MOLECULAR BIOLOGY *Techniques*

AN INTENSIVE LABORATORY COURSE

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Preface

We designed this intensive laboratory course to teach incoming graduate students the basic skills of molecular biology, so that students with varied research experience would understand the fundamental principles. To do this, we created an intensive 2-week course that includes techniques commonly used in molecular biology—methods that provide the foundation for most other procedures. In this course students learn how to prepare and analyze DNA, RNA, and proteins. The class must meet all day to cover the material in a 2-week period. This approach has several advantages. Students learn that the laboratory is open nights and weekends, and they discover that the day is not done until they complete their work. The students focus all their attention on learning molecular biology, and they learn to perform several tasks at once.

Soon, faculty, senior graduate students, technicians, and postdoctoral fellows joined our class, and beneficial interactions occurred among participants at different stages of their careers. Word of our course spread, and now each session includes faculty and staff from major research universities, teaching colleges, and biotechnology companies from around the world. Professionals unable to devote an entire term to a course often can spend 2 weeks away from home.

We based our choice of protocols for this book on their reliability and instructional value. For example, we use cesium chloride–ethidium bromide density-gradient centrifugation to purify large quantities of plasmid DNA, even though this method is seldom used in most laboratories. This method allows students to observe the differences in topological forms of DNA, it yields DNA of higher purity than other procedures, and students learn how to

use an ultracentrifuge, which is something few have done and many will need to do. We use radioactive probes in our course for similar reasons. Radioactive probes offer greater sensitivity, less background signal, and easier washing and detection than the nonradioactive methods we have tried. In addition, students learn to handle radioactive isotopes safely, which is something most graduate students must learn. Although in our current research we use polymerase chain reaction to create specific mutations, we still teach oligonucleotide-directed mutagenesis because it gives students hands-on experience with many concepts not covered elsewhere. Thus, this course includes seldom-used procedures with heuristic value together with methods used routinely in typical molecular biology laboratories.

Graduates of our course have successfully applied these approaches to their own experimental systems, and they have acquired the skills needed to teach themselves new procedures from *Current Protocols, Molecular Cloning,* and other sources. For example, one of our former students, William Proebsting, a professor of horticulture at Oregon State University, used skills he acquired in our class to clone the pea dwarfing gene studied by Mendel (*Proc. Natl. Acad. Sci. USA,* **94:** 8907–8911, August 1997). Thus, we have had the satisfaction of seeing this course prove beneficial to our students, and we have found it rewarding to teach. We hope you have the same experience.

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Course Synopsis

INTRODUCTION

Molecular biology, in particular recombinant DNA research, has transformed research in the biological and medical sciences. This technology currently influences all aspects of biological research, has far-reaching applications in clinical diagnosis, and has led to important developments in agriculture and biotechnology.

This course provides a hands-on introduction to molecular biological methods, including molecular cloning, polymerase chain reaction (PCR), Southern (DNA) blotting, Northern (RNA) blotting, DNA sequencing, oligonucleotide-directed mutagenesis, and protein expression, purification, and detection. You will work with a well-characterized gene (*virD2*) from the *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid. Virulence (*vir*) genes mediate transfer of a specific region of the Ti plasmid from *A. tumefaciens* into host plant cells; oncogenes contained in the transferred DNA integrate into the host nuclear genome where their expression causes tumorous growth. Because we have studied *virD2* previously, both starting materials and finished products for each experiment are available. All of the experiments you will do are "real," and most are published. This approach will demonstrate practical aspects of experimental design. Although the gene you will use is bacterial, the techniques apply to any research system.

Cloning, Restriction Analysis, Protein Expression, and Western Blots

You will begin with a plasmid (pWR160) that contains a portion of the *virD* operon inserted in a commonly used *E. coli* vector plasmid (pUC18). This plasmid contains *virD1* and *virD2*, which encode a 2-component, site-spe-

cific endonuclease that initiates transfer of DNA into plant cells.

To purify VirD2 protein, you will fuse the *virD2* gene to *gst*, a gene encoding glutathione-*S*-transferase. The Gst–VirD2 fusion protein retains the ability to bind glutathione and can be purified many-fold by affinity chromatography on glutathione–Sepharose. An expression vector plasmid (pGEX2) contains *gst* downstream from a strong promoter (the *tac* promoter); restriction endonuclease cleavage sites lie at the 3' end of the *gst* coding sequence.

Before you construct the gene fusion, you must purify the cloning vector plasmid (pGEX2) and the template for PCR DNA synthesis (pWR160). You will extract plasmid DNA from *E. coli* cells that harbor these plasmids and use CsCl–ethidium bromide density gradient centrifugation to purify the plasmid DNAs.

