EXERCISE

1

DNA Preparation, Polymerase Chain Reaction, and Molecular Cloning

Background

The goal of this experiment is to fuse *virD2* (a virulence gene from *Agrobacterium tumefaciens*) to *gst* (glutathione-*S*-transferase gene) to express and purify the fusion protein. To accomplish this, you need to create restriction sites on either side of *virD2*. You will use polymerase chain reaction (PCR) to make copies of *virD2* flanked by the required restriction sites. These restriction sites will allow you to insert *virD2* into the expression vector pGEX2. To start this and subsequent experiments, you need several different plasmids, which you will purify.

Steps of the experiment are

A. Prepare plasmid by cesium chloride density gradient centrifugation

B. Use PCR to synthesize *virD2* flanked with restriction sites

C. Perform restriction digests of plasmid pGEX2 and PCR products

D. Purify DNA fragments from agarose

E. Ligate PCR product to pGEX2

F. Transform *E. coli* with ligated plasmid

G. Make small-scale preparation of plasmid DNA from broth cultures

H. Perform restriction digests of DNAs; examine to confirm insert

A. CESIUM CHLORIDE–ETHIDIUM BROMIDE DENSITY GRADIENT CENTRIFUGATION

Introduction

The purpose of CsCl–ethidium bromide density gradient centrifugation is to separate supercoiled (covalently closed circular) plasmid DNA from other components present in lysates of bacterial cells: linear DNA (chromosomal fragments), nicked plasmid DNA (which lacks supercoils), RNA, proteins, and carbohydrates. Covalently closed circular plasmid DNA isolated from bacterial cells has one strand underwound (fewer helical turns per unit length) relative to the other. To relieve the strain caused by underwinding, the circular DNA twists into figure eight structures called negative supercoils. The axis of the DNA double helix crosses over itself multiple times, much like an overwound telephone cord.

After cell lysis, low-speed centrifugation removes debris and much of the chromosomal DNA from the lysate. High-speed centrifugation in the presence of CsCl and ethidium bromide separates components on the basis of their buoyant densities. During centrifugation, the CsCl solution forms a density gradient. Ethidium bromide is a planar molecule that intercalates between the bases in DNA. As ethidium intercalates into a negatively supercoiled plasmid, the superhelicity is relaxed; at this point, intercalation of additional ethidium molecules must introduce positive supercoils into the plasmid DNA, but this process requires energy. Therefore, closed circular plasmids intercalate less ethidium than linear DNA or nicked (relaxed) circular DNA. Because ethidium is less dense than DNA, the density of linear DNA–ethidium is less than that of closed circular DNA–ethidium; this difference in density provides the basis for separating these topological forms. (RNA, which also binds ethidium, is

more dense than DNA and forms a pellet, whereas protein is less dense than DNA.)

This procedure differs from most CsCl gradient protocols because it requires 10-fold less ethidium bromide. Use of such a small quantity of ethidium bromide is only possible on removal of ethidium-binding RNA by ribonuclease digestion. The DNA bands will appear pink against a colorless background (in subdued room light). Gradients that contain typical concentrations of ethidium will appear orange, and bands become visible only under UV light (which damages DNA). DNA prepared from lysates treated with RNase will contain significant ribonuclease activity. This is a problem only if the DNA is used for in vitro transcription, but phenol extraction will destroy the RNase activity.

We will prepare the following plasmid DNAs:

pWR160:	contains *virD2*; template for PCR synthesis (I); hybridization probe for Southern blot (V)
pGEX2:	expression vector plasmid (Pharmacia) (I and II)
pUC119:	phagemid vector for mutagenesis (III)
pCS64:	source of *virD2* restriction fragment; target for mutagenesis (III)
pMCB525:	oligonucleotide-directed mutagenesis template (III)
pRbcS:	contains *rbcS;* hybridization probe for Northern blots (VI)
pAD	yeast plasmid with transcription activator (TA) domain (VII)
pAD–E1	yeast plasmid with TA–*virE1* fusion (VII)
pBD	yeast plasmid with *lexA* DNA-binding domain (VII)
pBD–E2	yeast plasmid with *lexA–virE2* fusion (VII)

Before beginning any experiment involving bacterial cultures, streak the strain on the appropriate agar plates to obtain single colonies, assuring a pure culture. Then inoculate liquid medium (usually a rich broth, containing

antibiotics when appropriate) with a single colony, and incubate the culture with aeration at the proper temperature (usually 37°C for *E. coli*) overnight.

Safety Precautions

Ethidium bromide is mutagenic; avoid contact with it. Wear gloves.

Discard butanol in the organic waste bottle in the hood.

Technical Tips

RNases are extremely stable enzymes and are resistant to heating. Their presence will destroy RNA preparations. To avoid RNase contamination during future RNA work, use RNase only in one area under the hood and pipet RNase with the pipettor designated for RNase work.

Protocol

Beforehand (TAs)

1. Inoculate 200 mL of L broth (containing ampicillin at 50 μg/mL) with 1 mL of fresh overnight L broth culture of an *E. coli* strain harboring the desired plasmid. Incubate with aeration at 37°C overnight.

Day 1 (students start here)

2. Place 200 mL cells from an overnight culture in a 250-mL centrifuge bottle. Balance bottles and centrifuge cells 10 minutes, 5000 rpm, 4°C in Sorvall GSA.
3. While cells are in centrifuge, prepare fresh SDS–NaOH lysis buffer (200 mM NaOH; 1% SDS; mix

0.67 mL of 3 *M* NaOH + 0.4 mL of 25% SDS + 9 mL H_2O). Do not put lysis buffer on ice, or SDS will precipitate.

4. **Thoroughly** resuspend cells from 100 to 200 mL of culture in 2 mL of TE (25/10). Transfer to a sterile 50-mL "Oakridge" Nalgene tube.

5. Add 4 mL of SDS–NaOH lysis solution; mix **gently but thoroughly by inverting tube several times** (lyses cells). Incubate on ice 5 minutes and keep on ice from here on.

