EXERCISE

# 3

# Oligonucleotide-Directed Mutagenesis

## Background

The ability to create specific mutations is one of the most powerful techniques for analysis of well-defined genes. With this technique, we can create single-base changes or small insertions and deletions. For example, we can introduce or destroy a restriction site, alter a promoter, or change the coding sequence of a gene.

Oligonucleotide-directed mutagenesis allows us to alter DNA in a specific location by hybridizing a complementary oligonucleotide primer containing the desired mutation to a circular single-stranded vector containing the target sequence. DNA polymerase extends the primer in vitro; DNA ligase covalently joins the ends of the newly extended strand. This heteroduplex DNA now contains the mutation in its newly synthesized strand and the original sequence in the template strand. On transformation into *E. coli,* DNA repair and replication resolve this mismatch to produce colonies with either mutant or wild-type target DNA.

Several different methods can increase the chances of recovering mutant-containing clones. You will use uracil-containing template prepared from a mutant *E. coli* strain. You will provide dTTP for in vitro DNA synthesis, so that the mutant strand will contain thymine, not uracil. The uracil-containing template strand will be selectively destroyed when transformed into a wild-type host strain, leaving behind the mutant strand.

In this experiment, you will delete 58 bp from *virD2* and replace them with a 7-bp sequence that includes an *Nru*I site. You begin by inserting a portion of *virD2* into the phagemid vector pUC119 (*virD2* + pUC119 = pMCB525). A phagemid vector is a plasmid that contains both a plasmid (ColE1) and single-strand DNA phage (M13) origins of replication. When cells that harbor a phagemid are infected with an M13 helper phage (M13KO7), the cells produce phage particles that contain single-stranded DNA

composed of either phagemid DNA or helper phage DNA. Because M13KO7 replicates poorly in the presence of a phagemid (the helper phage has a slightly defective *ori*), most capsids contain phagemid DNA.

Experience shows that large restriction fragments inserted into phagemid vectors suffer rearrangements at a high frequency. Therefore, we will use a small restriction fragment, a 354-bp portion of *virD2* contained on a *Bam*HI–*Hin*dIII restriction fragment. To confirm the presence of the mutation, you will use these same restriction sites to excise the mutant portion of *virD2* from putative mutant derivatives of pMCB525. First you must insert this fragment into the corresponding sites of pUC119 to create the phagemid pMCB525. You will transform *E. coli* with pMCB525. The phagemid provides the host with antibiotic resistance, allowing you to select for transformed cells; when the host is plated on antibiotic-containing medium, only cells that contain the phagemid can grow. In addition, ligation of the insert into the phagemid disrupts *lacZ* present in the phagemid, allowing you to identify transformed cells that contain the insert.

You will prepare template for in vitro mutagenesis from a *dut*–*ung*– *E. coli* strain (CJ236) harboring the recombinant phagemid pMCB525. The *dut* mutation inactivates dUTPase, thereby increasing the cellular dUTP pool, which results in increased incorporation of uracil (in place of thymine) during DNA synthesis. The *ung* mutation inactivates uracil *N*-glycosylase, a DNA repair enzyme that removes uracil bases; this mutation prevents removal of uracil from the DNA. The uracil-containing phagemid DNA will serve as the template for in vitro DNA synthesis primed by the mutagenic oligonucleotide. The 5' end of this primer is the exact complement of a 14-base sequence in the template, and the 3' end is the complement of a separate 19-base region of the template. These 2 regions are separated by 58 bases in the template, but only 7 bases (which include an *Nru*I site) in the primer. Thus, successful mutagenesis will delete 58 bp from *virD2* and

replace them with a 7-bp sequence that includes an *Nru*I site. You will screen putative mutants for a net loss of 51 bp from the *Bam*HI–*Hin*dIII fragment.

On completion of DNA synthesis, you will transform the heteroduplex product into an *ung*+ *E. coli* strain. The nonmutant template DNA strand contains uracil, whereas the mutant DNA strand synthesized in vitro does not. Uracil repair, which occurs as the plasmid begins replication, causes selective loss of the parental nonmutant strand, thereby increasing your chances of isolating the oligonucleotide-directed mutation.