To fuse *virD2* to *gst*, you will cleave the vector plasmid (pGEX2) with the restriction endonucleases *Bam*HI and *Eco*RI. Polymerase chain reaction allows you to add appropriate restriction sites to the ends of the *virD2* coding sequence so that you can fuse it, in the same reading frame, to *gst*. The PCR primers contain restriction sites (at their 5' ends) adjacent to sequences complementary to the ends of *virD2*. You will anneal these primers to denatured template DNA (pWR160) and use PCR to amplify a DNA fragment consisting of *virD2* with a *Bgl*II restriction site at the 5' end and an *Eco*RI site at the 3' end of the gene.

Next treat the PCR product (containing *virD2*) with restriction enzymes *Bgl*II and *Eco*RI. After you inactivate the restriction enzymes, mix the DNAs and incubate them with T4 DNA ligase to join their cohesive ends. After ligation, transform *E. coli* cells with the ligated DNA. The vector plasmid confers ampicillin resistance on its host, allowing you to select transformed *E. coli* cells. To analyze the plasmids contained in ampicillin-resistant transformants, grow small liquid cultures of individual transformants and use an alkaline lysis method to prepare plasmid DNA for restriction endonuclease digestion and

agarose gel electrophoresis. Once you identify transformants that contain the *gst-virD2* gene fusion, you can express and purify the Gst-VirD2 fusion protein.

You will examine the Gst–VirD2 fusion protein using SDS-polyacrylamide gel electrophoresis and western blot immunological detection. To induce expression of the *gst–virD2* gene, grow *E. coli* containing this gene fusion in broth containing IPTG, which induces the *tac* promoter. Next, prepare crude extracts from these cells and allow the fusion protein to bind glutathione–Sepharose beads. Washes will remove most other proteins, resulting in a substantial purification of bound Gst–VirD2 protein. Examine the crude extracts and purified proteins on silver-stained SDS-polyacrylamide gels. You will also transfer proteins from gels to nylon filters and use antisera raised against Gst to detect the Gst–VirD2 fusion protein; this is the Western blot procedure.

Oligonucleotide-Directed Mutagenesis

You will create a specific mutation in *virD2* using an oligonucleotide primer that contains the mutation. To produce the single-stranded template, we will insert *virD2* sequences into a phagemid vector (pUC119). A phagemid contains origins of replication from a plasmid (ColE1) and a single-stranded DNA phage (M13); therefore, phagemid DNA can be isolated in either single- or double-stranded form. You will isolate uracil-containing single-stranded phagemid DNA from an *E. coli* strain deficient in dUTPase and uracil repair (*dut− ung−*). This DNA will serve as template for DNA synthesis (in vitro) primed by an oligonucleotide complementary (except for the desired mutation) to *virD2*. On completion, the uracil-containing template will be paired with newly synthesized thymine-containing DNA, except at the short heteroduplex region created by mismatches in the mutagenic primer oligonu-

cleotide. On transformation into a uracil repair-proficient (*ung*+) *E. coli* strain, the uracil-containing wild-type template will be degraded, and the thymine-containing mutant strand will give rise to transformants containing the mutation in *virD2*. You will confirm putative mutants by restriction analysis and DNA sequencing.

DNA Sequence Analysis

In vitro DNA synthesis can introduce unwanted mutations into DNA. To determine whether your mutant *virD2* contains mutations in addition to the oligonucleotide-directed mutation, you will determine its nucleotide sequence.

Southern (DNA) and Northern (RNA) Blotting

For Southern (DNA) blotting, you will use radiolabeled plasmid DNA containing *virD2* (pWR160) as a hybridization probe to examine genomic DNAs from *A. tumefaciens* for *virD2*. You will examine restriction fragment length polymorphisms (RFLPs) between *virD2* genes from different strains of *A. tumefaciens* and *A. rhizogenes*.

To examine the *virD2* region for RFLPs, you will isolate genomic DNAs from different strains of *A. tumefaciens*, digest these DNAs with restriction endonucleases, and separate the resulting fragments according to size by agarose gel electrophoresis. You will then denature the DNA and transfer it to a nylon filter. To the filter-bound DNA you will hybridize probe (pWR160) DNA labeled with ^{32}P by nick translation. Finally, you will wash the filter and detect hybridization by autoradiography.

Because bacterial messages are extremely difficult to detect by the Northern (RNA) blot procedure, we will depart from the sequence of experiments involving *virD2*. In-

stead, northern blot analysis will detect mRNA encoded by the *rbsS* (RuBisCO) gene among total RNA isolated from tobacco leaves. After extracting RNA from leaves, you will separate RNA molecules according to size by formaldehyde–agarose gel electrophoresis, transfer the RNA to a nylon filter, hybridize labeled probe DNA to the filter-bound RNA, wash the filter, and detect hybridization by autoradiography. The hybridization probe will be a pUC-based plasmid (pRbsS) containing part of the *rbsS* gene.

