 10 minutes. Centrifuge for 15 minutes at 12,000 rpm in a microcentrifuge at 4degC. Carefully decant the supernatant, and rinse the pellet by adding 300 ul of 70-80% ethanol. Vortex and centrifuge again for 15 minutes, and carefully decant the supernatant.

6. Repeat the rinse step to insure efficient removal of the unincorporated terminators. (Alternatively, after the first rinse step, droplets of supernatant can be removed by carefully absorbing them with a Q-tip cotton swab or a rolled up Kimwipe).

7. Dry the DNA for 5-10 minutes (or until dry) in the Speedy-Vac, and store the dried reactions at -20degC.

For double-stranded reactions:

1. Add the following to a 1.5 ml microcentrifuge tube:

5 ul ds DNA (5 ug)

4 ul 1 N NaOH

3 ul ddH2O

12 ul

2. Incubate the reaction at 65-70degC for 5 minutes, and then briefly centrifuge to reclaim condensation.

3. Add the following reagents to each reaction, vortex, and briefly centrifuge:

3 ul 8 uM primer

9 ul ddH2O

4 ul MOPS-Acid buffer

28 ul

4. To each reaction, add the following reagents and incubate for 10 minutes at 37degC. (For more than one reaction, a pot of the reagents should be made).

4 ul 10X Mn[2+]/isocitrate buffer

6 ul ABI terminator mix

2 ul diluted Sequenase[TM] (3.25 U/ul)

1 ul 2 mM [alpha]-S-dNTPs

22 ul

The undiluted Sequenase[TM] from United States Biochemicals is 13 U/ul and should be diluted 1:4 with USB dilution buffer prior to use resulting in a working dilution of 3.25 U/ul.

5. Add 60 ul 8 M ammonium acetate and 300 ul 95% ethanol to stop the reaction and vortex.

6. Precipitate the DNA in an ice-water bath for 10 minutes. Centrifuge for 15 minutes at 12,000 rpm in a microcentrifuge at 4degC. Carefully decant the supernatant, and rinse the pellet by adding 300 ul of 80% ethanol. Vortex and centrifuge again for 15 minutes, and carefully decant the supernatant.

7. Repeat the rinse step to insure efficient removal of the unincorporated terminators. (Alternatively, after the first rinse step, droplets of supernatant can be removed by carefully absorbing them with a Q-tip cotton swab or a rolled up Kim-wipe).

8. Dry the DNA for 5-10 minutes (or until dry) in the Speedy-Vac.

F. Fluorescent-labeled sequencing gel preparation, pre-electrophoresis, sample loading, electrophoresis, data collection, and analysis on the ABI 373A DNA sequencer

Polyacrylamide gels for fluorescent DNA sequencing are prepared as described above except that the gel mix is filtered prior to polymerization. Optically-ground, low fluorescence glass plates are carefully cleaned with hot water, distilled water, and ethanol to remove potential fluorescent contaminants prior to taping. Denaturing 6% polyacrylamide gels are poured into 0.3 mm X 89 cm X 52 cm taped plates and fitted with 36 well forming combs. After polymerization, the tape and the comb are removed from the gel and the outer surfaces of the glass plates are cleaned with hot water, and rinsed with distilled water and ethanol. The gel is assembled into an ABI sequencer, and the checked by laser-scanning. If baseline alterations are observed on the ABI-associated Macintosh computer display, the plates are recleaned. Subsequently, the buffer wells are attached, electrophoresis buffer is added, and the gel is pre-electrophoresed for 10-30 minutes at 30 W.

Prior to sample loading, the pooled and dried reaction products are resuspended in formamide/EDTA loading buffer by vortexing and then heated at 90degC. A sample sheet is created within the ABI data collection software on the Macintosh computer which indicated the number of samples loaded and the fluorescent-labeled mobility file to use for sequence data processing. After cleaning the sample wells with a syringe, the odd-numbered sequencing reactions are loaded into the respective wells using a micropipettor equipped with a flat-tipped gel-loading tip. The gel then is

electrophoresed for 5 minutes before the wells are cleaned again and the even numbered samples are loaded. The filter wheel used for dye-primers and dye-terminators is specified on the ABI 373A CPU, also where electrophoresis conditions are adjusted. Typically electrophoresis and data collection are for 10 hours at 30W on the ABI 373A that is fitted with a heat-distributing aluminum plate in contact with the outer glass gel plate in the region between the laser stop and the sample loading wells (26).

After data collection, an image file is created by the ABI software which related the fluorescent signal detected to the corresponding scan number. The software then determined the sample lane positions based on the signal intensities. After the lanes are tracked, the cross-section of data for each lane are extracted and processed by baseline subtraction, mobility calculation, spectral deconvolution, and time correction. On the Macintosh computer, the collected data can be viewed in several formats. The overall graphics image of the gel can be displayed to assess the accuracy of lane tracking, and the data from each sample lane can be viewed as either a four-color raw fluorescent signal versus scan number, as a chromatogram of processed sequence data, or as a string of nucleotides. After processing, the sequence data files are transferred to a SPARCstation 2 using NFS Share.

Protocol

1. Prepare 8 M urea, 4.75% polyacrylamide gels, as described above, using a 36-well forming comb. Alternatively, the recipe can be scaled up to one liter.

2. Prior to loading, remove the tape from around the entire gel and carefully clean the outer surface of the gel plates with hot water. Rinse the glass with distilled water and then with ethanol, and allow the ethanol to evaporate.

3. Assemble the gel plates into an ABI 373A DNA Sequencer by placing the plates on the ledge in the bottom buffer well and clamping the gel into place with the black clamps attached to the laser stop.

4. Check the glass plates by closing the ABI lid and selecting "Start Pre-run" and then "Plate Check" from the ABI display. Adjust the PMT on the ABI display ("Calibration", "PMT") so that the lower scan (usually the blue) line corresponds to an

intensity value of 800-1000 as displayed on the Macintosh computer data collection window. If the baseline of four-color scan lines is not flat, reclean the glass plates.

4. Attach the top buffer and the alignment brace, and fill both buffer wells with 1X MTBE electrophoresis buffer. Affix the aluminum heat distribution plate by setting it on the laser stop against the glass plates.

5. Pre-electrophorese the gel for 10-30 minutes by choosing "Start Pre-run" and "Pre-run Gel".

6. Use MakeSampleSheetOU to create a sample sheet or do this from within the ABI data collection software by entering the names and the fluorescent mobility file ("b920_21.mob" for fluorescent-labeled M13 -21 universal forward primer, "DyePrimer{M13RP1}" for fluorescent-labeled M13 universal reverse primer, "DyeTerm {any primer}" for AmpliTaq Terminators, and "DyeTerm{T7}-SetB" for Sequenase[TM] fluorescent-labeled dye terminators) to use for analysis. This Macintosh program and the related files are available from our ftp site at ftp://ftp.genome.ou.edu/ as a stuffit 1.5.1, binhexed file.

7. Prepare the samples for loading. Add 3 ul of FE to the bottom of each tube, vortex, heat at 90degC for 3 minutes, and centrifuge to reclaim condensation.

8. Abort the pre-electrophoresis, and flush the sample wells with electrophoresis buffer with a syringe. Using flat-tipped gel loading pipette tips, load each odd-numbered sample. Pre-electrophorese the gel for at least 5 minutes, flush the wells again, and then load each even-numbered sample.

9. Begin the electrophoresis (30 W for 10 hours) run by selecting "Start Run" on the ABI display and by choosing "Begin Data Collection" from the controller box within the ABI data collection software on the Macintosh.

10. After data collection, the ABI software will automatically open the data analysis software, which will create the imaged gel file, extract the data for each sample lane, and process the data. Check the imaged gel file for sample tracking, and then transfer the results folder containing the sequence trace files to a SPARCstation 2 where the hard disk is mounted on the ether-netted Macintosh computer via NFS Share.

G. Double-stranded sequencing of cDNA clones containing long poly(A) tails using

anchored poly(dT) primers

Sequencing double stranded DNA templates has become a common and efficient procedure (10) for rapidly obtaining sequence data while avoiding preparation of single stranded DNA. Double stranded templates of cDNAs containing long poly(A) tracts are difficult to sequence with vector primers which anneal downstream of the poly(A) tail. Sequencing with these primers results in a long poly(T) ladder followed by a sequence which is difficult to read. In an attempt to solve this problem we synthesized three primers which contain (dT)17 and either (dA) or (dC) or (dG) at the 3' end. We reasoned that the presence of these three bases at the 3' end would 'anchor' the primers at the upstream end of the poly(A) tail and allow sequencing of the region immediately upstream of the poly(A) region.

Using this protocol, over 300 bp of readable sequence could be obtained. We have applied this approach to several other poly(A)-containing cDNA clones with similar results. Sequencing of the opposite strand of these cDNAs using insert-specific primers occurred directly upstream of the poly(A) region. The ability to directly obtain sequence immediately upstream from the poly(A) tail of cDNAs should be of particular importance to large scale efforts to generate sequence-tagged sites (STSs) (11) from cDNAs (12,13).

Protocol 1. Synthesize anchored poly (dT)17 with anchors of (dA) or (dC) or (dG) at the 3' end on a DNA synthesizer and use after purification on Oligonucleotide Purification Cartridges.

2. For sequencing with anchored primers, denature 5-10 mg of plasmid DNA in a total volume of 50 ml containing 0.2 M sodium hydroxide and 0.16 mM EDTA by incubation at 65oC for 10 minutes.

3. Add the three poly(dT) anchored primers (2 pmol of each) and immediately place the mixture on ice. Neutralize the solution by adding 5 ml of 5 M ammonium acetate pH 7.0.

4. Precipitate the DNA by adding 150 ml of cold 95% ethanol and wash the pellet twice with cold 70% ethanol.

5. Dry the pellet for 5 minutes and then resuspend in MOPS-Acid buffer.

6. Anneal the primers by heating the solution for 2 minutes at 65oC followed by slow cooling to room temperature for 15-30 minutes.

7. Perform sequencing reactions, using modified T7 DNA polymerase and a-[32P]dATP (> 1000 Ci/mmole) using the protocol described earlier.

H. cDNA sequencing based on PCR and random shotgun cloning

The following is a rapid and efficient method for sequencing cloned cDNAs based on PCR amplification (14), random shotgun cloning (1,3,15), and automated fluorescent sequencing (16). This method was developed in our laboratory because once the sequence of a genomic DNA containing cosmid is obtained and putative exons are predicted, the corresponding cDNAs should be sequenced in a timely manner. However, the presently implemented directed cDNA sequencing strategies, i.e. primer walking (17) and exonuclease III deletion (18), are both time consuming and labor intensive, while the alternative, i.e. randomly shearing the intact plasmid followed by shotgun sequencing (1,3,15), leads to a significant number of clones containing the original cDNA cloning vector rather than the desired cDNA insert.

This is a PCR-based approach where the "universal" forward and/or reverse priming sites were excluded from the resulting PCR product by choosing a primer pair that lay between the usual "universal" forward and reverse priming sites and the multiple cloning sites of the Stratagene Bluescript vector. These two PCR primers, with the sequence 5'-TCGAGGTCGACGGTATCG-3' for the forward or -16bs primer and 5'-GCCGCTCTAGAACTAG TG-3' for the reverse or +19bs primer, now have been used to amplify sufficient quantities of cDNA inserts in the 1.2 to 3.4 kb size range so that the random shotgun sequencing approach described below could be implemented.

Protocol

1. Incubate four 100 ul PCR reactions, each containing approximately 100 ng of plasmid DNA, 100 pmoles of each primer, 50 mM KCl (dilute from 1 M stock), 10 mM Tris-HCl pH 8.5 (dilute from 1 M stock), 1.5 mM MgCl2 (dilute from 1 M stock), 0.2 mM of each dNTP (dilute from 100 mM stock), and 5 units of PE-Cetus Amplitaq in 0.5 ml snap cap tubes for 25 cycles of 95oC for 1 min., 55oC for 1 min. and 72oC for 2 min. in a PE-Cetus 48 tube DNA Thermal Cycler.

2. After pooling the four reactions to obtain sufficient quantities of PCR product for the subsequent steps the aqueous solution containing the PCR product is placed in an AeroMist nebulizer, brought to 2.0 ml by adding approximately 0.5 to 1.0 ml of glycerol, and equilibrated at -20oC by placing it in either an isopropyl alcohol/dry ice or saturated aqueous NaCl/dry ice bath for 10 min.

3. The sample is nebulized at -20oC by applying 25 - 30 psi nitrogen pressure for 2.5 min. Following ethanol precipitation to concentrate the sheared PCR product, the fragments were blunt ended and phosphorylated by incubation with the Klenow fragment of E. coli DNA polymerase and T4 polynucleotide kinase as described previously. Fragments in the 0.4 to 0.7 kb range were obtained by elution from a low melting agarose gel.

[NextPage]

V. Additional methods

A. Polymerase Chain Reaction (PCR)

The amplification of DNA fragments using the polymerase chain reaction (33) is performed in either the Perkin-Elmer Cetus DNA Thermal Cycler or the Perkin-Elmer Cetus Cycler 9600, by adding the following reagents to either a 0.2 ml thin-walled tube or a 1.5 ml tube, respectively: a small amount of the template DNA molecule (typically cosmid, plasmid, or genomic DNA), the two primers flanking the region to be amplified, nucleotides, buffer, and Taq DNA polymerase. The cycling protocol consisted of 25-30 cycles of three-temperatures: strand denaturation at 95degC, primer annealing at 55degC, and primer extension at 72deg C, typically 30 seconds, 30 seconds, and 60 seconds for the DNA Thermal Cycler and 4 seconds, 10 seconds, and 60 seconds for the Thermal Cycler 9600, respectively. For reactions performed in the DNA Thermal Cycler, the reaction mixtures are overlaid with two drops of mineral oil prior to temperature cycling to eliminate liquid evaporation and condensation. This is not necessary for the Thermal Cycler 9600, which is equipped with a heated lid, maintained at 100degC, that closely contacted the sample tube caps and eliminated liquid evaporation and condensation. After PCR, aliquots of the mixture typically are loaded onto an agarose gel and electrophoresed to detect

amplified product. In some instances where the yield from a single PCR is insufficient, the reaction is ethanol precipitated, resuspended, and an aliquot is used as template for a second round of PCR amplification.