6. Add 3 mL of potassium acetate solution; mix **gently but thoroughly** (neutralizes).

7. Centrifuge 13,000 rpm, 15 minutes, 4°C, in Sorvall SS34 (pellet contains cellular debris and chromosomal DNA).

8. Decant supernatant into weighed 50-mL "Oakridge" Nalgene tube.

9. Add 16 mL 95% ethanol to supernatant.

10. Centrifuge 10,000 rpm, 10 minutes, 4°C, in Sorvall SS34 (precipitates plasmid DNA).

11. During centrifugation, weigh 4.5 g of CsCl into a 12-mL Falcon tube.

12. Discard supernatant. Dissolve pellet in 3 mL TES buffer.

13. Add 5 μL of 5 mg/mL heat-treated RNase.

14. Add TES to bring resuspended pellet to 4 g.

15. Add 4.5 g of CsCl.

16. Use a Pasteur pipet to load solution in Beckman polyallomer 13- × 51-mm heat-seal tube (No. 342412).

17. Layer 30 μL of 10 mg/mL ethidium bromide on top of CsCl solution; do not mix yet; fill to base of neck with H_2O.

18. Weigh to find tubes that balance. Tube should weigh about 9.6 g. Balance tubes within 100 mg. Heat-seal tube; check seal; weigh again.

19. Centrifuge 65,000 rpm, 4 hours, 15°C, in Beckman vTi80 rotor.

20. Collect plasmid DNA band with 18-gauge syringe needle (this procedure will be demonstrated; also see diagram). Draw DNA into syringe and discard needle **before** you expel DNA into a 1.5-mL microfuge tube; this reduces shearing. To avoid spreading RNase around the lab, prepare a receptacle for the excess solution **before** you puncture the tube.

21. Extract ethidium 4 to 5 times with H_2O-saturated *n*-butanol. To extract, add 1 volume butanol, vortex, spin 2 seconds in microfuge. Butanol layer will be on top. Remove the butanol and repeat the extraction 3 or 4 more times.

22. Dilute with 2 volumes TE (10/0.1); if your sample volume is now greater than 500 μL divide it between 2 tubes. Add 2 volumes of ethanol (based on the volume of sample + TE together). Hold on ice for at least 10 minutes.

23. Centrifuge in microfuge at top speed for 5 minutes. Thoroughly remove supernatant and air dry pellet to evaporate remaining ethanol. If you divided your sample among different tubes, take up each pellet in a smaller volume and combine.

24. Measure optical density (OD) at 260 nm; concentration (μg/mL) = $OD_{260\ nm} \times$ dilution factor $\times$ 50. To measure OD, prepare 100 μL of a dilution of your sample, using 90 μL buffer and 10 μL sample for a

dilution factor of 10. Zero the spectrophotometer with the buffer used to prepare your dilution.

Solutions for Cesium Chloride Density Gradient Centrifugation

TE (25/10): 25 mM Tris, pH 7.5; 10 mM EDTA (autoclave)

SDS-NaOH lysis solution: 200 mM NaOH; 1% SDS
Mix 0.67 mL of 3 *M* NaOH + 0.4 mL of 25% SDS + 9 mL H_2O

Potassium acetate solution: 3 *M* K+/5 *M* CH_3CO_2- (autoclave)
Mix 60 mL of 5 *M* potassium acetate (29.45 g/60 mL) + 11.5 mL of acetic acid + 28.5 mL of H_2O

TE (10/0.1): 10 mM Tris, pH 7.5; 0.1 mM EDTA (autoclave)

TES: 50 mM Tris, pH 8.0; 5 mM EDTA; 50 mM NaCl (autoclave)

Ethidium bromide stock: 10 mg/ml in H_2O (store in dark) (avoid contact with this mutagen)

RNase solution: mix 5 mg/mL RNase A + 5 mg/mL RNase T1 in DNA buffer; heat at 15°C for 15 minutes (to inactivate DNases)

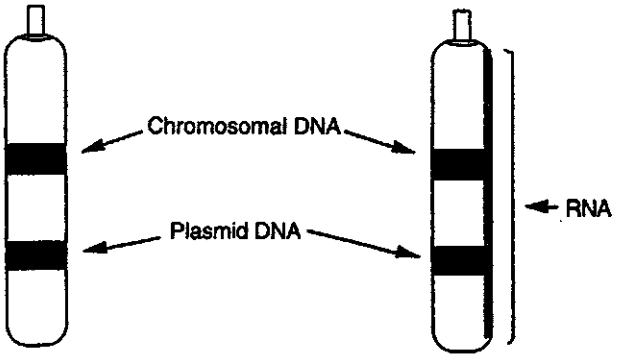
H_2O-saturated *n*-butanol: shake equal volumes of water and *n*-butanol

95% Ethanol

4 mL TES and 4.5 g CsCl (solution to top off tubes)

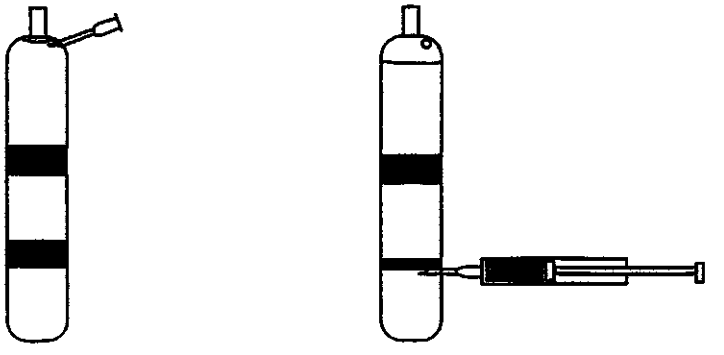
(Bacterial strains and plasmids are available from Walt Ream, Dept. of Microbiology, Oregon State University, Corvallis, OR 97330. Phone: 541-737-1791 E-mail: reamw@bee.orst.edu.)

IDENTIFICATION OF NUCLEIC ACID BANDS AFTER VERTICAL ROTOR CENTRIFUGATION



Procedure

1. Insert needle (without syringe) into top of tube; leave in place.
2. Insert needle (with syringe attached) through wall of tube 0.5 cm below the plasmid DNA band. **Do not** twist the needle; push it straight in with steady pressure. (Puncture the tube the way you hope the nurse does when drawing blood from your arm.) Keep the bevel up; place the bevel at the bottom of the band and draw the plasmid band into the syringe.