Steps of the procedure are

**A.** Perform restriction digests of *virD2* (in pCS64) and pUC119

**B.** Purify DNA fragments from agarose

**C.** Ligate restriction fragment to vector

**D.** Transform *E. coli* with the ligated phagemid

**E.** Prepare plasmid DNA

**F.** Perform restriction digest of DNAs; examine to confirm insert

**G.** Prepare single-stranded, uracil-containing DNA template

**H.** Phosphorylate oligonucleotide

**I.** Anneal oligonucleotide to template

**J.** Synthesize DNA in vitro by primer extension

**K.** Transform synthesis reactions into *E. coli*

**L.** Prepare plasmid DNA from putative mutants

**M.** Confirm mutants by restriction analysis of plasmid DNA

## Technical Tip

The proportion of mutant versus wild-type transformants will depend on the quality of each step. **Always** design site-directed mutagenesis experiments so that there is an efficient screen for the mutant, such as loss or creation of a restriction site.

## Notes

# CLONING INTO A PHAGEMID VECTOR

## Introduction

You will insert a portion of *virD2* into the phagemid vector pUC119. From the resulting phagemid, you can prepare single-stranded DNA to use as template for oligonucleotide-directed mutagenesis of *virD2*.

Phagemid pUC119 contains the origin of replication for single-strand DNA phage M13; this allows 1 strand of the phagemid (the plus strand) to package into M13 phage particles when a helper M13 phage is infected into the host cells. It also contains a ColE1 plasmid origin of replication, a β-lactamase (*bla;* ampicillin resistance) gene, and a multiple cloning sequence within a portion of the β-galactosidase (*lacZ*) gene. The presence of this portion of *lacZ* will allow you to identify transformants (in your cloning experiment) that harbor a recombinant plasmid with foreign DNA inserted into the multiple cloning site/*lacZ* region. Insertion of foreign DNA into *lacZ* will render the cells Lac−.

LacZ protein normally consists of 1021 amino acids, and the enzyme must form a tetramer to cleave lactose. A deletion mutation, *lacZΔM15*, removes amino acids 11 through 41 and prevents tetramer formation. A peptide (called the α peptide) contains the *N*-terminal 92 amino acids of LacZ, which includes the tetramerization domain. This peptide can interact with the LacZΔM15 protein and thereby restore tetramer formation and enzyme activity. This example of intragenic complementation is usually called alpha complementation. Many plasmid, phagemid, and phage lambda cloning vectors carry the alpha portion of *lacZ* to allow easy detection of inserted foreign DNA. These vectors are used with *E. coli* hosts (such as DH5α) that carry the *lacZΔM15* allele on an F'*lac* plasmid or in the chromosome (usually on a prophage);

note that these strains must not carry a wild-type copy of the *lacZ* gene.

The Lac phenotype of *E. coli* cells can be determined by plating on indicator agar that contains X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) to detect LacZ activity and IPTG (isopropylthiogalactoside) to bind the LacI repressor protein and allow expression of the *lac* operon. When LacZ cleaves the galactoside bond to free the indole moiety, the indole rings react to form indigo, a dark blue compound; before cleavage, X-gal is colorless. Thus, blue (Lac+) colonies contain a phagemid vector lacking an insert, whereas cells that contain a vector with foreign DNA inserted in *lacZα* will exhibit a Lac− (white) phenotype.

Your starting material is the plasmid pCS64, which contains the entire *virD2* coding sequence flanked by non-*virD2* sequences at each end. Digestion of this plasmid with *Hin*dIII and *Bam*HI will produce one large (vector) fragment, plus fragments of 259, 354, and 560 bp. The target for your oligonucleotide-directed mutagenesis experiment lies within the 354-bp *Bam*HI–*Hin*dIII fragment. To obtain the correct fragment, you will use agarose gel electrophoresis to separate the mixture of restriction fragments, excise the 354-bp fragment from the gel, and use the Geneclean procedure to purify it.