Protocol

1. Add the following reagents to a 0.5 ml flat-topped microcentrifuge tube :

1 ul	target DNA (10-20 ng)
2.5 ul	each primer (40 uM)
1 ul	AmpliTaq DNA Polymerase (5 U)
10 ul	2 mM dNTPs (2 mM each dNTP)
10 ul	10X PCR buffer
73 ul	ddH20
100 ul	

2. Cover the reaction with 2 drops of mineral oil, add a drop of oil to the heat-block well to ensure good contact between the heat-block and the tube, and place the tube in the wells of a Perkin-Elmer Cetus DNA Thermal Cycler which has been pre-heated to 95degC (Soak file #19).

3. Abort the soak file program and begin thermal-cycle program #43 for the amplification. This program has 25 cycles of a three temperature program and is linked to a 4degC soak file, which will hold indefinitely:

95degC for 1 minute

55degC for 1 minute

72degC for 2 minutes

4. Analyze a 10 ul aliquot on a 1-2% agarose gel.

B. Purification of PCR fragments for cloning

After an aliquot of the PCR mixture is analyzed on an agarose gel, the remainder of the reaction is concentrated by ethanol precipitation, resuspended in buffer, and subjected to a simultaneous fill-in/kinase reaction with the Klenow fragment of E. coli

DNA polymerase and T4 polynucleotide kinase, the four deoxynucleotides and rATP (34). The reaction then is loaded onto a preparative 1, 1.5, or 2% low-melting temperature agarose gel, depending on the size(s) of the fragment(s) as determined above, and after minimal separation is achieved between the product(s) and the excess primers, the DNA fragments are excised and eluted. After concentration by ethanol precipitation, amplified DNA fragments are ligated into blunt-ended cloning vector, such as SmaI-linearized, dephosphorylated double-stranded M13 replicative form or pUC.

It should be noted that several other methods to purify the DNA fragments for cloning have been investigated. These included standard ethanol-acetate precipitation (1), a 50% ethanol precipitation (35), spin-column purification (36), and precipitation with polyethylene glycol (PEG) (37). The first three methods did not remove sufficient unincorporated primer, and, during the subsequent ligation of the DNA fragment, the primers apparently competed for the blunt ended vector during ligation because the efficiency of ligation was significantly lower and the vast majority of recombinant clones contained only primer derived inserts. Precipitation by polyethylene glycol resulted in only an extremely small DNA pellet, removing the PEG supernatant is difficult, and yields of PCR product were variable.

Protocol

1. Ethanol precipitate the PCR reaction by adding 2.5 volumes of 95% ethanol containing 0.12 M sodium acetate, pH 4.8.

2. Perform the combined fillin-kinase reactions previously described. 3. Add 5 ul of agarose gel loading dye and load the reaction into a well of a 1.0% low-melting temperature agarose gel. Electrophorese for 30-60 minutes at 100-120 mA, and then excise the desired band visualized under UV light with a clean razor blade.

4. Elute the DNA from the gel by standard freeze-thaw methods, followed by a phenol extraction and concentrate by ethanol precipitation.

5. Resuspend the dried DNA in 10 ul of 10:0.1 TE buffer. Use this DNA in a standard blunt-ended ligation reaction. Typically, use 2-3 ul of this DNA in a 10 ul ligation reaction with 20 ng of pUC 18/SmaI-CIAP, although this will depend on the yield of

amplified DNA from the PCR reaction.

C. Preparation of SmaI-linearized, dephosphorylated double-stranded M13 replicative form cloning vector (10)

Several small batches of M13RF double stranded DNA are mixed with buffer, SmaI restriction endonuclease, and calf intestinal alkaline phosphatase (CIAP) and incubated for 2-4 hours at 37degC. The reactions then are pooled, ethanol precipitated, and resuspended in buffer to yield a final concentration of about 10 ng/ul. After characterization to determine the optimal concentration for shotgun cloning ligations and to assess the efficiency of SmaI digestion and CIAP dephosphorylation, aliquots of linearized vector are stored frozen at -70degC.

Protocol

1. Prepare 10-20 tubes with the following:

5 ug	M13RF
2 ul	NEB Buffer #4
4.5 ul	SmaI (16 U/ul)
3 ul	Calf Intestinal Alkaline Phosphatase (CIAP)
q.s.	ddH2O
20 ul	

SmaI from New England Biolabs (141L) and CIAP from Boehringer Mannheim (1097 075). NEB Buffer #4 (500 mM potassium acetate, 200 mM Tris-acetate, 100 mM magnesium acetate, 10 mM dithiothreitol, pH 7.9) included with SmaI from New England Biolabs.

2. Incubate at 37degC for 2-4 hours.

3. Pool the reactions, phenol extract, ethanol precipitate, and resuspend the dried DNA in 10:0.1 TE buffer to a final concentration of 10 ng/ul.

Alternatively: Preparation of M13 vector DNA for ligation M13 vectors should be digested with restriction enzymes as follows:

M13 DNA (1ug/ml)	1 衬
10x Assay buffer	1 衬

ddH2O	7 衬
Hinc II	1 衬
total volume	10 衬

(vector concentration of 100 ng/衬)

Incubate at 37?C for 2 to 4 hours. Inactivate restriction enzyme by heating at 75?C for 10 minutes. If dephosphorylation of the M13 vector DNA is desired, it may be performed at this point (see dephosphorylation procedure, above). Add 90 衬 water to give final concentration of 10 ng./衬; use 5 衬 (50 ng.) per ligation. The linearized vector may be stored at -20?C.

D. Synthesis and purification of oligonucleotides

Oligonucleotide primers, either to close gaps in a cosmid or plasmid sequencing project or for PCR, are chosen by manual observation using the following criteria:

1. A relatively even base distribution (about 50% GC) is desired, with no obvious repeated motifs.

2. When possible the 3' end of the primer contained either a G or a C residue.

3. To determine if the sequence chosen is unique, the sequence is compared to the available cosmid or plasmid sequence using Findpatterns.

Alternatively, several computer programs, the SPARCstation-based ospX , the Macintosh-based HYPER PCR and Primer, or the VAX-based Primer programs, could be employed and yield similar results.

Oligonucleotides are synthesized according to manufacturer's procedures on either the Beckman Oligo 1000 (38) or the ABI 392 synthesizer (39) using the phosphoramidite chemistry (40). The desired oligonucleotide sequence is entered into the CPU affixed to the respective synthesizer, the reagent bottles are attached, and the column is inserted which contains the respective 3' nucleotide base-specific linked by the 3-OH group to the solid support, controlled-pore-glass (CPG) silica beads (41,42). The 5'-OH group of the base is blocked with a dimethyloxytrityl (DMT) group. Typically, the columns are purchased for 30 nmoles of primer. For large scale synthesis, for lab stocks of universal primers, columns for 1 umole synthesis are obtained, and can only

be used on the ABI 392. The synthesizer automatically performs a cycle of base addition which consists of 5-detritylation to remove the 5-DMT blocking group with trichloroacetic acid and dimethylchloride, activating the phosphoramidite nucleoside monomer with tetrazole and coupling the activated phosphoramidite nucleoside to the column, capping chains that were not coupled during the previous step by acylation of the 5-OH end with acetic anhydride and 1-methylimidazole (43,44), and oxidizing the internucleotide phosphate linkage from the phosphite ester to the more stable phosphotriester with iodine and water (39). After each step in the synthesis, the column is washed with acetonitrile. For the synthesis of fluorescent 5'-end-labeled oligonucleotides, the last base added has an aminolink on the 5' end (45). After synthesis, the oligonucleotide is removed from the solid support, the protecting groups are removed, and the primer is used directly after concentration by butanol precipitation (46).

Protocol

1. Synthesize the oligonucleotide on either the ABI 392 or the Beckman Oligo 1000 automated DNA synthesizers according to manufacturers' instructions (38,39).

2a. After the cycles of base addition are complete, the ABI 392 automatically detaches the synthesized oligonucleotide from the solid support by adding of ammonium hydroxide and transfers the mixture into a 1.5 ml screw cap microcentrifuge tube.

2b. For the Beckman Oligo 1000, to detach the oligonucleotide from the solid support remove the column and affix it to a screw cap tube containing 1 ml of concentrated ammonium hydroxide, and with a syringe attached to the fluted end of the column, draw the ammonium hydroxide into the column. Allow this assembly to remain at room temperature for at least one hour, no longer than two hours, and after about 30 minutes mix the solution with the syringe. Push the liquid out of the column with the syringe into the tube.

3. Incubate the oligonucleotide in ammonium hydroxide at 70deg C for at least 2 hours (for 1 umole synthesis on the ABI 392, incubate overnight) to deprotect the bases.

4. Transfer 100 ul aliquots of the mixture into 9 microcentrifuge tubes, add 1.25 ml of

n-butanol (46), vortex twice for 10 seconds, and centrifuge at 4deg C for 10 minutes at 13,000 rpm.

5. Decant and dry in the Speed-Vac until dry (at least 2 hours or overnight).

6. Add 115 ul of 10:0.1 TE buffer into the first tube, resuspend by pipetting up and down, and then transfer into the second tube, etc, until the dried oligonucleotide in all nine tubes is contained in one tube in about 100 ul.

7. Remove 10 ul of oligonucleotide and dilute with 990 ul of ddH2O and read the A260 in the 1 ml cuvette. The amount of oligonucleotide in the solution in the cuvette is 100 X A260, but the amount of oligonucleotide in the remaining 100 ul of solution is 10 X A260.

For synthesis of fluorescent 5'-end-labeled oligonucleotides:

1. Primers were synthesized on an ABI 392 DNA Synthesizer [1] at the 1uM synthesis scale with the final detritylation and the end/cleave program DMT ON, AUTO (END-CE) employed (i.e. no final detritylation step, automatic cleaving of the oligo from the column delivering a final volume of 2 ml conc. ammonium hydroxide). The final base to be added is the Aminolink-2 diamine used to couple the fluorescent dye to the oligonucleotide primer. The procedure employed for the synthesis and purification of the aminolinked-primers is as follows:

a. Edit the primer sequence into the synthesizer the final 5' end base being the Aminolink-2 e.g.. 5' 5CA GGA AAC AGC TAT GAC C 3', the 5 representing the Aminolink-2 reagent placed in the bottle 5 position.

b. Dissolve the Aminolink-2 in 3.3 ml dry acetonitrile and place it in the bottle 5 position.

c. Place fresh conc. (30%) ammonium hydroxide solution in the ammonium hydroxide bottle (bottle 10) if needed.

d. START SYNTHESIS on the ABI selecting the relevant sequences to be synthesized, DMT ON, AUTO (END CE) and EXECUTE ABI BEGIN -- YES if synthesizer has not been used in the last 6 hrs.

e. Remember to place a 2 dram vial at the outlet port for collection of the synthesized oligo.

f. When the synthesis and cleaving steps are complete remove the vial, which should contain 2 ml of solution, cap it tightly and leave at 70deg C for 12 hrs. to remove the base protecting groups.

g. Precipitate the DNA using the method of Sawadogo and Van Dyke in which to 1 part DNA/ammonium hydroxide solution 10 parts of n-butanol is added. This is best achieved by aliquoting the DNA solution into 20x1.5ml ependorf tubes of about 100 ul each and adding 1.25 ml n-butanol to each tube, vortex each for 10 sec then centrifuge for 20 min at 13K and 40degC.