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B. PCR TO SYNTHESIZE *VIRD2* FLANKED WITH RESTRICTION SITES

Introduction

Polymerase chain reaction produces many copies of a particular template DNA sequence in vitro. This method takes advantage of thermally stable DNA polymerase, which is not denatured by temperatures high enough to melt apart duplex DNA, to allow repeated cycles of thermal template denaturation, primer annealing, and DNA synthesis. In theory, 30 cycles of amplification can produce 268,435,456 copies from a single template molecule. Because the yield rises geometrically with each cycle, it is extremely sensitive to factors that affect efficiency of the reaction (e.g., pH, hybridization temperature, magnesium ion concentration). PCR is also extremely sensitive to contamination by unwanted template DNA in reagents.

The reaction mix contains template DNA, polymerase, deoxynucleoside triphosphates, Mg^{2+}, buffer, and 2 primer oligonucleotides. One primer complements a site *upstream* from the sequence being amplified; the other primer complements a region on the *opposite* strand, *downstream* from the sequence. Primers are typically 18 to 30 bases long. The reaction temperature is raised to denature the template DNA (94°C), reduced to allow the primers to anneal to their target sequences (usually 45 to 55°C), and then raised to the optimum temperature for DNA polymerase activity (72°C). This cycle is repeated, usually 25 to 40 times. During the first cycle, the primers anneal to the target sequence and are extended by DNA polymerase beyond the other primer-binding site. In subsequent cycles newly extended strands, which start at either side of the gene, are used as templates, generating short strands that extend from primer to primer.

An additional sequence, such as a restriction endonuclease recognition site, can be added as a "tail" at the 5' end of a primer, provided the sequence complementary to the template is maintained. In our experiment, the 3' end of the left-hand primer (5'-CCC CTG ATC AAG ATC TAG ATC TAT GCC CGA TCG CGC TCA A-3') complements the first 18 bases of the *virD2* antisense strand. The 5' end of this primer contains two *Bgl*II restriction sites (AGATCT) and one *Bcl*I site (TGATCA). Similarly, the 3' end of the right-hand primer complements the last 18 bases of the *virD2* sense strand, and an *Eco*RI site (GAATTC) lies at its 5' end. The purpose is to produce the *virD2* coding sequence with restriction sites at both ends, allowing you to cut the PCR product and fuse it to the expression vector.

You will use a commercial PCR kit, which contains the buffer, deoxynucleoside triphosphates, and *Taq* DNA polymerase (from *Thermus aquaticus*), as well as primers and template for a positive-control reaction.

Safety Precautions

Protect eyes and skin from ultraviolet light; avoid contact with ethidium bromide.

Technical Tips

A single molecule of contaminating DNA can be amplified many times; thus, PCR requires unusual care to avoid contamination. Discard pipet tips after each transfer; a common source of contaminated solutions is "double-dipping". Use pipet tips with barriers to avoid contaminating the pipettors, reagent stocks, and reactions. Always include negative control reactions with no added template DNA to check for contaminating DNA; perform an equal

number of negative controls and template-containing reactions.

Practice opening and closing an empty thin-walled PCR tube to learn how to handle them without crushing.

Protocol

1. Turn on a heating block; set to 95°C.
2. Fill out a DNA Amplification Checklist (*see page* 32) for your reactions. Include a positive control with the primers and template supplied with the PCR kit, a negative control with no template DNA but all other reactants, a positive control using previously prepared pWR160 template provided by the TAs, and the experimental amplification using your prepared pWR160 template.

   These are the amounts for each amplification reaction:

   Reaction Cocktail:

10× buffer		10 µL
dNTPs, 2 mM stock		10 µL
Primer 1, 10 µM stock		2 µL
Primer 2, 10 µM stock		2 µL
Template DNA	10 ng =	µL
Taq polymerase	2.5 U =	0.5 µL
ddH_2O to bring total volume to 100 µL		µL

3. In 0.2-mL PCR tubes, add all the reactants except the *Taq* polymerase, starting with the water and then following the order listed above. Mix well.
4. Heat the reaction tubes to 95°C for 2 minutes.
5. Pulse the tubes in the microfuge.

6. Add the polymerase; mix without introducing bubbles.
7. Overlay the reaction with 2 drops of sterile mineral oil (omit oil if thermal cycler has a heated cover).
8. Place the tubes in the thermal cycler and run the following program:
   94°C for 1 minute; 55°C for 1 minute; 72°C for 3 minutes; 30 cycles; hold at 4°C (can be left overnight)
9. To estimate the yield of PCR product ("amplicon"), examine 5 μL of each reaction by agarose gel electrophoresis. Cast a 1% agarose gel (0.3 g agarose in 30 mL 1× TAE; heat in microwave until dissolved; replenish evaporated volume with distilled H_2O; add 1 μL of ethidium bromide; pour and insert comb).
10. Draw off 5 μL of each reaction from under the oil with a Pipetman and place in fresh tubes.
11. Add 1 μL of 6× load buffer; stir.
12. Prepare a size standard using 2 μL of DNA Mass Ladder, 2 μL ddH_2O, and 1 μL of 6× load buffer.
13. When gel has solidified, place in gel apparatus, add electrophoresis buffer, remove comb, and load. When loading an agarose gel, insert only the tip of the pipet tip into the well; you may pierce the bottom of the well if you insert the tip too far.
14. To turn on the power supply set rheostats to "0", turn on the power, and allow to warm up for a few minutes. Connect the leads to the gel apparatus and apply 100 V until bromphenol blue dye nears the bottom of the gel (approximately 30 minutes). DNA migrates toward the positive (red) electrode (anode). Set rheostats to "0" before you turn off the power supply. Examine under UV light (use a hand-held UV lamp and safety glasses) and continue electrophoresis as needed.
15. Photograph the gel using the UV transilluminator/ Polaroid camera apparatus. Please note that each

piece of film costs $1.25; take **1 picture only.** Make additional copies on the photocopier.