You will ligate the 354-bp *Bam*HI–*Hin*dIII fragment to pUC119 DNA cleaved with both enzymes. Note that the ends of the cut vector are incompatible with each other, so the vector cannot ligate into a circular molecule unless a *Bam*HI–*Hin*dIII fragment inserts into the multiple cloning sequence. Because the insert DNA has 2 different cohesive ends, it will insert into the vector in only 1 orientation. (Had we chosen to insert the target as a *Hin*dIII fragment, it could have ligated to the vector in either orientation.) For oligonucleotide-directed mutagenesis, the orientation of the target sequence relative to the vector is critical, because the mutagenic oligonucleotide must be the complement of the strand that becomes packaged when the phagemid replicates as a single-stranded

phage. For the phagemids pUC119 and pUC118, the *lacZ* coding (or sense) strand is also the phage M13 plus (or packaged) strand. We have inserted the *Bam*HI–*Hind*III fragment from *virD2* in the opposite orientation relative to *lacZ*: the coding strand of *virD2* is in the minus strand. Therefore, the packaged pUC119–*virD2* single-stranded DNA that you will use as template for the mutagenesis contains the noncoding (or antisense) strand of *virD2*, and the mutagenic oligonucleotide you will use corresponds to the sense strand of *virD2*.

## Notes

# A. RESTRICTION DIGESTS OF *virD2* (IN pCS64) AND pUC119

## Technical Tips

The volume of restriction endonucleases in a restriction digest must not exceed 10% of the total reaction volume because high concentrations of glycerol in enzyme preparations inhibit their activity.

Store restriction endonucleases at −20°C and keep them on ice for the short time you have them out of the freezer. Add them last to a restriction digest mixture, and begin the incubation immediately.

## Protocol

1. Mix: 2 μg of insert DNA (the plasmid pCS64)
   2 μL of 10× reaction buffer (NEB *Bam*HI)
   distilled water to give 18 μL total volume
2. Add 10 units each (usually 1 μL) of the restriction endonucleases *Bam*HI and *Hin*dIII. Incubate at 37°C for 1 to 2 hours.
3. Mix: 1 μg of CsCl-purified pUC119 DNA
   2 μL of 10× reaction buffer (NEB *Bam*HI)
   distilled water to give 18 μL total volume
4. Add 10 units each (usually 1 μL) of the restriction endonucleases *Bam*HI and *Hin*dIII. Incubate at 37°C for 1 to 2 hours.
5. Add 1 μL of heat-treated RNase (10 mg/mL).

## Solutions for Restriction Digests

1× NEBuffer *Bam*HI: 150 mM NaCl + 10 mM Tris, pH 7.9 + 10 mM $MgCl_2$ + 1 mM dithiothreitol
Heat-treated RNase (10 mg/mL)

## Notes

# B. PURIFICATION OF DNA FRAGMENTS FROM AGAROSE

## Introduction

The *Bam*HI–*Hin*dIII restriction digest of pCS64 resulted in a mixture of fragments; the one containing the target sequence for mutagenesis is 354 bp. It differs sufficiently in size from the other fragments such that it can be separated from the other fragments by agarose gel electrophoresis. Small DNA fragments cannot be separated on the kind of agarose gels you have used so far; instead use NuSieve 3:1 agarose (FMC BioProducts). You will identify the correct fragment by comparison with a size standard; excise it from the gel and use the Geneclean procedure to purify it.

## Protocol

1. Prepare a 2% NuSieve 3:1 agarose gel (0.8 g agarose in 40 mL 1× TAE; heat in microwave until dissolved; replace lost volume with $ddH_2O$; add 1.5 μL of ethidium bromide; pour and insert comb).
2. To the restriction digest of pCS64, add 4 μL 6× load buffer. Load the entire sample into 1 well. Separate DNA by electrophoresis at 100 V (toward positive electrode).
3. Follow the Geneclean procedure, Experiment I.D.