Protein Interaction Analysis in Yeast

You will use the yeast two-hybrid protein interaction assay to determine whether two *Agrobacterium* virulence proteins—VirE1 and VirE2—interact. The *virE2* gene is fused to a portion of *E. coli lexA* that encodes the sequence-specific DNA-binding domain of the LexA repressor. *virE1* is fused to a segment of the *Saccharomyces cerevisiae GAL4* gene that encodes the transcription activation domain of this yeast transcription factor. If VirE1 and VirE2 interact in vivo, the interaction will tether the Gal4 transcription activation domain to a LexA binding (operator) site located upstream from a reporter gene, which consists of the yeast *GAL1* promoter fused to the *E. coli lacZ* (β-galactosidase) gene. If *lacZ* is induced, the resulting β-galactosidase will turn the yeast blue in the presence of the chromogenic substrate X-gal. Controls needed to interpret the two-hybrid analyses are included.

SAFETY PRECAUTIONS

The following safety tips address common hazards encountered in molecular biology laboratories.

1. **Wear safety glasses when working with phenol and other caustic solvents**, including concentrated acids and bases.
2. **Never put bottles containing phenol or other caustic solvents on shelves over a lab bench.**
3. **Disconnect leads from power supplies after use**; capacitors can retain lethal electric charges, even when the unit is unplugged. When power supplies are in use, secure leads on the bench, not hanging down in front. To shut off power supplies, turn the rheostat to "0" first, then switch the power off. This will prevent the next user from blowing the unit's fuse, which can occur if the power supply is set to deliver full power the instant it is turned on.
4. **Put sharps in designated receptacles.** Razor blades, Pasteur pipets, needles, and other sharp objects will injure custodians if placed in the regular trash.
5. **Ethidium bromide (EtBr)** is a carcinogen. Wear gloves when handling it, and dispose of it in designated receptacles.
6. **Wear gloves and a lab coat when handling radioisotopes.** Use ^{32}P behind shields and limit body exposure to it. Scan hands, equipment and work areas after use. Dispose of radioactive material in radioactive waste receptacles.
7. **Autoclave materials containing bacterial cultures.** Dispose of culture plates in designated autoclave bags.

8. **Wear a lab coat.** It will protect you from chemical or isotopic contamination. Short pants and sandals are not safe attire in a laboratory.
9. **Use mechanical pipettors; avoid contact with reagents.**
10. **Do not eat or drink in the laboratory.**
11. **Protect eyes and skin from ultraviolet light.**

DAILY SCHEDULE

Day 1

8:00	Lab lecture
9:30	**I.A, steps 2–19.** Cesium chloride–ethidium bromide density gradient centrifugation: purification of plasmids; spin takes 4 hours
12:00	*Lunch break*
1:00	Lecture: Plasmids and Cosmids; Gene Cloning
2:30	Supplementary Lecture I: Gene Cloning II: Libraries
3:30	**I.A, steps 20–24.** Collect plasmid DNAs from the cesium chloride gradients, extract, precipitate, and quantitate

Day 2

8:00	Lab lecture
8:45	**I.C, steps 1–4.** Restriction digest of pGEX2 **III.A, steps 1–4.** Restriction digest of pCS64 and pUC119 **I.B, steps 1–8.** PCR to synthesize *virD2* flanked with restriction sites **I.D and III.B, steps 1–14.** Agarose gel and Geneclean restriction fragments
12:00	*Lunch break*
1:00	Lecture: Gene Amplification, RAPDs, Sequencing
2:00	**I.B, steps 9–14.** Agarose gel of PCR products **I.C, steps 5–7.** Restriction digest of PCR products **V.A, steps 1–17.** Prepare *A. tumefaciens* genomic DNAs **I.E, steps 1–5, III.C, steps 1–10.** Ligate DNA fragments to vectors

Day 3

8:00	Lab lecture
8:45	**V.B, steps 1–3.** Restriction digest of genomic DNAs
9:15	Supplementary Lecture II: Microbiological Techniques
9:45	**I.F, steps 1–12, III.D.** Transform *E. coli* DH5α with ligated plasmids
12:00	*Lunch break*
1:00	Lecture: Probes, Southern Blots, RFLPs
2:00	**V.C, steps 1–5.** Agarose gel of restriction fragments from genomic DNAs **V.D, steps 1–11.** Denature DNA and transfer to filter by blotting

Day 4

8:00	Lab lecture
8:45	**V.D, step 12.** Wash and UV cross-link DNA blot **V.F, step 1.** Prehybridization of Southern blot **V.E, steps 1–11.** Prepare probe **VI.A, steps 1–8.** Prepare total RNA from tobacco
12:00	*Lunch break*
1:00	Lecture: Cloning by Function, Two-Hybrid Screening
2:00	**V.F, step 2.** Hybridize Southern blot to probe **I.F, steps 13–14, III.D.** Pick colonies from transformations, inoculate broth