16. Estimate the yield of PCR reactions by comparing band intensity with the DNA Mass Ladder bands.

17. To remove mineral oil from the PCR product, withdraw DNA from under the oil with a pipet, place in fresh tubes, and extract with chloroform. Add 1 volume chloroform, vortex, and spin 2 minutes in microfuge. Remove the aqueous (top) layer to a fresh tube.

Solutions for PCR

10× PCR buffer (supplied by Perkin Elmer)
100 mM Tris-HCl, pH 8.3, at 25°C
500 mM KCl
15 mM $MgCl_2$
0.1% (w/v) gelatin

dNTPs: Deoxynucleoside triphosphates dissolved in ddH_2O and neutralized with NaOH. Stock solution contains 2 mM of each dNTP (in ddH_2O).

TAE: 0.04 *M* Tris-acetate, 0.002 *M* EDTA
1 L 50×: 242 g Tris base
57.1 mL glacial acetic acid
100 mL 0.5 *M* EDTA, pH 8.0

Ethidium Bromide stock = 10 mg/mL; store in dark at 4°C. Caution: mutagen; wear gloves and a mask while weighing; wear gloves while using.

6× Load buffer: 0.05% bromphenol blue;
40% (w/v) glycerol in H_2O (GIBCO/BRL)

DNA Mass Ladder (GIBCO/BRL)

Chloroform

Sterile mineral oil

Primer 1: 5'-CCC CTG ATC AAG ATC TAG ATC TAT GCC CGA TCG CGC TCA A-3'

Primer 2: 5'-CCC GAA TTC TAG GTC CCC CCG CGC CC-3'

DNA AMPLIFICATION CHECKLIST

DATE _____ NAME _____

No.	Template	H_2O	10× buffer	dNTPs	#1 primer	#2 primer	temp. DNA			*Taq* pol

Denaturation temp ____
time ____
Annealing temp ____
time ____
Elongation temp ____
time ____
Number of cycles ____

NOTES:

GEL PHOTO HERE

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C. RESTRICTION DIGESTS OF PLASMID pGEX2 AND PCR PRODUCTS

Introduction

Type II restriction endonucleases occur mainly in prokaryotes; these enzymes recognize and cleave specific sequences in double-stranded DNA. Type II restriction enzymes require Mg^{2+}, salt (often NaCl, sometimes KCl), and buffer for activity, but they do not require ATP.

Restriction endonucleases make double-stranded cuts at specific sequences in the phosphodiester backbone of DNA. Their recognition sequences usually contain 4 to 6 base pairs and are palindromes (they read the same, 5' to 3', on either strand). Most restriction endonucleases bind and cut DNA within the recognition sequence. For example, *Bam*HI recognizes

5'-G/GATC C-3'
3'-C CTAG/G-5'

and makes staggered cuts at the slashes. Note that the recognition sequence reads 5'-GGATCC-3' on each strand (a palindrome) and the cuts are staggered, leaving 4 bases unpaired at the 5' end:

5'-G -3'
3'-CCTAG-5'

This is called a 5' extension or overhang. Because of these unpaired (cohesive) ends, the termini of any *Bam*HI restriction fragments can pair with each other. These cohesive ends allow us to join any *Bam*HI fragments together in vitro (using an enzyme called DNA ligase). **This is the basis for molecular cloning.**

To perform a restriction digest, start with 0.5 to 1.0 μg of the DNA you will cut per 20 μL of reaction volume.

Each restriction enzyme comes with its reaction buffer. Manufacturer's instructions will indicate the buffer to use for simultaneous digestion with 2 enzymes.

You will cut the expression vector plasmid pGEX2 with restriction enzymes *Bam*HI (G/GATCC) and *Eco*RI (G/AATTC). These enzymes leave incompatible ends, so that completely cut plasmid will not recircularize. To check the efficiency of each enzyme, cut pGEX2 with each enzyme separately. Examine uncut, single-cut, and double-cut plasmid by agarose gel electrophoresis.

In a separate digestion, cut the PCR product with *Bgl*II (A/GATCT) and *Eco*RI, which will leave it with ends compatible with those of the cut plasmid.

Technical Tips

The volume of restriction endonucleases added must not exceed 10% of the total reaction volume because glycerol in the enzyme preparations inhibits their activity at high concentrations.

Keep enzyme stocks chilled (store at −20°C; not in frost-free freezer). Keep restriction enzymes on ice for the short period of time that you have them out of the freezer. Add them last to a restriction digest mixture, and then begin the incubation immediately.

Protocol

Digestion of pGEX2 plasmid

1. Mix: 2 μg of vector (pGEX2) DNA (from your CsCl preparation)
   2 μL of 10× restriction buffer (NEBuffer *Eco*RI)
   distilled water to give 18 μl total volume

2. Add 5 to 10 units (usually 0.5 to 1.0 μL each) of the restriction endonucleases (*Eco*RI and *Bam*HI).
3. Incubate at 37°C for at least 2 hours.
4. As controls, set up 3 more reactions, but omit both enzymes in one and add only 1 of the 2 restriction enzymes to the others (*Eco*RI to one, *Bam*HI to the other). Incubate as above.

Hint: To save time when setting up multiple restriction digests, prepare a "master mix". For example, for these 4 reactions, combine:

8 μg of vector (pGEX2) DNA (from your CsCl preparation)
8 μL of 10× restriction buffer (NEBuffer *Eco*RI)
distilled water to give 76 μL total volume

Divide this into 4 tubes and add the restriction enzymes to each (5 to 10 units of *Eco*RI and *Bam*HI to the 1st, *Eco*RI to the 2nd, *Bam*HI to the 3rd, water to the 4th).

Digestion of PCR product

5. After you estimate the yield of PCR product,
   Mix: 2 μg of insert DNA (your PCR product in this case)
   2 μL of 10× restriction buffer (NEBuffer *Eco*RI)
   distilled water to give 18 μL total volume
6. Add 10 units each (usually 1 μL) of the restriction endonucleases (*Eco*RI and *Bgl*II for our experiment).
7. Incubate at 37°C for 1 to 2 hours.