## Notes

# C. LIGATION OF RESTRICTION FRAGMENT AND VECTOR

## Introduction

Inactivation of restriction endonucleases prevents them from cutting restriction sites joined by DNA ligase. For many enzymes, including *Hin*dIII, incubation at 68°C for 15 minutes will destroy the enzyme activity. *Bam*HI is not susceptible to heat inactivation, but can be removed by phenol extraction followed by chloroform extraction and ethanol precipitation. Experience has shown us that *Bam*HI-digested DNAs can be successfully cloned using only a heat treatment before ligation; therefore, the phenol and chloroform extractions are optional. If you choose not to do them, incubate at 68°C instead.

## Protocol

Optional:

1. For each restriction digest, bring volume to 0.25 mL with TE (10/0.1). Add 0.2 mL phenol (equilibrated with 1 *M* Tris, pH 8.0); mix well, then add 0.12 mL $CHCl_3$.
2. Mix well, then centrifuge 10 minutes at full speed in microfuge.
3. Save aqueous (top) phase and extract with 0.2 mL $CHCl_3$:isoamyl alcohol (24:1).
4. Centrifuge 1 minute in microfuge; remove aqueous (top) phase to clean 1.5-mL tube.
5. Add 100 µL 7.5 *M* ammonium acetate. Add 0.6 mL of ethanol; mix and hold on ice for 5 minutes.

6. Centrifuge 3 minutes in microfuge.
7. Dissolve pellet in 20 μL TE (10/0.1).

If you choose not to extract with phenol and chloroform, do step 8 instead:

8. Heat DNAs at 68°C for 15 minutes to inactivate restriction endonucleases.
9. Mix: 0.5 μg of linear pUC119
   1 μg of the *Bam*HI–*Hin*dIII fragment from *virD2*
   5 μL of 5× ligase buffer (provided by the enzyme supplier)

   Bring to 25 μL with $H_2O$.
10. Add 0.5 μL of T4 DNA ligase; incubate at 15°C overnight.

## Solutions for Ligation

Phenol equilibrated with IM Tris, pH 8.0

Chloroform

Chloroform: isoamylalcohol (24:1)

7.5 M Ammonium acetate

Ethanol

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

T4 DNA ligase buffer (NEB):

50 mM Tris (pH 7.8), 10 mM Mg $Cl_2$,

1 mM ATP, 10 mM dithiothreitol, 25 μg/ml bovine serum albumin

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

## Notes

## Notes

## D. TRANSFORMATION OF *E. COLI* WITH THE LIGATED PLASMID AND RECOVERY OF CLONES

Follow the protocol for transformation in Experiment I, section F, except use *E. coli* strain DH5α, and plate transformations on LB-ampicillin plates with X-gal and IPTG.

## E. SMALL-SCALE PREPARATION OF PLASMID DNA FROM BROTH CULTURES

Follow the protocol for alkaline–SDS plasmid DNA preparation in Experiment I, section G, except add 1 μL of heat-treated RNase (10 mg/mL).

## F. RESTRICTION DIGEST OF DNAs: EXAMINATION TO CONFIRM INSERT

Follow the protocol for restriction digestion of plasmid DNA in Experiment III, section A (digestion with *Bam*HI and *Hin*dIII). Examine the restriction fragments on a 2% NuSieve 3:1 agarose gel, with 100-bp DNA ladder as a size standard.

## Notes

# G. PREPARATION OF SINGLE-STRANDED DNA TEMPLATE

## Introduction

For your mutagenesis experiment, you require single-stranded, uracil-containing template. You will prepare this template DNA (pMCB525) in *E. coli* strain CJ236 (*dut-1 ung-1 thi-1 relA1*; pCJ105 [$Cm^R$ = chloramphenicol resistance]).

The M13 phage that produces the single-stranded template infects *E. coli* by attaching to F pili, so you must use a strain of *E. coli* that produces these pili. The F' plasmid pCJ105 carries the *tra* (transfer) genes needed to produce F pili. These pili permit conjugal transfer of plasmid DNA from male (F+ or F') *E. coli* to female (F−) recipient strains. To ensure the presence of pCJ105, we will grow CJ236 in L broth containing 30 μg/mL chloramphenicol.