h. Pour off the supernatant is poured off, drain the tube on a paper towel for 5 minutes then dry the pellet in a Savant Speed-Vac for at least 2 hrs (overnight preferable to be certain that no ammonium hydroxide or n-butanol remains). i. To pool together and desalt the following procedure was employed: i Combine two samples into one (therefore the 20 microcentrifuge tubes are combined resulting in 10 tubes) by dissolving one of the two samples in 40 ml of 1M NaCl and transferring it into the second tube. Further wash the first tube with 32 ml of 1M NaCl and again transfer to the second tube giving a total volume of 72 ml. ii Then add 84 ml of 95% Ethanol and briefly vortex. iii Add a further 84 ml 95% Ethanol, vortex and then leave at -20degC for 30 minutes to precipitate. vi Centrifuge the precipitated samples for 20 minutes at 13K and 4degC, pour off the supernatant and dry the samples in a Savant Speed-Vac for 15 minutes j. The aminolink-primer oligonucleotide is now ready for coupling with the four fluorescent dyes and may be stored at -20degC until ready to couple. 2. The next step in the synthesis of the labeled primers is the coupling reaction between the fluorescent dye and the aminolinked-oligonucleotide primer followed by its eventual purification. The four dyes named FAM, JOE, ROX and TAMRA come from ABI, each as a solution in 60 ml DMSO. These dyes (which once diluted must all be used) are further diluted with 440 ml DMF to give a final volume of 500 ml (50 ml/reaction) and are therefore enough for 10 reactions each. Therefore it is best to prepare 4 x 1uM columns worth of aminolinked-oligonucleotide primers as described above which then will be distributed into 40 x 1.5 ml microcentrifuge tubes, 10 reactions for each dye. The following procedure was therefore employed to prepare

the fluorescently labeled primers from the aminolinked-oligonucleotides:- a. First prepare 4 x 1uM columns worth of aminolinked-oligonucleotide as described above yielding 40 x 1.5 ml microcentrifuge tubes of oligonucleotide ready for coupling. b. Dissolve each of the samples in 50 ml of double distilled water and 50 ml of a 0.5M NaHCO3/Na2CO3 pH=9. c. Add to each of the four dyes 440 ml DMF (to give a final volume of 500 ml), then briefly vortex and centrifuge. d. From each dye add 50 ml to each of 10 predissolved aminolinked-oligonucleotide reactions and leave overnight at room temperature in the dark (covered with aluminum foil is sufficient). e. Pool each of the 10 samples together and elute through a G-25 column to remove any unreacted dye as follows: i Prepare four G-25 columns by eluting with 100 ml 0.1M NH4OAc (dilute from 8.0M stock). ii Apply each sample to the column and elute with 0.1M NH4OAc collecting the leading colored band, the second colored band being the unreacted dye. iii Aliquot the primer fractions into approximately 400 ml lots and precipitate with 1 ml of ethanol/acetate at -20degC for 2 hrs. iv Centrifuge the samples for 20 minutes at 4degC and 13K, pour off the supernatant and dry the samples in a Savant Speed-Vac. f. To purify the dye labeled primers from the unlabeled primers the samples were electrophoresed on a 20% polyacrylamide gel as follows:- i For each of the three primers JOE, ROX and TAMRA prepare one, and for FAM prepare two 0.3 mm/20% polyacrylamide gel with 10 wells each capable of holding a minimum of 25 ml. ii Pool each of the four sets of primers together in 200 ml deionized formamide/50 ml double distilled water. iii Apply 25 ml of dye solution to each of the 10 wells of the respective gels and electrophorese at 2500 V, 22-25 mA for 2.5 hrs. iv Pry the gels apart and with a razor blade, cut away the colored material, place in a large falcon tube with 2 ml 1X TAE (dilute from 20X stock) and leave overnight at 37degC. v Remove the solution and aliquot evenly into two of approximately 1 ml each and then wash the residue with 2 ml 0.1M NH4OAc (dilute from 20X stock). vi Desalt each of the two samples for each of the four dye labeled primers by eluting with 0.1M NH4OAc through a G-25 column (prepared as above) collecting again the colored band. vii Pool the fractions, measure the A260 and Ax (FAM, 494 nm; JOE, 527 nm; ROX, 586 nm; TAMRA, 558 nm) (the A260 in the 0.1

cm cuvette with the UV lamp and the Ax in the 1 cm cell with the VIS lamp) and then aliquot the solutions into 1.5 ml microcentrifuge tubes, 300 ml per tube. Record the total volume to calculate the OD (see appendix). viii Dry the samples in a Savant Speed-Vac overnight or leave in a drawer in the dark until dry and then store at -20degC. Notes:

1. The Beckman Oligo 1000 DNA synthesizer can also be used to synthesize the primers at the 1uM scale In this case the Aminolink-2 reagent is again dissolved in 3.3 ml of dry acetonitrile and then quickly transferred to the Beckman X bottle and placed on the X port. On START SYNTHESIS the relevant sequence is chosen, synthesis SET SCALE(nmol) - 1000 and FINAL DETRITYLATION - NO set. On completion the aminolink- oligo is manually cleaved from the column using concentrated (30%) ammonium hydroxide 1 ml, the cleaving step taking 1 hr to complete. The resulting solution is then transferred to a 2 dram vial the volume brought up to 2 ml with conc. ammonium hydroxide and the left at 70degC for 12 hr. the remaining steps are as mentioned above.

PAGE Purification of synthetic, fluorescent 5'-end-labeled oligonucleotides

1. Remove DNA collection vial from DNA synthesizer following automatic cleavage from column. Bring the total volume up to 4 ml with fresh concentrated NH4OH. Cap tightly and place at 55deg. C for 4 to 12 hours.

2. Remove vial from 55deg. C water bath and place on ice for 10 to 15 minutes. Transfer the sample to three siliconized microfuge tubes and dry under vacuum for 6-10 hours (until completely dry).

3. Dissolve the contents of one tube in 100 ul of ddH2O. Determine the concentration of the sample by measuring the absorbance at 260 nm.

4. Remove an aliquot of the sample containing approximately 2 A260 and mix with 10-20 ul of dye/formamide/EDTA mix. This will be a sufficient amount to load four 1 cm wells (i.e., 0.5 A260 per well).

5. Prepare a 20% polyacrylamide gel containing 7M urea (20 cm x 40 cm x 0.4 mm). Load the samples and electrophorese at 25mA until the slow blue dye (XC) has migrated about 14 cm from the origin (for a 17-mer).

6. Remove the top glass plate and cover the gel with Saran Wrap. Carefully lift the Saran Wrap and the gel off of the bottom glass plate. Flip the gel over and cover the other side with a second sheet of Saran Wrap. Visualize the DNA bands by UV shadowing and photograph. A 17-mer will migrate halfway between the BP and XC dye bands. (Note: BP=8 nt, XC=28 nt for a 20% gel) Outline the oligonucleotide bands with a marker.

7. Excise the oligonucleotide bands from the gel and place each gel slice in a siliconized 0.5 ml microfuge tube. Add enough TAE or water to cover the gel slice (approximately 200 ul) and place in a dry 37degC incubator overnight.

8. Pool the eluant from all tubes and desalt on a small G-25 column (1 to 2 ml bed volume). Read the absorbance at 260 nm of all fractions. Pool the peak fractions, and re-measure the absorbance. Oligonucleotides may be used directly, or diluted for sequencing or labeling reactions, frozen in small aliquots or dried.

E. Rapid hybridization of complementary M13 inserts

M13 clones carrying complementary inserts (i.e.: in the opposite orientation) may be rapidly screened by clone-to-clone hybridization, followed by analysis on an agarose gel.

1. Set up hybridization reaction as follows:

M13 clone 1 1.0 ul

M13 clone 2 1.0 ul

5x Hind/DTT 1.0 ul

ddH2O 3.0 ul

Total Vol. = 6.0 ul

Incubate at 55deg. C for 30 minutes.

Note: M13 DNA approximately 0.5 - 1.0 ug/ul

2. Add 6 ul of 2x Ficoll/BP/XC dye mix, vortex briefly and load on 0.7% agarose gel. Electrophorese at 90 mA for 1 hour.

3. Visualize DNA bands under UV light. Positives will run slower as a duplex DNA.

Solutions

5x Hind/DTT buffer:

Mix equal volumes of 10x Hind with 0.1 M DTT. Store at 4degC.

10x Hind buffer:

0.5 ml 2M Tris-HCl, pH 7.6, 0.7 ml 1M MgCl2, 0.35 g NaCl,

distilled water to 10 ml. Store at -20oC

0.1 M DTT:

154 mg dithiothreitol in 10 ml distilled water. Store at -20oC.

[NextPage]

APPENDIX

Solutions

10X ABI TBE:

216 g Tris base

110 g boric acid

16.6 g EDTA

Add water to 2 liters.

40% Acrylamide/Bisacrylamide (40% A&B):

380 g Acrylamide (Kodak 5521)

20 g N,N-Methylene-bisacrylamide (Kodak 8383)

Dissolve in approx. 800 ml of double distilled water and then deionize by stirring with 50 g of Amberlite MB-1 (Sigma MB-1A) for 1 hour at room temperature. Suction filter to remove the Amberlite and adjust to a final volume of 1 liter with double distilled water. (store at 4deg.C).

10x Agarose gel loading dye: 15% Ficoll, 0.2% bromophenol blue, 0.2% xylene cyanol FF in double distilled water.

1.5 g Ficoll (Sigma F-2637)

0.02 g Bromophenol blue (Sigma B-0126)

0.02 g xylene cyanole FF (Kodak T-1579)

ddH2O to 10 ml (store at -20deg.C).

Alkaline lysis solution (NaOH/SDS): 0.2 N NaOH, 1% SDS in ddwater.

20 ml of 1 N NaOH (or 0.8 gms)

10 ml of 10% SDS (or 1.0 gms)

ddH2O to 100 ml (make fresh)

15% Ammonium persulfate (APS):

1.5 g APS (Kodak 11151)

ddH2O to 10 ml (store at 4deg.C).

Ampicillin (Amp): Stock of 5 mg/ml in sterile ddwater (sddH2O).

0.5 g Amp (Sigma A-9518)

sddH2O to 100 ml (Add to media for final conc. 100 ug/ml)

100 mM rATP (adenosine triphosphate):

619 mg dipotassium ATP (ICN 100004)

sddH2O to 10 ml (aliquot and store at -20deg.C).

1 mg/ml BSA (bovine serum albumin):

5 mg BSA (Sigma A-9647)

sddH2O to 5 ml (aliquot and store at -20deg.C)

Bst dilution buffer: 50 mM HEPES, pH 7.6, 10 mM MgCl2, 1 mM DTT, and 1 mg/ml BSA in sterile double distilled water.

500 ul 1 M HEPES, pH 7.6

100 ul 1 M MgCl2

10 ul 1 M DTT

10 mg BSA

sddH2O to 10 ml

Bst reaction buffer: 500 mM Tris-HCl, pH 8.5 and 150 mM MgCl2 in sterile double distilled water.

5 ml 1 M Tris-HCl, pH 8.5

1.5 ml 1 M MgCl2

sddH2O to 10 ml

Bst nucleotide extension mix: 15 uM dCTP, 7deaza-dGTP, and dTTP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA

3 ul 5 mM dCTP

3 ul 5 mM 7deaza-dGTP

3 ul 5 mM dTTP

100 ul 50:1 TE buffer

891 ul sddH2O

1 ml

Bst "short" termination "A" mix: 8 uM dATP, 164 uM dCTP, 164 uM 7deaza-dGTP, 164 uM dTTP, 660 uM ddATP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

8 ul 0.5 mM dATP

16.4 ul 5 mM dCTP

16.4 ul 5 mM 7deaza-dGTP

16.4 ul 5 mM dTTP

66 ul 5 mM ddATP

50 ul 50:1 TE buffer

326.8 ul sddH2O

500 ul

aliquot (18 ul for 6 reactions) and store at -70degC

Bst "short" termination "C" mix: 8 uM dCTP, 164 uM dATP, 164 uM 7deaza-dGTP, 164 uM dTTP, 400 uM ddCTP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

16.4 ul 5 mM dATP

8 ul 0.5 mM dCTP

16.4 ul 5 mM 7deaza-dGTP

16.4 ul 5 mM dTTP

40 ul 5 mM ddCTP

50 ul 50:1 TE buffer

352.8 ul sddH2O

500 ul

aliquot (18 ul for 6 reactions) and store at -70degC

Bst "short" termination "G" mix: 8 uM dGTP, 164 uM dATP, 164 uM dCTP, 164 uM dTTP, 540 uM ddGTP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

16.4 ul 5 mM dATP

16.4 ul 5 mM dCTP

8 ul 0.5 mM 7deaza-dGTP

16.4 ul 5 mM dTTP

54 ul 5 mM ddGTP

50 ul 50:1 TE buffer

338.8 ul sddH2O

500 ul

aliquot (18 ul for 6 reactions) and store at -70degC

Bst "short" termination "T" mix: 8 uM dTTP, 164 uM dATP, 164 uM dCTP, 164 uM dTTP, 600 uM ddTTP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

16.4 ul 5 mM dATP

16.4 ul 5 mM dCTP

16.4 ul 5 mM 7deaza-dGTP

8 ul 0.5 mM dTTP

60 ul 5 mM ddTTP

50 ul 50:1 TE buffer

332.8 ul sddH2O

500 ul

aliquot (18 ul for 6 reactions) and store at -70degC

Bst "long" termination "A" mix: 8 uM dATP, 164 uM dCTP, 164 uM 7deaza-dGTP, 164 uM dTTP, 110 uM ddATP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

8 ul 0.5 mM dATP

16.4 ul 5 mM dCTP

16.4 ul 5 mM 7deaza-dGTP

16.4 ul 5 mM dTTP

11 ul 5 mM ddATP

50 ul 50:1 TE buffer

381.8 ul sddH2O

500 ul

aliquot (18 ul for 6 reactions) and store at -70degC

Bst "long" termination "C" mix: 8 uM dCTP, 164 uM dATP, 164 uM 7deaza-dGTP,

164 uM dTTP, 65 uM ddCTP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

16.4 ul 5 mM dATP

8 ul 0.5 mM dCTP

16.4 ul 5 mM 7deaza-dGTP

16.4 ul 5 mM dTTP

6.5 ul 5 mM ddCTP

50 ul 50:1 TE buffer

386.3 ul sddH2O

500 ul

aliquot (18 ul for 6 reactions) and store at -70degC

Bst "long" termination "G" mix: 8 uM dGTP, 164 uM dATP, 164 uM dCTP, 164 uM dTTP, 70 uM ddGTP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

16.4 ul 5 mM dATP

16.4 ul 5 mM dCTP

8 ul 0.5 mM 7deaza-dGTP

16.4 ul 5 mM dTTP

7 ul 5 mM ddGTP

50 ul 50:1 TE buffer

385.8 ul sddH2O

500 ul

aliquot (18 ul for 6 reactions) and store at -70degC

Bst "long" termination "T" mix: 8 uM dTTP, 164 uM dATP, 164 uM dCTP, 164 uM dTTP, 150 uM ddTTP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

16.4 ul 5 mM dATP

16.4 ul 5 mM dCTP

16.4 ul 5 mM 7deaza-dGTP

8 ul 0.5 mM dTTP

15 ul 5 mM ddTTP

50 ul 50:1 TE buffer

377.8 ul sddH2O

500 ul

aliquot (18 ul for 6 reactions) and store at -70degC

100 mM calcium chloride (CaCl2):

1.48 g CaCl2-2H2O

ddH2O to 100 ml

autoclave to sterilize (store at 4deg.C).