Solutions for Restriction Digests

NEbuffer *Eco*RI: 50 mM NaCl, 100 mM Tris–HCl, 10 mM $MgCl_2$, 0.025% Triton X-100 (pH 7.5 at 25°C)

Notes

D. PURIFICATION OF DNA FRAGMENTS FROM AGAROSE

Introduction

Many vector plasmids, for example pUC119, are designed so that insertion of a foreign DNA fragment disrupts a gene with an easily detectable phenotype, for example *lacZ* (β-galactosidase). This enzyme converts colorless X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) to a dark blue indigo derivative. When included in selective agar plates, X-gal allows us to distinguish Lac+ transformants (blue colonies) that received a recircularized vector plasmid (with no foreign DNA inserted) from Lac− transformants (white colonies) that contain an insert in the vector plasmid.

Unfortunately, pGEX2 does not provide a marker that allows us to distinguish cells transformed with uncut or recircularized vector from those harboring plasmids with foreign DNA inserted. Because we cleaved pGEX2 with enzymes (*Eco*RI and *Bam*HI) that produce incompatible ends, recircularization of the vector will occur infrequently. However, if the digested vector sample contains uncut pGEX2 (even small quantities not detectable by agarose gel analysis), these uncut molecules will transform *E. coli* very efficiently and reduce the likelihood that the transformants you analyze contain plasmids with PCR product inserted.

To avoid these problems, you will use agarose gel electrophoresis to separate linear vector DNA from uncut plasmid. Excise the portion of the gel containing cut vector, dissolve the agarose in sodium iodide, and bind the DNA to glass beads in high-salt buffer. Wash the beads to remove contaminating material, and elute purified linear vector from the glass with low salt buffer. These reagents

are available in a commercial kit known as "Geneclean" (from Bio 101).

PCR products often are contaminated by additional DNAs that result from interactions between the primer and template at sites other than those intended. With luck, these unwanted products will be minor in quantity and have a different molecular weight than the desired DNA. If necessary, use agarose gel electrophoresis and the "Geneclean" procedure to isolate DNA of the proper size. We expect this step will not be necessary here.

Protocol

1. Prepare a 1% agarose gel (0.4 g agarose in 40 mL 1× TAE; heat in microwave until dissolved; add 1.5 μL of ethidium bromide; pour and insert comb).
2. To the restriction digests of pGEX2, add 4 μL 6× load buffer. Load the entire sample into 1 well. In separate lanes, load each single-cut control and uncut vector. Separate DNA by electrophoresis at 100 V (toward positive electrode).
3. Observe DNA under UV light. To protect DNA, use minimum fluence and long wavelength (365 nm). Set up a workstation with a long-wavelength UV light on a stand over a piece of clean glass. Place gel on glass and excise the portion containing the desired DNA band with a clean spatula and place it in a weighed 1.5-mL microfuge tube.
4. Weigh the agarose (in the tube).
5. Assume 1 g of agarose = 1 mL. Add 3 volumes of NaI solution (6 *M*).
6. Incubate at 55°C for 5 minutes, or until the agarose dissolves.

7. Add 5 μL of glass milk for solutions containing 5 μg or less of DNA. (Add 1 μL glass milk for each additional 1 μg of DNA.) Vortex.
8. **Important:** Incubate 5 to 10 minutes at room temperature to ensure that the DNA binds to the glass beads. Rock tube gently.
9. Centrifuge 5 seconds in microfuge.
10. Remove the supernatant with a Pipetman.
11. Wash (resuspend, centrifuge, decant supernatant, resuspend) the glass milk–DNA pellet 3 times with 0.5 mL (per wash) of NEW wash solution.
12. Resuspend the glass milk–DNA pellet in 10 μL of TE (10/0.1).
13. Incubate at 55°C for 5 minutes.
14. Centrifuge in microfuge for 30 seconds, then recover DNA contained in the supernatant.
15. To measure recovery, examine by gel electrophoresis (be sure you include the uncut plasmid on the gel for comparison), or determine the OD at 260 nm [OD_{260} nm × dilution factor × 50 = μg/mL DNA]. Recovery should average 80% of input.

Solutions for DNA Purification Using Glass Milk

Geneclean kit (Bio 101)

TAE:
0.04 *M* Tris–acetate, 0.002 *M* EDTA
1 L of 50×: 242 g Tris base
57.1 mL glacial acetic acid
100 mL 0.5 *M* EDTA pH 8.0

TE (10/0.1): 10 mM Tris, pH 7.5; + 0.1 mM EDTA

6× Load buffer: 0.05% bromphenol blue; 40% (w/v) glycerol in H_2O

To prepare your own reagents, if you do not want to use a commercial kit:

Glass milk solution: Silica 325 mesh: powdered flint glass from ceramic supply store; resuspend 250 mL powder in 750 mL water. Stir 1 hour then settle 1 hour. Take supernatant and repeat settling. Take second supernatant and centrifuge at 5000 rpm for 10 minutes in Sorvall GSA. Resuspend pellet in 200 mL water. Add nitric acid to 50%; steam for 4 to 5 hours. Wash 5 to 6 times (by centrifugation) with several volumes of water until supernatant is pH 7. Store at 4°C as a 50% slurry in water. Two hundred fifty milliliters of powder yields about 25 g of fines.

NaI solution (6.05 *M*): Mix 90.8 g NaI + 1.5 g Na_2SO_3 + water to 100 mL. Filter through Whatman #1 paper to remove undissolved sodium sulfite. Add another 0.5 g sodium sulfite; store in dark at 4°C.

Ethanol (NEW) wash: 50% ethanol + 0.1 *M* NaCl + 120 mM Tris, pH 7.5, + 1 mM EDTA

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E. LIGATION OF PCR PRODUCT TO pGEX2 VECTOR

Introduction

DNA restriction fragments with compatible cohesive ends (or blunt ends) can be joined by phage T4 DNA ligase to form recombinant DNA molecules. Two competing reactions occur during ligation: intermolecular joining of 2 fragments at 1 end to produce a linear product, and intramolecular joining of opposite ends of a single molecule to form a circular DNA. Successful insertion of a restriction fragment into a plasmid vector requires both types of ligation. First, one end of the desired restriction fragment must ligate to one end of the plasmid vector (which has also been made linear by cleavage with an appropriate restriction endonuclease). Next, the free ends of this 2-component ligation product must join to form a circular recombinant plasmid (vector + insert) capable of transforming *E. coli*.