Unlike most viruses, M13 does not kill host cells. M13 forms turbid plaques by slowing the growth of infected cells. M13 buds from the surface of host cells, so the medium of an infected culture contains virus particles. The M13 capsid contains lipids; therefore, chloroform destroys M13 particles.

Phage titer is measured in plaque-forming units (pfu). Bacterial titer is measured in colony-forming units (cfu). A cfu of $2 \times 10^8$ corresponds approximately to a Klett reading of 50 or an absorbance at 600 nm of 0.5 (these values are strain-specific and must be determined empirically). Multiplicity of infection (m.o.i.) is pfu divided by cfu.

## Note

Confine RNase to the hood; use designated pipettor.

Phenol is equilibrated with buffer before use, and is usually stored under buffer. Therefore, 2 phases are appar-

ent in the phenol bottle; **do not** mix these. A layer of aqueous buffer is on top. To obtain phenol, insert a pipet into the phenol layer beneath the buffer. After use, collect phenol for organic waste disposal.

## Protocol

**Beforehand (TAs):**

1. Prepare an M13KO7 stock:
   a. Grow a fresh overnight culture of a male (F' or F+) *E. coli* strain (JM101).
   b. Inoculate 2 mL of L broth with 20 μL of JM101 overnight culture and incubate with aeration for 1 hour at 37°C.
   c. Add 20 μL of phage ($10^{11}$ pfu) or one plaque of M13KO7 and incubate 1 hour at 37°C with slow shaking.
   d. Add kanamycin to 50 μg/mL. Incubate with aeration overnight at 37°C.
   e. Centrifuge 5 minutes at 3700 rpm in clinical centrifuge.
   f. Titer phage stock on male strain (JM101). Prepare serial dilutions of phage (in broth). Add 10 μL of each dilution to 0.1 mL of fresh overnight culture of JM101 (or another male strain). Incubate at room temperature for 5 minutes.
   g. Add 3 mL L top agar and plate on L agar plates; incubate 37°C overnight.
2. Grow CJ236 (containing pMCB525) overnight at 37°C with aeration in L broth with 50 μg/mL ampicillin and 30 μg/mL chloramphenicol.

**Day 1 (students start here):**

3. Inoculate 2 mL of L broth + 0.001% thiamine with 25 μL of overnight culture from step 2. Incubate at 37°C with aeration for 1 hour.
4. Add ampicillin to 25 μg/mL. Incubate at 37°C with aeration for 30 minutes. Titer should reach approximately $2 \times 10^8$ cfu at this time.
5. Add 30 to 50 μL of M13KO7 helper phage stock to give an m.o.i. of approximately 20.
6. Incubate at 37°C for 1 to 2 hours with gentle shaking.
7. Add entire 2 mL of infected culture to 8 mL of L broth + 0.001% thiamine + 25 μg/mL ampicillin + 50 μg/mL kanamycin. (The phagemid encodes ampicillin resistance and the helper phage encodes kanamycin resistance.) Incubate with aeration for 16 hours at 37°C.

**Day 2:**

8. Centrifuge at 10,000 rpm for 10 minutes at 4°C in the Sorval SS34 rotor.
9. Mix 6 mL supernatant with 720 μL 5 *M* NaCl + 960 μL 30% PEG 8000. Hold at 4°C **overnight;** do not skimp on time at 4°C.

**Day 3:**

10. Centrifuge at 10,000 rpm for 10 minutes at 4°C in the SS34 rotor; discard supernatant and save small beige pellet.
11. Resuspend pellet in 0.5 mL of TE 10/0.1 and transfer to a 1.5-mL microfuge tube. Add heat-treated RNase A to 20 μg/mL. Incubate for 20 minutes at 37°C.