50 mM calcium chloride:

0.74 g CaCl2-2H2O

ddH2O to 100 ml

autoclave to sterilize (store at 4deg.C).

Chloramphenicol 10 mg/ml stock:

2 g Chloramphenicol (Sigma C-0378)

200 ml 100% (or 95%) ethanol

Final volume 200 ml

Sterilize by filtering through a Nalgene filter (#8-0000-22 0301)

Store in the cold room in a foil wrapped screw cap Nalgene bottle

Use as an additive to microbial growth media at a final concentration of 15 ug/ml media.

For 200 ml media, add 300 ul of 10 mg/ml chloramphenicol stock.

For 1 liter media, add 1.5 ml of 10 mg/ml chloramphenicol stock.

Deionized formamide: Stir formamide (Schwarz/Mann Biotech 800686) with Amberlite MB resin, 10 g. per 100 ml, for one hour to deionize; filter through Whatman 3MM paper, store in a dark bottle at room temperature or 4deg.C.

10X denaturing buffer: 200 mM Tris-HCl, pH 9.5, 1 mM EDTA, 10 mM spermidine in double distilled water.

2 ml 1 M Tris-HCl, pH 9.5

20 ul 0.5 M EDTA, pH 8.0

1 ml 100 mM spermidine

ddH2O to 10 ml (aliquot and store at -20degC)

Diatomaceous earth (100 mg/ml): Suspend 10 g of diatomaceous earth (Sigma

D-5384) in 100 ml of distilled water in a 100 ml graduated cylinder, and let it settle down for 3 hours. Decant the supernatant, and resuspend the pellet in 100 ml of 6 M guanidine HCl, pH 6.4, containing 50 mM Tris-HCl, 20 mM EDTA.

Diatomaceous earth-wash buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 50% ethanol in double distilled water.

10 ml 1 M Tris-HCl, pH 8.0

2 ml 0.5 M EDTA, pH 8.0

500 ml 100% ethanol (McCormick Distilling Co., Inc.)

ddH2O to 1 L

1 M DTT (Dithiothreitol, Cleland's reagent):

1.54 g DTT (Calbiochem 233155)

ddH2O to 10 ml (aliquot and store at -20deg.C).

DNase-free RNase A: 20 mg/ml RNase A in 1 mM NaOAc, pH 4.5.

200 mg RNase A (Sigma R-5500)

3.3 ul 3 M NaOAc, pH 4.5

ddH2O to 10 ml

boil for 10 minutes (aliquot and store at -20deg.C).

0.5 M EDTA, pH 8.0 (disodium ethylenediamine tetraacetate):

186.1 g Na2EDTA

Dissolve in approx. 400 ml ddH2O, adjust pH to 8.0 with 10 N NaOH, and adjust to 1 liter final volume with distilled water.

100 mM EDTA:

20 ml 0.5 M EDTA

80 ml ddH2O

100 ml

25mM EDTA, 50 mg/ml Blue Dextran - ABI377 Loading Dye/Formamide mixture: Add 0.93g of EDTA to 90 ml water. Then, adjust the pH to 8.0. Bringthe final volume to 100 ml. Next, add 50 mg Blue Dextran to a 1 ml EDTA solution. Add 1 ul of a 1:5 solution of this loading dye:deionized formamide to each sample well for loading onto the ABI377

95% ethanol/0.12 M NaOAc (ethanol/acetate):

95 ml	100% ethanol
4 ml	3 M NaOAc pH 4.5
1 ml	ddH2O
100 ml	

5 mg/ml ethidium bromide (EtBr):

500 mg EtBr (Sigma E-8751)

ddH2O to 100 ml

10X Freezer Media (FM) for storing either shotgun plasmid-based sub-clone or cDNA clones in microtiter plates):

Final Concentration	1L	500 ml	250 ml
360 mM K2HPO4	62.7 gm	31.35 gm	15.68 gm
132 mM KH2PO4	17.96 gm	8.98 gm	4.49 gm
17 mM Sodium Citrate	5.0 gm	2.5 gm	1.25 gm
4 mM MgSO4.7H2O	0.98 gm	0.49 gm	0.24 gm
(or 1M MgSO4	4 ml	2 ml	1 ml)
68 mM (NH4)2SO4	8.98 gm	4.49 gm	2.25 gm
44% Glycerol	440 ml	220 ml	110 ml

Bring to volume with dH2O

Sterilize by filtration throught 0.2um filter

Then the final growth and storage media is prepared in a ratio of 9 volumes LB media and 1 volume of this 10X Freezer Media (FM).

FE (formamide/EDTA): 5:1 (v/v) formamide:50 mM EDTA

10 ul	ddH2O
10 ul	100 mM EDTA
100 ul	deionized formamide

make fresh

10X Fill-in/Kinase buffer: 500 mM Tris-HCl, pH 7.6, 100 mM MgCl2, 10 mM DTT,

and 50 ug/ml BSA in double distilled water.

5 ml 1 M Tris-HCl, pH 7.6

1 ml 1 M MgCl2

100 ul 1 M DTT

500 ul 1 mg/ml BSA

3.4 ml ddH2O

10 ml

Fill-in Deoxynucleotide Preparation: To make 4 ml of the fill-in nucleotides at a concentration of 0.25 mM of each nucleotide, combine the following:

500 ul PCR dNTPs (2 mM)

3500 ul ddH2O

Aliquot this into 0.5 ml eppendorf tubes with 10 ul in each tube.

To make 4 ml of these nucleotides at a final concentration of 0.25 mM from the stock 100 mM solutions, add the following:

10 ul 100 mM dATP

10 ul 100 mM dCTP

10 ul 100 mM dGTP

10 ul 100 mM dTTP

3.6 ml ddH2O

Aliquot into 0.5 eppendorf tubes with 10 ul in each tube.

To order these nucleotides, call Pharmacia at 1 800-526-3593 and use customer number 6933. Order the dNTP set: 27-2035-01 dNTP set (100mM each dATP, dCTP, dGTP, dTTP- each in 250 ul volume) $174.00 for the set.

20% glucose:

20 g d-glucose

ddH2O to 100 ml

filter sterilize

Sterile glycerin (sterile glycerol):

Either autoclave the bottle of ACS grade Glycerin (Fisher Scientific #G33-500) with loosened cap after putting autocalve tape on the bottle (to indicate that it has been

autoclaved) or we make a 50% solution of the same Glycerin with ultrapure water (which then is easier to work with) and autoclave that in a convenient size bottle.

6 M guanidine HCl, pH 6.4, containing 50 mM Tris-HCl, 20 mM EDTA:

573.18 g guanidine-HCl (Sigma G-4505)

50 ml 1 M Tris-HCl, pH 7.6

40 ml 0.5 M EDTA, pH 8.0

ddH2O to 1 liter

GET/lysozyme solution: 50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA, pH 8.0 in double distilled water,

0.9 g d-glucose

2.5 ml 1 M Tris-HCl, pH 8.0

2 ml 0.5 M EDTA, pH 8.0

ddH2O to 100 ml (filter sterilize and store at 4degC).

Add 2 mg/ml lysozyme (Sigma L-6876) just before use.

1 M HEPES, pH 7.5:

23.83 g HEPES (Sigma H-3375)

ddH2O to 100 ml

adjust pH to 7.5 with potassium hydroxide (KOH) (store at 4deg.C).

IPTG (isopropyl b-D-thiogalactopyranoside):25 mg/ml IPTG in double distilled water

250 mg IPTG (Sigma I-5502)

ddH2O to 10 ml (aliquot and store at -20degC)

1 M isocitrate (sodium salt-dihydrate):

29.41 g Na3isocitrate-2H2O (Sigma C-7254)

ddH2O to 100 ml

10x Kinase buffer: 500 mM Tris-HCl, pH 7.6, 100 mM MgCl2, and 100 mM DTT in sterile double distilled water.

5 ml 1 M Tris-HCl, pH 7.6

1 ml 1 M MgCl2

1 ml 1 M DTT

sddH2O to 10 ml (store in 25 ml aliquots at -20deg.C).

Kanamycin sulfate (Kan): Stock of 5 mg/ml in sterile double distilled water (sddH2O).

0.5 g Kanamycin (Boehringer Mannheim 106 801)

sddH2O to 100 ml (Add to media for final conc. 20 ug/ml)

1M KCl (potassium chloride):

7.5 g KCl

ddH2O to 100 ml

Lambda plates:

10 g Bacto-tryptone (Difco 0123-01-1)

15 g Bacto-agar (Difco 0140-01)

2.5 g NaCl

ddH2O to 1 L

autoclave to sterilize and pour into sterile petri dishes (approx. 20 ml/plate).

Lambda top agar:

10 g Bacto-tryptone (Difco 0123-01-1)

10 g Bacto-agar (Difco 0140-01)

5 g NaCl

ddH2O to 1 L

autoclave to sterilize

LB Medium:

10 g Bacto-Tryptone (Difco 0123-01-1)

5 g Bacto-yeast extract (Difco 0127-05-3)

10 g NaCl

ddH2O to 1 L

adjust the pH to 7.0 and then

autoclave to sterilize

(p> LB plates:

10 g Bacto-Tryptone (Difco 0123-01-1)

5 g Bacto-yeast extract (Difco 0127-05-3)

10 g NaCl

15 g Bacto-agar (Difco 0140-01)

ddH2O to 1 L

autoclave to sterilize, cool to 55deg.C, add antibiotic if desired, and pour into sterile petri dishes (approx. 20 ml/plate).

10x Ligation buffer: 50 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 10 mM DTT, 10 mM rATP, and 250 ug/ml bovine serum albumin (BSA) in sterile double distilled water.

5 ml 1 M Tris-HCl, pH 7.6

1 ml 1 M MgCl2

1 ml 1 M DTT

1 ml 100 mM rATP

2.5 mg BSA

sddH2O to 10 ml (store in 25 ml aliquots at -20deg.C)

Loading dye: 0.3% xylene cyanole FF, 0.3% bromophenol blue, 10 mM EDTA in deionized formamide.

3 g xylene cyanole FF

3 g bromophenol blue

0.2 ml 0.5 M EDTA

deionized formamide to 10 ml

Lysozyme solution: 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 5 mg/ml lysozyme in sterile double distilled water.

5 ml 1 M Tris-HCl, pH 8.0

2 ml 0.5 M EDTA

0.5 g lysozyme (Sigma L-6876)

sddH2O to 100 ml (make fresh)

M-9 Agar: Add 15 g. agar to 870 ml. double distilled water in a 2 L Ehrlenmeyer flask and autoclave. Also autoclave a 100 ml. graduate cylinder capped with aluminum foil to use for measuring the sterile M-9 salts later. Swirl the agar gently and carefully upon removal from autoclave to disperse any undissolved agar. Allow to cool in a 55degC water bath. When 55degC, add the ingredients called for in the M-9 liquid medium recipe, omitting the water. Be sure to use sterile pipettes or graduate

cylinders, as this mixture cannot be autoclaved. Immediately pour into sterile Petri dishes, using sterile technique.

M-9 Medium (liquid):

100 ml 10X M-9 salts

1 ml 1 M MgSO4 (autoclaved)

10 ml 20% glucose (filter sterilized),

1 ml 1% thiamine (filter sterilized)

10 ml 100 mM CaCl2 (autoclaved)

sddH2O to 1 L

10X M-9 Salts:

60 g Na2HPO4 (sodium phosphate, dibasic)

30 g KH2PO4 (potassium phosphate, monobasic)

5 g NaCl

10 g NH4Cl (ammonium chloride)

ddH2O to 1 liter (autoclave)

1 M MgCl2 (magnesium chloride):

20.33 g MgCl2-6H2O

ddH2O to 100 ml

1 M MgSO4 (magnesium sulfate):

12.04 g MgSO4

ddH2O to 100 ml (autoclave)

1 M MnCl2 (manganese chloride):

1.98 g MnCl2 (Sigma M-8530)

ddH2O to 10 ml (store protected from light)

1 M MOPS:

20.93 g MOPS (Sigma M-1254)

Dissolve in 80 ml ddH2O, adjust pH to 7.5 with 1 N NaOH, and bring volume to 100 ml.

10X MOPS buffer: 400 mM MOPS, pH 7.5, 500 mM NaCl, 100 mM MgCl2 in double distilled water.

400 ul 1 M MOPS, pH 7.5

170 ul 3 M NaCl

100 ul 1 M MgCl2

330 ul ddH2O

1 ml

2.7 M MOPS (acid form):

5.65 g MOPS (acid form)

ddH2O to 10 ml

MOPS-Acid buffer: 1.35 M MOPS (acid form), 100 mM MgCl2 in double distilled water.

500 ul 2.7 M MOPS (acid form)

100 ul 1 M MgCl2

400 ul ddH2O

1 ml

10X Mn2+/isocitrate buffer: 50 mM MnCl2, 150 mM isocitrate (Na salt), 25% glycerol in double distilled water

50 ul 1 M MnCl2

150 ul 1 M isocitrate

250 ul glycerol

550 ul ddH2O

1 ml

10x MTBE (Modified Tris-borate-EDTA buffer): 1.3 M Tris, 0.4 M Boric acid, and 0.02 M EDTA in double distilled water.