The total concentration of DNA termini in the ligation reaction is the most important factor controlling whether ligated DNAs will form primarily linear multimers or circular monomers. High concentrations favor linear multimers whereas low DNA concentrations favor circles. The likelihood that the ends of a single molecule will join to each other depends on its length: as their length increases, DNA molecules are less likely to form circles. The probability that the ends of a linear vector molecule will encounter the desired restriction fragment also depends on the relative abundance of each DNA and the number of other (competing) restriction fragments in the reaction. Thus, DNA concentration, fragment length, insert-to-vector ratio, and complexity of the DNA fragment population all affect the outcome of a ligation reaction.

Ligation requires ATP, which is usually included in the ligase buffer supplied by the manufacturer.

Protocol

1. Heat DNAs at 68°C for 15 minutes to inactivate restriction endonucleases (some other enzymes require phenol extraction followed by chloroform extraction and ethanol precipitation).
2. Mix: 100 ng of (linear) vector DNA
   500 ng of insert DNA (restricted PCR product; use one-fourth of the digest)
   2 μL of 10× ligase buffer
   H_2O to bring to 20 μL
3. As a control, prepare a ligation using 100 ng cut pGEX plasmid with no insert. This DNA should **not** transform *E. coli* because pGEX2 cut with both *Eco*RI and *Bam*HI **cannot** form circles.
4. Add 0.5 μL (200 units) of T4 DNA ligase to each.
5. Incubate at 15°C overnight.

Solutions for Ligation

Use ligase buffer provided by the supplier, store frozen, and thaw on ice to protect the ATP.

New England Biolabs (NEB) T4 DNA ligase buffer: 50 mM Tris-HCl, pH 7.8, + 10 mM $MgCl_2$ + 1 mM ATP + 10 mM dithiothreitol + 25 μg/mL bovine serum albumin

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F. TRANSFORMATION OF *E. COLI* WITH THE LIGATED PLASMID

Introduction

To introduce plasmid DNA into *E. coli*, grow the host cells to mid-log phase and incubate the bacteria (in the presence of plasmid DNA) in a buffer formulated to render the cell walls permeable to DNA. A heat shock completes the procedure. Such treatment stresses the bacteria; 30% survival (70% killing) is typical. This procedure does not work with linear DNA, which is sensitive to exonuclease attack. Different strains of *E. coli* vary greatly in transformation efficiency, and some strains (notably HB101) do not respond well to the RbCl method described here. For HB101, substitute 75 mM $CaCl_2$ for RbCl buffer. Note that "competent" cells (cells capable of taking up foreign DNA) can be prepared by this method and frozen for future use.

As host for the pGEX2 clones, you will use *E. coli* strain DH5α [F'/*supE44 hsdR17* (r_K- m_K+)*recA1 endA1 gyrA96* (Nal^r)*thi-1 relA1* Δ*(lacIZYA− argF)U169 deoR* (Φ80*dlac*Δ*(lacZ)M15)*. DH5α transforms at a high efficiency.

Do the following transformations and controls:

a. Control 1: follow transformation procedure, but with no added DNA

b. Control 2: transform with uncut pGEX2 plasmid (use 10 ng)

c. Control 3: transform with cut pGEX2 (unligated; use 100 ng)

d. Control 4: transform with cut pGEX2 (ligated; use 100 ng)

e. Experimental: transform with cut, ligated pGEX2 and PCR DNA

Safety Precautions

You will be working with cultures of bacteria. Review aseptic techniques. Do not contaminate the buffers or media.

Protocol

Beforehand (TAs)

1. Inoculate 0.1 to 0.2 mL of fresh overnight culture into 10 mL of L broth. Grow cells in L broth to 1×10^8cells/mL (a reading of 60 on a Klett colorimeter, or a spectrophotometer reading of 0.4 to 0.6 at 600 nm); prepare 5 mL for each transformation.

Students start here:

2. For 5 transformations, obtain 25 mL of cells (5 mL for each transformation). Centrifuge 5 minutes, 8000 rpm, 4°C in Sorvall SS-34 rotor. Use sterile 50-mL Nalgene tubes.
3. Discard supernatant. Resuspend pellet in 5 ml ice-cold RbCl buffer (sterile). Divide into 5 sterile 1.5-mL microfuge tubes; hold on ice 5 minutes.
4. Centrifuge 1 minute, 14,000 rpm, in microfuge.
5. Resuspend pellet in 0.2 mL ice-cold RbCl buffer.
6. Mix DNA with cells; use entire ligation, or controls as listed above. Hold on ice for 60 minutes.
7. Incubate in 42°C water bath for 2 minutes.
8. Add 1 mL of L broth (no antibiotics) to cells. Place in sterile test tube.
9. (optional) Incubate with aeration at 37°C for 45 minutes. Don't do this step if you want to avoid sib-

ling transformants, which are duplicate clones that arose from one transformed cell that divided during the 45-minute incubation. Because our goal in this experiment is to obtain clones, we do not need to avoid sibling transformants.

10. Pipet into microfuge tube. Pellet in microfuge; resuspend in 100 μL L broth.
11. Spread cells on selective agar plates.
12. Incubate at 37°C overnight.
13. Count colonies on each plate.
14. Use sterile toothpicks to pick single colonies and inoculate them into 2 ml of L broth (containing 100 μg/ml ampicillin). With the same toothpick, streak transformants on selective agar plates to purify single colonies from the transformants. You should always streak any transformants you intend to keep before storing them as permanent freezer stocks.

    Each team should pick 4 colonies: 1 from positive control 2 (uncut pGEX2) and 3 experimentals, which are putative recombinant clones.
15. Incubate the 2-mL cultures with aeration at 37°C overnight.