12. Extract twice with 1 volume of a 1:1 mixture of phenol:chloroform (add phenol:chloroform; vortex 1 minute; centrifuge 10 minutes at full speed in microfuge; remove and save aqueous (top) layer, leaving interphase behind). Repeat. Extract with chloroform:isoamyl alcohol (24:1). Thorough phenol extraction is crucial.

13. Add 0.5 volume of 7.5 *M* ammonium acetate, then add 2 volumes of ethanol. Hold on ice for 5 minutes, then centrifuge in microfuge for 5 minutes. Dissolve pellet in 25 μL TE (10/0.1) pH 8.0.

14. Measure optical density (OD) at 260 nm; concentration (μg/mL) = $OD_{260\ nm} \times 40$ (for single-stranded DNA).

15. Examine DNA on an agarose gel to estimate yield of single-stranded DNA. Include a size standard (1-kb ladder) and double-stranded DNA (the plasmid) on the gel for comparison.

## Solutions for Single-Stranded Template Preparation

| | | |
|---|---|---|
| L broth: | Tryptone (Difco) | 10 g/L |
| | Yeast extract (Difco) | 5 g/L |
| | NaCl | 10 g/L |
| | pH 7.0; autoclave 22 minutes | |

Ampicillin: 500× stock solution = 50 mg/mL

M13KO7 helper phage stock: titer = 0.5 to 5 × $10^{11}$ pfu/mL

Kanamycin: 1000× stock solution = 25 mg/mL

5 *M* NaCl

30% PEG 8000 (polyethylene glycol)

TE 10/0.1 (10 mM Tris, pH 8.0, 0.1 mM EDTA)

RNase A, heat-treated (see Experiment I)

Phenol:chloroform 1:1

Chloroform:isoamyl alcohol 24:1

7.5 *M* Ammonium acetate

Ethanol, 95%

Double-stranded pMCB525 for a size standard

## Notes

# H. PHOSPHORYLATION OF OLIGONUCLEOTIDE

## Introduction

DNA synthesis will extend from the 3' end of the primer, continue around the circular template, and reach the 5' end of the primer. DNA ligase can seal the "nick" between the 3' hydroxyl end of the nascent DNA and the adjacent 5' end of the primer, provided it has a 5' phosphate. Once ligase seals this nick, DNA synthesis cannot proceed farther. If the primer lacks a 5' phosphate, DNA synthesis will continue, thereby removing the mutagenic oligonucleotide. Therefore, you must add a phosphate to the 5' end of the mutagenic oligonucleotide primer before the DNA synthesis step. (Synthetic oligonucleotides lack a 5' phosphate.)

## Protocol

1. Mix:

| | |
|---|---|
| 10× polynucleotide kinase buffer | 3 μL |
| 1 mM ATP | 13 μL |
| mutagenic oligonucleotide | 200 pmol |
| distilled water to 30 μL | |

2. Add 4.5 units of T4 polynucleotide kinase.
3. Incubate at 37°C for 45 minutes.
4. Heat at 65°C for 10 minutes.
5. Add 3 μL of TE (10/0.1, pH 8.0) to dilute oligonucleotide to 6 pmol/μL. Store at −20°C.

## Solutions for Phosphorylation

NEB T4 polynucleotide kinase buffer: 70 mM Tris (pH7.6), 10 mM $MgCl_2$, 5 mM dithio threitol

1 mM ATP

200 pmol Mutagenic oligonucleotide: 5' CGC CAG CAG CGA TCT CGC GAT GCT GCG CAA GTT GAT TCC G 3'

T4 Polynucleotide kinase

TE 10/0.1, pH 8.0 (10 mM Tris, pH 8.0, 0.1 mM EDTA)

## Notes

## Notes

# I. ANNEALING MUTANT OLIGONUCLEOTIDE TO TEMPLATE

## Introduction

To anneal the mutagenic oligonucleotide to the target sequence, mix them together, heat, and allow to cool **slowly.** As a control, prepare a mixture that lacks the oligonucleotide primer.