162 g Tris base

27.5 g Boric acid

9.3 g Na2EDTA

ddH2O to 1 L

Nucleotide ordering information:

100 mM dATP 27-2050-01 Pharmacia

100 mM dCTP 27-2060-01 Pharmacia

100 mM dGTP	27-2070-01	Pharmacia
10 mM c7dGTP	988 537	Boehringer-Mannheim
100 mM dTTP	27-2080-01	Pharmacia
5 mM ddATP	27-2057-00	Pharmacia
5 mM ddCTP	27-2065-00	Pharmacia
5 mM ddGTP	27-2075-00	Pharmacia
5 mM ddTTP	27-2085-00	Pharmacia

20 mM dNTP stocks: Prepare from 100 mM stocks

80 ul	100 mM dNTP
40 ul	50:1 TE buffer
280 ul	ddH2O
400 ul	

5 mM dNTP stocks: Prepare from 20 mM stocks

25 ul	20 mM dNTP
10 ul	50:1 TE buffer
65 ul	ddH2O
100 ul	

2 mM dNTPs: 2 mM dATP, dCTP, dGTP, and dTTP in 5 mM Tris-HCl, pH 7.6, 0.1 mM EDTA

100 ul	20 mM dATP
100 ul	20 mM dCTP
100 ul	20 mM dGTP
100 ul	20 mM dTTP
100 ul	50:1 TE buffer
500 ul	ddH2O
1 ml	

2 mM [alpha]-S-dNTPs: 2 mM [alpha]-S-dATP, [alpha]-S-dCTP, [alpha]-S-dGTP, and [alpha]-S-dTTP in 5 mM Tris-HCl, pH 7.6, 0.1 mM EDTA

100 ul	20 mM [alpha]-S-dATP
100 ul	20 mM [alpha]-S-dCTP

100 ul 20 mM [alpha]-S-dGTP

100 ul 20 mM [alpha]-S-dTTP

100 ul 50:1 TE buffer

500 ul ddH2O

1 ml

3M NaCl (sodium chloride):

17.53 g NaCl

ddH2O to 100 ml

10N NaOH (sodium hydroxide):

40 g NaOH

ddH2O to 100 ml.

1N NaOH:

10 ml 10 N NaOH

ddH2O to 100 ml

9.5M NH4OAc (ammonium acetate):

73.23 g NH4OAc

ddH2O to 100 ml

8.0M NH4OAc:

61.69 g NH4OAc

ddH2O to 100 ml

10X PCR buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.5, 15 mM MgCl2 in sterile double distilled water

5 ml 1 M KCl

1 ml 1 M Tris-HCl, pH 8.5

150 ul 1 M MgCl2

ddH2O to 10 ml

PCR Deoxynucleotide Preparation: To make 12.5 ml of the PCR nucleotides at a concentration of 2 mM each nucleotide, combine the following:

250 ul 100 mM dATP

250 ul 100 mM dCTP

250 ul 100 mM dGTP

250 ul 100 mM dTTP

11.5 ml ddH2O

Aliquot this into 25 tubes with 500 ul in each tube. This will keep the nucleotides from being frozed and thawed too much.

To order these nucleotides, call Pharmacia at 1 800-526-3593 and use customer number 6933. Order the dNTP set: 27-2035-01 dNTP set (100mM each dATP, dCTP, dGTP and dTTP- each in 250 ul volume)$174.00 for the set.

20% PEG/2.5 M NaCl:

7.3 g	NaCl
10 g	PEG (MW=8000) (Fisher P156-3)

Dissolve in 40 ml double distilled water by stirring, and then adjust the volume to 50 ml.

50% PEG/0.5 M NaCl:

5.85 g	NaCl
100 g	PEG (MW=8000) (Fisher P156-3)

Dissolve in 100 ml double distilled water by stirring, and then adjust the volume to 200 ml.

PEG:TE rinse solution: 1:3 solution of 20% PEG containing 2.5M NaCl and 10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA in double distilled water.

250 ul	1 M Tris-HCl, pH 8.0
50 ul	0.5 M EDTA
12.5 ml	20% PEG/2.5 M NaCl.
ddH2O to 37.5 ml	

Phenol, TE-saturated: add an equal volume of 10 mM Tris-HCl, pH 7.5-8.0, 1 mM Na2EDTA to ultrapure phenol, mix well, allow phases to separate, remove and discard upper (aqueous) phase. Repeat until the pH of the aqueous phase is between 7.5-8.0 (store at 4deg. C).

Phenol/chloroform/isoamyl alcohol (25:25:1):

100 ml	TE-saturated phenol

100 ml chloroform

4 ml isoamyl alcohol

204 ml

2M NaOAc (sodium acetate):

27.22 g NaOAc-3H2O

ddH2O to 100 ml

3M NaOAc, pH 4.5:

408.24 g NaOAc-3H2O

Dissolve in approx. 800 ml ddH2O , adjust pH to 4.8 with glacial acetic acid and bring to a final volume of 1 L with ddH2O.

Restriction enzyme assay buffer, 10X Low Salt: 100 mM Tris-HCl, pH 7.6, 100 mM MgCl2, and 10 mM DTT in sterile double distilled water.

1 ml 1 M Tris-HCl, pH 7.6

1 ml 1 M MgCl2

0.1 ml 1 M DTT

ddH2O to 10 ml

Restriction enzyme assay buffer, 10X Medium Salt: 500 mM NaCl, 100 mM Tris-HCl, pH 7.6, 100 mM MgCl2, and 10 mM DTT in sterile double distilled water.

1.7 ml 3 M NaCl

1 ml 1 M Tris-HCl, pH 7.6

1 ml 1 M MgCl2

0.1 ml 1 M DTT

ddH2O to 10 ml

Restriction enzyme assay buffer, 10X High Salt: 1M NaCl, 500 mM Tris-HCl, pH 7.6, 100 mM MgCl2, and 10 mM DTT in sterile double distilled water.

3.3 ml 3 M NaCl

5 ml 1 M Tris-HCl, pH 7.6

1 ml 1 M MgCl2

0.1 ml 1 M DTT

ddH2O to 10 ml

Restriction enzyme assay buffer, 10X SmaI: 200 mM KCl, 100 mM Tris-HCl, pH 7.6, 100 mM MgCl2, and 10 mM DTT in sterile double distilled water.

2 ml	1 M KCl
1 ml	1 M Tris-HCl, pH 7.6
1 ml	1 M MgCl2
0.1 ml	1 M DTT
ddH2O to 10 ml	

RNase T1: 100 U/ul in 50 mM Tris-HCl, pH 7.6

100 ul	RNase T1 (Sigma R-8251) (100,000 U/0.2 ml)
25 ul	1 M Tris-HCl, pH 7.6
375 ul	ddH2O
500 ul	

10% SDS (sodium dodecyl sulfate):

10 g	SDS (Fisher S529-3)
ddH2O to 100 ml	

1X STB buffer: 25% sucrose and 50 mM Tris-HCl, pH 8.0 in double distilled water.

25 g	sucrose
5 ml	1 M Tris-HCl, pH 8.0
ddH2O to 100 ml (filter sterilize and store at 4degC)	

Silanizing reagent: 5% solution of dichloro dimethyl silane in 1,1,1-trichloroethane.

20X SSC (standard saline-citrate):

17.53 g	NaCl
8.82 g	sodium citrate

Dissolve in approx. 80 ml ddH2O, adjust pH to 7.0 with hydrochloric acid (HCl) and bring final volume to 100 ml.

1X SSC (standard saline-citrate):

5 ml	20X SSC
95 ml	ddH2O
100 ml	

5X Taq reaction buffer: 400 mM Tris-HCl, pH 9.0, 100 mM ammonium sulfate

((NH4)2SO4), pH 9.0, 25 mM MgCl2, and 5% dimethyl sulfoxide (DMSO) in sterile double distilled water.

16 ml	1 M Tris-HCl, pH 9.0
4 ml	1 M (NH4)2SO4, pH 9.0
1 ml	1 M MgCl2
2 ml	DMSO
17 ml	ddH2O
40 ml	

5X Taq dilution buffer: 400 mM Tris-HCl, pH 9.0, 100 mM (NH4)2SO4, pH 9.0, and 25 mM MgCl2 in sterile double distilled water.

16 ml	1 M Tris-HCl, pH 9.0
4 ml	1 M (NH4)2SO4, pH 9.0
1 ml	1 M MgCl2
19 ml	ddH2O
40 ml	

5X Taq "A" termination mix: 62.5 uM dATP, 250 uM dCTP, 375 uM c7dGTP, 250 uM dTTP and 1.5 mM ddATP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

20 ul	20 mM dATP
80 ul	20 mM dCTP
240 ul	10 mM 7deaza-dGTP
80 ul	20 mM dTTP
1920 ul	5 mM ddATP
640 ul	50:1 TE buffer
3420 ul	sddH2O
6.4 ml	

5X Taq "C" termination mix: 250 uM dATP, 62.5 uM dCTP, 375 uM c7dGTP, 250 uM dTTP and 0.75 mM ddATP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

80 ul	20 mM dATP
20 ul	20 mM dCTP
240 ul	10 mM 7deaza-dGTP

80 ul	20 mM dTTP
960 ul	5 mM ddCTP
640 ul	50:1 TE buffer
4380 ul	sddH2O
6.4 ml	

5X Taq "G" termination mix: 250 uM dATP, 250 uM dCTP, 94 uM c7dGTP, 250 uM dTTP and 0.125 mM ddGTP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

160 ul	20 mM dATP
160 ul	20 mM dCTP
120 ul	10 mM 7deaza-dGTP
160 ul	20 mM dTTP
320 ul	5 mM ddGTP
1280 ul	50:1 TE buffer
10600 ul	sddH2O
12.8 ml	

5X Taq "T" termination mix: 250 uM dATP, 250 uM dCTP, 375 uM c7dGTP, 62.5 uM dTTP and 1.25 mM ddTTP in 5 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA.

160 ul	20 mM dATP
160 ul	20 mM dCTP
480 ul	10 mM 7deaza-dGTP
40 ul	20 mM dTTP
3200 ul	5 mM ddTTP
1280 ul	50:1 TE buffer
7480 ul	sddH2O
12.8 ml	

20X TAE buffer: 0.8 M Tris, 0.4 M NaOAc, and 0.04 M Na2EDTA, and glacial acetic acid to pH 8.3 in double distilled water.

96.9 g	Tris base
32.8 g	NaOAc-3H2O
14.9 g	Na2EDTA

Dissolve in approx. 700 ml of double distilled water, adjust the pH to 8.3 with glacial acetic acid, and bring to 1 L with ddH2O.

TEMED (N,N,N',N'-tetramethylethylenediamine): Kodak T-7024, store protected from light at 15degC.

10xTB Salts:

2.31 g KH2PO4

12.54 g K2HPO4 (potassium phosphate, dibasic)

ddH2O to 100 ml autoclave)

Terrific Broth (TB):

12 g Bacto-tryptone

24 g yeast extract

4 ml glycerol

ddH2O to 900 ml

Autoclave, cool and add 100 ml of 10xTB Salts and adjust the final volume to 1 L with sddH2O.

TE (10:0.1) buffer:10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA

10 ml 1 M Tris-HCl, pH 7.6

0.2 ml 0.5 M EDTA

ddH2O to 1 L

TE (10:1) buffer: 10 mM Tris-HCl, pH 7.6, 1 mM EDTA

10 ml 1 M Tris-HCl, pH 7.6

2 ml 0.5 M EDTA

ddH2O to 1 L

TE (100:10) buffer: 100 mM Tris-HCl, pH 7.6, 10 mM EDTA

100 ml 1 M Tris-HCl, pH 7.6

20 ml 0.5 M EDTA

ddH2O to 1 L

TE (50:1) buffer: 50 mM Tris-HCl, pH 7.6, 1 mM EDTA

0.5 ml 1 M Tris-HCl, pH 7.6

0.1 ml 100 mM EDTA

9.4 ml ddH2O

10 ml

TE-RNase solution: 50:10 TE buffer containing 40 ug/ml RNase A and 40 U/ml RNase T1

1.2 ml 1 M Tris-HCl, pH 7.6

480 ul 0.5 M EDTA

50 ul 20 mg/ml RNase A

10 ul 100U/ul RNase T1

22.3 ml ddH2O

24.0 ml

Tetracycline stock (Tet): Stock of 10 mg/ml in 50% ethanol + sddwater.

1 g Tetracycline (Sigma T-3383)

50 ml 100% ethanol

sddH2O to 100 ml (store at 4deg.C in the absence of light)

Add to media for final conc. 20 ug/ml.

1% thiamine:

100 mg thiamine (Sigma T-4625)

sddH2O to 10 ml (filter sterilized)

1M Tris-HCl, pH 7.6, 8.0, 8.5, 9.0, 9.5:

121.1 g Tris base

ddH2O to 800 ml

Adjust pH with concentrated HCl and then add ddH2O to 1 L.

10X TM buffer: 500 mM Tris-HCl, pH 8.0, 150 mM MgCl2 in sterile double distilled water.

5 ml 1 M Tris-HCl, pH 8.0

1.5 ml 1 M MgCl2

sddH2O to 10 ml

50:2:10 TTE: 50 mM Tris-HCl, pH 8.0, 2% Triton X-100, and 10 mM EDTA in double distilled water.

5 ml 1 M Tris-HCl, pH 8.0

2 ml 0.5 M EDTA

2 ml Triton X-100 (Sigma X-100)

ddH2O to 100 ml

TTE: 10 mM Tris-HCl, pH 8.0, 0.5% Triton X-100, and 0.1 mM EDTA in double distilled water.