Day 5

8:00	Lab lecture
8:45	**I.G, steps 1–11, III.E.** Prepare plasmid DNA **V.F, steps 3–5.** Wash Southern blot
10:15	**III.G, steps 3–6.** Template preparation: inoculate, add ampicillin, add helper phage **I.H, steps 1–13, III.F.** Restriction analysis of recombinant plasmids **VI.A, steps 9–12.** Precipitate RNA, dissolve, and reprecipitate
12:00	*Lunch break*
1:00	Lecture: Protein Isolation, Fusion Proteins, Expression Vectors
2:00	**I.H, steps 4–10, III.F.** Restriction analysis of recombinant plasmids: gels **III.G, step 7.** Template preparation: add ampicillin and kanamycin, incubate **V.F, steps 6–7.** Southern blot: autoradiography **VI.A, steps 13–15.** Precipitate RNA, dissolve and quantify

Day 6

8:00	Lab lecture
8:45	**VI.B, steps 1–2.** Pour agarose-formaldehyde gel for Northern blot
9:45	**VI.B, steps 3–6.** Formaldehyde-agarose gel electrophoresis of RNA
11:30	**VI.B, steps 7–8, VI.C, steps 1–11.** RNA transfer by blotting
12:00	*Lunch break*
1:00	Lecture: Northern Blots and Other RNA Techniques
2:00	**VI.D, steps 1–11.** Prepare probe **VI.C, step 12.** Rinse and cross-link RNA blot **III.G, steps 8–9.** Template preparation: centrifuge, begin PEG precipitation

Day 7

8:00	Lab lecture
8:45	**III.G, steps 10–15.** Finish single-stranded template preparation **III.H, steps 1–5.** Phosphorylate oligonucleotide primer
12:00	*Lunch break*
1:00	Lecture: Oligo-Directed Mutagenesis
2:00	**III.I, steps 1–4.** Anneal primer to template **III.J, steps 1–3.** DNA synthesis by primer extension **III.K, step 1.** Transform synthesis reactions into *E. coli* DH5α **VI.E, step 1.** Begin Northern blot prehybridization; incubate overnight

Day 8

8:00	Lab lecture
8:30	**VII.A.** Transform yeast **III.K, step 2.** Pick colonies, inoculate broth
10:45	**II.B, steps 1–2.** Pour resolving gel
11:30	**II.A, step 2.** Induce with IPTG, incubate
12:00	*Lunch break*
1:00	Lecture: In situ Hybridization
2:00	**II.B, steps 3–4.** Pour stacking gel
2:30	**II.A, steps 3–11.** Purify fusion protein on glutathione sepharose
3:00	**II.B, steps 5–8.** SDS-polyacrylamide gel electrophoresis **II.C, step 1–2.** Store gel overnight in gel fix **VI.E, step 2.** Begin Northern hybridization; incubate overnight

Day 9

8:00	Lab lecture
8:30	**III.L.** Minipreps of plasmid DNA
9:30	**III.M, step 1.** Restriction digest of plasmid DNA with *Hin*dIII and *Bam*HI
10:30	**II.C, steps 1–13.** Silver staining of protein gel **II.B, steps 1–3.** Pour resolving part of SDS-polyacrylamide gel; store in refrigerator
12:00	*Lunch break*
1:00	Lecture: Computer Analysis of Gene Sequence Data
2:00	Group 1: Computer analysis of nucleic acid sequences Group 2: **III.M, steps 2–8.** Gel of restriction digest to confirm mutants **VI.E.** Northern blot washes
3:30	Group 1 switch with Group 2

Day 10

8:00	Lab lecture
8:45	**IV.A, steps 1–3.** Pour gels for sequencing **IV.B, steps 1–13.** Sequencing reactions **IV.A, steps 4–6.** Sequencing gel electrophoresis
12:00	*Lunch break*
1:00	Lecture: Methods for Analysis of Gene Expression
2:00	**II.B, steps 4–8.** Pour stacking gel; perform electrophoresis **IV.A, steps 7–8.** Dry sequencing gels, put on film **II.D, steps 1–4.** Electrophoretic transfer of proteins **II.D, steps 5–9.** Antibody incubation of Western blot
3:30	Lecture: DNA-Protein Interactions

Day 11

8:00	Lab lecture
8:45	**II.D, steps 10–15.** Secondary antibody incubation, color development **IV.** Develop films from Northern blot, examine, and discuss **VII.B.** β-galactosidase assays
12:00	*Lunch break*
1:00	Lecture: Antibodies, Immunological Techniques
2:00	Develop all films; examine and discuss

Lab Cleanup

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