## Protocol

1. On ice, mix:
   1 μg (0.8 pmol) of your single-stranded template (phagemid) DNA
   1 μL (6 pmol) phosphorylated oligonucleotide primer
   1 μL 10× T4 DNA polymerase buffer
   distilled water to 10 μL.

The primer:template ratio should be 7.5:1.

2. Also on ice, mix:
   1 μg (0.8 pmol) of your single-stranded template (phagemid) DNA
   1 μL 10× T4 DNA polymerase buffer
   distilled water to 10 μL

   (control without oligonucleotide primer)

3. Incubate the tubes at 70°C for 1 minute.
4. Cool to room temperature **slowly** by placing the tubes in a styrofoam float and setting them in a beaker containing 400 mL of 70°C water. Allow the water to gradually reach room temperature.

## Solutions for Annealing

Single-stranded template (phagemid) DNA—student preparations

Phosphorylated oligonucleotide primer

NEB T4 DNA polymerase buffer: 0.5 M NaCl, 0.1 M Tris (pH 7.9), 0.1 mM $MgCl_2$, 10 mM dithiothreitol

## Notes

## Notes

# J. IN VITRO DNA SYNTHESIS BY PRIMER EXTENSION

## Introduction

DNA synthesis will require single-stranded pMCB525 DNA as a template, DNA polymerase and dNTPs. After DNA polymerase copies the entire template, DNA ligase will join the 3' end of the newly synthesized strand to the 5' end of the primer, preventing further synthesis, which would replace the mutagenic oligonucleotide. Ligation requires ATP.

## Protocol

1. To each 10 μL annealed primer/template add:

| | |
|---|---|
| 10× T4 DNA polymerase buffer | 1 μL |
| 10 mM ATP | 1.5 μL (to give 0.75 mM) |
| 2.5 mM dNTP mix | 3.2 μL (0.4 mM final concentration) |
| T4 DNA ligase | 1 μL |
| T4 DNA polymerase | 1.2 μL |

2. Incubate 5 minutes at room temperature, then 75 minutes at 37°C.

3. Examine 2 μL of the synthesis product by agarose gel electrophoresis. Include: 1-kb ladder, double-stranded plasmid, single-stranded template, and no-primer control.

## Solutions for in Vitro DNA Synthesis

10× T4 DNA polymerase buffer

10 mM ATP

2.5 mM dNTP mix

T4 DNA ligase (400 units/μL; NEBiolabs)

T4 DNA polymerase (3 units/μL)

## Notes

## Notes

## K. TRANSFORM SYNTHESIS REACTION INTO *E. COLI* DH5α

### Introduction

Use the following DNAs to transform *E. coli* strain DH5α:

1. Experimental
2. Negative control: no primer synthesis reaction
3. Transformation-positive control: double-stranded pMCB525

### Note

Use aseptic technique to avoid contamination of bacterial cultures.

### Protocol

1. Transform 2 μL of each synthesis reaction and 1 ng pMCB525 into DH5α according to the protocol in Experiment I.F.
2. Pick 5 putative mutants and 1 colony (pMCB525) from the transformation-positive control; inoculate broth + ampicillin.

## L. SMALL-SCALE PREPARATION OF PLASMID DNA

### Protocol

Obtain a culture containing a confirmed mutant plasmid. This will be your mutant-positive control. You will prepare

7 DNAs: 2 controls (mutant and transformation) and 5 putative mutants. Follow the procedure in Experiment I.G.

Note: Use RNase. Do 2 ethanol precipitations.

## M. CONFIRMATION OF MUTANTS BY RESTRICTION ANALYSIS

### Introduction

You will digest plasmid DNA with *Hin*dIII and *Bam*HI and examine samples by gel electrophoresis. The *Bam*HI and *Hin*dIII digestions cleave at the termini of the insert. The parental plasmid will yield one large (>3200 bp) vector fragment and a 354-bp fragment. Mutants will have a 303-bp fragment in place of the 354-bp fragment. To detect these small fragments, use either a polyacrylamide gel or agarose such as NuSieve 3:1 or MetaPhor (FMC BioProducts). We give the protocol for an acrylamide gel; if you prefer, use a 2% NuSieve 3:1 agarose gel.