500 ul 1 M Tris-HCl, pH 8.0

250 ul Triton X-100 (Sigma X-100)

10 ul 0.5 M EDTA

ddH2O to 50 ml

X-gal (5-bromo-4-chloro-3-indolyl b-D-galactopyranoside):

200 mg x-gal (Sigma B-4252)

dimethylformamide (DMF) to 10 ml

Aliquot and store protected from light at -20degC)

2xTY medium:

16 g Bacto-tryptone (Difco 0123-01-1)

10 g Bacto-yeast extract (Difco 0127-05-3)

5 g NaCl

ddH2O to 1 L (autoclave)

[NextPage]

Primers:

ABI Forward primer sequence-

20mer 5' GACGTTGTAAAACGACGGCC 3'

18mer 5' TGTAAAACGACGGCCAGT 3'

ABI Forward Aminolink-primer sequence-

5' 5TG TAA AAC GAC GGC CAG T 3'

ABI Reverse primer sequence-

20mer 5' CACAGGAAACAGCTATGACC 3'

18mer 5' CAGGAAACAGCTATGACC 3'

ABI Reverse Aminolink-primer sequence-

5' 5CA GGA AAC AGC TAT GAC C 3'

Taq Cycle Sequencing Reagent Preparation

1. 5X Taq Reaction buffer

400 mM Tris-HCl, pH 9.0 16 ml 1 M Tris-HCl, pH 9.0

100 mM (NH4)2SO4, pH 9.0 4 ml 1 M (NH4)2SO4, pH 9.0

25 mM MgCl2, pH 7.0 1 ml 1 M MgCl2, pH 7.0

5% DMSO 2 ml DMSO

17 ml ddH2O

40 ml

The 5X Taq reaction buffer will be added separately with the A, C, G, and T nucleotide mixes for ease in reaction pipetting. One 40 ml preparation of buffer will be sufficient for one batch (about 200 tubes) of A, C, G, and T mix aliquots.

2. Taq Dilution Buffer

400 mM Tris-HCl, pH 9.0 16 ml 1 M Tris-HCl, pH 9.0

100 mM (NH4)2SO4, pH 9.0 4 ml 1 M (NH4)2SO4, pH 9.0

25 mM MgCl2, pH 7.0 1 ml 1 M MgCl2, pH 7.0

19 ml ddH2O

40 ml

This is routinely distributed into 30 ul aliquots in clear, unlabeled 0.5 ml microcentrifuge tubes (about 200 per batch).

3. 50:1 TE

50 mM Tris-HCl, pH 7.6 0.5 ml 1 M Tris-HCl, pH 7.6

1 mM Na2EDTA, pH 8.0 0.1 ml 0.1 M Na2EDTA, pH 8.0

9.6 ml ddH2O

10 ml

4. Fluorescent Labeled Primers

Prepare a 100 X stock solution (40 uM); an example calculation for a dry tube of an 18mer with an O.D. of 1.00 is shown below (remembering that Joe is the dye-labeled primer for the A reaction, Fam is for C, Tamra is for G, and Rox is for T):

1.00 OD(37 ug/OD)(mol x mer/320 g)(10+12pmol/mol)(g/10+6ug)

(1/18 mer)(ul/40 pmoles)=x ul

In this example x=160 ul, and 160 ul of ddH20 should be added to the dried tube of fluorescent primer for a concentration of 40 uM (40 pmol/ul). From this 100X stock of 40 uM make 1:100 dilutions. To make the amount of primer aliquoted the same as the amount of mixes per batch:

Dilute either fluorescent forward or reverse primers as follows:

64 ul of 40 uM A or C primer	128 ul of 40 uM G or T primer
6.3 ml ddH2O	12.7 ml ddH2O
6.4 ml	12.8 ml

For the A and C primers, distribute the 1X (0.4 uM solution) into 30 ul aliquots, and for the G and T primers, distribute them into 60 ul aliquots. The primers aliquots are stored in clear 0.5 ml microcentrifuge tubes which are labeled with blue, green, red, or yellow markers for A, C, G, or T primers, respectively. (Note: The current primers work optimally at the effective concentration of 0.4 uM, however with each new fluorescent primer preparation, the optimal concentration must be determined). The primers should be stored at 20 or -70degC.

5. 5X Taq Cycle Sequencing Mixes Working dilutions of 20 mM are made for dATP, dCTP, and dTTP based on using one complete tube of 20 mM stock per batch of mixes. 7deaza-dGTP is purchased at a concentration of 10 mM (1080 ul are needed for one batch each of A, C, G, and T mixes, so slightly more than five tubes will be needed-each tube contains 200 ul).

20 mM dATP	20 mM dCTP	20 mM dTTP
95 ul of 100 mM dATP	95 ul of 100 mM dCTP	80 ul of 100 mM dTTP
47.5 ul of 50:1 TE	47.5 ul of 50:1 TE	40 ul of 50:1 TE
332.5 ul ddH2O	332.5 ul ddH2O	280 ul ddH2O

475 ul 475 ul 400 ul

The concentration of deoxy and dideoxy nucleotides in the mixes are shown below, followed by the recipe for one 200 tube batch of each of the four mixes.

	A	C	G	T
dATP	62.5 uM	250 uM	250 uM	250 uM
dCTP	250 uM	62.5 uM	250 uM	250 uM
7-dGTP	375 uM	375 uM	94 uM	375 uM
dTTP	250 uM	250 uM	250 uM	62.5 uM
ddATP	1.5 mM	--	--	--
ddCTP	--	0.75 mM	--	--
ddGTP	--	--	0.125 mM	--
ddTTP	--	--	--	1.25 mM

For one batch (200 tubes) of each nucleotide mix:

	A	C	G	T
20 mM dATP	20 ul	80 ul	160 ul	160 ul
20 mM dCTP	80 ul	20 ul	160 ul	160 ul
10 mM 7-dGTP	240 ul	240 ul	120 ul	480 ul
20 mM dTTP	80 ul	80 ul	160 ul	40 ul
5 mM ddATP	1920 ul	--	--	--
5 mM ddCTP	--	960 ul	--	--
5 mM ddGTP	--	--	320 ul	--
5 mM ddTTP	--	--	--	3200 ul
50:1 TE	640 ul	640 ul	1280 ul	1280 ul
ddH2O	3420 ul	4380 ul	10600 ul	7480 ul
	6400 ul	6400 ul	12800 ul	12800 ul

To each of these mix solutions, and equal volume of 5X Taq reaction buffer is added (with DMSO), so 6.4 ml is added to A and C, and 12.8 ml is added to G and T. This

mix/buffer solution is distributed into 0.5 ml colored microcentrifuge tubes (blue for A, green for C, purple for G, and yellow for T) in 60 or 120 ul aliquots (60 for A and C/120 for G and T). The simplest way to distribute the 60 ul aliquots is 2 x 30 ul using the Eppendorf repeat pipettor set on 3 with the 0.5 ml Combitips, and for the 120 ul aliquots use 1 x 100 ul with the 5 ml Combitip plus 1 x 20 ul with the 0.5 ml Combitip. The mixes should be stored at -20 or -70degC.

Ordering information:

100 mM dATP	27-2050-01	$48	Pharmacia	25 umoles	250 ul
100 mM dCTP	27-2060-01	$48	Pharmacia	25 umoles	250 ul
10 mM c7dGTP	988 537	$98	Boehringer	2 umoles	200 ul
100 mM dTTP	27-2080-01	$48	Pharmacia	25 umoles	250 ul
5 mM ddATP	27-2057-00	$25	Pharmacia	0.5 umoles	100 ul
5 mM ddCTP	27-2065-00	$25	Pharmacia	0.5 umoles	100 ul
5 mM ddGTP	27-2075-00	$25	Pharmacia	0.5 umoles	100 ul
5 mM ddTTP	27-2085-00	$25	Pharmacia	0.5 umoles	100 ul
Micro PCR tubes	1044-20-0	$90	Robbins	1000/bag	10 rxn/bag
StripEase caps	1044-10-0	$65	Robbins	300/bag	25 rxn/bag

Bulk reagents from Pharmacia (cust. no. 6933) (1-800-526-3593) are ordered, with the usual $750 ceiling, and these bulk orders sometimes require a week or two to be filled. Reagents from Boehringer Mannheim (cust. no. 66155-01) (1-800-262-1640) are usually processed overnight. Cycle sequencing tubes from Robbins Scientific (cust. no. 19800-3) (1-800-752-8585):

Oligonucleotide universal primers used for DNA sequencing

At present, we are using the following primers:

Universal Forward 20mer 5' GTTGTAAAACGACGGCCAGT 3'

Universal Reverse 20mer 5' CACAGGAAACAGCTATGACC 3'

The following primers also have been used in the past:

ABI Forward primer sequence-

20mer 5' GACGTTGTAAAACGACGGCC 3'

18mer 5' TGTAAAACGACGGCCAGT 3'

ABI Reverse primer sequence-

20mer 5' CACAGGAAACAGCTATGACC 3'

18mer 5' CAGGAAACAGCTATGACC 3'

T7: 5'-TAA-TAC-GAC-TCA-CTA-TAG-GG-3'

SP6:5'-ATT-TAG-GTG-ACA-CTA-TAG-AA-3'

M13 (-21) universal forward 5'-TGT-AAA-ACG-ACG-GCC-AGT-3'

M13 (-40) universal forward 5'-GTT-TTC-CCA-GTC-ACG-AC-3'

M13/pUC reverse primer 5'-CAG-GAA-ACA-GCT-ATG-ACC-3'

T7 primer 5'-TAA-TAC-GAC-TCA-CTA-TAG-GG-3'

SP6 primer 5'-ATT-TAG-GTG-ACA-CTA-TAG-3'

-16bs 5'-TCG-AGG-TCG-ACG-GTA-TCG-3'

+19bs 5'-GCC-GCT-CTA-GAA-CTA-GTG-3'

Listing of M13 (pUC) cloning sites

As they are read on DNA sequencing gels using the Universal primer:

M13mp7

.....EcoR1....BamH1.SalI..PstI..SalI..BamH1....EcoR1

GGCCAGTGAATTCCCCGGATCCGTCGACCTGCAGGTCGACGGATCCGGGG

AATTC

M13mp8

.....HindIII.PstI.SalI...BamH1.SmaI.EcoR

GGCCAGTGCCAAGCTTGGCTGCAGGTCGACGGATCCCCGGGAATTCGTAAT

CATG

M13mp9

.....EcoR1.SmaI.BamH1..SalI..PstI..HindIII

GGCCAGTGAATTCCCGGGGATCCGTCGACCTGCAGCCAAGCTTGGCGTAAT

CATG

M13mp10

...HindIII..PstI..SalI..XbaI..BamH1..SmaI..SstI..EcoR1

GCCAAGCTTGGGCTGCAGGTCGACTCTAGAGGATCCCCGGGCGAGCTCGA

ATTCG

M13mp11

...EcoR1..SstI..SmaI..BamH1..XbaI..SalI..PstI..HindIII

GTGAATTCGAGCTCGCCCGGGGATCCTCTAGAGTCGACCTGCAGCCCAAGC

TTGG

M13mp18

HindIII.SphI..PstI..SalI.XbaI.BamH1.SmaI.KpnI.SstI.EcoR1

AAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTC

GAATTC

M13mp19

EcoR1.SstI.KpnI.SmaI.BamH1.XbaI.SalI.PstI..SphI..HindIII

GAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGC

AAGCTT

Commonly used restriction enzymes and assay buffers

Common Assay Incub. Recognition

Enzyme isoschizomers buffer temp. site Cloning sites

Aat II med37 GACGT/C Aat II

Acc I med37 GT/(AC)(GT)AC Acc I, Cla I

Aha III Dra I med37 TTT/AAA blunt

Alu I med37 AG/CT blunt

Asu II 37 TT/CGAA Acc I, Cla I

Ava I med37 C/YCGRG Sal I, Xho I, Xma I

Ava II med37 G/G(AT)CC

Bal I		low	37	TGG/CCA	blunt
BamH1		med	37	G/GATCC	BamH1, Bgl II
Bgl I		med	37	GCCN4/NGGC	
Bgl II		low	37	A/GATCT	BamH1, Bgl II
BstE II		high	60	G/GTNACC	
BstN I		low	55	CC/(AT)GG	
Cla I		low	37	AT/CGAT	Acc I, Cla I
Dra I	Aha III	low	37	TTT/AAA	blunt
EcoR1		high	37	G/AATTC	EcoR1
EcoR1*		low	37	/AATT	EcoR1
EcoRV		med	37	GAT/ATC	blunt
Hae I		low	37	(AT)GG/CC(TA)	blunt
Hae II		low	37	RGCGC/Y	
Hae III		med	37	GG/CC	blunt
Hha I	Cfo I, HinP1	med	37	GCG/C	Hha I
Hinc II		med	37	GTY/RAC	blunt
Hind III		med	37-55	A/AGCTT	Hind III
Hinf I		med	37	G/ANTC	
HinP1	Cfo I, Hha I	low	37	G/CGC	Acc I, Cla I
Hpa I		low	37	GTT/AAC	blunt
Hpa II	Msp I	low	37	C/CGG	Acc I, Cla I
Kpn I		low	37	GGTAC/C	Kpn I
Mbo I	Sau3A	med	37	/GATC	BamH1, Bgl II
Msp I		med	37	C/CGG	Acc I, Cla I
Mst I	Fsp I	high	37	TGC/GCA	blunt
Mst II	Bsu36 I	high	37	CC/TNAGG	
Nae I		med	37	GCC/CCG	blunt
Nco I		high	37	C/CATGG	Nco I
Nde I		med	37	CA/TATG	Nde I
Not I		high	37	GC/GGCCGC	

Nru I		med	37	TCG/CGA	blunt
Pst I		med	21-37	CTGCA/G	Pst I
Pvu I		high	37	CGAT/CG	Pvu I
Pvu II		med	37	CAG/CTG	blunt
Rsa I		med	37	GT/AC	blunt
Sac I	Sst I	low	37	GAGCT/C	Sac I, Sst I
Sal I		high	37	G/TCGAC	Ava I, Sal I, Xho I
Sau3A I	Mbo I	med	37	/G*ATC	BamH1, Bgl II
Sfi I			50	GGCCN4/NGGCC	
Sma I	Xma I	(1)	37	CCC/GGG	blunt
Sph I		high	37	GCATG/C	Sph I
Sst I	Sac I	med	37	GAGCT/C	Sst I, Sac I
Sst II	Sac II	med	37	CCGC/GG	Sst II
Taq I		low	37-55	T/CGA	AccI, Cla I
Tha I	FnuD II, Acc II	low	37-60	CG/CG	blunt
Xba I		high	37	T/CTAGA	Xba I
Xho I	Ccr I	high	37	C/TCGAG	Ava I, Cla I
Xma I	Sma I	low	37	C/CCGGG	Ava I, Xma I

Assay buffers (see enzyme vendors catalogs for additional information)

10x Low salt buffer	10x Core buffer
100mM Tris-HCl, pH 7.6	500mM NaCl
100mM MgCl2	500mM Tris-HCl, pH 7.6
10mM DTT	100mM MgCl2

10x Medium salt buffer	10x Hind buffer
500mM NaCl	600mM NaCl
100mM Tris-HCl, pH 7.6	100mM Tris-HCl, pH 7.6
100mM MgCl2	70mM MgCl2

10mM DTT

10x High salt buffer	10x Sma I buffer (1)
1.0M NaCl	200mM KCl
500mM Tris-HCl, pH 7.6	100mM Tris-HCl, pH 7.6
100mM MgCl2	100mM MgCl2
10mM DTT	10mM DTT

The following enzymes CAN be heat inactivated by incubation at 65 deg. C for 10 min.