Solutions for Transformation

RbCl buffer: 100 mM RbCl + 45 mM $MnCl_2$ + 10 mM $CaCl_2$ + 35 mM KCH_3CO_2 + 15% sucrose, pH 6.0; filter sterilize

Mix 1.21 g RbCl + 0.89 g $MnCl_2 \cdot 4\ H_2O$ + 0.147 g $CaCl_2 \cdot 2\ H_2O$ + 0.344 g KCH_3CO_2 + 15 g sucrose in 100 mL total volume; adjust pH; filter through sterile 0.2-μm filter (Gelman HT-200) into sterile vacuum flask

L broth: 10 g/L tryptone (Difco) + 5 g/L yeast extract (Difco) + 10 g/L NaCl, pH 7.0 (autoclave 22 minutes)

L agar: add 15 g Difco agar to 1 L of L broth; autoclave

Selective agar plates: from 100× or 1000× stock, add sterile ampicillin to 100 μg/mL. Dissolve ampicillin in sterile water and store frozen at −20°C.

To store competent cells: proceed through step 5. **Do not** add transforming DNA. Incubate on ice for 60 minutes. Add an equal volume of ice-cold 30% glycerol. Freeze at −70°C. To use remove from freezer, add transforming DNA, incubate on ice for 30 to 60 minutes, and proceed with heat shock (step 7).

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G. SMALL-SCALE PREPARATION OF PLASMID DNA BY THE ALKALINE LYSIS METHOD

Introduction

Use this procedure to purify small amounts of plasmid DNA for restriction endonuclease analysis. This rapid method allows us to prepare DNA from as many as 72 bacterial cultures at one time. The preparation will also contain RNA, some protein and cellular debris, and some sheared linear fragments of chromosomal DNA. Usually these impurities do not interfere with restriction analysis and many other procedures.

For many purposes, plasmid DNA is treated with RNase. You will not add RNase because the restriction fragments are larger than 1000 base pairs (bp), and the RNA will not interfere. Avoid RNase if future experiments will use RNA. Because your DNA preparations will contain RNA, you will not quantify them; RNA also absorbs at 260 nm.

DNA preparation kits are available from biotechnology companies. These kits produce DNA suitable for sequencing; however, the alkaline lysis method is much less expensive than a kit.

Protocol

1. Centrifuge 1.5 mL of overnight culture for 1 minute at 14,000 rpm in microfuge (use 1.5-mL tubes).
2. Discard supernatant and resuspend cells in 0.1 mL of TE (25/10).
3. Add 0.2 mL of alkaline lysis buffer (make fresh from NaOH and SDS). Mix **gently** (invert tube several times); excess vigor will increase contamination by sheared chromosomal DNA fragments.

4. Add 0.15 mL of 3 *M* potassium acetate buffer; mix **gently.**
5. Centrifuge 3 minutes in microfuge at full speed (14,000 rpm).
6. Remove supernatant to a 1.5-mL microfuge tube and mix with 1 mL 95% ethanol; hold 10 minutes at **room** temperature (or 4°C).
7. Centrifuge 3 minutes in microfuge.
8. Discard supernatant; dissolve pellet in 100 μL TE (10/0.1); add 50 μL of 7.5 *M* ammonium acetate.
9. Precipitate with 300 μL of 95% ethanol (on ice, 10 minutes).
10. Centrifuge 3 minutes in microfuge; remove all ethanol. Wash the pellet with 70% ethanol: add 0.5 mL ethanol to the tube, invert **gently,** then remove ethanol. Air dry pellet to remove traces of ethanol.
11. Dissolve pellet in 60 μL of TE (10/0.1). Use 2 to 5 μL per sample for restriction analysis and agarose gel electrophoresis; treat with 1 μL RNase **(not needed here)** to examine DNA restriction fragments smaller than 1000 bp.

Note: Many protocols omit the 2nd ethanol precipitation. Follow the procedure through step 7. Discard supernatant. Wash the pellet with 70% ethanol and air dry. Dissolve the pellet in 60 μL TE.

Solutions for Plasmid DNA Preparation (Alkaline Lysis)

TE (25/10): 25 mM Tris, pH 7.5; 10 mM EDTA (autoclave)

SDS–NaOH lysis solution (make fresh before use):
200 mM NaOH; 1% SDS

Mix 0.67 mL of 3 *M* NaOH + 0.4 mL of 25% SDS + 9 mL H_2O
(3 *M* NaOH = 12 g/100 mL)

3 *M* potassium acetate solution: 3 *M* K+ 5 *M* CH_3CO_2– (autoclave)
Mix 60 mL of 5 *M* potassium acetate (29.45 g/60 mL) + 11.5 mL of acetic acid + 28.5 mL of H_2O

Ammonium acetate solution: 7.5 *M* ammonium acetate = 57.8 g/100 mL (autoclave)

TE (10/0.1): 10 mM Tris, pH 7.5; 0.1 mM EDTA (autoclave)

95% Ethanol

Notes

H. RESTRICTION ANALYSIS

Introduction

Analysis of recombinant plasmids (created by restriction and ligation in vitro) to determine whether cloning was successful involves: 1) digestion of recombinant plasmid DNAs with appropriate restriction endonucleases; 2) separation of restriction fragments according to their size via electrophoresis through an agarose gel; and 3) estimation of restriction fragment molecular weights.

Usually we perform more than one restriction analysis to confirm the structure of a particular recombinant plasmid. For example, we often choose to excise the insert from the vector with the same enzymes used to make the recombinant plasmid. This will show whether the putative recombinant plasmid contains an inserted restriction fragment of the proper size. We can also map a restriction site found inside the inserted fragment relative to a site in the vector to determine the orientation of the insert in the vector. This provides independent evidence that the recombinant plasmid has the expected structure.