### Protocol

| 1. | | Each digestion: | Master mix: |
|---|---|---|---|
| 10 | µL | DNA preparation | — |
| 2 | µL | 10× *Hin*dIII buffer (NEBuffer 2) | 16 µL |
| 1 | µL | 1 µg/µL RNase A solution | 8 µL |
| 6 | µL | water | 48 µL |
| 0.5 | µL | *Hin*dIII | 4 µL |
| 0.5 | µL | *Bam*HI | 4 µL |

Prepare a master mix (on ice) containing everything except the DNAs. Pipet 10 µL of master mix into 7 tubes, which already contain 10 µL of DNA.

2. Assemble glass plates and spacers.

3. Combine: 1 mL 5× TAE buffer
   2.5 mL 40% acrylamide stock (19:1 acrylamide:bis)
   6.5 mL $H_2O$
   in a small flask and swirl gently to mix.

   Add: 10 μL TEMED
   40 μL 10% ammonium persulfate
   and swirl gently to mix. Pour gel using automatic pipettor. Insert comb.

4. After gel has polymerized, pipet water onto top of gel, remove comb, and rinse wells thoroughly with distilled water.

5. Place gel in apparatus and add electrophoresis buffer (0.5× TAE).

6. Sample loading:

   Lanes 1–7: 20 μL restriction digest mixed with 5 μL 5× load buffer*

   Lane 8: 5 μL 100-bp DNA ladder, 0.2 μg/μL (premixed with load buffer)

   *Note: use the load buffer containing 2 tracking dyes, bromphenol blue and xylene cyanol.

7. Secure electrodes and apply 150 V (constant voltage) until xylene cyanol (turquoise dye) migrates almost to the bottom of the gel (about 2 hours).

8. Staining: Place gel in tray containing 50 mL $H_2O$ and 5 μL 10 mg/ml ethidium bromide. Stain 5 minutes. Pour stain into collection flask. Rinse gel in distilled water and photograph.

## Solutions for Restriction Analysis

NEBuffer 2: 50 mM NaCl, 10mM Tris (pH 7.9), 10 mM Mg $Cl_2$, 1 mM dithiothreitol

1 μg/μL RNaseA

5 × TAE: 0.2 M Tris-acetate, 0.1 M EDTA

40% acrylamide stock (19:1 acrylamide:bis)

TEMED

10% ammonium persulfate (fresh)

100-bp DNA ladder

5 × load buffer: 0.05% bromphenol blue, 0.05% xylene cyanol, 40% glycerol

10 mg/mL ethidium bromide

## EXERCISE 3. STUDY QUESTIONS

1. The sense strand (which corresponds to the sequence of the mRNA) of the region of *virD2* affected by the oligonucleotide-directed mutation is shown separated into codons; bases found in both *virD2* and the mutagenic oligonucleotide are underlined. The sequence below is the mutagenic oligonucleotide. Note that the single-stranded pMCB525 template DNA that you prepared is the complement of the strand shown here.

   A portion of the *virD2* coding sequence:

   5' etc AAG CGC CAG CAG CGA TCA AAA CGA CGT AAT GAC GAG GAG GCA GGT CCG AGC GGA GCA AAC CGT AAA GGA TTG AAG GCT GCG CAA GTT GAT TCC GAG GCA etc 3'

   Mutagenic oligonucleotide:

   5' CGC CAG CAG CGA TCT CGC GAT GCT GCG CAA GTT GAT TCC G 3'

   Is it important for each end of the mutagenic oligonucleotide to anneal stably to the template DNA? Explain.

2. What is the melting temperature ($T_m$) for each portion of the primer/template duplex?

3. The mutagenesis should create a new *NruI* site. Where is it?

4. How will this mutation alter the VirD2 protein?

5. Both ends of the primer were designed to form a number of G:C base pairs with the template. Why? Explain why a G:C base pair has greater thermal stability than an A:T base pair.

## Notes