Alu I, Apa I, Ava II, Bal I, Bgl I, Cvn I, Dpn I, Dra I, Eco R II, Eco RV, Hae II, Hha I, Hinc II, Kpn I, Mbo I, Msp I, Nar I, Nde II, Rsa I, Sau 3a, Sca I, Sfi I, Spe I, Sph I, Ssp I, Sst I, Stu I, and Sty I.

The following enzymes are ONLY PARTIALLY heat inactivated by incubation at 65 deg.C for 10 min.

Ava I, Cfo I, Cla I, Cvn I, Eco RI, Mbo II, Mlu I, Nci I, Nru I, Pst I, Pvu II, Sma I and Xma III

The following enzymes CANNOT be heat inactivated by incubation at 65 deg. C for 10 min.

Acc I, Bam HI, Bcl I, Bgl II, BstE II, Dde I, Hae III, Hind III, Hinf I, Hpa I, Hpa II Nde I, Nhe I, Nsi I, Pvu I, Sal I, Sau 96 I, Sst II, Taq I, Tha I, Xba I, Xho I, and Xor II.

Bacterial Transformation and Transfection

Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. If the foreign DNA has an origin of replication recognized by the host cell DNA polymerases, the bacteria will replicate the foreign DNA along with their own DNA. When transformation is coupled with antibiotic selection techniques, bacteria can be induced to uptake certain DNA molecules, and those bacteria can be selected for that incorporation. Bacteria which are able to uptake DNA are called "competent" and are made so by treatment with calcium chloride in the early log phase of growth.

The bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA. The exact mechanism of this uptake is unknown. It is known, however, that the calcium chloride treatment be followed by heat. When E. coli are subjected to 42degC heat, a set of genes are expressed which aid the bacteria in surviving at such temperatures. This set of genes are called the heat shock genes. The heat shock step is necessary for the uptake of DNA. At temperatures above 42degC, the bacteria's ability to uptake DNA becomes reduced, and at extreme temperatures the bacteria will die.

Plasmid Transformation and Antibiotic Selection

The process for the uptake of naked plasmid and bacteriophage DNA is the same; calcium chloride treatment of bacterial cells produces competent cells which will uptake DNA after a heat shock step. However, there is a slight, but important difference in the procedures for transformation of plasmid DNA and bacteriophage M13 DNA. In the plasmid transformation, after the heat shock step intact plasmid DNA molecules replicate in bacterial host cells. To help the bacterial cells recover from the heat shock, the cells are briefly incubated with non-selective growth media. As the cells recover, plasmid genes are expressed, including those that enable the production of daughter plasmids which will segregate with dividing bacterial cells. However, due to the low number of bacterial cells which contain the plasmid and the potential for the plasmid not to propogate itself in all daughter cells, it is necessary to select for bacterial cells which contain the plasmid. This is commonly performed with antibiotic selection. E. coli strains such as GM272 are sensitive to common antibiotics such as ampicillin. Plasmids used for the cloning and manipulation of DNA have been engineered to harbor the genes for antibiotic resistance. Thus, if the bacterial transformation is plated onto media containing ampicillin, only bacteria which possess the plasmid DNA will have the ability to metabolize ampicillin and form colonies. In this way, bacterial cells containing plasmid DNA are selected.

Bacteriophage M13 Transformation and Viral Transfection

The transformation of bacteriophage M13 into bacterial cells is identical to plasmid DNA transformation through the heat shock step. After the heat shock step, single stranded M13 DNA begins replicating in the host cell through use of the host cell machinery. During the life cycle of this virus, however, M13 replicative form is created and daughter phages are packaged and extruded from the bacterial cell. These intact phage molecules then infect neighboring bacteria in a process called transfection. When these transformed and transfected bacteria are plated with non-infected cells onto growth media, the non-infected cells form a background cell lawn which covers the plate. In regions of M13 transfection, areas of slowed growth, called plaques, can be identified as opaque regions which interrupt the lawn.

Bacterial Strains

Since M13 viral transfection is a critical part of the transformation of bacterial cells with M13, it is absolutely necessary to use a strain of E. coli which harbors the episome for the F pilus. When M13 phages infect bacterial cells they attach to the F pilus, and the loss of this pilus is a common reason for a failed or poor transformation/transfection of M13. JM101 is a strain of E. coli which possesses the F pilus if the culture is maintained under appropriate conditions. Since the F pilus is not necessary for plasmid DNA transformation, it is advisable to use GM272, a much healthier, F- strain of E. coli for this procedure. To avoid confusion between the similar procedures, bacterial transformation with plasmid DNA is termed a "Transformation", and a bacterial transformation with naked M13 followed by a transfection with intact M13 phage is called a "Transfection."

Plasmid Transformation and Antibiotic Selection

Lac Z Operon

An additional level of selection can be achieved during transformation and transfections. Bacterial cells containing plasmids with the antibiotic resistance gene are selected in bacterial transformations, and cells in an area of M13 infection are recognized as plaques against a lawn of non-infected cells. However, the object of most transformations and transfections is to clone foreign DNA of interest into a known plasmid or viral vector and to isolate cells containing those recombinant

molecules from each other and from those containing the non-recombinant vector. The E. coli lacZ operon has been incorporated into several cloning vectors, including plasmid pUC and bacteriophage M13. The polylinker regions of these vectors was engineered inside of the lacZ gene coding region, but in a way not to interrupt the reading frame or the functionality of the resultant lacZ gene protein product. This protein product is a galactosidase. In recombinant vectors which have an insert DNA molecule cloned into one of the restriction enzyme sites in the polylinker, this insert DNA results in an altered lacZ gene and a non-functional galactosidase. The presence or absence of this protein can easily be determined through the use of a simple chromogenic assay using IPTG and X-Gal. IPTG is the lacZ gene inducer and is necessary for the production of the galactosidase. The usual substrate for the lacZ gene protein product is galactose, which is metabolized into lactose and glucose. X-Gal is a colorless, modified galactose sugar. When this molecule is metabolized by the galactosidase, the resultant products are a bright blue color.

When IPTG and X-Gal are included in a plasmid DNA transformation, blue colonies represent bacteria harboring non-recombinant pUC vector DNA since the lacZ gene region is intact. IPTG induces production of the functional galactosidase which cleaves X-Gal and results in a blue colored metobolite. It follows that colorless colonies contain recombinant pUC DNA since a nonfunctional galactosidase is induced by IPTG which is unable to cleave the X-Gal. Similarly, for bacteriophage transfections, colorless plaques indicate regions of infection with recombinant M13 viruses, and blue plaques represent infection with non-recombinant M13.

Host Mutation Descriptions:

ara Inability to utilize arabinose.

deoR Regulatory gene that allows for constitutive synthesis for genes involved in deoxyribose synthesis. Allows for the uptake of large plasmids.

endA DNA specific endonuclease I. Mutation shown to improve yield and quality of DNA from plasmid minipreps.

F' F' episome, male E. coli host. Necessary for M13 infection.

galK Inability to utilize galactose.

galT Inability to utilize galactose.

gyrA Mutation in DNA gyrase. Confers resistance to nalidixic acid.

hfl High frequency of lysogeny. Mutation increases lambda lysogeny by inactivating specific protease.

lacI Repressor protein of lac operon. LacI[q]is a mutant lacI that overproduces the repressor protein.

lacY Lactose utilization; galactosidase permease (M protein).

lacZ b-D-galactosidase; lactose utilization. Cells with lacZ mutations produce white colonies in the presence of X-gal; wild type produce blue colonies.

lacZdM15 A specific N-terminal deletion which permits the a-complementation segment present on a phagemid or plasmid vector to make functional lacZ protein.

Dlon Deletion of the lon protease. Reduces degradation of b-galactosidase fusion proteins to enhance antibody screening of l libraries.

malA Inability to utilize maltose.

proAB Mutants require proline for growth in minimal media.

recA Gene central to general recombination and DNA repair. Mutation eliminates general recombination and renders bacteria sensitive to UV light.

rec BCD Exonuclease V. Mutation in recB or recC reduces general recombination to a hundredth of its normal level and affects DNA repair.

relA Relaxed phenotype; permits RNA synthesis in the absence of protein synthesis.

rspL 30S ribosomal sub-unit protein S12. Mutation makes cells resistant to streptomycin. Also written strA.

recJ Exonuclease involved in alternate recombination pathways of E. coli.

strA See rspL.

sbcBC Exonuclease I. Permits general recombination in recBC mutants.

supE Supressor of amber (UAG) mutations. Some phage require a mutation in this gene in order to grow.

supF Supressor of amber (UAG) mutations. Some phage require a mutation in this gene in order to grow.

thi-1 Mutants require vitamin B1(thiamine) for growth on minimal media.

traD36 mutation inactivates conjugal transfer of F' episome.

umuC Component of SOS repair pathway.

uvrC Component of UV excision pathway.

xylA Inability to utilize xylose.

Restriction and Modification Systems

dam DNA adenine methylase/ Mutation blocks methylation of Adenine residues in the recognition sequence 5'-G*ATC-3' (*=methylated)

dcm DNA cytosine methylase/Mutation blocks methylation of cytosine residues in the recognition sequences 5'-C*CAGG-3' or 5'-C*CTGG-3' (*=methylated)

hsdM E. coli methylase/ Mutation blocks sequence specific methylation A[N6]*ACNNNNNNGTGC or GC [N6]*ACNNNNNNGTT (*=methylated). DNA isloated from a HsdM[-] strain will be restricted by a HsdR[+]host.

hsd R17 Restriction negative and modification positive.

(rK[-], mK[+]) Allows cloning of DNA without cleavage by endogenous restriction endonucleases. DNA prepared from hosts with this marker can efficiently transform rK[+]E. coli hosts.

hsdS20 Restriction negative and modification negative.

(rB[-,] mB[-]) Allows cloning of DNA without cleavage by endogenous restriction endonucleases . DNAprepared from hosts with this marker is unmethylated by the hsdS20 modificationsystem.

mcrA E. coli restriction system/ Mutation prevents McrA restriction of methylated DNA of sequence 5'-C*CGG (*=methylated).

mcrCB E. coli restriction system/ Mutation prevents McrCB restriction of methylated DNA of sequence 5'-G[5]*C, 5'-G[5h]*C, or 5'-G[N4]*C (*=methylated).

mrr E. coli restriction system/ Mutation prevents Mrr restriction of methylated DNA of sequence 5'-G*AC or 5'-C*AG (*=methylated). Mutation also prevents McrF restriction of methylated cytosine sequences.

Other Descriptions:

cm[r] Chloramphenicol resistance

kan[r] Kanamycin resistance

Tetracycline resistance

Streptomycin resistance

Indicates a deletion of genes following it.

Tn10

A transposon that normally codes for tetrTn5

A transposon that normally codes for kan[r]

spi[-] Refers to red[-]gam[-]mutant derivatives of lambda defined by their ability to form plaques on E. coli P2 lysogens.

Reference: Bachman, B.J. (1990) Microbiology Reviews 54: 130- 197.

Commonly used bacterial strains

C600 - F-, e14, mcrA, thr-1 supE44, thi-1, leuB6, lacY1, tonA21, [[lambda]] [-]

-for plating lambda (gt10) libraries, grows well in L broth, 2x TY, plate on NZYDT+Mg.

-Huynh, Young, and Davis (1985) DNA Cloning, Vol. 1, 56-110.

DH1 - F[-], recA1, endA1, gyrA96, thi-1, hsdR17 (rk[-], mk[+], supE44, relA1, [[lambda]][-]

]-for plasmid transformation, grows well on L broth and plates.

-Hanahan (1983) J. Mol. Biol. 166, 557-580.

XL1Blue-MRF' - D(mcrA)182, D(mcrCB-hsdSMR-mrr)172,endA1, supE44, thi-1, recA, gyrA96, relA1, lac, l-, [F'proAB, lac I[q]ZDM15, Tn10 (tet[r])] -For plating or glycerol stocks, grow in LB with 20 ug/ml of tetracycline. For transfection, grow in tryptone broth containing 10 mM MgSO4 and 0.2% maltose. (No antibiotic--Mg2+ interferes with tetracycline action.) For picking plaques, grow glycerol stock in LB to an O.D. of 0.5 at 600 nm (2.5 hours?). When at 0.5, add MgSO4 to a final concentration of 10 mM.

SURE Cells - Stratagene - e14(mcrA), D(mcrCB- hsdSMR-mrr)171, sbcC, recB, recJ, umuC::Tn5 (kan[r]), uvrC, supE44, lac, gyrA96, relA1, thi-1, end A1[F'proAB, lacI[q]DM15, Tn10(tet[r])]. An uncharacterized mutation enhances the a[-] complementation to give a more intense blue color on plates containing X-gal and IPTG.