The pGEX2–*virD2* plasmid you constructed should contain a single *Eco*RI site at one junction between the PCR-produced 1280-bp DNA fragment and the 4948-bp vector. We expect digestion with *Eco*RI to produce a single fragment of 6218 bp. The other vector–insert junction is a hybrid *Bam*HI/*Bgl*II site, which cannot be cleaved by either enzyme. The insert fragment contains a single *Pst*I site 121 bp from its *Bgl*II end and 1159 bp from its *Eco*RI end. The vector contains a single *Pst*I site 957 bp from its *Eco*RI site and 3981 bp from its *Bam*HI site. Thus, *Pst*I digestion of the plasmid we constructed should produce fragments of 2116 and 4102 bp.

Technical Tip

Keep enzymes chilled (store at −20°C in non-frost-free freezer).

Protocol

1. Mix 2 of the following reactions, one for *Eco*RI and one for *Pst*I:

   Mix:
   5 μL of miniprep DNA (200 to 800 ng)
   1.5 μL of 10× reaction buffer (supplied by manufacturer) (NEBuffer *Eco*RI for *Eco*RI; NEBuffer 3 for *Pst*I);

   Bring to 15 μL with water (8.5 μL).

2. Add 2 to 5 units of restriction endonuclease (usually about 0.5 μL)

3. Incubate at proper temperature (37°C for most enzymes) for 1 or more hours.

4. Mix agarose with 1× TAE buffer (0.8% = 0.2 g/25 mL for minigel); melt in microwave; add 1.25 μL of 10 mg/mL ethidium bromide. Pour molten agarose into bed with comb (well-former) in place; allow to solidify; cover with buffer and remove comb.

5. Place 5 μL of each restriction digest in fresh tubes. Add 1 μL 6× load buffer to each and load in individual wells. Note: one-third of your restriction digest should form visible bands. However, if your DNA yield was low, the bands may appear faint; you can use the remaining two-thirds to repeat the electrophoresis.

6. Fill buffer chambers with 1× TAE; apply 100 V; DNA will migrate to the positive electrode.

7. When bromphenol blue dye reaches bottom of gel, observe DNA under UV light; photograph.

Solutions for Restriction Analysis

TAE: 0.04 *M* Tris–acetate, 0.002 *M* EDTA
1 L of 50×: 242 g Tris base
57.1 mL glacial acetic acid
100 mL 0.5 *M* EDTA pH 8.0

NEBuffer *Eco*RI: 50 mM NaCl, 100 mM Tris–HCl, 10 mM $MgCl_2$, 0.025% Triton X-100 (pH 7.5 at 25°C)

NEBuffer 3: 50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol (pH 7.9 at 25°C)

6× Load buffer: 0.05% bromphenol blue; 40% glycerol in water

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EXERCISE 1. Study Questions

Cesium Chloride Gradient

1. Sometimes undigested plasmid DNA preparations isolated from CsCl gradients show 4 bands (in the upper half of the gel) on agarose gel electrophoresis. What form of plasmid DNA is in each band? How could you test whether the plasmid DNA in a particular band is supercoiled (also called covalently closed circular), relaxed circular (also called nicked circular or open circular), or linear?

2. Why does each DNA form migrate differently during agarose gel electrophoresis?

3. What is the extremely bright broad band of material near the bottom of the gel? How does its presence affect your estimate of the plasmid DNA concentration based on OD_{260} readings? What would you do to eliminate this problem?

PCR

1. What does each control in the PCR reaction tell you? What other controls might be useful?

2. Several parameters affect the melting temperature (T_m) of DNA:

   a. base composition

   b. salt concentration

   c. formamide: 1% formamide lowers T_m by 0.65°C

   d. DNA sequence similarity: every 1% decrease in similarity lowers T_m by 1.5°C

   e. length of DNA: T_m (short duplex) = T_m (long duplex) − (500 ÷ bp of short duplex)

   The following equation is used to estimate the melting point of duplex DNA.

   $$T_m = 16.6 \log [Na^+] + 0.41 (\% G+C) + 81.5$$

   Does an increase in salt concentration raise or lower the T_m of DNA? Why?

3. a. Look at your gel analysis of the PCR products. Is the brightest band in each lane the desired prod-

uct? How can you tell? What else could you do to confirm the identity of the PCR product? Is such confirmation important?

b. Estimate the molecular weight of the major PCR product; do a semilog plot of molecular weight versus migration distance for the 1-kb ladder marker.

c. Did the kit primer and template give a PCR product of the anticipated molecular weight?

d. On some gels, the no-template control produced a faint band the same size as the desired PCR product. Explain what probably led to the appearance of this band and what steps you could take to prevent this problem in the future.

e. What are the other bands? How might you prevent synthesis of these minor PCR products?

Cloning

1. During the cloning you cut pGEX2 (4948 bp) with *Eco*RI and *Bam*HI (which cut next to each other), and you attempted to insert your PCR product (1280 bp), after cleavage with *Eco*RI and *Bgl*II, into the compatible cohesive ends of the cut pGEX2. Why are the ends of these DNAs compatible even though they are cut with different restriction endonucleases?

2. Why did you heat the restriction digest at 65°C for 10 minutes before you added T4 DNA ligase?

3. Which of the enzymes you used can be inactivated by heat (65°C) and which cannot (see the appendix in the New England Biolabs [NEB] catalog)? Does it matter? Explain.

4. Some restriction endonucleases do not cut well when the target site is at or near the end of a DNA molecule, such as the PCR product you used. Does this observation apply to your cloning experiment? What is the likely consequence if one of the enzymes failed to cut its target in the PCR product?

5. Why did we design a *Bgl*II site into the PCR product instead of a *Bam*HI site? (Use your imagination.)

6. What is the molar ratio between vector and insert in the ligation you did?

7. What is the purpose of each control in the transformation procedure?

8. What was your transformation efficiency? How does the efficiency of your cells compare with commercial competent cells?

9. Make a drawing of the recombinant plasmids we made. Include restriction enzyme recognition sites, and label the restriction fragments with their lengths in base pairs.

10. Look at the gel of the restriction analysis of plasmid DNAs isolated from transformants in your cloning experiment. Measure the distance each band has migrated. Use the known molecular weight standards on your gel to plot a molecular weight versus migration distance curve, and use this curve to estimate the molecular weight of each unknown restriction fragment. Do any of the lanes contain the desired clones? Can you explain what may have happened in the lanes that do not?

Notes