GM272 - F[-], hsdR544 (rk[-], mk[-]), supE44, supF58, lacY1 or [[Delta]]lacIZY6, galK2, galT22, metB1m, trpR55, [[lambda]][-]

-for plasmid transformation, grows well in 2x TY, TYE, L broth and plates.

-Hanahan (1983) J. Mol. Biol. 166, 557-580.

HB101 - F[-], hsdS20 (rb[-], mb[-]), supE44, ara14, galK2, lacY1, proA2, rpsL20 (str[R]), xyl-5, mtl-1, [[lambda]][-], recA13, mcrA(+), mcrB(-)

-for plasmid transformation, grows well in 2x TY, TYE, L broth and plates.

-Raleigh and Wilson (1986) Proc. Natl. Acad. Sci. USA 83, 9070-9074.

JM101 - supE, thi, [[Delta]](lac-proAB), [F', traD36, proAB, lacIqZ[[Delta]]M15], restriction: (rk[+], mk[+]), mcrA+

-for M13 transformation, grow on minimal medium to maintain F episome, grows well in 2x TY, plate on TY or lambda agar.

-Yanisch-Perron et al. (1985) Gene 33, 103-119.

XL-1 blue recA1, endA1, gyrA96, thi, hsdR17 (rk[+], mk[+]), supE44, relA1, [[lambda]][-], lac, [F', proAB, lacIqZ[[Delta]]M15, Tn10 (tet[R])]

-for M13 and plasmid transformation, grow in 2x TY + 10 ug/ml Tet, plate on TY agar + 10 ug/ml Tet (Tet maintains F episome).

-Bullock, et al. (1987) BioTechniques 5, 376-379.

GM2929 - from B. Bachman, Yale E.coli Genetic Stock Center (CSGC#7080); M.Marinus strain; sex F[-];(ara-14, leuB6, fhuA13, lacY1, tsx-78, supE44, [glnV44], galK2, galT22, l[-], mcrA, dcm-6, hisG4,[Oc], rfbD1, rpsL136, dam-13::Tn9, xyl-5, mtl-1, recF143, thi-1, mcrB, hsdR2.)

MC1000 - (araD139, D[ara-leu]7679, galU, galK, D[lac]174, rpsL, thi-1). obtained from the McCarthy lab at the University of Oklahoma.

ED8767 (F-,e14-[mcrA],supE44,supF58,hsdS3[rB[-]mB[-]], recA56, galK2, galT22,metB1, lac-3 or lac3Y1 , obtained from Nora Heisterkamp and used as the host for abl and bcr cosmids.

Notes on Restriction/Modification Bacterial Strains:

1. EcoK (alternate=EcoB)-hsdRMS genes=attack DNA not protected by adenine methylation. (ED8767 is EcoK methylation minus). (1)

2. mcA (modified cytosine restriction), mcrBC, and mrr=methylation requiring systems that attack DNA only when it IS methylated (Ed8767 is mrr+, so methylated adenines will be restricted. Clone can carry methylation activity.) (1)

3. In general, it is best to use a strain lacking Mcr and Mrr systems when cloning genomic DNA from an organism with methylcytosine such as mammals, higher plants , and many prokaryotes. (2)

4. The use of D(mrr-hsd-mcrB) hosts=general methylation tolerance and suitability for clones with N6 methyladenine as well as 5mC (as with bacterial DNAs). (3)

5. XL1-Blue MRF'=D(mcrA)182, D(mcrCB-hsdSMR-mrr)172,endA1, supE44, thi-1, recA, gyrA96, relA1, lac, l-, [F' proAB, lacI[q]ZDM15, Tn10(tet[r]

REFERENCES:

1. Bickle, T. (1982) in Nucleases eds Linn, S.M. and Roberts, R.G. (CSH, NY) p. 95-100.

2. Erlich, M. and Wang, R.Y. (1981) Science 212, 1350-1357.

3. Woodcock, D.M. et al, (1989) Nucleic Acids Res., 17,3469-3478.

[NextPage]

Units and formulas

Units:

1 mg = 10-3 g.

1 ug = 10-6 g.

1 ng = 10-9 g.

1 pg = 10-12 g.

1 kb of DNA= 6.5 x10+5 Daltons of duplex DNA (sodium salt)

= 3.3 x10+5 Daltons of single-stranded DNA (sodium salt)

= 3.4 x10+5 Daltons of single-stranded RNA (sodium salt)

Average MW of a deoxynucleotide base = 324.5 Daltons

Average MW of a deoxynucleotide base pair = 649 Daltons

1 ug/ml of DNA = 3.08 uM phosphate

1 ug/ml of 1 kb of DNA = 3.08 nM 5' ends

1 mol of pBR322 = 2.83 x10+6 g.

1 pmol of linear pBR322, 5' ends = 1.4 ug

1 A260 unit of duplex DNA = 50 ug

1 A260 unit of single-stranded DNA = 37 ug

1 A260 unit of single-stranded RNA = 50 ug

1 kb of DNA= 333 amino acids of coding capacity
= 37,000 daltons

Densities (50% GC):

RF I (supercoiled)	ds DNA 1.709 g/ml
RF II (nicked)	ds DNA 1.54 g/ml
ss DNA	1.726 g/ml
ss RNA	1.90 g/ml
protein	1.33 g/ml

Formulas

DNA melting point:

For duplex DNA >50 bp:

Tm = 81.5deg. C +16.6 log (M of NaCl) + 0.41(% GC)
- [500/bp of shortest chain in duplex]
- [0.65(% formamide)]

For duplex DNA <50 bp:

Add 2deg. C for each A or T

Add 4deg. C for each G or C

Picomoles of ends:

pmol ends per ug linear DNA = 3030/number of bases

DNA mobility in gels

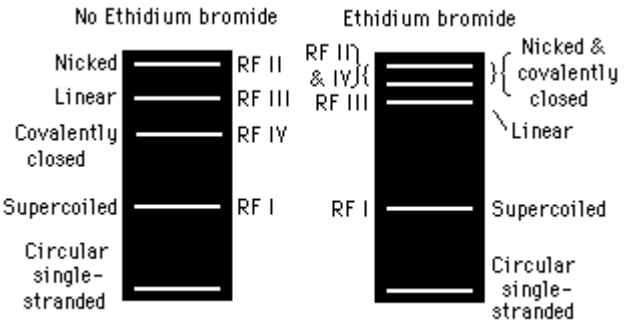
1. Migration of marker dyes in native polyacrylamide non-denaturing gels

Gel %	Bromophenol blue (BP)	Xylene cyanole (XC)
3.5	100	460
5.0	65	260
8.0	45	160
12.0	20	70
20.0	12	45

2. Migration of marker dyes in polyacrylamide denaturing gels

Gel %	Bromophenol blue (BP)	Xylene cyanole (XC)
5.0	35	130
6.0	26	106
8.0	19	75
10.0	12	55
20.0	8	28

3. Relative positions of different DNA forms on Tris-acetate agarose gels



The exact distance between bands is influenced by percentage of agarose, time of electrophoresis, concentration of Ethidium bromide, degree of supercoiling and the size and complexity of the DNA.

Codon chart and amino acid symbols

Second base

First base	U		C		A		G		Third base
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC		UCC		UAC		UGC		C
	UUA	Leu	UCA		UAA	Ocr	UGA	Opl	A
	UUG		UCG		UAG	Amb	UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	Gln	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	Lys	AGA	Arg	A
	AUG	Met	ACG		AAG		AGG		G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	Glu	GGA		A
	GUG		GCG		GAG		GGG		G

One and three-letter amino acid symbols

Alanine Ala A

Arginine Arg R

Asparagine Asn N

Aspartic acid Asp D

Asparagine or Aspartic acid Asx B

Cysteine Cys C

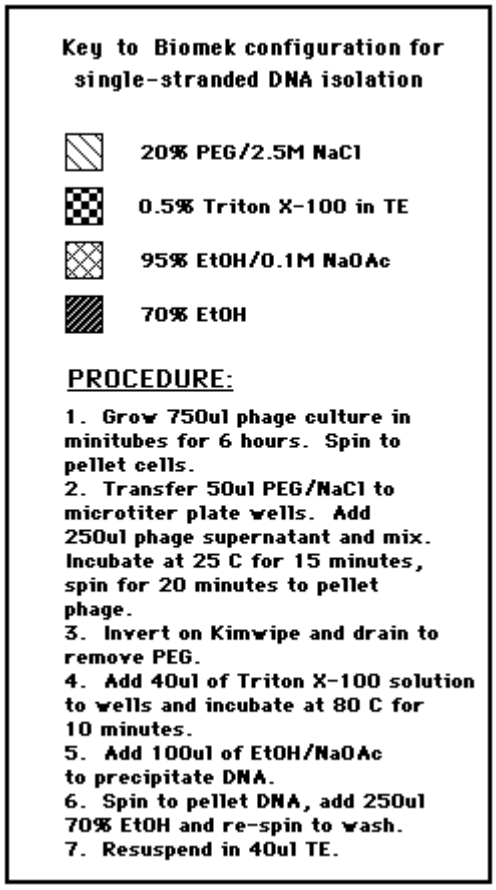
Glutamine Gln Q

Glutamic acid Glu E

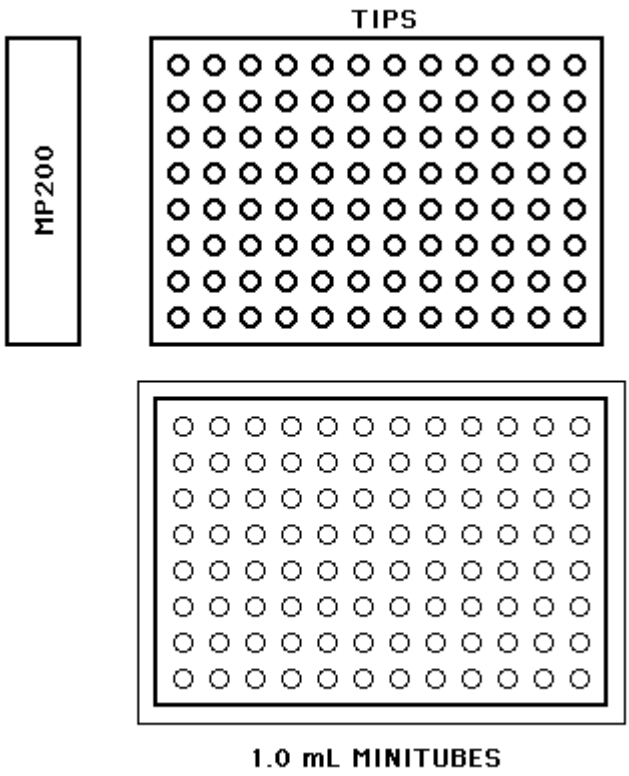
Glutamine or Glutamic acid Glx Z

Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

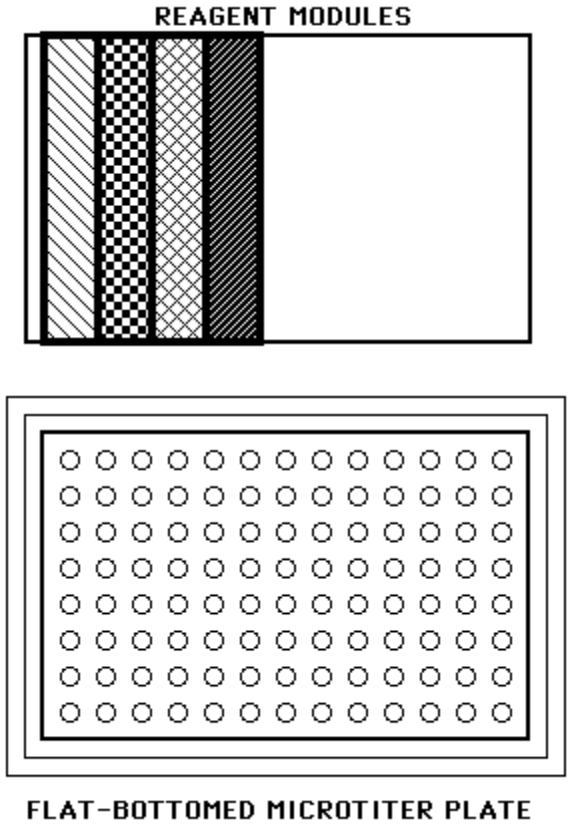
Biomek configuration for single stranded DNA isolation



Biomek configuration for single-stranded DNA isolation

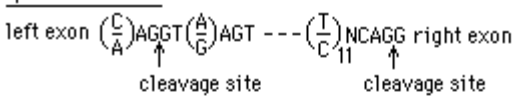


Biomek configuration for single-stranded DNA isolation



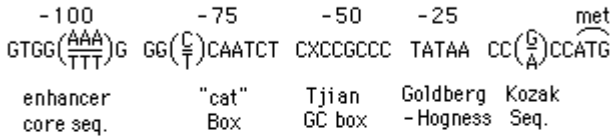
Consensus sequences in nucleic acids

Splice Junctions:

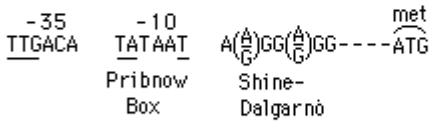


Note: In some instances the cleavage site contains one or more A's

Eucaryote Promoters:



Procaryote Promoters:



PolyA signal: AATAAA

Note: The Shine-Dalgarno sequence is complementary to the 3' end of 16S r RNA whose sequence is.

3' AUUCCUCCACUA

The Kozak sequence may be involved in forming a hairpin in the mRNA to bring CAP close to the AUG start codon.